BOTANY

POST GRADUATE DEGREE PROGRAMME
(CBCS CURRICULUM)

SEMESTER: IV

PAPER: BOET 4.1

Genetics & Molecular genetics

Directorate of Open and Distance Learning
UNIVERSITY OF KALYANI
Kalyani, Nadia
West Bengal

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POST GRADUATE DEGREE PROGRAMME (CBCS)
IN
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Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI
KALYANI – 741 235,
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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks are also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof. Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

SYLLABUS
COURSE – BOET 4.1
Genetics & Molecular Genetics
(Full Marks – 100)

Course	Group	Details Contents Structure		Study hour
BOET 4.1	Genetics & Molecular Genetics	**Unit 1. Genetic engineering-I**	1. Genetic engineering: Restriction enzymes; cloning vectors (plasmids, cosmid, phagmids, YAC, BAC); cloning strategies; polymerase chain reaction;	1
		Unit 2. Genetic engineering-II	2. Genetic engineering: Sequencing strategies; DNA gelelectrophoresis; blotting techniques; c-DNA library; DNA foot printing; DNA finger printing.	1
		Unit 3. Transgenics-I	3. Transgenics: Development strategies; Transgenics in relation to insect, herbicide, stress resistance	1
		Unit 4. Transgenics-II	4. Transgenics: Delayed fruit ripening; golden rice; vaccine development; male sterility; molecular farming; flower colour; terminator gene sequence.	1
		Unit 5. Genetic recombination	5. Genetic recombination: Molecular basis of chromosome pairing; Rec BCD pathway; double strand model in yeast; gene conversion in bread mold; site specific recombination	1
		Unit 6. Genetic diseases and gene knockout mutation	6. Genetic diseases. Pedigree analysis. Gene therapy and genetic counselling. 7. Gene knockout mutation in reference to mice and yeast models.	1
		Unit 7. Gene regulation-I	8. Gene regulation at the level of transcription in eukaryotes organism	1
		Unit 8. Gene regulation-II	9. Gene regulation at the level of transcription and translation in eukaryotes organism	1
		Unit 9. Dosage compensation	10. Dosage compensation	1
		Unit 10. RNA biology	11. RNA biology: RNA editing and evolutionary significance; antisense RNA technology and gene silencing; ribozyme; different categories of small non coding RNAs; biogenesis and functions of small RNAs in posttranscriptional gene silencing; application of RNAi in crop quality improvement.	1
		Unit 11. Cancer-I	12. Cancer: Properties of cancer cells. Transfection test. Genetic basis of cancer. Characterization of p 53 and its role in regulation of cancer	1
		Unit 12. Cancer-II	13. Cancer: Role of gene mutation, reciprocal translocation, insertion of retroviral genome and constitutive amplification in cancer development; environmental carcinogenesis; therapy and side effects.	1

Course	Group	Details Contents Structure		Study hour
BOET4.1	**Genetics & Molecular Genetics**	**Unit 13. T-DNA technology**	14. T-DNA technology: T-DNA transfer, disarming of T-DNA, cointegrates; direct and indirect methods of gene transfer. Binary vector. Shuttle vector.	1
		Unit 14. Cell Signaling	15. Cell Signaling: Intracellular and cell surface; receptor proteins: ion channel linked, G-protein linked and enzyme linked	1
		Unit 15. Genomics	16. Genomics: Structural genomics, molecular markers and mapping of genome using - RFLP, RAPD, AFLP, ESTS and micro-satellite markers; chromosome walking; Functional genomics: DNA microarray and chip technology; a brief idea on Human Genome Project.	1
		Unit 16. Proteomics	17. Proteomics: Concept of proteome; basic principles of 2-DE; advantages and limitations of 2-DE; gel free proteomics; mass spectrometry	1

Content

COURSE – BOET 4.1

(Genetics & Molecular Genetics)

Hard Core Theory Paper **Credit: 4**

Content Structure

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1. Introduction
2. Course Objectives
3. Genetic engineering: Restriction enzymes; cloning vectors (plasmids, cosmid, phagmids, YAC, BAC); cloning strategies; polymerase chain reaction; sequencing strategies; DNA gelelectrophoresis; blotting techniques; c-DNA library; DNA foot printing; DNA finger printing.
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5. Genetic recombination: Molecular basis of chromosome pairing; Rec BCD pathway; double strand model in yeast; gene conversion in bread mold; site specific recombination.
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7. Gene knockout mutation in reference to mice and yeast models.
8. Gene regulation at the level of transcription and translation in eukaryotes.
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15. Proteomics: Concept of proteome; basic principles of 2-DE; advantages and limitations of 2-DE; gel free proteomics; mass spectrometry.
16. Let us sum up
17. Suggested Reading
18. Assignment

1. Introduction

Genetics has always been concerned with the problem of how the hereditary information in DNA controls what an organism looks like and how it works. Classically this involved the use of genetic variants (mutants) to upset the biological function of the cells or organisms and, from the effect of these mutations, to make deductions about the way cells and organisms worked. At the molecular end of the subject, the availability of sequence information and genomic analysis, together with sophisticated techniques for gene replacement, and analysis of gene expression patterns. gives us much more powerful tools for looking at the way genes work to make us what we are. At the other extreme of the subject, a knowledge of genetics is fundamental to an understanding of how organisms, populations and species evolve.

2. Course Objectives

At the end of the course the learners will be able to:

- To know the methods and techniques of genetics are applicable throughout the spectrum of biological activity.
- Gather knowledge about restriction enzyme, cloning vector, gel electrophoresis and other technique.
- Identify the genetic disease and that can help in genetic counselling.
- To understand transgenic application in our daily life
- Be able to look at a pedigree chart and discern the most likely mode of inheritance.
- Describe gene knockout, dosage compensation, genomics and proteomics
- Distinguish between loss-of-function mutations and gain-of-function mutations
- Describe variation both in DNA and chromosomal level.
- Explain cell signalling

3. Genetic engineering: Restriction enzymes; cloning vectors (plasmids, cosmid, phagmids, YAC, BAC); cloning strategies; polymerase chain reaction; sequencing strategies; DNA gelelectrophoresis; blotting techniques; c-DNA library; DNA foot printing; DNA finger printing

Recombinant DNA (rDNA) is a form of artificial DNA that is created by combining two or more sequences that would not normally occur together through the process of gene splicing.

Recombinant DNA technology is a technology which allows DNA to be produced via artificial means. The procedure has been used to change DNA in living organisms and may have even more practical uses in the future.

Stanley N. Cohen, who received the Nobel Prize in Medicine in 1986 for his work on discoveries of growth factors. **Stanley N. Cohen** and **Herbert Boyer** constructed the first recombinant DNA using bacterial DNA and plasmids.

Restriction enzymes:

These are also known as molecular scissors, used for cutting of DNA. The cutting of DNA at specific locations became possible with the discovery of molecular scissors, i.e., restriction enzymes. In the year 1963, the two enzymes responsible for restricting the growth of bacteriophage in E. coli were isolated. One of these added methyl groups to DNA, while the other cuts the DNA. Later was termed as restriction endonucleases.

The first restriction endonuclease was isolated by Smith Wilcox and Kelley in 1968 was Hind II. It was found that it always cuts DNA molecules at a particular point by recognising a specific sequence of six base pairs known as recognition sequence for Hind II. Today, more than 900 restriction enzymes have been isolated from over 230 bacterial strains each of which recognise different recognition sequences.

Restriction endonuclease enzymes occur naturally in bacteria as a chemical weapon against the invading viruses. They cut both strands of DNA when certain foreign nucleotides are introduced in the cell. Endonucleases break strands of DNA at internal positions in random manner.

The first observations on the existence of restriction enzymes was made by Arber and Dussoix in 1962, and proposed model to explain the restriction phenomenon. Their views on restriction enzymes affirmed that certain bacterial strains contained endonucleases able to cleave unprotected DNA. In addition, several other strains contained a modification system responsible for protecting their own DNA.

Some of the observations were made by W. Arber and his associates while studying the efficiency of plating of the bacteriophage lambda on different strains of Escherichia coli. They even demonstrated that restriction endonucleases were able to cleave DNA from other strains while exempting that of the original strains.

They are also associated with modifying enzymes, which methylate the DNA. Methylated DNA escape cleavage by endonucleases, and prevents the cell from degrading its own DNA. Thus, invading foreign DNA in bacteria that has not been correctly methylated will be degraded.

In 1970, Smith, Wilcox and Kelly have characterized and purified restriction enzymes and elucidated their recognition and cleavage site of a more useful restriction enzyme, Hind II.

Naming of Restriction Enzymes:

The convention for naming these enzymes proceeds in a way that the first letter of the name comes from the genes and the second two letters come from the species of prokaryotic cell, from which they were isolated, e.g., Eco RI comes from E. coli RY13. The letter 'R' is derived from the name of strain. Roman numbers following the names, indicate the order in which the enzymes were isolated from that strain of bacteria.

Naming exercise of RE enzymes is based on following rules:

1. Each RE enzyme is named by a three-letter code.
2. The first letter of this code is derived from the first epithet (first letter of name) of the genus name. It is printed in italics.
3. The second and third letters are from the first two letters of its species name. They are also printed in italics.
4. This is followed by the strain number. If a particular strain has more than one restriction enzyme, these will be identified by Roman numerals as I, II, III, etc.

For example, the enzyme Eco RI was isolated from the bacterium *Escherichia* (E) *coli* (co) strain RY13 (R) and it was the first endonuclease (I). R also indicates antibiotic resistant plasmid of the bacterium. Likewise, Hind II from *Haemophilus influenzae* strain Rd and Bgl I from *Bacillus globigii*. A few restriction endonuclease enzymes and their sources are given below:

Table 55.3. A few restriction endonuclease enzymes, their sources and recognition sites.

Name of the restriction endonuclease enzyme	Source (Microorganisms)	Recognition sequence and cleavage site
1. *Aat* II	*Acetobacter aceti*	GACGT↓C
2. *Bcl* I	*Bacillus Caldoyticus*	T↓GATCA
3. *Cvn* I	*Chromatium vinosum*	CC↓TNAGG
4. *Eco* RI	*Escherichia coli* RY13	G↓AATTC
5. *Eco* RII	*Escherichia coli R*245	↓CCTGG
6. *Hind* II	*Haemophilus influenzae* Rd	GTP_{γ}↓PuAC*
7. *Hind* III	*Haemophilus influenzae* Rd	A↓AGCTT
8. *Kpn* I	*Klebsiella pneumoniae* OK	GGTAC↓C
9. *Nop* I	*Nocardia opaca*	G↓TC GAC
10. *Nsp* B II	*Nostoc*	C(A/C)G↓C(T/G)G

Restriction enzymes belong to a larger class of enzymes called nucleases, which are of two types:

(a) Exonucleases remove nucleotides from the ends of the DNA either (5′ or 3′) in one strand of duplex.

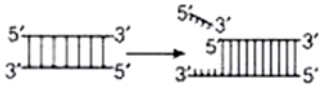
(b) Endonucleases make cuts at specific positions within DNA. Each restriction endonucleases function by 'inspecting' the length of a DNA sequence.

Once it finds its specific recognition sequence, it will bind to the DNA and cut each of the two strands of the double helix at specific points in their sugar phosphate backbones.

Differences between Exonucleases and Endonucleases

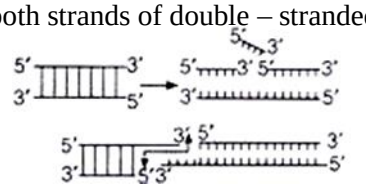
Exonucleases:

1. These nucleases cleave base pairs of DNA at their terminal ends
2. They act on single – strand of DNA or gaps in double –stranded DNA. They do not cut RNA



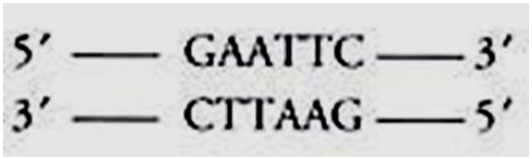
Endonucleases:

1. They cleave DNA at any Pont except the terminal ends
2. They cleave one strand or both strands of double – stranded DNA. They may cut RNA

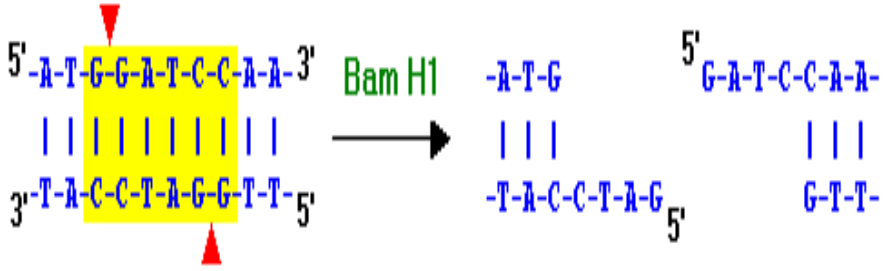


Each restriction endonuclease recognizes a specific palindromic nucleotide sequences in the DNA. Recognition sites of most restriction enzymes have a twofold rotational symmetry. Restriction enzymes have corresponding symmetry to facilitate recognition and usually cleave the DNA on the axis of symmetry.

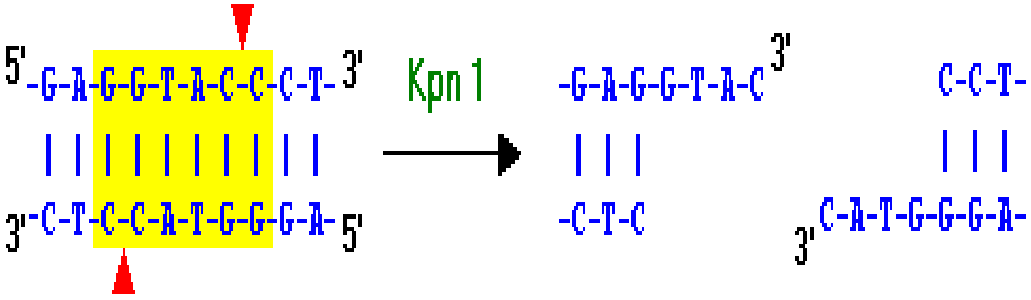
Palindrome in the DNA is a group of letters that forms the same words when read both forward and backward. For example, the following sequences read the same on the two strands in 5′ → 3′ direction as well as 3′ → 5′ direction.



5' overhangs: The enzyme cuts asymmetrically within the recognition site such that a short single-stranded segment extends from the 5' ends. Bam HI cuts in this manner.

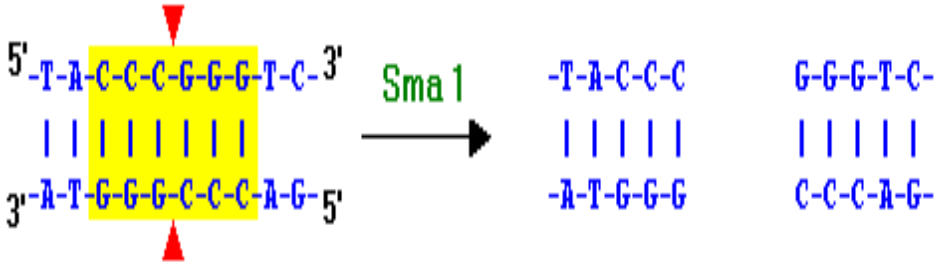


3' overhangs: Again, we see asymmetrical cutting within the recognition site, but the result is a single-stranded overhang from the two 3' ends. KpnI cuts in this manner.



Blunt cut ends:

Enzymes that cut at precisely opposite sites in the two strands of DNA generate blunt ends without overhangs. SmaI is an example of an enzyme that generates blunt ends.



Isoschizomers: Different enzymes identified from different bacteria may have the same recognition sequence, with the same cleavage site. Such restriction enzymes are known as isoschizomers. For example,SphI (CGTAC/G) and BbuI (CGTAC/G) are isoschizomers that recognize the same recognition sequence.

If two enzymes have the same recognition site but different cleavage sites, they are known as **neoschizomers**. For example SmaI (CCC/GGG) and XmaI (C/CCGGG) are neoschizomers, where SmaI produces blunt ends, whereas XmaI produces cohesive ends. Some enzymes recognize similar (not same) sequences but produce the same sticky ends. Such enzymes are known as **isocaudomers**. For example, NotI and Bsp120I

Types:

Naturally occurring restriction endonucleases are categorized into four groups (Types I, II III, and IV) based on their composition and enzyme cofactor requirements, the nature of their target sequence, and the position of their DNA cleavage site relative to the target sequence. All types of enzymes recognize specific short DNA sequences and carry out the endonucleolytic cleavage of DNA to give specific fragments with terminal 5'-phosphates. They differ in their recognition sequence, subunit composition, cleavage position, and cofactor requirements, as summarised below:

Type-I Restriction Endonucleases (EC 3.1.21.3):

These are the complex type of endonucleases which cleave only one strand of DNA. These enzymes have the recognition sequences of about 15 bp length.

They require Mg^{++} ions and ATP for their functioning. Such types of restriction endonucleases cleave the DNA about 1000 bp away from the 5′ end of the sequence 'TCA' located within the recognition site. Important examples of Type-I restriction endonuclease enzyme are EcoK, EcoB, etc.

- Capable of both restriction and modification activities
- The cofactors S-Adenosyl methionine (AdoMet), ATP, and Mg2+, are required for their full activity
- Contain,
    - two R (restriction) subunits,
    - two M (methylation) subunits and
    - one S (specificity) subunit
- Cleave DNA at random length from recognition site

Type-II Restriction Endonucleases (EC 3.1.21.4):

These are most important endonucleases for gene cloning and hence for rec DNA technology. These enzymes are most stable. They show cleavage only at specific sites and therefore they produce the DNA fragments of a defined length. These enzymes show cleavage in both the strands of DNA, immediately outs.de then- recognition sequences. They require Mg^{++} ions for their functioning.

Such enzymes are advantageous because they don't require ATP for cleavage and they cause cleavage in both strands of DNA. Only Type II Restriction Endonucleases are used tor gene cloning due to their suitability.

The recognition sequences for Type-II Restriction Endonuclease enzymes are in the form of palindromic sequences with rotational symmetry, i.e., the base sequence .n the first half of one strand of DNA is the mirror image of the second half of other strand of that DNA double helix. Important examples of Type-II Restriction endonucleases include Hinfl, EcoRI, PvuII, Alul, Haelll etc

- Mostly used for gene analysis and cloning
- More than 3500 REs
- Recognize 4-8 bp sequences
- Need Mg^{2+} as cofactor
- Cut in close proximity of the recognition site
- Single function (restriction digestion) enzymes independent of methylase.
- Homodimers
- ATP hydrolysis is not required
- Examples: EcoRI, EcoRII, BamHI, HindIII

Type III enzymes (EC 3.1.21.5)

Type-III Restriction Endonucleases:

These are not used for gene cloning. They are the intermediate enzymes between Type-I and Type-II restriction endonuclease. They require Mg^{++} ions and ATP for cleavage and they cleave the DNA at well-defined sites in the immediate vicinity of recognition sequences, e.g. Hinf III, etc.

- Large enzymes
- Combination restriction-and-modification
- Cleave outside of their recognition sequences
- Require two recognition sequences in opposite orientations within the same DNA moleculecle.
- Require ATP (but do not hydrolyse it); S-adenosyl-L-methionine stimulates the reaction but is not required

Table 55.2. Characteristics of restriction endonuclease enzymes.

Characteristics	Type I	Type II	Type III
1. Restriction and modification activities	Single multifunctional enzyme	Separate endonuclease and methylase	Separate enzymes with a subunit is common
2. Protein structure of enzyme	Three different subunits	Simple	Two different subunits
3. Requirements for restriction	ATP, Mg^{2+} S–adenosyl-Mg^{2+} methionine		ATP, Mg^{2+} (S-adenosyl methionine)
4. Sequence of host specificity sites	*Eco* B: TGAN*$_8$TGGT *Eco* K: AA N*$_6$ GTGC	Usually rotational symmetry	*Eco* P1: AGACC *Eco* P15: CAGCAG
5. Cleavage sites	Possibly random at least 1000 bp from host specificity site	At or near host specificity site	24-26 bp to 3' of host specificity site
6. Enzymatic turnover	No	Yes	Yes
7. DNA translocation	Yes	No	No
8. Site of methylation	Host specificity site	Host specificity site	Host specificity site

Type IV enzymes

- Cleave only normal and modified DNA (methylated, hydroxymethylated and glucosyl-hydroxymethylated bases).
- Recognition sequences have not been well defined
- Cleavage takes place ~30 bp away from one of the sites

Artificial Restriction Enzymes

- Generated by fusing a natural or engineered DNA binding domain to a nuclease domain
- Can target large DNA sites (up to 36 bp)
- Can be engineered to bind to desired DNA sequences

Table 9.8 : Characteristics of some restriction enzymes

Name of the enzyme	Source	Recognition site and cleavage site	Nature of cut ends
Eco R1	*E. coli* RY13	5'–G|AATTC–3' 3'–CTTAA|G–5'	Sticky
Hind III	*Haemophilus influenzae* Rd	5'–A|AGCTT–3' 3'–TTCGA|A–5'	Sticky
Bam HI	*Bacillus amyloliquifaciens* H	5'–G|GATCC–3' 3'–CCTAG|G–5'	Sticky
Sal I	*Streptomyces albus* G	5'–G|TCGAC–3' 3'–CAGCT|G–5'	Sticky
Bal I	*Brevibacterium albidum*	5'–TGG|CCA–3' 3'–ACC|GGT–5'	Blunt
Hae III	*Haemophilus aegiptius*	5'–GG|CC–3' 3'–CC|GG–5'	Blunt
Sma I	*Serratia marcescens*	5'–CCC|GGG–3' 3'–GGG|CCC–5'	Blunt

Mechanism of Action of Restriction Enzymes:

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded DNA. The enzyme makes two incisions, one through each of the sugar- phosphate backbones (i.e., each strand) of the double helix without damaging the nitrogenous bases.

The chemical bonds that the enzymes cleave can be reformed by other enzymes known as ligases, so that restriction fragments carved from different chromosomes or genes can be spliced together, provided their ends are complementary.

Restriction enzymes cut the strand of DNA a little away from the center of the palindrome sites, but between the same two bases on the opposite strands. This leaves single-stranded portion at the ends. There are overhanging stretches called sticky ends on each strand as given in above figure.

Restriction endonucleases are also used in genetic engineering to form recombinant molecules of DNA, which are composed of DNA from different sources or genomes. The resultant DNA fragments have the same sticky ends, which are complementary to each other, therefore can be joined together (end-to-end) using DNA ligases, when cut by the same restriction enzyme.

Many of the procedures of molecular biology and genetic engineering rely on restriction enzymes. The term restriction comes from the fact that these enzymes were discovered in *E. coli* strains that appeared to be restricting the infection by certain bacteriophages.

Cloning vectors:

Vectors are those DNA molecules that can carry a foreign DNA fragment when inserted into it. A vector must possess certain minimum qualifications to be an efficient agent for the transfer, maintenance and amplification of the passenger DNA.

Criteria of an Ideal Vector:

1. The vector should be small and easy to isolate.
2. They must have one or more origins of replication so that they will stably maintain themselves within host cell.
3. Vector should have one or more unique restriction sites into which the recombinant DNA can be inserted.
4. They should have a selectable marker (antibiotic resistance gene) which allows recognition of transformants.
5. Vector DNA can be introduced into a cell.
6. The vector should not be toxic to host cell.

History of Cloning Vectors:

Herbert Boyer, Keiichi Itakura, and Arthur Riggs were three scientists working in the Boyer's lab, University of California, where they recognized a general cloning vector. This cloning vector had restriction sites for cloning foreign DNA and also, the expression of antibiotic resistance genes for the screening of recombinant/ transformed cells. The first vector used for cloning purposes was pBR322, a plasmid. It was small in size, nearly 4kB, and had two selectable markers.

The cloning vectors must possess the following general characteristics:

- It should small in size.
- It must have an origin of replication.
- It must also be compatible with the host organism.
- It must possess a restriction site.
- The introduction of donor fragment must not intervene with the self-replicating property of the cloning vector.
- A selectable marker, possibly an antibiotic resistance gene, must be present to screen the recombinant cells.
- It should be capable of working under the prokaryotic as well as the eukaryotic system.
- Multiple cloning sites should be present.

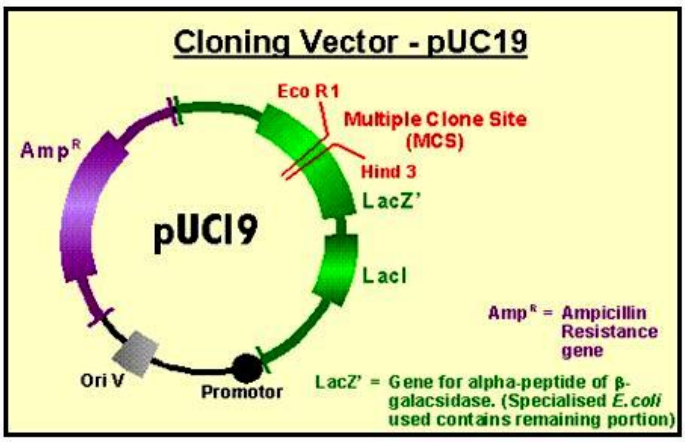
Features of Cloning Vectors

1. Origin of Replication (ori)

- A specific set/ sequence of nucleotides where replication initiates.
- For autonomous replication inside the host cell.
- Foreign DNA attached to ori also begins to replicate.

2. Cloning Site

- Point of entry or analysis for genetic engineering.
- Vector DNA at this site is digested and foreign DNA is inserted with the aid of restriction enzymes.



Cloning vector

- Recent works have discovered plasmids with multiple cloning sites (MCS) which harbour up to 20 restriction sites.

3. Selectable Marker

- Gene that confers resistance to particular antibiotics or selective agent which, under normal conditions, is fatal for the host organism.
- Confers the host cell the property to survive and propagate in culture medium containing the particular antibiotics.

4. Marker or Reporter Gene

- Permits the screening of successful clones or recombinant cells.
- Utilised extensively in blue-white selection.

5. Inability to Transfer via Conjugation

Vectors must not enable recombinant DNA to escape to the natural population of bacterial cells.

Types of Cloning Vectors:

A. Plasmids

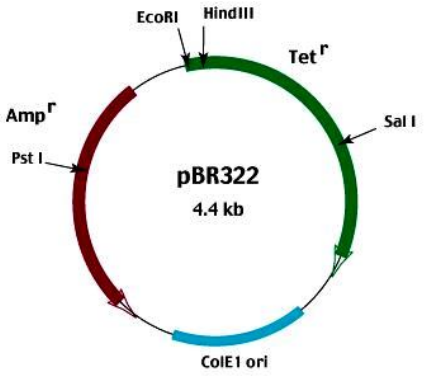
Plasmids are the extra-chromosomal, self-replicating, and double stranded closed and circular DNA molecules present in the bacterial cell. A number of properties are specified by plasmids such as antibiotic and heavy metal resistance, nitro-gen fixation, pollutant degradation, bacteriocin and toxin production, colicin factors, etc.

- Plasmids were the first vectors to be used in gene cloning.
- They are present in bacteria, archaea, and eukaryotes.
- The size of plasmids ranges from 1.0 kb to 250 kb.
- DNA insert of up to 10 kb can be cloned in the plasmids.
- The plasmids have high copy number which is useful for production of greater yield of recombinant plasmid for subsequent experiments.
- The low copy number plasmids are exploited under certain conditions like the cloned gene produces the protein which is toxic to the cells.
- Plasmids only encode those proteins which are essential for their own replication. These protein-encoding genes are located near the ori.

Examples: pBR322, pUC18, F plasmid, Col plasmid.

pBR 322 and pUC Vectors:

pBR322 is a derived plasmid from a naturally occurring plasmid col El, composed of 4362 bp DNA and its replication may be more faster. The plasmid has a point of origin of replication 114 (ori), two selectable marker genes conferring resistance to antibiotics, e.g., ampicillin (ampr), tetracycline (tetr) and unique recognition sites for 20 restriction endonucleases.



pBR322 plasmid

Tetracycline resistance gene has a cloning site and insertion of foreign segment of DNA will inactivate the tetr gene. The recombinant plasmid will allow the cells to grow only in presence of ampicillin but will not protect them against tetracycline .

Nomenclature of plasmid cloning vector: pBR322 cloning vector has the following elements:

p= plasmid

B= Bolivar (name of the scientist)

R= Rodriguez (name of the scientist)

322= number of plasmid discovered in the same lab

Another plasmid used in gene cloning is pUC vector available in pairs with reverse orders of restriction sites relative to lacz promoter. This is a synthesized plasmid possessing ampicillin resistance gene (ampr), origin of replication from pBR322(on) and lacz J gene from E. coli. pUC 8 and pUC 9 make one such pair.

Nomenclature of plasmid cloning vector: pUC 9 cloning vector has the following elements:

p= plasmid

U= University of

C= California

9= number of plasmid discovered in the same lab

Advantages of using Plasmids as vectors:

- Easy to manipulate and isolate because of small size.
- More stable because of circular configuration.
- Replicate independent of the host.
- High copy number.
- Detection easy because of antibiotic-resistant genes.

Disadvantages of using Plasmids as vectors:

- Large fragments cannot be cloned.
- Size range is only 0 to 10kb.
- Standard methods of transformation are inefficient.

B. Bacteriophage:

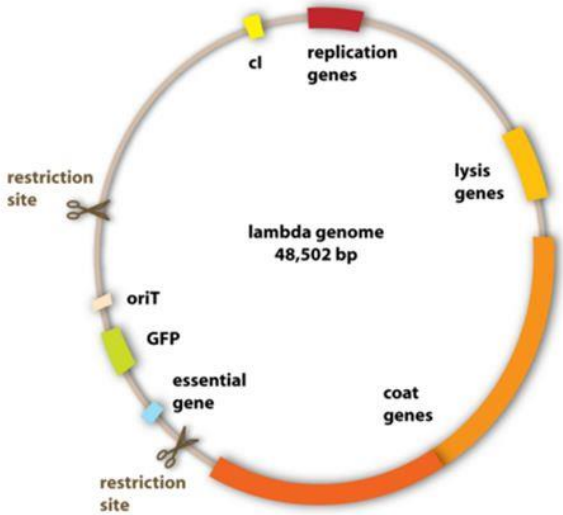
The bacteriophage has linear DNA molecule, a single break will generate two fragments, foreign DNA can be inserted to generate chimeric phage particle. But as the capacity of phage head is limited, some segments of phage DNA, not having essential genes, may be removed. This technique has been followed in λ (Lambda) phage vectors to clone large foreign particle**.**

- Bacteriophages or phages are viruses which infect bacterial cells.
- The most common bacteriophages utilized in gene cloning are Phage λ and M13 Phage.
- A maximum of 53 kb DNA can be packaged into the phage.
- If the vector DNA is too small, it cannot be packaged properly into the phage.

Examples: Phage Lambda, M13 Phage, etc.

Phage Lambda λ:

- It has head, tail, and tail fibers.
- Its genome consists of 48.5 kb of DNA and 12 bp ss DNA which comprise of sticky ends at both the terminals. Since these ends are complementary, they are cohesive and also referred to as cos sites.
- Infection by λ phage requires adsorption of tail fibers on the cell surface, contraction of the tail, and injection of the DNA inside the cell.



Bacteriophage λ

M13 Phage

- These vectors are used for obtaining single-stranded copies of the cloned DNA.
- They are utilized in DNA sequencing and in vitro mutagenesis.
- M13 phages are derived from filamentous bacteriophage M13. The genome of M13 is 6.4 kb.

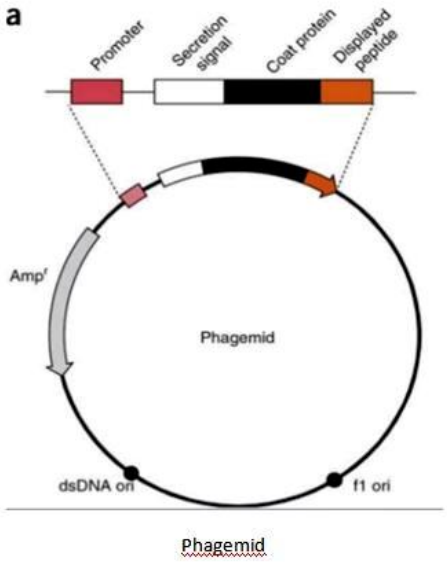
- DNA inserts of large sizes can be cloned.
- From the double-stranded inserts, pure single-stranded DNA copies are obtained.

Advantages of using Phage Vectors

- They are way more efficient than plasmids for cloning large inserts.
- Screening of phage plaques is much easier than identification of recombinant bacterial colonies.

C. Phagemids or Phasmid

A phagemids is a hybrid of a plasmid and a filamentous coliphage that can be propagated in either form. The coliphage could be either of the three virtually identical phages, M13 fd or f1. These are male specific phages that contain single stranded circular DNA as their genome. Upon infection of *E. coli* by the bacteriophage, double stranded DNA is first formed as the replicative intermediate.



Phagemid

Finally single stranded DNA is packaged into the virion. Both the replication origins of the plasmid and the coliphage are incorporated in the phagemid. The auxiliary replication functions necessary in trans for the coliphage replication are not, however, incorporate in the phagemid. Hence, replication from the coliphage origin can take place only in the presence of a helper phage.

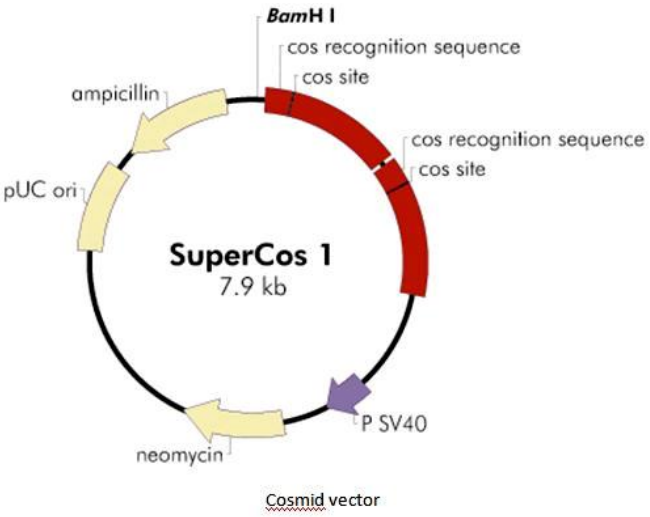
- They are prepared artificially.
- Phasmid contains the F1 origin of replication from F1 phage.
- They are generally used as a cloning vector in combination with M13 phage.
- It replicates as a plasmid and gets packaged in the form of single-stranded DNA in viral particles.

Advantages of using Phagemids:

- They contain multiple cloning sites.
- An inducible lac gene promoter is present.
- Blue-white colony selection is observed.

D. Cosmids

The DNA incorporated into phage heads by bacteriophage λ packaging systems must satisfy only a few criteria. It must possess a 14-bp sequence known as cos (which stands for cohesive end site) at each of its ends, and these cos sequences must be separated by no fewer than 36 kbp and no more than 51 kbp of DNA. Essentially any DNA satisfying these minimal requirements will be packaged and assembled into an infective phage particle.



Cosmid vector

- Cosmids are plasmids.
- They are capable of incorporating the bacteriophage λ DNA segment. This DNA segment contains cohesive terminal sites (cos sites).

- Cos sites are necessary for efficient packaging of DNA into λ phage particles.
- Large DNA fragments of size varying from 25 to 45 kb can be cloned.
- They are also packaged into λ This permits the foreign DNA fragment or genes to be introduced into the host organism by the mechanism of transduction.

Advantages of using cosmids as vectors:

- They have high transformation efficiency and are capable of producing a large number of clones from a small quantity of DNA.
- Also, they can carry up to 45 kb of insert compared to 25 kb carried by plasmids and λ.

Disadvantages of using cosmids as vectors:

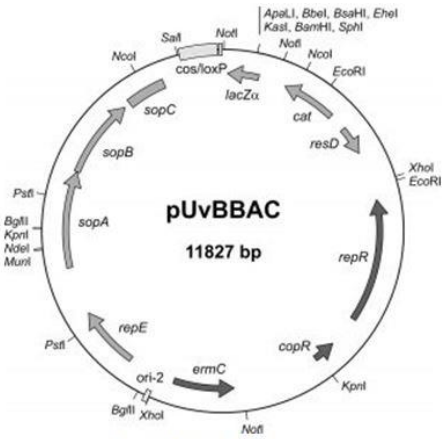
- Cosmids cannot accept more than 50 kb of the insert.
- Cosmid VectorCosmid Vector

E. Fosmids:

Vector containing the single copy E.coli F-factor replicon, developed as an improved method for constructing libraries of cosmid-sized (approximately 40 kb) clones. The stability of inserts cloned into fosmid vectors has been shown to be substantially greater than in high copy vectors. Copy control fosmids, e.g., pCC1fos, contain both the E. coli F-factor replicon and the oriV high-copy origin of replication, thus providing the user the clone stability afforded by single-copy fosmid cloning and the high yields of DNA that can be realized from cosmid clones.

F. Bacterial Artificial Chromosomes (BACs)

- Bacterial artificial chromosomes are similar to *E. coli* plasmid vectors.
- They contain ori and genes which encode ori binding proteins. These proteins are critical for BAC replication.
- It is derived from naturally occurring F' plasmid.
- The DNA insert size varies between 150 to 350 kb.



Bacterial Artificial Chromosome

Advantages of BACs:

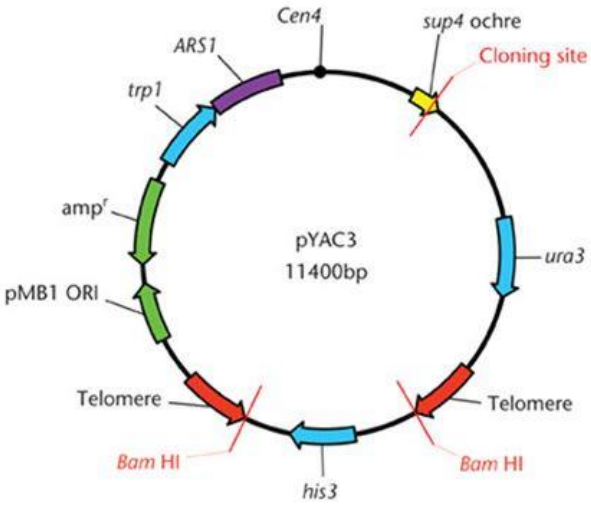
- They are capable of accommodating large sequences without any risk of rearrangement.
- BACs are frequently used for studies of genetic or infectious disorders.
- High yield of DNA clones is obtained.

Disadvantages of BACs:

- They are present in low copy number.
- The eukaryotic DNA inserts with repetitive sequences are structurally unstable in BACs often resulting in deletion or rearrangement.

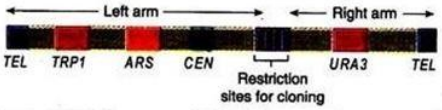
F. Yeast Artificial Chromosomes (YACs)

- A large DNA insert of up to 200 kb can be cloned.
- They are used for cloning inside eukaryotic cells. These act as eukaryotic chromosomes inside the host eukaryotic cell.



Yeast Artificial Chromosome

- It possesses the yeast telomere at each end.
- A yeast centromere sequence (CEN) is present which allows proper segregation during meiosis.
- The ori is bacterial in origin.
- Both yeast and bacterial cells can be used as hosts.



Structure of yeast artificial chromosome (YAC) cloning vector. It contains a telomere (TEL) at each end, a yeast centromere sequence (CEN), a yeast selectable marker for each arm (*TRP1* and *URA3*), a sequence that allows autonomous replication in yeast (ARS) and restriction sites for cloning

Yeast Artificial Chromosome

Advantages of using YACs:

- A large amount of DNA can be cloned.
- Physical maps of large genomes like the human genome can be constructed.

Disadvantages of using YACs:

- Overall transformation efficiency is low.
- The yield of cloned DNA is also low.

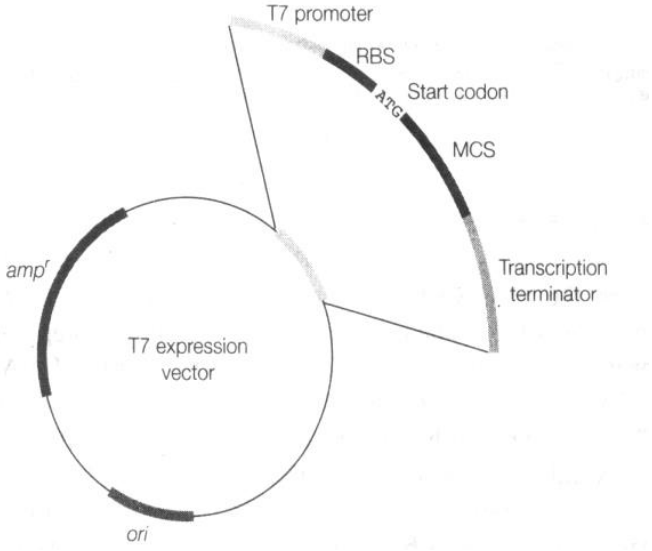
Advantages of BACs over YACs

- Comparatively stable.
- Easy to transform.
- Simple purification required.
- User- friendly.
- Aid in the development of vaccines.

G. Expression Vectors

A vector that has been constructed in such a way that inserted DNA molecule is put under appropriate promoter and terminator sequences for high level expression through efficient transcription and translation. Example: Use of promoters ('nos' from T-DNA) or expression cassettes (pRT plasmids)

- Express the DNA insert producing specific protein.
- They have prokaryotic promoter.
- Ribosome binding site.
- Origin of replication.
- Antibiotic resistance gene.
- Expression vectors with strong promoters.
- Inducible Expression Vectors.
- Eukaryotic expression vectors.



H. Human Artificial Chromosome (HACs)

- Human artificial chromosomes are artificially synthesized.
- They are utilized for gene transfer or gene delivery into human cells.
- It can carry large amounts of DNA inserts.
- They are used extensively in expression studies and determining the function of the human chromosomes.

Advantages of using HACs:

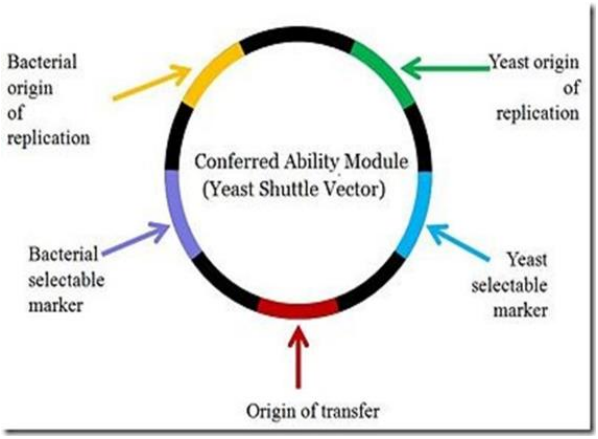
- No upper limit on DNA that can be cloned.
- it avoids the possibility of insertional mutagenesis.

Summary of vectors and what they can carry.

The size of DNA that vector can carry	Vector
0 – 10 kb	Standard plasmid
0 – 23 Kb	Lambda Bacteriophage
30 – 44 Kb	Cosmid
70 – 100 Kb	Bacteriophage P1
130 – 150 Kb	P1 Artificial chromosome PAC
Maximum 300 Kb	Bacterial Artificial Chromosome BAC
0.2 – 2 Mb	Yeast Artificial Chromosome YAC

Shuttle vectors:

There are plasmids capable of propagating and transferring genes between two organisms (e.g., *E. coli* and *A. tumefciciens*). It has unique origins of replication for each cell type as well as different selectable markers. It can, therefore, be used to shuttle gene from prokaryotes to eukaryotes. Example: pBin19.



Vectors contain sequences required for replication and selection in both *E. coli* and the desired host cells, so that the construction and many other manipulations of the recombinant plasmids can be completed in *E. coli*. Most of the eukaryotic vectors are constructed as shuttle vectors.

Yeast episomal plasmids (YEps):

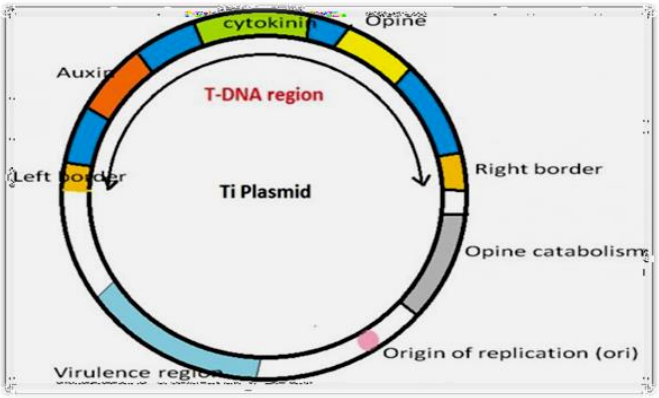
Vectors used for the cloning and expression of genes in *Saccharomyces cerevisiae*.

- Based on 2 micron (2m) plasmid which is 6 kb in length.
  - One origin
  - Two genes involved in replication
- •A site-specific recombination protein FLP, homologous to l Int. 2. Normally replicate as plasmids, and may integrate into the yeast genome.

Recombinant Ti plasmid:

1. Place the target gene in the T-DNA region of a Ti plasmid, then transform the recombinant Ti plasmid.

2. Recombinant T-DNA transformed into the A. tumefaciens cell carrying a modified Ti plasmid without T-DNA (T-DNA that are responsible for crown gall formation. The deleted T-DNA is called disarmed T-DNA shuttle vector).



Mammalian viral vectors

1. SV40: 5.2 kb, can pack DNA fragment similar to phage l.
2. Retroviruss:

•single-stranded RNA genome, which copy to dsDNA after infection.
•Have some strong promoters for gene expression
•Gene therapy

Retroviral vectors:

- ❖ Retroviral vectors are used to introduce new or altered genes into the genomes of human and animal cells.
- ❖ Retroviruses are RNA viruses.
- ❖ The viral RNA is converted into DNA by the viral reverse transcriptase and then is efficiently integrated into the host genome
- ❖ Any foreign or mutated host gene introduced into the retroviral genome will be integrated into the host chromosome and can reside there practically indefinitely.
- ❖ Retroviral vectors are widely used to study oncogenes and other human genes.

Gene Cloning:

Principal:

A fragment of DNA, containing the gene to be cloned, is inserted into a suitable vector, to produce a recombinant DNA molecule. The vector acts as a vehicle that transports the gene into a host cell usually a bacterium, although other types of living cell can be used. Within the host cell the vector multiplies, producing numerous identical copies not only of itself but also of the gene that it carries. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place. After a large number of cell divisions, a colony, or clone, of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule; the gene carried by the recombinant molecule is now said to be cloned.

Requirements for Gene Cloning:

Gene Cloning utilizes a number of biological tools to achieve its objectives, most important of them being the enzymes. Important biological tools for rec DNA technology are:

A. Enzymes:
    a) Restriction Endonucleases
    b) Exonucleases
    c) DNA ligases
    d) DNA polymerase
B. Cloning Vector
C. Host organism

D. DNA insert or foreign DNA
E. Linker and adaptor sequences

Enzymes:

A number of specific enzymes are utilized to achieve the objectives of rec DNA technology.

The enzymology of genetic engineering includes the following types of enzymes:

(a) Restriction Endonuclease:

These enzymes serve as important tools to cut DNA molecules at specific sites, which is the basic need for rec DNA technology.

These are the enzymes that produce internal cuts (cleavage) in the strands of DNA, only within or near some specific sites called recognition sites/recognition sequences/ restriction sites 01 target sites. Such recognition sequences are specific for each restriction enzyme. Restriction endonuclease enzymes are the first necessity for rec DNA technology.

(b) Exonucleases:

Exonuclease is an enzyme that removes nucleotides from the ends of a nucleic acid molecule. An exonuclease removes nucleotide from the 5′ or 3′ end of a DNA molecule. An exonuclease never produces internal cuts in DNA.

In rec DNA technology, various types of exonucleases are employed like Exonuclease Bal31, *E. coli* exonuclease III, Lambda exonuclease, etc.

Exonculease Bal31 are employed for making the DNA fragment with blunt ends shorter from both its ends.

(c) DNA ligase:

The function of these enzymes is to join two fragments of DNA by synthesizing the phosphodiester bond. They function to repair the single stranded nicks in DNA double helix and in rec DNA technology they are employed for sealing the nicks between adjacent nucleotides. This enzyme is also termed as molecular glue.

(d) DNA polymerases:

These are the enzymes which synthesize a new complementary DNA strand of an existing DNA or RNA template. A few important types of DNA polymerases are used routinely in genetic engineering. One such enzyme is DNA polymerase, which prepared from *E coli*. The Klenow fragment of DNA polymerase-I .s employed to make the protruding ends double-stranded by extension of the shorter strand.

Reverse transcriptase is also an important type of DNA polymerase enzyme for genetic engineering. It uses RNA as a template for synthesizing a new DNA strand called as cDNA a e complementary DNA). Its main use is in the formation of cDNA libraries. Apart from all these above mentioned enzymes, a few other enzymes also mark their importance in genetic engineering.

(e) Terminal deoxynucleotidyl transferase enzyme:

It adds single stranded sequences to 3′-terminus of the DNA molecule. One or more deoxynbonucleotides (dATP, dGTP, dl IP, dCTP) are added onto the 3′-end of the blunt- ended fragments. This enzyme has a molecular weight of 32000 and consists of two subunits each with a molecular weight of 26500 and 8000. This enzyme is isolated from calf thymus.

Uses of Terminal Transferase Enzyme:

1. The enzyme terminal transferase is used to add homopolymer tails of DNA fragments. Using a technique called homopolymer tailing, sticky ends can be built up on blunt-ended DNA 102 molecules. For examples, one preparation of DNA could be treated with the enzyme terminal transferase in the presence of dATP, resulting in the addition of a poly (dA) chain to each DNA strand. There is another preparation of DNA which provides 3 tails of poly (T) using same enzyme with TTP. When both types of DNA preparations DNA fragments with poly A tails and DNA

fragments with poly T tails, are mixed, there takes place base pairing between complementary sticky ends, which could then be ligated. One advantage of this method is that ligation does not take place between fragments from the same DNA preparation.

2. Terminal transferase enzyme is used for 3′-end labelling of DNA fragments

3. Terminal transferase enzyme is also used for the addition of single nucleotides to the 3- end of DNA for in vitro mutagenesis.

(f) Alkaline Phosphatase Enzyme:

It functions to remove the phosphate group from the 5′-end of a DNA molecule. This enzyme is isolated from bacteria (BAP) or calf intestine (CAP). This enzyme is a dimeric glycoprotein with a molecular weight 14,000. It is made up of two identical or similar subunitsb each with a molecular weight of 6900. It is a zinc-containing enzyme with four atoms of Zn^{2+} per molecule.

Uses of Alkaline Phosphatase Enzyme:

1. Linearized cloning vectors can be prevented from recircularizing by dephosphorylation with Alkaline phosphatase enzyme.

2. The free 5′-OH can be phosphorylated with polynucleotide kinase and ϒ-32P ATP to produce 32P end labelled nucleic acid.

3. AP enzyme is used for mapping and DNA fingerprinting studies

(g) Polynucleotide Kinase Enzyme:

It has an effect reverse to that of Alkaline Phosphatase, i.e. it functions to add phosphate group to the 5′-terminus of a DNA molecule.

(h) Taq Polymerase:

Taq polymerases are DNA dependent DNA polymerase from *Thermus aquaticus*, primarily used for synthesis of longer stretches of DNA. Thermus aquaticus is the source for this enzymewhich is an extreme thermophile, living in hot springs.

(i) S1 Nuclease Enzyme:

The S1 nuclease enzyme is single- strand specific endonuclease which cleaves DNA to release 5′-mono and 5′-oligonucleotides. Normally, double- stranded DNA, double- stranded RNA and DNA-RNA hybrids are resistant to action of S1 nuclease enzyme.

However, very large amounts of S1 nuclease enzyme can completely hydrolyze doublestranded nucleic acids. The enzyme hydrolyzes single stranded regions in duplex DNA such as loops and gaps. S1 nuclease enzyme can also cleave single stranded areas of super helical DNA at torsional stress points where DNA may be unpaired or weakly hydrogen bonded. Once the super-helical DNA is nicked, S1 nuclease enzyme can cleave the second strand near the nick to generate linear DNA. S1 nuclease enzyme is a monomeric protein with 3800 Dalton molecular weight. It requires Zn2+ for its activity and is relatively stable against denaturing reagents such as urea, SDS and formamide. The optimum pH requirement lies between 4 to 4.5.

Uses of S1 Nuclease Enzyme:

1. S1 nuclease enzyme is used to analyse DNA-RNA hybrid structures to map transcripts.

2. It can be used to remove singles stranded tails from DNA fragments to produce blunt ends.

3. Hair pin loop structures formed during synthesis of double-stranded cDNA is digested by this enzyme. 101

4. S1 nuclease enzyme is also used for DNA mapping, called SI nuclease mapping Turner.

(j) Ribonuclease:

Generally RNase A and RNase T1 enzymes are used in genetic engineering techniques. Both enzymes cleave the phosphodiester bond between adjacent ribonucleotides. RNase A cleaves next to uracil (U) and cytosine (C) in such a way that phosphate remains with these pyrimidines.

The nucleotide present on the other side of phosphate is dephosphorylated. RNase A enzyme is isolated from the bovine pancreas. RNase T1 cleaves specifically next to guanine. The phosphate group at the 3′ end of the nucleotide remains with the cut end. This enzyme is isolated from *Aspergillus oryzae.*

Ribonuclease H (RNase H):

The enzyme RNase H is an endoribonuclease that degrades the RNA portion of the RNA- DNA hybrids. RNase H enzyme cuts the RNA into short fragments.

Applications of RNase H:

a. RNase H is the key enzyme in the cDNA cloning technique. In this case, it is used to remove the mRNA from the RNA-DNA hybrid.

b. RNase H enzyme is used to detect the presence of RNA-DNA hybrid.

c. RNase H enzyme is used to remove poly (A) tails on mRNA.

(k) Deoxyribonuclease I (Dnase I):

The enzyme DNase I is an endonuclease enzyme which digests either single or double-stranded DNA, producing a mixture of mononucleotides and oligonucleotides. DNase I hydrolyses each strand of double-stranded DNA independently and at random. Addition of Mg2+ to reaction mixture ensures random cleavage while addition of Mn^{2+} gives cleavage nearly at the same place on both strands. DNase enzyme is obtained mostly from bovine pancreas.

Uses of DNase I Enzyme:

DNase 1 enzyme is useful for a variety of applications including nick translation, DNA foot printing, bisulphite mediated mutagenesis and RNA purification.

Cloning Vectors:

It is another important natural tool which geneticists use in rec DNA technology. The cloning vector is the DNA molecule capable of replication in a host organism, into which the target DNA is introduced producing the rec DNA molecule.

A cloning vector may also be termed as a cloning vehicle or earner DNA or simply as a vector or a vehicle a great variety of cloning vectors are present for use with E. coli is the host organism. Different types of DNA molecules may be used as cloning vehicles such as they may be plasmids, bacteriophages, cosmids, phasmids or artificial chromosomes.

Host Organism:

A good host organism is an essential tool tor genetic engineering. Most widely used host for rec DNA technology is the bacterium E. coli. because cloning and isolation of DNA inserts is very easy in this host. A good host organism is the one winch easy to transform and in which the replication of rec DNA is easier. There should not be any interfering element against the replication of rec DNA in the host cells

DNA Insert or Foreign DNA:

The desired DNA segment which is to be cloned is called as DNA insert or foreign DNA or target DNA. The selection of a suitable target DNA is the very first step of rec DNA technology. The target DNA (gene) may be of viral, plant, animal or bacterial origin.

Following points must be kept in mind while selecting the foreign DNA:

1. It can be easily extracted from source.
2. It can be easily introduced into the vector.
3. The genes should be beneficial for commercial or research point of view.

Linker and Adaptor Sequences:

Linkers and adaptors are the DNA molecules which help in the modifications of cut ends of DNA fragments. These can be joined to the cut ends and hence produce modifications as desired.

Both are short, chemically synthesized, double stranded DNA sequences. Linkers have (within them) one or more restriction endonuclease sites and adaptors have one or both sticky ends. Different types of linkers and adaptors are used for different purposes.

Steps/strategies of gene cloning:

The basic 7 steps involved in gene cloning are:

1. Isolation of DNA [gene of interest] fragments to be cloned.
2. Insertion of isolated DNA into a suitable vector to form recombinant DNA.
3. Introduction of recombinant DNA into a suitable organism known as host.
4. Selection of transformed host cells and identification of the clone containing the gene of interest.
5. Multiplication/Expression of the introduced Gene in the host.
6. Isolation of multiple gene copies/Protein expressed by the gene.
7. Purification of the isolated gene copy/protein

Isolation of the DNA fragment or gene:

- The target DNA or gene to be cloned must be first isolated. A gene of interest is a fragment of gene whose prod-uct (a protein, enzyme or a hormone) interests us. For example, gene encoding for the hormone insulin.
- The desired gene may be isolated by using restriction endonuclease (RE) enzyme, which cut DNA at specific recognition nucleotide se-quences known as restriction sites towards the inner region (hence endonuclease) producing blunt or sticky ends.
- Sometimes, reverse transcriptase enzyme may also be used which synthesizes complementary DNA strand of the desired gene using its mRNA.

Selection of suitable cloning vector

- The vector is a carrier molecule which can carry the gene of interest (GI) into a host, replicate there along with the GI making its multiple copies.
- The cloning vectors are limited to the size of insert that they can carry. Depending on the size and the application of the insert the suitable vector is selected.
- The different types of vectors available for cloning are plasmids, bacteriophages, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs) and mammalian artificial chromosomes (MACs).
- However, the most commonly used cloning vectors include plasmids and bacteriophages (phage λ) beside all the other available vectors.

Formation of Recombinant DNA

- The plasmid vector is cut open by the same RE enzyme used for isolation of donor DNA fragment.
- The mixture of donor DNA fragment and plasmid vector are mixed together.
- In the presence of DNA ligase, base pairing of donor DNA fragment and plasmid vector occurs.
- The result-ing DNA molecule is a hybrid of two DNA molecules – the GI and the vector. In the ter-minology of genetics this intermixing of dif-ferent DNA strands is called recombination.
- Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and the technology is referred to as the recom-binant DNA technology.

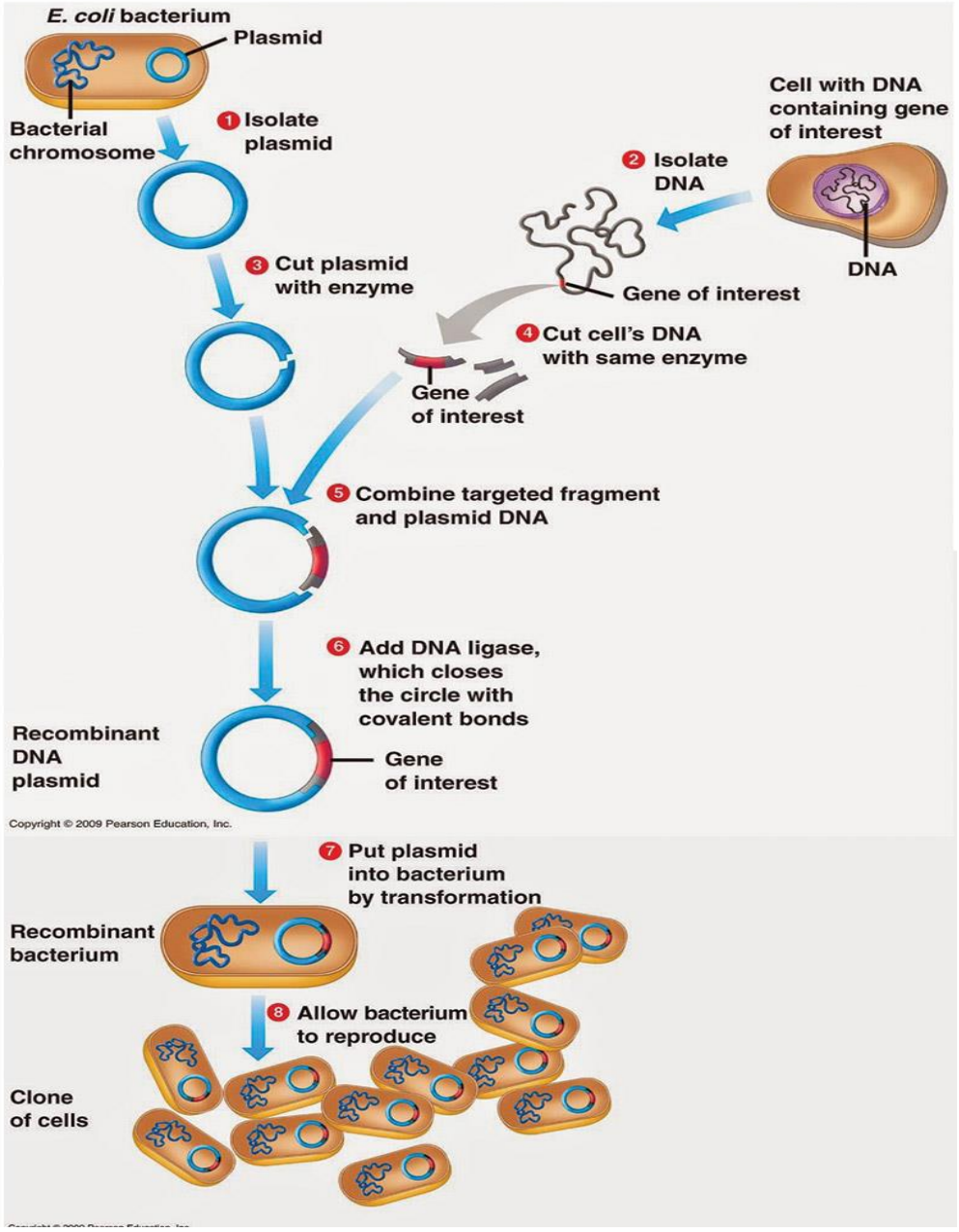
Transformation of recombinant vector into suitable host

- The recombinant vector is transformed into suitable host cell mostly, a bacterial cell.
- This is done either for one or both of the following reasons:

- To replicate the recombinant DNA mol-ecule in order to get the multiple copies of the GI.
- To allow the expression of the GI such that it produces its needed protein product.
- Some bacteria are naturally transformable; they take up the recombinant vector automatically.
- For example: *Bacillus*, *Haemophillus*, *Helicobacter pylori*, which are naturally competent.
- Some other bacteria, on the other hand require the incorporation by artificial methods such as Ca^{++} ion treatment, electroporation, etc.

Isolation of Recombinant Cells

- The transformation process generates a mixed population of transformed and non-transformed host cells.
- The selection process involves filtering the transformed host cells only.
- For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed.
- For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is used it knock out Ampicillin resistant gene from the plasmid, so that the recombinant cell become sensitive to Ampicillin.



Multiplication of Selected Host Cells

- Once transformed host cells are separated by the screening process; becomes necessary to provide them optimum parameters to grow and multiply.
- In this step the transformed host cells are introduced into fresh culture media .
- At this stage the host cells divide and re-divide along with the replication of the recom-binant DNA carried by them.
- If the aim is obtaining numerous copies of GI, then simply replication of the host cell is allowed. But for obtaining the product of interest, favourable conditions must be provided such that the GI in the vector expresses the product of interest.

Isolation and Purification of the Product

- The next step involves isolation of the multiplied GI attached with the vector or of the protein encoded by it.
- This is followed by purification of the isolated gene copy/protein.

Polymerase chain reaction:

Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Developed in 1983 by Kary Mullis, who was an employee of the Cetus Corporation, and also the winner of Nobel Prize in Chemistry in 1993, it is an easy, cheap, and reliable way to repeatedly replicate a focused segment of DNA, a concept which is applicable to numerous fields in modern biology and related sciences. PCR is probably the most widely used technique in molecular biology. This technique is used in biomedical research, criminal forensics, and molecular archaeology.

PCR is now a common and often indispensable technique used in clinical and research laboratories for a broad variety of applications. These include DNA cloning for sequencing, gene cloning and manipulation, gene mutagenesis; construction of DNA-based phylogenies, or functional analysis of genes; diagnosis and monitoring of hereditary diseases; amplification of ancient DNA; analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing); and detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.

Requirements of PCR:

(a) DNA Template:

The original DNA molecule that is to be copied is called the DNA template and the segment of it that will actually be amplified is known as the target sequence. A trace amount of the DNA template is sufficient. This can be obtained by any one of the DNA isolation techniques discussed before.

(b) PCR Primers:

Two PCR primers are needed to initiate DNA synthesis. These are short pieces of single-stranded DNA that match the sequences at either end of the target DNA segment. PCR primers are made by chemical synthesis of DNA.

There are several computer programs available to suggest suitable primers for the process of PCR, and some of the general guidelines are listed below:

1. Length:

Shorter primers have a tendency to go and anneal to the non-target sequence of the DNA template. This will result in production of DNA copies of having non-target sequence. The greater the complexity of the template DNA, the more likely this is to happen.

Thus, a Short primer may offer sufficient specificity when amplifying using a simple template such as a small plasmid, but a long primer may be required when using eukaryotic genomic DNA as template. In practice, 20-30 nucleotides is generally satisfactory.

2. Mismatches:

Primers do not need to match the template completely, although the 3′ end of the primer should be correctly base-paired to the template, otherwise the polymerase will not be able to extend it. It is often beneficial to have C or G as the 3′ terminal nucleotide. This makes the binding of the 3′ end of the primer to the template more stable than it would be with A or T at the 3′ end.

3. Melting Temperature:

The temperatures at which the two primers can associate with the template should be relatively similar to ensure that they both bind at about the same time as temperatures are being lowered during annealing. The similarity of melting temperatures is likely to mean that the primers have a similar nucleotide composition.

4. Internal Secondary Structure:

This should be avoided in order to prevent the primer to fold back on itself and not be available to bind to the template.

5. Primer-Primer Annealing:

It is also important to avoid the two primers being able to anneal to each other. Extension by DNA polymerase of two self-annealed primers leads to formation of a primer dimer.

(c) Thermo-Stable DNA Polymerase:

The enzyme DNA polymerase is needed to manufacture the DNA copies. The Klenow fragment was the first DNA polymerase enzyme used in PCR. The Klenow fragment is a large protein fragment produced when DNA polymerase I from E. coli is enzymatically cleaved by the protease subtilisin.

After enzymatic modification it retains the 5′-3′ polymerase activity and the 3′ → 5′ exonuclease activity for removal of pre-coding nucleotides and proofreading, but loses its 5′ → 3′ exonuclease activity.

Klenow fragment failed to play a successful role as a polymerase enzyme for lacking a stability at high temperature. As we know that the PCR procedure involves several temperature steps, in this situation we had to replenish the Klenow fragment during each cycle.

To solve this issued heat resistant DNA polymerase was required. This came originally from heat resistant bacteria living in hot springs at temperatures up to 90°C. Today Taq polymerase from Thermusaquaticus is the most widely used PCR DNA polymerase enzyme. It is generally produced by expression of the gene in E. coli.

(d) Deoxy Nucleotide Triphosphates:

A supply of four deoxynucleotide triphosphates, dATP, dCTP, dGTP and dTTP, are needed by the polymerase to make the new DNA.

(e) PCR Machine:

Finally we need a PCR machine to keep changing the temperature. The PCR process requires cycling through several different temperatures. Because of this, PCR machines are sometimes called thermo-cyclers.

Procedure

Typically, PCR consists of a series of 20–40 repeated temperature changes, called cycles, with each cycle commonly consisting of two or three discrete temperature steps (see figure below). The cycling is often preceded by a single temperature step at a very high temperature (>90 °C (194 °F)), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters, including the enzyme used for DNA synthesis, the concentration of bivalent ions and dNTPs in the reaction, and the

melting temperature (Tm) of the primers. The individual steps common to most PCR methods are as follows:

Initialization: This step is only required for DNA polymerases that require heat activation by hot-start PCR.[17] It consists of heating the reaction chamber to a temperature of 94–96 °C (201–205 °F), or 98 °C (208 °F) if extremely thermostable polymerases are used, which is then held for 1–10 minutes.

Denaturation: This step is the first regular cycling event and consists of heating the reaction chamber to 94–98 °C (201–208 °F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.

Annealing: In the next step, the reaction temperature is lowered to 50–65 °C (122–149 °F) for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.

It is critical to determine a proper temperature for the annealing step because efficiency and specificity are strongly affected by the annealing temperature. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should bind only to a perfectly complementary part of the strand, and nowhere else. If the temperature is too low, the primer may bind imperfectly. If it is too high, the primer may not bind at all. A typical annealing temperature is about 3–5 °C below the Tm of the primers used. Stable hydrogen bonds between complementary bases are formed only when the primer sequence very closely matches the template sequence. During this step, the polymerase binds to the primer-template hybrid and begins DNA formation.

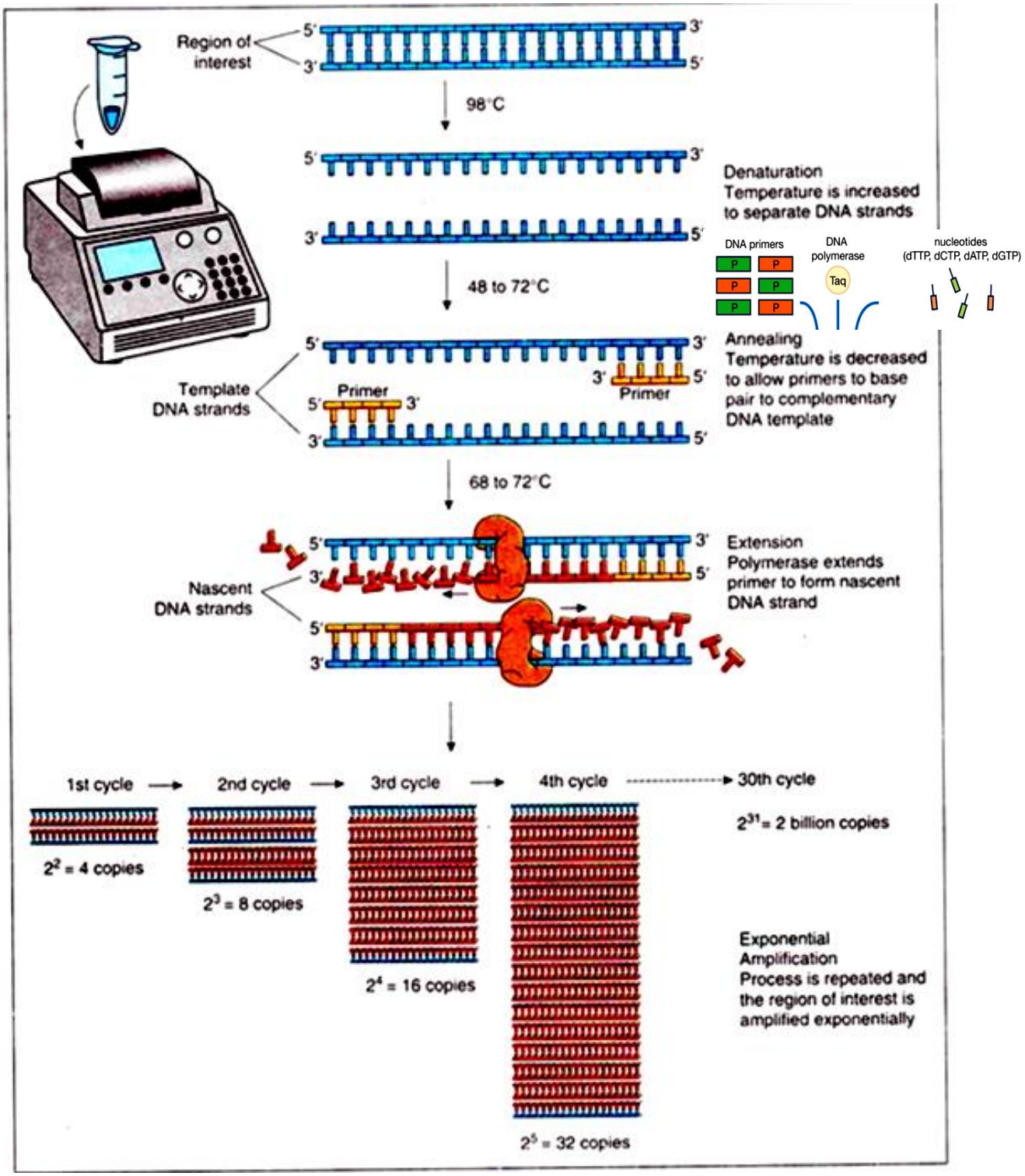
Extension/elongation: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for the thermostable DNA polymerase of Taq (Thermus aquaticus) polymerase is approximately 75–80 °C (167–176 °F), though a temperature of 72 °C (162 °F) is commonly used with this enzyme. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxy group at the end of the nascent (elongating) DNA strand. The precise time required for elongation depends both on the DNA polymerase used and on the length of the DNA target region to amplify. As a rule of thumb, at their optimal temperature, most DNA polymerases polymerize a thousand bases per minute. Under optimal conditions (i.e., if there are no limitations due to limiting substrates or reagents), at each extension/elongation step, the number of DNA target sequences is doubled. With each successive cycle, the original template strands plus all newly generated strands become template strands for the next round of elongation, leading to exponential (geometric) amplification of the specific DNA target region.

The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2n, where n is the number of cycles. Thus, a reaction set for 30 cycles results in 230, or 1073741824, copies of the original double-stranded DNA target region.

Final elongation: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–

15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

Final hold: The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products



Applications of PCR:

PCR has a number of applications especially where speed and the number of samples to be processed are important or where the amount of DNA available is very limited. Here are some of the applications.

a. DNA Sequencing:

PCR in the presence of di-deoxynucleoside triphosphates (ddNTPs), used for DNA sequencing, allows DNA sequencing reactions to be run successfully with very small amounts of template.

b. Diagnostic:

PCR is useful as a diagnostic tool, e.g., in the identification of specific genetic traits or for the detection of pathogens or food contaminants. One of the first applications of PCR to genetic diagnosis was for sickle cell anaemia.

c. Forensic:

The ability to amplify DNA from regions of the genome that are highly polymorphic (and which are variable between individuals) starting with samples containing very small amounts of DNA (e.g., single hairs or traces of body fluids, such as blood and semen) leads to applications in forensic work.

d. Present-Day Population Genetics:

It allows for the determination of frequencies of particular alleles in a large collection of individuals. A particular advantage of using PCR in population genetic studies is that, with appropriately designed specific primers, it may be possible to amplify DNA from one organism that cannot be separated from others, such as a particular bacterial strain in a mixed population.

(Such primers will anneal to the target DNA from the organism of interest, but not to DNA from others.)

e. Archaeology and Evolution:

PCR can be used with old material as well as more recent samples, and it is often possible to amplify ancient DNA from museum specimens and archaeological remains. Mostly mitochondrial DNA or chloroplast DNA is used. This allows inferences to be made about the origins of particular populations or species.

f. The amplification of gene fragments as fast alternative of cloning:

(a) Inserts of bacterial plasmids can be amplified with primers.

(b) DNA from known sequence can be obtained by designing primers.

(c) PCR helps in identification of homologous sequences from related organisms.

(d) Using RT-PCR the 3′ end of cDNA can be amplified (RACE: Rapid Amplification of cDNA Ends).

(e) Reverse PCR helps to know the flanking sequences of a known DNA clone.

g. Detection of Mutation Relevant for Inherited Diseases:

Any point mutation, a deletion or an insertion and expanded tandem trinucleotide repeat can be detected by PCR. Somatic mutations in oncogenes or tumour repressor genes can also be detected by PCR with primers flanking the insertions or deletions.

h. Analysis of Genetic Markers for Forensic Applications, for paternity testing and for the mapping of hereditary traits.

(a) Amplification of SSR.

(b) RAPD (Random Amplified Polymorphic DNA) with arbitrary, often short (10 bp) primers.

j. Species-Specific Amplification of DNA Segments between interspersed repeat elements (IRS) using the primer based on the SINE sequence (Short Interspersed Nuclear Elements).

k. Genetic Engineering using PCR:

Using PCR we can incorporate alteration or mutation in the ultimate product by choice altering, removing or adding sequences to the primer at the 5′ end. By recombinant PCR technique, it is possible to join two DNA fragments at a specific site through complementary overlaps (This technique is termed as splicing). By synthesizing two mutagenic primers, spanning the internal site to be changed, it is possible to introduce mutations within a fragment.

Modified Forms of PCR:

The conventional PCR is the symmetrical PCR technique. There are some other modified forms of PCR which are used for various purposes:

Nested PCR:
Nested PCR increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are being used in two successive PCR reactions. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non- specifically amplified DNA fragments.
The product(s) (sometimes after gel purification after electrophoresis of the PCR product) are then used in a second PCR reaction with a set of primers whose binding sites are completely or partially different from the primer pair used in the first reaction, but are completely within the DNA target fragment. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.
Real Time PCR:
It is possible to use PCR to estimate the abundance of a particular nucleic acid molecule in a sample. This can be done by real time PCR. This can be done in two ways.
In the first, a fluorescent, double-stranded DNA (dsDNA)-binding dye (such as SYBR green) is present in the PCR. As dsDNA product accumulates, the amount of fluorescence from the dye increases, and this can be detected.
The second approach to real-time PCR allows detection of a specific product, rather than dsDNA in general, and uses a specially synthesized probe oligonucleotide. This probe is designed to anneal within the region to be amplified and carries a fluorescent reporter dye at one end and a quencher at the other end of the molecule. If the quencher and the reporter are in close proximity (i.e., attached to the same oligonucleotide), then the quencher stops the reporter from fluorescing.
During PCR, the probe will anneal to single-stranded DNA within the target region. When the poly-merase meets the annealed probe, the 5′-3′ exonuclease activity of the enzyme degrades the probe, liberating the reporter from the quencher. Thus, the fluorescent reporter accumulates during the course of the PCR. This type of PCR mechanism is shown in the diagram given above.
Quantitative PCR:
Q-PCR (Quantitative PCR) is used to measure the quantity of a PCR product (preferably real-time). It is the method of choice to quantitatively measure starting amounts of DNA, cDNA or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample.
The method with currently the highest level of accuracy is Quantitative real-time PCR. It is often confusingly known as RT-PCR (Real Time PCR) or RQ-PCR. QRT-PCR or RTQ-PCR is more appropriate contractions. RT-PCR commonly refers to reverse transcription PCR (see below), which is often used in conjunction with Q- PCR. QRT-PCR methods use fluorescent dyes, such as Sybr Green, or fluorophore- containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time.
Hot-start PCR:
Hot-start PCR is a technique that reduces non-specific amplification during the initial set up stages of the PCR. The technique may be performed manually by simply heating the reaction components briefly at the melting temperature (e.g., 95°C) before adding the polymerase.
Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.
Inter-sequence specific (ISSR) PCR:
A PCR method for DNA fingerprinting that amplifies regions between some simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.
8. Inverse PCR:
Inverse PCR is a method used to allow PCR when only one internal sequence is known. This is especially useful in identifying flanking sequences to various genomic inserts. This involves a series of DNA digestions and self-ligation, resulting in known sequences at either end of the unknown sequence.
RT-PCR:

RT-PCR (Reverse Transcription PCR) is a method used to amplify, isolate or identify a known sequence from a cellular or tissue RNA. The PCR reaction is preceded by a reaction using reverse transcriptase to convert RNA to cDNA.

RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites and, if the genomic DNA sequence of a gene is known, to map the location of exons and introns in the gene. The 5′ end of a gene (corresponding to the transcription start site) is typically identified by a RT-PCR method, named RACE-PCR, short for Rapid Amplification of cDNA Ends.

TAIL-PCR:

Thermal asymmetric interlaced PCR is used to isolate unknown sequence flanking a known sequence. Within the known sequence TAIL-PCR uses a nested pair of primers with differing annealing temperatures; a degenerate primer is used to amplify in the other direction from the unknown sequence.

Touchdown PCR:

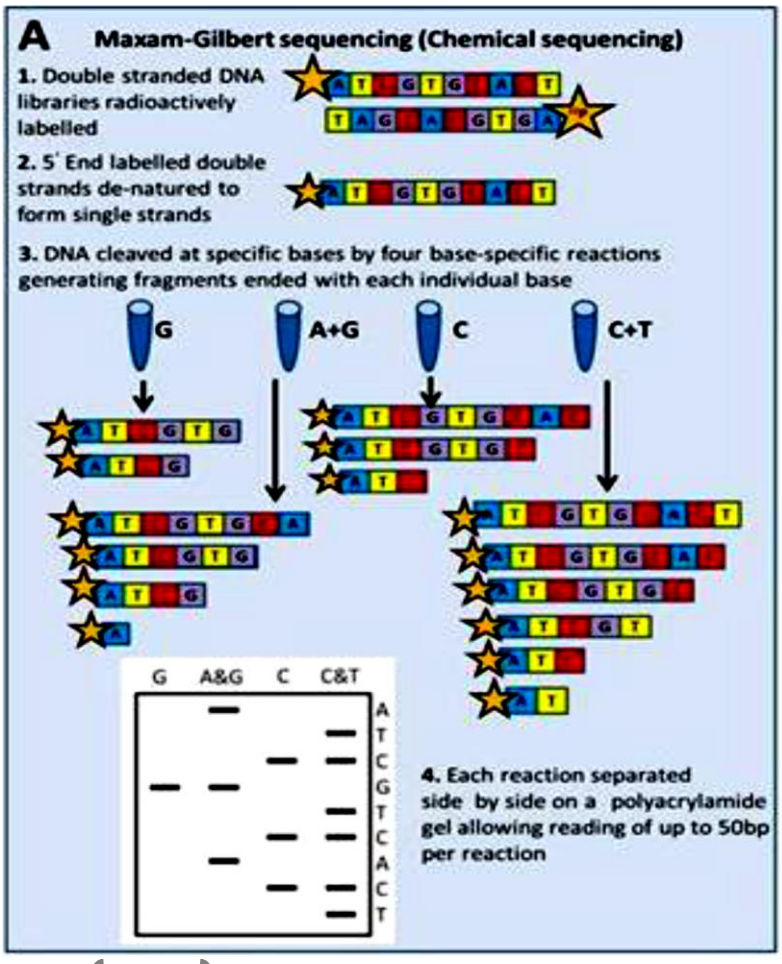
Touchdown PCR is a variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees above the T_m of the primers used, while at the later cycles, it is a few degrees below the primer T_m. The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycle.

Asymmetrical PCR:

Target sequences of one strand may be amplified in several orders of magnitude more as compared to its complementary strand. This approach is particularly useful for generating single stranded DNA fragment to be used for sequencing of DNA.

Sequencing strategies:

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. Before the development of direct DNA sequencing methods, DNA sequencing was difficult and indirect. The DNA had to be converted to RNA, and limited RNA sequencing could be done by the existing cumbersome methods. Thus, only shorter DNA sequences could be determined by this method. Using this method, Walter Gilbert and Alan Maxam at Havard University determined that the Lac operator is a 27 bp long sequence.



Maxam & Gilbert's Chemical Degradation Method:

Allan Maxam and Walter Gilbert published a DNA sequencing method in 1977 based on chemical modification of DNA and subsequent cleavage at specific bases. Also known as chemical sequencing, this method allowed purified samples of double-stranded DNA to be used without further cloning. This method's use of radioactive labeling and its technical complexity discouraged extensive use after refinements in the Sanger methods had been made.

In this method the following steps are involved

1. The 3’ ends of DNA fragments are labelled.
2. The labelled strands are then separated, of which both strands are labelled at 3’ ends.
3. The mixture is divided into four samples, each treated with a different reagent having the property of destroying either only G or only C or A and G, T and C; the concentration is adjusted in such a way that 50% of target base is destroyed.
4. Fragments of different sizes having ^{32}P are produced.
5. Electrophoresis is done using each of the four samples in four different lanes of the gel.

Sanger and Coulson’s Dideoxynucleotide Synthetic Method:

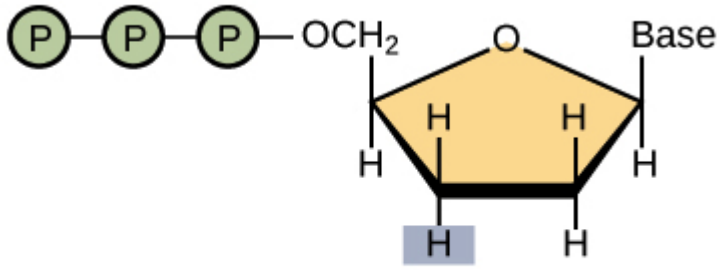
This method was described by Fred Sanger in 1977. In this technique, the DNA is sequenced using an enzymatic method which polymerizes the DNA fragments complimentary to the DNA of interest. P^{32} is used to label the synthetically designed primer that binds to the DNA template at a known sequence. The synthesis occurrs with DNA polymerases and dNTPs (deoxynucleotide triphosphate) until a ddNTP (dideoxynucleotide triphosphate) is incorporated which terminates the reaction due to the absence of the deoxy-group. **Ingredients for Sanger sequencing**

Sanger sequencing involves making many copies of a target DNA region. Its ingredients are similar to those needed for DNA replication in an organism, or for polymerase chain reaction (PCR), which copies DNA *in vitro*. They include:

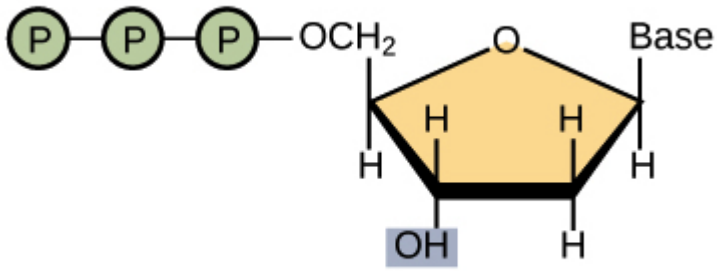
- A DNA polymerase enzyme
- A primer, which is a short piece of single-stranded DNA that binds to the template DNA and acts as a "starter" for the polymerase
- The four DNA nucleotides (dATP, dTTP, dCTP, dGTP)
- The template DNA to be sequenced

However, a Sanger sequencing reaction also contains a unique ingredient:

- Dideoxy, or chain-terminating, versions of all four nucleotides (ddATP, ddTTP, ddCTP, ddGTP), each labeled with a different color of dye



Dideoxynucleotide (ddNTP)



Deoxynucleotide (dNTP)

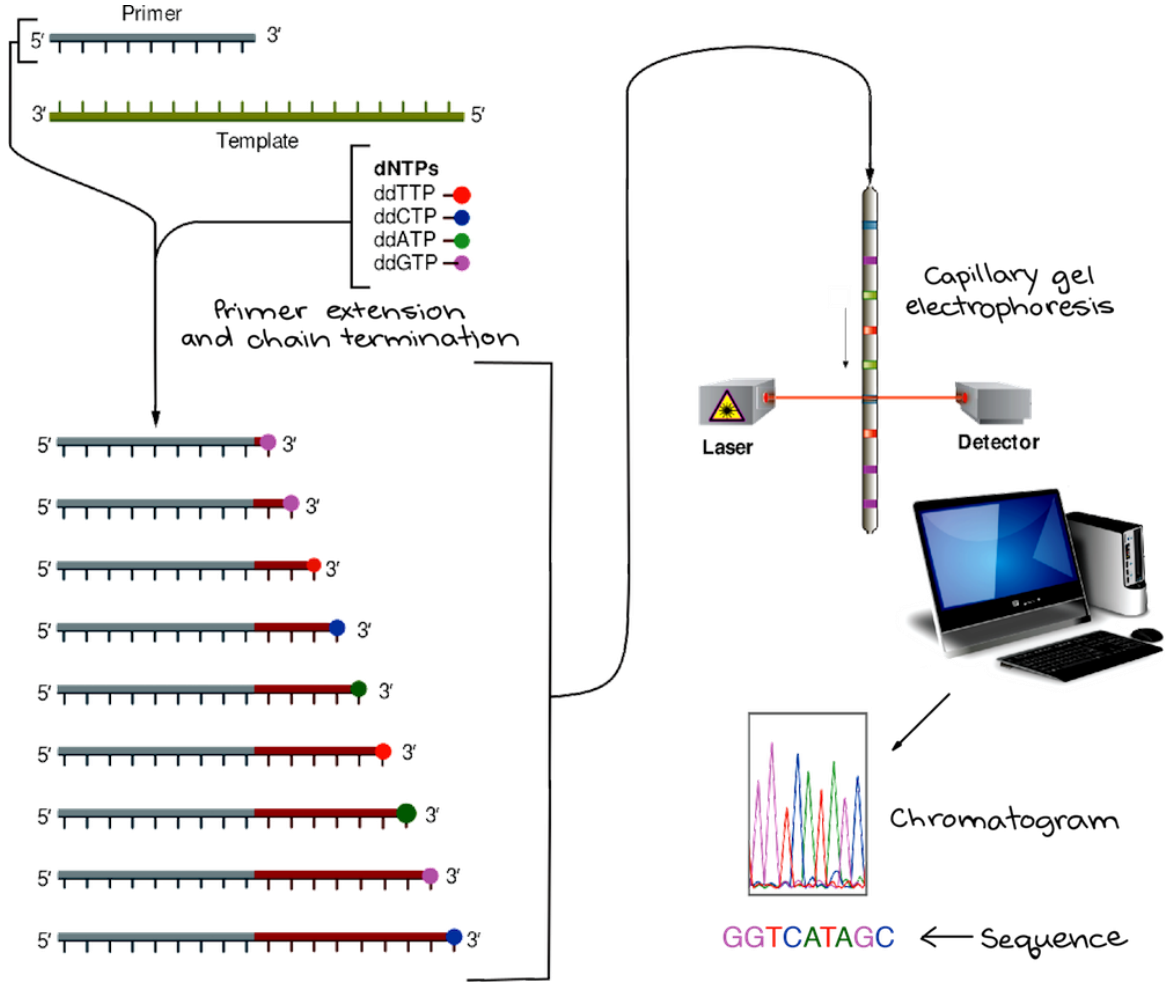
Dideoxy nucleotides are similar to regular, or deoxy, nucleotides, but with one key difference: they lack a hydroxyl group on the 3’ carbon of the sugar ring. In a regular nucleotide, the 3’ hydroxyl group acts as a “hook," allowing a new nucleotide to be added to an existing chain.

Once a dideoxy nucleotide has been added to the chain, there is no hydroxyl available and no further nucleotides can be added. The chain ends with the dideoxy nucleotide, which is marked with a particular color of dye depending on the base (A, T, C or G) that it carries.

Method:

The method involves the following steps:

1. Four reaction tubes are set up each containing single stranded DNA sample to be sequenced, all four dNTPs (radioactively labelled) and an enzyme for DNA syn-thesis (DNA polymerase I).
2. Each tube also contains a small amount of (much smaller amount relative to four dNTPs) one of the ddNTP, so that four tubes have each different ddNTP, bring-ing about termination at a specific base - Adenine (A), Cytosine (C), Thymine (T), and Guanine (G).
3. The fragments generated by random incorporation of ddNTP leads to termi-nation of reaction and so the different fragments are produced which can be separated by high resolution polyacrylamide gel, four adjoining lanes are loaded by four different samples.
4. The gel is then auto-radiographed; the position of different bands in each lane can be visualized, and based on the position of the bands, the DNA sequence can be read out very easily.



Whole-genome shotgun sequencing:

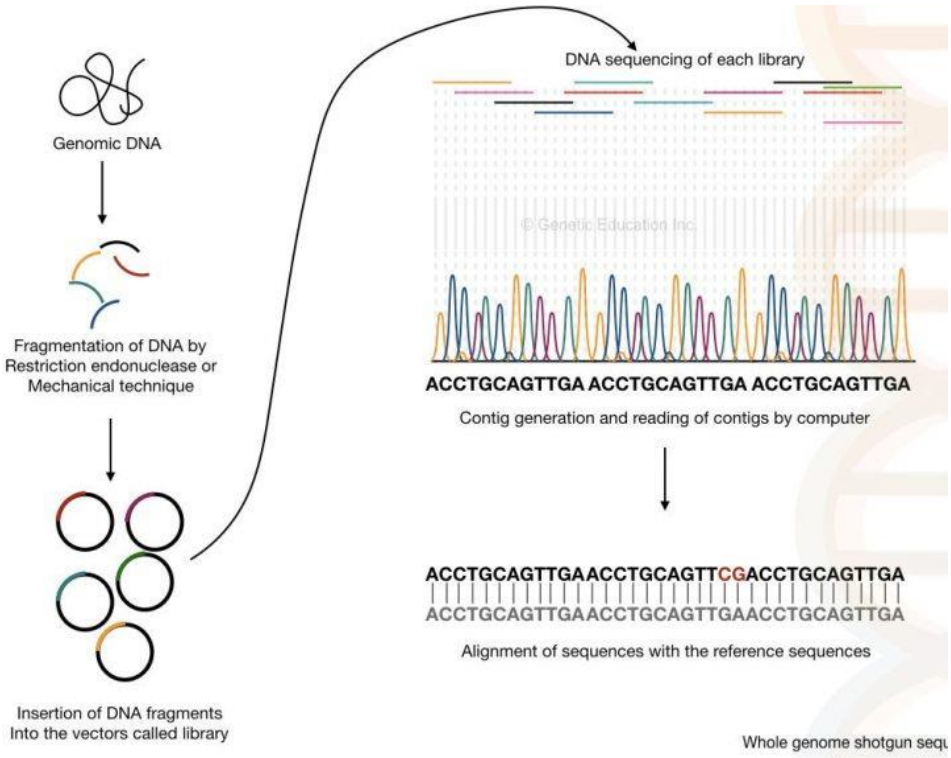
Yet another modification of the Sanger's chain termination method is the whole-genome shotgun sequencing. However, instead of a single gene or few basepair, the present method is powerful enough to sequence the entire genome of an organism.

The principle of the shotgun is the same as Sanger's method, one additional step of DNA fragmentation allows to read multiple fragments.

The entire genome of an organism is fragmented with the help of endonuclease enzymes or by the mechanical techniques. After that, the smaller fragments of DNA sequenced individually into the machine.
The computer-based software analyses each and every overlapping fragment and reassembled it to generate the complete sequence of the entire genome.
The method can be divided into four steps:

1. Fragmentation of a DNA: with the help of restriction endonucleases or physical method
2. Formation of libraries of the subfragments: the fragments are ligated in vectors and an entire library for various vectors are generated
3. Sequencing the subfragments: each library is sequenced individually.
4. Generation and reading the contigs: the overlapping fragments called contigs are read by the computer.

The fragments generated by the lysis or restriction digestion are around 2 to 20kb.
Importantly, the shotgun sequencing reads both the sequences (it sequence the double-stranded DNA) based on that contigs data, it identifies the gaps remained sequenced. The brief overview of present method is given below,



Next-generation sequencing:
The principle behind Next Generation Sequencing (NGS) is similar to that of Sanger sequencing, which relies on capillary electrophoresis. The genomic strand is fragmented, and the bases in each fragment are identified by emitted signals when the fragments are ligated against a template strand. The NGS method uses array-based sequencing which combines the techniques developed in Sanger sequencing to process millions of reactions in parallel, resulting in very high speed and throughput at a reduced cost.

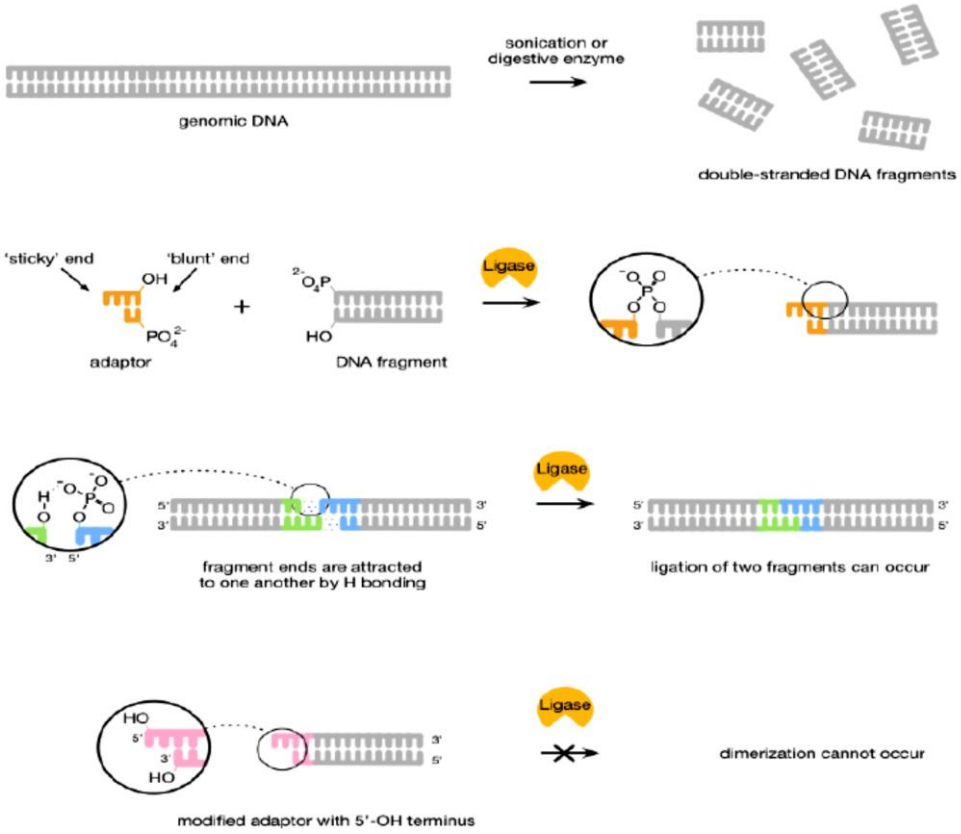
Three general steps in NGS

1. Library preparation: libraries are created using random fragmentation of DNA, followed by ligation with custom linkers
2. Amplification: the library is amplified using clonal amplification methods and PCR
3. Sequencing: DNA is sequenced using one of several different approaches

Library preparation

- Firstly, DNA is fragmented either enzymatically or by sonication (excitation using ultrasound) to create smaller strands.

- Adaptors (short, double-stranded pieces of synthetic DNA) are then ligated to these fragments with the help of DNA ligase, an enzyme that joins DNA strands.
- The adaptors enable the sequence to become bound to a complementary counterpart.
- Adaptors are synthesized so that one end is 'sticky' whilst the other is 'blunt' (non-cohesive) with the view to joining the blunt end to the blunt ended DNA.
- This could lead to the potential problem of base pairing between molecules and therefore dimer formation.
- To prevent this, the chemical structure of DNA is utilised, since ligation takes place between the 3′-OH and 5′-P ends.
- By removing the phosphate from the sticky end of the adaptor and therefore creating a 5′-OH end instead, the DNA ligase is unable to form a bridge between the two termini.



In order for sequencing to be successful, the library fragments need to be spatially clustered in PCR colonies or ‘colonies' as they are conventionally known, which consist of many copies of a particular library fragment.

Since these colonies are attached in a planar fashion, the features of the array can be manipulated enzymatically in parallel.

This method of library construction is much faster than the previous labour intensive procedure of colony picking and *E. coli* cloning used to isolate and amplify DNA for Sanger sequencing, however, this is at the expense of read length of the fragments.

Amplification

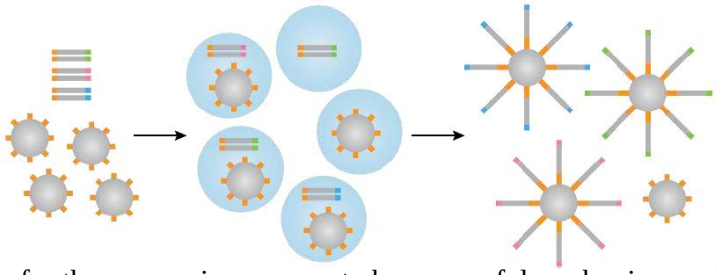
Library amplification is required so that the received signal from the sequencer is strong enough to be detected accurately.

With enzymatic amplification, phenomena such as 'biasing' and 'duplication' can occur leading to preferential amplification of certain library fragments.

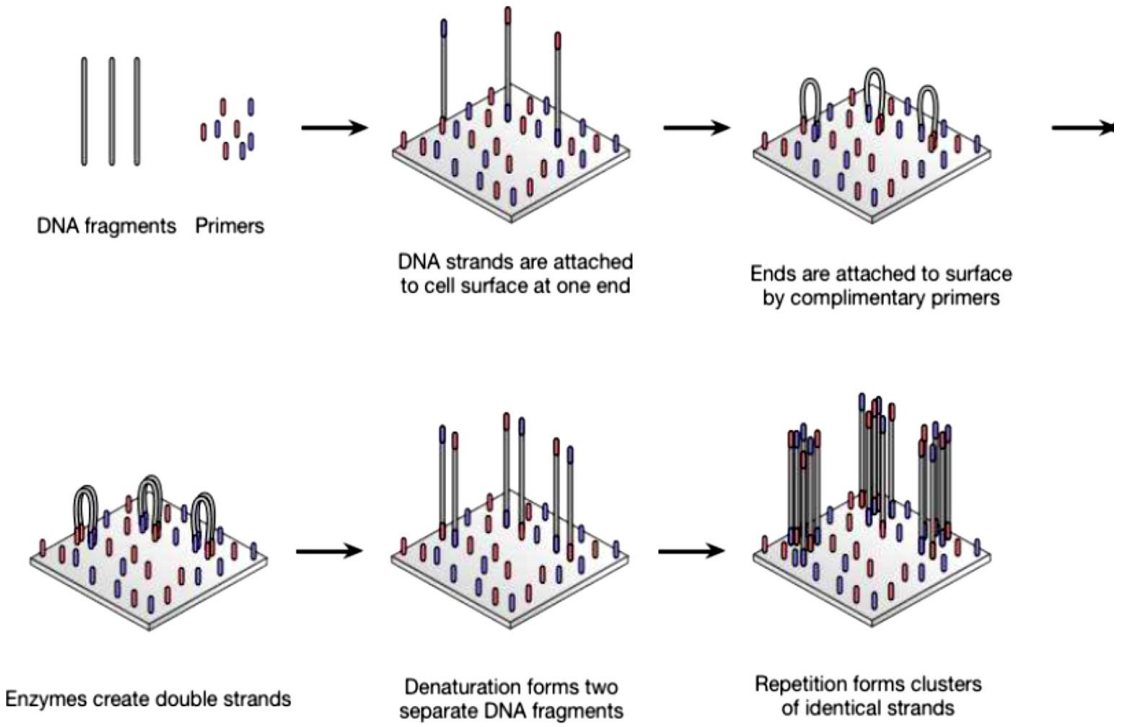
Instead, there are several types of amplification process which use PCR to create large numbers of DNA clusters.

Emulsion PCR

Emulsion oil, beads, PCR mix and the library DNA are mixed to form an emulsion which leads to the formation of micro wells.



- In order for the sequencing process to be successful, each micro well should contain one bead with one strand of DNA (approximately 15% of micro wells are of this composition).
- The PCR then denatures the library fragment leading two separate strands, one of which (the reverse strand) anneals to the bead.
- The annealed DNA is amplified by polymerase starting from the bead towards the primer site.
- The original reverse strand then denatures and is released from the bead only to re-anneal to the bead to give two separate strands.
- These are both amplified to give two DNA strands attached to the bead.
- The process is then repeated over 30-60 cycles leading to clusters of DNA.
- This technique has been criticized for its time consuming nature, since it requires many steps (forming and breaking the emulsion, PCR amplification, enrichment etc) despite its extensive use in many of the NGS platforms.
- It is also relatively inefficient since only around two thirds of the emulsion micro reactors will actually contain one bead.
- Therefore an extra step is required to separate empty systems leading to more potential inaccuracies.



Bridge PCR

The surface of the flow cell is densely coated with primers that are complementary to the primers attached to the DNA library fragments.

The DNA is then attached to the surface of the cell at random where it is exposed to reagents for polymerase based extension.

On addition of nucleotides and enzymes, the free ends of the single strands of DNA attach themselves to the surface of the cell via complementary primers, creating bridged structures.
Enzymes then interact with the bridges to make them double stranded, so that when the denaturation occurs, two single stranded DNA fragments are attached to the surface in close proximity.
Repetition of this process leads to clonal clusters of localized identical strands.
In order to optimize cluster density, concentrations of reagents must be monitored very closely to avoid overcrowding.
Several competing **methods of Next Generation Sequencing** have been developed by different companies.
1. 454 Pyrosequencing
2. Ion torrent semiconductor sequencing
3. Sequencing by ligation (SOLiD)
4. Illumina (Solexa) sequencing

Pyrosequencing

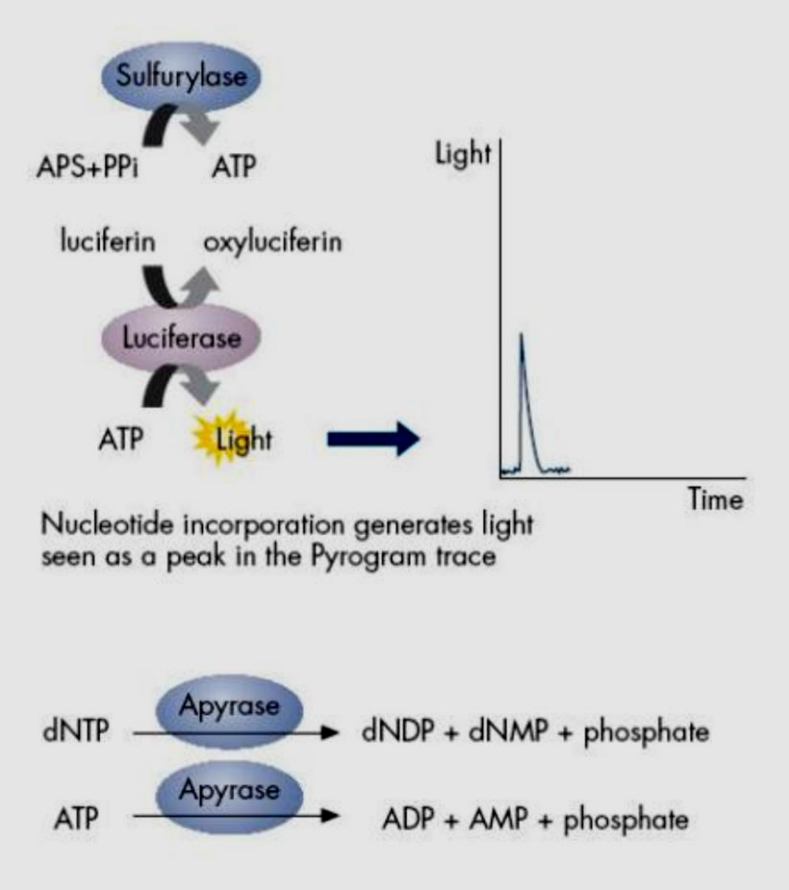
This method is based on the release of PPi during the DNA polymerization reaction. The addition of a DNTP is accompanied by release of a molecule of pyrophosphate.

$$(DNA)_n + dNTP \xrightarrow{\text{DNA polymerase}} (DNA)_{n+1} + PPi$$

$$PPi + APS \xrightarrow{\text{ATP sulphurylase}} ATP + SO_4^{-2}$$

$$ATP + \text{luciferin} + O_2 \xrightarrow{\text{luciferase}} AMP + PPi + \text{oxyluciferin} + CO_2 + h\nu$$

The DNA is denatured into ssDNA (single stranded DNA) and is added to a mixture containing DNA polymerase, adenosinesulphate, ATP sulphurylase, luciferin and luciferase.
As and when a nucleotide is added PPi is released. The PPi released is detected by the amount of light emitted by luciferase and corresponds to the nucleotide added in the reaction.

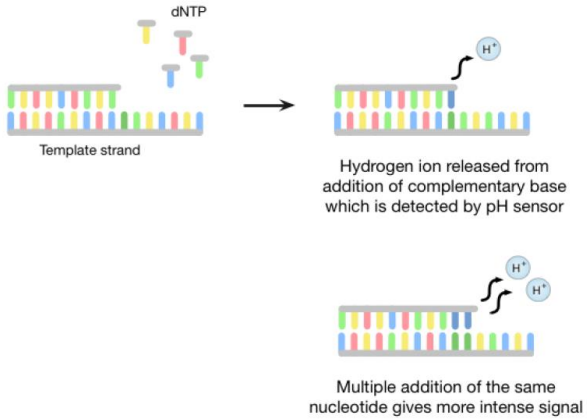


Principle of pyrosequencing

Pyrosequencing, developed by 454 Life Sciences, was one of the early successes of Next-generation sequencing; indeed, 454 Life Sciences produced the first commercially available Next-generation sequencer. However, the method was eclipsed by other technologies and, in 2013, new owners Roche announced the closure of 454 Life Sciences and the discontinuation of the 454 pyrosequencing platform.

Ion torrent semiconductor sequencing

- Ion torrent sequencing uses a "sequencing by synthesis" approach, in which a new DNA strand, complementary to the target strand, is synthesized one base at a time.
- A semiconductor chip detects the hydrogen ions produced during DNA polymerization
- Following colony formation using emulsion PCR, the DNA library fragment is flooded sequentially with each nucleoside triphosphate (dNTP), as in pyrosequencing.
- The dNTP is then incorporated into the new strand if complementary to the nucleotide on the target strand.
- Each time a nucleotide is successfully added, a hydrogen ion is released, and it detected by the sequencer's pH sensor.

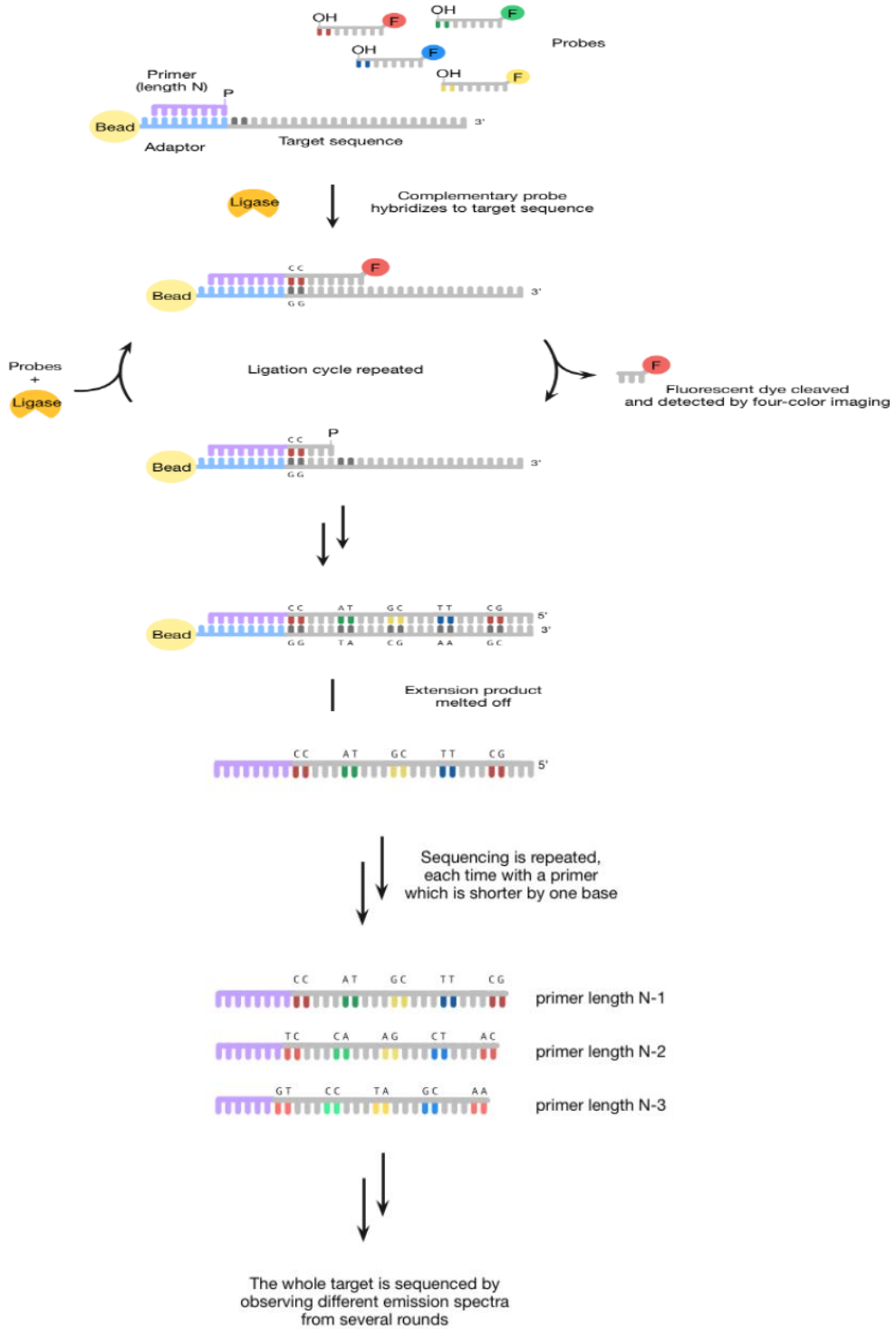


- As in the pyrosequencing method, if more than one of the same nucleotide is added, the change in pH/signal intensity is correspondingly larger.
- Ion torrent sequencing is the first commercial technique not to use fluorescence and camera scanning.
- It is therefore faster and cheaper than many of the other methods.
- Unfortunately, it can be difficult to enumerate the number of identical bases added consecutively.
- For example, it may be difficult to differentiate the pH change for a homo repeat of length 9 to one of length 10, making it difficult to decode repetitive sequences.

Sequencing by ligation (SOLiD)

- SOLiD is an enzymatic method of sequencing that uses DNA ligase, an enzyme used widely in biotechnology for its ability to ligate double-stranded DNA strands.
- Emulsion PCR is used to immobilize/amplify a ssDNA primerbinding region (known as an adapter) which has been conjugated to the target sequence (i.e. the sequence that is to be sequenced) on a bead.
- These beads are then deposited onto a glass surface − a high density of beads can be achieved which in turn, increases the throughput of the technique.
- Once bead deposition has occurred, a primer of length N is hybridized to the adapter, then the beads are exposed to a library of 8-mer probes which have different fluorescent dye at the 5' end and a hydroxyl group at the 3' end.
- Bases 1 and 2 are complementary to the nucleotides to be sequenced whilst bases 3-5 are degenerate and bases 6-8 are inosine bases.
- Only a complementary probe will hybridize to the target sequence, adjacent to the primer.
- DNA ligase is then uses to join the 8-mer probe to the primer.
- A phosphorothioate linkage between bases 5 and 6 allows the fluorescent dye to be cleaved from the fragment using silver ions.

- This cleavage allows fluorescence to be measured (four different fluorescent dyes are used, all of which have different emission spectra) and also generates a 5'-phosphate group which can undergo further ligation.
- Once the first round of sequencing is completed, the extension product is melted off and then a second round of sequencing is performed with a primer of length N−1.
- Many rounds of sequencing using shorter primers each time (i.e. N−2, N−3 etc) and measuring the fluorescence ensures that the target is sequenced.
- Due to the two-base sequencing method (since each base is effectively sequenced twice), the SOLiD technique is highly accurate (at 99.999% with a sixth primer, it is the most accurate of the second generation platforms) and also inexpensive.
- It can complete a single run in 7 days and in that time can produce 30 Gb of data.
- Unfortunately, its main disadvantage is that read lengths are short, making it unsuitable for many applications.



Illumina (Solexa) sequencing:

Solexa, now part of Illumina, was founded by Shankar Balasubramanian and David Klenerman in 1998, and developed a sequencing method based on reversible dye-terminators technology, and engineered polymerases. The reversible terminated chemistry concept was invented by Bruno Canard and Simon Sarfati at the Pasteur Institute in Paris.

In this method, DNA molecules and primers are first attached on a slide or flow cell and amplified with polymerase so that local clonal DNA colonies, later coined "DNA clusters", are formed. To determine the sequence, four types of reversible terminator bases (RT-bases) are added and non-incorporated nucleotides are washed away. A camera takes images of the fluorescently labeled nucleotides. Then the dye, along with the terminal 3' blocker, is chemically removed from the DNA, allowing for the next cycle to begin. Unlike pyrosequencing, the DNA chains are extended one nucleotide at a time and image acquisition can be performed at a delayed moment, allowing for very large arrays of DNA colonies to be captured by sequential images taken from a single camera.

Applications of DNA sequencing

- In medical science, DNA sequencing can be used in the identification of genes responsible for hereditary disorders. New mutation can also be detected with the help of the DNA sequencing
- In forensic science, it is used for parental verification, criminal investigation and identification of individuals through any of the available samples such as hair, nail, blood or tissue.
- In the agriculture industries, identification of GMO species can be possible with the help of the DNA sequencing methods. Any of minor variations into the plant genome can be detected with the help of the DNA sequencing.
- It is used to construct maps such as whole chromosomal maps, restriction digestion maps and genome maps.
- Open reading frames, non-open reading frames and protein-coding DNA sequences can be identified by the present method.
- DNA sequencing is used in exon/ intron, repeat sequence and tandem repeat identification and detection.
- Furthermore, the present method is employed in gene manipulation and gene editing. New variations in nature can also be determined through the sequencing.
- Metagenomic studies are nowadays possible by sequencing methods such as pyrosequencing.
- It is further used in the Microbial identification and study of the new bacterial species. The sequencing technique advances the microbial identification by eliminating the traditional and time consuming culturing methods.
- Nowadays microbial identification and characterisation become more rapidly and accurately done using sequencing. By comparing the sequence of the target microbes with the available data, scientists can identify new mutations and new strains.
- The sequencing techniques specifically, the NGS has a great application in the oncology and cancer studies. various cancer-causing genes are identified and characterised by the present method.
- The advancement in evolutionary studies is only possible because of DNA sequencing. By comparing various genes and sequences, an evolutionary map can be generated. Also, new variations through evolution can be encountered.
- sequencing helps in studying the asymptomatic high-risk population, prior to occurrence of disease. And thus preventive steps can be taken earlier.

DNA gel electrophoresis:

Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar. The proteins may be separated by charge and/or size (isoelectric focusing agarose electrophoresis is essentially size independent), and the DNA and RNA fragments by length. Biomolecules are separated by applying

an electric field to move the charged molecules through an agarose matrix, and the biomolecules are separated by size in the agarose gel matrix.
Agarose gel is easy to cast, has relatively fewer charged groups, and is particularly suitable for separating DNA of size range most often encountered in laboratories, which accounts for the popularity of its use. The separated DNA may be viewed with stain, most commonly under UV light, and the DNA fragments can be extracted from the gel with relative ease. Most agarose gels used are between 0.7–2% dissolved in a suitable electrophoresis buffer.

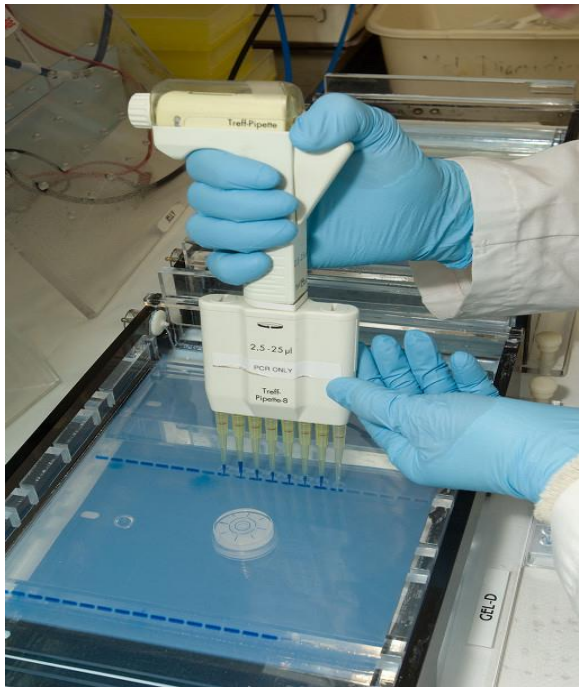
Principle

Gel electrophoresis separates DNA fragments by size in a solid support medium such as an agarose gel. Sample (DNA) are pipetted into the sample wells, followed by the application of an electric current at the anodal, negative end which causes the negatively-charged DNA to migrate (electrophorese) towards the bottom (cathodal, positive) end. The rate of migration is proportional to size: smaller fragments move more quickly, and wind up at the bottom of the gel.
DNA is visualized by including in the gel an intercalating dye, ethidium bromide. DNA fragments take up the dye as they migrate through the gel. Illumination with ultraviolet lightc auses the intercalated dye to fluoresce.
The larger fragments fluoresce more intensely. Although each of the fragments of a single class of molecule are present in equimolar proportions, the smaller fragments include less mass of DNA, take up less dye, and therefore fluoresce less intensely. A "ladder" set of DNA fragments of known size can be run simultaneously and used to estimate the sizes of the other unknown fragments.

General procedure

Casting of gel

The gel is prepared by dissolving the agarose powder in an appropriate buffer, such as TAE or TBE, to be used in electrophoresis. The agarose is dispersed in the buffer before heating it to near-boiling point, but avoid boiling. The melted agarose is allowed to cool sufficiently before pouring the solution into a cast as the cast may warp or crack if the agarose solution is too hot. A comb is placed in the cast to create wells for loading sample, and the gel should be completely set before use.



The concentration of gel affects the resolution of DNA separation. For a standard agarose gel electrophoresis, a 0.8% gives good separation or resolution of large 5–10kb DNA fragments, while 2% gel gives good resolution for small 0.2–1kb fragments. 1% gels is often used for a standard electrophoresis. The concentration is measured in weight of agarose over volume of buffer used (g/ml). High percentage gels are often brittle and may not set evenly, while low percentage gels (0.1-0.2%) are fragile and not easy to handle. Low-melting-point (LMP) agarose gels are also more fragile than normal agarose gel. Low-melting point agarose may be used on its own or simultaneously with standard agarose for the separation and isolation of DNA. PFGE and FIGE are often done with high percentage agarose gels.

Loading of samples

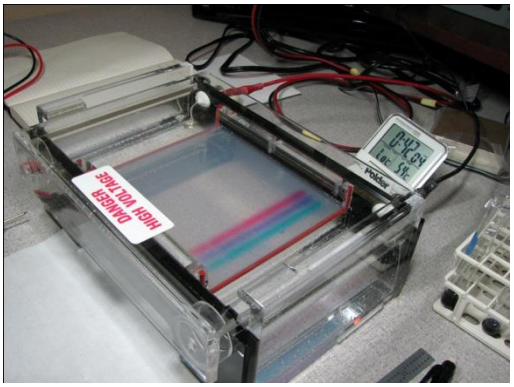
Once the gel has set, the comb is removed, leaving wells where DNA samples can be loaded. Loading buffer is mixed with the DNA sample before the mixture is loaded into the wells. The loading buffer contains a dense compound, which may be glycerol, sucrose, or Ficoll, that raises the density of the sample so that the DNA sample may sink to the bottom of the well. If the DNA sample contains residual ethanol after its preparation, it may float out of the well. The loading buffer also includes

colored dyes such as xylene cyanol and bromophenol blue used to monitor the progress of the electrophoresis. The DNA samples are loaded using a pipette.

Electrophoresis

Agarose gel electrophoresis is most commonly done horizontally in a submarine mode whereby the slab gel is completely submerged in buffer during electrophoresis. It is also possible, but less common, to perform the electrophoresis vertically, as well as horizontally with the gel raised on agarose legs using an appropriate apparatus. The buffer used in the gel is the same as the running buffer in the electrophoresis tank, which is why electrophoresis in the submarine mode is possible with agarose gel.

For optimal resolution of DNA greater than 2 kb in size in standard gel electrophoresis, 5 to 8 V/cm is recommended (the distance in cm refers to the distance between electrodes, therefore this recommended voltage would be 5 to 8 multiplied by the distance between the electrodes in cm). Voltage may also be limited by the fact that it heats the gel and may cause the gel to melt if it is run at high voltage for a prolonged period, especially if the gel used is LMP agarose gel. Too high a voltage may also reduce resolution, as well as causing band streaking for large DNA molecules. Too low a voltage may lead to broadening of band for small DNA fragments due to dispersion and diffusion.

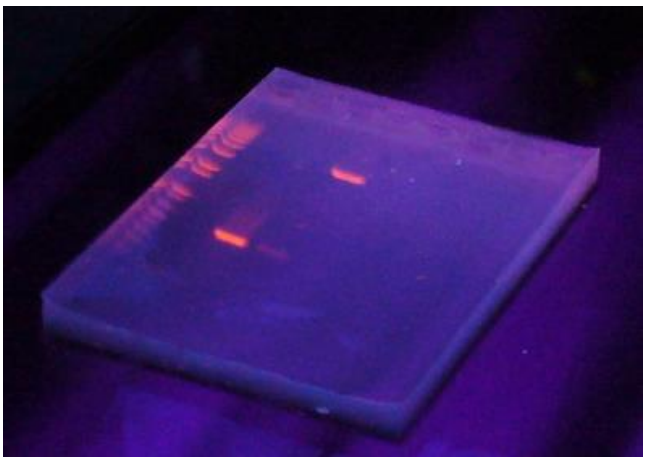


Agarose gel slab in electrophoresis tank with bands of dyes indicating progress of the electrophoresis. The DNA moves towards anode

Since DNA is not visible in natural light, the progress of the electrophoresis is monitored using colored dyes. Xylene cyanol (light blue color) comigrates large DNA fragments, while Bromophenol blue (dark blue) comigrates with the smaller fragments. Less commonly used dyes include Cresol Red and Orange G which migrate ahead of bromophenol blue. A DNA marker is also run together for the estimation of the molecular weight of the DNA fragments. Note however that the size of a circular DNA like plasmids cannot be accurately gauged using standard markers unless it has been linearized by restriction digest, alternatively a supercoiled DNA marker may be used.

Staining and visualization

DNA as well as RNA are normally visualized by staining with ethidium bromide, which intercalates into the major grooves of the DNA and fluoresces under UV light. The intercalation depends on the concentration of DNA and thus, a band with high intensity will indicate a higher amount of DNA compared to a band of less intensity. The ethidium bromide may be added to the agarose solution before it gels, or the DNA gel may be stained later after electrophoresis. Destaining of the gel is not necessary but may produce better images. Other methods of staining are available; examples are SYBR Green, GelRed, methylene blue, brilliant cresyl blue, Nile blue sulphate, and crystal violet. SYBR Green, GelRed and other similar commercial products are sold as safer alternatives to ethidium bromide as it has been shown to be mutagenic in Ames test, although the carcinogenicity of ethidium bromide has not actually been established. SYBR Green requires the use of a blue-light transilluminator. DNA stained with crystal violet can be viewed under natural light without the use of a UV transilluminator which is an advantage, however it may not produce a strong band.



When stained with ethidium bromide, the gel is viewed with an ultraviolet (UV) transilluminator. The UV light excites the electrons within the aromatic ring of ethidium bromide, and once they return to the ground state, light is released, making the DNA and ethidium bromide complex fluoresce. Standard transilluminators use wavelengths of 302/312-nm (UV-B), however exposure of DNA to UV radiation for as little as 45 seconds can produce damage to DNA and affect subsequent procedures, for example reducing the efficiency of transformation, in vitro transcription, and PCR. Exposure of the DNA to UV radiation therefore should be limited. Using a higher wavelength of 365 nm (UV-A range) causes less damage to the DNA but also produces much weaker fluorescence with ethidium bromide. Where multiple wavelengths can be selected in the transillumintor, the shorter wavelength would be used to capture images, while the longer wavelength should be used if it is necessary to work on the gel for any extended period of time.

The transilluminator apparatus may also contain image capture devices, such as a digital or polaroid camera, that allow an image of the gel to be taken or printed. For gel electrophoresis of protein, the bands may be visualised with Coomassie or silver stains.

Downstream procedures

The separated DNA bands are often used for further procedures, and a DNA band may be cut out of the gel as a slice, dissolved and purified. Contaminants however may affect some downstream procedures such as PCR, and low melting point agarose may be preferred in some cases as it contains fewer of the sulphates that can affect some enzymatic reactions. The gels may also be used for blotting techniques.

Buffers:

In general, the ideal buffer should have good conductivity, produce less heat and have a long life.[33] There are a number of buffers used for agarose electrophoresis; common ones for nucleic acids include Tris/Acetate/EDTA (TAE) and Tris/Borate/EDTA (TBE). The buffers used contain EDTA to inactivate many nucleases which require divalent cation for their function. The borate in TBE buffer can be problematic as borate can polymerize, and/or interact with cis diols such as those found in RNA. TAE has the lowest buffering capacity, but it provides the best resolution for larger DNA. This means a lower voltage and more time, but a better product.

Many other buffers have been proposed, e.g. lithium borate (LB), iso electric histidine, pK matched goods buffers, etc.; in most cases the purported rationale is lower current (less heat) and or matched ion mobilities, which leads to longer buffer life. Tris-phosphate buffer has high buffering capacity but cannot be used if DNA extracted is to be used in phosphate sensitive reaction. LB is relatively new and is ineffective in resolving fragments larger than 5 kbp; However, with its low conductivity, a much higher voltage could be used (up to 35 V/cm), which means a shorter analysis time for routine electrophoresis. As low as one base pair size difference could be resolved in 3% agarose gel with an extremely low conductivity medium (1 mM lithium borate).

Other buffering system may be used in specific applications, for example, barbituric acid-sodium barbiturate or Tris-barbiturate buffers may be used for in agarose gel electrophoresis of proteins, for example in the detection of abnormal distribution of proteins

Applications

- Estimation of the size of DNA molecules following digestion with restriction enzymes, e.g. in restriction mapping of cloned DNA.
- Analysis of products of a polymerase chain reaction (PCR), e.g. in molecular genetic diagnosis or genetic fingerprinting
- Separation of DNA fragments for extraction and purification.
- Separation of restricted genomic DNA prior to Southern transfer, or of RNA prior to Northern transfer.
- Separation of proteins, for example, screening of protein abnormalities in clinical chemistry.

Agarose gels are easily cast and handled compared to other matrices and nucleic acids are not chemically altered during electrophoresis. Samples are also easily recovered. After the experiment is finished, the resulting gel can be stored in a plastic bag in a refrigerator.

Electrophoresis is performed in buffer solutions to reduce pH changes due to the electric field, which is important because the charge of DNA and RNA depends on pH, but running for too long can

exhaust the buffering capacity of the solution. Further, different preparations of genetic material may not migrate consistently with each other, for morphological or other reasons.

Blotting techniques

Blotting is the technique in which nucleic acids or proteins are immobilized onto a solid support generally nylon or nitrocellulose membranes. Blotting of nucleic acid is the central technique for hybridization studies. Nucleic acid labeling and hybridization on membranes have formed the basis for a range of experimental techniques involving understanding of gene expression, organization, etc.
Identifying and measuring specific proteins in complex biological mixtures, such as blood, have long been important goals in scientific and diagnostic practice. More recently the identification of abnormal genes in genomic DNA has become increasingly important in clinical research and genetic counseling. Blotting techniques are used to identify unique proteins and nucleic acid sequences. They have been developed to be highly specific and sensitive and have become important tools in both molecular biology and clinical research.

General principle

The blotting methods are fairly simple and usually consist of four separate steps: electrophoretic separation of protein or of nucleic acid fragments in the sample; transfer to and immobilization on paper support; binding of analytical probe to target molecule on paper; and visualization of bound probe. Molecules in a sample are first separated by electrophoresis and then transferred on to an easily handled support medium or membrane. This immobilizes the protein or DNA fragments, provides a faithful replica of the original separation, and facilitates subsequent biochemical analysis. After being transferred to the support medium the immobilized protein or nucleic acid fragment is localized by the use of probes, such as antibodies or DNA, that specifically bind to the molecule of interest. Finally, the position of the probe that is bound to the immobilized target molecule is visualized usually by autoradiography. Three main blotting techniques have been developed and are commonly called Southern, northern and western blotting.

Southern blot:

Southern blot is a method used to check for the presence of a DNA sequence in a DNA sample. The method is named after its inventor, the British biologist Edwin Southern. In Southern blotting, DNA is transferred from a gel to a membrane for hybridization analysis.

Principle:

- Southern blotting is an example of RFLP (restriction fragment length polymorphism). It was developed by Edward M. Southern (1975). Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization.
- Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane; the desired DNA is detected using specific DNA probe that is complementary to the desired DNA.
- A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

Procedure/ Steps

1. Restriction digest: by RE enzyme and amplification by PCR
2. Gel electrophoresis: SDS gel electrophoresis
3. Denaturation: Treating with HCl and NaOH
4. Blotting
5. Baking and Blocking with casein in BSA
6. Hybridization using labelled probes
7. Visualization by autoradiogram

Step I: Restriction digest

▪ The DNA is fragmentized by using suitable restriction enzyme. RE cuts the DNA at specific site generating fragments

▪ The number of fragments of DNA obtained by restriction digest is amplified by PCR

Step II: Gel electrophoresis

▪ The desired DNA fragments is separated by gel electrophoresis
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Step III: Denaturation

▪ The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCl) to denature the double stranded DNA fragments.

▪ DNA strands get separated

Step IV: Blotting

▪ The separated strands of DNA is then transferred to positively charged membrane nylon membrane (Nitrocellulose paper) by the process of blotting.

Step V: Baking and blocking

▪ After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.

▪ The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

Step VI: Hybridization with labelled probes

▪ The DNA bound to membrane is then treated with labelled probe

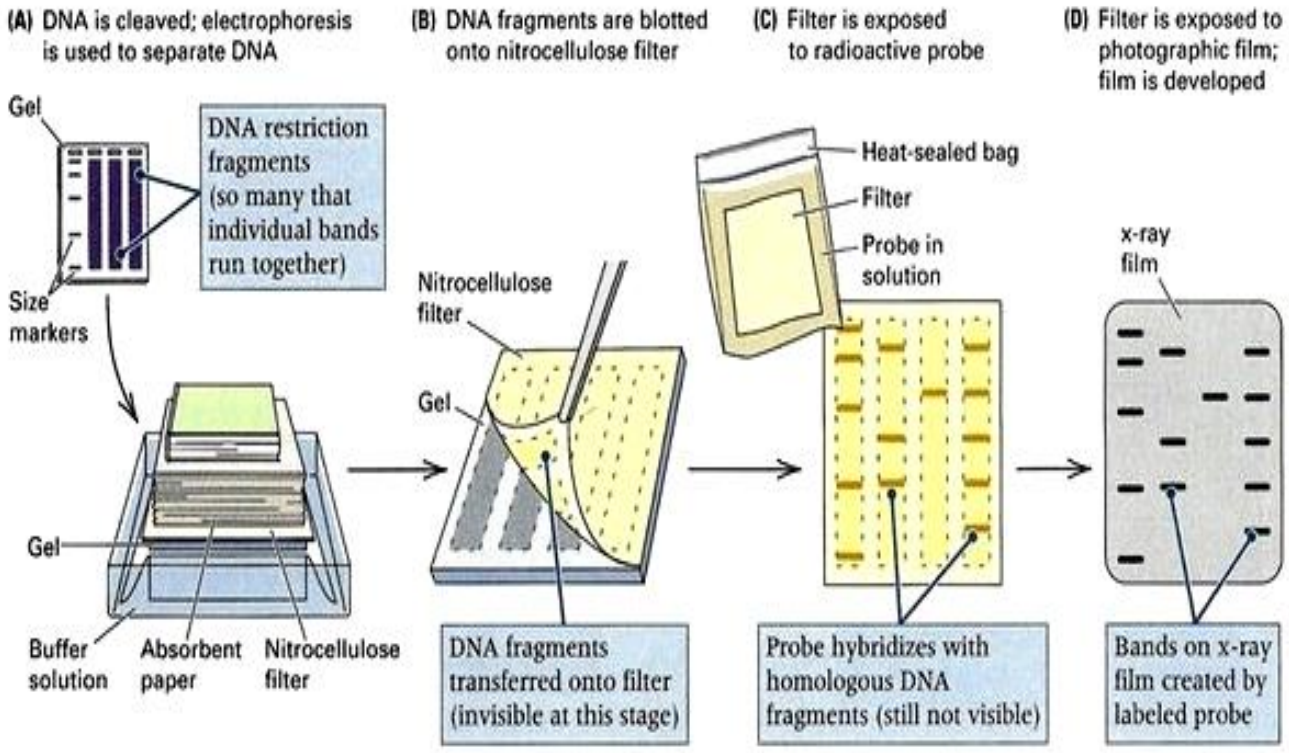
▪ The labelled probe contains the complementary sequences to the gene of interest

▪ The probe bind with complementary DNA on the membrane since all other non-specific binding site on the membrane has been blocked by BSA or casein.

Step VII: Visualization by Autoradiogram

▪ The membrane bound DNA labelled with probe can be visualized under autoradiogram which give pattern of bands.

Southern blot



Application of Southern blotting:

1. Southern blotting technique is used to detect DNA in given sample.
2. DNA finger printing is an example of southern blotting
3. Used for paternity testing, criminal identification, victim identification
4. To isolate and identify desire gene of interest.
5. Used in restriction fragment length polymorphism
6. To identify mutation or gene rearrangement in the sequence of DNA
7. Used in diagnosis of disease caused by genetic defects
8. Used to identify infectious agents

Significance:

Southern blotting is useful for detecting major gene arrangements. This technique plays important role in DNA finger print, identification of novel gene, identification of structurally related genes in the species etc.

Northern blot

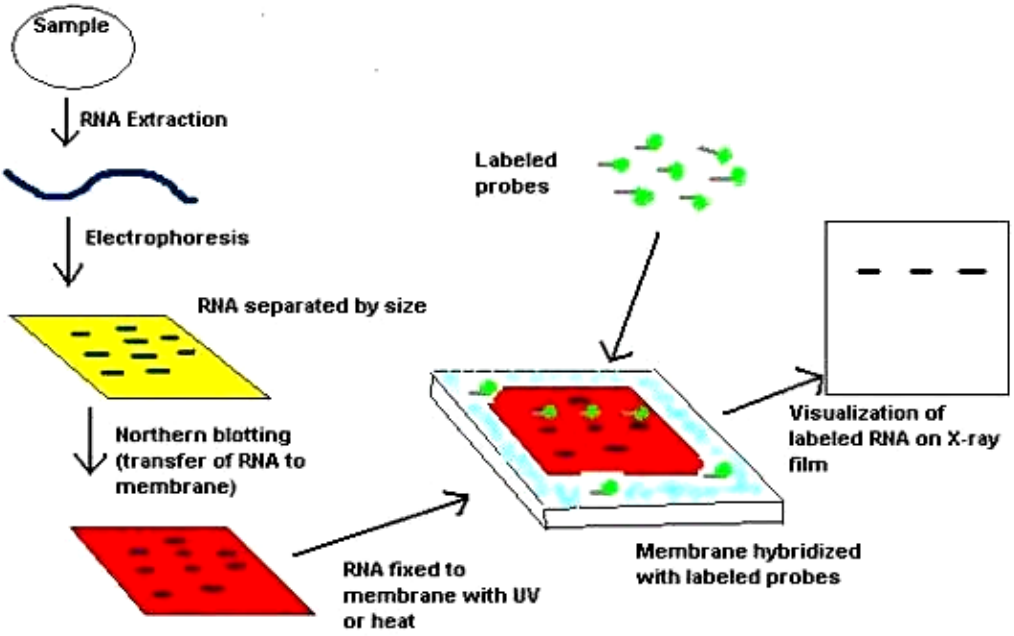
Northern blotting is used to detect RNA. Cells can be broken open to release their RNA. The RNA from different cell types can be run on separate lanes on a gel. The gel spreads the different RNA by size. These neat, parallel rows of RNA allow a researcher to compare which cell type has how much of which RNA. This method allows a researcher to determine if cells from a certain disease have more of this RNA or less of that RNA. Northern blotting may reveal how a disease is working at the level of RNA production.

Principle

As all normal blotting technique, northern blotting starts with the electrophoresis to separate RNA samples by size. Electrophoresis separates the RNA molecules based on the charge of the nucleic acids. The charge in the nucleic acids is proportional to the size of the nucleic acid sequence. Thus the electrophoresis membrane separates the Nucleic acid sequence according to the size of the RNA sequence. In cases where our target sequence is an mRNA, the sample can be isolated through oligo cellulose chromatographic techniques, as mRNA are characterized by the poly(A)-tail. Since gel molecules are fragile in nature, the separated sequences are transferred to the nylon membranes. The selection of nylon membrane is contributed to the factor that nucleic acids are negatively charged in nature. Once the RNA molecules are transferred it is immobilized by covalent linkage. The probe is then added, the probe can be complementary an ss DNA sequence. Formamide is generally used as a blotting buffer as it reduces the annealing temperature.

Procedure:

1. The tissue or culture sample collected is first homogenized. The samples may be representative of different types of culture for comparison or it can be for the study of different stages of growth inside the culture.
2. The RNA sequence is separated in the electrophoresis unit an agarose gel is used for the purpose of the nucleic acid separation.
3. Now the separated RNA sequence is transferred to the nylon membrane. This is done by two mechanisms capillary action and the ionic interaction.
4. The transfer operation is done by keeping the gel in the following order. First, the agarose gel is placed on the bottom of the stack, followed by the blotting membrane. On top of these paper towels a mild weight (glass plate) is placed. The entire setup is kept in a beaker containing transfer buffer.
5. RNA transferred to the nylon membrane is then fixed using UV radiation.
6. The fixed nylon membrane is then mixed with probes. The probes are specifically designed for the gene of interest, so that they will hybridize with RNA sequences on the blot corresponding to the sequence of interest.
7. The blot membrane is washed to remove unwanted probe
8. Labeled probe is detected by chemiluminescence or autoradiography. The result will be dark bands in x ray film.



Applications:

Northern blotting allows one to observe a particular gene's expression pattern between tissues, organs, developmental stages, environmental stress levels, pathogen infection, and over the course of treatment. The technique has been used to show overexpression of oncogenes and downregulation of tumor-suppressor genes in cancerous cells when compared to 'normal' tissue, as well as the gene expression in the rejection of transplanted organs. If an upregulated gene is observed by an abundance of mRNA on the northern blot the sample can then be sequenced to determine if the gene is known to researchers or if it is a novel finding.

The expression patterns obtained under given conditions can provide insight into the function of that gene. Since the RNA is first separated by size, if only one probe type is used variance in the level of each band on the membrane can provide insight into the size of the product, suggesting alternative splice products of the same gene or repetitive sequence motifs.The variance in size of a gene product can also indicate deletions or errors in transcript processing. By altering the probe target used along the known sequence it is possible to determine which region of the RNA is missing.

BlotBase is an online database publishing northern blots. BlotBase has over 700 published northern blots of human and mouse samples, in over 650 genes across more than 25 different tissue types.Northern blots can be searched by a blot ID, paper reference, gene identifier, or by tissue. The results of a search provide the blot ID, species, tissue, gene, expression level, blot image (if available), and links to the publication that the work originated from. This new database provides sharing of information between members of the science community that was not previously seen in northern blotting as it was in sequence analysis, genome determination, protein structure, etc.

Advantages and disadvantages

Analysis of gene expression can be done by several different methods including RT-PCR, RNase protection assays, microarrays, RNA-Seq, serial analysis of gene expression (SAGE), as well as northern blotting. Microarrays are quite commonly used and are usually consistent with data obtained from northern blots; however, at times northern blotting is able to detect small changes in gene expression that microarrays cannot. The advantage that microarrays have over northern blots is that thousands of genes can be visualized at a time, while northern blotting is usually looking at one or a small number of genes.

A problem in northern blotting is often sample degradation by RNases (both endogenous to the sample and through environmental contamination), which can be avoided by proper sterilization of glassware and the use of RNase inhibitors such as DEPC (diethylpyrocarbonate). The chemicals used in most northern blots can be a risk to the researcher, since formaldehyde, radioactive material,

ethidium bromide, DEPC, and UV light are all harmful under certain exposures. Compared to RT-PCR, northern blotting has a low sensitivity, but it also has a high specificity, which is important to reduce false positive results.
The advantages of using northern blotting include the detection of RNA size, the observation of alternate splice products, the use of probes with partial homology, the quality and quantity of RNA can be measured on the gel prior to blotting, and the membranes can be stored and reprobed for years after blotting.
For northern blotting for the detection of acetylcholinesterase mRNA the nonradioactive technique was compared to a radioactive technique and found as sensitive as the radioactive one, but requires no protection against radiation and is less time consuming.

Western blot:
Western blotting is a common technique for separating proteins by size, but in straight columns. These parallel columns allow researchers to compare the amount of a protein across different samples that are run right next to each other, like bowling lanes. For example, if you were testing the effect of different amounts of a drug on cell growth, you would treat four different groups of cells with a different amount of drug. Then you could break the cells open and run the proteins of each group in separate lanes on a gel. Spreading the proteins out in this way allows you to see what an increasing concentration of drugs does to a certain protein.

Principle

- Western blotting technique is used for identification of particular protein from the mixture of protein.
- In this method labelled antibody against particular protein is used identify the desired protein, so it is a specific test. Western blotting is also known as immunoblotting because it uses antibodies to detect the protein.

Procedure/Steps

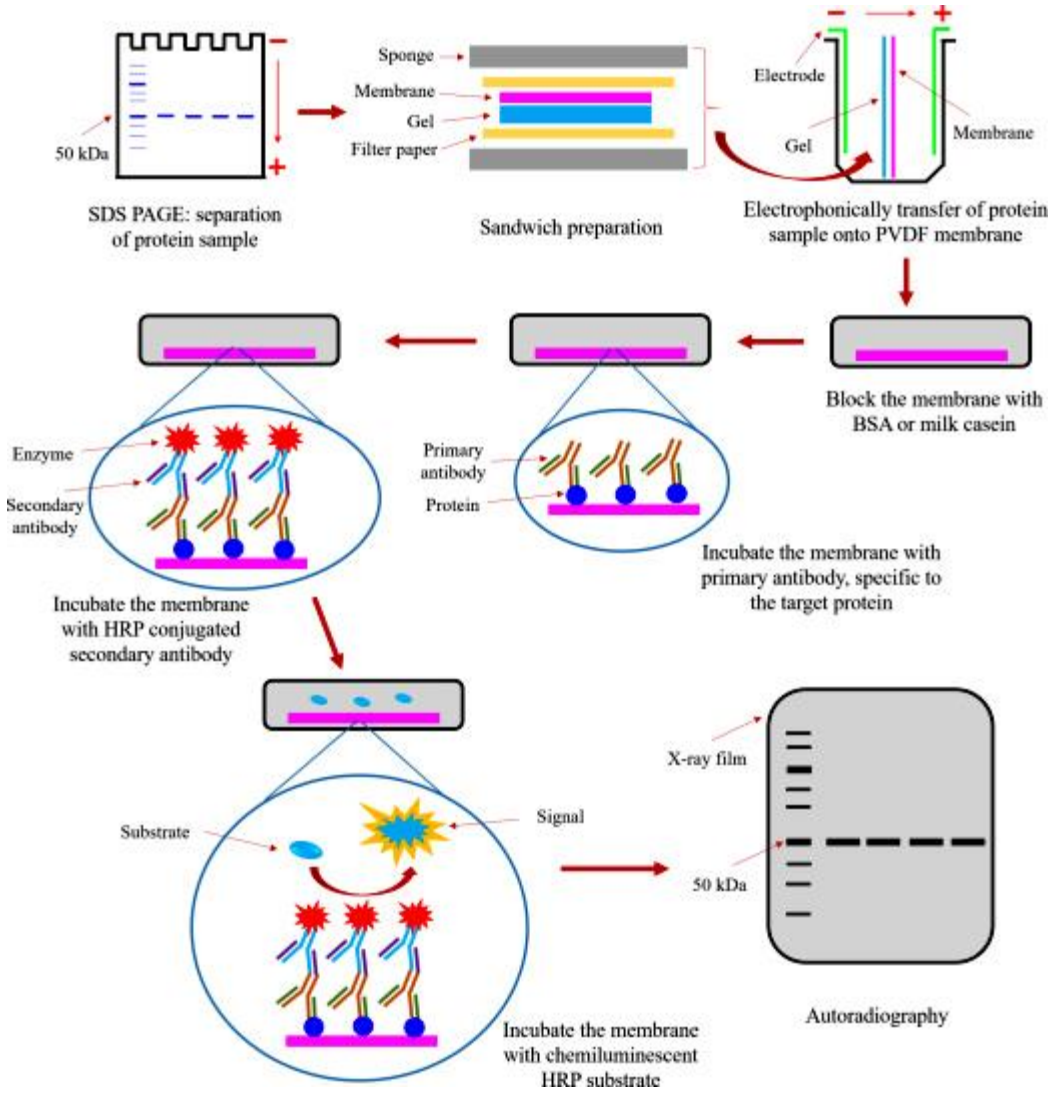
1. Extraction of protein
2. Gel electrophoresis: SDS PAGE
3. Blotting: electrical or capillary blotting
4. Blocking: BSA
5. Treatment with primary antibody
6. Treatment with secondary antibody(enzyme labelled anti Ab)
7. Treatment with specific substrate; if enzyme is alkaline phosphatase, substrate is p-nitro phenyl phosphate which give color.

Step I: Extraction of Protein

- ❖ Cell lysate is most common sample for western blotting.
- ❖ Protein is extracted from cell by mechanical or chemical lysis of cell. This step is also known as tissue preparation.
- ❖ To prevent denaturing of protein protease inhibitor is used.
- ❖ The concentration of protein is determined by spectroscopy.
- ❖ When sufficient amount of protein sample is obtained, it is diluted in loading buffer containing glycerol which helps to sink the sample in well.
- ❖ Tracking dye (bromothymol blue) is also added in sample to monitor the movement of proteins.

Step II: Gel electrophoresis

- ❖ The sample is loaded in well of SDS-PAGE Sodium dodecyl sulfate- poly-acrylamide gel electrophoresis.
- ❖ The proteins are separated on the basis of electric charge, isoelectric point, molecular weight, or combination of these all.
- ❖ The small size protein moves faster than large size protein.
- ❖ Protein is negatively charged, so they move toward positive (anode) pole as electric current is applied.



Step III: Blotting

- The nitrocellulose membrane is placed on the gel. The separated protein from gel get transferred to nitrocellulose paper by capillary action. This type of blotting is time consuming and may take 1-2 days
- For fast and more efficient transfer of desired protein from the gel to nitrocellulose paper electro-blotting can be used.
- In electro-blotting nitrocellulose membrane is sandwich between gel and cassette of filter paper and then electric current is passed through the gel causing transfer of protein to the membrane.

Step IV: Blocking

- Blocking is very important step in western blotting.
- Antibodies are also protein so they are likely to bind the nitrocellulose paper. So before adding the primary antibody the membrane is non-specifically saturated or masked by using casein or Bovine serum albumin (BSA).

Step V: Treatment with Primary Antibody

- The primary antibody (1° Ab) is specific to desired protein so it form Ag-Ab complex

Step VI: Treatment with secondary antibody

- The secondary antibody is enzyme labelled. For eg. alkaline phosphatase or Horseradish peroxidase (HRP) is labelled with secondary antibody.
- Secondary antibody (2° Ab) is antibody against primary antibody (anti-antibody) so it can bind with Ag-Ab complex.

Step VII: Treatment with suitable substrate

- ❖ To visualize the enzyme action, the reaction mixture is incubated with specific substrate.
- ❖ The enzyme convert the substrate to give visible colored product, so band of color can be visualized in the membrane.
- ❖ Western blotting is also a quantitative test to determine the amount of protein in sample.

Application:

1. To determine the size and amount of protein in given sample.
2. Disease diagnosis: detects antibody against virus or bacteria in serum.
3. Western blotting technique is the confirmatory test for HIV. It detects anti HIV antibody in patient's serum.
4. Useful to detect defective proteins. For eg Prions disease.
5. Definitive test for Creutzfeldt-Jacob disease, Lyme disease, Hepatitis B and Herpes.

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South-Western Blotting:

South-western blotting is the combination of Southern blotting and Western blotting. It was first described by Bowen et al. and was used to identify DNA-binding proteins that specifically interact with a chosen DNA fragment in a sequence-specific manner.

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In this technique, mixtures of proteins such as crude nuclear extracts or partially purified preparations are first fractionated on a sodium dodecyl sulfate (SDS) denaturing gel; the gel is then equili-brated in a SDS-free buffer to remove deter-gent and the proteins transferred by electro blotting to an immobilizing membrane.

During the transfer, proteins re-nature and hence DNA-binding proteins may subse-quently be detected on the membrane by their ability to bind radio-labelled DNA.

Fractionation of crude nuclear extracts on an SDS gel followed by electro blotting and analysis for sequence-specific DNA binding directly on the blot combines the advantages of a high-resolution fractionation step with the ability to rapidly analyse for a large number of differ-ent DNA-binding specificities.

North-Western Blotting:

North-western blotting technique is the com-bination of northern blotting and western blot-ting. This technique is used for identification of protein-RNA interactions in which protein is run on a gel, blotted, and probed with a la-belled RNA of interest. Interactions are de-tected as hot-spots on the filter.

Dot Blotting:

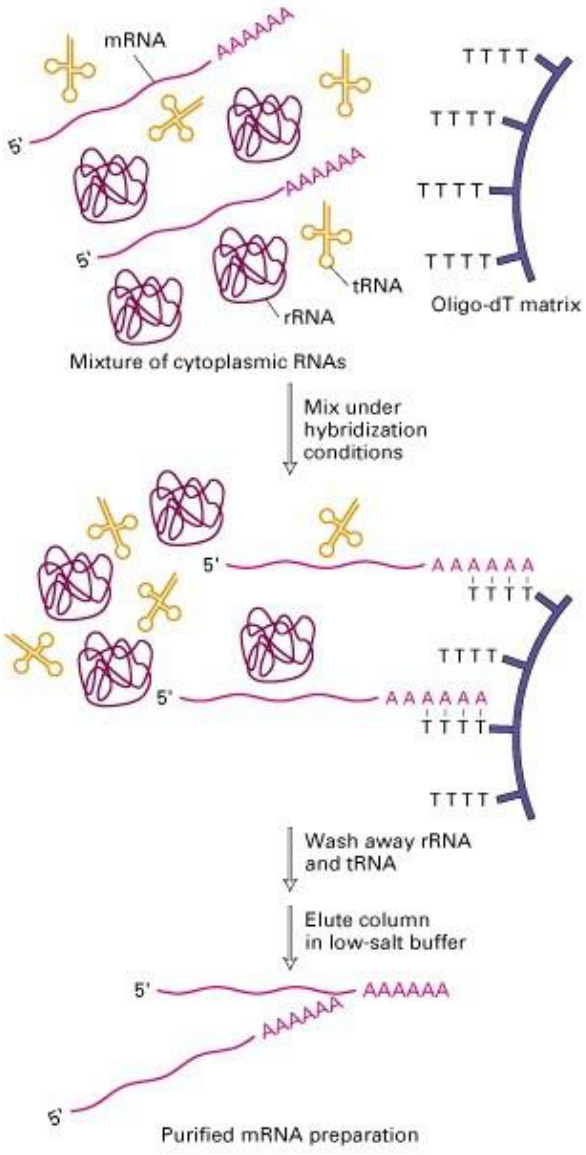
It is a modified version of Western blotting which is used for identification and analysis of protein of interest. Dot blot methodology differs from traditional Western blot tech-niques by not separating protein samples us-ing electrophoresis. Sample proteins are ins-tead spotted onto membranes and hybridized with an antibody probe.

Zoo Blotting:

Zoo blotting is based on the principles of South-ern blotting. In the genome of any organisms there are two regions – the coding and non- coding regions. It is the coding region that causes interest for most of the researchers as it is associated with the genetic information for a specific protein.

cDNA library

cDNA library is a combination of cloned cDNA (complementary DNA) fragments inserted into a collection of host cells, which together constitute some portion of the transcriptome of the organism. cDNA is produced from fully transcribed mRNA found in the nucleus and therefore contains only the expressed genes of an organism. In eukaryotic cells the mature mRNA is already spliced, hence the cDNA produced lacks introns and can be readily expressed in a bacterial cell. While information in cDNA libraries is a powerful and useful tool since gene products are easily identified, the libraries lack information about enhancers, introns, and other regulatory elements found in a genomic DNA library.



Steps:

cDNA Library preparation

cDNA is created from a mature mRNA from a eukaryotic cell with the use of an enzyme known as reverse transcriptase. In eukaryotes, a poly-(A) tail (consisting of a long sequence of adenine nucleotides) distinguishes mRNA from tRNA and rRNA and can therefore be used as a primer site for reverse transcription.

mRNA extraction

Firstly, the mRNA is obtained and purified from the rest of the RNAs. Several methods exist for purifying RNA such as trizol extraction and column purification . Column purification is done by using oligomeric dT nucleotide coated resins where only the mRNA having the poly-A tail will bind. The rest of the RNAs are eluted out. The mRNA is eluted by using eluting buffer and some heat to separate the mRNA strands from oligo-dT.

Purification can be performed by binding mRNAs on a solid matrix to which short strings of thymidylate residues are attached (oligo dT matrix). The mRNAs are removed again by washing in a low salt buffer.

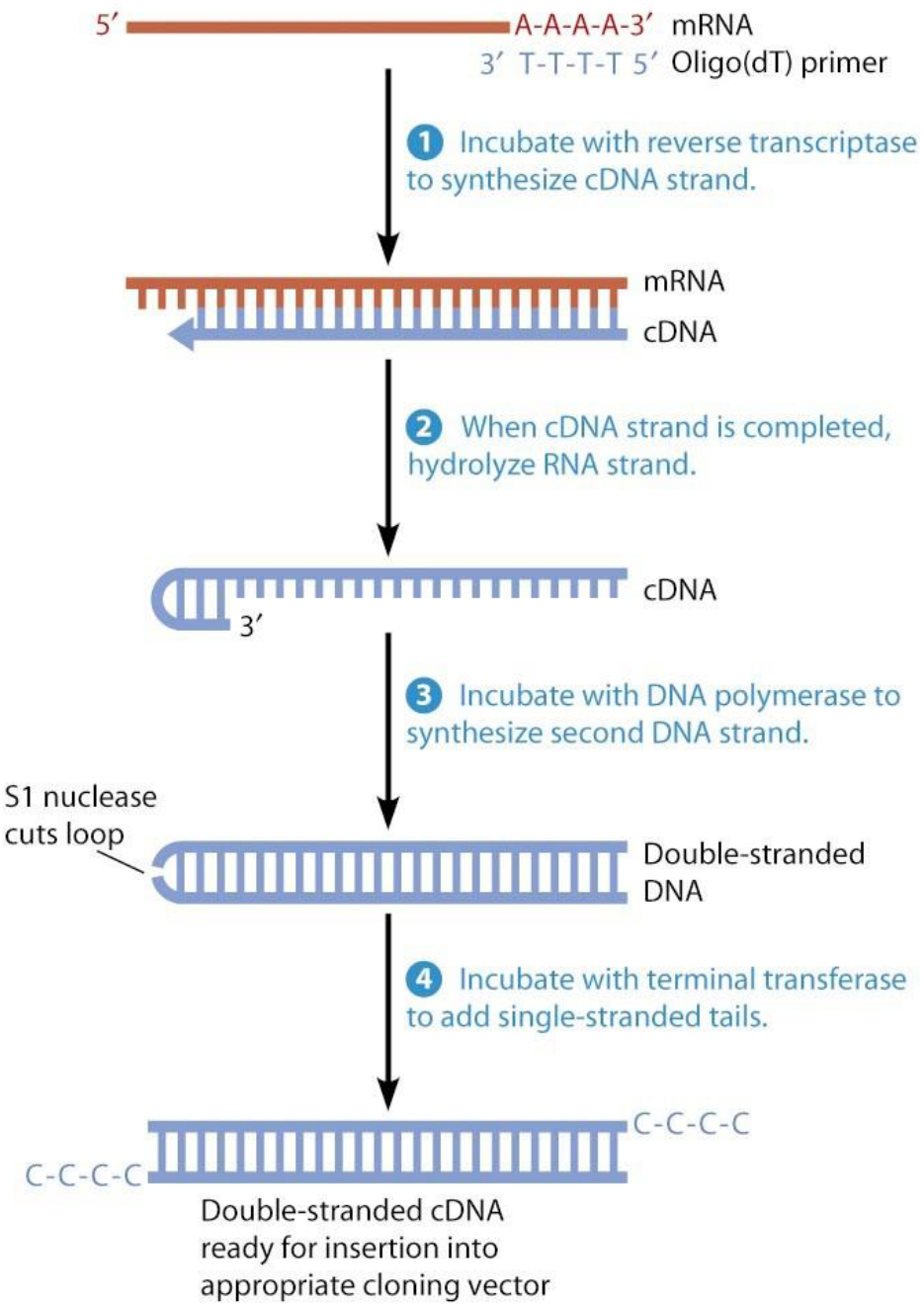
cDNA construction:

Once mRNA is purified, oligo-dT is tagged as a complementary primer which binds to the poly-A tail providing a free 3'-OH end that can be extended by reverse transcriptase to create the complementary DNA strand. Now, the mRNA is removed by using an RNAse enzyme leaving a single stranded cDNA (sscDNA). This sscDNA is converted into a double stranded DNA with the help of DNA polymerase. However, for DNA polymerase to synthesize a complementary strand a free 3'-OH end is needed.

This is provided by the sscDNA itself by generating a hair pin loop at the 3' end by coiling on it.

The polymerase extends the 3'-OH end and later the loop at 3' end is opened by the scissoring action of S1 nuclease. Restriction endonucleases and DNA ligase are then used to clone the sequences into bacterial plasmids.

The cloned bacteria are then selected, commonly through the use of antibiotic selection. Once selected, stocks of the bacteria are created which can later be grown and sequenced to compile the cDNA library.



Other strategies for the construction of a cDNA:

These are discussed as follows:

The RNase Method:

The principle of this method is that a complementary DNA strand is synthesized using reverse tran-scriptase to make an RNA: DNA duplex. The RNA strand is then nicked and replaced by DNA. In this method the first step is to anneal a chemically synthesized oligo-dT primer to the 3′ polyA-tail of the RNA.

The primer is typically 10-15 resi-dues long, and it primes (by providing a free 3′ end) the synthesis of the first DNA strand in the presence of reverse trans-criptase and deoxyribonucleotides. This leaves an RNA: DNA duplex.

The next step is to replace the RNA strand with a DNA strand. This is done by using RNase H enzyme which removes the RNA from RNA: DNA duplex. The DNA strand thus left behind is then considered as the template and the second DNA strand is synthesized by the action of DNA poly-merase II.

Land et al. Strategy:
After first-strand synthesis, which is primed with an oligo- dT primer as usual, the cDNA is tailed with a string of cytidine residues using the en-zyme terminal transferase. This artificial oligo-dC tail is then used as an annealing site for a synthetic oligo-dG primer, allow-ing synthesis of the second strand.

Homopolymer Tailing:
This approach uses the enzyme terminal transferase, which can polymerize nucleotides onto the 3′-hydroxyl of both DNA and RNA mol-ecules. We carry out the synthesis of the first DNA strand essentially as before, to produce an RNA: DNA hybrid.
We then use terminal transferase and a single deoxyribonucleotide to add tails of that nucleotide to the 3′ ends of both RNA and DNA strands. The result of this is that the DNA strand now has a known sequence at its 3′ end Typically, dCTP or dATP are used.
A complementary oligomer (synthesized chemically) can now be annealed and used as a primer to direct second strand syn-thesis. This oligomer (and also the one used for first strand synthesis) may addi-tionally incorporate a restriction site, to help in cloning the resulting double- stranded cDNA.

Rapid Amplification of cDNA Ends (RACE):
It is sometimes the case that we wish to clone a particular cDNA for which we already have some sequence data, but with particular emphasis on the integrity of the 5′ or 3′ ends. RACE techniques (Rapid Amplification of cDNA Ends) are available for this. The RACE methods are divided into 3'RACE and 5'RACE, accord-ing to which end of the cDNA we are in-terested in.

3'RACE:
In this type of RACE, reverse transcriptase synthesis of a first DNA strand is carried out using a modified oligo-dT primer. This primer comprises a stretch of unique adaptor se-quence followed by an oligo-dT stretch. The first strand synthesis is followed by a second strand synthesis using a primer internal to the coding sequence of interest.

5'RACE:
In this type of RACE first cDNA strand is synthesized with re- verse transcriptase and a primer from within the coding sequence. Unincor-porated primer is removed and the cDNA strands are tailed with oligo-dA. A second cDNA strand is then synthe-sized with an adaptor-oligo-dT primer.

Cloning the c-DNA:

(a) Linkers:
The RNaseH and homopolymer tailing methods ultimately generate a col-lection of double-stranded, blunt-ended cDNA molecules. They must now be at-tached to the vector molecules. This could be done by blunt-ended ligation, or by the addition of linkers, digestion with the rel-evant enzyme and ligation into vector.

(b) Incorporation of Restriction Sites:
It is possible to adapt the homopolymer tailing method by using primers that are modi-fied to incorporate restriction. In the dia-gram shown next page, the oligo-dT primer is modified to contain a restriction site (in the figure, a Sail site GTCGAC).

The 3′ end of the newly synthesized first cDNA strand is tailed with C's. An oligo-dG primer, again preceded by a Sail site within a short double-stranded region of the oligonucleotide, is then used for sec-ond-strand synthesis.
Note that this method requires the use of an oligonucleo-tide containing a double-stranded region. Such oligonucleotides are made by synthe-sizing the two strands separately and then allowing them to anneal to one another.

(c) Homopolymer Tailing of cDNA:
Another option is to use terminal transferase again. Treatment of the blunt-ended double-stranded cDNA with terminal transferase and dCTP leads to the poly-merization of several C residues (typically 20 or so) to the 3′ hydroxyl at each end.
Treatment of the vector with terminal transferase and dGTP leads to the incor-poration of several G residues onto the ends of the vector. (Alternatively, dATP and dTTP can be used.) The vector and cDNA can now anneal, and the base-paired region is often so extensive that treatment with DNA ligase is unnecessary.

In fact, there may be gaps rather than nicks at the vector insert boundaries, but these are re-paired by physiological processes once the recombinant molecules have been intro-duced into a host.

Uses of cDNA library:

- cDNA libraries are commonly used when reproducing eukaryotic genomes, as the amount of information is reduced to remove the large numbers of non-coding regions from the library.
- cDNA libraries are used to express eukaryotic genes in prokaryotes.
- Prokaryotes do not have introns in their DNA and therefore do not possess any enzymes that can cut it out during transcription process. cDNA do not have introns and therefore can be expressed in prokaryotic cells.
- cDNA libraries are most useful in reverse genetics where the additional genomic information is of less use.
- Also, it is useful for subsequently isolating the gene that codes for that mRNA.
- Discovery of novel genes.
- Clonning of full length cDNA molecules for in vitro study of gene function.
- Study of the repertoire if mRNAs expressed in different cells or tissues.
- Study of alternative splicing in different cells or tissues.

DNA footprinting

DNA footprinting is a method of investigating the sequence specificity of DNA- binding proteins in vitro. This technique can be used to study protein-DNA interactions both outside and within cells.

The regulation of transcription has been studied extensively, and yet there is still much that is not known. Transcription factors and associated proteins that bind promoters, enhancers, or silencers to drive or repress transcription are fundamental to understanding the unique regulation of individual genes within the genome. Techniques like DNA footprinting help elucidate which proteins bind to these associated regions of DNA and unravel the complexities of transcriptional control.

In 1978, David Galas and Albert Schmitz developed the DNA footprinting technique to study the binding specificity of the lac repressor protein. It was originally a modification of the Maxam-Gilbert chemical sequencing technique.

Methods and Procedure

The simplest application of this technique is to assess whether a given protein binds to a region of interest within a DNA molecule. Polymerase chain reaction (PCR) amplifies and label region of interest that contains a potential protein-binding site, ideally amplicon is between 50 and 200 base pairs in length. Add protein of interest to a portion of the labeled template DNA; a portion should remain separate without protein, for later comparison. Add a cleavage agent to both portions of DNA template. The cleavage agent is a chemical or

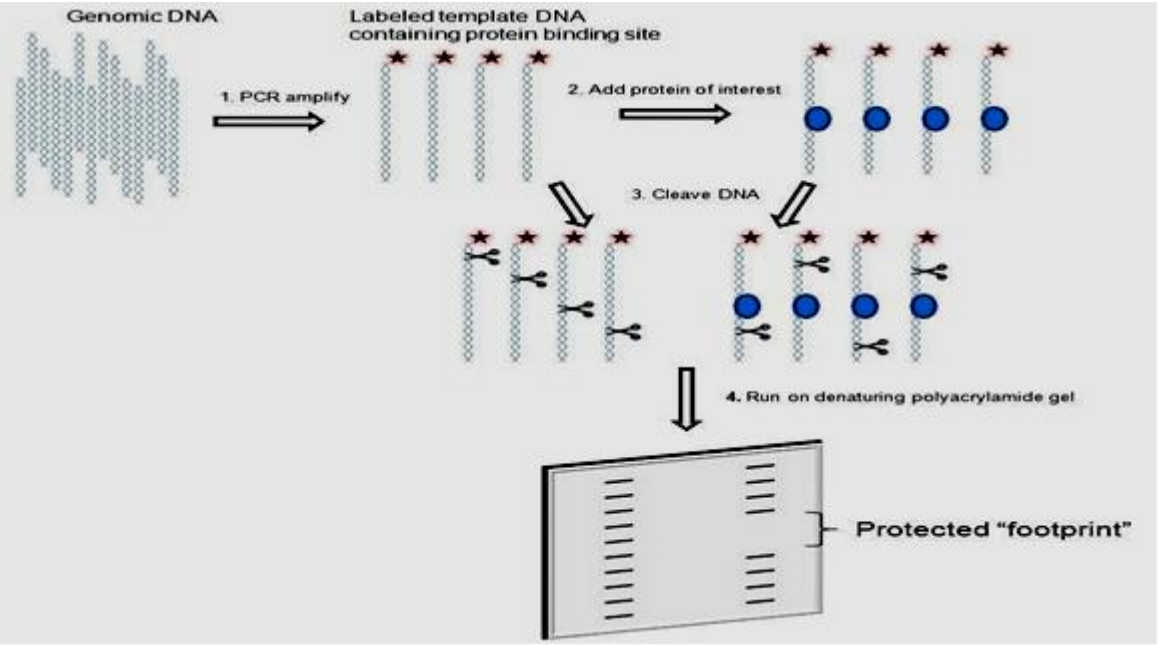
enzyme that will cut at random locations in a sequence independent manner. The reaction should occur just long enough to cut each DNA molecule in only one location. A protein that specifically binds a region within the DNA template will protect the DNA it is bound to from the cleavage agent. Run both samples side by side on a polyacrylamide gel electrophoresis. The portion of DNA template without protein will be cut at random locations, and thus when it is run on a gel, will produce a ladder-like distribution. The DNA template with the protein will result in ladder distribution with a break in it, the "footprint", where the DNA has been protected from the cleavage agent. Note: Maxam-Gilbert chemical DNA sequencing can be run alongside the samples on the polyacrylamide gel to allow the prediction of the exact location of ligand binding site.

Labeling

The DNA template labeled at the 3' or 5' end, depending on the location of the binding site(s). Labels that can be used are: radioactivity and fluorescence. Radioactivity has been traditionally used to label DNA fragments for footprinting analysis, as the method was originally developed from the Maxam-Gilbert chemical sequencing technique. Radioactive labeling is very sensitive and is optimal for visualizing small amounts of DNA. Fluorescence is a desirable advancement due to the hazards of using radio-chemicals. However, it has been more difficult to optimize because it is not always sensitive enough to detect the low concentrations of the target DNA strands used in DNA footprinting experiments. Electrophoretic sequencing gels or capillary electrophoresis have been successful in analyzing footprinting of fluorescent tagged fragments.

Cleavage agent

A variety of cleavage agents can be chosen. a desirable agent is one that is sequence neutral, easy to use, and is easy to control. Unfortunately no available agents meet all of these standards, so an appropriate agent can be chosen, depending on your DNA sequence and ligand of interest. The following cleavage agents are described in detail:DNase I is a large protein that functions as a double-strand endonuclease. It binds the minor groove of DNA and cleaves the phosphodiester backbone. It is a good cleavage agent for footprinting because its size makes it easily physically hindered. Thus is more likely to have its action blocked by a bound protein on a DNA sequence. In addition, the DNase I enzyme is easily controlled by adding EDTA to stop the reaction. There are however some limitations in using DNase I. The enzyme does not cut DNA randomly; its activity is affected by local DNA structure and sequence and therefore results in an uneven ladder. This can limit the precision of predicting a protein's binding site on the DNA molecule. Hydroxyl radicals are created from the Fenton reaction, which involves reducing Fe^{2+} with H_2O_2 to form free hydroxyl molecules. These hydroxyl molecules react with the DNA backbone, resulting in a break. Due to their small size, the resulting DNA footprint has high resolution. Unlike DNase I they have no sequence dependence and result in a much more evenly distributed ladder. The negative aspect of using hydroxyl radicals is that they are more time consuming to use, due to a slower reaction and digestion time. Ultraviolet irradiation can be used to excite nucleic acids and create photoreactions, which results in damaged bases in the DNA strand. Photoreactions can include: single strand breaks, interactions between or within DNA strands, reactions with solvents, or crosslinks with proteins. The workflow for this method has an additional step, once both your protected and unprotected DNA have been treated, there is subsequent primer extension of the cleaved products. The extension will terminate upon reaching a damaged base, and thus when the PCR products are run side-by-side on a gel; the protected sample will show an additional band where the DNA was crosslinked with a bound protein. Advantages of using UV are that it reacts very quickly and can therefore capture interactions that are only momentary. Additionally it can be applied to in vivo experiments, because UV can penetrate cell membranes. A disadvantage is that the gel can be difficult to interpret, as the bound protein does not protect the DNA, it merely alters the photoreactions in the vicinity



Advance application

1. In vivo footprinting

In vivo footprinting is a technique used to analyze the protein-DNA interactions that are occurring in a cell at a given time point. DNase I can be used as a cleavage agent if the cellular membrane has been permeabilized. However the most common cleavage agent used is UV irradiation because it penetrates the cell membrane without disrupting cell state and can thus capture interactions that are

sensitive to cellular changes. Once the DNA has been cleaved or damaged by UV, the cells can be lysed and DNA purified for analysis of a region of interest. Ligation-mediated PCR is an alternative method to footprint in vivo. Once a cleavage agent has been used on the genomic DNA, resulting in single strand breaks, and the DNA is isolated, a linker is added onto the break points. A region of interest is amplified between the linker and a gene-specific primer, and when run on a polyacrylamide gel, will have a footprint where a protein was bound. In vivo footprinting combined with immunoprecipitation can be used to assess protein specificity at many locations throughout the genome. The DNA bound to a protein of interest can be immunoprecipitated with an antibody to that protein, and then specific region binding can be assessed using the DNA footprinting technique.

2. Quantitative footprinting

The DNA footprinting technique can be modified to assess the binding strength of a protein to a region of DNA. Using varying concentrations of the protein for the footprinting experiment, the appearance of the footprint can be observed as the concentrations increase and the proteins binding affinity can then be estimated.

3. Detection by capillary electrophoresis

To adapt the footprinting technique to updated detection methods, the labelled DNA fragments are detected by a capillary electrophoresis device instead of being run on a polyacrylamide gel. If the DNA fragment to be analyzed is produced by polymerase chain reaction (PCR), it is straightforward to couple a fluorescent molecule such as carboxyfluorescein (FAM) to the primers. This way, the fragments produced by DNaseI digestion will contain FAM, and will be detectable by the capillary electrophoresis machine.

DNA-fingerprinting:

DNA-fingerprinting (also called DNA typing or DNA profiling). It is a technique of determin-ing nucleotide sequences of certain areas of DNA which are unique to each individual. Each person has a unique DNA fingerprint.

Unlike a conventional fingerprint that occurs only on the fingertips and can be altered by surgery, a DNA fingerprint is the same for every cell, tissue and organ of a person. It cannot be changed by any known treatment. The ideal way to distinguish an individual — from other people would be his or her entire genomic DNA sequence.

It has been a subject of human interest since primitive times when man used to hunt for his food with the help of animal's foot prints. Science of fingerprinting was first used by Sir William Herschel as a method of identification in 1858. In India the science of fingerprints was discovered by chance during a murder investigation in Jalpaiguri in 1897.

Alec Jeffreys (1984) invented the DNA fingerprinting technique at Leicester University, United Kingdom. Dr. V.K. Kashyap and Dr. Lalji Singh started the DNA fingerprinting technology in India at CCMB (Centre for Cell and Molecular Biology) Hyderabad.

Principle of DNA Fingerprinting:

By their differences, about 0.1% or 3 x 106 base pairs (out of 3 x 109 bp) provide individuality to each human being. Human genome pos-sesses numerous small noncoding but inheritable sequences of bases which are repeated many times. These sequences occur near telomere, centromeres, Y chromosome and hetero-chromatic area. The area with same sequence of bases repeated several times is called repetitive DNA.

They can be separated as satellite from the bulk DNA during density gradient centrifugation and hence called satellite DNA. In satellite DNA, repetition of bases is in tandem. Depending upon length, base composition and numbers of tandemly re-petitive units, satellite DNAs have subcat-egories like microsatellites and mini-satellites. Satellite DNAs show poly-morphism. The term polymorphism is used when a variant at a locus is present with a frequency of more than 0.01 population.

Variations occur due to mutations. While mutations in genes produce alleles with dif-ferent expressions, mutations in noncoding repetitive DNA have no immediate impact.

These mutations in the noncoding sequences have piled up with time and form the basis of DNA polymorphism (variation at genetic level arises due to mutations). DNA polymorphism is the basis of genetic mapping of human genome as well as DNA finger printing.

Technique*:*

1. The first step is to obtain DNA sample of the individual in question.
2. DNA is also isolated from bloodstains, semen stains or hair root from the body of the victim or from victim's cloth even after many hours of any criminal offence. Even it can be obtained from vaginal swabs of rape victims. The amount of DNA needed for developing fingerprints is very small, only a few nanograms.
3. The DNA is digested with a suitable restriction endonuclease enzyme, which cuts them into fragments.
4. The fragments are subjected to gel electrophoresis by which the fragments are separated according to their size.
5. The separated fragments are copied onto a nitrocellulose filter membrane by Southern blotting technique.
6. Special DNA probes are prepared in the laboratory and made radioactive by labeling with radioactive isotopes. These probes contain repeated sequences of bases complimentary to those on mini satellites.
7. The DNA on the nitrocellulose filter membrane is hybridized with the radioactive probes and the free probes are washed off.
8. The bands to which the radioactive probes have been hybridized are detected through autoradiography. This is a technique where an X-ray film is exposed to the nitrocellulose membrane to mark the places where the radioactive DNA probes have bound to the DNA fragments. These places are marked as dark bands when X-ray film is exposed.
9. The dark bands on the X-ray film represent the DNA fingerprints or DNA profiles.
10. Comparison is made between the banding pattern of collected DNA sample and suspected human subject to confirm the criminal with hundred percent accuracy

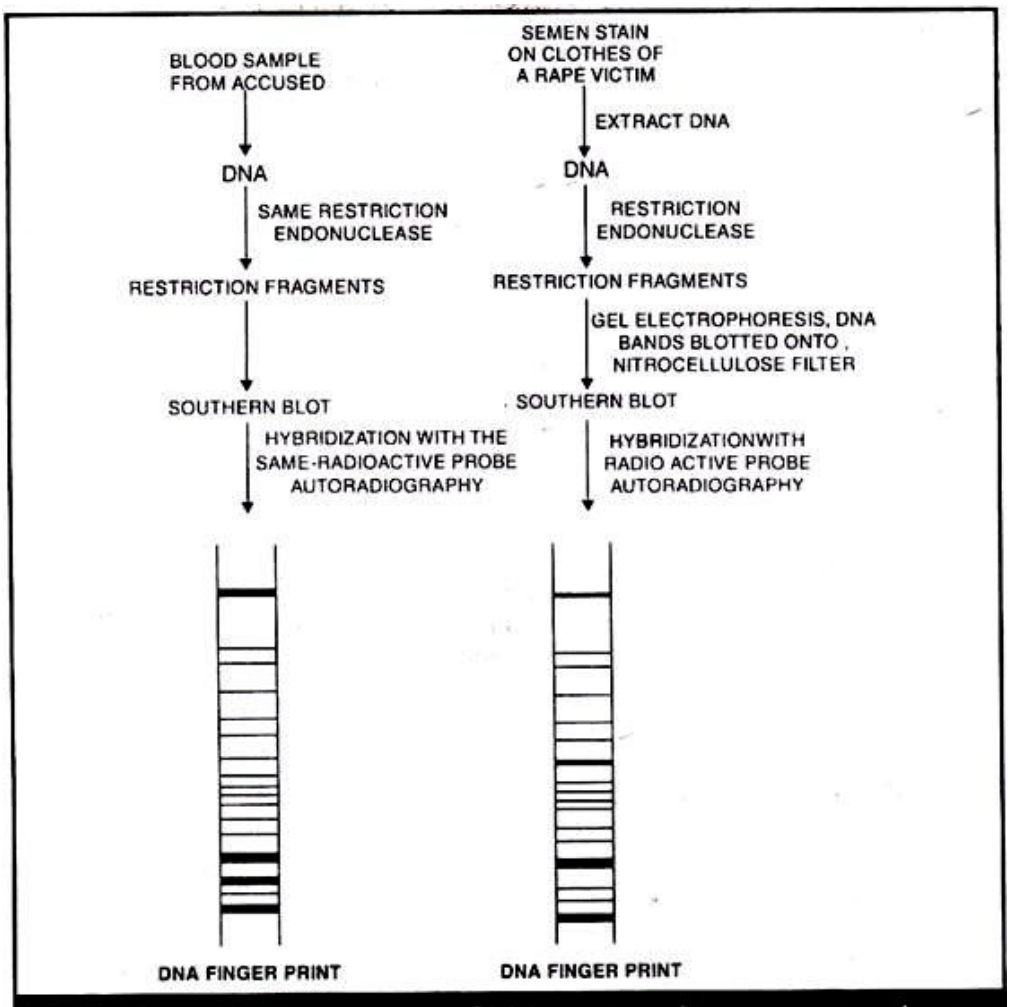
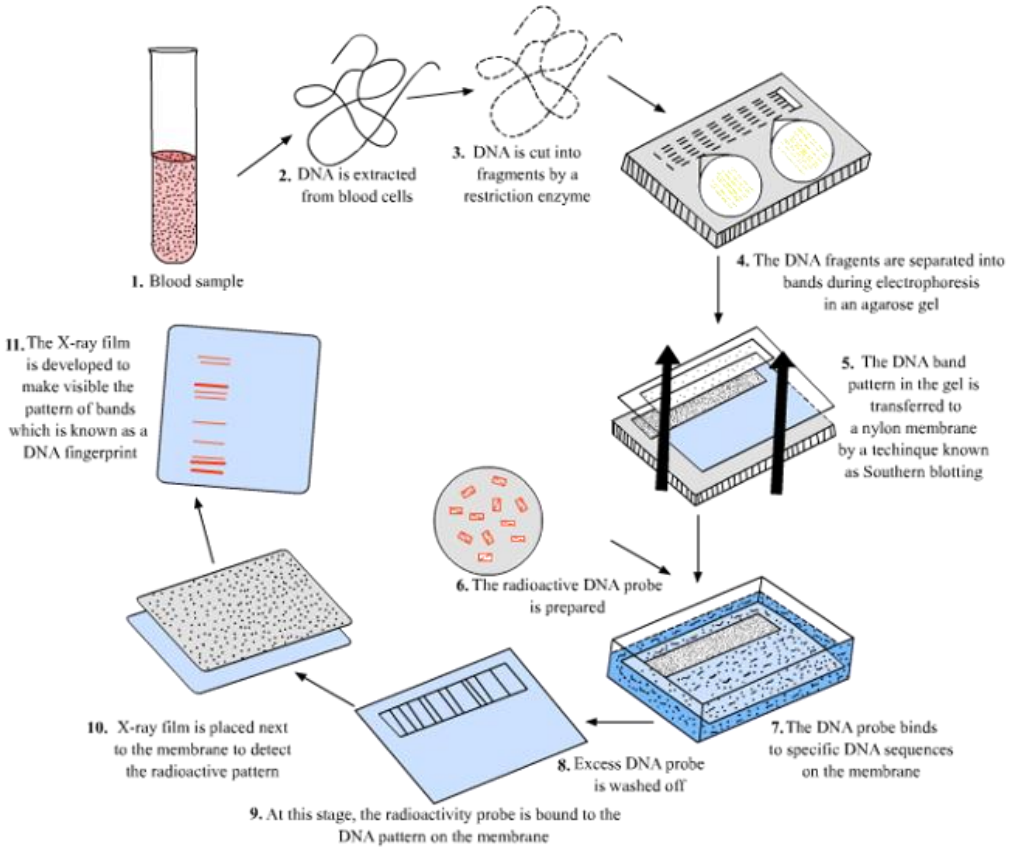


Fig. 5.24 A schematic representation of DNA fingerprinting of a person accused of rape, and of the semen stain recovered from the clothes of rape victim.



Steps in DNA fingerprinting techinque

Application:

DNA Fingerprinting is used by scientists to distinguish between individuals of the same species using only samples of their DNA. It is a primary method for identifying an individual.

1. **Forensic Science:**

Biological materials used for DNA profiling are: Blood, Hair, Saliva, Semen, Body tissue cells etc. DNA isolated from the evidence sample can be compared through VNTR (Variable number of tandem repeats) prototype. It is useful in solving crimes like murder and rape.

2. **Paternity and Maternity Determination:**

A Person accedes to his or her VNTRs from his or her parents. Parent-child VNTR prototype analysis has been used to solve disputed cases. This information can also be used in inheritance cases, immigration cases.

3. **Personal Identification:**

It utilizes the concept of using DNA fingerprints as a sort of genetic bar code to pinpoint individuals.

4. **Diagnosis of Inherited Disorders:**

It is also useful in diagnosing inherited disorders in both prenatal and newborn babies. These disorders may include cystic fibrosis, hemophilia, Huntington's disease, familial Alzheimer's, sickle cell anemia, thalassemia, and many others.

5. **Development of Cures for Inherited Disorders:**

By studying the DNA fingerprints of relatives who have a history of some particular disorder, DNA prototypes associated with the disease can be ascertained.

6. **Detection of AIDS:**

By comparing the band of HIV "RNA" (converted to DNA using RTPCR) with the bands form by the man's blood, person suffering with AIDS can be identified.

7. **Breeding Program:**

Breeders conventionally use the phenotype to evaluate the genotype of a plant or an animal.As it is difficult to make out homozygous or heterozygous dominance from appearance, the DNA fingerprinting allows a fastidious and precise determination of genotype. It is basically useful in breeding race horses and hunting dogs.

4. Transgenics: Development strategies; Transgenics in relation to insect, herbicide, stress resistance; delayed fruit ripening; golden rice; vaccine development; male sterility; molecular farming; flower colour; terminator gene sequence.

Transgene- It is a foreign gene or genetic material that has been transferred naturally or by any of a number of genetic engineering techniques from one organism to another.

Transgenesis- The phenomenon of introduction of exogenous DNA into the genome to create and maintain a stable and heritable character.

Transgenic plants- The plant whose genome is altered by adding one or more transgenes are known as transgenic plants.

The ultimate goal of transgenic (involving introduction, integration, and expression of foreign genes) is to improve the crops, with the desired traits.

i. Resistance to biotic stresses i.e. resistance to diseases caused by insects, viruses, fungi and bacteria.
ii. Resistance to abiotic stresses-herbicides, temperature (heat, chilling, freezing), drought, salinity, ozone, intense light.
iii. Improvement of crop yield, and quality e.g. storage, longer shelf life of fruits and flowers.
iv. Transgenic plants with improved nutrition.
v. v. Transgenic plants as bioreactors for the manufacture of commercial products e.g. proteins, vaccines, and biodegradable plastics.

Development of transgenic:

i. Identification of useful Genes:

The desirable genes may be located in wild species, unrelated plant species, unrelated organisms i.e., micro-organisms (bacteria, viruses or fungi) and animals. This work is carried out in the research laboratory.

ii. Designing Gene for Insertion:

The gene of interest is isolated from the donor source and cloned in the laboratory. The cloning is done generally using plasmids.

iii. Insertion of Gene into Target Plant:

The cloned gene i.e., multiple copies of the gene of interest are inserted into the host plant or the recipient plant. Two methods, viz. agrobacterium mediated and gene gun or particle bombardment methods are used for gene transfer. This is done in the research laboratory. Protoplast culture for plasmid method and meristematic tissues or embryogenic callus is used for gene gun method.

iv. Identification of Transgenic Cells:

Transformed cells are identified using selectable marker (Kanamycin) and are regenerated into whole plant in nutrient medium. The regenerated plant is compared with parent variety. It should look like parent variety except gene of interest. This work is done in the laboratory and glass house.

v. Small Scale Field Trials:

The transgenic plants are evaluated for their performance in small scale field trials. The seed of the transgenic plant such as cotton, soybean, rapeseed, etc. is tested in laboratory for biosafety i.e., allergen city and toxicity. These tests are conducted with animals such as rats, rabbits, poultry, goats etc.

vi. Larger Field Trials:

Transgenic plants which are passed by regulatory authority are evaluated in multiplication trials for their performance for the gene of interest. Superior performing genotypes are released after testing and stable performance for three years.

Insect (Pest) Resistance:

It is estimated that about 15% of the world's crop yield is lost to insects or pests. The damage to crops is mainly caused by insect larvae and to some extent adult insects.

The majority of the insects that damage crops belong to the following orders (with examples):

i. Lepidoptera (bollworms).
ii. Coleoptera (beetles).
iii. Orthoptera (grasshoppers).
iv. Homoptera (aphids).

Till some time ago, chemical pesticides are the only means of pest control. Transgenic plants with insect resistance transgenes have been developed. About 40 genes obtained from microorganisms of higher plants and animals have been used to provide insect resistance in crop plants.

Resistance Genes from Microorganisms:

***Bacillus thuringiensis* (Bt) toxin:**

Bacillus thuringiensis was first discovered by Ishiwaki in 1901, although its commercial importance was ignored until 1951. *B. thuringiensis* is a Gram negative, soil bacterium. This bacterium produces a parasporal crystalline proteinous toxin with insecticidal activity. The protein produced by B .thuringiensis is referred to as insecticidal crystalline protein (ICP). ICPs are among the endotoxins produced by sporulating bacteria, and were originally classified as δ-endotoxins (to distinguish them from other classes of α-, β- and y-endotoxins).

Bt toxin genes:

Several strains of B. thuringiensis producing a wide range of crystal (cry) proteins have been identified. Further, the structure of cry genes and their corresponding toxin (δ-endotoxin) products have been characterized. The cry genes are classified into a large number of distinct families (about 40) designated as cry 1…… cry 40, based on their size and sequence similarities. And within each family, there may be sub-families. Thus, the total number of genes producing Bt toxins (Cry proteins) is more than 100.

There are differences in the structure of different Cry proteins, besides certain sequence similarities. The molecular weights of Cry proteins may be either large (~130 KDa) or small (~70KDa). Despite the differences in the Cry proteins, they share a common active core of three domains.

Different cry protein produced by Bacillus:

Cry I : kills butterflies and moths
Cry II : kills butterflies and flies
Cry III : kills beetles
Cry IV : kills only flies

Mode of action of Cry proteins:

Most of the Bt toxins (Cry proteins) are active against Lepidopteran larvae, while some of them are specific against Dipteran and Coleopteran insects. The pro-toxin of Cry I toxin group has a molecular mass of 130 kilo Daltons (130 KDa).

When this parasporal crystal is ingested by the target insect, the pro-toxin gets activated within its gut by a combination of alkaline pH (7.5 to 8.5) and proteolytic enzymes. This results in the conversion of pro-toxin into an active toxin with a molecular weight of 68 KDa.

The active form of toxin protein gets itself inserted into the membrane of the gut epithelial cells of the insect. This result in the formation of ion channels through which there occurs an excessive loss of

cellular ATP. As a consequence, cellular metabolism ceases, insect stops feeding, and becomes dehydrated and finally dies.

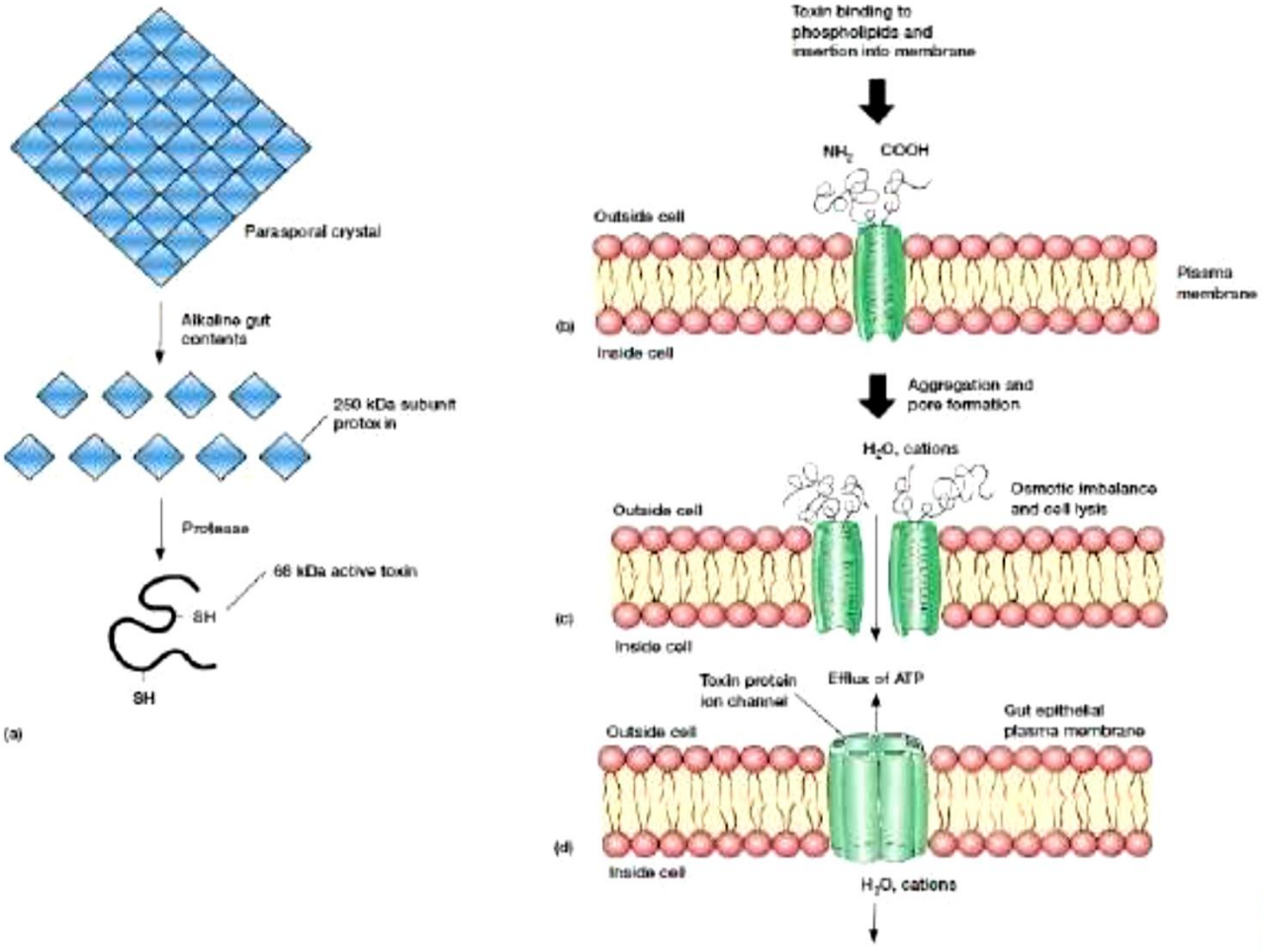
Some workers in the recent years suggest that the Bt toxin opens cation-selective pores in the membranes, leading to the inflow of cations into the cells that causes osmotic lysis and destruction of epithelial cells (and finally the death of insect larvae). The Bt toxin is not toxic to humans and animals since the conversion of pro-toxin to toxin requires alkaline pH and specific proteases.

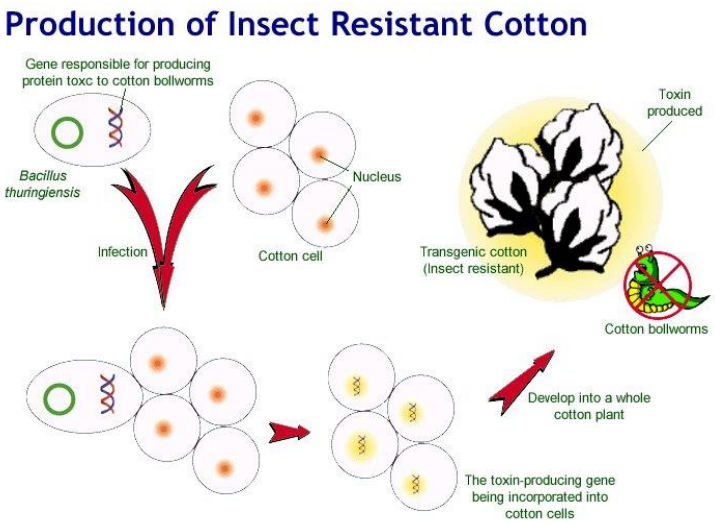
The usage Bt is commonly used for a transgenic crop with a cry gene e.g. Bt cotton. In the same way, Cry proteins are also referred to as Bt proteins. It may also be stated here that the authors use four different names for the same group of proteins-δ-endotoxin, insecticidal crystal protein (ICP), Cry and now Bt.

Bt-based genetic transformation of plants:

It has been possible to genetically modify (GM) plants by inserting Bt genes and provide pest resistance to these transformed plants. For an effective pest resistance, the bacterial gene in transgenic plants must possess high level expression. This obviously means that the transgene transcription should be under the effective control of promoter and terminator sequences. The early attempts to express cry 1A and cry 3A proteins under the control of CaMV 35S or Agrobacterium T-DNA promoters resulted in a very low expression in tobacco, tomato and potato plants.

In March 1995, the first Bt crop deregulated in the U.S. were seven lines of Colorado Potato Beetle Resistant Bt Potato by Monsanto. Since then, many more Bt crops have been deregulated, engineered to produce a variety of different Bt proteins from various subspecies of Bt. Bt crops include:





Corn:

European Corn Borer Resistant Corn (first deregulated in the U.S. in May 1995)

Corn Rootworm Resistant Corn (first deregulated in the U.S. in October 2002)

Cotton:

Lepidopteran Resistant Cotton (first deregulated in the U.S. in June 1995)

Potato:

Colorado Potato Beetle Resistant Bt Potato (first deregulated in the U.S. in March 1995)

Potato Tuber Moth Resistant Bt Potato (being developed in South Africa)

Soybean:

Bt Soybean (first deregulated in the U.S. in October 2011, not yet sold commercially)

Tomato:

Lepidopteran Resistant Tomato (first deregulated in the U.S. in March 1998, not yet sold commercially)

Resistance Genes from Higher Plants:

Certain genes from higher plants were also found to result in the synthesis of products possessing insecticidal activity. Some authors regard them as non-Bt insecticidal proteins. A selected list of plant insecticidal (non-Bt) genes used for developing transgenic plants with insect resistance is given Table. Some of them are briefly described

TABLE 50.3 A selected list of plant insecticidal (non-*Bt*) genes used for developing transgenic plants with insect resistance

Plant gene	*Transgenic plant(s)*	*Encoded protein*	*Resistance to insect(s)*
Protease inhibitors			
CpTi	Potato, apple, rice, sunflower, wheat, tomato	Trypsin	Coleoptera, Lepidoptera
CII	Tobacco, potato	Serine protease	Coleoptera, Lepidoptera
PI-IV	Potato, tobacco	Serine protease	Lebidoptera
OC-1	Tobacco, oilseed rape	Cysteine protease	Coleoptera, Homoptera
CMe	Tobacco	Trypsin	Lebidoptera
α-Amylase inhibitors			
α-A1-Pv	Pea, tobacco	α-Amylase	Coleoptera
WMAI-1	Tobacco	α-Amylase	Lepidoptera
Lectins			
GNA	Potato, rice, sugarcane sweet potato, tobacco	Lectin	Homoptera, Lepidoptera
WGA	Maize	Agglutin	Lepidoptera, Coleoptera

Herbicide Resistance:

Weed infestations destroy about 10 percent of crops worldwide. To combat weeds, farmers often apply herbicides before seeding a crop and between rows after the crops are growing. As the most efficient broad-spectrum herbicides also kill crop plants, herbicide use may be difficult and limited. Farmers also use tillage to control weeds; however, tillage damages soil structure and increases erosion.

A good or an ideal herbicide is expected to possess the following characteristics:

i. Capable of killing weeds without affecting crop plants.
ii. Not toxic to animals and microorganisms.
iii. Rapidly translocated within the target plant.
iv. Rapidly degraded in the soil.

Glyphosate Resistance:

Glyphosate, is a glycine derivative. It acts as a broad-spectrum herbicide and is effective against 76 of the world's worst 78 weeds. Glyphosate is less toxic to animals and is rapidly degraded by microorganisms. In addition, it has a short half-life. The American chemical company Monsanto markets glyphosate as **Round up**.

Mechanism of action of glyphosate:

Glyphosate is rapidly transported to the growing points of plants. It is capable of killing the plants even at a low concentration. Glyphosate acts as a competitive inhibitor of the enzyme 5-enoylpyruvylshikimate 3-phosphate synthase (EPSPS). This is a key enzyme in shikimic acid pathway that results in the formation of aromatic amino acids (tryptophan, phenylalanine and tyrosine), phenols and certain secondary metabolites.

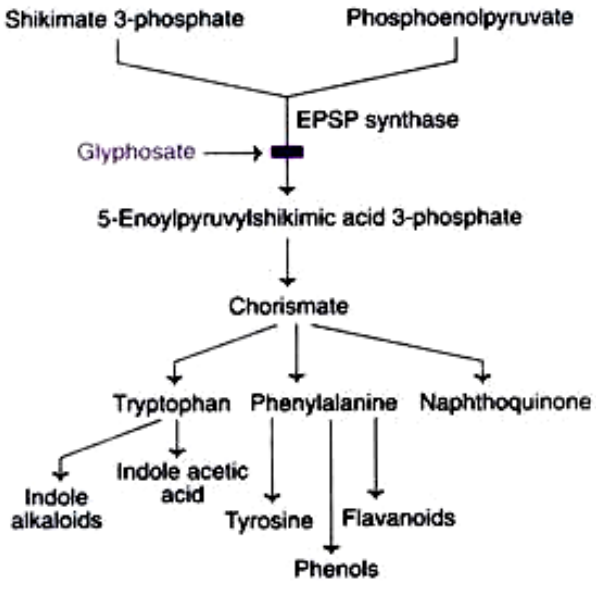


Fig. 50.5 : Shikimate pathway indicating the action of the herbicide glyphosate (EPSP synthase-5-Enolylpyruvylshikimate 3-phosphate synthase)

The enzyme EPSPS catalyses the synthesis of 5-enoylpyruvylshikimate 3-phosphate from shikimate 3-phosphate and phosphoenoylpyruvate. Glyphosate has some structural similarly with the substrate phosphoenol pyruvate. Consequently, glyphosate binds more tightly with EPSPS and blocks the normal shikimic acid pathway. Thus, the herbicide glyphosate inhibits the biosynthesis of aromatic amino acids and other important products.

This results in inhibition of protein biosynthesis (due to lack of aromatic amino acids). As a consequence, cell division and plant growth are blocked. Further, the plant growth regulator indole acetic acid (an auxin) is also produced from tryptophan. The net result of glyphosate is the death of the plants. Glyphosate is toxic to microorganisms as they also possess shikimate pathway.

Glyphosate is non-toxic to animals (including humans), since they do not possess shikimate pathway.

Strategies for glyphosate resistance:

There are three distinct strategies to provide glyphosphate resistance to plants:

1. Overexpression of crop plant EPSPS gene:

An overexpressing gene of EPSPS was detected in *Petunia*. This expression was found to be due to gene amplification rather than an increased expression of the gene. EPSPS gene from *Petunia* was isolated and introduced into other plants. The increased synthesis of EPSPS (by about 40 fold) in transgenic plants provides resistance to glyphosate. These plants can tolerate glyphosate at a dose of 2-4 times higher than that required to kill wild-type plants.

2. Use of mutant EPSPS genes:

An EPSPS mutant gene that conferred resistance to glyphosate was first detected in the bacterium Salmonella typhimurium. It was found that a single base substitution (C to 7) resulted in the change of an amino acid from proline to serine in EPSPS. This modified enzyme cannot bind to glyphosate, and thus provides resistance.

The mutant EPSPS gene was introduced into tobacco plants using *Agrobacterium* Ti plasmid vectors. The transgene produced high quantities of the enzyme EPSPS. However, the transformed tobacco plants provided only marginal resistance to glyphosate. The reason for this was not immediately identified.

3. Detoxification of glyphosate:

The soil microorganisms possess the enzyme glyphosate oxidase that converts glyphosate to glyoxylate and aminomethylphosponic acid. The gene encoding glyphosate oxidase has been isolated from a soil organism Ochrobactrum anthropi. With suitable modifications, this gene was introduced into crop plants e.g. oilseed rape. The transgenic plants were found to exhibit very good glyphosate resistance in the field.

Use of a combined strategy:

More efficient resistance of plants against glyphosate can be provided by employing a combined strategy. Thus, resistant (i.e. mutant) EPSPS gene in combination with glyphosate oxidase gene are used. By this approach, there occurs glyphosate resistance (due to mutant EPSPS gene) as well as its detoxification (due to glyphosate oxidase gene).

Phosphinothricin Resistance:

Phosphinothricin (or glufosinate) is also a broad spectrum herbicide like glyphosate. Phosphinothricin is more effective against broad-leafed weeds but least effective against perennials.

Phosphinothricin-a natural herbicide:

Phosphinothricin is an unusual herbicide, being a derivative of a natural product namely bialaphos.

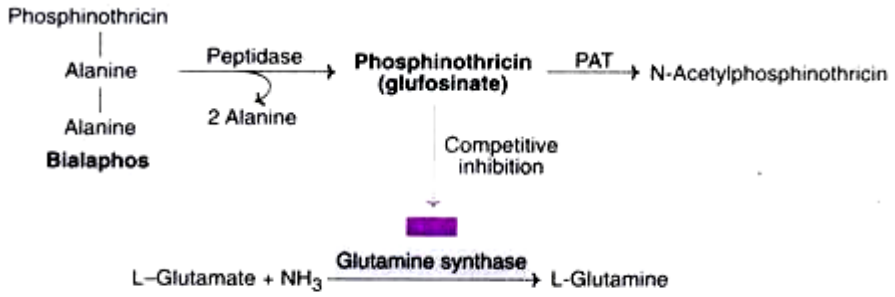


***Fig. 50.7** : The formation, mode of action and detoxification of phosphinothricin (PAT-Phosphinothricin acetyl transferase).*

Certain species of Streptomyces produce bialaphos which is a combination of phosphinothricin bound to two alanine residues, forming a tripeptide. By the action of a peptidase, bialaphos is converted to active phosphinothricin.

Mechanism of action of phosphinothricin:

Phosphinothricin acts as a competitive inhibitor of the enzyme glutamine synthase. This is possible since phosphinothricin has some structural similarity with the substrate glutamate. As a consequence of the inhibition of glutamine synthase, ammonia accumulates and kills the plant cells. Further, disturbance in glutamine synthesis also inhibits photosynthesis. Thus, the herbicidal activity of phosphinothricin is due to the combined effects of ammonia toxicity and inhibition of photosynthesis.

Strategy for phosphinothricin resistance:

The natural detoxifying mechanism of phosphinothricin observed in Streptomyces sp has prompted scientists to develop resistant plants against this herbicide. The enzyme phosphinothricin acetyl transferase (of *Streptomyces* sp) acetylates phosphinothricin, and thus inactivates the herbicide.

The gene responsible for coding phosphinothricin acetyl transferase (bar gene) has been identified in *Streptomyces hygroscopicus*. Some success has been reported in developing transgenic maize and oilseed rape by introducing bar gene. These plants were found to provide resistance to phosphinothricin.

Bromoxynil

Tobacco plants have been engineered to be resistant to the herbicide bromoxynil.

Glufosinate

Crops have been commercialized that are resistant to the herbicide glufosinate, as well. Crops engineered for resistance to multiple herbicides to allow farmers to use a mixed group of two, three, or four different chemicals are under development to combat growing herbicide resistance.

2,4-D

In October 2014 the US EPA registered Dow's Enlist Duo maize, which is genetically modified to be resistant to both glyphosate and 2,4-D, in six states. Inserting a bacterial aryloxyalkanoate dioxygenase gene, aad1 makes the corn resistant to 2,4-D. The USDA had approved maize and soybeans with the mutation in September 2014.

Herbicides are simply chemical compounds that kill or inhibit the growth of plants without deleterious effects on animals. Herbicides usually inhibit processes that are unique to plants, e.g. photosynthesis. Mostly herbicides act as inhibitors of essential enzyme reactions. Any change which can reduce the

Table 17.3. Gene based herbicide resistance in plants

Herbicide	Mode of development of herbicide resistance
Triazines	Resistance is due to an alteration in the psbA gene, which codes for the target of this herbicide, chloroplast protein D1.
Sulphonylureas	Genes encoding resistant version of the enzyme acetolactate synthetase have been introduced into poplar, canola, flax, and rice.
Glyphosate Bromoxynil	Resistance is from overproduction of EPSPS, the target of this herbicide. Resistance to this photosystem II inhibitor has been created by transforming tobacco and cotton plants with a bacterial nitrilase gene, which encodes an enzyme that degrades this herbicide.
Phenoxy carboxylic acids (e.g., 2, 4-D and 2, 4, 5-T)	Resistant cotton and tobacco plants have been created by transformation with the rfdA gene from Alcaligenes, which encodes a dioxygenase that degrades this herbicide.
Gluphosinate (Phosphinothricin)	Over 200 different plants have been transformed with either the bar gene from *Streptomyces hygroscopicus* or the pat gene from *S. viridochromogenes*. The phosphinothricin acetyltransferase that these genes encode, detoxifies this herbicide.
Cyanamide	Resistant tobacco plants were produced when cyanamide hydrates gene from the fungus *Myrothecium verrucarla* was introduced. The enzyme encoded by this gene converts cyanamide to urea.

inhibitory effect of herbicide will provide increased herbicide tolerance.

Transgenic strategy of stress in plants

In Arabidopsis, overexpression of cDNA encoding DREB1 and DREB2 triggered expres-sion of many stress tolerance genes under normal growing conditions. Thus, transgenic strat-egy resulted in increased tolerance to drought, salinity and cold stress. Researchers from Japan International Research Center for Agricultural Science (JIRCAS) identified 12 stress-inducible genes which are targeted by DREBIA transcription factor protein.

In the transgene construct DREBIA) was driven under the control of Camv 35S promoter (35S:DREB1A). In the notable observation, all the transgenic plants carrying 35S:DREB1A transgene exhibited severe retar-dation under normal growth condition. This negative effect is probably due to different levels of expression of DREBIA transgenes.

Over production of transcription factor proteins resulted in the expression of all stress inducible target genes, rd29A, rdl7, cor 6.6, cor 15a, erd 10 and kinl. Under normal conditions, plants under this situation suffer heav-ily due to unnecessary drenching of energy for the production of several hundreds of proteins. This problem was effectively tackled by using stress inducible rd 29A promoter.

In an extended transgenic strategy DREBIA gene expressed under the control of stress inducible rd 29A pro-moter provides protection and shows minimal effect on plant growth. In addition, it provides high degree of tolerance to stress condition rather when it is expressed under 35S Camv pro-moter. Since rd 29A promoter is stress inducible, it did not cause expression of the transcription factor gene (DREB).

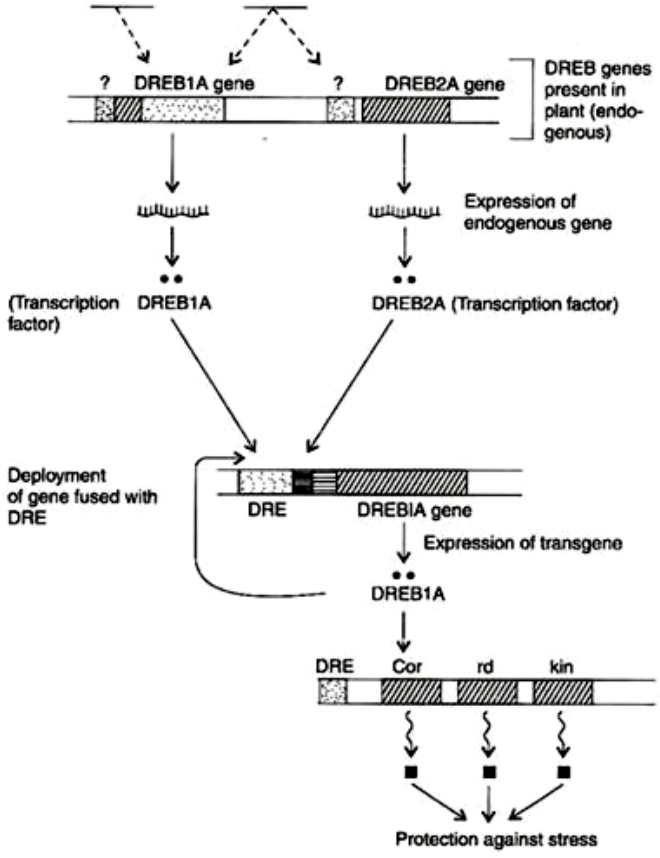


Fig. 15.5 Overexpression of stress inducible transcription factor protein and amplification of signalling pathway by overexpressing the introduced gene (DREB1) fused with DRE-*cis*-acting sequence (after Smirnoff and Bryant, 1999.)

Under unstressed conditions, instead, it rapidly enhances the expression of DREBIA transgene only under dehydration, high salt and low temperature conditions . Overall rd 29A promoter may be useful to improve stress tolerance of agriculturally important crops by gene transfer. In the earlier transgenic work, Shinozaki and coworkers have amplified signalling pathway by overexpressing gene containing fusion of a DRE-containing promoter with a DREB gene.

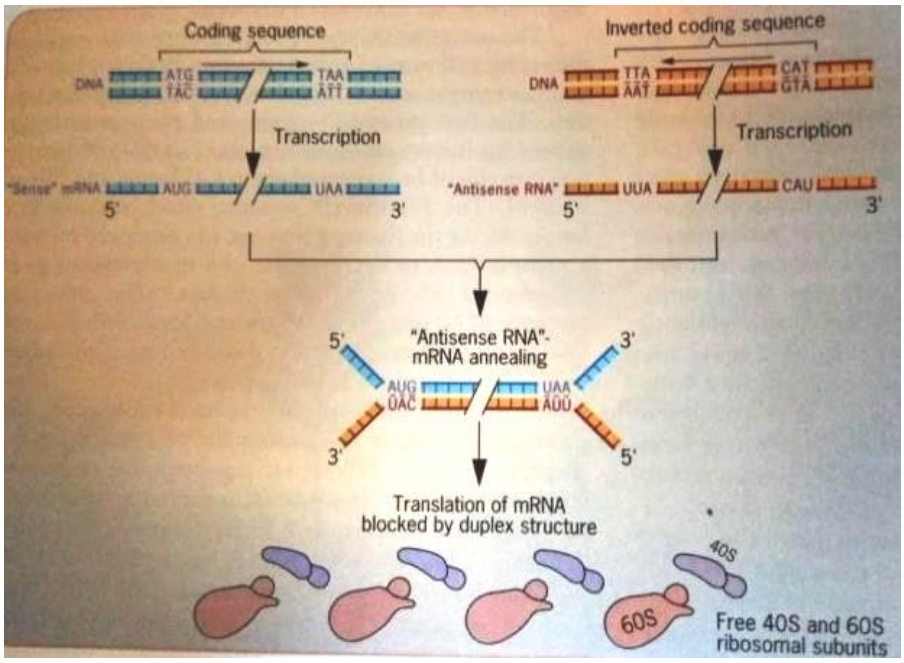
This enhances further DREBIA expression in response to stress. This amplified strategy led to strong induction of DRE-containing target genes. Some contrasting experiments show that DREBIA cannot

induce the expression of other drought-responsive genes (such as PSCS, erdl, rd22 and rd 29B) which contain no DRE element in their promoter region.

Delayed fruit ripening:

Antisense RNA approach has proven useful to improve shelf life of fruits and vegetables. It has also been a commercial success. The antisense RNA method involves the synthesis of the RNA molecules that are complementary to the mRNA molecules produced by transcription of a given gene. The antisense is produced by inverting the orientation of the protein encoding region of a gene in relation to its promoter. A s result, the reference sense strand of the gene becomes oriented 3'-5' direction with reference to its promoter and is transcribed. The RNA produced by this gene has the same sequence as the antisense strand of the normal gene and is therefore known as antisense RNA.

A major problem in agriculture is perishing ability of its products. Various methods have been initiated and developed to increase the shelf life of crops especially for fruits and vegetables. One such successful technique is delaying the ripening of fruits and vegetables by manipulating the genes through genetic engineering.



Flavr Savr is a genetically modified tomato, was the first commercially grown genetically engineered food to be granted a license for human consumption. It was produced by Californian Company Calgene 1992. Calgene introduced a gene in plant which synthesizes a complementary mRNA to PG gene and inhibiting the synthesis of PG enzyme. On May 21, 1994, the genetically engineered Flavr Savr tomato was introduced.

Fruit ripening is an active process characterized by increased respiration accompanied by a rapid increase in ethylene synthesis. As the chlorophyll gets degraded, the green color of fruit disappears and a red pigment, lycopene is synthesized. The fruit gets softened as a result of the activity of cell wall degrading enzymes namely polygalacturonase (PG) and methyl

esterase. The phyto hormone ethylene production is linked to fruit ripening as the same is known to trigger the ripening effect. The breakdown of starch to sugars and accumulation of large number of secondary products improves the flavor, taste and smell of the fruits.

Genes involved in tomatoes ripening:

i) **pTOM5** encodes for phytoene synthase which promote lycopene synthesis that gives red coloration
ii) **pTOM6** gene encodes for polygalacturonase. This enzyme degrades the cell wall, resulting in fruit softening.
iii) **pTOM** gene encodes for ACC oxidase. This enzyme catalyzes the ethylene formation that triggers the fruit ripening.

Development of flavr savr tomato

Softening of fruits is largely due to degradation of cell wall (pectin) by enzyme polygalacturonase (PG). The gene encoding PG has been isolated and cloned (pTOM6).

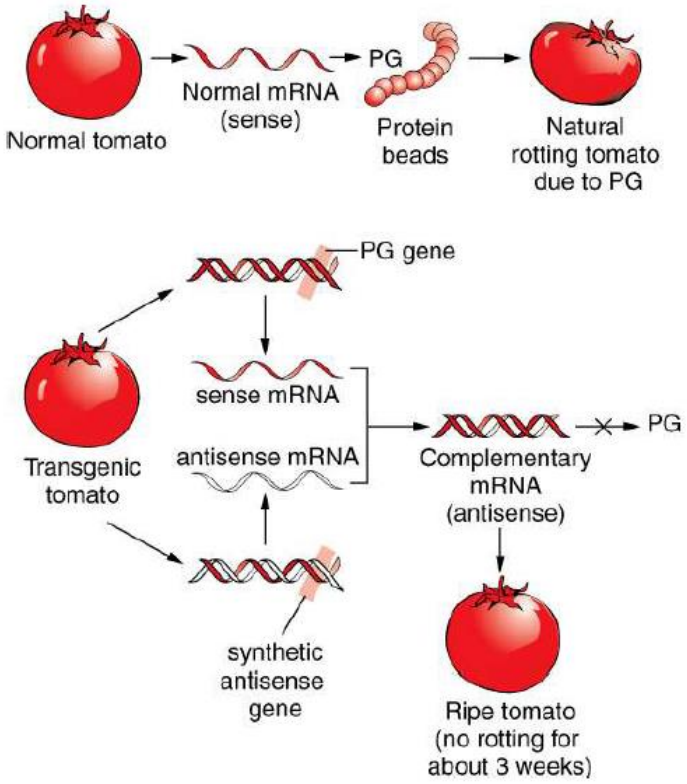
Procedure involves: -

1) Isolation of DNA from tomato plant that encodes the enzyme polygalacturonase (PG).
2) Transfer of PG gene to a vector bacteria and production of complementary DNA (cDNA) molecules.
3) Introduction of cDNA into a fresh tomato plant to produce transgenic plant.

Mechanism of pg antisense RNA approach

In normal plants, PG gene encodes a normal or sense mRNA that produce the enzyme PG and it is actively involved in fruit ripening.

- The cDNA of PG encodes for antisense mRNA, which is complementary to sense mRNA.
- The hybridization between sense and antisense mRNA render the sense mRNA ineffective.Consequently, no polygalacturonase is produced hence fruit ripening is delayed.



Advantages:

1. Slower ripen rate
2. Ripen longer on vine
3. Fully developed flavors
4. Increase the shelf life

The rise and fall of Flavr Savr Tomato:

The genetically engineered tomato, known as Flavr Savr (pronounced flavour saver) by employing PC antisense RNA was approved by U.S. Food and Drug Administration on 18th May 1994. The FDA ruled that Flavr Savr tomatoes are as safe as tomatoes that are bred by conventional means, and therefore no special labeling is required. The new tomato could be shipped without refrigeration too far off places, as it was capable of resisting rot for more than three weeks (double the time of a conventional tomato).

Although Flavr Savr was launched with a great fanfare in 1995, it did not fulfill the expectation for the following reasons:

i. Transgenic tomatoes could not be grown properly in different parts of U.S.A.
ii. The yield of tomatoes was low.
iii. The cost of Flavr Savr was high.

It is argued that the company that developed Flavr Savr, in its overenthusiasm to become the first Biotech Company to market a bioengineered food had not taken adequate care in developing the transgenic plant. And unfortunately, within a year after its entry, Flavr Savr was withdrawn, and it is now almost forgotten

Golden Rice —The Provitamin A Enriched Rice:

About one-third of the world's population is dependent on rice as staple food. The milled rice that is usually consumed is almost deficit in P-carotene, the pro-vitamin A. As such, vitamin A deficiency (causing night blindness) is major nutritional disorder world over, particularly in people subsisting on rice.

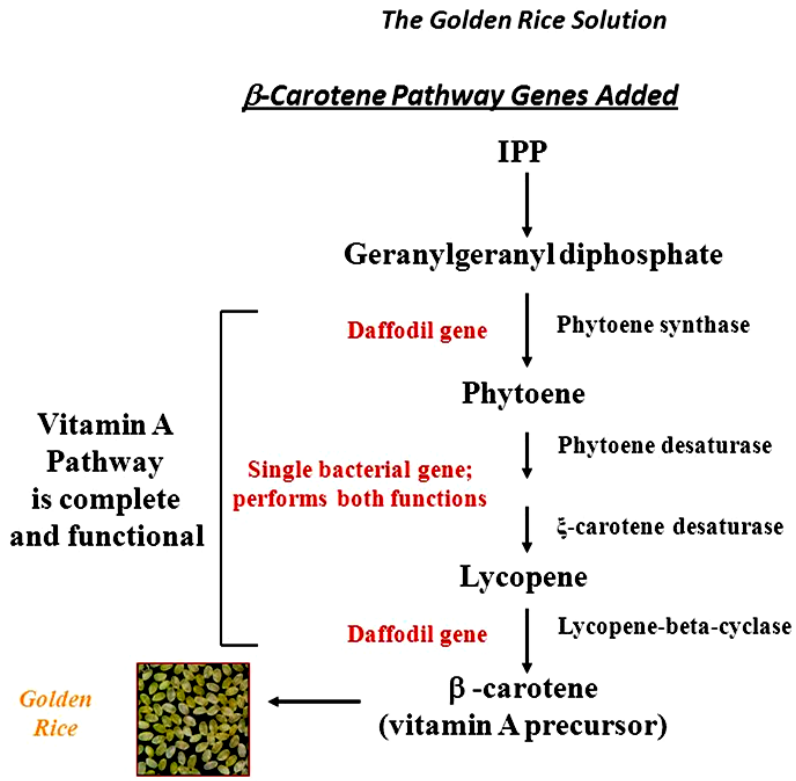
To overcome vitamin A deficiency, it was proposed to genetically manipulate rice to produce β-carotene, in the rice endosperm. The presence of β-carotene in the rice gives a characteristic yellow/orange colour, hence the pro-vitamin A-enriched rice are appropriately considered as Golden Rice.

The genetic manipulation to produce Golden Rice required the introduction of three genes encoding the enzymes phytoene synthase, carotene desaturase and lycopene β-cyclase. It took about 7 years to insert three genes for developing Golden Rice.

Golden Rice has met almost all the objections raised by the opponents of GM foods. However, many people are still against the large scale production of Golden Rice, as this will open door to the entry of many other GM foods.

Another argument put forth against the consumption of Golden Rice is that it can supply only about 20% of daily requirement of vitamin A. But the proponents justify that since rice is a part of a mixed diet consumed (along with many other foods), the contribution of pro-vitamin A through Golden Rice is quite substantial.

Recently (in 2004), a group of British scientists have developed an improved version of Golden Rice. The new strain, Golden Rice 2 contains more than 20 times the amount of pro-vitamin A than its predecessor. It is claimed that a daily consumption of 70 g rice can meet the recommended dietary allowance for vitamin A.



Vaccine development:

Potatoes have been studied using a portion of the *E. coli* enterotoxin in mice and humans and then transgenic potatoes were produced. Ingestion of this transgenic potato resulted in satisfactory vaccinations and no adverse effects.

Other candidates for edible vaccines include banana and tomato, and alfalfa, corn, and wheat are possible candidates for use in livestock.

Edible vaccines are vaccines produced in plants that can be administered directly through the ingestion of plant materials containing the vaccine. Eating the plant would then confer immunity against diseases. In the edible vaccine, Transgenic plants are used as vaccine production systems. The genes encoding antigens of bacterial and viral pathogens can be expressed in plants in a form in which they retain native immunogenic properties. Initially thought to be useful only for preventing infectious diseases, it has also found application in prevention of autoimmune diseases, birth control , cancer therapy, etc. Edible vaccines are currently being developed for a number of human and animal diseases.

One focus of current vaccine effort is on hepatitis B. Transgenic tobacco and potatoes were engineered to express hepatitis B virus vaccine.

Transgenic potato with CT-B gene of *Vibrio cholerae was* shown to be effective in mice.

Male sterility:

Male sterility (dominant genetic) can be produced by transferring certain gene from other species (as against endogenous genes, e.g., *rolB* and *rolC* genes from *Agrobacterium rhizogenes*, *barnase* gene from *Bacillus amyloliquefaciens* etc.

Barnase-Barstar System:

TA 29 Barnase Male Sterility:

One of the most effective and classic case of male sterility induction was carried out by the complete destruction of RNA molecule in tapetal cells. It was demon-strated that chimaeric RNase and barnase gene driven under the control of tapetum specific promoter TA 29.

Where it can induce male sterility in tobacco and oil seed rape. The tissue specific TA 29 gene is highly regulated and specifically expressed in tapetum cells of anther. Expression of TA 29 barnase fused gene consequently acts as cytotoxic and selectively destroys the tapetal cell layer by complete elimination of RNA molecule.

Tapetal cell is a nutritive layer which surrounds the pollen sac in the anther pollen development requiring nutrition obtained from tapetal cells. Therefore, destruction of tapetal cells cut off food supply, arrest pollen devel-opment or non-functional pollen will be formed to produce sterile plants.

The destruction of tapetum by RNase and RNase T1 gene derived from bacterium Bacillus amyloliquefaciens and fungus *Aspergillus oryzae*, respectively. The same TA 29 RNase gene combination induced ste-rility was also produced in maize and other vegetable species.

TA 29 Barstar Male Fertility:

Expression of a novel gene known as barstar derived from the same bacterium Bacillus amyloliquefaciens lead to the production of normal male fertile plant without interfering its pollen development. *Bacillus amyloliquefaciens* expressing barnase gene has corresponding inhibitor protein called barstar. Barstar is produced intracellularly and protect bacteria from the harmful role played by barnase by forming stable complex with the barnase in the cyto-plasm.

The gene 1.5 kilobase barstar was fused to tapetum specific TA 29 gene upstream frag-ment that comprises all regulatory segments targeted for tapetum specific expression. Mariani et al., 1992 introduced TA 29 barstar into oil seed rape by employing Agrobacterium Ti plasmid and biolophase

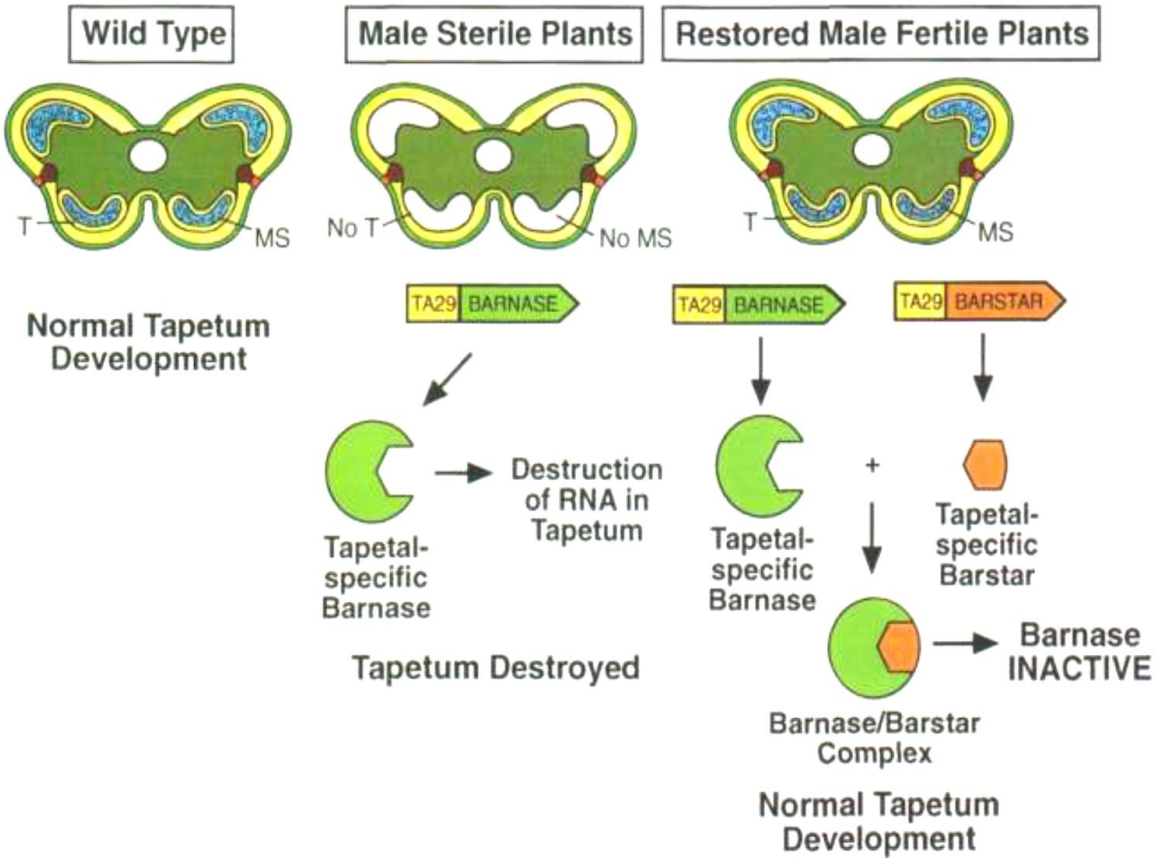
selectable marker gene. Barstar expressing transformants were male fertile, produced normal anther with well-developed anther tapetal cells.

Fertility Restoration by Barnase-Barstar System:

Once male sterility is induced, plants are then can be exploited for introduction of hybrid seed production and plants are maintained as male sterile lines. Restoration of fertility has been accomplished by crossing male sterile plants with male fertile plants expressing barstar gene.

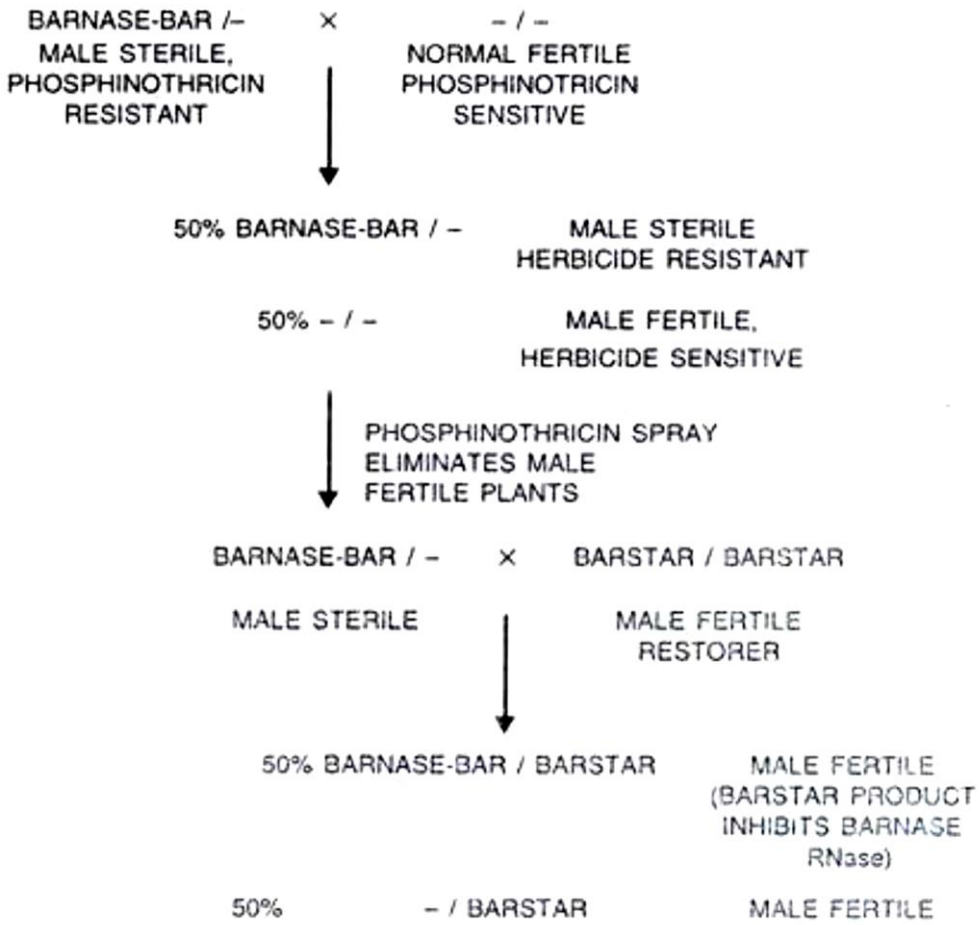
To determine whether TA 29 barstar gene expression could inhibit barnase activity in tapetal cells and led to male fertile restoration, Mariami selected TA 29 barstar gene containing male fertile plants and crosses with male sterile parental plants expressing TA 29 baranse single copy germlines.

Both barnase and barstar gene co-expressed specifically in the tapetal cells of anther led to the formation of barstar-barnase stable complex. In the Fi progeny ratio of fertile to sterile would be 2:1 ratio. All F1 progeny from the crosses that were expressing both chimeric genes were male fertile led to the conclusion that TA 29 barstar gene functioned as a dominant suppressor of TA 29 barnase gene activity.



Plants which are restored to fertility are indistinguish from those of wild-type plants develop normally and exhibited nor-mal anther dehiscent process. They have well developed tapetal cell layers and produce vast amount of functional pollen grain. Thus, effectiveness of chimeric TA 29 barnase and TA 29 barstar genes system in the male-sterility induction and male fertility restoration may permit the breeding of genetic engineering hybrid plant.

Transgenic lines exhibiting the barstar gene have also been developed in Indian Mus-tard, Brassica juncea, to develop a complete male sterility and restoration system for heterosis in this crop. Transgenic mustard lines containing a modified barstar were also raised using modified sequence of the barstar gene.



Another example of obtaining parental male sterility in transgenic tobacco by blocking the synthesis of flavanols and starch in pollen grain has been reported by Mutsuda (1996). Reduction in levels of flavanols and sporopollenin of the pollen wall has been demon-strated to be associated with abnormal microspores and abnormal growth of pollen tube led to reduced fertility.

Pollen cells have plenty of actin which is an essential protein in cell function, blocking the production of actin by antisense actin gene expression leading to male sterile plants in crop plants.

Phenyl ammonia lyase, key enzyme (PAL) in phenyl propanoid pathway, responsible for the synthesis of flavanoids. Introduction of sense or antisense PAL cDNA of sweet potato under the control of tapetum specific rice promoter into tobacco generated transgenic plants. This exhibited reduced pollen fertility. The pollen fertility of these plants droped from 8% to 6%.

Reduction of PAL activity in anther was correlated with the number of fertile pollen grain at flowering stage. These results demonstrated that manipulation of phenyl propanoid pathway by transgene provides a powerful tool for a alternative of phenyl propanoid in pollen and lead to reduced fertility.

Molecular control of male fertility has been studied in Brassica. The gene, BcP1, essen-tial for production of functional pollen grain has been manipulated by their expression using antisense approach. The specific down regulation of BcP1 further demonstrated the importance of this gene during pollen development.

Transgenic Arabidopsis plants in which the BcP1 gene expression is blocked either in tapetum or in developing microspore using Lat52 promoter show arrest in pollen development leading to pollen abortion and exhibit male sterile phenotype.

Apart from antisense approach or production of degradative enzymes for flavanoid biosynthesis, deploying unedited gene targeted to tapetal mitochondria induces male sterility. The expression of transgene, designed to contain the unedited atp 9 mitochondrial gene (u atp 9) fused to the yeast COXTV led to the production of male sterile plants. In contrast expression of edited transgene in the same plant led to the production of male fertile plants with normal pollen development.

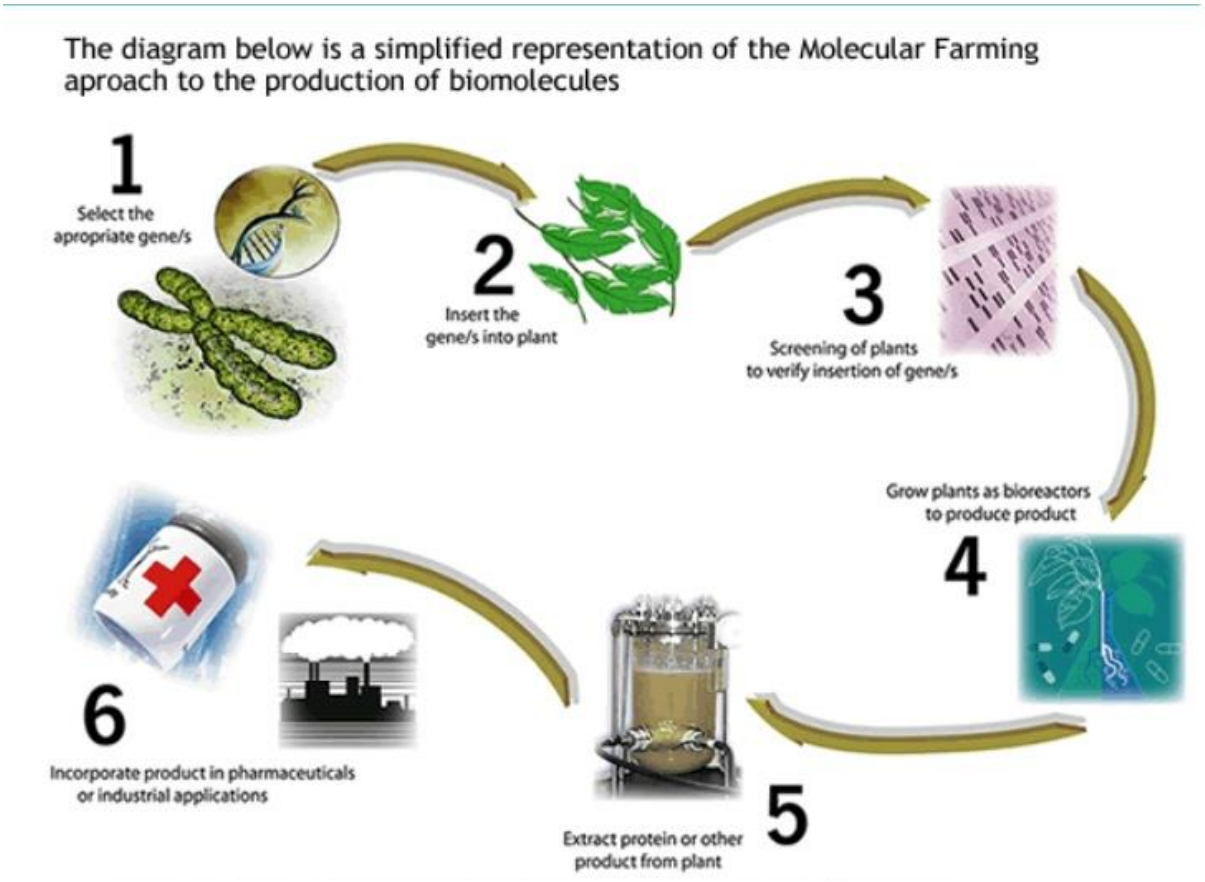
RNA editing in plant mitochondria is a post-transcription process improves protein synthesis of mitochondrial proteins which are basically derived from edited mRNA. However, several exceptions have been recorded. Expression of unedited gene led to the production of non-functional proteins and produce male sterile plants.

The modification of mitochondria was correlated with presence of translated product of unedited atp 9 and a significant decrease in oxygen consumption in non-photosynthetic tissue. The high reduction of CO2 consumption in root meristems of male sterile plants confirms improvement of mitochodrial function.

Molecular farming

Molecular farming is a new technology that uses plants to produce large quantities of pharmaceutical substances such as vaccines and antibodies. It relies on the same method used to produce genetically modified (GM) crops – the artificial introduction of genes into plants**.**

Plant molecular farming is the production of pharmaceutically important and commercially valuable diagnostic proteins and/or industrial enzymes in plants. Combination of biotechnology and agriculture to produce new biomolecules for the benefits of human being It is also known as **biopharming or gene pharming**. Molecular farming started about 20 years ago with the promise to produce therapeutic molecules. Some therapeutic molecules are very expensive to produce. Falls under the category of green biotechnology.



Applications:

Monoclonal antibody (mAb):

Antibody that is produced by genetically engineered Plant i.e. insertion of antibodies into a transgenic plant; referred to as **plantibody.** Biolex (North Carolina) is the trademark for Monoclonal antibody No risk of spreading diseases to humans. Hiatt. et al (1989): First time demonstrate the production of antibodies in tobacco as therapeutic protein and plant protection against diseases. Daniel (2002) was reported that due to the lack of glycosylation, chloroplast transformation is ideal for single chain fragment(scFv). Agrofiltration is ideal for transient expression of heavy and light chain genes. All current therapeutic antibodies are of the IgG class. Purification is done through processes such as filtration, immunofluorescence, and chromatography.

Plant	Antibody type	Purpose
Tobacco	IgG	Catalytic antibodies
Tobacco	IgG-colon cancer	Systemic injection
Alfalfa	IgG-human	Diarrhea Diagnostic
Tobacco	IgG-rabies virus	Anti rabies virus
Tobacco	IgG-hepatitis B virus	hepatitis B surface antigen
Tobacco	IgG-Anthrax	Monoclonal antibody
Tobacco	IgG-rabies virus	Human anti rabies virus
Tobacco	BoNT antidotes	Botulinum neurotoxins (BoNTs)
Tobacco	mAb 2F5	Activity against HIV-1

Edible vaccines:

Crop plants offer cost-effective bioreactors to express antigens which can be used as edible vaccines. The genes encoding antigenic proteins can be isolated from the pathogens and expressed in plants and such transgenic plants or their tissues producing antigens can be eaten for immunization (edible vaccines).

The expression of such antigenic proteins in crops such as banana and tomato are useful for immunization of humans since both of these fruits can be eaten raw. Such edible vaccines of transgenic plants have the following advantages: lessening of their storage problems, their easy delivery system by feeding and low cost as compared to the recombinant vaccines produced by bacteria.

Pathogen	Antigen	Plant
Major capsid protein	VP6	Potato
Hepatitis B virus	Surface antigen	Potato
Human immunodeficiency virus (HIV-1)	p24-Nef	Tobacco
HIV-1	C4(V3)6 multi-epitopic protein	Lettuce
Human papillomavirus (HPV)	HPV16-L1	Tobacco
HPV	HPVL1-E6/E7	Tomato
Influenza virus	H3N2 nucleoprotein	Maize seeds
Vibrio cholerae	CTB	Rice seeds
Rabies virus	G protein	Tomato hairy roots

Industrial enzymes & proteins produced in different plant host system:

Industrial enzymes	Potential use	Host
α- amylase	Industry	Tobacco
Phytase	Industry	Alfalfa, Tobacco
Cellulase	Industry	Alfalfa, Tobacco, potato
Manganese peroxidase	Industry	Alfalfa, Tobacco
β- (1,4) xylanase	Industry	Tobacco, Canola
β-(1,3-1,4)glucanase	Industry	Tobacco, Barley
Avidin	Research reagent	Maize
Glucuronidase	Research reagent	Maize

Biodegradable plastic:

Plant seeds may be a potential source for plastics that could be produced and easily extracted. A type of PHA (polyhydroxylalkanoate) polymer called "poly-beta-hydroxybutyrate", or PHB, is produced in *Arabidopsis* or mustard plant. PHB can be made in canola seeds by the transfer of three genes from the bacterium *Alcaligenes eutrophus*, which codes for enzymes in the PHB synthesis pathway. A polymer called PHBV produced through *Alcaligenes* fermentation, which is sold under the name Biopol.

Flower Pigmentation:

There are continuous attempts in flower industry to make the ornamental flowers more attractive (by improving or creating new colours), besides prolonging post-harvest lifetime. The cut flower industry is mostly (about 70%) dominated by four plants—roses, tulips, chrysanthemums and carnations.

The most common type of flower pigments are anthocyanin's, a group of flavonoids. They are synthesized by a series of reactions, starting from the amino acid phenylalanine. The colour of the flower is dependent on the chemical nature of the anthocyanin produced.

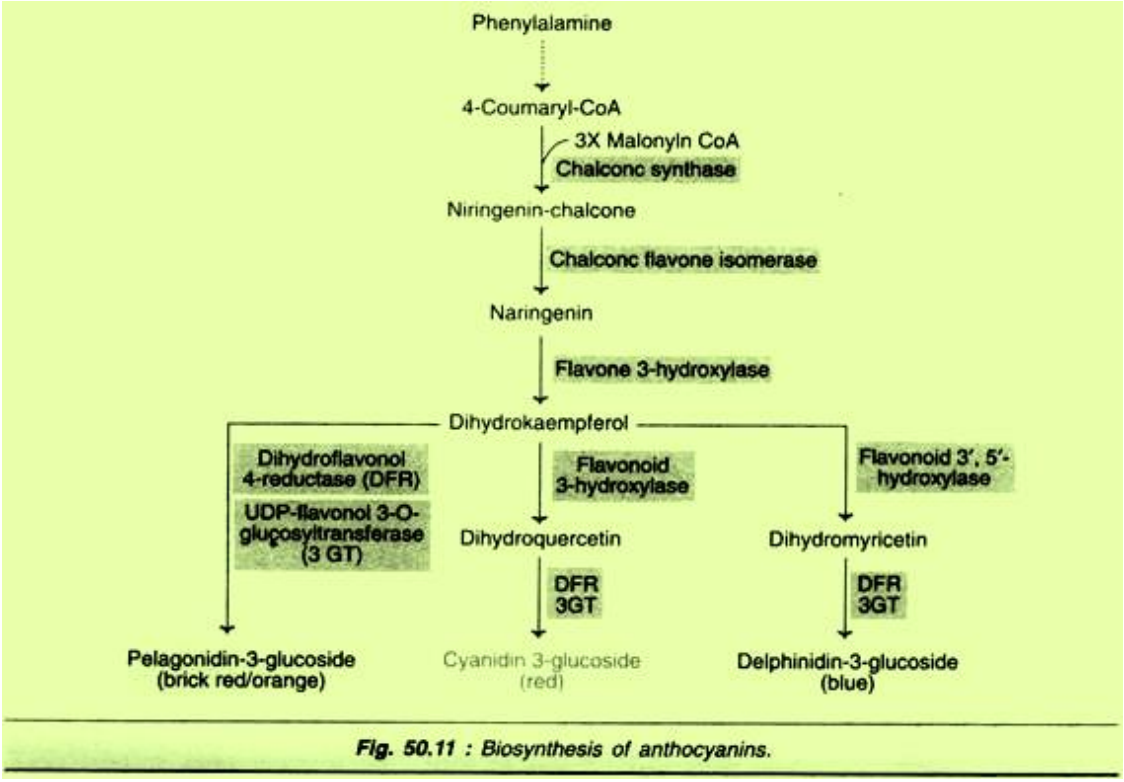


Fig. 50.11 : Biosynthesis of anthocyanins.

i. Pelagonidin 3-glucoside — brick red/orange.
ii. Cyanidin 3-glucoside — red.
iii. Delphinidin 3-glucoside — blue to purple.

Manipulation of anthocyanin pathway enzymes:

The enzymes responsible for different reactions, in the anthocyanin pathway have been identified. By genetic manipulations and mutations, it is possible to develop flowers with the desired colours. Most of the flowers (roses, carnations chrysanthemums) lack blue colour due to the absence of the key enzyme flavonoids 3′, 5′- hydroxylase (F 3′ 5′ H) that produces delphinidine 3 glucoside. One company, by the name Florigene, has genetically manipulated and introduced the gene encoding the enzyme F 3′ 5′ H (from Petunia hybrida) into the following plants.

The world's first genetically modified (GM) flower was introduced in 1996. It was a mauve (bluish) coloured carnation with a trade name Moondust™. Subsequently, many other flowers have been produced and marketed.

Terminator gene technology:

Terminator technology designed to preclude unauthorised seed saving by farmers. In other words, terminator genes prevent crops from producing fertile seeds because seed will not germinate if re-planted a second time which means that farmers growing them would have to buy new seeds each year instead of saving harvested seeds to plant for next year's crop.

Terminator technology is the biggest and most controversial subject contributed by US based companies. According to Rural Advancement Foundation International (RAFI), this tech-nology was dubbed as terminator or traitor technology. If technology is commercially viable, it could have far-reaching implications for farmers, commercial seed industry, socio economic and environmental implications.

The real consequence of this technology cannot be appreciated without an understanding of the science behind this scientic endeavour. Thanks to civil society organizations, farmers, scientist and governmental authorities all over the world who have waged effective anti-terminator campaign for several months. The US based Monsanto (pine land was the then sold to Monsanto) surrender suicide seeds armed with terminator gene but continue to work on other Traitor technology.

There are several major crops which usually are not grown from hybrid seeds. These include wheat, rice, soybeans, and cotton. Collection and saving of seeds normally done from these plants by farmers and may not go back to the seed company for several years to purchase a new variety.

Therefore, it would be a big economic boon to a seed company. If people, who now grow non-hybrid crops would have to buy new seed every year. This is one of the probable reasons for developing terminator technology. Generally this technology can be effectively im-plicated for non-hybrid crops, for example, cotton is not often sold as hybrid seed, and is thus a likely candidate for Terminator protection.

On the contrary, corn is planted as a hybrid, and thus it has some variety protection. The terminator technology cannot be implicated to hybrids because the first generation of a hybrid is genetically uniform, and when these hybrids make seeds, the second generation is extensively variable because of the shuffling of genes that occur during sexual reproduction.

5. Genetic recombination: Molecular basis of chromosome pairing; Rec BCD pathway; double strand model in yeast; gene conversion in bread mold; site specific recombination.

Molecular Basis of Chromosome Pairing:

A new era in the studies of chromosome pairing was ushered in with the introduction of electron microscope to biological research. Working with the spermatocytes of crayfish, Moses in 1956 discovered a tripartite ribbon at the site of synapsis; this structure is called **synaptonemal complex** or synaptinemal **complex**.

This complex occupies the space between the paired homologous chromosomes and appears to be a advice to bring together and allign the DNA molecules from paired homologous chromosomes which facilitates crossing over between them.

Synaptinemal Complex:

Synaptinemal complexes have been detected in almost all the eukaryotic cells undergoing meiosis. Synapsis starts at the beginning of zygotene when synaptinemal complex begins to develop between the homologous chromosomes. Synapsis is preceded by the formation of a rope-like proteinaceous axis along each of the homologous chromosomes.

As pairing advances, the axes of homologous chromosomes appear to adhere to each other to become the lateral elements of the synaptinemal complex. These lateral elements of the two chromosomes together form a third band called the central element.

The formation of the central element is preceded by the development of protein connections between the chromosomes undergoing synapsis. Close association of chromosomes is found to start at the chromosome ends attached to the nuclear membrane. The three bands (two lateral and one central) from the synaptinemal complex.

Synaptinemal complexes vary in size and organization in different eukaryotic cells. In tomatoes, the overall width of the complex is approximately 160 nm, whereas in mammals, and crickets it is 180 nm and 200 nm, respectively.

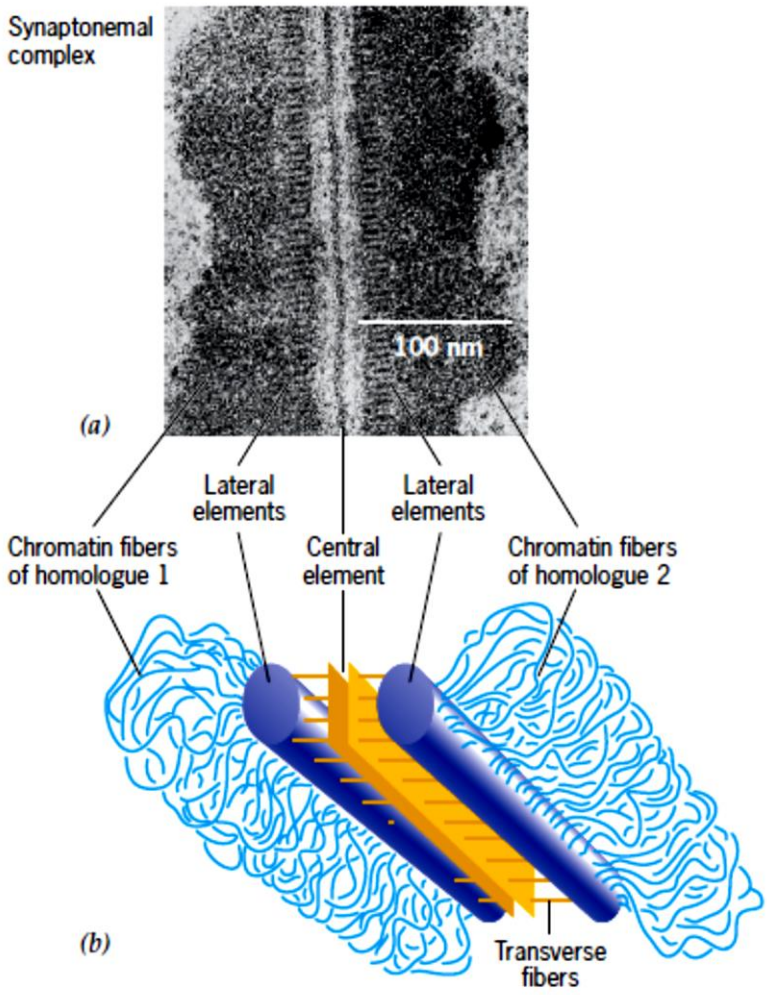
The lateral elements may be spaced from 20-30 nm to 100-125 nm. The central element generally ranges from 12 to 20 nm in diameter; it is separated from the lateral elements by a space of about 40 nm.

The presence of the central element fibres of about 2 nm diameter is revealed in cross sections. The two lateral element seem to be composed of granules and fibres (synaptomeres) that are slightly wider than 10 nm.

The major part of synaptinemal complex is protein as revealed by the cytochemical analyses. The lateral elements contain a small amount of DNA which is thought to be derived from the chromatids with which they are associated.

DNA is also present in the transverse fibres that cross the space between the central and lateral elements. It seems that the synaptinemal complex is made up of a framework of protein within which DNA containing chromosomal fibres is interspersed

After pachytene, the synaptinemal complex dissolves and the homologous chromosomes begin to de-synapse; this stage is called diplotene during which homologues are associated by chiasmata. Synaptinemal complex may not get fully dissolved in some cells and its remnants may persist through the second meiotic division and form aggregates called poly-complexes.



Electron micrograph (a) and diagram (b) showing the structure of the synaptonemal complex that forms between homologous chromosomes during prophase I of meiosis.

Synaptinemal complex brings the homologous chromosomes close together so that crossing over may occur between them. In D. melanogaster females homozygous for the mutant gene c3G, genetic crossing over is suppressed and synaptinemal complex does not form. Similarly, crossing over does not occur in male Drosophila and synaptinemal complex also is absent.

Synaptomere-Zygosome Hypothesis of the Formation of Synaptonemal Complex:

This hypothesis states that homologous chromosomes are attached to the nuclear membrane with their ends and that two substances, namely, synaptomere (made up of polysegments) and zygosomes (composed of protein molecules) are involved in chromosome pairing, as shown by King in 1970.

Synaptomeres are distributed along the length of synapsed chromosomes; each synaptomere consists of three segments designated as A (lateral), B (central) and C (lateral segments arranged in the following order, ABC, CBA, ABC, CBA,. The lateral segment A pairs with A and C pairs with C of the same chromosome, and it causes the chromosome to become shorter and thicker due to folding.

The B segment of the synaptomere is directed towards the central element and acts as the site for zygosome attachment. The zygosome is rod-shaped subunit and its one end (head) is attached to the B segment, while the other end (tail) is attached to the tail of the other zygosome attached to the homologous chromosome generating a ladder-like arrangement.

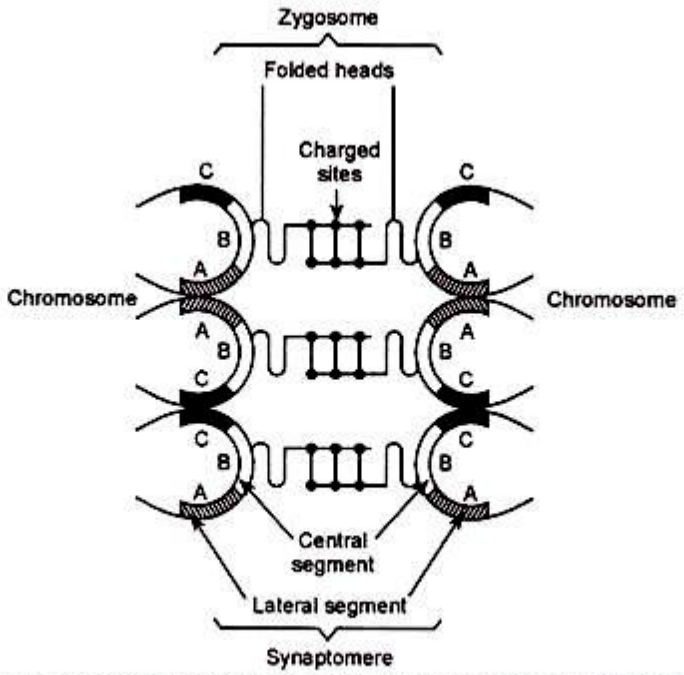


Fig. 11.3. Schematic representation of synaptinemal complex, according to the synatpomere-zygosome hypothesis.

The mechanism and force due to which the homologous chromosomes become precisely aligned for the paring are not exactly known. Some of the investigators are of the view that the homologous chromosomes are prepared for synapsis by the attachment of their telomeres to the attachment sites on the nuclear membrane.

There is an increase in the content of "colchicine binding protein" during the leptotene-pachytene period; this increase may have some role in synapsis.

Recombination Nodules:

The occurrence of certain modifications in the synaptinemal complex at the crossover sites has been observed. These modifications have been called "nodes" or "recombination nodules" by Carpenter in 1975; they are considered to be involved in the active process of recombination.

Recombination nodules are approximately 90 nm in diameter and may be of varying shapes, such as, spherical, ellipsoidal or bar-like, and are composed of protein. They are placed between the homologous chromatids on the synaptinemal complex.

Recombination nodules are considered to mark the site of multi-enzyme recombination machine that brings local region of DNA on the maternal and paternal chromatids together across the synaptinemal complex, as suggested by Alberts et al. in 1983.

The available indirect evidences for the function of recombination nodules in recombination is summarised below:

(i) Total number of recombination nodules is about equal to the number of chiasmata observed during prophase I.

(ii) The distribution pattern of recombination nodules and crossover events along the synaptinemal complex are comparable,

(iii) Certain mutants of Drosophila show reduced recombination frequencies; these mutants also exhibit relatively fewer recombination nodules,

(iv) The radioactive DNA precursors are known to be preferentially incorporated into pachytene DNA at or near the recombination nodules.

RecBCD pathway:

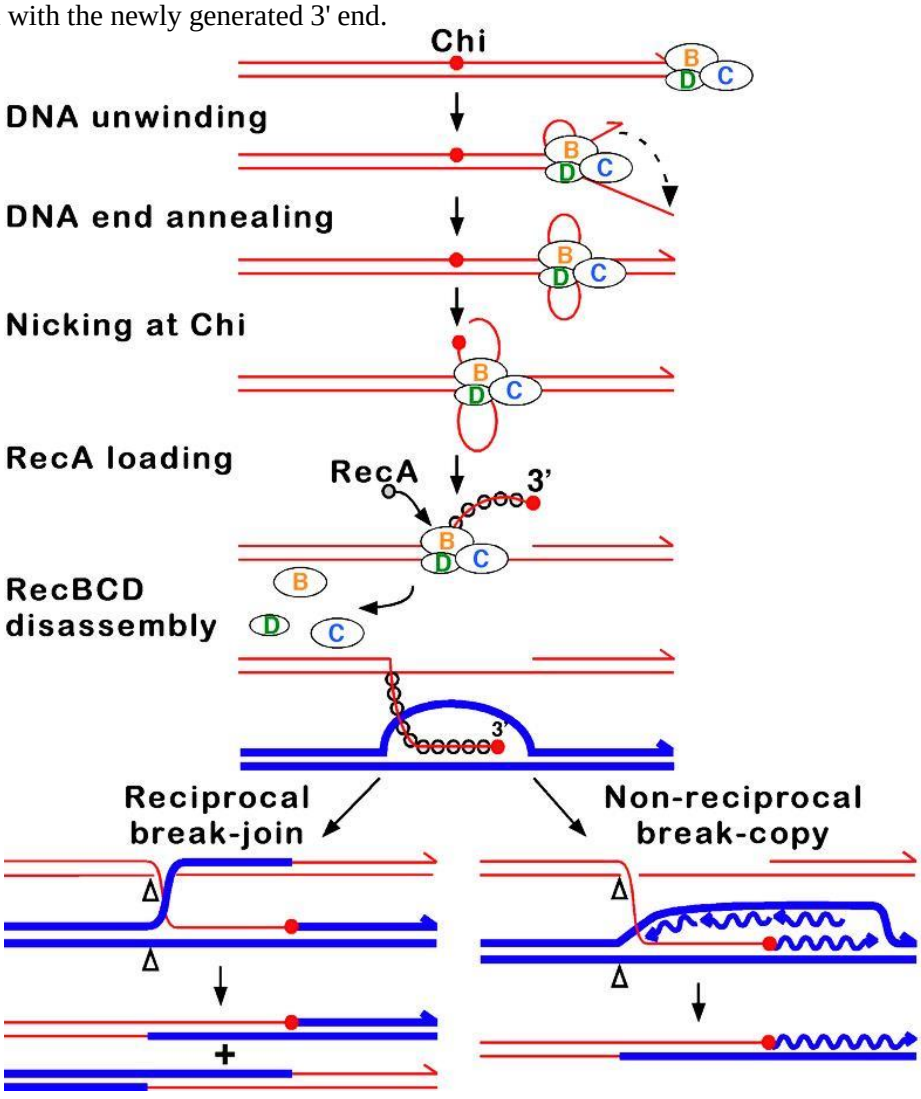
RecBCD (EC3.1.11.5, Exonuclease V, Escherichia coli exonuclease V, E. coli exonuclease V, gene recBC endoenzyme, RecBC deoxyribonuclease, gene recBC DNase, gene recBCD enzymes) is an enzyme of the E. coli bacterium that initiates recombinational repair from potentially lethal double strand breaks in DNA which may result from ionizing radiation, replication errors, endonucleases,

oxidative damage, and a host of other factors. The RecBCD enzyme is both a helicase that unwinds, or separates the strands of DNA, and a nuclease that makes single-stranded nicks in DNA.

The RecBCD pathway is the main recombination pathway used in many bacteria to repair double-strand breaks in DNA, and the proteins are found in a broad array of bacteria. These double-strand breaks can be caused by UV light and other radiation, as well as chemical mutagens. Double-strand breaks may also arise by DNA replication through a single-strand nick or gap. Such a situation causes what is known as a collapsed replication fork and is fixed by several pathways of homologous recombination including the RecBCD pathway.

In this pathway, a three-subunit enzyme complex called RecBCD initiates recombination by binding to a blunt or nearly blunt end of a break in double-strand DNA. After RecBCD binds the DNA end, the RecB and RecD subunits begin unzipping the DNA duplex through helicase activity. The RecB subunit also has a nuclease domain, which cuts the single strand of DNA that emerges from the unzipping process. This unzipping continues until RecBCD encounters a specific nucleotide sequence (5'-GCTGGTGG-3') known as a Chi site.

Upon encountering a Chi site, the activity of the RecBCD enzyme changes drastically. DNA unwinding pauses for a few seconds and then resumes at roughly half the initial speed. This is likely because the slower RecB helicase unwinds the DNA after Chi, rather than the faster RecD helicase, which unwinds the DNA before Chi. Recognition of the Chi site also changes the RecBCD enzyme so that it cuts the DNA strand with Chi and begins loading multiple RecA proteins onto the single-stranded DNA with the newly generated 3' end.



RecBCD pathway

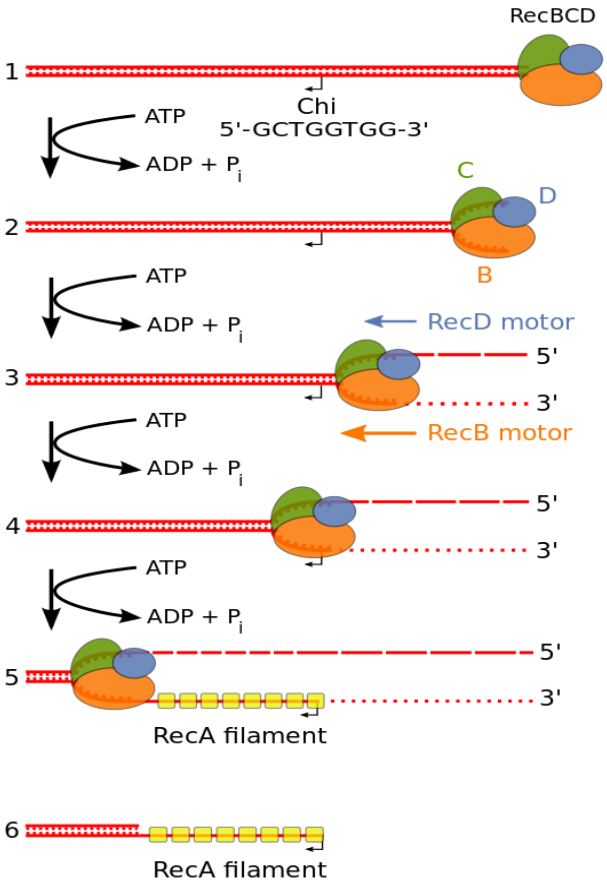
The resulting RecA-coated nucleoprotein filament then searches out similar sequences of DNA on a homologous chromosome. The search process induces stretching of the DNA duplex, which enhances homology recognition (a mechanism termed conformational proofreading). Upon finding such a sequence, the single-stranded nucleoprotein filament moves into the homologous recipient DNA duplex in a process called **strand invasion**. The invading 3' overhang causes one of the strands of the recipient DNA duplex to be displaced, to form a D-loop. If the D-loop is cut, another swapping of strands forms a cross-shaped structure called a Holliday junction. Resolution of the Holliday junction by some combination of RuvABC or RecG can produce two recombinant DNA molecules with reciprocal genetic types, if the two interacting DNA molecules differ genetically. Alternatively, the invading 3’ end near Chi can prime DNA synthesis and form a replication fork. This type of resolution produces only one type of recombinant (non-reciprocal).

Mechanism of action:

During unwinding the nuclease in RecB can act in different ways depending on the reaction conditions, notably the ratio of the concentrations of Mg2+ ions and ATP. (1) If ATP is in excess, the enzyme simply nicks the strand with Chi (the strand with the initial 3' end) (Figure 2). Unwinding continues and produces a 3' ss tail with Chi near its terminus. This tail can be bound by RecA protein, which promotes strand exchange with an intact homologous DNA duplex. When RecBCD reaches the end of the DNA, all three subunits disassemble and the enzyme remains inactive for an hour or more, a RecBCD molecule that acted at Chi does not attack another DNA molecule. (2) If Mg2+ ions are in excess, RecBCD cleaves both DNA strands endonucleolytically, although the 5' tail is cleaved less often (Figure 3). When RecBCD encounters a Chi site on the 3' ended strand, unwinding pauses and digestion of the 3' tail is reduced. When RecBCD resumes unwinding, it now cleaves the opposite strand (i.e., the 5' tail) and loads RecA protein onto the 3’-ended strand. After completing reaction on one DNA molecule, the enzyme quickly attacks a second DNA, on which the same reactions occur as on the first DNA.

Although neither reaction has been verified by analysis of intracellular DNA, due to their transient nature, genetic evidence indicates that the first reaction more nearly mimics that in cells. For example, RecBCD mutants lacking detectable exonuclease activity retain high Chi hotspot activity in cells and nicking at Chi outside cells. A Chi site on one DNA molecule in cells reduces or eliminates Chi activity on another DNA, perhaps reflecting the Chi-dependent disassembly of RecBCD observed in vitro under conditions of excess ATP and nicking of DNA at Chi.

Under both reaction conditions, the 3' strand remains intact downstream of Chi. TheRecA protein is then actively loaded onto the 3' tail by RecBCD. At some undetermined point RecBCD dissociates from the DNA, although RecBCD can unwind at least 60 kb of DNA without falling off. RecA initiates exchange of the DNA strand to which it is bound with the identical, or nearly identical, strand in an intact DNA duplex; this strand exchange generates a joint DNA molecule, such as a D-loop (Figure 2). The joint DNA molecule is thought to be resolved either by replication primed by the invading 3’ ended strand containing Chi or by cleavage of the D-loop and formation of a Holliday junction. The Holliday junction can be resolved into linear DNA by the RuvABC complex or dissociated by the RecG protein. Each of these events can generate intact DNA with new combinations of genetic markers by which the parental DNAs may differ. This process, homologous recombination, completes the repair of the double-stranded DNA break.



Double strand model in yeast:

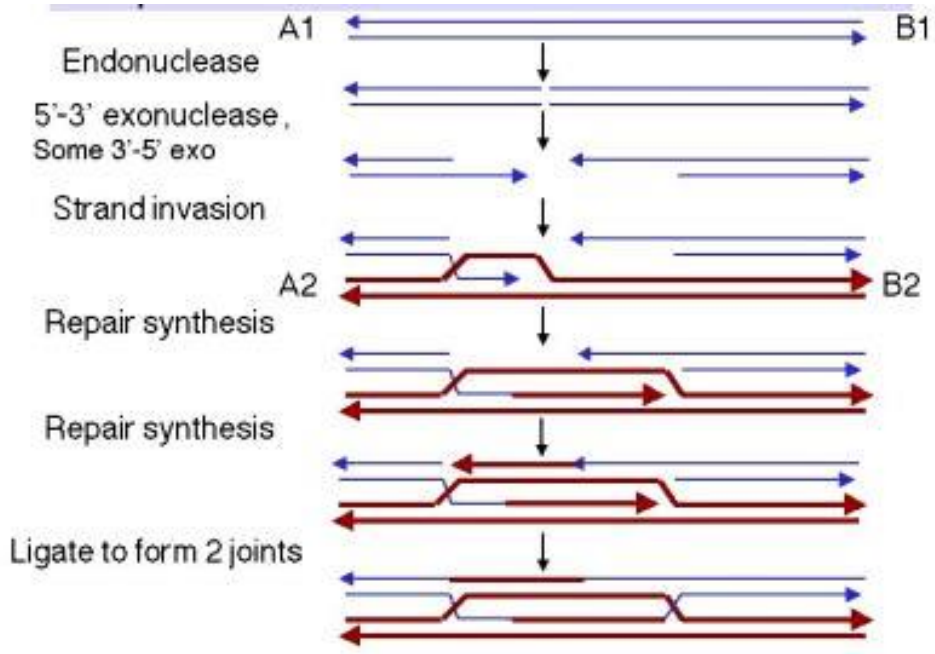
A substantial body of evidence indicates that homologous recombination occurs by more than one mechanism—probably by several different mechanisms. In S. cerevisiae, the ends of DNA molecules produced by double-strand breaks are highly recombinogenic. This fact and other evidence suggest that recombination in yeast often involves a double-strand break in one of the parental double helices. Thus, in 1983, Jack Szostak, Franklin Stahl, and colleagues proposed **a double-strand break model** of crossing over. According to their model, recombination involves a double-strand break in one of the parental double helices, not just single-strand breaks as in the Holliday model. The initial breaks are then enlarged to gaps in both strands. The two single-stranded termini produced at the double-stranded gap of the broken double helix invade the intact double helix and displace segments of the homologous strand in this region. The gaps are then filled in by repair synthesis. This process yields two homologous chromosomes joined by two single-strand bridges. The bridges are resolved by endonucleolytic cleavage, just as in the Holliday model. Both the double-strand-break model and the Holliday model nicely explain the production of chromosomes that are recombinant for genetic markers flanking the region in which the crossover occurs.

The steps in the double-strand-break model up to the formation of the joint molecules are diagrammed in Figure.

1. An endonuclease cleaves both strands of one of the homologous DNA duplexes, shown as thin blue lines in Figure. This is the **aggressor duplex**, since it initiates the recombination. It is also the **recipient** of genetic information, as will be apparent as we go through the model.

2. The cut is enlarged by an exonuclease to generate a gap with 3' single-stranded termini on the strands.

3. One of the free 3' ends invades a homologous region on the other duplex (shown as thick red lines), called the **donor duplex**. The formation of heteroduplex also generates a **D-loop** (a displacement loop), in which one strand of the donor duplex is displaced.
4. The D-loop is extended as a result of **repair synthesis** primed by the invading 3' end. The D-loop eventually gets large enough to cover the entire gap on the aggressor duplex, i.e. the one initially cleaved by the endonuclease. The newly synthesized DNA uses the DNA from the invaded DNA duplex (thick red line) as the template, so the new DNA has the sequence specified by the invaded DNA.
5. When the displaced strand from the donor (red) extends as far as the other side of the gap on the recipient (thin blue), it will anneal with the other 3' single stranded end at that end of the gap. The displaced strand has now filled the gap on the aggressor duplex, donating its sequence to the duplex that was initially cleaved. Repair synthesiscatalyzed by DNA polymerase converts the donor D-loop to duplex DNA. During steps 4 and 5, the duplex that was initially invaded serves as the donorduplex; i.e. it provides genetic information during this phase of repair synthesis. Conversely, the aggressor duplex is the recipient of genetic information. Note that the single strand invasion models predict the opposite, where the initial invading strand is the donor of the genetic information.
6. DNA ligase will seal the nicks, one on the left side of the diagram in Figure and the other on the right side. Although the latter is between a strand on the bottom duplex and a strand on the top duplex, it is equivalent to the ligation in the first nick (the apparent physical separation is an artifact of the drawing). In both cases, sealing the nick forms a **Holliday junction**.

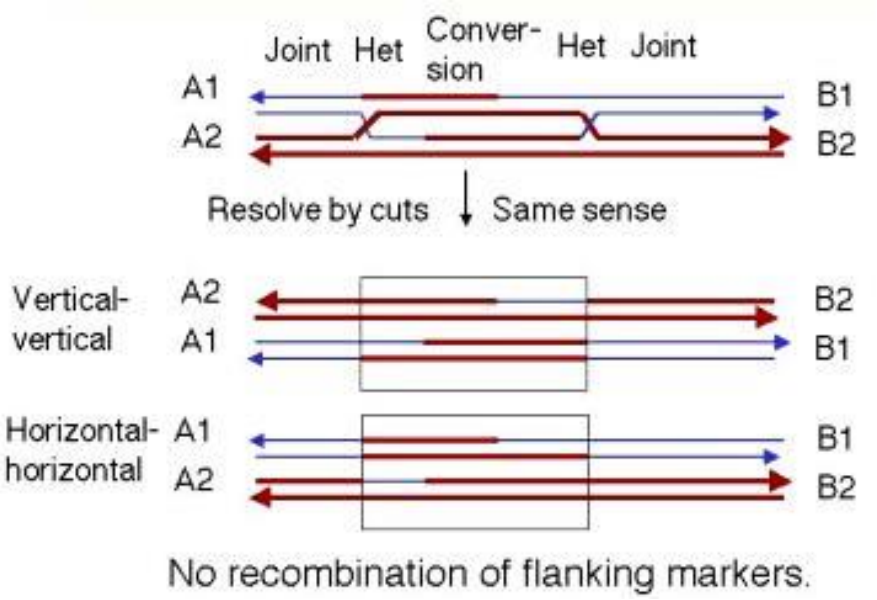


Steps in the double-strand-break model for recombination

At this point, the recombination intermediate has **two recombinant joints** (Holliday junctions). The original gap in the aggressor duplex has been filled with DNA donated by the invaded duplex. The filled gap is now **flanked by heteroduplex**. The heteroduplexes are arranged **asymmetrically**, with one to the left of the filled gap on the aggressor duplex and one to the right of the filled gap on the donor duplex. Branch migration can extend the regions of heteroduplex from each Holliday junction.

The recombination intermediate can now be resolved. The presence of two recombination joints adds some complexity, but the process is essentially the same as discussed for the Holliday model. Each joint can be resolved horizontally or vertically. The key factor is whether the joints are resolved in the same mode or sense (both horizontally or both vertically) or in different modes.

If both joints are resolved the same sense, the original duplexes will be released, each with a region of altered genetic information that is a "footprint" of the exchange event. That region of altered information is the original gap, plus or minus the regions covered by branch migration. For instance, if both joints are resolved by cutting the originally cleaved strands ("horizontally" in our diagram of the Holliday model), then you have no crossover at either joint. If both joints are resolved by cleaving the strands not cut originally ("vertically" in our diagram of the Holliday model), then you have a crossover at both joints. This closely spaced double crossover will produce no recombination of flanking markers.



Resolution of intermediates in the double-strand-break model by cutting the recombinant joints in the same mode or sense.

In contrast, if each joint is resolved in opposite directions, then there will be recombination between flanking markers. That is, one joint will not give a crossover and the other one will.

Gene conversion:

Analysis of tetrads of meiotic products of certain fungi reveals that genetic exchange is not always reciprocal. For example, if crosses are performed between two closely linked mutations in the mold Neurospora, and asci containing wild-type recombinants are analyzed, these asci frequently do not contain the reciprocal, double-mutant recombinant.

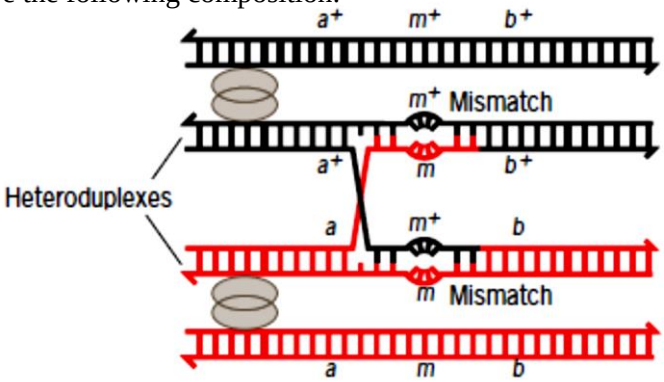
Consider a cross involving two closely linked mutations, m_1 and m_2. In a cross of $m_1m_2^+$ with $m_1^+m_2$, asci of the following type are observed:

Spore pair 1: $m_1^+ m_2$
Spore pair 2: $m_1^+ m_2^+$
Spore pair 3: $m_1 \quad m_2^+$
Spore pair 4: $m_1 \quad m_2^+$

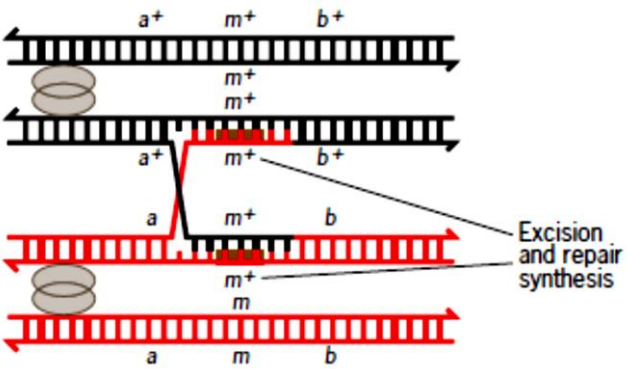
Wild type spores are present, but the $m_1^+m_2^+$ double-mutant spores are not present in the ascus. Reciprocal recombination would produce an m_1 m_2 chromosome whenever an $m_1^+m_2^+$ chromosome was produced. In this ascus, the m_2:m_2 ratio is 3:1 rather than 2:2 as expected. One of the m_2 alleles appears to have been "converted" to the m_2 allelic form. Thus, this type of nonreciprocal recombination is called **gene conversion**. We might assume that gene conversion results from mutation, except that it occurs at a higher frequency than the corresponding mutation events, always produces the allele present on the homologous chromosome, not a new allele, and is correlated about 50 percent of the time with reciprocal recombination of flanking markers. The last observation strongly suggests that gene conversion results from events that occur during crossing over. Indeed, gene conversion is now believed to result from DNA repair synthesis associated with the breakage, excision, and reunion events of crossing over.

With closely linked markers, gene conversion occurs more frequently than reciprocal recombination. In one study of the his1 gene of yeast, 980 of 1081 asci containing his+ recombinants exhibited gene conversion, whereas only 101 showed classical reciprocal recombination. The most striking feature of gene conversion is that the input 1:1 allele ratio is not maintained. This can be explained easily if short segments of parental DNA are degraded and then resynthesized with template strands provided by DNA carrying the other allele. Given the mechanisms of excision repair discussed earlier in this chapter, the Holliday model of crossing over explains gene conversion for genetic markers located in the immediate vicinity of the crossover. In Figure, there is a segment of DNA between the a+ andb+ loci where complementary strands of DNA from the two homologous chromosomes are base-paired. If a third pair of alleles located within this segment were segregating in the cross, mismatches in the two double helices would be present. DNA molecules containing such mismatches, or different alleles in the two complementary strands of a double helix, are called **heteroduplexes**. Such heteroduplex molecules occur as intermediates in the process of recombination.

If Figure were modified to include a third pair of alleles, and the other two chromatids were added, the tetrad would have the following composition:

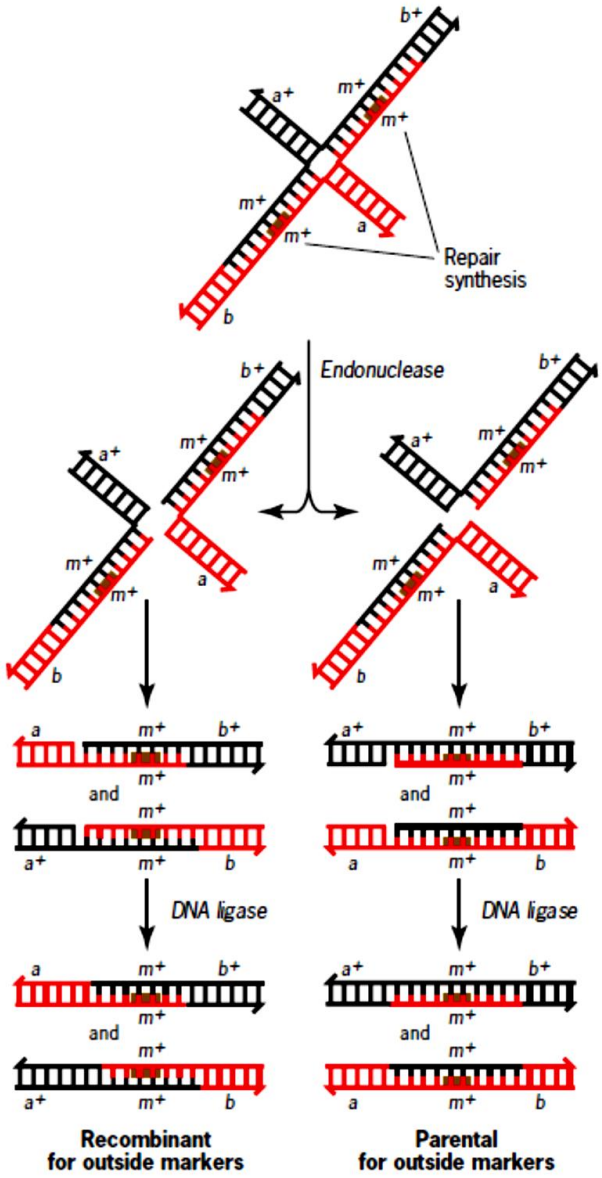


If the mismatches are resolved by nucleotide excision repair, in which the m strands are excised and resynthesized with the complementary M^+ strands as templates, the following tetrad will As a result:



As a result of semiconservative DNA replication during the subsequent mitotic division, this tetrad will yield an ascus containing six m^+ ascospores and two m ascospores, the 3:1 gene conversion ratio. Suppose that only one of the two mismatches in the tetrad just described is repaired prior to the mitotic division. In this case, the semiconservative replication of the remaining heteroduplex will yield one m^+ homoduplex and one m homoduplex, and the resulting ascus will contain a $5m^+$:3m ratio of ascospores. Such 5:3 gene conversion ratios do occur. They result from postmeiotic (mitotic) segregation of unrepaired heteroduplexes.

Gene conversion is associated with the reciprocal recombination of flanking markers approximately 50 percent of the time. If the two recombinant chromatids of the tetrad just diagrammed are drawn in a form equivalent to that shown in Figure, the association of gene conversion with reciprocal recombination of flanking markers can easily be explainedmarkers. Thus, if cleavage occurs in the vertical plane half of the time and in the horizontal plane half of the time, gene conversion will be associated with reciprocal recombination of flanking markers about 50 percent of the time, as observed.



Site-Specific Recombination:

Site specific recombination alters the relative position of nucleotide sequences in chromosome. The base pairing reaction depends on protein mediated recognition of the two DNA sequences that will combine. Very long homologous sequence is not required.

Unlike general recombination, site specific recombination is guided by a recombination enzyme that recognises specific nucleotide sequences present on one of both recombining DNA molecules. Base pairing is not involved, however, if occurs the heteroduplex joint is only a few base pair long.

It was first discovered in phage λ by which its genome moves into and out of the E. coli chromosome. After penetration phage encoded an enzyme, lambda integrase which catalyses the recombination process. Lambda integrase binds to a specific attachment site of DNA sequence on each chromosome. It makes cuts and breaks a short homologous DNA sequences. The integrase switches the partner strands and rejoins them to form a heteroduplex joint of 7 bp long. The integrase resembles a DNA topoisomerase in rejoining the strands which have previously been broken.

Site specific recombination is of the following two types:

(a) Conservative site-specific recombination:

Production of a very short heteroduplex by requiring some DNA sequence that is the same on the two DNA molecules is known as conservative site-specific recombination.

b) Trans-positional site-specific recombination:

There is another type of recombination system known as trans-positional site-specific (TSS) recombination. The TSS recombination does not produce heteroduplex and requires no specific sequences on the largest DNA.

There are several mobile DNA sequences including many viruses and transposable elements that encode integrates. The enzyme integrates by involving a mechanism different from phage λ insert its DNA into a chromosome. Each enzyme of integrates recognises a specific DNA sequence like phage λ.

K. Mizuuchi (1992a) reviewed the mechanism of trans-positional recombination based on the studies of bacteriophage Mu and the other elements. The enzyme integrase was first purified from Mu. Similar to integrase of phage λ, the Mu integrase also carries out of its cutting and rejoining reactions without requirement of ATP. Also they do not require a specific DNA sequence in the target chromosome and do not form a joint of heteroduplex.

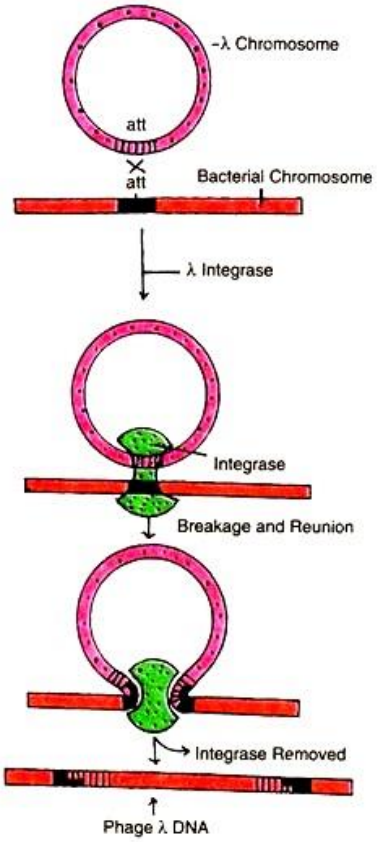


Fig. 8.28 : Diagrammatic representation of site specific genetic recombination.

Different steps of TSS recombinational events are shown in Fig. 8.29. The integrase makes a cut in one strand at each end of the viral DNA sequences, and exposes the 3′-OH group that protrudes out. Therefore, each of these 3′-OH ends directly invades a phosphodiester bond on opposite strands of a randomly selected site on a target chromosome.

This facilitates to insert the viral DNA sequence into the target chromosome, leaving two short single stranded gaps on each side of recombinational DNA molecule.

These gaps are filled in later on by DNA repair process (i.e. DNA polymerase) to complete the recombination process. This mechanism results in formation of short duplication (short repeats of about 3 to 12 nucleotide long) of the adjacent target DNA sequence. Formation of short repeats is the hall-marks of a TSS recombination.

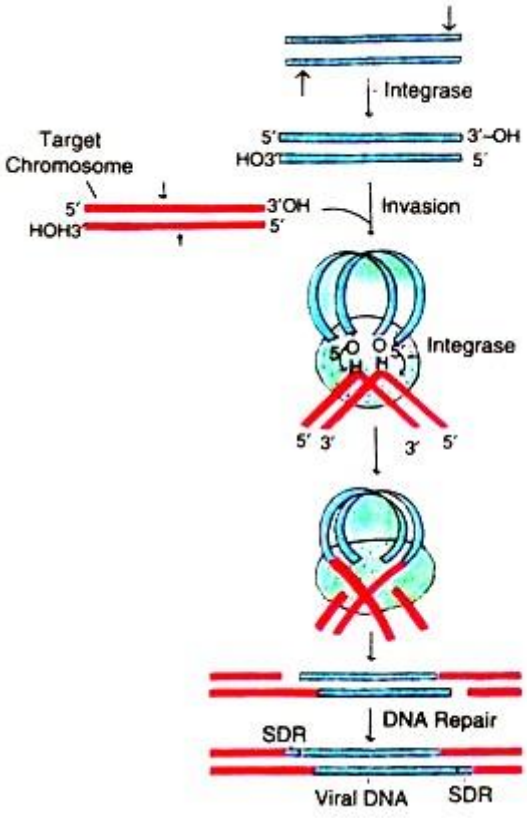


Fig. : Mechanism of trans-positional site-specific recombination

6. Genetic diseases. Pedigree analysis. Gene therapy and genetic counselling

Genetic disease is a disorder that is caused by an abnormality in an individual's DNA. Abnormalities can range from a small mutation in DNA or addition or subtraction of an entire chromosome or set of chromosomes. Most Genetic disease are quite rare and affect one person in every several thousands or millions.

Genetic disease may results by

- Point mutation, or any insertion/deletion entirely inside one gene
- Deletion of a gene or genes
- Whole chromosome extra, missing, or both

Classification:

- Single gene disorder
- Chromosomal genetic disorder
- Multifactorial genetic disorder

1. Single gene disorder

These disorders involve mutations in the DNA sequences of single genes. As a result, the protein the gene codes for is either altered or missing. Over 4000 human diseases caused by single gene defects.

Some Example: Haemophilia, Phenylketonuria, Cystic fibrosis, Alkaptonuria, Sickle cell anemia, Thalasemia, Huntington's disease, etc.

Haemophilia:

It is a sex-linked recessive disease, which is transmitted from an unaffected carrier female to some of the male offsprings. Due to this, patient continues bleeding even on a minor injury because of defective blood coagulation. The gene for haemophilia is located on X-chromosome. The defective alleles produce non-functional proteins, which later form a non-functional cascade of proteins involved in blood clotting.

The possibility of a female becoming a haemophilic is extremely rare because mother of such a female has to be at-least carrier and father should be haemophilic. For example, females suffer from this disease only in homozygous condition, i.e., X^CX^C. Queen Victoria was a carrier of haemophilia and produced haemophilic individuals

A Carrier Woman marries a Normal man. A carrier Woman for haemophilia (XX^h) marries a normal man. The carrier woman produces ova of two types, one with X and other with X^h. The normal male also produces sperms of two types, one with X and other with Y chromosome.

The marriage can produce four types of children of combinations XX, XX^h, X^hY, XY. Among the daughters 50% are normal and the remaining 50% are carriers. Among the sons 50% are normal and rest 50% arc haemophilic. The carrier daughters are normal but transmits their haemophilic gene to 50% their children.

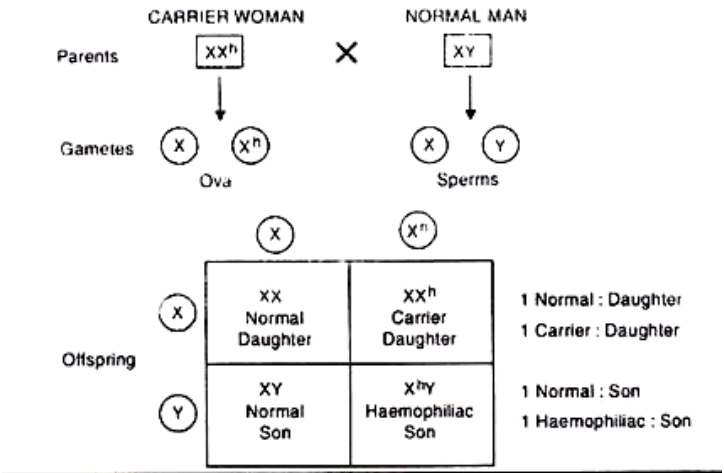


Fig. 5.27 Inheritance of haemophilia by 50% of the male children when the mother is carrier and the father is normal

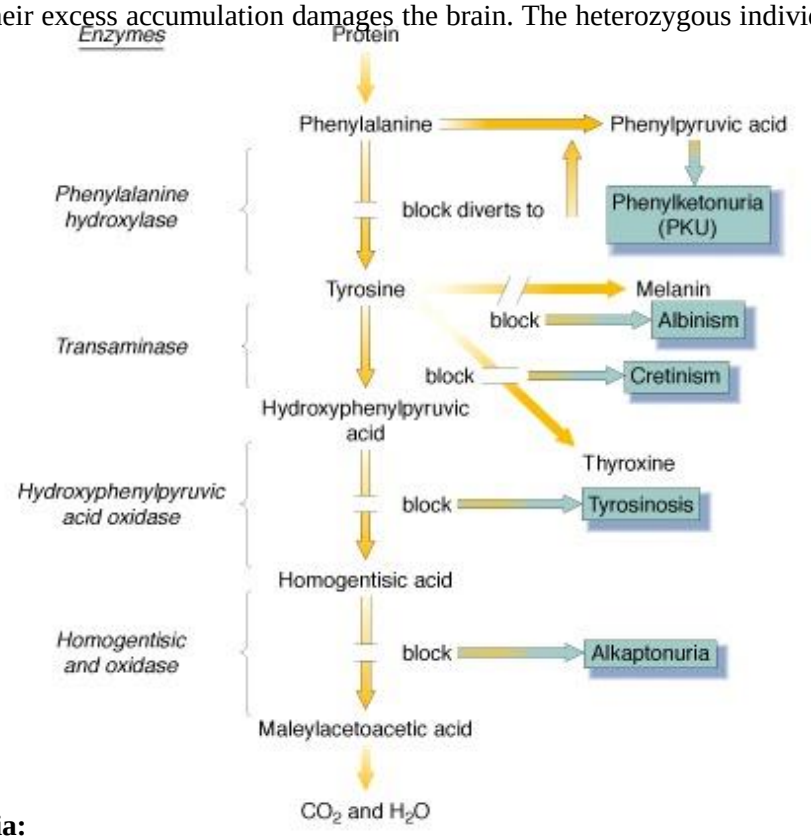
Phenylketonuria (PKU):

This genetic disease was discovered by the Norwegian physician A. Foiling in 1934. It is a single gene disorder caused by the mutation (= change) of a gene on chromosome 12. PKU results when there is deficiency of liver enzyme phenylalanine hydroxylase which converts phenylalanine (an amino acid)

into tyrosine (amino acid). Thus, there is high level of phenyl alanine in the blood and tissue fluid of the patient causing disease.

Lack of enzyme phenyl alanine hydroxylase (an inborn metabolic disorder) is due to the homozygous recessive gene. Affected babies are normal at birth but within a few weeks there is rise in plasma phenylalanine level which damages the development of brain. Generally by six months severe mental retardation is observed. If these affected children are not treated properly one third of them are unable to walk and two-thirds cannot talk.

Besides mental retardation other symptoms of the disease include decreased pigmentation of hair and skin and eczema. Large amount of phenylalanine and its metabolites although excreted through urine and sweat, their excess accumulation damages the brain. The heterozygous individuals are normal but carriers.



Alkaptonuria:

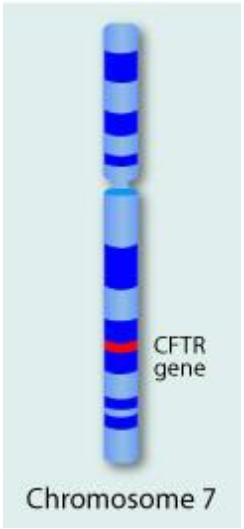
It occur when the absence of an enzyme prevents the breakdown of homogentisic acid. A large amount of homogentisic acid excreted in the urine causes it to turn black upon exposure to air. The acid is oxidized to a black pigment, alkapton. The black pigment is also deposited in the cartilaginous tissue turning them grey to bluish black and arthritis begins at a usually early age.

Albinism:

It is a recessive condition caused by a defect in Phenylalanine tyrosine metabolism. It is characterized by absence of black pigment, melanin.One out of twenty thousand (1/20,0000) is affected by this disease

Cystic fibrosis

Cystic fibrosis is a genetic disorder that affects the respiratory and digestive systems. People with cystic fibrosis inherit a defective gene on chromosome 7 called CFTR (cystic fibrosis transmembrane conductance regulator). The protein produced by this gene normally helps salt (sodium chloride) move in and out of cells.



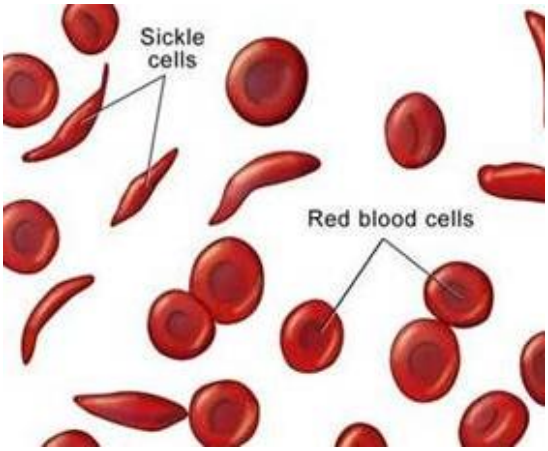
Sickle-Cell Anaemia:

It is a genetic disease of human beings, found especially in Negroes. This disease is caused by a recessive gene Hb^s. The normal gene Hb^A present on chromosome 11 has undergone mutation to produce the recessive Hb^s gene which cause sickle-cell anaemia in homozygous condition (Hb^s Hb^s)

and the patient dies. In heterozygous condition ($Hb^A Hb^s$) the patient survives, only few R.B.Cs are affected. The R.B.Cs in this disease become sickle-shaped in venous blood owing to the lower concentration of oxygen. This causes rupture of cells and severe haemolytic anaemia.

Haemoglobin is a conjugate protein of heme and globulin. It is formed of about 600 amino acids, two identical a chains and two identical β chains. The sixth amino acid in chain of normal haemoglobin is glutamic acid. In sickle-cell haemoglobin glutamic acid is replaced by valine. The children homozygous for sickle-cell gene (Hb^sHb^s) produce rigid chains. When oxygen level of blood drops below certain level, R.B.Cs undergo sickling.

Such cells do not transport oxygen efficiently they are removed by spleen causing severe anaemia. Individuals with Hb^AHb^A genotype are normal, those with Hb^sHb^s genotype have sickle-cell disease and those with Hb^AHB^s geno-type have the sickle-cell trait. Two individual with sickle-cell trait can produce children with all three phenotypes. Individuals of sickle-cell trait are immune to malaria.



	β chain of Haemoglobin-A	β chain of Haemoglobin-S	
1.	Valine	Valine	1.
2.	Histidine	Histidine	2.
3.	Leucin	Leucin	3.
4.	Threonine	Threonine	4.
5.	Proline	Proline	5.
6.	glutamic acid	Valine	6.
7.	Glutamic acid	Glutamic acid	7.
8.	Lysine	Lysine	8.

Fig. 5.30. β chain of normal haemoglobin and sickle shaped haemoglobin showing difference in the arrangement of amino acids.

Thalassemia:

It is an autosome-linked recessive disease, which occurs due to either mutation or deletion, resulting in reduced rate of synthesis of one of the globin chains of haemoglobin. Anaemia is the main feature of this disease.

Thalassemia is classified into three types on the basis of globin chain affected:

(i) Alpha (α) Thalassemia:

It is controlled by the closely linked genes HBA1 and HBA2 on chromosome 16. In this, the production of globin gene is affected due to the mutation or deletion of one or more of the four alleles.

(ii) Beta (β) Thalassemia:

It is controlled by a single gene HBB on chromosome 11. It occurs due to the mutation in one or both the alleles of the gene. Hence, there is a reduced synthesis of beta globin of haemoglobin.

(iii) Delta (δ) Thalassemia:

As well as alpha and beta chains present in haemoglobin about 3% of adult haemoglobin is made up of alpha and delta chains. Just like beta thalassemia mutations can occur which affect the ability of this gene to produce delta chains.

Huntington's disease

An inherited condition in which nerve cells in the brain break down over time

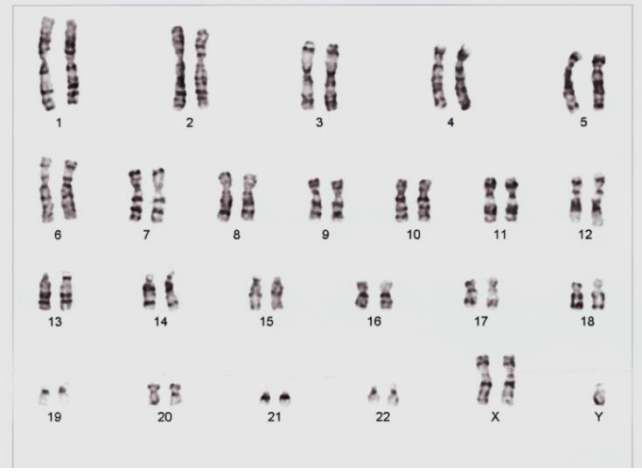
2. Chromosomal Genetic Disorder

In these disorders, entire chromosomes, or large segments of them, are missing, duplicated, or otherwise altered.

Can be organized into two basic groups:

1) Numerical Abnormalities: When an individual is missing either a chromosome from a pair (monosomy) or has more than two chromosomes of a pair (trisomy)
2) Structural Abnormalities: When the chromosome's structure is altered.

Klinefelters Syndrome (2n = 47 or 44 + XXY):

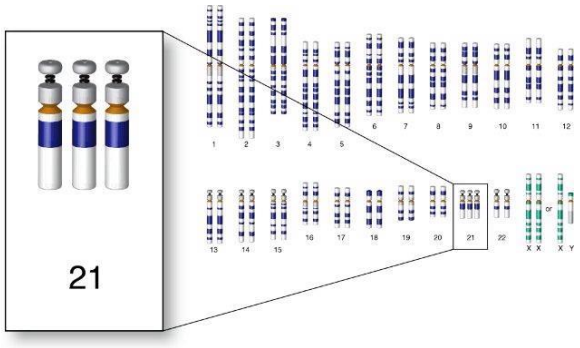


H.F. Klinefelter first described this genetic disorder in 1942. The chromosome number of the patient is 2n = 47 and the chromosome formula is 44A + XXY. Thus, there is an extra chromosome in male. This syndrome originates when ovum with XX chromosomes (ab normal egg) unites with a normal sperm with an Y chromosome or a normal ovum (with an X chromosome) unites with an abnormal sperm carrying XY chromosomes. The individual has 47 chromosomes (44 + XXY).

Such persons are sterile males with undeveloped testes, mental retardation, thinly scattered body hair, and long limbs and with some female characteristics such as enlarged breasts (gynaecomastia) and feminine pitched voice. It is reported that the more the X chromosomes, the greater is the mental defect klinefelter's syndrome is generally seen in one out of every 500 male births. It arises by the nondisjunction of sex chromosomes during meiosis.

Down's syndrome (=Monogolian Idiocy, Monogolism, 21-trisomy):

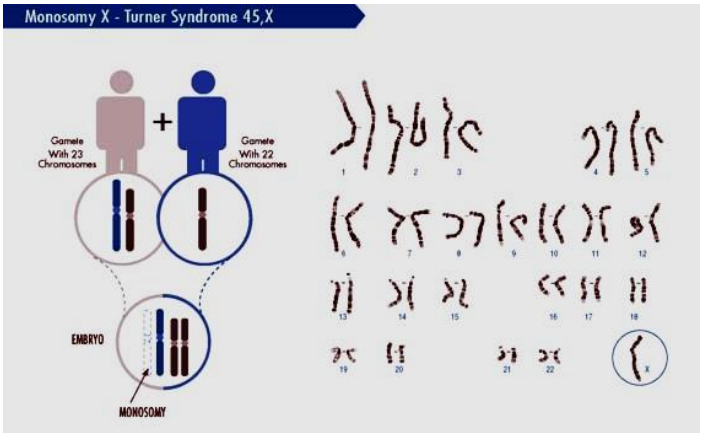
Down's syndrome is one of the most common chromosome abnormality of man. It was first reported by a British physician Langdon Down in 1866. It is caused by the presence of an extra chromosome 21. During normal oogenesis a chromosome of the pair 2] enters into an ovum.



But due to non-disjunction (= non-separation) of chromosomes of pair 21 during meiosis both the chromosomes of pair 21 pass into a single ovum. Thus the ovum possesses 24 chromosomes in stead of 23 and offspring has 47 chromosomes (45 + XY in male, 45 + XX in female) in stead of 46. One in every 600 children is a victim of this genetic disorder. Persons suffering from Down syndrome resemble Mongolians.

They have broad fore head, short and broad neck, short and stubby fingers, permanently open mouth, protruding tongue, projecting lower lip, fiat hands. The victim suffers from severe mental retardation (IQ generally below 40) because of malformation of central nervous system, heart and other organs may be defective.

Gonads and genitalia are underdeveloped. Women around 45 years of age are more likely to produce children having Down's syndrome. Translocation of a portion of chromosome 21 on autosome 14 also results in Down's syndrome. In translocation Down's syndrome chromosome number is 2n – 46.



Turner Syndrome (2 n = 45 or 44A + X):

Henry H. Turner first described this genetic disorder in 1938. The chromosome number is 2n = 45, the chromosomal formula is 44A + XO. It is caused by the absence of one X

chromosome in female When an abnormal ovum (without X chromosome) unites with a normal sperm (having X chromosome) or a normal ovum (having X) fuses with an abnormal sperm (without X) a female individual is formed having 45 chromosomes i.e. one less chromosome than the normal 46.

These are sterile female (incapable of reproduction) with poorly developed ovaries, under developed breasts, small uterus, short stature and abnormal intelligence. They have webbed neck and broad chest.

Patau syndrome

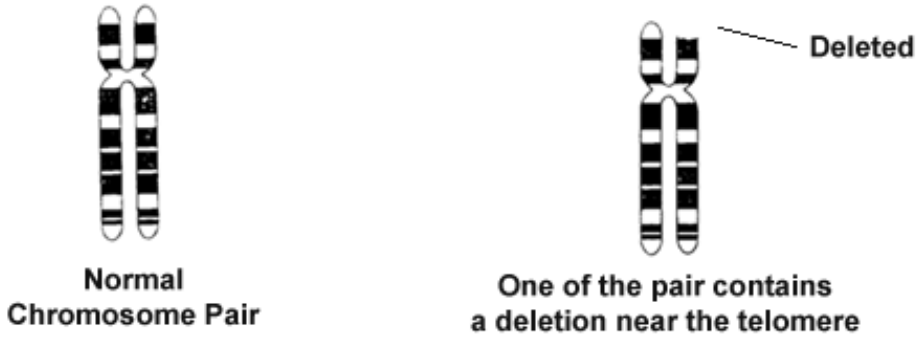
Trisomy at chromosome 13 causing Intellectual disability and motor disorder, Structural eye defects, polydactyly, abnormal genitalia, kidney defects

Edwards syndrome

Trisomy at chromosome 18, 47,XX,+18, Multiple malformation of many organs lowest, Malformed ears, small mouth and nose, mental deficiency

Cri-du-chat:

Cat like mewing of infants, microcephaly (small head), broad face and saddle nose, widely spaced eyes, Individuals die in infancy/childhood, I.Q. range 20-40, Deletion of short arm of chromosome 5; translocation of the short arm of chromosome 15 producing a deficiency for 5S.



3. Multifactorial Disorder

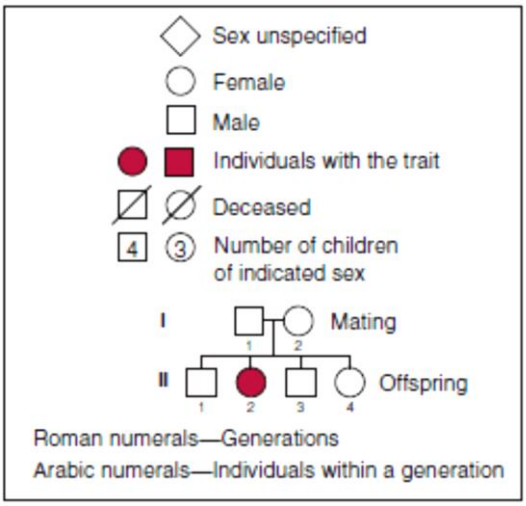
These disorders involve variations in multiple genes, often coupled with environmental causes.

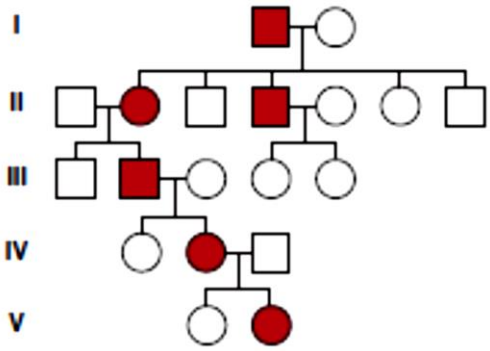
Some example: Alzheimer's Disease, Breast/Ovarian Cancer, Colon Cancer, Hypothyroidism. Asthma, cancers, cleft palate, diabetes, heart disease, hypertension, inflammatory bowel disease, mood disorder, obesity, refractive error, infertility

Pedigree Analysis:

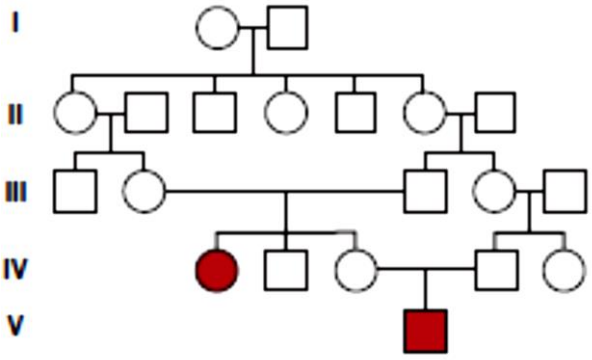
Pedigrees are diagrams that show the relationships among the members of a family. It is customary to represent males as squares and females as circles. A horizontal line connecting a circle and a square represents a mating. The offspring of the mating are shown beneath the mates, starting with the first born at the left and proceeding through the birth order to the right. Individuals that have a genetic condition are indicated by coloring or shading. The generations in a pedigree are usually denoted by Roman numerals, and particular individuals within a generation are referred to by Arabic numerals following the Roman numeral. Traits caused by dominant alleles are the easiest to identify. Usually, every individual who carries the dominant allele manifests the trait, making it possible to trace the transmission of the dominant allele through the pedigree. Every affected individual is expected to have at least one affected parent, unless, of course, the dominant allele has just appeared in the family as a result of a new mutation—a change in the gene itself. However, the frequency of most new mutations is very low—on the order of one in a million; consequently, the spontaneous appearance of a dominant condition is an extremely rare event. Dominant traits that are associated with reduced viability or fertility never become frequent in a population. Thus, most of the people who show such traits are heterozygous for the dominant allele. If their spouses do not have the trait, half their children should inherit the condition.

Recessive traits are not so easy to identify because they may occur in individuals whose parents are not affected. Sometimes several generations of pedigree data are needed to tracethe transmission of a recessive allele. Nevertheless, a large number of recessive traits have been observed in humans—at last count, over 4000. Rare recessive traits are more likely to appear in a pedigree when spouses are related to each other—for example, when theyare first cousins. This increased incidence occurs because relatives share alleles by virtue of theircommon ancestry. Siblings share one-half their alleles, half siblings one-fourth their alleles, and firstcousins one-eighth their alleles. Thus, when such relatives mate, they have a greater chance of producing a child who is homozygous for a particular recessive allele than do unrelated parents. Many of the classical studies in human genetics have relied on the analysis of matings between relatives, principallyfirst cousins.





(b) Dominant trait



(c) Recessive trait

Expected patterns for various types of inheritance in pedigrees

Autosomal recessive inheritance:

- Trait often skips generations.
- An almost equal number of affected males and females.
- If both parents are affected, all children should be affected.
- In most cases of unaffected people matingwith affected individuals, all children produced are unaffected. When at least one child is affected (indicating that the unaffected parent is heterozygous), then approximately half of the children should be affected.
- Most affected individuals have unaffected parents.

Autosomal dominant inheritance:

- Trait should not skip generations.
- An affected person mating with an unaffected person should be produced approximately 50% affected offsprings (indicating also that the affected individual is heterozygous).
- The distribution of the trait among sexes should be equal.
- Transmitted by the either sex

X-linked recessive inheritance:

- Most affected individuals are males.
- Affected males result from mothers who have affected or who are known to be carrier (heterozygous).
- Affected females comes from affected fathers and affected or carrier mothers
- The sons of affected females should be affected.
- Approximately half of the sons of carrier should be affected.

X-linked dominant inheritance:

- ❖ The trait does not skip generations.
- ❖ Affected males must come from affected mothers.
- ❖ Approximately half of the children of an affected heterozygous female are affected.
- ❖ Affected females come from affected fathers or mothers
- ❖ All of the daughters, but none of the sons, of an affected fathers are affected.

Y-linked dominant inheritance:

- ❖ Affectes only males.
- ❖ Affected males always have an affected fathers
- ❖ All sons of an affected man are affected.

Gene therapy:

Cell membranes are impermeable to large macromolecules such as proteins; thus, enzymes must be synthesized in the cells where they are needed. Therefore, treatment of inherited diseases is largely restricted to those cases where the missing metabolite is a small molecule that can be distributed to the appropriate tissues of the body through the circulatory system, or the symptoms can be controlled by modifying the individual's diet. For many other inherited diseases, gene therapy offers the most promising approach to successful treatment. Gene therapy involves adding a normal (wild-type) copy of a gene to the genome of individual carrying defective copies of the gene. A gene that has been introduced into a cell or organism is called a transgene (for transferred gene) to distinguish it from endogenous genes, and the organism carrying the introduced gene is said to be transgenic. If gene therapy is successful, the transgene will synthesize the missing gene product and restore the normal phenotype.

Types:

Gene therapy may be classified into two types:

Somatic:

In somatic cellgene therapy (SCGT), the therapeutic genes are transferred into any cell other than a gamete, germ cell, gametocyte, or undifferentiated stem cell. Any such modifications affect the individual patient only, and are not inherited by offspring. Somatic gene therapy represents mainstream basic and clinical research, in which therapeutic DNA (either integrated in the genome or as an external episome or plasmid) is used to treat disease.

Germline:

In germline gene therapy (GGT), germ cells (sperm or egg cells) are modified by the introduction of functional genes into their genomes. Modifying a germ cell causes all the organism's cells to contain the modified gene. The change is therefore heritable and passed on to later generations.

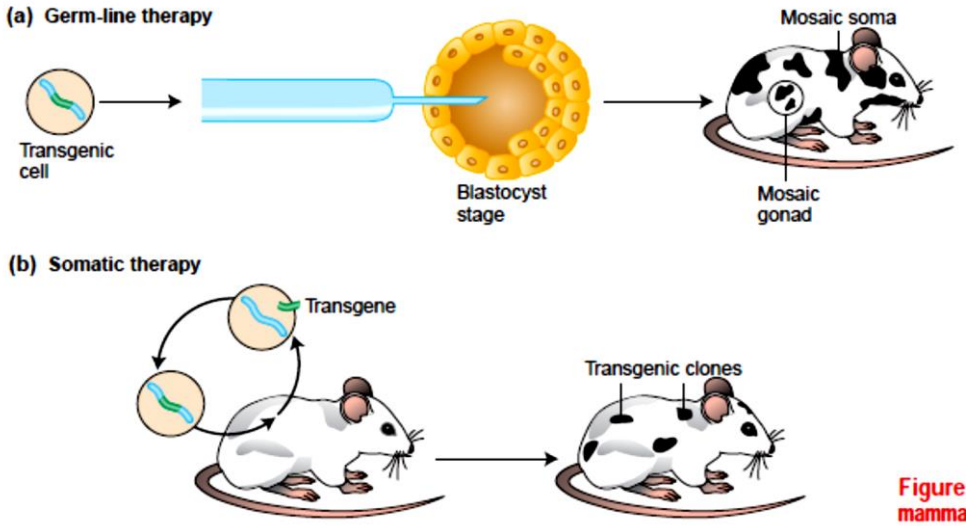


Figure
mamma

Gene Therapy Strategies

Gene Augmentation Therapy (GAT)

In GAT, simple addition of functional alleles is used to treat inherited disorders caused by genetic deficiency of a gene product, e.g. GAT has been applied to autosomal recessive disorders. Dominantly inherited disorders are much less amenable to GAT.

Targeted Killing of Specific Cells

It involves utilizing genes encoding toxic compounds (suicide genes), or prodrugs (reagents which confer sensitivity to subsequent treatment with a drug) to kill the transfected/ transformed cells. This general approach is popular in cancer gene therapies.

Targeted Inhibition of Gene Expression

This is to block the expression of any diseased gene or a new gene expressing a protein which is harmful for a cell. This is particularly suitable for treating infectious diseases and some cancers.

Targeted Gene Mutation Correction

It is used to correct a defective gene to restore its function which can be done at genetic level by homologous recombination or at mRNA level by using therapeutic ribozymes or therapeutic RNA editing.

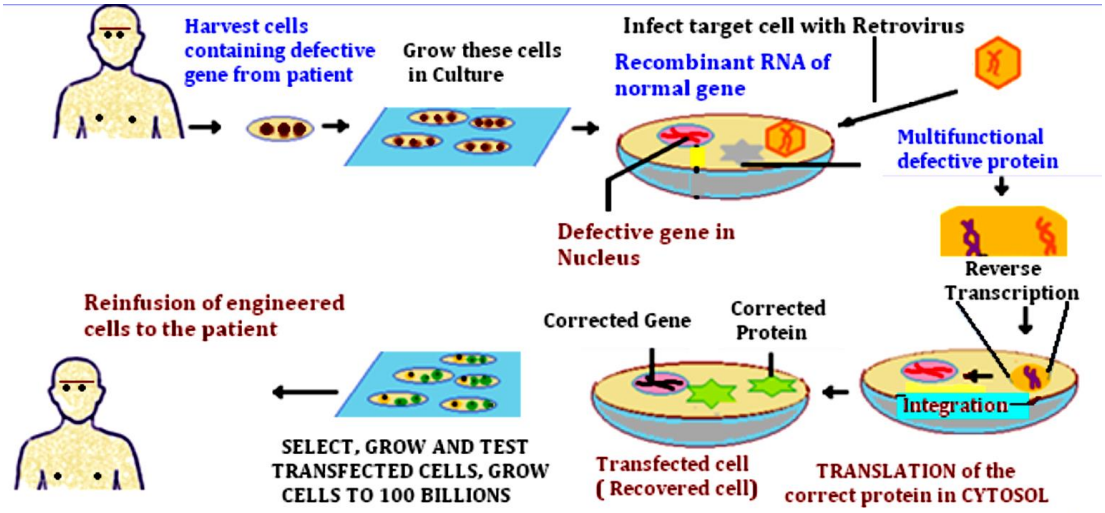
Methods of gene therapy

There are mainly two approaches for the transfer of genes in gene therapy:

1. Transfer of genes into patient cells outside the body (*ex vivo gene therapy*)
2. Transfer of genes directly to cells inside the body (*in vivo*).

***Ex vivo* gene therapy**

- In this mode of gene therapy genes are transferred to the cells grown in culture, transformed cells are selected, multiplied and then introduced into the patient.
- The use of autologous cells avoids immune system rejection of the introduced cells.
- The cells are sourced initially from the patient to be treated and grown in culture before being reintroduced into the same individual.
- This approach can be applied to the tissues like hematopoietic cells and skin cells which can be removed from the body, genetically corrected outside the body and reintroduced into the patient body where they become engrafted and survive for a long period of time.



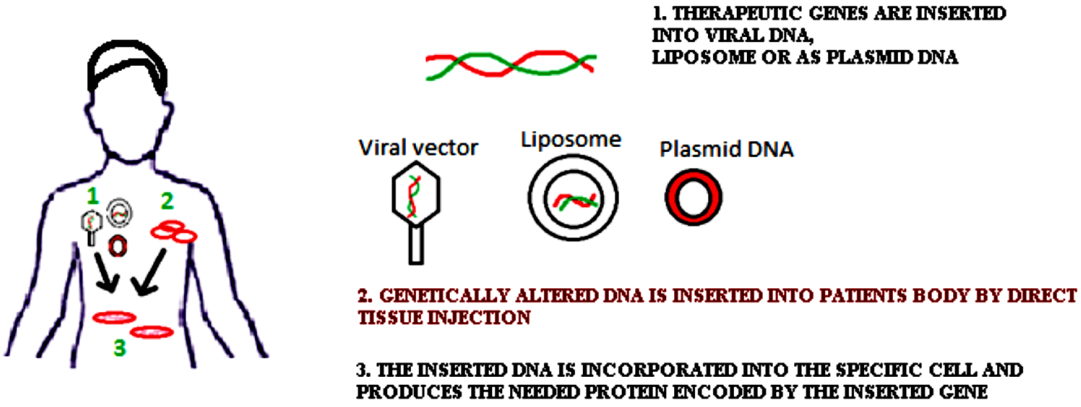
***Ex vivo* therapy involves tightly regulated cellular manipulation in harvested cells**

In Vivo Gene Therapy

- In vivo method of gene transfer involves the transfer of cloned genes directly into the tissues of the patient.
- This is done in case of tissues whose individual cells cannot be cultured in vitro in sufficient numbers (like brain cells) and/or where re-implantation of the cultured cells in the patient is not efficient.
- Liposomes and certain viral vectors are employed for this purpose because of lack of any other mode of selection.
- In case of viral vectors such type of cultured cells were often used which have been infected with the recombinant retrovirus in vitro to produce modified viral vectors regularly. These

cultured cells will be called as vector-producing cells (VPCs)). The VPCs transfer the gene to surrounding disease cells.

- The efficiency of gene transfer and expression determines the success of this approach, because of the lack of any way for selection and amplification of cells which take up and express the foreign gene.



***In vivo* therapy various step**

Potential advantages

Gene therapy can potentially be used to treat genetic disorders with single or few administrations rather than frequent dosing, improving quality of life and reducing the need for physician visits. Gene therapy also offers the potential to specifically target the affected tissues within the body.

Speculative Uses:

Speculated uses for gene therapy include:

Fertility:

Gene therapy techniques have the potential to provide alternative treatments for those with infertility. Recently, successful experimentation on mice has proven that fertility can be restored by using the gene therapy method, CRISPR. Spermatogenical stem cells from another organism were transplanted into the testes of an infertile male mouse. The stem cells re-established spermatogenesis and fertility.

Gene doping:

Athletes might adopt gene therapy technologies to improve their performance.Gene doping is not known to occur, but multiple gene therapies may have such effects. Many argue that gene doping could level the playing field if all athletes receive equal access. Critics claim that any therapeutic intervention for non-therapeutic/enhancement purposes compromises the ethical foundations of medicine and sports.

Human Genetic engineering:

Genetic engineering could be used to cure diseases, but also to change physical appearance, metabolism, and eve improve physical capabilities and mental faculties such as memory and intelligence. Ethical claims about germline engineering include beliefs that every fetus has a right to remain genetically unmodified, that parents hold the right to genetically modify their offspring, and that every child has the right to be born free of preventable diseases. For parents, genetic engineering could be seen as another child enhancement technique to add to diet, exercise, education, training, cosmetics, and plastic surgery. Another theorist claims that moral concerns limit but do not prohibit germline engineering.

Human gene therapy is performed under strict guidelines developed by the National Institutes of Health (NIH). Each proposed gene-therapy procedure is scrutinized by review committees at both the local (institution or medical center) and national (NIH) levels. Several requirements must be fulfilled before a gene-therapy procedure will be approved:

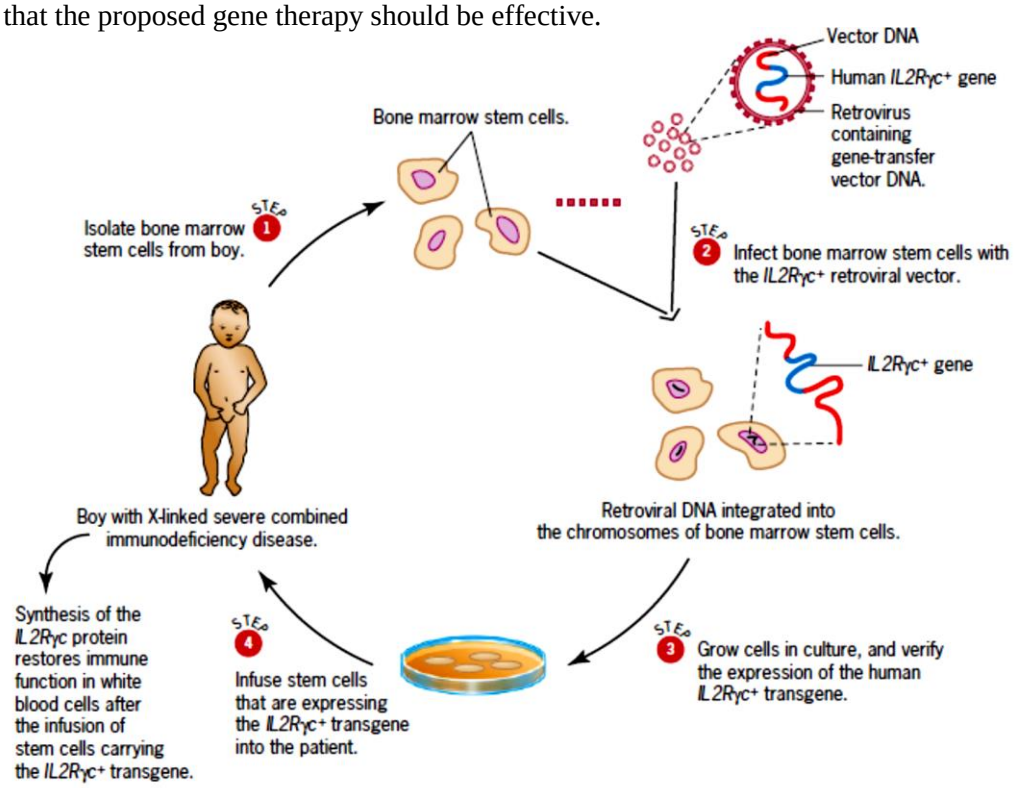
1. The gene must be cloned and well characterized; that is, it must be available in pure form.

2. An effective method must be available for delivering the gene into the desired tissue(s) or cells.

3. The risks of gene therapy to the patient must have been carefully evaluated and shown to be minimal.

4. The disease must not be treatable by other strategies.

5. Data must be available from preliminary experiments with animal models or human cells and must indicate that the proposed gene therapy should be effective.



Disadvantages:

Long lasting therapy is not achieved by gene therapy; Due to rapid dividing of cells benefits of gene therapy is short lived.

Immune response to the transferred gene stimulates a potential risk to gene therapy.

Viruses used as vectors for gene transfer may cause toxicity, immune responses, and inflammatory reactions in the host.

Disorders caused by defects in multiple genes cannot be treated effectively using gene therapy.

Genetic Counseling

Genetic counselingis a field that provides information to patients and others who are concerned about hereditary conditions. It is an educational process that helps patieand family members deal with many aspects of a genetic condition including a diagnosis, information about symptoms and treatment, and information about the mode of inheritance. Genetic counseling also helps a patient and the family cope with the psychological and physical stress that may be associated with the disorder. Clearly, all of these considerations cannot be handled by a single person; so most genetic counseling is done by a team that can include counselors, physicians, medical geneticists, and laboratory personnel.

Genetic counseling usually begins with a diagnosis of the condition. On the bases of a physical examination, biochemical tests, DNA testing, chromosome analysis, family history, and other information, a physician determines the cause of the condition. An accurate diagnosis is critical, because treatment and the probability of passing the condition

on may vary, depending on the diagnosis. For example, there are a number of different types of dwarfism, which may be caused by chromosome abnormalities, single-gene mutations, hormonal imbalances, or environmental factors. People who have dwarfism resulting from an autosomal dominant gene have a 50% chance of passing the condition

on to their children, whereas people who have dwarfism caused by a rare recessive gene have a low likelihood of passing the trait on to their children.

Genetic counselors:

The National Society of Genetic Counselors (NSGC) officially defines genetic counseling as the understanding and adaptation to the medical, psychological and familial implications of genetic contributions to disease. This process integrates:

- Interpretation of family and medical histories to assess the chance of disease occurrence or recurrence.
- Education about inheritance, testing, management, prevention, resources
- Counseling to promote informed choices and adaptation to the risk or condition.

Genetic counselors work as members of a health care team, providing information and support to families with congenital disorders. A genetic counselor is an expert with a Master of Science degree in genetic counseling.

Types of genetic counseling:

1. Prenatal

Prenatal counseling is focused on **pregnant women** with the high risk of having a child with some genetic problems.

- It is often based on abnormal screening findings.
- It is recommended to women, who already have an affected child, during her next pregnancy.
- The most important technique is **amniocentesis**.
- In some very serious cases it is recommended to interrupt the pregnancy.

2. Postnatal

- Postnatal counseling is important for people, who discover some serious inherited disease in their family (**positive family history**).
- Another group are families with **disabled newborns** (inborn syndromes).
- Pairs with two or more **pregnancy losses** can be also refered to the counseling.

Steps of genetic counseling:

- Diagnosis - based on accurate family history, medical history, examination and investigations
- Mode of inheritance and risk of developing/transmitting the disease
- Communicating the information to the families so that they make their own decisions in view of their risk and family goals and their ethical and religious standards
- Management and prognosis
- Options available for dealing with the risks
- Maintaining contact with families and follow up

Common reason of seeking genetic counselling:

1. A person knows of a genetic disease in the family.
2. A couple has given birth to a child with a genetic disease, birth defect, or chromosomal abnormality.
3. A couple has a child who is mentally retarded or has a close relative who is mentally retarded.
4. An older woman becomes pregnant or wants to become pregnant. There is disagreement about the age at which a prospective mother who has no other risk factor should seek genetic counseling; many experts suggest that any prospective mother age 35 or older should seek genetic counseling.
5. Husband and wife are closely related (e.g., first cousins).
6. A couple experiences difficulties achieving a successful pregnancy.
7. A pregnant woman is concerned about exposure to an environmental substance (drug, chemical, or virus) that causes birth defects.
8. A couple needs assistance in interpreting the results of a prenatal or other test.
9. Both prospective parents are known carriers for a recessive genetic disease.

7. Gene knockout mutation in reference to mice and yeast models.

Gene knockout:

The gene knockout method is one of the traditional and most trusted methods used since long for studying the function of a gene or a group of function for different genes.

A gene is a functional piece of a DNA which encodes a protein, by inactivating a gene either by removing DNA sequence, altering DNA sequence or introducing a mutation inhibits the normal function of a gene- loss of function.

That gene is then inserted into the germline cells of a model organism and allows growing. Using artificial vectors it can be inserted into the growing embryo.

If a gene knockout performed well, a visible phenotypic variation can be observed or alteration in the biochemical phenotype can be reported. This is the simple explanation of gene knockout.

"Suppressing the function of a gene or inactivating it using gene manipulation methods in a DNA sequence of a gene is called a gene knockout process."

"Using either gene manipulation or artificial mutagenesis, loss of function of a gene can be caused to study the function of a particular gene in an animal model organism is called gene knockout method."

Let's take an example, suppose we wish to study how mice coat hairs are developed. For let say an *MCH* gene encodes mice coat hair.

If we wish to study how the *MCH* gene works we have to suppress its activity or inactivate it. For that, we can use different techniques,

We can introduce a mutation into the DNA sequence of the *MCH* gene, we can inactivate some of the promoter sequences which regulate its gene expression or we can remove the entire *MCH* gene.

Then the final version of the (inactive) gene is introduced into the vector and which are inserted into the embryonic stem cells.

The model organism- transgenic mice developed from the homozygous cell types may not have hairs on its coat; we can call it "naked mice".

The *MCH* gene might also be linked to some other function as well, that functions are also suppressed in the homozygous mice and can be studied.

The genetically altered mice are called gene knockout mice or gene knockout organism or gene knockouts.

Different Methods for gene knockout

Homologous Recombination- Homologous recombination is the conventional method for gene knockout and widely used in genome engineering. This method comprises of nucleotide exchange between DNA sequences which are either similar or identical. Homologous recombination method includes a DNA construct with the mutation of choice and a drug resistance cassette to be interchanged in place of knockout gene. Additionally, the construct also includes a homologous region of nearly 2Kb with the target gene. Microinjection or electroporation are the most common methods which are next applied to transfer the construct into desired organism. The incorporation of vector construct into target site depends on the DNA repair mechanism of the organism. Once incorporated the vector construct will result in alternation of wild ype gene and eventually production of non-functional protein. However the efficiency of homologous recombination accounts only upto 10^{-2} to 10^{-3} integration of DNA.

Site-Specific Nucleases- There are namely three methods, zinc fingers, TALENS and CRISPER which is known to introduce double stranded breaks in DNA. Following DNA damage, the cells own

repair mechanism get functional through non-homologous end joining (NHEJ), to ligate two open ends. The repair mechanism being finished imperfectly generates insertion or deletion mutation which results in frame shift mutation. Following the mutation the gene produces non-functional protein and generates a knockout for the gene of interest.

Zinc-Fingers:

Zinc finger nucleases (ZFNs) are restriction enzymes widely used in genome engineering for initiating double stranded breaks. They are known to act as site specific endonuclease which has the ability to bind to DNA at a specific site. Zinc finger nucleases (ZFNs) contain two part, a zinc finger DNA binding domain fused with a DNA cleavage part. The DNA bonding domain is consisting of 3-6 zinc finger repeats which can further recognize and bind 9-18 base pairs in a specific DNA sequence. On the other hand the cleavage domain is comprised of a type II restriction endonuclease FokI. When both the domains are assembled together, the zinc finger endonuclease protein acts as a highly effective genomic scissors. When delivered in cell, Zinc finger nucleases (ZFNs) are producing site specific DNA double strand break followed by homologous recombination. As ZFN plasmids are able to express ZFN and effectively target a double stranded break, they provide an excellent opportunity to predesigned therapeutic constructs on a preselected location in genome. ZFNs provides several benefits in targeting genome editing, such as-

- ZFNs are able to integrate or disrupt any location of genome rapidly.
- Mutations created by ZFNs are permanent and can be inherited.
- Genome editing are possible with the help of single transfection.
- They can be used for a wide range of mammalian cell lines
- No antibiotic selections are required for positive clone selection
- Knock-out/ knock-in effects are seen to exists nearly for two months

Applications-

- Zinc finger nucleases (ZFNs) are used for creating complete knockout in several cell lines.
- They can be used in cell based screening methods by generating knock-in cells lines where endogenous target genes can be tagged with promoter, reporter or fusion tag proteins.
- They are also useful for generating cell lines to produce specific protein or antibodies in higher amount.

Although Zinc finger nucleases (ZFNs) shows several promising effects, but there are also chance of having potential side effects. When the ZFN construct do not target or not specific off target cleavage takes place. In such scenarios DNA repair mechanism cannot control the overproduction of double stranded breaks and consequently chromosomal rearrangement or cell death takes place. Off-target cleavage also generates random integration in the host genome increasing the risk of immunological response developed by cells in response to therapeutic agents.

TALENS

TALEN or Transcription Activator-Like Effector Nucleases (TALENs) are enzymes which are genetically engineered to edit gene. These enzymes are basically isolated from bacteria of Xanthomonas species and in bacteria they participate in binding and activating the host promoter. In a similarity to ZFNs, TALENS are also consisting of two groups fusing ranscription activator-like (TAL) proteins with Fok1 nuclease. TAL protein consisting of repeating motifs composed of 33to 34 amino acids which can strongly recognize nucleotides of interest. They are also known as Repeat Variable Diresidue (RVD) due to their variable nature. As in TALEN there exist direct relation within DNA and amino acid recognition, it is possible to engineered a specific domain to bind DNA by changing the combination of repeating motif with specific RVD. By fusing TAL with FokI, it can effectively cutting the genome. In presence of two domain of TALEN, FokI create a double stranded break. After constructing TALEN construct they are transfected to host cell with the help of plasmid.

Inside the host cell the construct get expressed and get access to the genome by entering into nucleus. Additionally, in cells TALENs are also being transferred as mRNA which does not require genomic integration process. In comparison to Zinc finger nucleases (ZFNs) TALENS shows advantages, such as-

- The design of TALEN is much easy in comparison to ZFNs. Many TAL repeats can be created with RVD code which will bind strongly with the genomic DNA with high affinity.
- The amount of TALE repeats can be extended based on desired length.

Applications-

- TALENs are used widely for plant genome modification.
- They are useful for the production of biofuels.
- They have been used to generate knockout in organisms such as zebrafish, rat , mice or c.elegance
- It also been used to treat genetic diseases such as xeroderma pigmentation ot sickle cell.

However like ZFNs, TALENs also display off target effect. The offtarget effect will eventually generate chromosomal rearrangement due to presence of unwanted breaks in DNA double strands.

CRISPER/cas9

CRISPER/cas9 is a rapid genome editing methods which is used to delete or modify specific sequences of DNA. CRSIPER is known as Clustered Regularly Interspaced Short Palindromic Repeats which can be found naturally in certain types of bacteria. While invaded by phage viruses, bacteria use CRIPER/Cas9 method to cut and disintegrate the viral DNA. In bacteria there exists three types of CRISPER method, among them type II is most widely studied. At this method, once cut into small parts, the invading DNA gets incorporated into CRIPER locus. Once transcribed, small repeats RNA or crRNA/ CRISPER RNAs are generated. crRNA next get joined by another noncoding RNA known as trans-activating CRISPR RNA or tracrRNA and activates the endonuclease Cas9 to target the invading viral DNA. crRNA together with tracrRNA forms a single guide RNA or sgRNA .CRISPER/Cas9 generates DNA double stranded breaks which can be repaired by two mechanism-i) nonhomologous end joining or NHEJ, where homologous double strands are absent and ii) homology-directed repair which is occurred in presence of synthetic DNA repair template.

Applications-

- CRISPER/Cas9 gene editing system is useful for Homology-directed repair (HDR) mechanism.
- They can be used for gene silencing
- They are functional for transiently activate endogenous genes.
- It is useful for DNA free gene editing methods.
- They are used for transiently silenced expression of genes.
- Preparation of transgenic animals as well as embryonic stem cells.

Following the discovery of CRISPER/Cas9 system, it has been extensively used in several organisms for gene targeting such as plants, humans, c.elegance, zebra fish, yeast, drosophila or mouse. They are now days used to produce single point mutations in presence of single gRNA. In comparison to TALENs and ZNFs, CRISPER/Cas9 is easy to use as here only crRNA required redesigning to target any gene specifically. Additionally, this method also enables genome wide analysis of gene function by producing libraries of large gRNA.

What is Gene knock in?

In genetic engineering, gene knock in refers to insertion or one to ne substitution of any locus which is normally absent in the target organism. By this method any gene, exon with mutation or tag can get inserter or knocked in and they are mostly performed in mice. Commonly knock-in method is applied to create a model organism to study functional aspects of a specific disease. Here the exogenous gene

also gets added through the mechanism of homologous recombination. To perform a gene knock-in following steps are performed-i) a mutated or reporter gene is first cloned into a vector. ii) These genes contain a flanking region known as loxP which are able to go an inverse recombination with a cre recombinase enzyme which is present in the target site. iii) Recombination of loxP with cre recombinase takes out the intervening target DNA and instead the mutated or reporter gene gets introduced in the target site. iii) Embryonic stem cells having the modified gene next placed into early mouse embryo cavity following transfer into a surrogate female mouse. .iv) Further the mouse embryo developed to produce chimeric mouse having the germ line expression of the mutated or reported gene. The transgene get expressed in following generations also. These methods do not show random integration in target genome and rather gives tissue specific expression of the transgene.

Applications-

Knock-in strategy is useful in areas, such as-

- Modelling human disease- Knock-in strategies are used for the generation of skin disease like mice with mutant keratin 10(K10) gene resembles human patients where epidermolytic hyperkeratosis are seen. Knock-in mice are also generated for studying Huntington's disease, which is a hereditary condition resulting in brain cell degeneration.
- **therapeutic compounds safety and efficacy- Preclinical use of wild type mice are prohibited for the tasting of efficacy of therapeutic compounds. To combat the problem mice model of certain human diseases are used.**

Advantage of knock-in methods- Knock-in methods shows several advantages, such as-

- to mimic a disease state of human gene of interest get replaced by a mutated form.
- Integration sites can be identified easily.
- Both conditional and constitutive expression of transgene is possible
- The method is useful for the study of genes where the function has been changed.

However, knock-in methods show some limitations, such as-

- There can be presence of unexplained phenotype due to complex interaction of inserted gene with other genomic regions generating side effects.
- Forced gene overexpression generates complex pattern of protein-protein interactions.
- Knock-in mouse do not show complete resemblance to human disease state.
- Additionally generation of Knock-in mice is expensive.

Conditional gene knockout

Conditional knockouts are performed to delete a gene in a specific tissue in specific time. This method is required for the functional study of individual gene at living organism. In comparison to gene knockout, conditional knockouts are created at adult animals rather than in embryonic stage where a mutation can show lethal effect. In mammalian cell conditional knock outs are created through homologous recombination and following strategies are used.

Recombination with Cre-loxP – cre or cyclization recombination enzyme is able to recognize and cut DNA sequence specifically which is further followed by recombination with second enzyme loxP.Within DNA, Cre recombinase is able to bind with two loxP site resulting a recombination between. Depending on the arrangement of loxP site, the recombination produces deletion or inversions of the target genes. Due to the inducible nature of cre-loxP, the knockout can be induced by chemicals such as tetracyclin and tamoxifane. Tetracyclin function in activating the cre recombinase transcription whereas, tamoxifane is responsible for nuclear transport.

Applications

Conditional gene becomes widely used methods to study human disease in different mammal model such as cancer. Genes for breast cancer like BRCA1 has been studied through gene knockout mice with BRCA1 deletion in tissues of mammary gland to confirm the tumour suppression role.

Conditional mouse models are favoured for the study of human disease as both shows resemblance in phenotype while specific genes are deleted.

RNAi mediated gene silencing

RNAi or RNA intereference is a posttranslational modification which is initiated by double stranded RNAs (dsRNA). At the time of RNA intereference, double stranded RNAs are cut out or 'diced' out by an enzyme Dicer, member of RNase III family into smaller parts and those small fragments are known as interfering RNA or siRNA. Short siRNA sequences are composed of two strands namely a guide strand and a passenger strand. Following generation of small siRNA fragments, they get attached with the special protein called Argonaute protein. Argonaute 2 or Ago 2 is an endonuclease which is responsible for unwinding of guide strand and a passenger strand. Once the double strands are separated, the guide strand gets attached with RNA Interference Specificity Complex or RISC. RISC altogether with guide strand attached to mRNA with a complementary sequence and results in endonucleolytic cleavage degrading target mRNA. SiRNA are transferred to target cells by transfection agents such as cationic lipid or polymer based or electroporation.

Although RNAi mechanism shows gene known down with target specificity, however off target effect r knock down of unintended genes may take place. To reduce such off target effect several strategies has been identified, such as-siRNAs are chemically modified to preferentially induce the transfer of guide strand to RISC complex or use of pool of siRNA to target single gene to reduce the effect of gene targets which are non-specific and shows off target phenotypes.

Applications

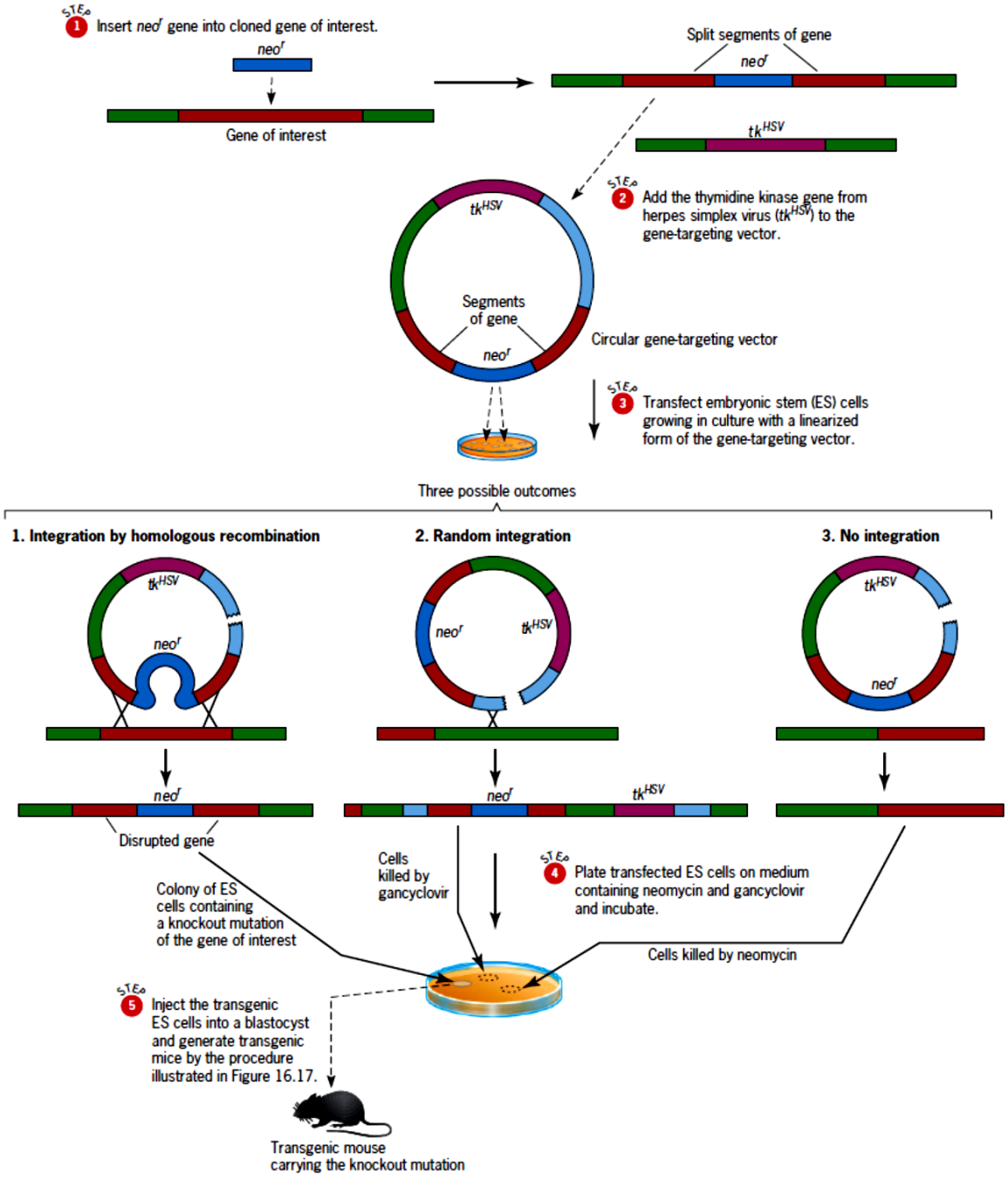
To analyse the function of individual gene, siRNAs are studied in several cellular mechanisms such as apoptosis, insulin signalling or cytokinesis. They have also been used to novel pathways identification and target validation in diseases such as cancer, hepatitis or HIV. Additionally in vivo application of RNAi has also applied in animal disease model for the verification of specific targets so they can be used to develop therapeutic agents.

Knockout mutations in the mouse

Normally, the transgenes are inserted into the genome at random sites. However, if the injected or transfected DNA contains a sequence homologous to a sequence in the mouse genome, it will sometimes be inserted into that sequence by homologous recombination. The insertion of this foreign DNA into a gene will disrupt or "**knock out**" the function of the gene just like the insertion of a transposable genetic element. Indeed, this approach has been used to generate knockout mutations in hundreds of mouse genes.

The first step in the production of mice carrying a knockout mutation in a gene of interest is to construct a gene-targeting vector, a vector with the potential to undergo homologous recombination with one of the chromosomal copies of the gene and, in so doing, insert foreign DNA into the gene and disrupt its function. A gene (neor) that confers resistance to the antibiotic neomycin is inserted into a cloned copy of the genemof interest, splitting it into two parts and making it nonfunctional (step 1). The presence of the neor gene in the vector will allow neomycin to be used to eliminate cells not carrying an integrated copy of the gene-targeting vector or the neor gene. The segments of the gene retained on either side of the inserted neor gene provide sites of homology for recombination with chromosomal copies of the gene. The thymidine kinase gene (tkHSV) from herpes simplex virus is inserted into the cloning vector for subsequent use in eliminating transgenic mouse cells resulting from the random integration of the vector. The thymidine kinase from herpes simplex virus (HSV) phosphorylates the drug gancyclovir, and when this phosphorylated nucleotide-analog is incorporated into DNA, it kills the host cell. In the absence of the HSV thymidine kinase, gancyclovir is harmless to the host cell. The next step is to transfect embryonic stem (ES) cells (from dark-colored mice)

growing in culture with linear copies of the gene-targeting vector (step 3) and subsequently plate them on medium containing neomycin and gancyclovir (step 4). Three different events will occur in the transfected ES cells. (1)



Homologous recombination may occur between the split sequences of the gene in the vector and a chromosomal copy of the gene inserting the neor gene into the chromosomal gene and disrupting its function. When this event occurs, the tkHSV gene will not be inserted into the chromosome. As a result, these cells will be resistant to neomycin, but not sensitive to gancyclovir. (2) The gene-targeting vector may integrate at random into the host chromosome. When this occurs, both the neor gene and the tkHSV gene will be present in the chromosome. These cells will be resistant to neomycin, but killed by gancyclovir. (3) There may be no recombination between the gene-targeting vector and the chromosome and, thus, no integration of any kind. In this case, the cells will be killed

by neomycin. Thus, only the ES cells with the knockout mutation produced by the insertion of the neor gene into the gene of interest on the chromosome will be able to grow on medium containing both neomycin and gancyclovir.

The generation of knockout mutations in the mouse by homologous recombination

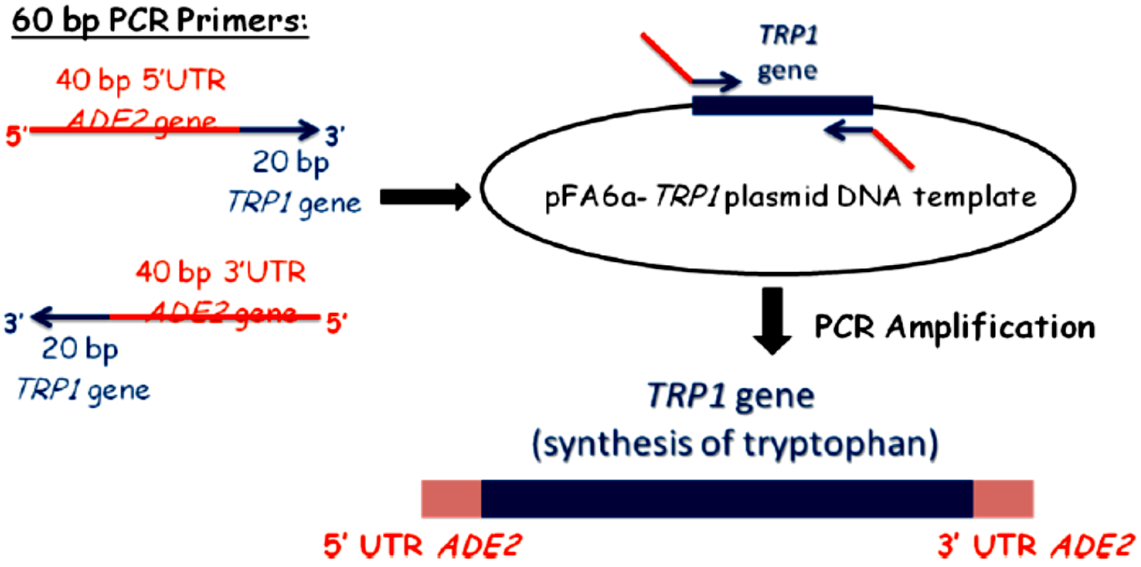
The selected ES cells containing the knockout mutation are injected into blastocysts from light-colored parents, and the blastocysts are implanted into light-colored females. Some of the offspring will be chimeric with patches of light and dark fur. The chimeric offspring are mated with light-colored mice, and any dark-colored progeny produced by this mating are examined for the presence of the knockout mutation. In the last step, male and female offspring that carry the knockout mutation are crossed to produce progeny that are homozygous for the mutation. Depending on the function of the gene, the homozygous progeny may have normal or abnormal phenotypes. Indeed, if the product of the gene is essential early during development, homozygosity for the knockout mutation will be lethal during embryonic development. In other cases, for example, when there are related genes with overlapping or identical functions, mice that are homozygous for the knockout mutation may have wild-type phenotypes, and PCR or Southern blots will have to be performed to verify the presence of the knockout mutation. Knockout mice have been used to study a wide range of processes in mammals including development, physiology, neurobiology, and immunology. Knockout mice have provided model systems for studies of numerous inherited human disorders from sickle-cell anemia to heart disease to many different types of cancer.

Because of the value of knockout mice for studies of processes related to human health, the National Institutes of Health initiated the Knockout Mouse Project in 2006 with the goal of producing knockout mutations in as many mouse genes as possible. All of the knockout strains produced by this collaborative effort are being made available to researchers throughout the world.

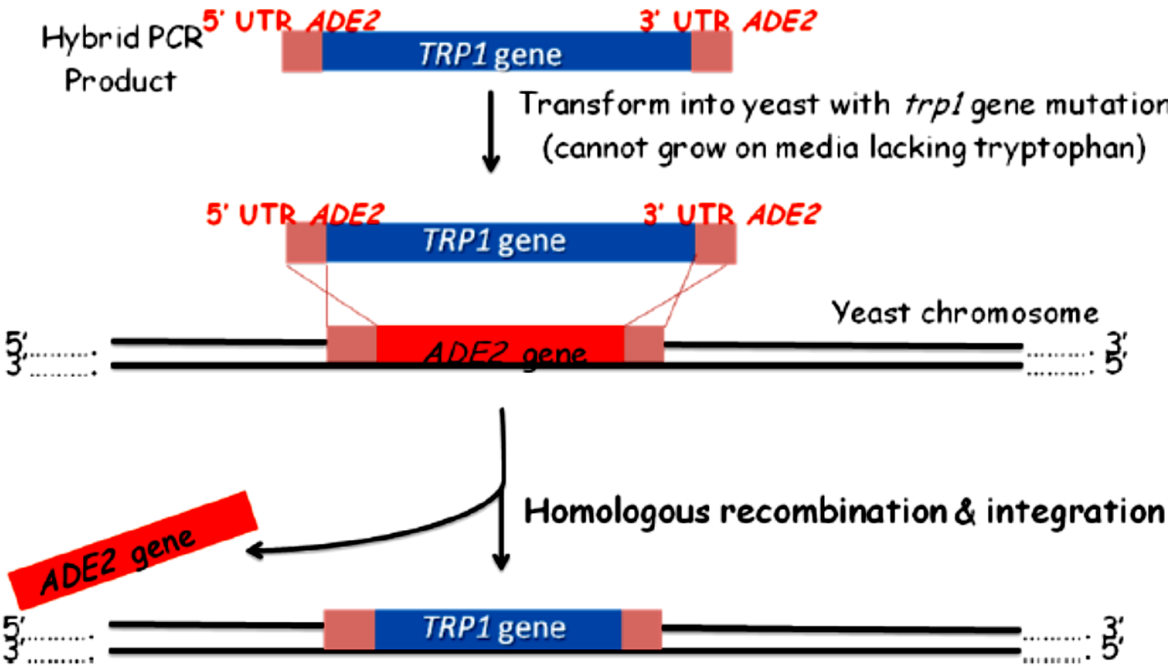
Yeast model:

In the 'Yeast Gene Knockout/Gene Therapy' laboratory exercise, use homologous recombination to target gene deletion in a unicellular, haploid organism: the brewer's yeast Saccharomyces cerevisae. Since mice are multi-cellular and diploid, both copies of the gene must be deleted to visualize a possible phenotype, making for quite a long experiment (months to years). In contrast, yeasts are unicellular and haploid, thus only one copy of a given gene need be deleted to allow phenotype manifestation in a timely manner: 2-3 days. Specifically, students will aim to delete the ADE2 gene, important for purine biosynthesis, using a hybrid piece of DNA generated via a PCR reaction. The hybrid DNA contains 5' and 3' untranslated regions (UTRs) of the ADE2 gene and the protein coding sequence of the TRP1 gene. Through a process of homologous recombination similar to that shown in the Nobel Prize work, the 5' and 3' UTRs of the ADE2 gene found in the hybrid PCR fragment allow specific gene targeting of the ADE2 gene located within the yeast genome. During the gene-targeting event, the ADE2 gene is replaced by the TRP1 gene. TRP1 is important for tryptophan biosynthesis. The strain of yeast that the students will be using for this experiment has a defective trp1 gene in its genome; thus, the yeast is incapable of producing its own supply of tryptophan and must depend on the media for this essential amino acid. If these yeast are plated on media lacking tryptophan, the yeast will die. Therefore, by inserting a wildtype copy of the TRP1 gene into the yeast genome, students are simultaneously 'knocking-out' one gene, ADE2, and inserting a functional TRP1 gene into yeast cells. The visible phenotype of the ADE2 gene removal is transformation from cream-colored yeast to red/pink-colored yeast. The visible phenotype of the TRP1 gene therapy is a transformation from tryptophan-dependence to tryptophanindependence.

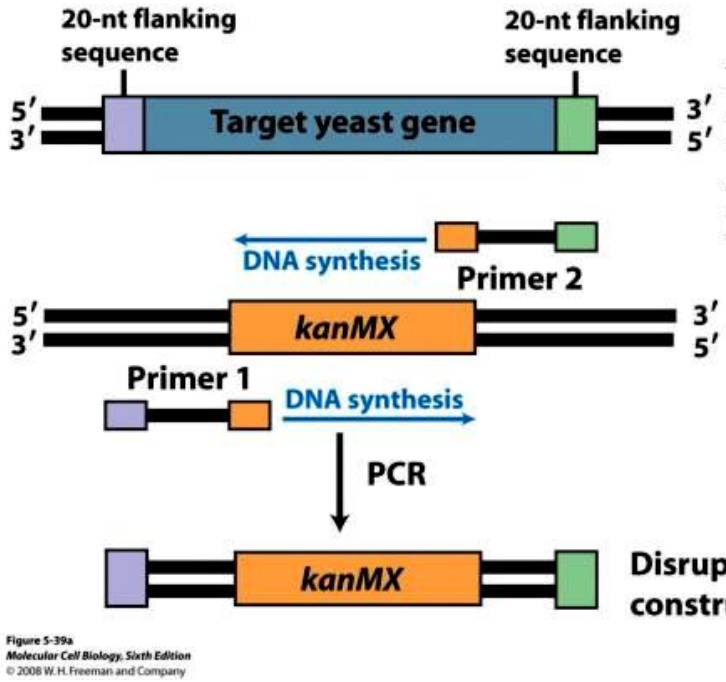
Knockout/Gene Therapy using Hybrid PCR Product



Using Homologous Recombination to Knockout a Specific Gene



Gene Knockout



EXPERIMENTAL FIGURE 5-39(a) Homologous recomnination with fransfected disruption constructs can inactivate specific target genes in yeast

Gene knockout Vs gene knockdown

Making a gene non-functional is called gene knockout, as we are discussed in the entire article while reducing the expression of a gene is known as gene knockdown.

In a simple language, we can say in the gene knockout method we are making a gene inactive totally, while in the gene knockdown, the gene is active but the expression of a gene is reduced to know its activity in a particular cell type.

One of the best methods for inactivating a gene is by introducing a mutation- gene knockout.

Using the RNA interference scientists can reduce gene expression using siRNA or shRNA- gene knockdown.

Applications of gene knockout:

- One of the important application of gene knockout is to study the function of a particular gene.
- It also enables scientists to monitor and control the effect of a gene.
- Gene knockout method is used for constructing genetically modified organism such as GM plants, GM bacteria and GM animals.
- It is also used to study the effect and contribution of a particular gene and its role in the development of a disease.
- It is likewise employed in drug discovery: using gene knockout like genetic engineering tools, drug screening can be done.

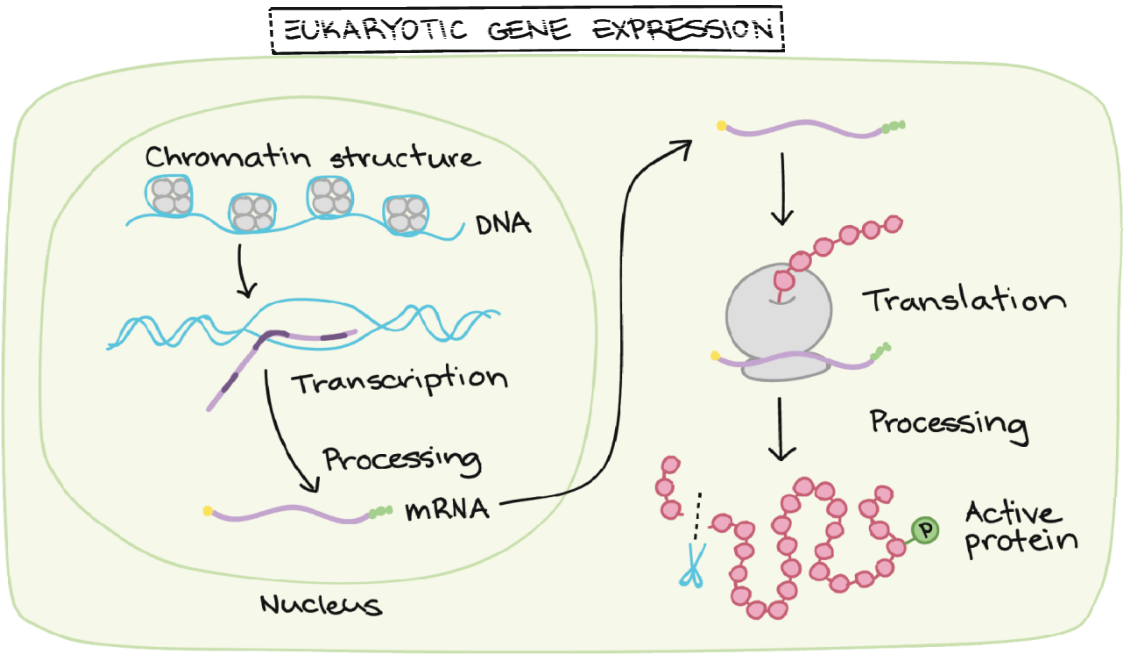
8. Gene regulation at the level of transcription and translation in eukaryotes.

Gene expression is more complicated in eukaryotes than it is in prokaryotes because eukaryotic cells are compartmentalized by an elaborate system of membranes. This compartmentalization subdivides the cells into separate organelles, the most conspicuous one being the nucleus; eukaryotic cells also possess mitochondria, chloroplasts and an endoplasmic reticulum. Each of these organelles performs a different function. The nucleus stores the genetic material, the mitochondria and chloroplasts recruit energy, and the reticulum transports materials within the cell.

The subdivision of eukaryotic cells into organelles physically separates the events of gene expression. The primary event, transcription of DNA into RNA, occurs in the nucleus. RNA transcripts are also modified in the nucleus by capping, polyadenylation, and the removal of introns. The resulting messenger RNAs are then exported to the cytoplasm where they become associated with ribosomes, many of which are located on the membranes of the endoplasmic reticulum. Once associated with ribosomes, these mRNAs are translated into polypeptides. This physical separation of the events of gene expression makes it possible for regulation to occur in different places. Regulation can occur in the nucleus at either the DNA or RNA level, or in the cytoplasm at either the RNA or polypeptide level.

Eukaryotic gene expression involves many steps, and almost all of them can be regulated. Different genes are regulated at different points, and it's not uncommon for a gene (particularly an important or powerful one) to be regulated at multiplesteps.

- **Chromatinaccessibility**:Thestructureofchromatin(DNAanditsorganizingproteins)can be regulated. More open or "relaxed" chromatin makes a gene more available for transcription.
- **Transcription**:Transcriptionisakeyregulatorypointformanygenes.Setsoftranscription factorproteinsbindtospecificDNAsequencesinorneargeneandpromoteorrepressits transcription into anRNA.
- **RNA processing**: Splicing, capping, and addition of a poly-A tail to an RNA molecule can be regulated, and so can exit from the nucleus. Different mRNAs may be made from the same pre-mRNA by alternativesplicing.
- **RNA stability**. The lifetime of an mRNA molecule in the cytosol affects how many proteins can be made from it. Small regulatory RNAs called **miRNAs** can bind to target mRNAs and cause them to be chopped up.
- **Translation**. Translation of an mRNA may be increased or inhibited by regulators. For instance, miRNAs sometimes block translation of their target mRNAs (rather than causing them to be chopped up).
- **Protein activity**. Proteins can undergo a variety of modifications, such as being chopped up or tagged with chemical groups. These modifications can be regulated and may affect the activity or behavior of the protein.



Transcription: The key control point

Transcription is the process where a gene's DNA sequence is copied (transcribed) into an RNA molecule. Transcription is a key step in using information from a gene to make a protein. If you're not familiar with those ideas yet, you might consider watching the central dogma video for a solid intro from Sal.

Gene expression is when a gene in DNA is "turned on," that is, used to make the protein it specifies. Not all the genes in your body are turned on at the same time, or in the same cells or parts of the body. For many genes, transcription is the key on/off control point:

- If a gene is not transcribed in a cell, it can't be used to make a protein in that cell.
- If a gene does get transcribed, it is likely going to be used to make a protein (expressed). In general, the more a gene is transcribed, the more protein that will be made.

Various factors control how much a gene is transcribed. For instance, how tightly the DNA of the gene is wound around its supporting proteins to form chromatin can affect a gene's availability for transcription.

Proteins called transcription factors, however, play a particularly central role in regulating transcription. These important proteins help determine which genes are active in each cell of your body.

Transcriptional Activators and Coactivators

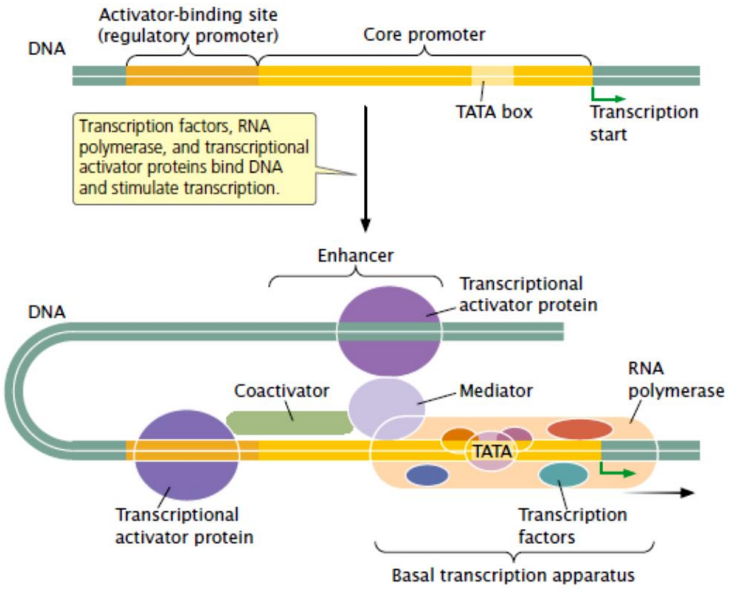
Transcriptional activator proteins stimulate and stabilize the basal transcription apparatus at the core promoter. The activators may interact directly with the basal transcription apparatus or indirectly through protein coactivators. Some activators and coactivators, as well as the general transcription factors, also have acteyltransferase activity and so further stimulate transcription by altering chromatin structure.

Transcriptional activator proteins have two distinct functions. First, they are capable of binding DNA at a specific base sequence, usually a consensus sequence in a regulatory promoter or enhancer; for this function, most transcriptional activator proteins contain one or more DNA-binding motifs. A

second function is the ability to interact with other components of the transcriptional apparatus and influence the rate of transcription.

Within the regulatory promoter are typically several different consensus sequences to which different transcriptional activators can bind. Among different promoters, activator-binding sites are mixed and matched in different combinations, and so each promoter is regulated by a unique combination of transcriptional activator proteins.

Transcriptional activator proteins bind to the consensus sequences in the regulatory promoter and affect the assembly or stability of the basal transcription apparatus at the core promoter. One of the components of the basal transcription apparatus is a complex of proteins called the **mediator**. Transcriptional activator proteins binding to sequences in the regulatory promoter (or enhancer) make contract with the mediator and affect the rate at which transcription is initiated. Some regulatory promoters also contain sequences that are bound by proteins that lower the rate of transcription through inhibitory interactions with the mediator.



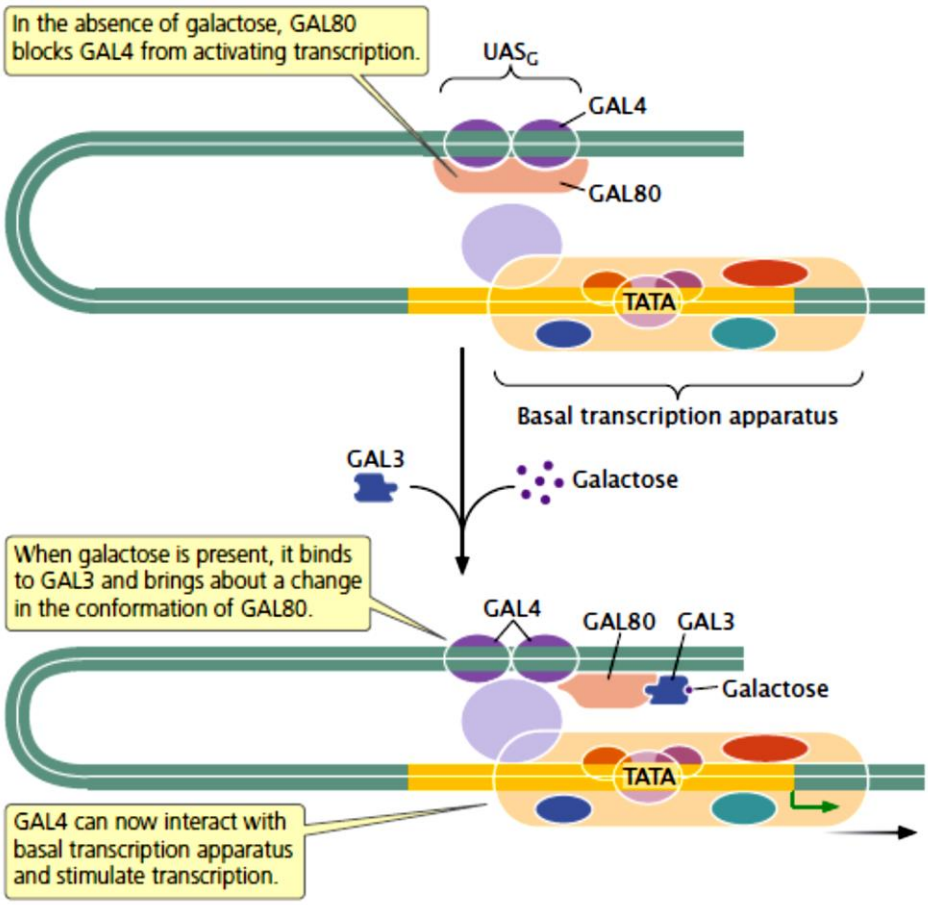
17.5 Transcriptional activator proteins bind to sites on DNA and stimulate transcription. Most act by stimulating or stabilizing the assembly of the basal transcription apparatus.

Regulation of galactose metabolism through GAL4

An example of a transcriptional activator protein is GAL4, which regulates the transcription of several yeast genes whose products metabolize galactose. Like the genes in the lac operon, the genes that control galactose metabolism are inducible: when galactose is absent, these genes are not. transcribed and the proteins that break down galactose are not produced; when galactose is present, the genes are transcribed and the enzymes are synthesized. GAL4 contains several zinc fingers and binds to a DNA sequence called UASG (upstream activating sequence for GAL4). UASG exhibits the properties of an enhancer—a regulatory sequence that may be some distance from the regulated gene and is independent of the gene in position and orientation. When bound to UASG, GAL4 activates the transcription of yeast genes needed for metabolizing galactose.

A particular region of GAL4 binds another protein called GAL80, which regulates the activity of GAL4 in the presence of galactose. When galactose is absent, GAL80 binds to GAL4, preventing GAL4 from activating transcription. When galactose is present, however, it binds to another protein called GAL3, which interacts with GAL80, causing a conformational change in GAL80 so that it can no longer bind GAL4. The GAL4 protein is then free to activate the transcription of the genes, whose products metabolize galactose.

GAL4 and a number of other transcriptional activator proteins contain multiple amino acids with negative charges that form an *acidic activation domain.* These acidic activators stimulate transcription by enhancing the assembly of the basal transcription apparatus.



Transcription is activated by GAL4 in response to galactose.

Transcriptional Repressors

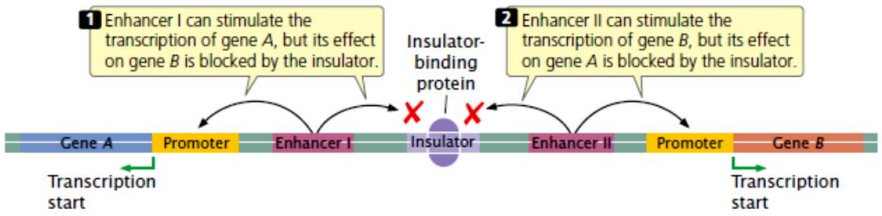
Some regulatory proteins in eukaryotic cells act as repressors, inhibiting transcription. These repressors bind to sequences in the regulatory promoter or to distant sequences called **silencers**, which, like enhancers, are position and orientation independent. Unlike repressors in bacteria, most eukaryotic repressors do not directly block RNA polymerase. These repressors may compete with activators for DNA binding sites: when a site is occupied by an activator, transcription is activated, but, if a repressor occupies that site, there is no activation. Alternatively, a repressor may bind to sites near an activator site and prevent the activator from contacting the basal transcription apparatus. A third possible mechanism of repressor action is direct interference with the assembly of the basal transcription apparatus, thereby blocking the initiation of transcription.

Enhancers and Insulators

Enhancers are capable of affecting transcription at distant promoters. For example, an enhancer that regulates the gene encoding the alpha chain of the T-cell receptor is located 69,000 bp downstream of the gene's promoter. Furthermore, the exact position and orientation of an enhancer relative to the promoter can vary. How can an enhancer affect the initiation of transcription taking place at a promoter that is tens of thousands of base pairs away? In many cases, regulator proteins bind to the enhancer and cause the DNA between the enhancer and the promoter to loop out, bringing the promoter and enhancer close to each other, and so the transcriptional regulator proteins are able to directly interact with the basal transcription apparatus at the core promoter. Some enhancers may be

attracted to promoters by proteins that bind to sequences in the regulatory promoter and "tether" the enhancer close to the core promoter. A typical enhancer is some 500 bp in length and contains 10 binding sites for proteins that regulate transcription.

Most enhancers are capable of stimulating any promoter in their vicinities. Their effects are limited, however, by **insulators** (also called boundary elements), which are DNA sequences that block or insulate the effect of enhancers in a position-dependent manner. If the insulator lies between the enhancer and the promoter, it blocks the action of the enhancer; but, if the insulator lies outside the region between the two, it has no effect. Specific proteins bind to insulators and play a role in their blocking activity. Some insulators also limit the spread of changes in chromatin structure that affect transcription.



17.8 An insulator blocks the action of an enhancer on a promoter when the insulator lies between the enhancer and the promoter.

Alternative splicing:

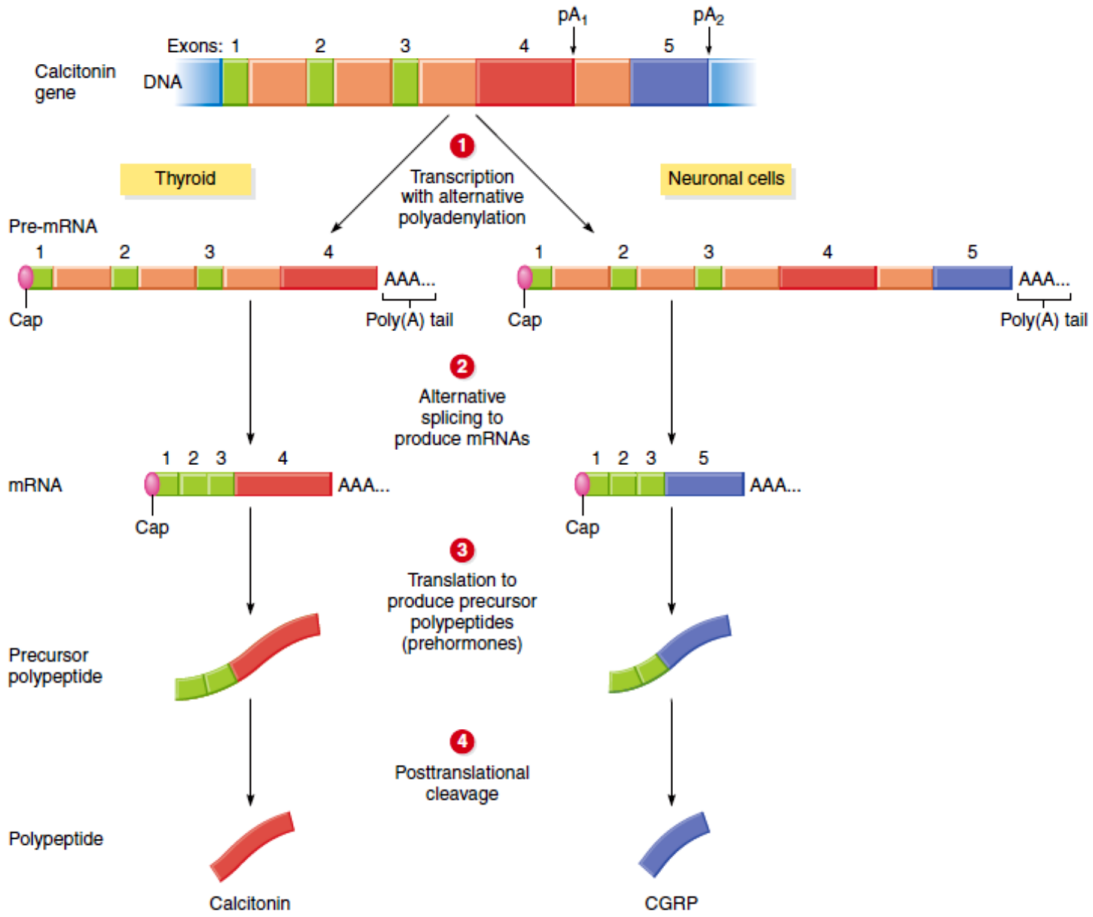
RNA processing control regulates the production of mature mRNA molecules from precursor-RNA (pre-mRNA) molecules. The "textbook" RNA processing steps do not take place in every instance. For example, there are many cases in which alternative polyadenylation sites may be used to produce different pre-mRNA molecules and **alternative splicing** (also called **differential splicing**) may be used to produce different functional mRNAs. Which product is generated depends on regulatory signals. The products of alternative polyadenylation or alternative splicing are proteins that are encoded by the same gene, but that differ structurally and functionally. Such proteins are called protein isoforms, and their synthesis may be tissue specific. Alternative polyadenylation is independent of alternative splicing.

Figure 18.14 gives an example of how alternative polyadenylation *and* alternative splicing together result in tissue-specific products of the human calcitonin gene (*CALC*). *CALC* consists of five exons and four introns and is transcribed in certain cells of the thyroid gland and in certain neurons of the brain. Alternative polyadenylation occurs with the polyadenylation site next to exon 4, used in thyroid cells, and the polyadenylation site next to exon 5, used in the neuronal cells (Figure 18.14, part 1).

Alternative splicing occurs at the next stage of intron removal (Figure 18.14, part 2). The pre-mRNA in the thyroid is spliced to remove the three introns and bring together exons 1, 2, 3, and 4. The pre-mRNA in the neuronal cells is spliced to remove introns and to bring together introns 1, 2, 3, and 5; exon 4 is excised and discarded. The mRNAs that are produced are translated to yield precursor polypeptides (prehormones; see Figure 18.14, part 3), from which the functional hormones are generated posttranslationally by protease cleavage (Figure 18.14, part 4). The two products are calcitonin in the thyroid, with its amino acid sequence encoded by exon 4, and CGRP (calcitonin gene-related peptide), with its amino acid sequence encoded by part of exon 5. (The remainder of exon 5 is the trailer part of the mRNA.) The outcome of alternative polyadenylation and alternative splicing in this case is two different polypeptides encoded by the same gene and synthesized in two different tissues. The thyroid hormone calcitonin is a circulating calcium ion homeostatic hormone that aids the kidney in retaining calcium. CGRP is found in the hypothalamus and appears to have neuromodulatory and trophic (growth-promoting) activities.

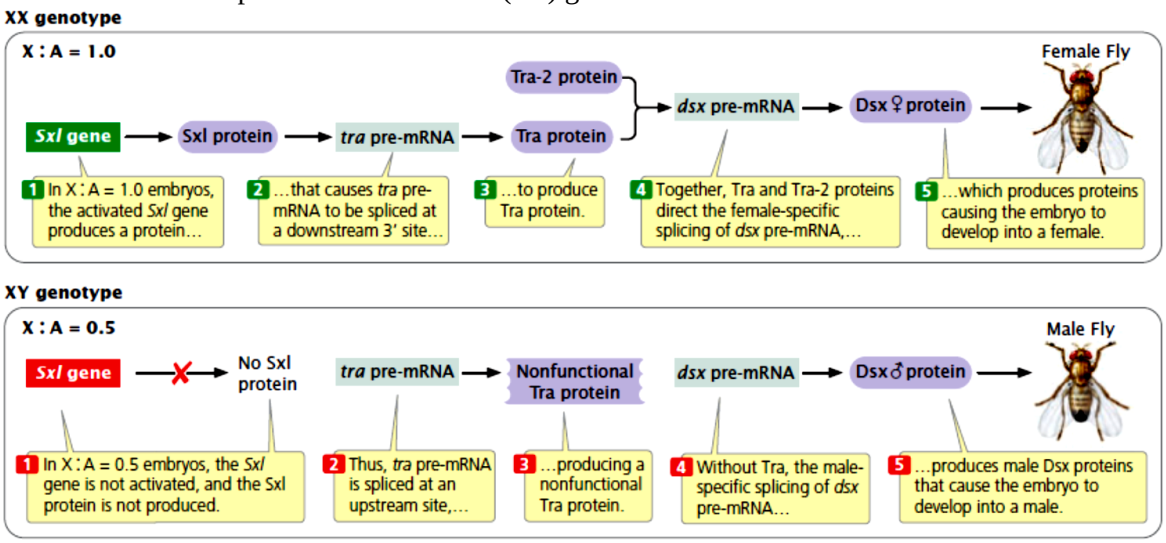
Figure 18.14

Alternative polyadenylation and alternative splicing resulting in tissue-specific products of the human calcitonin gene *CALC*. In the thyroid gland, calcitonin is produced, whereas in certain neurons, CGRP (calcitonin-gene-related peptide) is produced.



Alternative splicing in *Drosophila* sexual development

Another example of alternative mRNA splicing that regulates the expression of genes controls whether a fruit fly develops as male or female. Sex differentiation in *Drosophila* arises from a cascade of gene regulation. When the ratio of X chromosomes to the number of haploid sets of autosomes (the X : A ratio; see Chapter 4) is 1, a female-specific promoter is activated early in development and stimulates the transcription of the *sex-lethal* (*Sxl*) gene.



17.11 Alternative splicing controls sex determination in *Drosophila*.

The protein encoded by *Sxl* regulates the splicing of the premRNA transcribed from another gene called *transformer* (*tra*). The splicing of *tra* pre-mRNA results in the production of the Tra protein. Together with another protein (Tra-2), Tra stimulates the female-specific splicing of pre-mRNA from yet another gene called *doublesex* (*dsx*). This event produces a female-specific Dsx protein, which causes the embryo to develop female characteristics.

In male embryos, which have an X : A ratio of 0.5, the promoter that transcribes the *Sxl* gene in females is inactive; so no Sxl protein is produced. In the absence of Sxl protein, *tra* pre-mRNA is spliced at a different 3′ splice site to produce a nonfunctional form of Tra protein. In turn, the presence of this nonfunctional Tra in males causes *dsx* pre-mRNAs to be spliced differently from that in females, and a male-specific Dsx protein is produced.

RNA Interference Is an Important Mechanism of Gene Regulation

The expression of a number of eukaryotic genes is controlled through RNA interference, also known as RNA silencing and posttranscriptional gene silencing. Recent research suggests that as much as 30% of human genes are regulated by RNA interference. Although many of the details of this mechanism are still being investigated, RNA interference is widespread in eukaryotes, existing in fungi, plants, and animals. This mechanism is also widely used as a powerful technique for artificially regulating gene expression in genetically engineered organisms.

Small Interfering RNAs and MicroRNAs

Both are considered as interfering RNA. Historically miRNA was discovered in 1993 by Ambros and his coworkers where as siRNAs concept came in 1999 from another discovery in which a dsRNA showed its role in post-transcriptional gene silencing (PTGS) in plants by David Baulcombe's group. During PTGS at one stage there is a role of ∼20 − 25 nt RNAs in silencing which was produced by the dsRNA. miRNAs is considered as regulators of cellular self genes(i.e. endogenous genes), and siRNAs act as guards of foreign or invasive genes coming from viruses, transposons, and transgenes etc. which try to get integrated into host genome.

The miRNA gene is always present in the host gnome which gets transcribed into primary-miRNA (pri-miRNA) first with the help of RNA polymerase II. This pri-miRNA is cleaved by an enzyme called Drosha which is a type of ribonuclease III enzyme. It liberates approximately 60 to 70 nt looped structure which is consider as precursor miRNA or pre- miRNA. This pre-miRNA is the transported with the help of Exportin 5 present in cytoplasm. Once the pre-miRNA is exported into cytoplasmic space the another dsRNA specific enzyme called Dicer helps in duplexing with other miRNA. The unwinding of the duplexed miRNA is done by helicase. Now the both dsRNA-specific endonucleases enzymes (Drosha and Dicer) help to generate 2-nucleotide-long-3’ overhangs near the cleavage site. After unwinding of the double stranded miRNA the generation of target specific Guide strand and the passenger stand. Now the miRNA (i.e. Guide strand) is considered as mature miRNA which is then incorporated with the RNA-induced silencing complex (RISC). The target specific miRNA now bind with the mRNA and stop the translation. Finally the gene is silenced with the help of miRNA and the cell undergo self destruction pathway.

MicroRNAs (miRNAs) mediated gene silencing

- The transcript containing an miRNA is called the primary miRNA transcript or pri-miRNA.
- The pri-miRNA molecule contains a hairpin structure about 70 nt long, within which is the eventual miRNA.
- The hairpin is cut out of the pri-miRNA in the nucleus by the dsRNA-specific endonuclease Drosha complexed to an accessory protein (Pasha in Drosophila).
- Drosha makes staggered cuts resulting in a ~2 nt 3’ single-stranded overhang.
- The excised hairpin- pre-miRNA is exported rapidly to the cytoplasm.

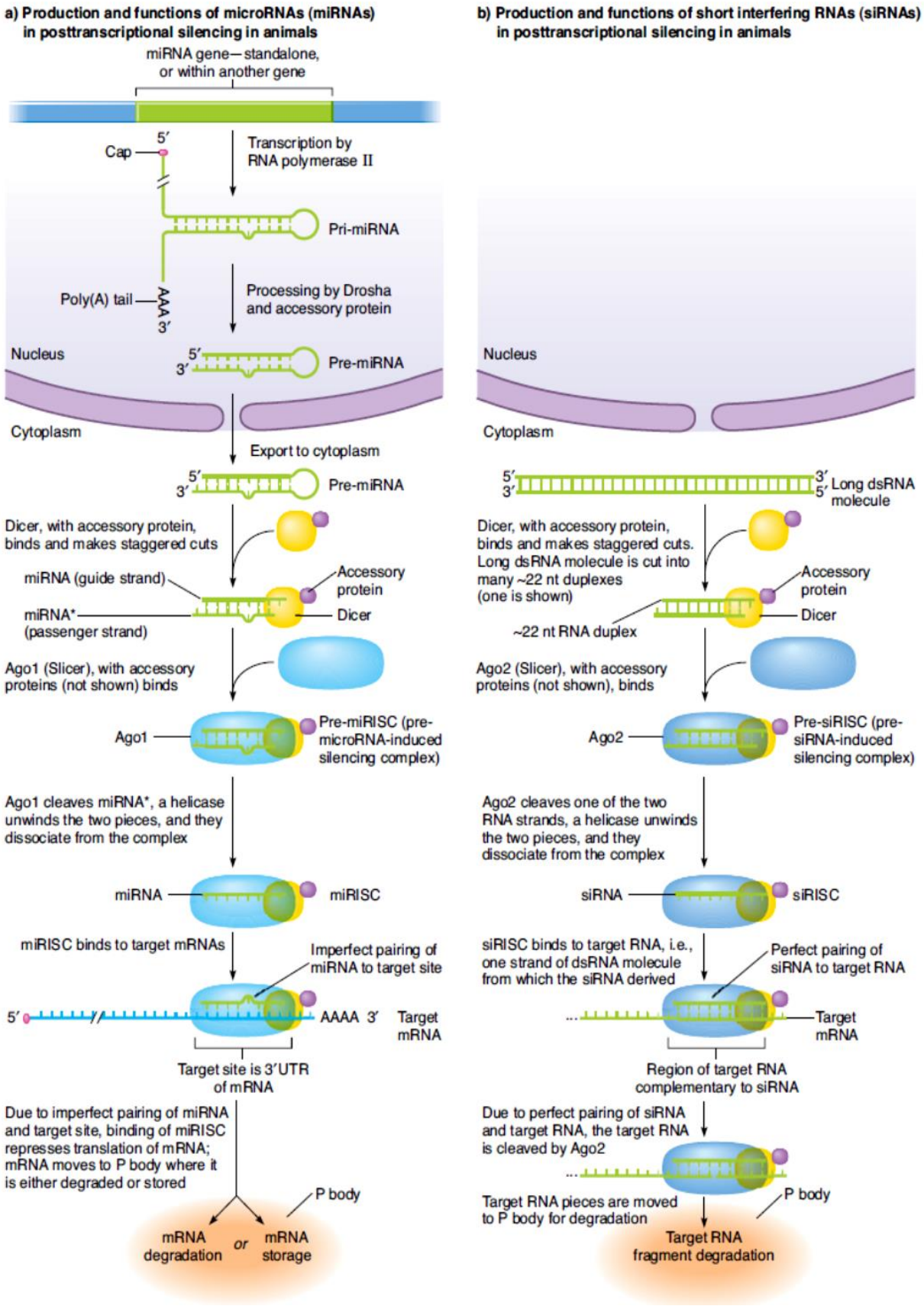
- In the cytoplasm, another dsRNA-specific endonuclease, Dicer, complexed to an accessory protein (Loq in Drosophila), makes staggered cuts in the pre-miRNA, releasing a short miRNA:miRNA* dsRNA consisting of some the former paired sides of the hairpin.
- The two RNA strands are imperfectly paired: "miRNA" is the mature miRNA strand that subsequently functions in the cell for RNA silencing, while miRNA* is its partial complement and does not function in RNA silencing.
- Because the miRNA directs RNA silencing, it is termed the guide strand, while the miRNA* is termed the passenger strand.
- Next the dsRNA, Dicer and accessory protein bind to Ago1, a member of the Argonaute family of protein and other proteins to form the pre-microRNA-induced silencing complex, or pre-miRISC.
- Ago1 is another RNA endonuclease; more generally called Slicer. It makes a single cut within the miRNA* passenger strand.
- A helicase that is part of the pre-miRISC then unwinds to two pieces from the miRNA guide strand, and they dissociate from the complex.
- The result is the mature miRISC, the ribonucleoprotein complex that can silence gene expression.

How does a miRISC function in posttrancriptional gene silencing?

- The miRNA in the miRISC is a trans-acting RNA regulatory molecule, meaning that it targets mRNAs that are not the same as the RNA molecules from which the miRNA is derived. This is one distinguishing feature of miRNAs compared with siRNAs.
- An miRISC binds to a target mRNA through complementary base pairing involving the miRNA.
- Usually, the sequence to which the miRNA binds are short sequence in the 3' UTR of the mRNA
- An mRNA molecule may have one or more sequences in its 3' UTR to which the same miRNA can bind and/or it may have several sequences in its 3' UTR to which several different miRNAs can bind. The latter raises the possibility of regulating the expression of the same gene (through its mRNA) by various combinations of miRNA regulator molecules.
- Here, one miRISC is shown binding to a 3' UTR sequence for simplicity.
- Binding of most of the miRISC to their target mRNAs involves imperfect pairing between the miRNA and the 3' UTR region of the mRNA.
- Such pairing triggers translational repression – translation of that mRNA becomes inhibited.
- The translationally repressed mRNA with its associated miRISC(s) is then sequestered from the translation machinery by becoming or moving into a P body.
- P body is a cytoplasmically located aggregate of translationally repressed mRNAs complexed with proteins, and proteins for mRNA decapping and mRNA degradation.
- The mRNAs is P bodies may be degraded using the contained mRNA degradation machinery or stored in ribonucleoprotein complexes.
- Stored mRNAs can be returned to translation at a later time. Whether degraded or stored the effect of miRNA action is to reduce the expression of the gene encoding the targeted mRNA at the translational level.
- In plants, binding of most miRISCs to their target mRNAs involves perfect or near-perfect pairing between much of the miRNA and the 3' UTR region of the mRNA.
- Perfect pairing triggers mRNA degradation rather than translational repression.
- Here, the Ago1 Slicer protein cuts the target mRNA into two and the mRNA-miRISC complex forms, or it is moved to a P body where degradation of the mRNA is completed.

Figure 18.15

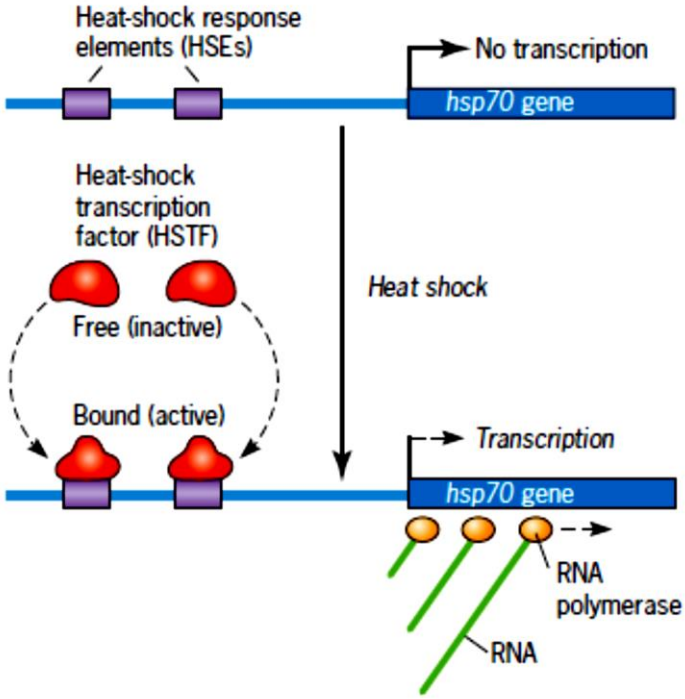
RNA interference (RNAi) by small regulatory RNAs.



Temperature: the heat-shock genes

When organisms are subjected to the stress of high temperature, they respond by synthesizing a group of proteins that help to stabilize the internal cellular environment. These **heat-shock proteins**, found in both prokaryotes and eukaryotes, are among the most conserved polypeptides known. Comparisons of the amino acid sequences of heat-shock proteins from organisms as diverse as E. coli and Drosophila show that they are 40 to 50 percent identical—a remarkable finding considering the length of evolutionary time separating these organisms. The expression of the heat-shock proteins is regulated at the transcriptional level; that is, heat stress specifically induces the transcription of the genes encoding these proteins.

In Drosophila, for example, one of the heat-shock proteins called HSP70 (for heat-shock protein, molecular weight 70 kilodaltons) is encoded by a family of genes located in two nearby clusters on one of the autosomes. Altogether, there are five to six copies of these hsp70 genes in the two clusters. When the temperature exceeds 33ºC, each of the genes is transcribed into RNA, which is then processed and translated to produce HSP70 polypeptides. This heat induced transcription of the hsp70 genes is mediated by a polypeptide called the heat-shock transcription factor, or HSTF, which is present in the nuclei of Drosophila cells. When Drosophila are heat stressed, the HSTF is chemically altered by phosphorylation. In this altered state, it binds specifically to nucleotide sequences upstream of the hsp70 genes and makes the genes more accessible to RNA polymerase II, The sequences to which the phosphorylated HSTF binds are called heat-shock response elements (HSEs).



Induction of transcription from the Drosophila hsp70 gene by heat shock.

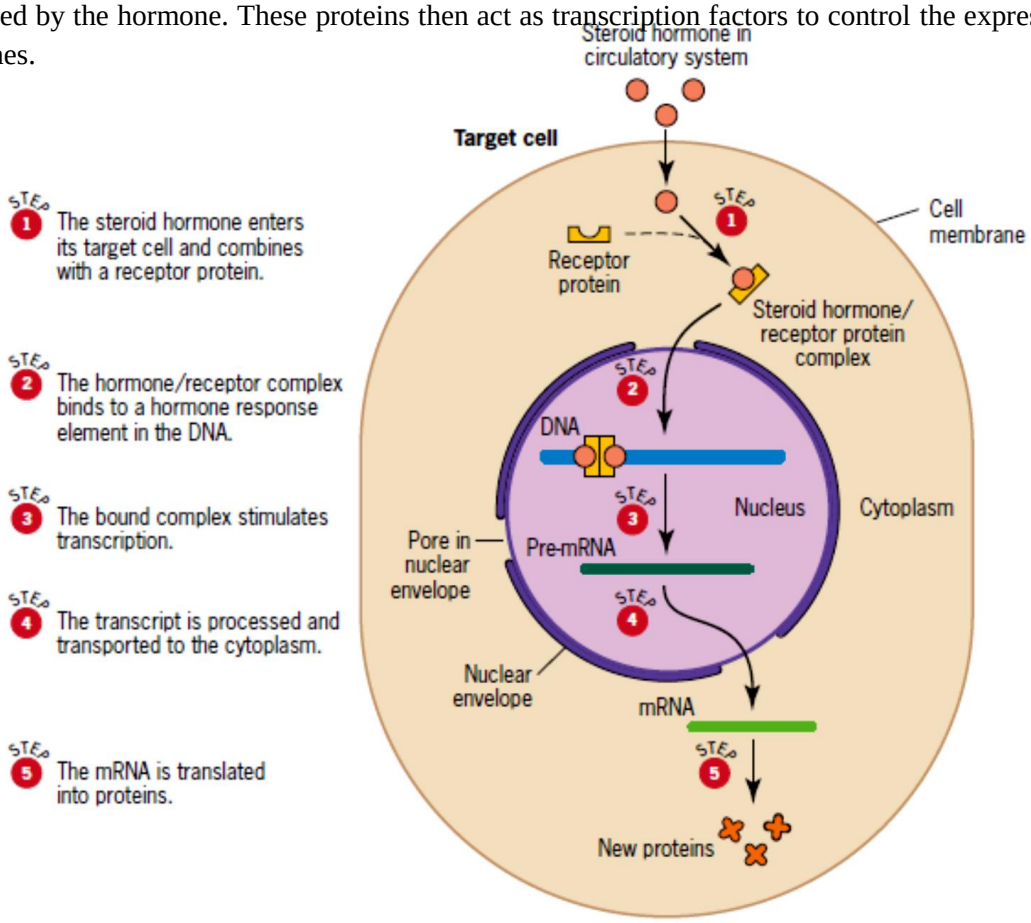
Signal molecules: genes that respond to hormones

In multicellular eukaryotes, one type of cell can signal another by secreting a **hormone.** Hormones circulate through the body; make contact with their target cells, and theninitiate a series of events that regulate the expression of particular genes. In animalsthere are two general classes of hormones. The first class, **the steroid hormones**, aresmall, lipid-soluble molecules derived from cholesterol. Because of their lipid nature, they have little or no trouble passing through cell membranes, examples are estrogenand progesterone. Once these hormones have entered a cell, they interact with cytoplasmic or nuclear proteins called hormone receptors. The receptor/hormone complex that is formed then

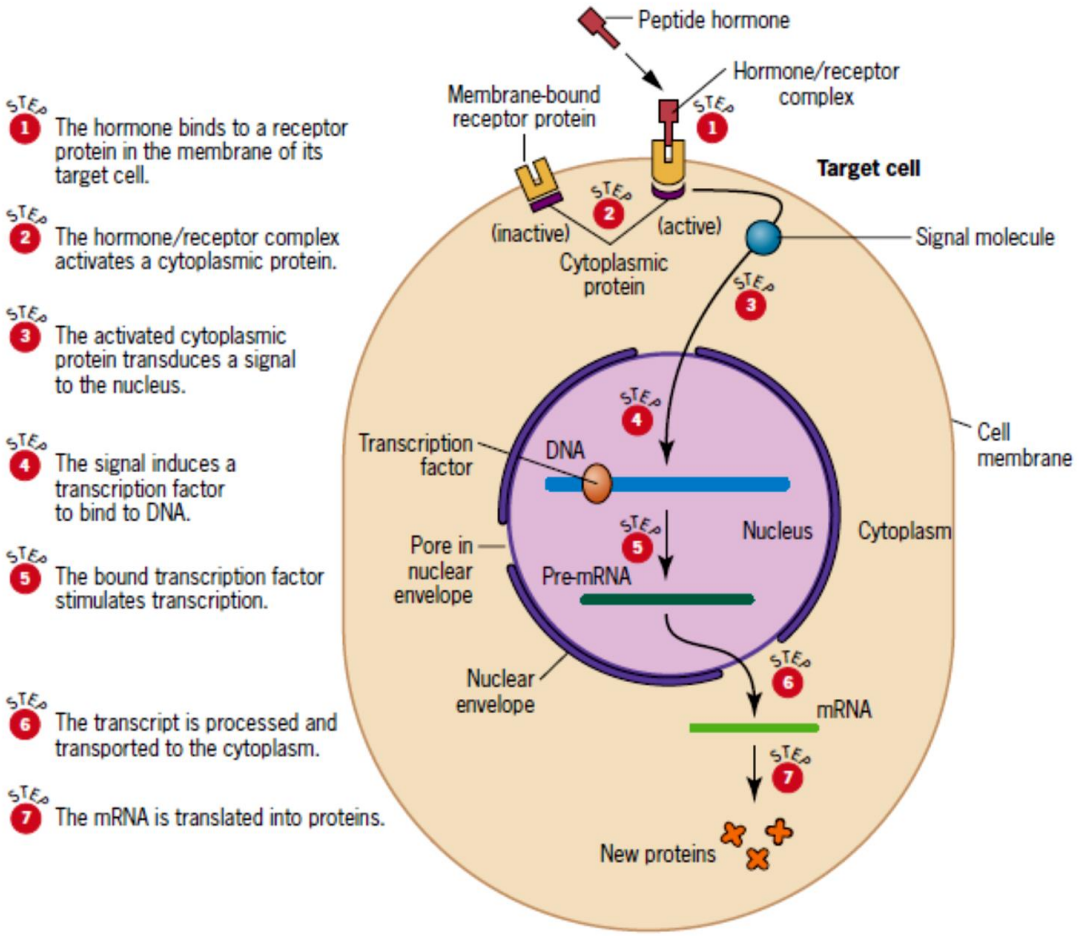
interacts with the DNA where it acts as a transcription factor to regulate the expression of certain genes

The second class of hormones, the *peptide hormones*, are linear chains of amino acids. Like all other polypeptides, these molecules are encoded by genes. Examples are insulin, which regulates blood sugar levels, somatotropin, which is a growth hormone, and prolactin, which targets tissues in the breasts of female mammals. Because peptide hormones are typically too large to pass freely through cell membranes, the signals they convey must be transmitted to the interior of cells by *membrane-bound receptor proteins*. When a peptide hormone interacts with its receptor, it causes a conformational change in the receptor that eventually leads to changes in other proteins inside the cell. Through a cascade of such changes, the hormonal signal is transmitted through the cytoplasm of the cell and into the nucleus, where it ultimately has the effect of regulating the expression of specific genes. This process of transmitting the hormonal signal through the cell and into the nucleus is called **signal transduction.**

Hormone-induced gene expression is mediated by specific sequences in the DNA. These sequences, called *hormone response elements* (*HREs*), are analogous to the heat-shock response elements discussed earlier. They are situated near the genes they regulate and serve to bind specific proteins, which then act as transcription factors. With steroid hormones such as estrogen, the HREs are bound by the hormone/ receptor complex, which then stimulates transcription. The vigor of this transcriptional response depends on the number of HREs present. When there are multiple response elements, hormone/receptor complexes bind cooperatively with each other, significantly increasing the rate of transcription; that is, a gene with two response elements is transcribed more than twice as vigorously as a gene with only one. With peptide hormones, the receptor usually remains in the cell membrane, even after it has formed a complex with the hormone. The hormonal signal is therefore conveyed to the nucleus by other proteins, some of which bind to sequences near the genes that are regulated by the hormone. These proteins then act as transcription factors to control the expression of the genes.



Regulation of gene expression by steroid hormones.



Regulation of gene expression by peptide hormones.

DNase I Hypersensitivity

Several types of changes are observed in chromatin structure when genes become transcriptionally active. As genes become transcriptionally active, regions around the genes become highly sensitive to the action of DNase I. These regions, called **DNase I hypersensitive sites**, frequently develop about 1000 nucleotides upstream of the start site of transcription, suggesting that the chromatin in these regions adopts a more open configuration during transcription. This relaxation of the chromatin structure allows regulatory proteins access to binding sites on the DNA. Indeed, many DNase I hypersensitive sites correspond to known binding sites for regulatory proteins. At least three different processes affect gene regulation by altering chromatin structure: (1) the modification of histone proteins; (2) chromatin remodeling; and (3) DNA methylation. Each of these mechanisms will be discussed in the sections that follow.

Histone Modification

Histones in the octamer core of the nucleosome have two domains: (1) a globular domain that associates with other histones and the DNA and (2) a positively charged tail domain that probably interacts with the negatively charged phosphate groups on the backbone of DNA. The tails of histone proteins are often modified by the addition or removal of phosphate groups, methyl groups, or acetyl groups. These modifications have sometimes been called the **histone code**, because they encode information that affects how genes are expressed.

Methylation of histones One type of histone modification is the addition of methyl groups to the tails of histone proteins. These modifications can bring about either the activation or the repression of transcription, depending on which particular amino acids in the histone tail are methylated. A common modification is the addition of three methyl groups to lysine 4 in the tail of the H3 histone

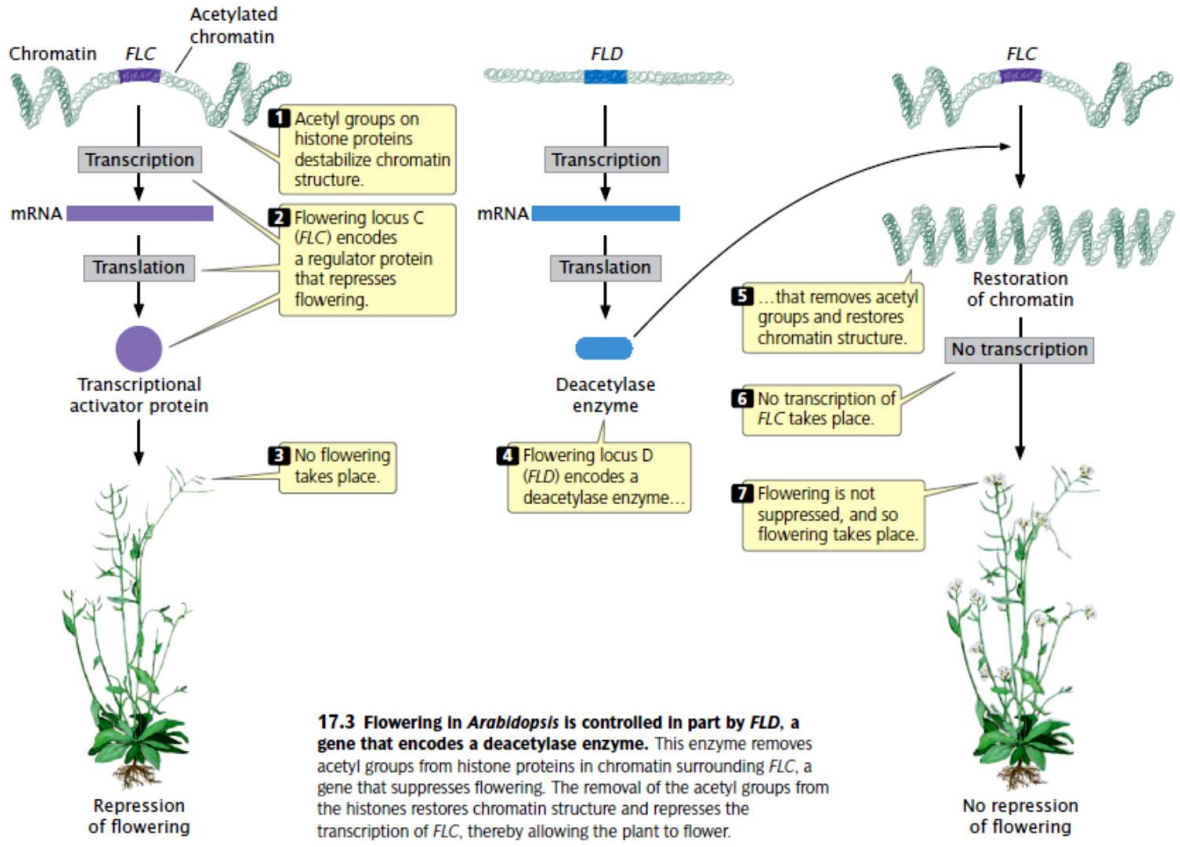
protein, abbreviated H3K4me3 (K is the abbreviation for lysine). The H3K4me3 modification is frequently found in promoters of transcriptionally active genes in eukaryotes.

Recent studies have identified proteins that recognize and bind to H3K4me3, including a protein called nucleosome remodeling factor (NURF). NURF and other proteins that recognize H3K4me3 have a common protein-binding domain that binds to the H3 histone tail and then alters chromatin packing, allowing transcription to take place.

Acetylation of histones Another type of histone modification that affects chromatin structure is acetylation, the addition of acetyl groups (CH3CO) to histone proteins. The acetylation of histones usually stimulates transcription. For example, the addition of a single acetyl group to lysine 16 in the tail of the H4 histone prevents the formation of the 30-nm chromatin fiber causing the chromatin to be in an open configuration and available for transcription. In general, acetyl groups destabilize chromatin structure, allowing transcription to take place. Acetyl groups are added to histone proteins by acetyltransferase enzymes; other enzymes called deacetylases strip acetyl groups from histones and restore chromatin structure, which represses transcription.

Acetylation of histones controls flowering in *Arabidopsis*

The importance of histone acetylation in gene regulation is demonstrated by the control of flowering in *Arabidopsis*, a plant with a number of characteristics that make it an excellent genetic model for plant systems. The time at which flowering takes place is critical to the life of a plant; if flowering is initiated at the wrong time of year, pollinators may not be available to fertilize the flowers or environmental conditions may be unsuitable for the survival and germination of the seeds. Consequently, flowering time in most plants is carefully regulated in response to multiple internal and external cues, such as plant size, photoperiod, and temperature.



17.3 Flowering in *Arabidopsis* is controlled in part by *FLD*, a gene that encodes a deacetylase enzyme. This enzyme removes acetyl groups from histone proteins in chromatin surrounding *FLC*, a gene that suppresses flowering. The removal of the acetyl groups from the histones restores chromatin structure and represses the transcription of *FLC*, thereby allowing the plant to flower.

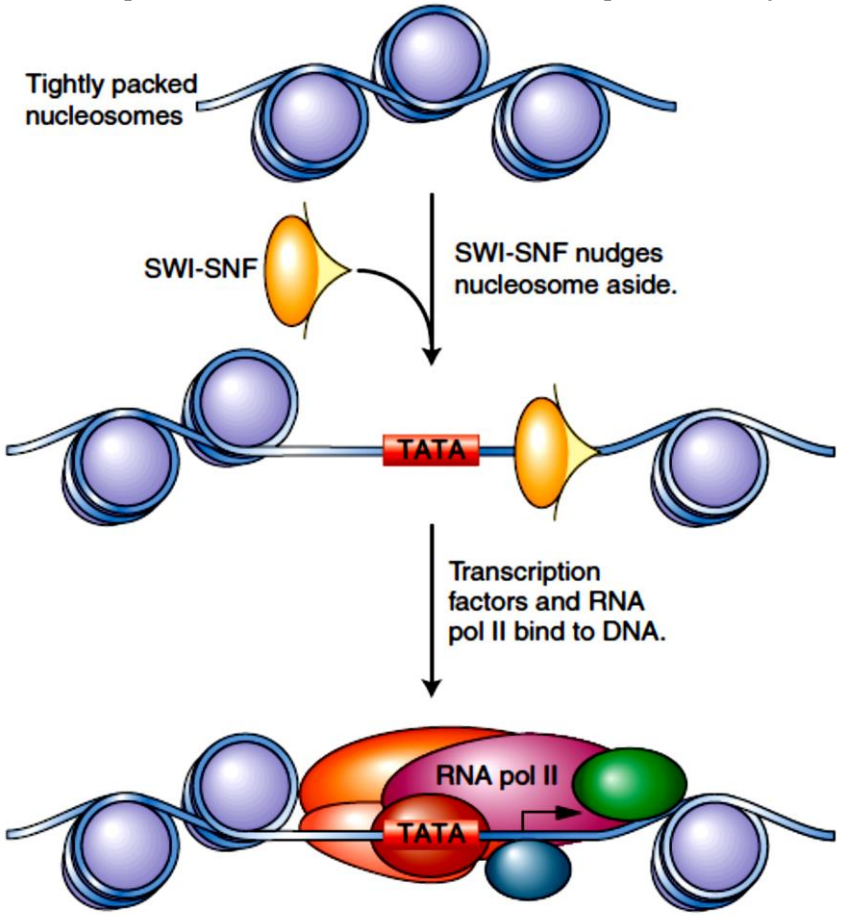
Among the many genes that control flowering in *Arabidopsis* is *flowering locus C* (*FLC*), which plays an important role in suppressing flowering until after an extended period of coldness (a process called

vernalization). The *FLC* gene encodes a regulator protein that represses the activity of other genes that affect flowering. As long as *FLC* is active, flowering remains suppressed. The activity of *FLC* is controlled by another locus called *flowering locus D* (*FLD*), the key role of which is to stimulate flowering by repressing the action of *FLC*. In essense, flowering is stimulated because *FLD* represses the repressor. How does *FLD* repress *FLC? FLD* encodes a deacetylase enzyme, which removes acetyl groups from histone proteins in the chromatin surrounding *FLC*. The removal of acetyl groups from histones alters chromatin structure and inhibits transcription. The inhibition of transcription prevents *FLC* from being transcribed and removes its repression on flowering. In short, *FLD* stimulates flowering in *Arabidopsis* by deacetylating the chromatin that surrounds *FLC*, thereby removing its inhibitory effect on flowering.

Chromatin remodeling

Experiments that assess the sensitivity of DNA to digestion with DNase I have established that transcribed DNA is more accessible to in transcribed DNA, the nucleosomesare altered by multiprotein complexes that ultimately facilitate the action of the RNA polymerase. This alteration of nucleosomes in preparation for transcription is called **chromatin remodeling.**

Two general types of chromatin-remodeling complexes have been identified. One type is composed of enzymes that transfer acetyl groups to the amino acid lysine at specific positions in the histones of the nucleosomes. As a class, these enzymes are called histone acetyl transferases (HATs). Numerous studies have shown that acetylation of histones is correlated with increased gene expression, perhaps because the addition of the acetyl groups loosens the association between the DNA and the histone octamers in the nucleosomes. Kinases—enzymes that transfer phosphate groups to molecules—may also play a role along with these chromatin-remodeling complexes. Together, these two modifications of histone H4 seem to "open" the chromatin for increased transcriptional activity.

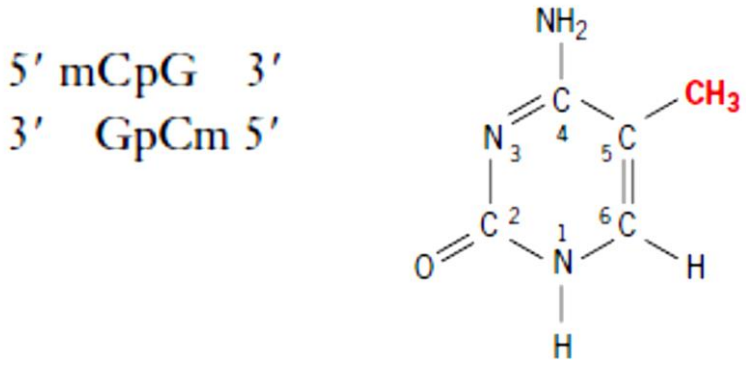


Chromatin remodeling. The movement ofnucleosomes in response to SWI-SNF activity is shown.

Another type of chromatin-remodeling complex disrupts nucleosome structure in the vicinity of a gene's promoter. The most intensively studied of these complexes is the SWI/SNF complex found in baker's yeast. This complex is named for the two types of mutations (switching-inhibited and sucrose nonfermenter) that led to the discovery of its constituent proteins. Related complexes have been found in the cells of other organisms, including humans. The SWI/SNF complex consists of at least eight proteins. It regulates transcription by sliding histone octamers along the associated DNA in nucleosomes; it can also transfer these octamers to other locations on a DNA molecule. The nucleosome shifting catalyzed by the SWI/SNF complex apparently gives transcription factors access to the DNA. These factors then stimulate a gene's expression.

DNA Methylation

Another change in chromatin structure associated with transcription is the methylation of cytosine bases, which yields 5-methylcytosine. The methylation of cytosine in DNA is distinct from the methylation of histone proteins mentioned earlier. Heavily methylated DNA is associated with the repression of transcription in vertebrates and plants, whereas transcriptionally active DNA is usually unmethylated in these organisms. Abnormal patterns of methylation are also associated with some types of cancer.



Structure of 5-methylcytosine

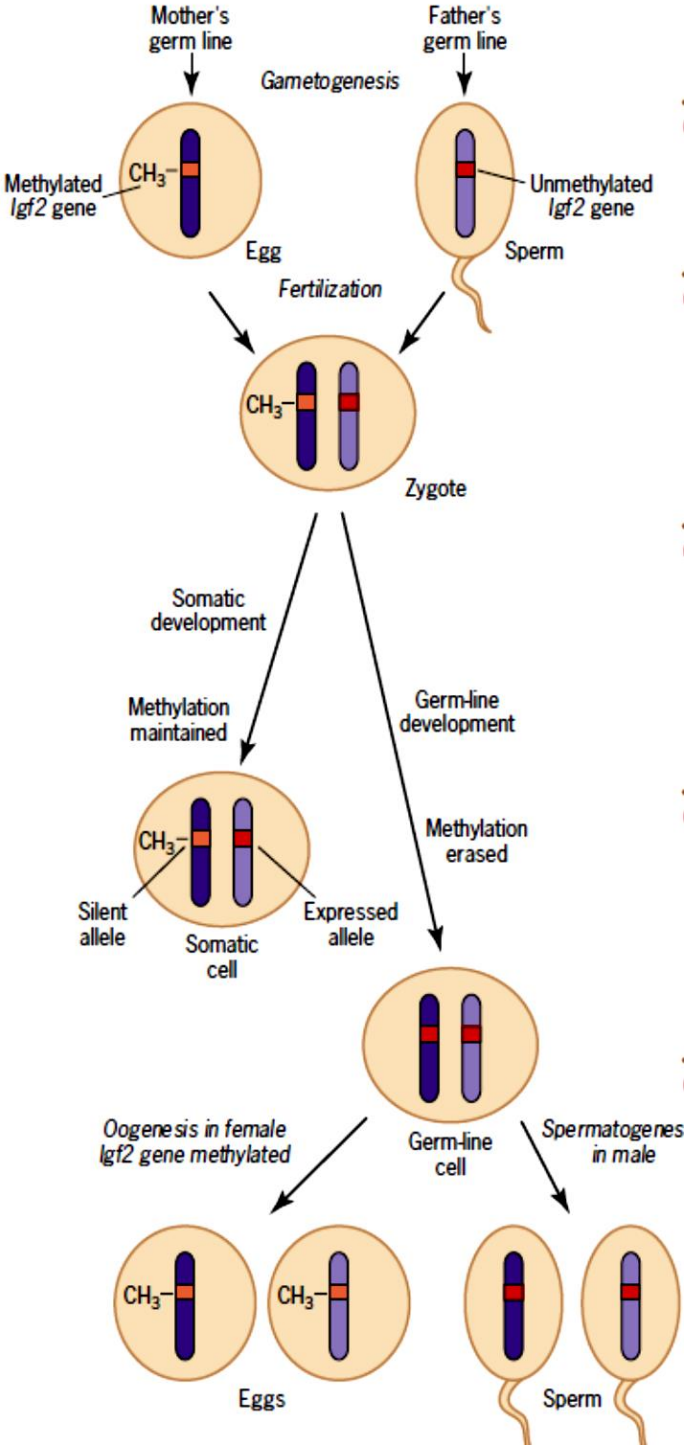
DNA methylation is most common on cytosine bases adjacent to guanine nucleotides (CpG, where p represents the phosphate group in the DNA backbone); so two methylated cytosines sit diagonally across from each other on opposing strands: DNA regions with many CpG sequences are called **CpG islands** and are commonly found near transcription start sites. While genes are not being transcribed, these CpG islands are often methylated, but the methyl groups are removed before the initiation of transcription. CpG methylation is also associated with long-term gene repression, such as on the inactivated X chromosome of female mammals.

Imprinting

DNA methylation in mammals is also responsible for unusual cases in which the expression of a gene is controlled by its parental origin. For example, in mice, the Igf 2 gene, which encodes an insulin-like growth factor, is expressed when it is inherited from the father but not from the mother. By contrast, a gene known as H19 is expressed when it is inherited from the mother but not from the father. Whenever the expression of a gene is conditioned by its parental origin, geneticists say that the gene has been **imprinted**—a term intended to convey the idea that the gene has been marked in some way so that it "remembers" which parent it came from.

Recent molecular analysis has demonstrated that the mark that conditions the expression of a gene is methylation of one or more CpG dinucleotides in the gene's vicinity. These methylated dinucleotides

are initially formed in the parental germ line Thus; for example, the Igf 2 gene is methylated in the female germline but not in the male germ line.



Methylation and imprinting of the *Igf*2 gene in mice.

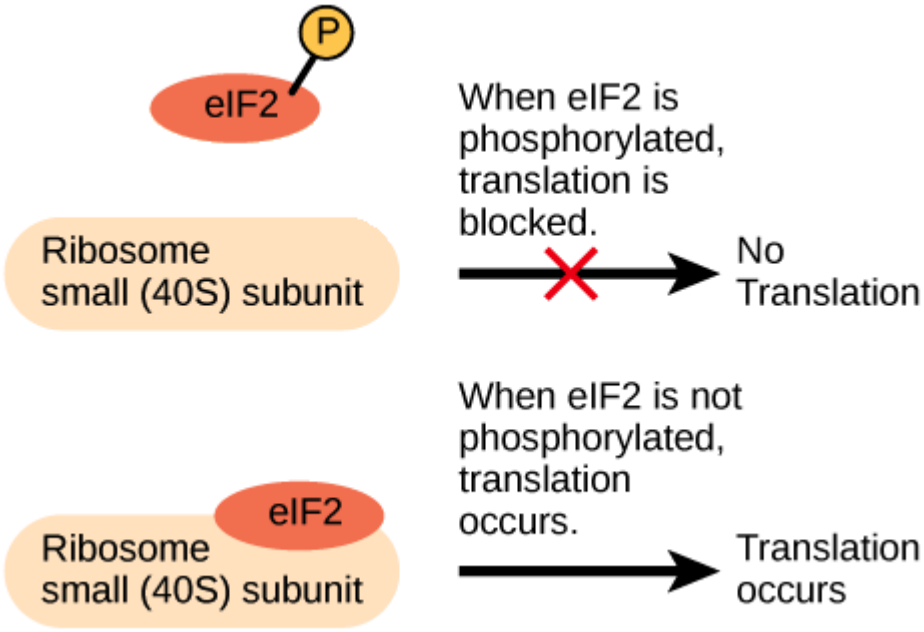
Regulation of translation

We already saw how miRNAs can inhibit translation, but there are a number of other ways that translation of an mRNA can also be regulated in a cell. One key step for regulation is translation initiation.

In order for translation to begin, the ribosome, an RNA-and-protein complex that houses translation, must assemble on the mRNA. This process involves many “helper” proteins, which make sure the ribosome is correctly positioned. Translation can be regulated globally (for every mRNA in the cell) through changes in the availability or activity of the “helper” proteins.

For example, in order for translation to begin, a protein called eukaryotic initiation factor-2 (eIF-2) must bind to a part of the ribosome called the small subunit. Binding of eIF-2 is controlled by phosphorylation, or addition of a phosphate group to the protein.

When eIF-2 is phosphorylated, it's turned "off"—it undergoes a shape change and can no longer play its role in initiation, so translation cannot begin. When eIF-2 is not phosphorylated, in contrast, it's "on" and can carry out its role in initiation, allowing translation to proceed.



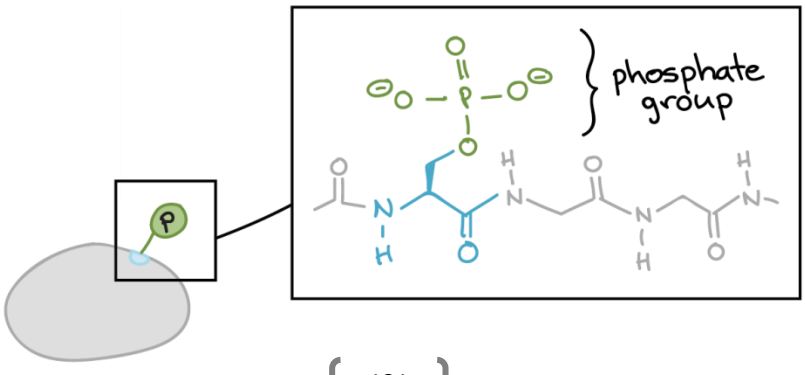
In this way, phosphorylation of eIF-2 acts as a switch, turning translation on or off. Inactivation of translation can be a good strategy in periods when the cell can't “afford” to make new proteins (e.g., when the cell is starved for nutrients).

Proteins can be regulated after translation

There are also regulatory mechanisms that act on proteins that have already been made. In these cases, an "edit" to the protein – such as removal of amino acids, or addition of a chemical modification – can lead to a change in its activity or behavior. These processing and modification steps can be targets for regulation

Phosphorylation

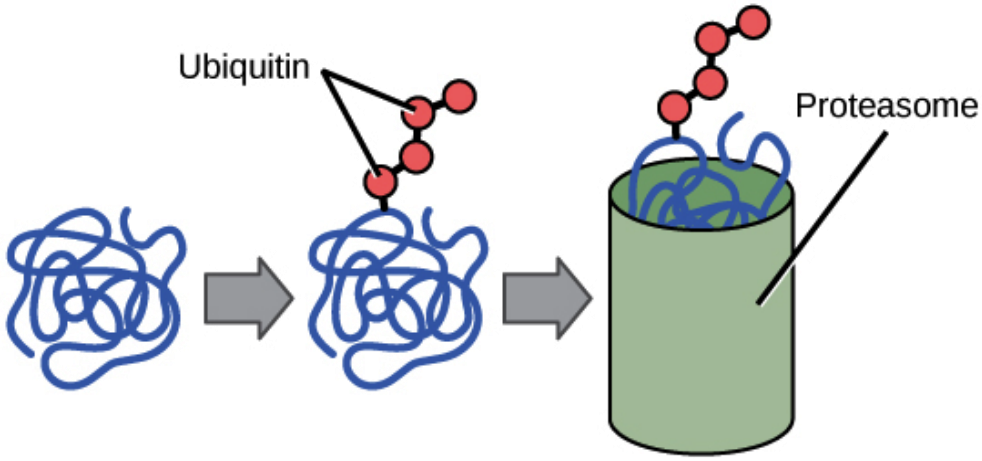
One of the most common post-translational modifications is phosphorylation, in which a phosphate group is attached to a protein. The effect of phosphorylation varies from protein to protein: some are activated by phosphorylation, while others are deactivated, and others yet simply change their behavior (interacting with a different partner, or going to a different part of the cell).



We saw one example of this above, when we examined how eIF-2 is inactivated by addition of a phosphate group (blocking translation). However, many different proteins can be selectively phosphorylated, producing various effects depending on the protein's role in the cell.

Ubiquitination

Proteins can be tagged for degradation by the addition of a chemical marker called ubiquitin. Ubiquitin-tagged proteins are taken to the proteasome, or "recycling center" of the cell, and broken down into their component parts. Ubiquitination is an important way of controlling the persistence of a protein in the cell.



9. Dosage compensation

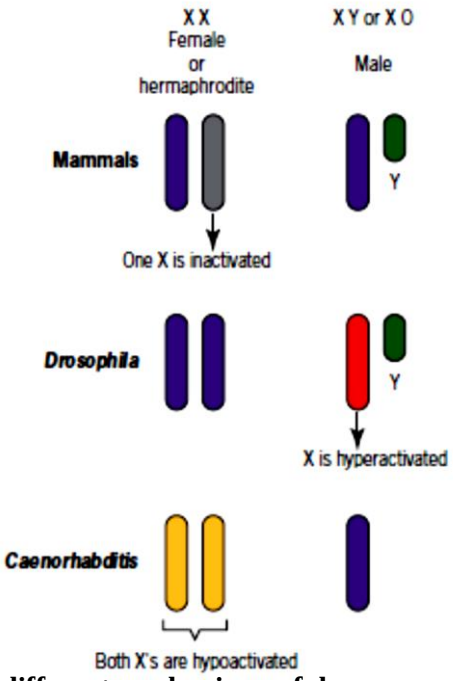
Dosage compensation

In species with XX-XY sex determination, the difference in the number of X chromosomes possessed by males and females presents a special problem in development. Because females have two copies of every X-linked gene and males have only one copy, the amount of gene product (protein) encoded by X-linked genes would differ in the two sexes: females would produce twice as much gene product as that produced by males. This difference could be highly detrimental because protein concentration plays a critical role in development. Animals overcome this potential problem through **dosage compensation,** which equalizes the amount of protein produced by X-linked genes in the two sexes. In fruit flies, dosage compensation is achieved by a doubling of the activity of the genes on the X chromosome of the male.

Normally, each gene is present in two copies. Departures from this condition, either up or down, can cause abnormal phenotypes, and sometimes even death. It is therefore puzzling

that so many species should have a sex-determination system based on females with two X chromosomes and males with only one. In these species, how is the numerical difference of X-linked genes accommodated? A priori, three mechanisms may compensate for this difference: (1) each X-linked gene could work twice as hard in males as it does in females, or (2) one copy of each X-linked gene could be inactivated in females, or (3) each X-linked gene could work half as hard in females as it does in males. Extensive research has shown that all three mechanisms are utilized, the first in Drosophila, the second in mammals, and the third in the nematode Caenorhabditis elegans.

These three different mechanisms of **dosage compensation**—inactivation, hyperactivation, and hypoactivation—have an important feature in common: many differentgenes are coordinately regulated because they are on the same chromosome. Thischromosomewide regulation is superimposed on all other regulatory mechanismsinvolved in the spatial and temporal expression of these genes.



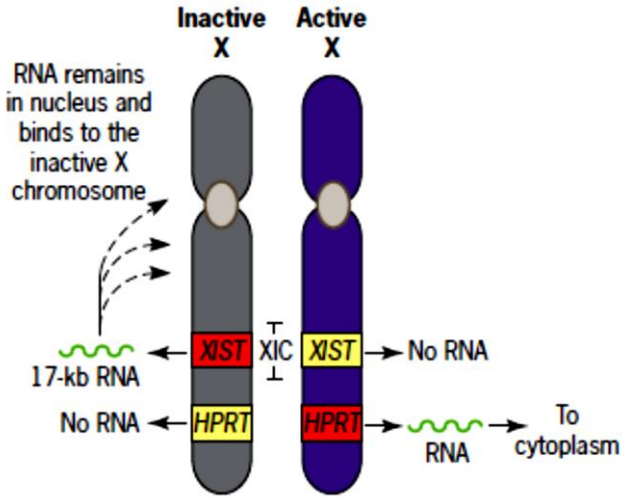
Three different mechanisms of dosage compensation

Inactivation of X chromosomes in mammals

In mammals, X chromosome inactivation begins at a particular site called the X inactivation center (XIC) and then spreads in opposite directions toward the endsof the chromosome. Curiously, not all genes on an inactivated X chromosome aretranscriptionally silent. One that remains active is called XIST (for X inactive specific transcript); this gene is located within the XIC. In human beings the XIST gene encodes a 17-kb transcript devoid of any significant open reading frames.It therefore seems unlikely that the XIST gene codes for a protein. Instead, the RNAitself is probably the functional product of the XIST gene. Though polyadenylated,this RNA is restricted to the nucleus and is specifically localized to inactivated X chromosomes;it does not appear to be associated with active X chromosomes in eithermales or females.

In mice, where fairly detailed experimental analysis has been possible, researchers have found that the homologue of the human XIST gene is transcribed during the early stages of embryonic development at a low level from both of the X chromosomes that are present in females. The transcripts from each of a female mouse's Xist genes are unstable and remain closely associated with their respective genes. As development proceeds, the transcripts from one of the genes stabilize and eventually envelop the entire X chromosome on which that gene is located; the transcripts from the other Xist gene disintegrate, and further transcription from that gene is repressed by methylation of nucleotides in the gene's promoter. Thus,

in the female mouse, one X chromosome—the one whose Xist gene continues to be transcribed—becomes coated with Xist RNA and the other does not. The choice of the chromosome that becomes coated is apparently random.



Expression of Xist gene in inactive X chromosomes of human females

Inactive X chromosomes are readily identified in mammalian cells. During interphase, they condense into a darkly staining mass associated with the nuclear membrane. This mass, the Barr body, decondenses during S phase to allow the inactive X chromosome to be replicated. However, because decondensation takes some time, the inactive X replicates later than the rest of the chromosomes. Inactive X chromosomes must therefore have a very different chromatin structure than that of other chromosomes. This difference is partly determined by the kinds of histones associated with the DNA. One of the four core histones, H4, can be chemically modified by the addition of acetyl groups to any of several lysines in the polypeptide chain. Acetylated H4 is associated with all the chromosomes in the human genome. However, on the inactive X it seems to be restricted to three fairly narrow bands, each corresponding to a region that contains some active genes. Acetylated H4 is also depleted in

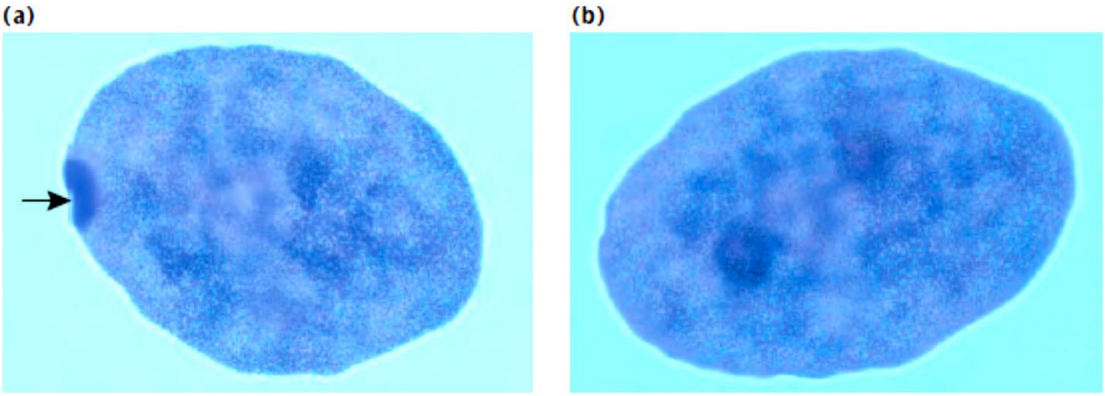
areas of heterochromatin on the other chromosomes. These findings suggest that the depletion of acetylated H4 is a key feature of the inactive X chromosome.

Lyon hypothesis:

In 1949, Murray Barr observed condensed, darkly staining bodies in the nuclei of cells from female cats; this darkly staining structure became known as a **Barr body.**Mary Lyon proposed in 1961 that the Barr body was an inactive X chromosome; her hypothesis has become known as the **Lyon hypothesis.** She suggested that, within each female cell, one of the two X chromosomes becomes inactive; which X chromosome is inactivated is random. If a cell contains more than two X chromosomes, all but one of them areinactivated. The number of Barr bodies present in human cells with different complements of sex chromosomes is shown in Table.

Table 4.3 Number of Barr bodies in human cells with different complements of sex chromosomes

Sex Chromosomes	Syndrome	Number of Barr Bodies
XX	None	1
XY	None	0
XO	Turner	0
XXY	Klinefelter	1
XXYY	Klinefelter	1
XXXY	Klinefelter	2
XXXXY	Klinefelter	3
XXX	Triplo-X	2
XXXX	Poly-X female	3
XXXXX	Poly-X female	4



A Barr body is an inactivated X chromosome.

(a) Female cell with a Barr body (indicated by arrow). (b) Male cell without a Barr body

As a result of X inactivation, females are functionally hemizygous at the cellular level for X-linked genes. In females that are heterozygous at an X-linked locus, approximately 50% of the cells will express one allele and 50% will express the other allele; thus, in heterozygous females, proteins encoded by both alleles are produced, although not within the same cell. This functional hemizygosity means that cells in females are not identical with respect to the expression of the genes on the X chromosome; females are mosaics for the expression of X-linked genes.

Random X inactivation takes place early in development—in humans, within the first few weeks of development. After an X chromosome has become inactive in a cell, it remains inactive and is inactive in all somatic cells that descend from the cell. Thus, neighboring cells tend to have the same X chromosome inactivated, producing a patchy pattern (mosaic) for the expression of an X-linked characteristic in heterozygous females. This patchy distribution can be seen in tortoiseshell and calico cats. Although many genes contribute to coat color and pattern in domestic cats, a single X-linked locus determines the presence of orange color. There are two possible alleles at this locus: X^+, which produces nonorange (usually black) fur, and X^o, which produces orange fur. Males are hemizygous and thus may be black (X^+Y) or orange (X^oY) but not black and orange. (Rare tortoiseshell males can arise from the presence of two X chromosomes, X^+X^oY.) Females may be black (X^+ X^+), orange (X^oX^o), or tortoiseshell (X^+X^o), the tortoiseshell pattern arising from a patchy mixture of black and orange fur. Each orange patch is a clone of cells derived from an original cell in which the black allele is inactivated, and each black patch is a clone of cells derived from an original cell in which the orange allele is inactivated.

The patchy distribution of color on tortoiseshell cats results from the random inactivation of one X chromosome in females.

Hyperactivation of x chromosomes in Drosophila

In Drosophila, dosage compensation requires the protein products of at least five different genes. Null mutations in these genes result in male-specific lethality because the single X chromosome in males is not hyperactivated. Mutant males usually die during the late larval or early pupal stages. These dosage compensation genes are therefore called male-specific lethal (msl) loci, and their products are called the MSL proteins. Antibodies prepared against these proteins have been used as probes to localize the proteins inside cells. The remarkable finding is that each of the MSL proteins binds specifically to the X chromosome in males. These proteins do not bind to the other chromosomes in the male's genome, and they do not bind to any of the chromosomes, including the X's, in a female's genome. The binding of the MSL proteins to the male's X chromosome is facilitated by two types of RNA molecules called roX1 and roX2 (for RNA on the X chromosome) that are transcribed from genes on the X chromosome.

The current model proposes that the MSL proteins form a complex that is joined by the roX RNAs. This complex then binds to 30 to 40 sites along the male's X chromosome, including the loci that contain the two roX genes. From each of these entry sites, the MSL/roX complex spreads bidirectionally until it reaches all the genes on the male's X chromosome that need to be hyperactivated. The process of hyperactivation may involve chromatin remodeling by the MSL/roX complex. One of the MSL proteins is a histone acetyl transferase, and a particular acetylated version of histone H4 is exclusively associated with hyperactivated X chromosomes.

Hypoactivation of x chromosomes in *Caenorhabditis*

In C. elegans, dosage compensation involves the partial repression of X-linked genes in the somatic cells of hermaphrodites. The mechanism is not fully understood, but the products of several genes are

involved. Like the MSL proteins in Drosophila, the proteins encoded by these genes bind specifically to the X chromosome. However, unlike the situation in Drosophila, they bind only when two X chromosomes are present. The proteins apparently do not bind to the single X chromosome in males, nor do they bind to any of the autosomes in either males or hermaphrodites. Dosage compensation in C. elegans therefore seems to involve a mechanism exactly opposite to the one in Drosophila. A protein complex binds to the X chromosomes and represses rather than enhances transcription.

10. RNA biology: RNA editing and evolutionary significance; antisense RNA technology and gene silencing; ribozyme; different categories of small non coding RNAs; biogenesis and functions of small RNAs in posttranscriptional gene silencing; application of RNAi in crop quality improvement.

RNA editing:

RNA editing is a molecular process through which some cells can make discrete changes to specific nucleotide sequences within an RNA molecule after it has been generated by RNA polymerase. RNA editing may include the insertion, deletion, and base substitution of nucleotides within the RNA molecule. RNA editing is relatively rare, with common forms of RNA processing (e.g. splicing, 5'-capping, and 3'-polyadenylation) not usually considered as editing.

RNA editing takes place in cell nucleus and cytosol as well as mitochodria and plastids.

RNA-editing processes show great molecular diversity, and some appear to be evolutionarily recent acquisitions that arose independently. The diversity of RNA editing phenomena includes nucleobase modifications such as cytidine (C) to uridine (U) and adenosine (A) to inosine (I) deaminations, as well as non-template nucleotide additions and insertions. RNA editing in mRNAs effectively alters the amino acid sequence of the encoded protein so that it differs from that predicted by the genomic DNA sequence.

RNA editing events can **modify RNA molecules in several cellular contexts** causing:

- the modulation of gene expression pathways during translation;
- the gain or loss of miRNA/siRNA recognition elements during mRNA targeting
- nuclear/cytoplasmic sequestration
- endonucleolytic cleavage by Tudor-SN
- inhibition of miRNA/siRNA processing
- the creation and/or destruction of splicing sites;

RNA editing: Two general types:

Two general types:

a) Base modification (deaminase)

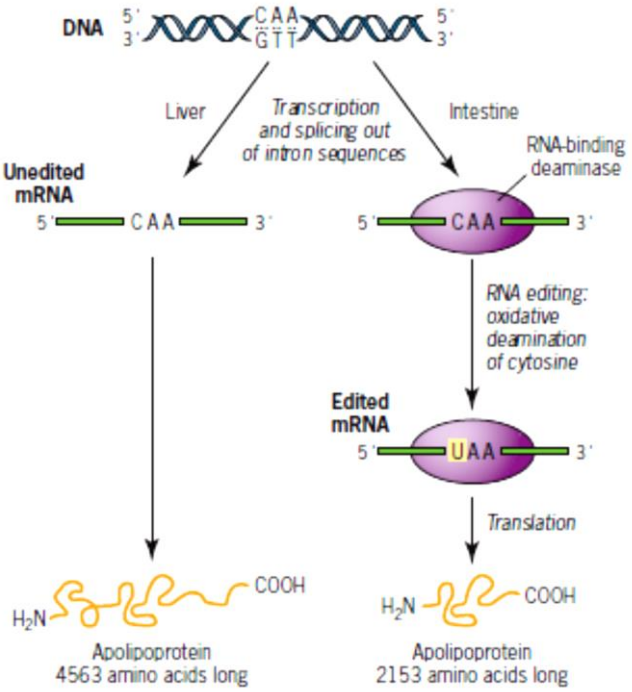
- A to I double-stranded mechanism, seen in viruses, human genes.
- C to U, U to C seen in chloroplasts, plant mitochondria, human genes.

b) Insertion/deletion

U insertion/deletion, seen in kinetoplastid protozoa mono/di nucleotide insertion, seen in *Physarum* nucleotide replacement, seen in *Acanthamoeba* tRNAs

C-to-U editing:

The first type of RNA editing, which results in the substitution of one base for another base, is rare. This type of editing was discovered in studies of the apolipoprotein-B (*apo-B*) genes and mRNAs in rabbits and humans. Apolipoproteins are blood proteins that transport certain types of fat molecules in the circulatory system. In the liver, the *apo-B* mRNA encodes a large protein 4563 amino acids long. In the intestine, the *apo-B* mRNA directs the synthesis of a protein only 2153 amino acids long. Here, a C residue in the pre-mRNA is converted to a U, generating an internal UAA translation– termination codon, which results in the truncated apolipoprotein.



A-to-I editing:

Adenosine-to-inosine (A-to-I) modifications contribute to nearly 90% of all editing events in RNA. The deamination of adenosine is catalyzed by the double-stranded RNA-specific adenosine deaminase (ADAR), which typically acts on pre-mRNAs. The deamination of adenosine to inosine disrupts and destabilizes the dsRNA base pairing, therefore rendering that particular dsRNA less able to produce siRNA, which interferes with the RNAi pathway.

The wobble base pairing causes deaminated RNA to have a unique but different structure, which may be related to the inhibition of the initiation step of RNA translation. Studies have shown that I-RNA (RNA with many repeats of the I-U base pair) recruits methylases that are involved in the formation of heterochromatin and that this chemical modification heavily interferes with miRNA target sites. There is active research into the importance of A-to-I modifications and their purpose in the novel concept of epitranscriptomics, in which modifications are made to RNA that alter their function. A long established consequence of A-to-I in mRNA is the interpretation of I as a G, therefore leading to functional A-to-G substitution, e.g. in the interpretation of the genetic code by ribosomes. Newer studies however, have weakened this correlation by showing that I's can also be decoded by the ribosome (although in a lesser extent) as A's and U's. Furthermore it was shown that I's lead to the stalling of ribosomes on the I-rich mRNA

The development of high-throughput sequencing in recent years has allowed for the development of extensive databases for different modifications and edits of RNA. RADAR (Rigorously Annotated Database of A-to-I RNA editing) was developed in 2013 to catalog the vast variety of A-to-I sites and tissue-specific levels present in humans, mice, and flies.

Editing by insertion or deletion:

RNA editing through the addition and deletion of uracil has been found in kinetoplasts[A kinetoplast is a network of circular DNA (called kDNA) inside a large mitochondrion] from the mitochondria of ***Trypanosoma brucei*** Because this may involve a large fraction of the sites in a gene, it is sometimes called **"pan-editing"** to distinguish it from topical editing of one or a few sites.

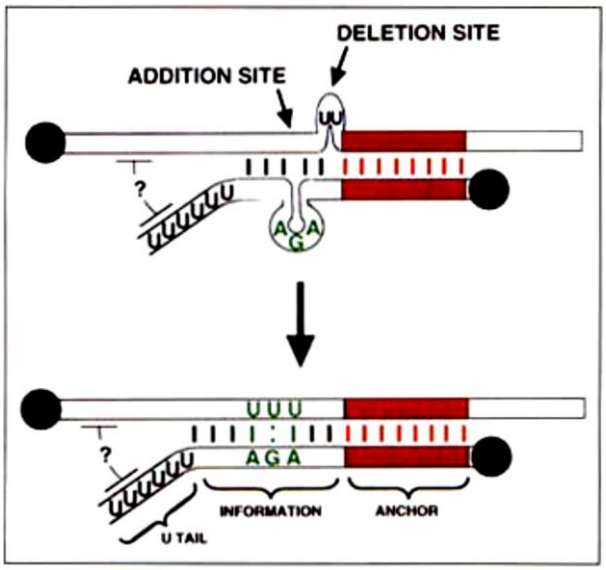
Pan-editing starts with the base-pairing of the unedited primary transcript with a guide RNA (gRNA), which contains complementary sequences to the regions around the insertion/deletion points. The

newly formed double-stranded region is then enveloped by an editosome, a large multi-protein complex that catalyzes the editing. The editosome opens the transcript at the first mismatched nucleotide and starts inserting uridines. The inserted uridines will base-pair with the guide RNA, and insertion will continue as long as A or G is present in the guide RNA and will stop when a C or U is encountered. The inserted nucleotides cause a frameshift, and result in a translated protein that differs from its gene.

The mechanism of the editosome involves an endonucleolytic cut at the mismatch point between the guide RNA and the unedited transcript. The next step is catalyzed by one of the enzymes in the complex, a **terminal U-transferase**, which adds Us from UTP at the 3' end of the mRNA. The opened ends are held in place by other proteins in the complex. Another enzyme, a U-specific exoribonuclease, removes the unpaired Us. After editing has made mRNA complementary to gRNA, an RNA ligase rejoins the ends of the edited mRNA transcript. As a consequence, the editosome can edit only in a 3' to 5' direction along the primary RNA transcript. The complex can act on only a single guide RNA at a time. Therefore, a RNA transcript requiring extensive editing will need more than one guide RNA and editosome complex.

Guide RNA (gRNA):

In general, RNA editing mechanisms are based on protein or protein-RNA complexes and require a "**guide RNA**" molecule, which, through base-pairing with the target RNA, determines the editing site.



Guide RNAs (gRNAs) direct editing

– gRNAs are small (40-70 nt) and complementary to portions of the mRNA

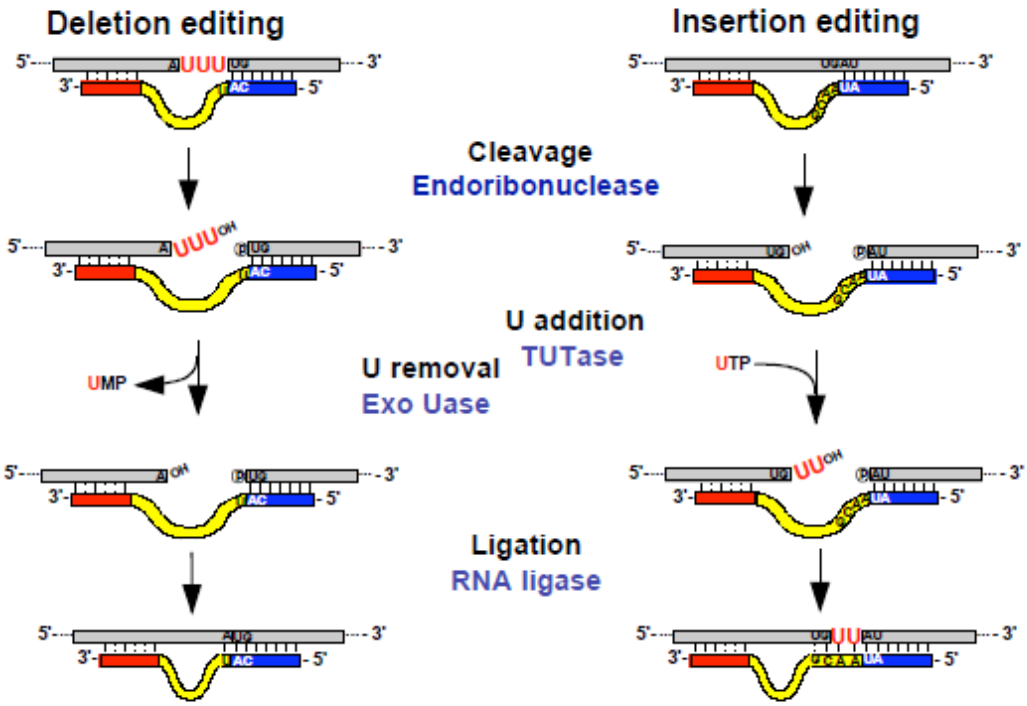
– Structural elements: anchor, informational part and Oligo(U)tail

– Base-pairing of gRNA with unedited RNA gives mismatched regions,

which are recognized by the editing machinery

– Machinery includes an **Endonuclease**, a **Terminal Uridylyl Transferase (TUTase),**and a **RNA ligase**

• Editing is **directional**, from 3' to 5'



Editing is catalyzed by a multiprotein complex

- Editing is catalyzed by a multiprotein complexes that have not been fully defined yet.
- A complex has been purified from glycerol gradients that contains the four key enzyme activities: **20S editosome**
- **Endonuclease:** cleavage in vitro occurs at an unpaired nucleotide immediately upstream of the gRNA-mRNA anchor duplex.
- **Exonuclease:** exoUase removes non-base-paired U nucleotides after cleavage of deletion editing sites
- **TUTase:In insertion editing, Us are added to the 3' end of the 5' pre-mRNA fragment by a terminal uridyl transferase as specified by the gRNA.**
- **RNA ligase:** the natural editing ligase substrates are nicked dsRNAs that are completely base-paired after the correct addition or removal of U nucleotides
- **Helicase:** each gRNA must be displaced from the sequence that it creates to enable binding by the subsequent gRNA and also from the mRNA completely before translation
- **Other 20S editosome proteins**

Significance of RNA Editing

- It is essential in **regulating gene expression** of organisms.
- **RNA editing mutant** was reported with strong defects in organelle development.
- Deficiency causes **diseases**.
- It is a mechanism to **increase the number of different proteins** available without the need to increase the number of genes in the genome.
- May help **protect the genome** against some viruses.

Evolutionary significance:

Trypanosomes are primitive single-celled eukaryotes that diverged from other eukaryotes early in evolution. Some evolutionists have speculated that RNA editing was common in ancient cells, where many reactions are thought to have been catalyzed by RNA molecules instead of proteins. Another view is that RNA editing is a primitive mechanism for altering patterns of gene expression. For whatever reason, RNA editing plays a major role in the expression of genes in the mitochondria of trypanosomes and plants.

The RNA-editing system seen in the animal may have evolved from mononucleotide deaminases, which have led to larger gene families that include the apobec-1 and adar genes. These genes share close identity with the bacterial deaminases involved in nucleotide metabolism. The adenosine deaminase of *E. coli* cannot deaminate a nucleoside in the RNA; the enzyme's reaction pocket is too small for the RNA strand to bind to. However, this active site is widened by amino acid changes in the corresponding human analog genes, *APOBEC1* and *ADAR*, allowing deamination. The gRNA-mediated pan-editing in trypanosome mitochondria, involving templated insertion of U residues, is an entirely different biochemical reaction. The enzymes involved have been shown in other studies to be recruited and adapted from different sources. But the specificity of nucleotide insertion via the interaction between the gRNA and mRNA is similar to the tRNA editing processes in the animal and Acanthamoeba mithochondria. Eukaryotic ribose methylation of rRNAs by guide RNA molecules is a similar form of modification.

Thus, RNA editing evolved more than once. Several adaptive rationales for editing have been suggested. Editing is often described as a mechanism of correction or repair to compensate for defects in gene sequences. However, in the case of gRNA-mediated editing, this explanation does not seem possible because if a defect happens first, there is no way to generate an error-free gRNA-encoding region, which presumably arises by duplication of the original gene region. This thinking leads to an evolutionary proposal called "constructive neutral evolution" in which the order of steps is reversed, with the gratuitous capacity for editing preceding the "defect"

Gene Silencing

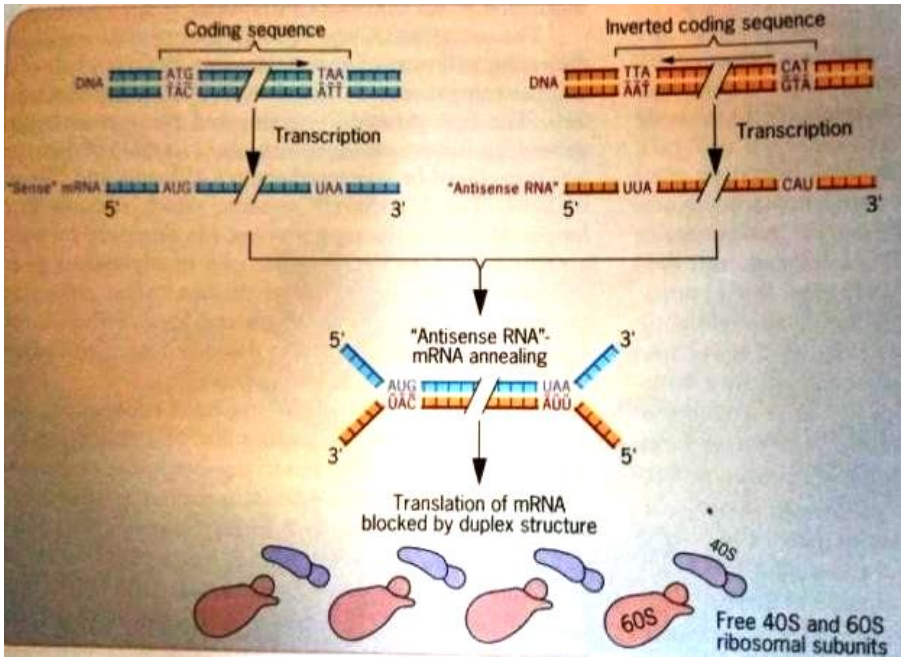
Gene silencing is a term of gene regulation used to describe the "switching off" of a gene by a mechanism without introducing any genetic modification. DNA is transcribed into mRNA but the mRNA is never translated into proteins. Gene regulation can be done either at the transcriptional level

or at post-transcriptional level. In case of transcriptional level it is done by inducing modification in histone protein, changing the environment for the binding of transcriptional machineries such as RNA polymerase, transcription factors, etc. However in case of post-transcriptional level of gene regulations the transcribed mRNA by a particular gene is being blocked or destroyed. The post-transcriptional level of gene silencing is achieved by

1. Antisense Technology
2. RNA interference (RNAi)

Antisense RNA technology:

Antisense RNA (asRNA), also referred to as antisense transcript, natural antisense transcript (NAT) or antisense oligonucleotide, is a single stranded RNA that is complementary to a protein coding messenger RNA (mRNA) with which it hybridizes, and thereby blocks its translation into protein. asRNAs (which occur naturally) have been found in both prokaryotes and eukaryotes,[1] antisense transcripts can be classified into short (<200 nucleotides) and long (>200 nucleotides) non-coding RNAs (ncRNAs).[4] The primary function of asRNA is regulating gene expression. asRNAs may also be produced synthetically and have found wide spread use as research tools for gene knockdown. They may also have therapeutic applications.



Antisense Technology

Antisense technology talks about the production of complementary nucleic acid molecules against the mRNA molecule transcribed from the DNA in order to stop the translation into protein. These complementary molecules can be synthetically produced and delivered inside the cell to block the expression of diseased protein. It can be a short length of either RNA or DNA which commonly termed as Antisense oligonucleotides (AON). Here antisense refers the complementary nature of the synthetic molecule with respect to mRNA. When these AON inserted inside the cell it forms RNA duplex (i.e. double stranded RNA or RNA-DNA duplex). The formation of double stranded RNA inhibits gene expression at translation level as protein synthesis requires single stranded mRNA molecule as a template. This phenomenon is still not well understood but the current hypothesis about this is following-:

- blocking RNA splicing,
- accelerate the degradation process of the RNA and it also prevents the introns from splicing
- preventing the migration of mRNA from nucleolus to cytoplasm
- stopping the translation of diseased protein, and

- If complementary DNA molecule is used there may be a formation of triplex in DNA template.

Mechanism of Antisense Technology

- The synthetic AON introduced inside the cell according to the gene of interest.
- If it is a DNA molecule it binds with the DNA inside the nucleus to form a triplex which inhibits the transcription and finally translation. Sometimes RNA-DNA heterodimer is also formed to stop the translation.
- In case of antisense RNA it binds with mRNA to stop the translation.

Applications of Antisense RNA Technology:

Antisense strategies have been applied to plant systems as well as animal systems not only for production of novel mutants but also for studying the steps involved in particular metabolic pathways, identifying gene function, plant development, crop improvement and other novel uses.

Antisense RNA provides an opening in the study of regulation of viral genes, as an antisense inhibition can be taken to be a leaky mutation which would be useful in studying genes, mutations in which are lethal and their partial inhibition also leads to a significant change in phenotype.

Such partial inhibition was used to create a tobacco mutant deficient in NADH-hydroxypyruvate reductase to study the role of photo-respiration in stress protection (Oliver et al. 1993). Besides unravelling the vital gene functions, antisense RNA inhibition has been used to observe various steps in metabolic pathways. Majeau et al. (1994) modified the activity of carbonic anhydrase which had no significant impact on CO_2 assimilation but it brought forward the effect of decline in carbonic anhydrase activity on stomatal conductance and susceptibility to water stress.

Antisense mutants of tobacco with drastic decrease in Rubisco content resulted in low photosynthetic rate; however, the leaf development was normal and independent of Rubisco content though leaf development was delayed (Jiang and Rodermel 1995). The biochemical target of various herbicides is acetolactate synthase, and this was confirmed by raising transgenic potato plants expressing antisense acetolactate synthase which were inviable without amino acid supplementation, thus an in vivo model for herbicide action was put forward (Hofgen et al. 1995).

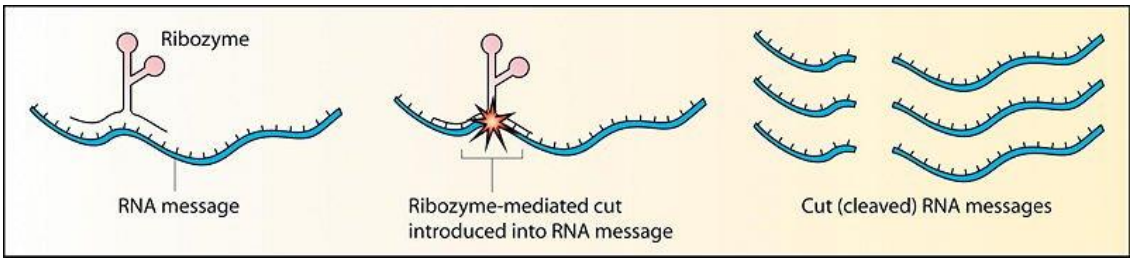
Similarly, the effect of ethylene on shoot morphogenesis was studied via the production of transgenic mustard plants expressing antisense 1-aminocyclopropane -1-carboxylic acid (ACC) oxidase gene, and such plants showed marked increase in regeneration potential and corresponding decrease in ethylene production (Pua and Lee, 1995). Cotton fibre protein genes have also been characterized using antisense RNA inhibition of a particular gene (Hohn, 1996). Antisense inhibition has been utilized to work out the role of lipoxygenase (LOX) in lentil protoplast by the introduction of antisense LOX gene (Maccarone et al. 1995).

The antisense RNA technology has formed the basis for elucidating the flavonoid biosynthetic pathway, and, as a matter of fact, CHS gene was the first endogenous gene targeted by antisense RNA in plants. Antisense CHS petunia plants produced flowers with pale corolla pigmentation but the steady state levels of mRNA of other flavonoid-specific genes were not affected (Van der Krol et al. 1990).

Ribozymes:

Ribozymes (ribonucleic acid enzymes) are RNA molecules that have the ability to catalyze specific biochemical reactions, including RNA splicing in gene expression, similar to the action of protein enzymes. The 1982 discovery of ribozymes demonstrated that RNA can be both genetic material (like DNA) and a biological catalyst (like protein enzymes), and contributed to the RNA world hypothesis, which suggests that RNA may have been important in the evolution of prebiotic self-replicating systems. The most common activities of natural or in vitro-evolved ribozymes are the cleavage or ligation of RNA and DNA and peptide bond formation. Within the ribosome, ribozymes function as part of the large subunit ribosomal RNA to link amino acids during protein synthesis. They also participate in a variety of RNA processing reactions, including RNA splicing, viral replication, and transfer RNA biosynthesis. Examples of ribozymes include the hammerhead ribozyme, the VS ribozyme, Leadzyme and the hairpin ribozyme.

Investigators studying the origin of life have produced ribozymes in the laboratory that are capable of catalyzing their own synthesis from activated monomers under very specific conditions, such as an RNA polymerase ribozyme.

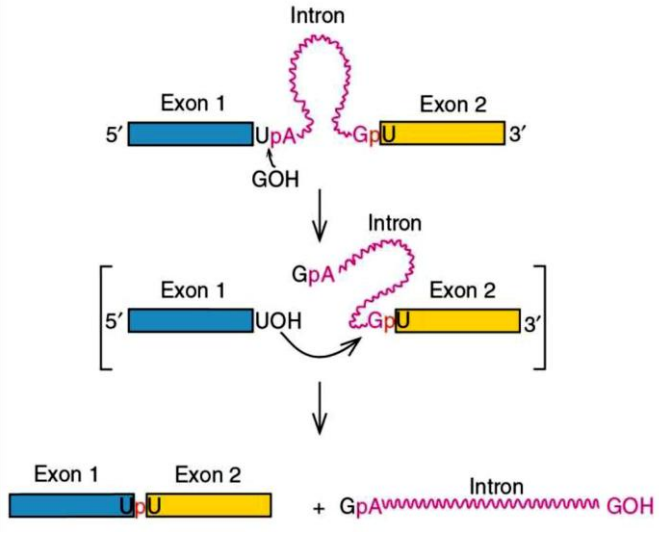


Types:

- Group I and group II intron splicing ribozymes
- RNase P
- Hammerhead Ribozyme
- Hairpin ribozyme
- Ribozome

Group I intron:

Group I intron ribozymes constitute one of the main classes of ribozymes. Found in bacteria, lower eukaryotes and higher plants. Group I introns are also found inserted into genes of a wide variety of bacteriophages of Gram-positive bacteria. However, their distribution in the phage of Gram-negative bacteria is mainly limited to the T4, T-even and T7-like like bacteriophages.

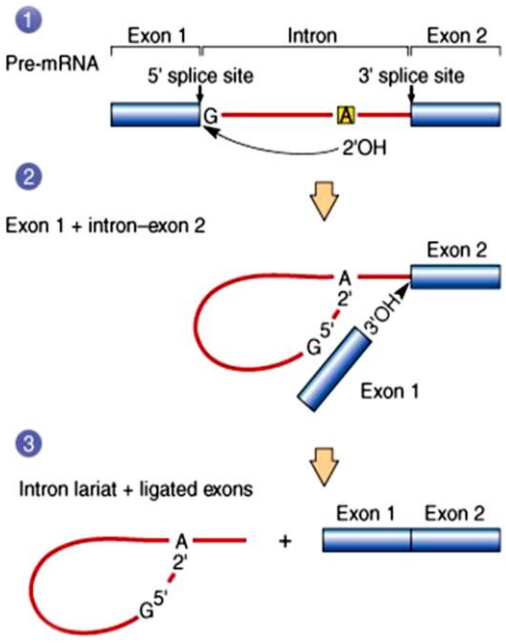


Mechanism:

The group I splicing reaction requires a guanine residue cofactor, the 3’ OH group of guanosine is used as a nucleophile. The 3’ OH group attacks the 5’ phosphate of the intron and a new phosphordiester bond is formed. The 3’ OH of the exon that is displaced now acts as the nucleophile in a similar reaction at the 3’ end of the intron. So the intron is precisely excised and exons are joined together.

Group II introns

Group II introns have been found in bacteria and in the mitochondrial and chloroplast genomes of fungi, plants, protists, and an annelid worm.



Mechanism:

The 2’OH of a specific adenosine acts as a nucleophile and attacks the 5’ splice site creating a branched intron structure. The 3’ OH of the 5’ exon attacks the 3’ splice site, ligating the exons and releasing the intron as a lariat structure.

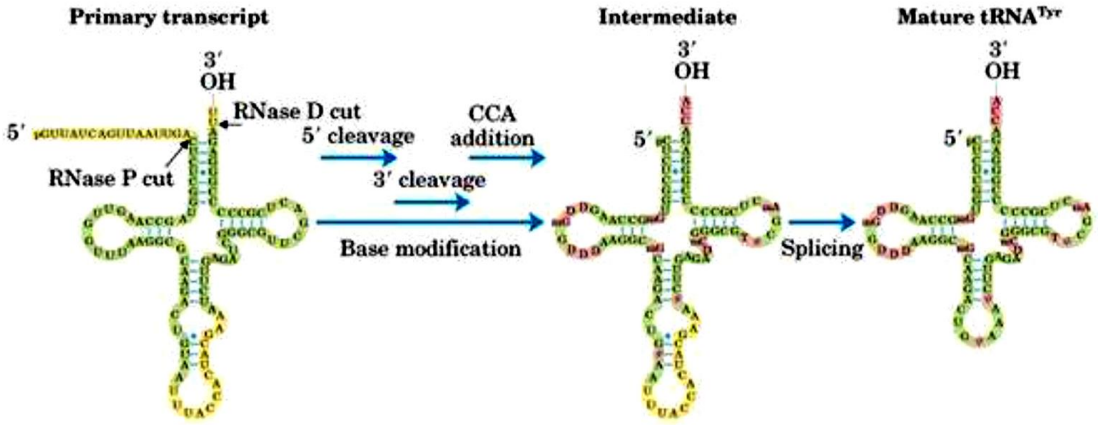
Ribonuclease P

Ribonuclease P (RNaseP), a ribonucleoprotein, is an essential tRNA processing enzyme found in all living organisms. Since its discovery almost 40 years ago, research on RNase P has led to the discovery of the

catalytic properties of RNA, and of the only known, naturally occurring RNA enzymes.

Mechanism:

All RNase P enzymes are ribonucleoproteins [bacteria: 1RNA + 1 protein subunit; eukaryotes: 1 RNA + many protein subunits (11 in human)], In Ribonulease – P, protein component is facilitates binding between RNase and t-RNA substrate. It requires divalent metal ions (like Mg2+) for its activity. Endo-ribonuclease is responsible for generating 5' end of matured tRNA molecules. Cleavage via nucleophilic attack on the phosphodiester bond is leaving a 5'-phosphate and 3'-hydroxyl at the cleavage site.



Hammerhead Ribozyme:

Hammerhead ribozyme is another small RNA which performs self cleavage reaction. It is found in viroids, which have RNA as genetic material and they infect the plants. When viroid replicates by rolling circle, it produces a continuous RNA chain, which consists of multiple copies of RNA. This continuous chain RNA undergoes cleavage to form single viroids or monomers. The self-cleaving RNA sequence at the junction of monomers is called hammerhead because of the shape of its secondary structure.

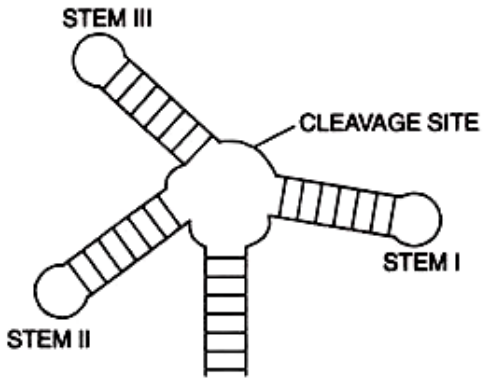


Fig. 10.4. Hammerhead Ribozyme

It consists of three complementary base paired stems. These are stem I, stem II and stem III surrounding a core of non-complementary nucleotides. This core lies at their junction and undergoes self-cleavage reaction.

The catalytic reaction centre lies between stem 2/3 and stem I. This centre contains a magnesium ion that initiates hydrolysis reaction. The reaction breaks the RNA chain producing a 2′, 3′ cyclic phosphate and a free 5′-hydroxyl end.

Hairpin ribozyme

The hairpin ribozyme is an RNA motif that catalyzes RNA processing reactions essential for replication of the satellite RNA molecules in which it is embedded. These reactions are selfprocessing, i.e. a molecule rearranging its own structure. Both cleavage and end joining reactions are mediated by the ribozyme motif. In contrast to the hammerhead and Tetrahymena ribozyme reactions, hairpin-mediated cleavage and ligation proceed through a catalytic mechanism that does not require direct coordination of metal cations to phosphate or water oxygens.

Non-coding RNA

A non-coding RNA (ncRNA) is an RNA molecule that is not translated into a protein. The DNA sequence from which a functional non-coding RNA is transcribed is often called an RNA gene. Abundant and functionally important types of non-coding RNAs include transfer RNAs (tRNAs) and

ribosomal RNAs (rRNAs), as well as small RNAs such as microRNAs, siRNAs, piRNAs, snoRNAs, snRNAs, exRNAs, scaRNAs and the long ncRNAs such as Xist and HOTAIR.
non-coding RNAs can be divided into two main types: infrastructural and regulatory ncRNAs. Infrastructural ncRNAs seem to have a housekeeping role in translation and splicing and include species such as ribosomal, transfer and small nuclear RNAs. Regulatory ncRNAs are more interesting from an epigenetic point of view as they are involved in the modification of other RNAs. They can be further classified into the following:

microRNAs (miRNAs)

miRNAs are small single-stranded molecules (20 – 24 nt) that derive from transcripts forming distinctive hairpin structures called pre-miRNA. The hairpin is processed into mature miRNA and forms the RNA induced silencing complex (RISC), which contains miRNA-interacting proteins such as Dicer. The miRNAs will pair with complementary sequences on target mRNAs transcripts through the 3'UTR, leading to gene silencing of the target.

Piwi-interacting RNAs (piRNAs)

piRNAs are small ncRNA (24–31 nt) that are able to form complexes with Piwi proteins of the Argonaute family. piRNAs are characterized by a uridine at the 5'end and a 2'-O-methyl modification at the 3' end. Their main role is the silencing of transposable elements during germ line development.

Small interfering RNAs (siRNAs)

siRNAs are long linear dsRNA processed by Dicer into mature 20–24 nt siRNAs that direct silencing when loaded onto RISC. They mediate post-transcriptional silencing similarly to miRNA by a process called RNA interference (RNAi), where siRNA interferes with the expression of a complementary nucleotide sequence.

Long non-coding RNAs (lncRNAs)

lncRNAs are considered as non-protein coding transcripts >200 nt in length. The majority of non-coding RNAs belong to this group. Many of the lncRNAs are subject to splicing, polyadenylation and other post-transcriptional modifications, and can be classified according to their proximity to protein coding genes. A subgroup of lncRNAs, named large intergenic non-coding RNAs (lincRNAs) are marked by trimethylation of K4 on histone H3 (H3K4me3) at their promoter and trimethylation of K36 on histone H3 (H3K36me3) along the transcribed region. LincRNAs are involved in epigenetic gene silencing, such as the role of Xist (X-inactive specific transcript) and in tumor development by promoting expression of genes involved in metastasis and angiogenesis

RNA interference

RNAi has shown its importance in the analysis of gene functions and silencing of gene for therapeutic purpose. It was first reported by Andrew Fire and his team in the year of 1997 while studying the introduction of dsRNA into *C. elegans* for silencing a gene unc-22 gene. RNA interference (RNAi) is basically a post-transcriptional phenomenon which may be triggered by providing a double-stranded RNA (dsRNA) which is known as double RNA activation. Two types of small RNA molecules – microRNA (miRNA) and small interfering RNA (siRNA) plays a central role in RNA interference based gene silencing. RNAi looks very similar to plant posttranscriptional gene-silencing (i.e.PTGS) and quelling in case of fungi. Functions of RNAi are as follows:

• Immunity: the immune response to viruses and other foreign genetic material

(In case of plants)

• Down regulation of genes through mi RNA (micro RNA)

• Up-regulation of genes by using both siRNA and miRNA complementary to parts of a promoter

Micro RNA(miRNA) and Small Interfering RNA (siRNA)

Both are considered as interfering RNA. Historically miRNA was discovered in 1993 by Ambros and his coworkers where as siRNAs concept came in 1999 from another discovery in which a dsRNA showed its role in post-transcriptional gene silencing (PTGS) in plants by David Baulcombe's group. During PTGS at one stage there is a role of ~20 − 25 nt RNAs in silencing which was produced by the dsRNA. miRNAs is considered as regulators of cellular self genes(i.e. endogenous genes), and siRNAs act as guards of foreign or invasive genes coming from viruses, transposons, and transgenes etc. which try to get integrated into host genome.

	Occurrence	Configuration	Length	Complementary to target mRNA	Biogenesis	Action	Function
miRNA	Occur naturally in plants and animals	Single stranded	19-25 nt	Not exact, and therefore a single miRNA may target up to hundreds of mRNAs	Expressed by genes whose purpose is to make miRNAs, but they regulate genes (mRNAs) other than the ones that expressed them	Inhibit translate-ion of mRNA	Regulators (inhibitors) of genes (mRNAs)
siRNA	Occur naturally in plants and lower animals. Whether or not they occur naturally in mammals is an unsettled question	Double stranded	21-22 nt	100% perfect match, and therefore siRNAs knock down specific genes, with minor off-target exceptions	Regulate the same genes that express them	Cleave mRNA	Act as gene-silencing guard -ians in plants and animals that do not have antibody-or cell-mediated immunity

Biogenesis of miRNAs and Role in Protein Regulation

The miRNA gene is always present in the host gnome which gets transcribed into primary-miRNA (pri-miRNA) first with the help of RNA polymerase II. This pri-miRNA is cleaved by an enzyme called Drosha which is a type of ribonuclease III enzyme. It liberates approximately 60 to 70 nt looped structure which is consider as precursor miRNA or pre- miRNA. This pre-miRNA is the transported with the help of Exportin 5 present in cytoplasm. Once the pre-miRNA is exported into cytoplasmic space the another dsRNA specific enzyme called Dicer helps in duplexing with other miRNA. The unwinding of the duplexed miRNA is done by helicase. Now the both dsRNA-specific endonucleases enzymes (Drosha and Dicer) help to generate 2-nucleotide-long-3' overhangs near the cleavage site. After unwinding of the double stranded miRNA the generation of target specific Guide strand and the passenger stand. Now the miRNA (i.e. Guide strand) is considered as mature miRNA which is then incorporated with the RNA-induced silencing complex (RISC). The target specific miRNA now bind with the mRNA and stop the translation. Finally the gene is silenced with the help of miRNA and the cell undergo self destruction pathway.

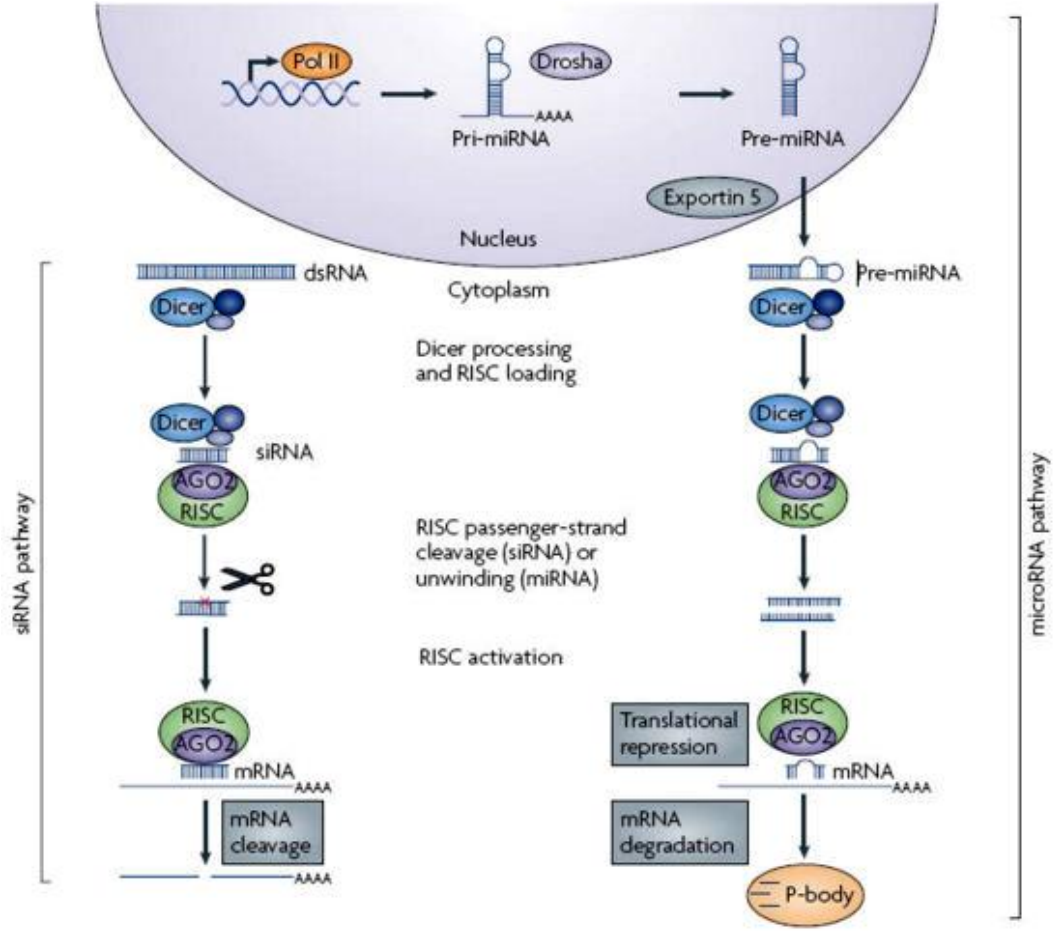
MicroRNAs (miRNAs) mediated gene silencing

- The transcript containing an miRNA is called the primary miRNA transcript or pri-miRNA.
- The pri-miRNA molecule contains a hairpin structure about 70 nt long, within which is the eventual miRNA.
- The hairpin is cut out of the pri-miRNA in the nucleus by the dsRNA-specific endonuclease Drosha complexed to an accessory protein (Pasha in Drosophila).
- Drosha makes staggered cuts resulting in a ~2 nt 3' single-stranded overhang.
- The excised hairpin- pre-miRNA is exported rapidly to the cytoplasm.

- In the cytoplasm, another dsRNA-specific endonuclease, Dicer, complexed to an accessory protein (Loq in Drosophila), makes staggered cuts in the pre-miRNA, releasing a short miRNA:miRNA* dsRNA consisting of some the former paired sides of the hairpin.
- The two RNA strands are imperfectly paired: "miRNA" is the mature miRNA strand that subsequently functions in the cell for RNA silencing, while miRNA* is its partial complement and does not function in RNA silencing.
- Because the miRNA directs RNA silencing, it is termed the guide strand, while the miRNA* is termed the passenger strand.
- Next the dsRNA, Dicer and accessory protein bind to Ago1, a member of the Argonaute family of protein and other proteins to form the pre-microRNA-induced silencing complex, or pre-miRISC.
- Ago1 is another RNA endonuclease; more generally called Slicer. It makes a single cut within the miRNA* passenger strand.
- A helicase that is part of the pre-miRISC then unwinds to two pieces from the miRNA guide strand, and they dissociate from the complex.
- The result is the mature miRISC, the ribonucleoprotein complex that can silence gene expression.

How does a miRISC function in posttrancriptional gene silencing?

- The miRNA in the miRISC is a trans-acting RNA regulatory molecule, meaning that it targets mRNAs that are not the same as the RNA molecules from which the miRNA is derived. This is one distinguishing feature of miRNAs compared with siRNAs.
- An miRISC binds to a target mRNA through complementary base pairing involving the miRNA.
- Usually, the sequence to which the miRNA binds are short sequence in the 3' UTR of the mRNA
- An mRNA molecule may have one or more sequences in its 3' UTR to which the same miRNA can bind and/or it may have several sequences in its 3' UTR to which several different miRNAs can bind. The latter raises the possibility of regulating the expression of the same gene (through its mRNA) by various combinations of miRNA regulator molecules.
- Here, one miRISC is shown binding to a 3' UTR sequence for simplicity.
- Binding of most of the miRISC to their target mRNAs involves imperfect pairing between the miRNA and the 3' UTR region of the mRNA.
- Such pairing triggers translational repression – translation of that mRNA becomes inhibited.
- The translationally repressed mRNA with its associated miRISC(s) is then sequestered from the translation machinery by becoming or moving into a P body.
- P body is a cytoplasmically located aggregate of translationally repressed mRNAs complexed with proteins, and proteins for mRNA decapping and mRNA degradation.
- The mRNAs is P bodies may be degraded using the contained mRNA degradation machinery or stored in ribonucleoprotein complexes.
- Stored mRNAs can be returned to translation at a later time. Whether degraded or stored the effect of miRNA action is to reduce the expression of the gene encoding the targeted mRNA at the translational level.
- In plants, binding of most miRISCs to their target mRNAs involves perfect or near-perfect pairing between much of the miRNA and the 3' UTR region of the mRNA.
- Perfect pairing triggers mRNA degradation rather than translational repression.
- Here, the Ago1 Slicer protein cuts the target mRNA into two and the mRNA-miRISC complex forms, or it is moved to a P body where degradation of the mRNA is completed.



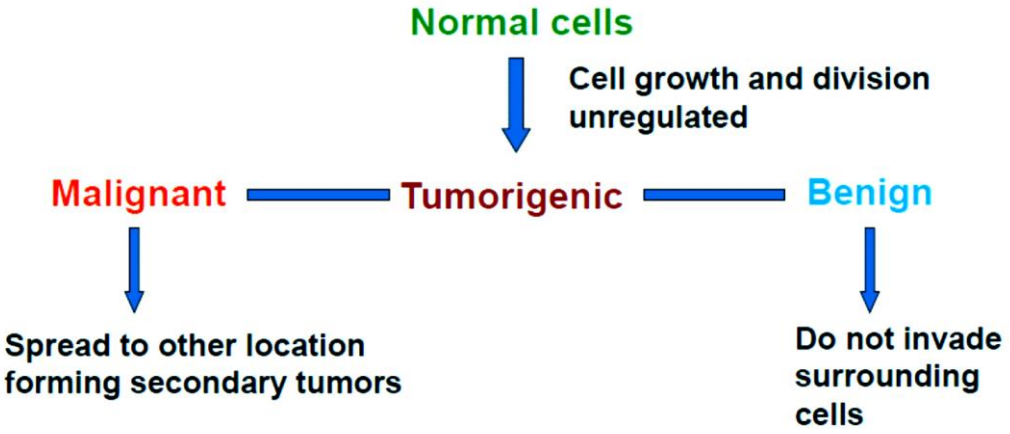
Applications of RNAi in crop quality improvement

- Improvement in the crop quality has been done through conventional breeding, but this approach is time consuming and labor intensive.
- RNAi, being a novel approach has great potential to modify the gene expression in plants for better quality traits and nutritional improvement in different crops.
- RNAi enables repression of gibberellic acid and auxin signal pathways after a reduction in the level of *SlARF7 transcript responsible* for pollination and fertilization in tomato plants (DeJong et al., 2009). These results by-pass the auxin signaling-fertilization pathway that leads to the development of parthenocarpic fruits having great commercial value.
- Carotenoid's production such as β-carotene and lutein were reported higher in potato through gene silencing of β-carotene hydroxylase (Eck et al., 2007).
- The post-harvest life can enhance by knowing-out genes responsible for ethylene production in tomato (Xiong et al., 2005). This was achieved through introducing dsRNA and blocking the gene expression of ACC-oxidase which significantly reduced the ethylene formation and enhanced shelf-life in tomato.
- RNAi suppression of α-mannosidase and β-acetylhexosaminidase associated with fruit softening also increased the shelf-life in tomato fruits (Meli et al., 2010).
- Increase in amylose contents in wheat by suppressing two genes (*SBEIIa and SBEIIb) meant for* starch-branching enzyme was well demonstrated by (Regina et al., 2006).
- RNAi could be exploited as a metabolic engineering tool for the production and synthesis of commercially valuable plant products such as alkaloid production (codeine, quinine, vincristine, scopolamine), biosynthesis of essential oil and flavoring agents (vanillin) (Saurabh et al., 2014).

11. **Cancer: Properties of cancer cells. Transfection test. Genetic basis of cancer. Characterization of p ^{53} and its role in regulation of cancer. Role of gene mutation, reciprocal translocation, insertion of retroviral genome and constitutive amplification in cancer development; environmental carcinogenesis; therapy and side effects.**

Cancer:

Cancer is a group of disease characterized by uncontrolled cell growth and division.Cancers arise when critical genes are mutated. These mutations can cause biochemical processes to go awry and lead to the unregulated proliferation of cells.Without regulation, cancer cells divide ceaselessly, piling up on top of each other toform tumors. When cells detach from a tumor and invade the surrounding tissues, the tumor is **malignant**. When the cells do not invade the surrounding tissues, the tumor is **benign**. Malignant tumors may spread to other locations in the body, forming secondarytumors. This process is called **metastasis.**



Possible causes:

1. Clonally inherited – genetic basis.
2. Some viruses can induce tumors experimentally.
3. Mutagenic agents, chemicals, ionizing radiations can induce tumor in experimental animals.
4. Colon cancer / cancer of eye – simple dominant inheritance.
5. Leukemias and lymphomas are associated with chromosomal aberrations.
6. Accumulation of spontaneous mutations in the somatic tissues.

Characteristics of cancer cells

1. Immortalization: the property of indefinite cell growth
2. Loss of contact inhibition
3. Reduced cellular adhesion
4. Invasiveness – Malignant cells generally secrete Proteases that digest extra cellular matrix components, allowing the cancer cells to invade adjacent normal tissues. eg. Collagenase digest and penetrate through basal laminae to invade the underlying connective tissue.
5. Fail to differentiate – (Loss of size and shape of cells)
6. Autostimulation of cell division
7. Apoptosis – loss

8. High nucleus to cytoplasm ratio
9. Depolymerization of cytoskeleton – microtubules
10. Chromosomal changes – high degree of ploidy and aneuploidy
11. Interaction with Immune System.

Classification of cancer:

Carcinoma: malignant tumors derived from epithelial cells. This group represent the most common cancers, including the common forms of breast, prostate, lung and colon cancer.

Lymphoma and Leukemia: malignant tumors derived from blood and bone marrow cells.

Sarcoma: malignant tumors derived from connective tissue, or mesenchymal cells.

Mesothelioma: tumors derived from the mesothelial cells lining the peritoneum and the pleura.

Glioma: tumors derived from glia, the most common type of brain cell.

Germinoma: tumors derived from germ cells, normally found in the testicle and ovary.

Choriocarcinoma: malignant tumors derived from the placenta.

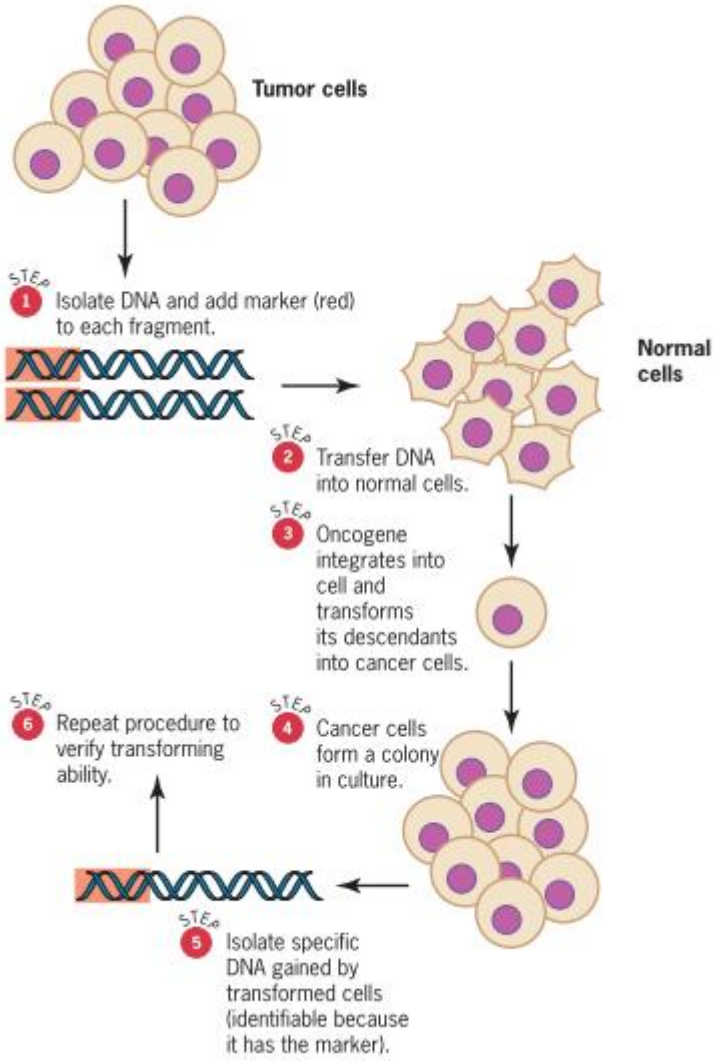
Genetic basis for cancer

The recent great advances in understanding cancer have come through application of molecular genetic techniques. However, before these techniques were available to researchers, there was strong evidence that the underlying causes of cancer are genetic. First, it was known that the cancerous state is clonally inherited. When cancer cells are grown in culture, their descendants are all cancerous. The cancerous condition is therefore transmitted from each cell to its daughters at the time of division—a phenomenon indicating that cancer has a genetic (or epigenetic) basis. Second, it was known that certain types of viruses can induce the formation of tumors in experimental animals. The induction of cancer by viruses implies that the proteins encoded by viral genes are involved in the production of the cancerous state. Third, it was known that cancer can be induced by agents capable of causing mutations. Mutagenic chemicals and ionizing radiation had been shown to induce tumors in experimental animals. In addition, a wealth of epidemiological data had implicated these agents as the causes of cancer in humans. Fourth, it was known that certain types of cancer tend to run in families. In particular, susceptibility to retinoblastoma, a rare cancer of the eye, and susceptibility to some forms of colon cancer appeared to be inherited as simple dominant conditions, albeit with incomplete penetrance and variable expressivity. Because susceptibility to these special types of cancer is inherited, it seemed plausible that all cancers might have their basis in genetic defects—either inherited mutations or somatic mutations acquired during a person's lifetime. Finally, it was known that certain types of white blood cell cancers (leukemias and lymphomas) are associated with particular chromosomal aberrations. Collectively, these diverse observations strongly suggested that cancer is caused by genetic malfunctions.

Transfection test:

The first evidence linking cancer to a mutant c-onc came from the study of a human bladder cancer. The mutation responsible for this bladder cancer was isolated by Robert Weinberg and colleagues using a transfection test. DNA was extracted from the cancerous tissue and fragmented into small pieces; then each of these pieces was joined to a segment of bacterial DNA, which served as a molecular marker. The marked DNA fragments were then introduced, or transfected, into cells growing in culture to determine if any of them could transform the cells into a cancerous state. This state could be recognized by the tendency of the cancer cells to form small clumps, or foci, when grown on soft agar plates. The DNA from such cells was extracted and screened to see if it carried the molecular marker that was linked to the original transfecting fragments. If it did, this DNA was retested for its ability to induce the cancerous state. After several tests, Weinberg's research team

identified a DNA fragment from the original bladder cancer that reproducibly transformed cultured cells into cancer cells.



Oncogenes

Genes that promote autonomous cell growth in cancer cells are called **oncogenes**, and their normal cellular counterparts are called **proto-oncogenes**. Proto-oncogenes are physiologic regulators of cell proliferation and differentiation while oncogenes are characterised by the ability to promote cell growth in the absence of normal mitogenic signals. Their products, oncoproteins, resemble the normal products of proto-oncogenes with the exception that oncoproteins are devoid of important regulatory elements. Their production in the transformed cells becomes constitutive, that is, not dependent on growth factors or other external signals. Proto-oncogenes can be converted to oncogenes by several mechanisms, such as,

1. Mutational change in the protein.
2. Constitutive activation.
3. Gene amplification resulting in over expression of gene(s)
4. Reciprocal translocation and position effect.
5. Mutation in tumour suppressor gene(s).

Resulting in:

- Overproduction of growth factors

- Flooding of the cell with replication signals
- Uncontrolled stimulation in the intermediary pathways
- Cell growth by elevated levels of transcription factors

Tumour suppressor genes

Tumour suppressor genes encode proteins that are:

- receptors for secreted hormones that function to inhibit cell proliferation
- negative regulators of cell cycle entry or progression
- negative regulators of growth signalling pathways (e.g. APC or PTEN)
- checkpoint-control proteins that arrest the cell cycle if DNA is damaged or chromosomes are abnormal
- proteins that promote apoptosis DNA repair enzymes. The transformation of a normal cell to a cancer cell is accompanied by the loss of function of one or more tumour suppressor genes and both gene copies must be defective in order to promote tumour development.

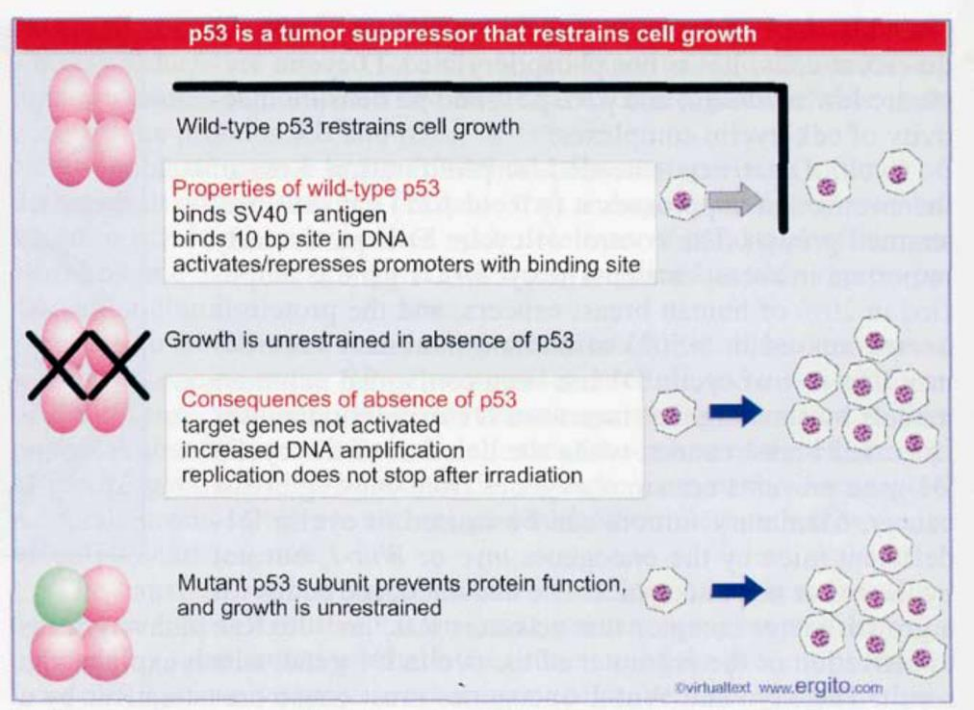
TABLE 18.2 SOME PROTO-ONCOGENES AND TUMOR SUPPRESSOR GENES

Proto-oncogene	Normal Function	Alteration in Cancer	Associated Cancers
Ha-ras	Signal transduction molecule, binds GTP/GDP	Point mutations	Colorectal, bladder, many types
c-erbB	Transmembrane growth factor receptor	Gene amplification, point mutations	Glioblastomas, breast cancer, cervix
c-myc	Transcription factor, regulates cell cycle, differentiation, apoptosis	Translocation, amplification, point mutations	Lymphomas, leukemias, lung cancer, many types
c-fos	Transcription factor, responds to growth factors	Overexpression	Osteosarcomas, many types
c-kit	Tyrosine kinase, signal transduction	Mutation	Sarcomas
c-raf	Cytoplasmic serine-threonine kinase, signal transduction	Gene rearrangements	Stomach cancer
RARα	Hormone-dependent transcription factor, differentiation	Chromosomal translocations with PML gene, fusion product	Acute promyelocytic leukemia
E6	Human papillomavirus encoded oncogene, inactivates p53	HPV infection	Cervical cancer
MDM2	Binds and inactivates p53, abrogates cell cycle checkpoints	Gene amplification, over-expression	Osteosarcomas, liposarcomas
Cyclins	Bind to CDKs, regulate cell cycle	Gene amplification, over-expression	Lung, esophagus, many types
CDK2, 4	Cyclin-dependent kinases, regulate cell cycle phases	Overexpression, mutation	Bladder, breast, many types
Tumor Suppressor	**Normal Function**	**Alteration in Cancer**	**Associated Cancers**
p53	Cell cycle checkpoints, apoptosis	Mutation, inactivation by viral oncogene products	Brain, lung, colorectal, breast, many types
RB1	Cell cycle checkpoints, binds E2F	Mutation, deletion, inactivation by viral oncogene products	Retinoblastoma, osteosarcoma, many types
APC	Cell–cell interaction	Mutation	Colorectal cancers, brain, thyroid
Bcl2	Apoptosis regulation	Overexpression blocks apoptosis	Lymphomas, leukemias
XPA–XPG	Nucleotide excision repair	Mutation	Xeroderma pigmentosum, skin cancers
BRCA2	DNA repair	Point mutations	Breast, ovarian, prostate cancers

p53

The 53-kilodalton tumor suppressor protein p53 was discovered through its role in the induction of cancers by certain DNA viruses. This protein is encoded by a tumor suppressor gene called *TP53*. Inherited mutations in *TP53* are associated with the Li-Fraumeni syndrome, a rare dominant condition in which any of several different types of cancer may develop. Somatic mutations that inactivate both

copies of the *TP53* gene are also associated with a variety of cancers. In fact, such mutations are found in a majority of all human tumors. Loss of p53 function is therefore a key step in carcinogenesis. The most common form of a dominant negative mutant is one that forms a heteromeric protein containing both mutant and wild-type subunits, in which the wild-type subunits are unable to function. p53 exists as a tetramer. When mutant and wild-type subunits of p53 associate, the tetramer takes up the mutant conformation. **Figure 30.32** shows that the same phenotype is produced either by the deletion of both alleles or by a missense point mutation in one allele that produces a dominant negative subunit. Both situations are found in humancancers. Mutations in p53 accumulate in many types of human cancer, probably because loss of p53 provides a growth advantage to cells; that is, wild-type p53 restrains growth. The diversity of these cancers suggests that p53 is not involved in a tissue-specific event, but in some general and rather common control of cell proliferation; and the loss of this control may be a secondary event that occurs to assist the growth of many tumors. p53 is defined as a tumor suppressor also by the fact that wild-type p53 can suppress or inhibit the transformation of cells in culture by various oncogenes. Mutant p53 cells also have an increased propensity to amplify DNA, which is likely to reflect p53's role in the characteristic instability of the genome that is found in cancer cells.



Mutation in p53 is a cause of Li-Fraumeni syndrome, which is a rare form of inherited cancer. Affected individuals display cancers in a variety of tissues. They are heterozygotes that have missense mutations in one allele. These mutations behave as dominant negatives, overwhelming the function of the wild-type allele. This explains the occurrence of the disease as an autosomal dominant. All normal cells have low levels of p53. A paradigm for p53 function is provided by systems in which it becomes activated, the most usual cause being irradiation or other treatments that damage DNA. This results in a large increase in the amount of p53. Two types of event can be triggered by the activation of p53: growth arrest and apoptosis (cell death). The outcome depends in part on which stage of the cell cycle has been reached. **Figure 30.33** shows that in cells early in Gl, p53 triggers a checkpoint that blocks further progression through the cell cycle. This allows the damaged DNA to be repaired before the

cell tries to enter S phase. But if a cell is committed to division, then p53 triggers a program of cell death. The typical results of this apoptosis are the collapse of the cell into a small heteropycnotic mass and the fragmentation of nuclear DNA. The stage of the cell cycle is not the only determinant of the outcome; for example, some cell types are more prone to show an apoptotic response than others.

p^{53} is a DNA-binding protein:

p53 is a DNA-binding protein that recognizes an interrupted palindromic 10 bp motif. The ability to bind to its specific target sequences is conferred by the central domain.

p53 activates transcription at promoters that contain multiple copies of this motif. The immediate N-terminal region provides the transactivator domain. p53 may repress other genes; the mechanism is unknown.

p53 also has the ability to bind to damaged DNA. The C-terminal domain recognizes single-stranded regions in DNA.

p53 is a tetramer (oligomerization is a prerequisite for mutants to behave in a dominant negative manner). Oligomerization requires the C-terminal region.

A (putative) signaling domain contains copies of the sequence PXXP, which forms a binding site for SH3 domains. Mutations in p53 have various effects on its properties, including increasing its half-life from 20 minutes to several hours, causing a change in conformation that can be detected with an antibody, changing its location from the nucleus to the cytoplasm, preventing binding to SV40 T antigen, and preventing DNA-binding. As shown in Figure 30.34, the majority of these mutations map in the central DNA-binding domain, suggesting that this is an important activity. p53 activates various pathways through its role as a transcription factor. The pathways can be divided into the three groups summarized in **Figure 30.35.** The major pathway leading to inhibition of the cell cycle at Gl is mediated via activation of p21, which is a CKI (cell cycle inhibitor) that is involved with preventing cells from proceeding through Gl (see Figure 29.30 and Figure 30.30). Activation of GADD45 identifies the pathway that is involved with maintaining genome stability.

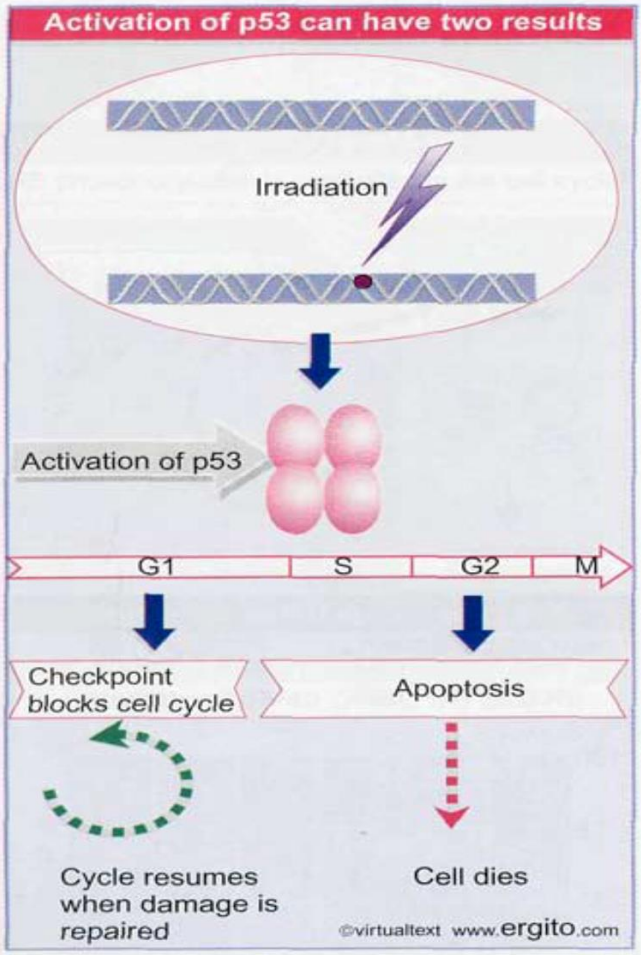


Figure 30.33 Damage to DNA activates p53. The outcome depends on the stage of the cell cycle. Early in the cycle, p53 activates a checkpoint that prevents further progress until the damage has been repaired. If it is too late to exercise the checkpoint, p53 triggers apoptosis.

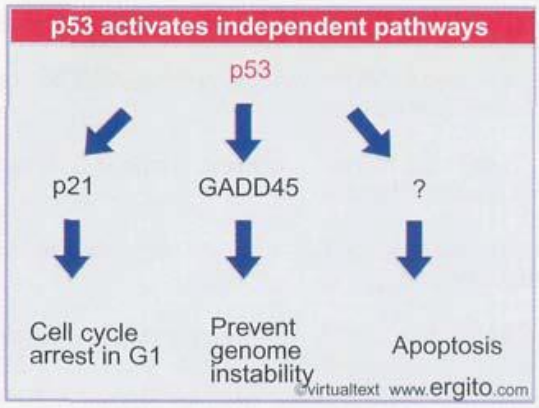


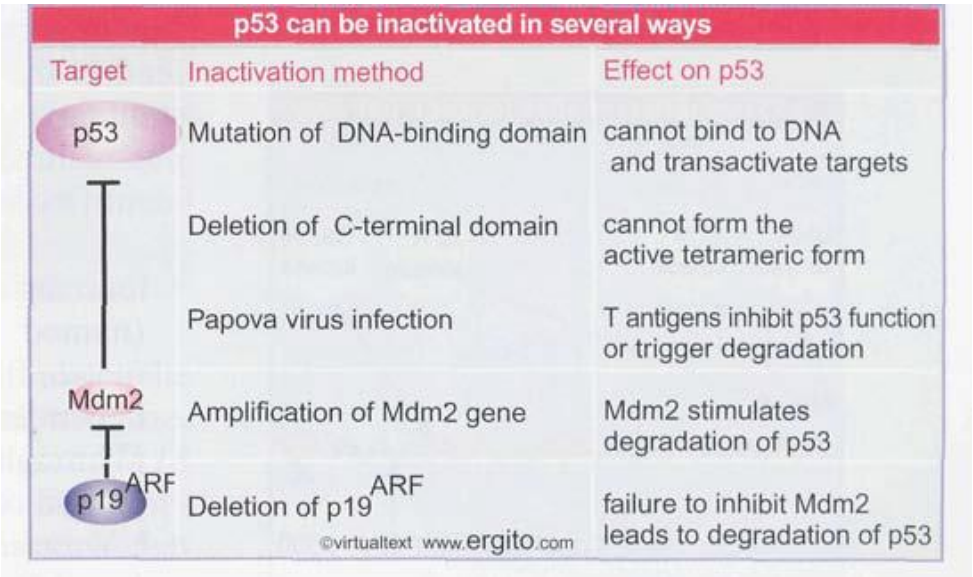
Figure 30.35 p53 activates several independent pathways. Activation of cell cycle arrest together with inhibition of genome instability is an alternative to apoptosis.

GADD45 is a repair protein that is activated also by other pathways that respond to irradiation damage.

P 53 does not function correctly in most human tumors, but the cause of the problem lies with the gene itself in only about half of the cases. **Figure 30.36** summarizes the causes of p53 deficiency:

The mutations in p53 itself most often lie in the DNA-binding region, and prevent the protein from binding to promoters to activate the protective response. In some cases, the mutations lie in the C-terminal region that is responsible for forming tetramers, so that active proteins are not produced.

The major pathway controlling p53 is mediated through the protein Mdm2, which inactivates p53, so the Mdm2 locus behaves as an oncogene. Amplification of the Mdm2 gene causes an increase in expression of the protein, which reduces p53 function.



Mdm2 is itself inactivated by the protein pl9ARF, so deletions of the pl9ARF gene lead to increase in Mdm2 and thus to decrease in p53.

Because p53 inhibits growth or triggers apoptosis when it is activated, it is obviously crucial for the cell to restrain the activity unless it is needed. The circuitry that controls p53's activity is illustrated in the upper part of **Figure 30.37.** Proteins that activate p53 behave as tumor suppressors; proteins that inactivate p53 behave as oncogenes. A major feature in controlling p53 activity is its interaction with Mdm2 (which was originally identified as the product of an oncogene). Mdm2 inhibits p53 activity in two ways: Mdm2 affects p53's stability by acting as an E3 ubiquitin ligase that causes p53 to be targeted by the degradation apparatus.

Mdm2 also acts directly at the N-terminus to inhibit the transactivation activity of p53. In the reverse direction, p53 induces transcription of Mdm2. The consequence of this circuit is that Mdm2 limits p53 activity; and the activation of p53 increases the amount of Mdm2, so the interaction between p53 and Mdm2 forms a negative feedback loop in which the two components limit each other's activities.INK4A-ARF is an important locus that controls both p53 and RB. The transcript of the INK4A-ARF gene is alternatively spliced to give two mRNAs that code for proteins with no sequence relationship. pjgiNK4a j s UpSt r e am of RB. The second protein is called pl9ARF in mouse and pl4ARF in man. We will use pl9ARF to describe it irrespective of source. As we have just seen, pl9 F is upstream of p53. Deletions of the locus are common in human cancers (almost as common as mutations in p53), and have a highly significant effect, because they eliminate both pl6INK4a and pl9 and therefore lead to loss of both the RB and p53 tumor suppressor pathways. p16iNK4a ijj^bits t n e C(jk4/6 kinase. So it prevents the kinase from phosphorylating RB. In the absence of this

phosphorylation, progress through the cell cycle (and therefore growth) is inhibited. pl61N is often inhibited by point mutations in human tumors. pl9ARF antagonizes Mdm2, as shown in the lower part of Figure 30.37. pl9ARF binds to Mdm2 and directly prevents it from ubiquitinating p53. This stabilizes p53 and allows it to accumulate. In effect, therefore, pl9ARF functions as a tumor suppressor by inhibiting the inhibitor of the p53 tumor suppressor. Loss of pl9ARF or loss of p53 have similar effects on cell growth (and tumors usually lose one or the other but not both), suggesting that they function in the same pathway, that is, pl9ARF in effect functions exclusively through p53. The cellular oncogene c-myc, and the adenoviral oncogene El, both act via pl9ARF to activate p53- dependent pathways.

The p53 protein can also mediate another response to cell stress. Instead of orchestrating efforts to repair damage within a cell, p53 may trigger a suicidal response in which the damaged cell is programmed for destruction. The way in which p53 programs cell death is not well understood. One mechanism seems to involve the protein product of the *BAX* gene. The BAX protein is an antagonist of another protein called BCL-2, which normally suppresses the apoptotic, or cell-death, pathway. When the *BAX* gene is activated by p53, its protein product releases the BCL-2 protein from its suppressing mode. This release then opens the apoptotic pathway, and the cell proceeds to its own destruction.

RB:

Retinoblastoma is a human childhood disease, involving a tumor of the retina. It occurs both as a heritable trait and sporadically (by somatic mutation). It is often associated with deletions of band ql4 of human chromosome 13. The *RB* gene has been localized to this region by molecular cloning.

Figure 30.28 summarizes the situation. Retinoblastoma arises when both copies of the *RB* gene are inactivated. In the inherited form of the disease, one parental chromosome carries an alteration in this region. A somatic event in retinal cells that causes loss of the other copy of the *RB* gene causes a tumor. In the sporadic form of the

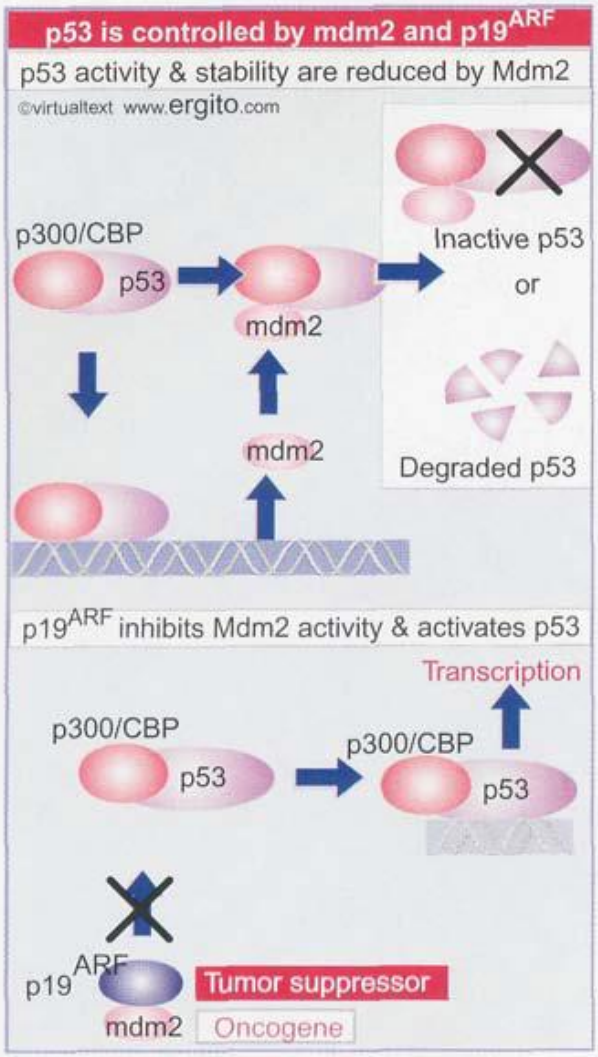


Figure 30.37 p53 activity is antagonized by Mdm2, which is

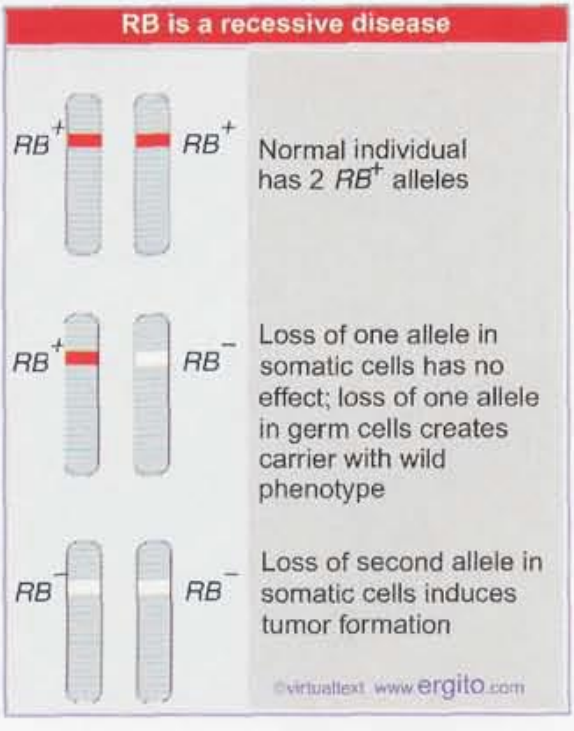


Figure 30.28 Retinoblastoma is

disease, the parental chromosomes are normal, and both *RB* alleles are lost by (individual) somatic events.

The cause of retinoblastoma is therefore loss of protein function, usually resulting from mutations that prevent gene expression (as opposed to point mutations that affect function of the protein product).

Loss of *RB* is involved also in other forms of cancer, including osteosarcomas and small cell lung cancers.

RB is a nuclear phosphoprotein that influences the cell cycle (see *29.17 G0/G1 and Gl/S transitions involve cdk inhibitors).* In resting (G0/G1) cells, RB is not phosphorylated. RB is phosphorylated during the cell cycle by cyclin/cdk complexes, most particularly at the end of Gl; it is dephosphorylated during mitosis. The nonphosphorylated form of RB specifically binds several proteins, and these interactions therefore occur only during part of the cell cycle (prior to S phase). Phosphorylation releases these proteins.

The target proteins include the E2F group of transcription factors, which activate target genes whose products are essential for S phase. Binding to RB inhibits the ability of E2F to activate transcription, which suggests that RB blocks the expression of genes dependent on E2F. In this way, RB indirectly prevents cells from entering S phase. Also, the RB-E2F complex directly represses some target genes, so itsdissociation allows them to be expressed.

Certain viral tumor antigens bind specifically to the nonphosphorylated form of RB. The best characterized are SV40 T antigen and adenovirus El A. This suggests the model shown in **Figure 30.29.** Nonphosphorylated RB prevents cell proliferation; this activity must be suppressed in order to pass through the cell cycle, which is accomplished by the cyclic phosphorylation. And it may also be suppressed when a tumor antigen sequesters the nonphosphorylated RB. Because the RB-tumor antigen complex does not bind E2F, the E2F is permanently free to allow entry into S phase (and the RB-E2F complex is not available to repress its target genes).

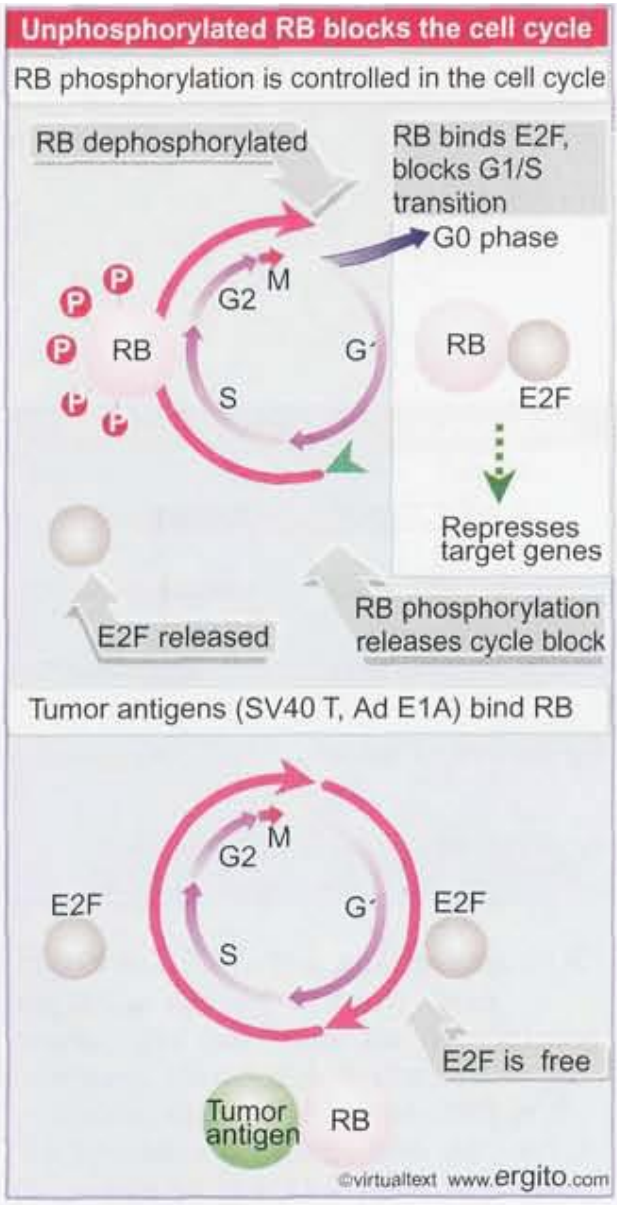


Figure 30.29 A block to the cell cycle is released when RB is phosphorylated or when it is sequestered by a tumor antigen.

Overexpression of RB impedes cell growth. An indication of the importance of RB for cell proliferation is given by the properties of an osteosarcoma cell line that lacks RB; when RB is introduced into this cell line, its growth is impeded. However, the inhibition can be overcome by expression of D cyclins, which form cdk-cyclin

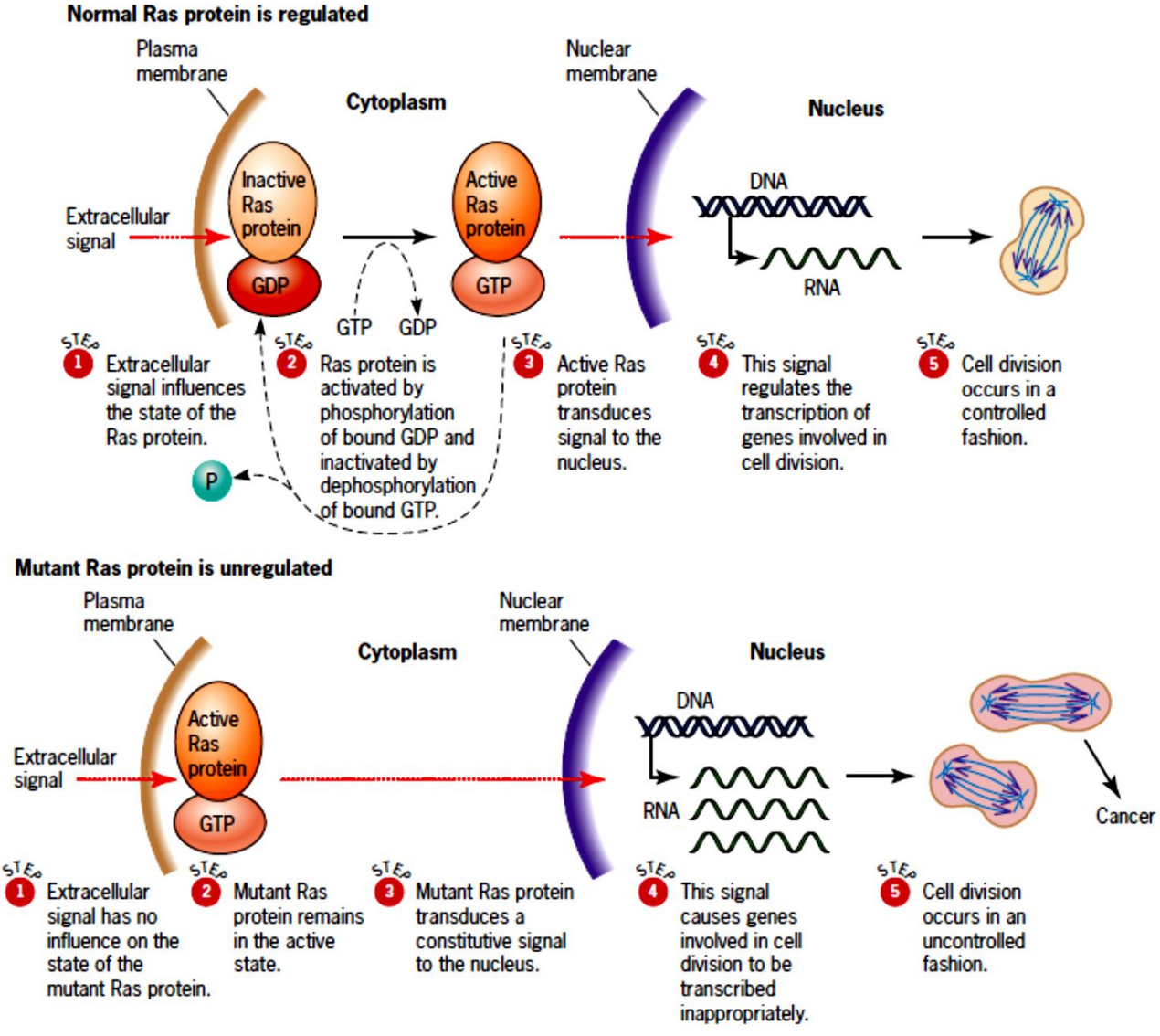
combinations that phosphorylate RB. RB is not the only protein of its type: proteins with related sequences, called pi07 and pi30, have similar properties.

Gene mutation:

The products of the *c-onc*s play key roles in regulating cellular activities. Consequently, a mutation in one of these genes can upset the biochemical balance within a cell and put it on the track to becoming cancerous. Studies of many different types of human cancer have demonstrated that mutant cellular oncogenes are associated with the development of a cancerous state.

After severaltests, Weinberg's research team identifi ed a DNA fragment from the original bladder cancer that reproducibly transformed cultured cells into cancer cells. This fragment carried an allele of the *c-H-ras* oncogene, a homologue of an oncogene in the Harvey strain of the rat sarcoma virus. DNA sequence analysis subsequently showed that a nucleotide in codon 12 of this allele had been mutated, with a substitution of a valine for the glycine normally found at this position in the c-H-ras protein.

Geneticists now have some understanding of how this mutation causes cells to become cancerous. Unlike viral oncogenes, the mutant c-H-ras gene does not synthesize abnormally large amounts of protein. Instead, the valine-for-glycine substitution at position 12 impairs the ability of the mutant c-H-ras protein to hydrolyze one of its substrates, guanosine triphosphate (GTP). Because of this impairment, the mutant protein is kept in an active signaling mode, transmitting information that ultimately stimulates the cells to divide in an uncontrolled way.



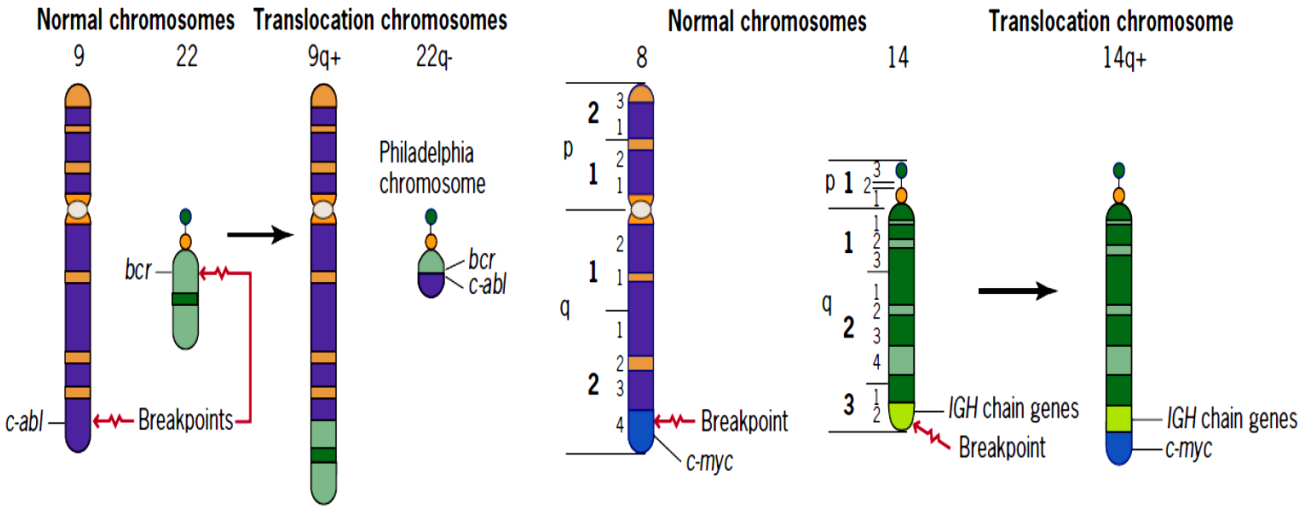
Mutant versions of the *c-ras* oncogenes have now been found in a large number of different human tumors, including lung, colon, mammary, prostate, and bladder tumors, as well as neuroblastomas (nerve cell cancers), fi brosarcomas (cancers of the connective tissues), and teratocarcinomas (cancers that contain different embryonic cell types). In all cases, the mutations involve amino acid changes in one of three positions—12, 59, or 61. Each of these amino acid changes impairs the ability of the mutant Ras protein to switch out of its active signaling mode. These types of mutations therefore stimulate cells to grow and divide.

In these types of cancer, only one of the two copies of the *c-ras* gene has been mutated. The single mutant allele is dominant in its ability to bring about the cancerous state. Mutations in *c-ras* and other cellular oncogenes that lead to cancer in this way are therefore *dominant activators* of uncontrolled cell growth.

Reciprocal translocation

chronic myelogenous leukemia (CML) is associated with an aberration of chromosome 22. Initially it was thought to have a simple deletion in its long arm; however, subsequent analysis using molecular techniques has shown that the Philadelphia chromosome is actually the result of a reciprocal translocation between chromosomes 9 and 22. In the Philadelphia translocation, the tip of the long arm of chromosome 9 has been joined to the body of chromosome 22, and the distal portion of the long arm of chromosome 22 has been joined to the body of chromosome 9. The translocation breakpoint on chromosome 9 is in the c-abl oncogene, which encodes a tyrosine kinase, and the breakpoint on chromosome 22 is in a gene called bcr. Through the translocation, the bcr and c-abl genes have been physically joined, creating a fusion gene whose polypeptide product has the amino terminus of the Bcr protein and the carboxy terminus of the c-Abl protein.

The mechanism may involve the tyrosine kinase activity of the c-Abl protein, which is tightly controlled in normal cells but is deregulated in cells that produce the fusion polypeptide. In effect, the tyrosine kinase function of the c-Abl protein has been constitutively activated by the *bcr/c-abl* gene fusion. This fusion is therefore a dominant activator of the c-Abl tyrosine kinase. Deregulation of the c-Abl tyrosine kinase leads to abnormal phosphorylation of other proteins, including some that are involved in controlling the cell cycle. In their phosphorylated state, these proteins cause cells to grow and divide uncontrollably.



Translocations implicated in human cancers. (*a*) The reciprocal translocation involved in the Philadelphia chromosome that is associated with chronic myelogenous leukemia. (*b*) A reciprocal translocation involved in Burkitt's lymphoma

Burkitt's lymphoma is another example of a white blood cell cancer associated with reciprocal translocations. These translocations invariably involve chromosome 8 and one of the three chromosomes (2, 14, and 22) that carry genes encoding the polypeptides that form immunoglobulins. Translocations involving chromosomes 8 and 14 are the most common. In these translocations, the *c-myc* oncogene on chromosome 8 is juxtaposed to the genes for the immunoglobulin heavy chains (*IGH*) on chromosome 14. This rearrangement results in the overexpression of the *c-myc* oncogene in cells that produce immunoglobulin heavy chains—that is, in the B cells of the immune system. The *c-myc* gene encodes a transcription factor that activates genes involved in promoting cell division. Consequently, the overexpression of *c-myc* that occurs in cells that carry the *IGH*/ *c-myc* fusion created by the t8;14 translocation causes those cells to become cancerous.

Insertion of retroviral genome:

One common mechanism is the insertion of a nondefective retrovirus in the vicinity of the gene. The ability of a retrovirus to transform without expressing a *v-onc* sequence was first noted during analysis of the bursal lymphomas caused by the transformation of B lymphocytes with avian leukemia virus. Similar events occur in the induction of T-cell lymphomas by murine leukemia virus. In each case, the transforming potential of the retrovirus is due to the ability of its LTR (the long terminal repeat of the integrated form) to cause expression of cellular gene(s).

In many independent tumors, the virus has integrated into the cellular genome within or close to the *c-myc* gene. **Figure 30.17** summarizes the types of insertions. The retrovirus may be inserted at a variety of locations relative to the *c-myc* gene.

The gene consists of three exons; the first represents a long nontranslated leader, and the second two code for the c-Myc protein. The simplest insertions to explain are those that occur within the first intron. The LTR provides a promoter, and transcription reads through the two coding exons. Transcription of *c-myc* under viral control differs from its usual control: the level of expression is increased (because the LTR provides an

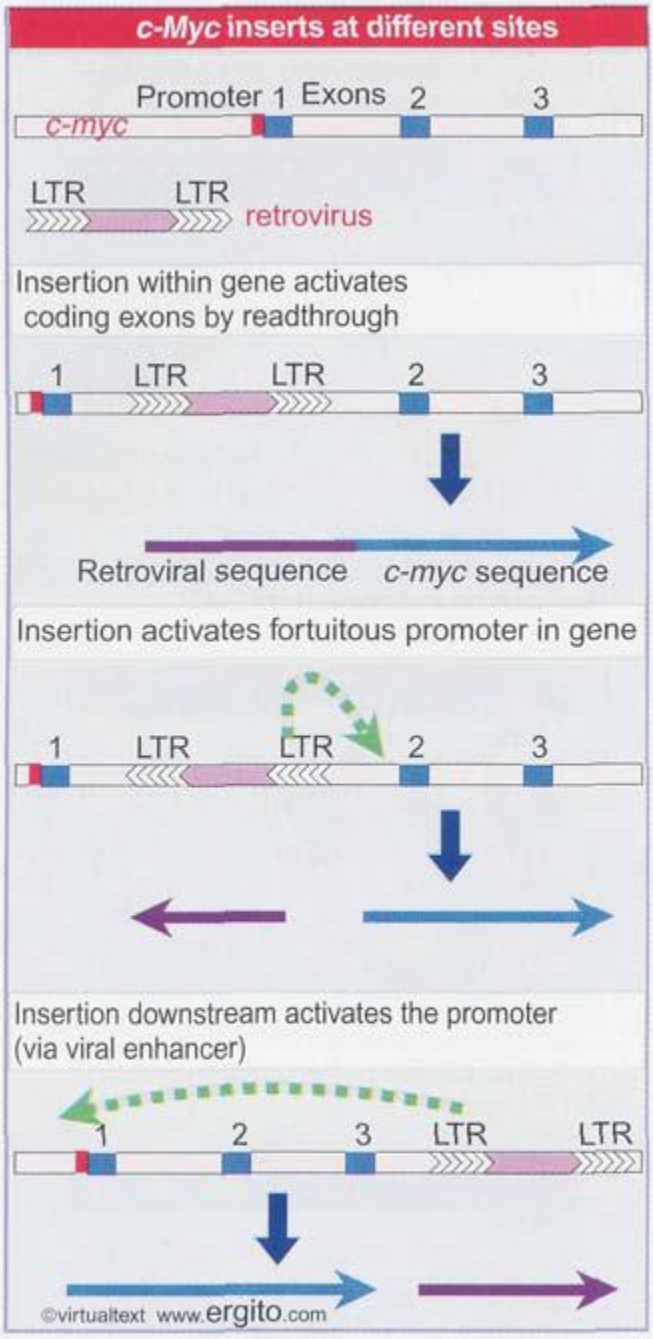


Figure 30.17 Insertions of ALV at the c-myc locus occur at various positions, and activate the gene in different ways.

efficient promoter); expression cannot be switched off in B or T cells in response to the usual differentiation signals; and the transcript lacks its usual nontranslated leader (which may usually limit expression). All of these changes add up to increased constitutive expression. Activation of *c-myc* in the other two classes of insertions reflects different mechanisms. The retroviral genome may be inserted within or upstream of the first intron, but in reverse orientation, so that its promoter points in the wrong direction. The retroviral genome also may be inserted downstream of the *c-myc* gene. In these cases, the enhancer in the viral LTR may be responsible for activating transcription of c-Myc, either from its normal promoter or from a fortuitous promoter. In all of these cases, the coding sequence o/c-myc is unchanged, so oncogenicity is attributed to the loss of normal control and increased expression of the gene.

Other oncogenes that are activated in tumors by the insertion of a retroviral genome include *c-erbB, c-myb, c-mos, c-H-ras,* and *c-raf* Up to 10 other cellular genes (not previously identified as oncogenes by their presence in transforming viruses) are implicated as potential oncogenes by this criterion. The best characterized among this latter class are *wntl* and *int2.* The *wntl* gene codes for a protein involved in early embryogenesis that is related to the *wingless* gene of *Drosophila; int2* codes for an FGF (fibroblast growth factor).

Gene amplification

Gene amplification refers to the expansion in copy number of a gene within the genome of a cell. Gene amplification was first discovered as a mechanism by which some tumor cell lines can acquire resistance to growth-inhibiting drugs. The process of gene amplification occurs through redundant replication of genomic DNA, often giving rise to karyotypic abnormalities called double-minute chromosomes (DMs) and homogeneous staining regions (HSRs). DMs are characteristic minichromosome structures without centromeres. HSRs are segments of chromosomes that lack the normal alternating pattern of light- and dark-staining bands. Both DMs and HSRs represent large regions of amplified genomic DNA containing up to several hundred copies of a gene. Amplification leads to the increased expression of genes, which in turn can confer a selective advantage for cell growth.The frequent observation of DMs and HSRs in human tumors suggested that the amplification of specific protooncogenes may be a common occurrence in neoplasia. Studies then demonstrated that three protooncogene families-myc, erb B, and ras-are amplified in a significant number of human tumors. About 20% to 30% of breast and ovarian cancers show c-myc amplification, and an approximately equal frequency of c-myc amplification is found in some types of squamous cell carcinomas. N-myc was discovered as a new member of the mycprotooncogene family through its amplification in neuroblastomas. Amplification of N-myc correlates strongly with advanced tumor stage in neuroblastoma, suggesting a role for this gene in tumor progression. L-myc was discovered through its amplification in small-cell carcinoma of the lung, a neuroendocrine-derived tumor.Amplification of erb B, the epidermal growth factor receptor,

is found in up to 50% of glioblastomas and in 10% to 20% of squamous carcinomas of the head and neck. Approximately 15% to 30% of breast and ovarian cancers have amplification of the erbB-2 (HER-2/neu) gene.

Environmental carcinogens

Environmental carcinogens are broadly defined as compounds that humans are exposed to through diet, lifestyle, infectious agents, and occupation. They are considered as nongenetic factors that contribute to cancer risk. A subset of *known* and *reasonably anticipated* human carcinogens can be classified as environmental carcinogens and include such compounds as dioxins, metals, components of pesticides, the polyaromatic hydrocarbon (PAH) benzo(*a*)pyrene (BaP), and mineral fibers such as erionite and asbestos2. These contaminants are major constituents of indoor and outdoor air pollution, water, soil, and food products.

Example:
Aflatoxins
Arsenic compound inorganic
Asbestos
Benzene Benzo(a)pyrenea
Cadmium and cadmium compounds
Chromium hexavalent compounds
Coal tars and coal tar pitches
Coke oven emissions Diethylstilbestrol
Environmental tobacco smoke
Erionite Ethylene oxide
Nickel compounds
Pesticide components: dichlorodiphenyltrichloroethane (DDT)a
Radon Silica, crystalline (respirable size)
Solar radiation

Treatment of cancer

Cancer can be treated by

- surgery,
- chemotherapy,
- radiation therapy,
- immunotherapy,
- monoclonal antibody therapy or
- other methods.

Surgery

Cancer can be cured if entirely removed by surgery, but this is not always possible. When the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible.

Examples of surgical procedures for cancer:
mastectomy for breast cancer and
prostatectomy for prostate cancer.

Chemotherapy

Chemotherapy is the treatment of cancer with drugs ("anticancer drugs") that can destroy cancer cells. It interferes with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes. Most forms of chemotherapy target all rapidly dividing cells and are not specific for cancer cells. Hence, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g. intestinal lining). These cells usually repair themselves after chemotherapy.

The majority of chemotherapeutic drugs can be divided in to:

1. alkylating agents,
2. antimetabolites,
3. anthracyclines,
4. plant alkaloids,
5. topoisomerase inhibitors,
6. monoclonal antibodies, and
7. other antitumour agents.

All of these drugs affect cell division or DNA synthesis and function in some way.

Side-effects

Important common side-effects include (dependent on the agent):

- Hair loss
- Nausea and vomiting
- Diarrhea or constipation
- Anemia
- Malnutrition
- Depression of the immune system, hence (potentially lethal) infections and sepsis
- Hemorrhage
- Secondary neoplasms
- Cardiotoxicity
- Hepatotoxicity
- Nephrotoxicity
- Ototoxicity
- Death

Common drug:
Cyclophosphamide (Cytoxan),
Methotrexate,
5-Fluorouracil (5-FU) and
Doxorubicin (Adriamycin).

Adriamycin

Generic Name: Doxorubicin

Mode of Action: Adriamycin prevents DNA replication. The exact mechanism is still being studied, but it may be a "topoisomerase inhibitor". Topoisomerases are enzymes that temporarily cut one strand of DNA during replication to help unwind the double helix.. Adriamycin prevents the topoisomerase from reattaching the cut ends. A new version of doxorubicin 'wrapped up' in a lipid coat (liposome) called Doxil has been recently approved as a Kaposi's sarcoma treatment.

Common Side Effects: hair loss, mouth sores, nausea, vomiting, lowered blood counts (WBCs, RBCs and platelets), damage to the heart muscle, skin damage if drug leaks out of vein during infusion.

Cytoxan

Generic Name: Cyclophosphamide

Mode of Action: Interferes with DNA synthesis and replication. Cytoxan is a drug that cross-links with the nucleotides of DNA. When cross-linked with Cytoxan, the DNA double helix is unable to unwind, DNA replication does not occur, and the cells die.

Common side effects: Cytoxan is one of the nastier chemo drugs, with lots of side effects. Side effects include lowered blood counts (WBCs, Platelets, RBCs), nausea, vomiting, loss of appetite, hair loss, loss of menstrual periods, decreased sex drive, bladder irritation, metallic taste in mouth during injection.

5FU or Adrucil

Generic Name: 5-Fluorouracil

Mode of Action: Stops DNA synthesis and replication by inhibiting the enzyme thymidylate synthetase, involved in the incorporation of the nucleotide T into the DNA during replication.

Common Side Effects: mild nausea, vomiting, loss of appetite, thinning or loss of hair, skin rash and itching, skin darkening, weakness.

Mexate, Emtexate, Metatrexan, Methopterin, orFolex

Generic Name: Methotrexate

Mode of Action: Blocks actively dividing cells from making nucleotides. Methotrexate competes in the cell for an enzyme called folic acid reductase, and prevents the synthesis of tetrahydrofolate, a chemical used by the cell for making nucleotides. Without nucleotides, DNA synthesis cannot proceed.

Common side effects: mouth sores, nausea, vomiting, loss of appetite, upset stomach, skin and eye sensitivity to sunlight, abnormal liver function tests, hair loss, anemia.

Taxol

Generic Name: Paclitaxel

Mode of Action: Taxol (Paclitaxel) was first isolated from the from the bark of the Pacific Yew tree (Taxus brevifolia). Taxol disrupts the balance between tubulin and microtubule fibers, causing the formation of abnormal microtubule bundles. This prevents dividing cells from moving chromosomes to their "daughter" cells by interfering with spindle fiber formation.

Common side effects: Allergic reaction such as low blood pressure, shortness of breath, rash; loss of hair; low blood cell counts; nerve pain.

Oncovin, Vincasar, Vincrex, or Leurocristine

Generic Name: Vincristine

Mode of Action: Prevents the formation of spindle fibers during metaphase of mitosis. Vincristine is an alkaloid isolated from the Madagascar periwinkle, Catharantus roseus, formerly classified as Vinca rosea, which led to it being called a Vinca alkaloid, and thus vincristine. It binds to tubulin, the protein that makes up the spindle fiber microtubules, and prevents metaphase of mitosis.

Common side effects: bloating, nausea and vomiting, skin rash, temporary loss of hair, neurological problems

Leukerin, Mercaleukin, or Puri-Nethol

Generic Name: 6-Mercaptopurine, or 6-MP

Mode of Action: Inhibits the synthesis of the purine nucleotides A and G necessary for DNA synthesis; may also mimic A and G during DNA replication and stop further DNA synthesis.

Common side effects: low blood counts, mouth sores, skin rash/acne, mild nausea, abnormal liver function.

Ara-C, Arabitin, Aracytine, Cytarbel, or Cytosar

Generic Name: Cytosine arabinoside

Mode of Action: Ara-C is a DNA synthesis inhibitor. Its structure is very similar to the nucleotide C, but instead of having a ribose (for RNA) or a deoxyribose (for DNA) sugar, its consists of an arabinose sugar - a 5 carbon sugar that competes for the enzymes in DNA synthesis, but cannot function in DNA or RNA.

Common Side effects: decreased blood counts with risk of infection, bleeding and anemia which may require blood transfusion, nausea and diarrhea, abdominal pains, mouth sores, liver injury (which gets better after the drug is stopped).

Monoclonal antibody therapy:

Immunotherapy is the use of immune mechanisms against tumors. These are used in various forms of cancer, such as breast cancer (trastuzumab/Herceptin®) andleukemia (gemtuzumabozogamicin/Mylotarg®).

The agents are monoclonal antibodies directed against proteins that are characteristic to the cells of the cancer in question, or cytokines that modulate the immune system's response.

Radiation therapy

Radiation therapy (also called radiotherapy, X-ray therapy, or irradiation) is the use of ionizing radiation to kill cancer cells and shrink tumors.

Radiation therapy can be administered externally via external beam radiotherapy (EBRT) or internally via brachytherapy.

Radiation therapy injures or destroys cells in the area being treated (the "target tissue") by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. Hence, it is given in many fractions, allowing healthy tissue to recover between fractionsin many fractions, allowing healthy tissue to recover between fractions.

Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, stomach, uterus, or soft tissue sarcomas.

Radiation is also used to treat leukemia and lymphoma.

Radiation dose to each site depends on a number of factors, including the radio sensitivity of each cancer type and whether there are tissues and organs nearby that may be damaged by radiation.

Immunotherapy

Cancer immunotherapy refers to a diverse set of therapeutic strategies designed to induce the patient's own immune system to fight the tumor. Contemporary methods for generating an immune response against tumours include intravesical BCG immunotherapy for superficial bladder cancer, and use of interferons and other cytokines to induce an immune response in renal cell carcinoma and melanoma patients. Cancer vaccines to generate specific immune responses are the subject of intensive research for a number of tumours, notably malignant melanoma and renal cell carcinoma. Sipuleucel-T is a vaccine-like strategy in late clinical trials for prostate cancer in which dendritic cells from the patient are loaded with prostatic acid phosphatase peptides to induce a specific immune response against prostate-derived cells.

Hormonal therapy

The growth of some cancers can be inhibited by providing or blocking certain hormones. Common examples of hormone-sensitive tumors include certain types of breast and prostate cancers. Removing or blocking estrogen or testosterone is often an important additional treatment. In certain cancers, administration of hormone agonists, such as progestogens may be therapeutically beneficial.

12. T-DNA technology: T-DNA transfer, disarming of T-DNA, cointegrates; direct and indirect methods of gene transfer. Binary vector. Shuttle vector.

T-DNA technology

T-DNA transfer

The transfer DNA (abbreviated T-DNA) is the transferred DNA of the tumor-inducing (Ti) plasmid of some species of bacteria such as *Agrobacterium tumefaciens* and [*Agrobacterium rhizogenes*] (actually an Ri plasmid)]. The T-DNA is transferred from bacterium into the host plant's nuclear DNA genome. The capability of this specialized tumor-inducing (Ti) plasmid is attributed to two essential regions required for DNA transfer to the host cell. As the T-DNA is bordered by 25-base-pair repeats on each end. Transfer is initiated at the right border and terminated at the left border and requires the vir genes of the Ti plasmid.

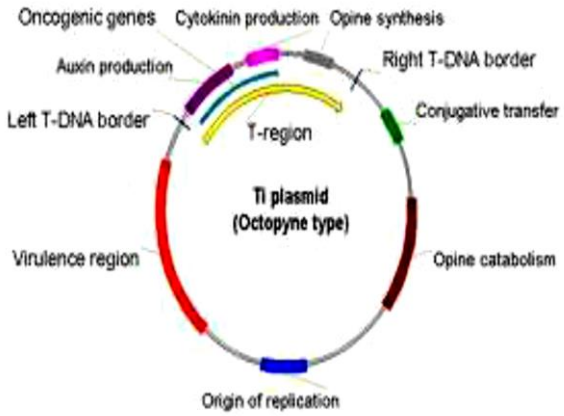
Ti Plasmid:

The Ti plasmid is a large conjugative plasmid or megaplasmid of about 200 kb (range 150-250 kb). *p*Ti is lost when *Agrobacterium* is grown above 28°C; such cured bacteria do not induce crowngalls, i.e. they become avirulent. *p*Ti and *p*Ri share little sequence homology, but arc functionally rather similar. When *p*Ri is introduced into a *p*Ti-curcd *A. tumefaciens*, the bacteria acquire the hairy root induction property of *A. rhizogenes* and *vice-versa*. Clearly, the crowngall/hairy root induction property resides in the *p*Ti/*p*Ri and not in the *Agrobacterium* chromosome. Many strains of *Agrobacterium* contain very large cryptic plasmids, which do not induce crown gall or hairy root diseases.

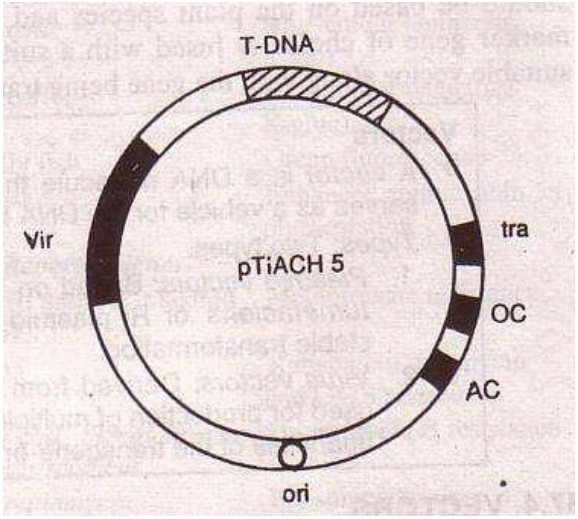
The *p*Ti and *p*Ri are unique bacterial plasmids in the following two respects. (1) They contain some genes (dhe genes locatcd within their T-DNA), which have regulatory sequences recognised by plant cells, while their remaining genes have prokaryotic regulatory sequences. As a result, the former are cxpressed only in plant cells (and not in the *Agrobacterium*), while the latter are expressed only in the bacterium. (2) These plasmids naturally transfer their T-DNA into the host plant genome, which makes *Agrobacterium* a **natural genetic engineer**.

The Ti plasmids are classificd into dilferent types based on the type of opine produced by their genes. The different opines specified by *p*Ti are octopine, nopaline, succinamopine and leucinopine. The different Ti plasmids can be grouped into two general categories: octopine type and nopaline type they differ mainly in the organisation of their T-DNAs. The succinamopine and leucinopine specifying *p*Ti are similar to the nopalıne type *p*Ti, while ctopine type of pTi forms a separate group. Both octopine and nopaline type pTi contain the following important functional regions

1. T-DNA contains oncogenes and opine synthesis genes, and is transferred into the host plant genome
2. *vir* region regulates the transter of T-DNA into plant cells.
3. Opine catabolism regions produce enzymes necessary for the utilization of opines by *Agrobacterium*.
4. Conjugative transfer (*ori*T or *tra*) region functions in conjugative transfer of the plasmid; it can also function in T-DNA transfer when the T-DNA borders or deleted.
5. Origin of replication for propagation in *Agrobacterium*.



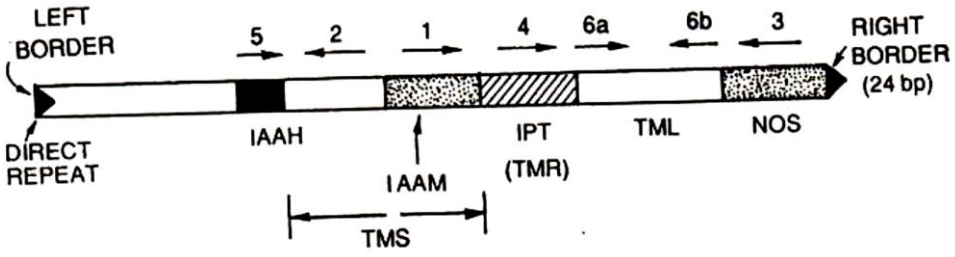
Components of Ti Plasmid



Structure of Ti Plasmid

Organisation of T-DNA:

T-DNA (transferred DNA) is that Ca. 23 kb segment of Ti/Ri plasmids, which is transferred into the plant genome during *Agrobacterium* infection. T-DNA is defined on both its sides by a 24 bp direct repeat border sequence, and contains the genes for tumour/hairy root induction and those for opine biosynthesis. *p*Ti has three genes, which are involvedin crowngall formation. Two of these genes (*iaaM* and *iaaH*) cncode enzymes that together convert trypophan into IAA (indole-3-acetic acid). A deletion of these two genes produces *shooty crowngalls*; therefore, the locus was earlier called *shooty locus*, and the genes were designated as *tms*l (tumour with shoots) and *tms*2. The third gene, *ipt* encodes an enzyme, which produces the zeatin-typeeytokinin iseopentenyl adenine. A deletion or abolition of *ipt* results in rooty crowngall; as a result, this locus was carlier designated as *rooty locus* ànd denoted by *tmr* (tumour having roots). Another locus concerned with tumour production is *tml*; a deletion of this locus results in large tumours. In addition, TDNA also contains genes involved in opine biosynthesis; these genes are located near the right border of T-DNA



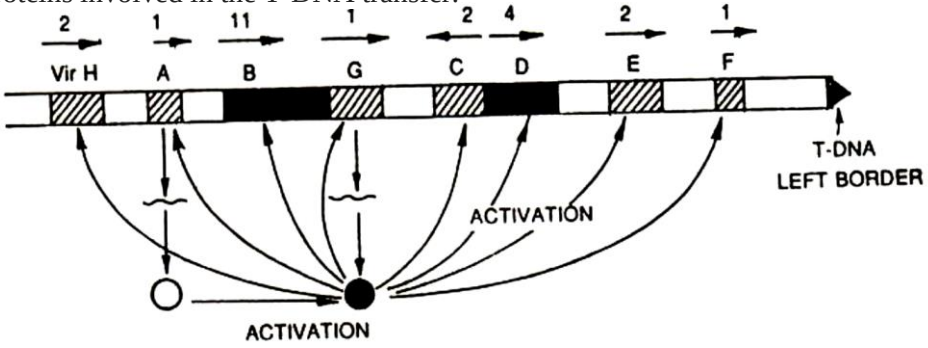
ORGANISATION OF T-DNA OF Ti PLASMID

The T-DNA is organised in two distinct regions called **TL (left T-DNA)** and **TR (right T-DNA).** In case or nopaline type of plasmids, both TL and TR are always transferred together and integrated into the plant genome as a single segment. But in case of octopine type of plasmids, the TL and TR are transferred independently so that a single cell may contain one or both of these segments. The TL region of octopine *p*Ti carries the genes for auxin and cytokinin biosynthesis and the gene *ocs* encoding octopine synthase; this enzyme synthesizes octopine from arginine and pyruvic acid. The TR, on the other hand, contains genes for agropine and mannopine biosyntheses. Transtormed plant cells usually contain only one copy/cell of TL (upto 10 copies/cell have been found); but TR may be present in high copy numbers. Therefore, all tumours produced by octopine type pTi contain TL, but they may or may not contain TR.

The *p*Ri T-DNA also consists of TL and TR regions. The TL region has the *rol* locus (root locus), which contains *rol*A, *rol*B and *rol*C genes; these genes increase the auxin sensitivity of transformed

plant cells. The TR segments have genes for auxin and opine biosyntheses. The TR and TL segments are transferred independent of each other as is the case for octopine type *p*Ti. AS a rule, the hairy roots produced due to *A. rhizogenes* intection always contain TL, but they may or may not contain TR. All the genes present in T-DNA contain eukaryotic regulatory sequençes. As a result, these genes are expressed only in plant cells, and they are not expressed in the *Agrobacterium*.

Organisation of vir Region: The vir region of a nopaline type Ti plasmid contains 8 operons (designated as virA, virB, virC, virD, virE, virF, virG and virH), which together span about 40 Kb of DNA and have 25 genes. This region mediates the transfer of T-DNA into plant genomes, and hence is essential for **virulence**, that is, production of crowngall/hairy root disease; therefore, it is called the **virulence region or vir region**. The genes of vir region are **not transferred** themselves; they only induce the transfer of T-DNA. On the other hand, the genes present in T-DNA are not required for its transfer: only the 24 bp direct repeat left and right borders of T-DNA are essentual for the tansfer. Of the 8 vir operons, 4 operons, viz., virA, virB, virD and virG, are essential Tor virulence, while the remaining 4 operons play an accessory role. The operons virA and virG are constitutive, encode one protein each, and are concerned with the regulation of all the vir operons. The other vir operons encode various proteins involved in the T-DNA transfer.

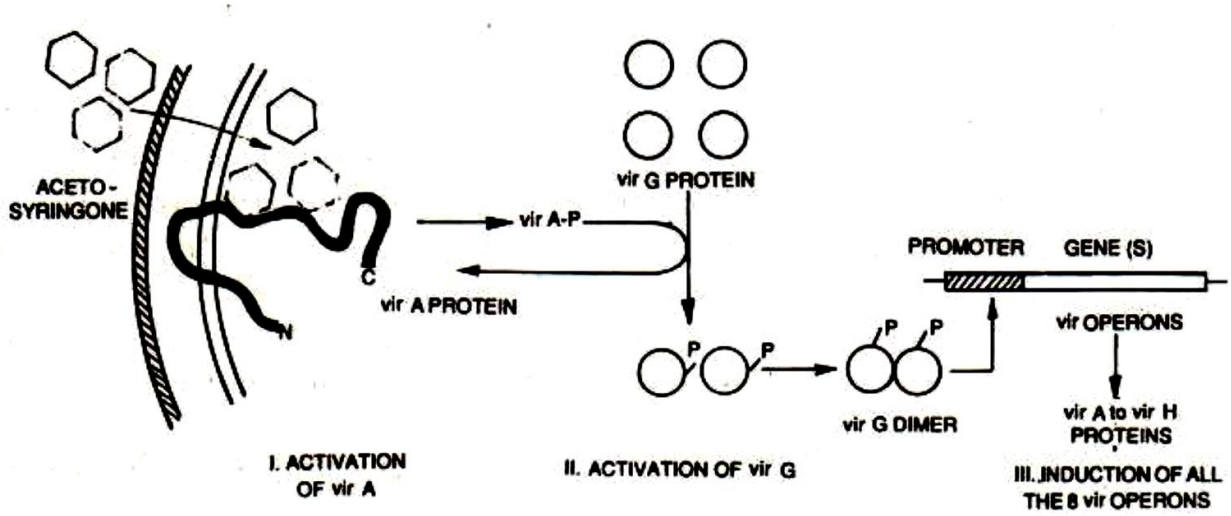


ORGANISATION OF Vir REGION OF Ti PLASMID
8 operons having 24 genes

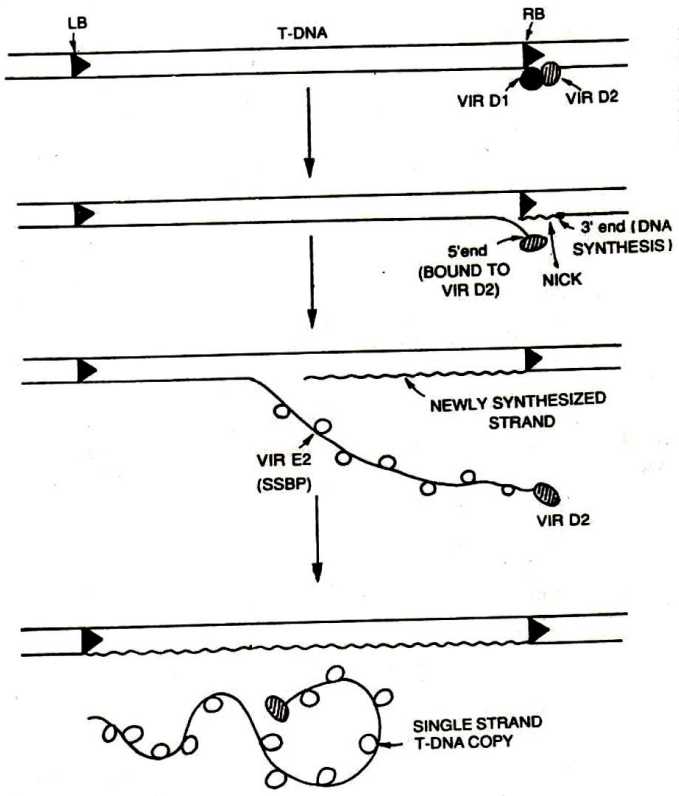
Gene/Operon	Function
	T-DNA
iaaM (*aux1, tms1*)*	Auxin biosynthesis; encodes the enzyme tryptophan-2-mono-oxygenase, which converts tryptophan into indole-3-acetamide (IAM).
iaaH (*aux2, tms2*)	Auxin biosynthesis; encodes the enzyme indole-3-acetamide hydrolase, which converts IAM into IAA (indole-3-acetic acid)
ipt (*tmr, Cyt*)	Cytokinin biosynthesis; encodes the enzyme isopentenyl transferase, which catalyzes the formation of isopentenyl adenine
nos	Nopaline biosynthesis; encodes the enzyme nopaline synthase, which produces nopaline from arginine and pyruvic acid
24 bp left and right	*Sites of endonuclease action during T-DNA transfer; the only sequences of T-DNA essential*
border sequences	*for its transfer*
	vir *Region* (vir *Regulon*)**
virA (1)*	Encodes a sensor protein; receptor for acetosyringone and functions as an autokinase; also phosphorylates VirG protein; *constitutive expression*
virB (11)	Membrane proteins; combine with VirD4 to form a channel for T-DNA transport (conjugal tube formation); VirB11 has ATPase activity
virC (2)	Helicase; binds to the overdrive region just outside the right border; involved in unwinding of T-DNA
virD (4)	VirD1 has topoisomerase activity; it binds to the right border of T-DNA; VirD2 is an endonuclease; it nicks the right border
virE (2)	VirE2 is a single-strand binding protein (SSBP); it binds to T-DNA during its transfer; VirE1 stabilizes VirE2
virF (1)	Presumed to mark some host plant proteins for proteolysis
virG (1)	DNA binding protein; probably forms dimer after phosphorylation by VirA, and induces the expression of all *vir* operons (operons *A* to *H*); *constitutive expression*
virH (2)	Detoxification of the phenolics produced by plant cells at the wound site

Mechanism of transfer of T-DNA

- Transfer of T-DNA is a step wise process.
- Vir region of Ti plasmid becomes activated by the phenolic signal molecules.
- Acetosyringone and α-hydroxyacetosyringone released by wounded tissue of dicot plants which constitute wound response as follows



- Acetosyringone and α-hydroxyacetosyringone bind with Vir A protein (located in the inner membrane) and activates it. It start functioning as autokinase to phosphorylate itself by ATP. Phosphorylated Vir A protein then phosphorylates Vir G protein which then dimerises.
- Phosphorylated Vir G protein has DNA binding function. It induces expression of rest of Vir operons
- Vir D1 protein has topoisomerase and endonuclease activity.
- It binds to right border sequence of T-DNA and facilitate the action of Vir D2 protein which is also endonuclease and nicks at the right border and remains bound to 5'end so generated.



Production of T-DNA copy (single stranded for transfer into plant cells)

LB- Left Border
RB- Right Border
SSBP-Single stranded binding proteins

- The 3'end produced at the site of nick serves as a primer for DNA synthesis in 5'----3' direction as a result of which one strand of
- T-DNA is displaced from the DNA duplex.
- The T-DNA strand is again nicked at the left border to generate a single strand copy of T-DNA.
- To this single strand copy Vir E 2 protein (single strand DNA binding proteins) bind for its protection against exonucleases
- Vir B operon consisting of 11 genes encode membrane bound Vir B proteins. These along with Vir D4 proteins participate in conjugal tube formation between bacterial and plant cells for transfer of T-DNA
- Vir D2 which remains bound to 5'end of T DNA has a signal sequence which drives it into the nucleus of plant cell.

Integration of T-dna into plant genome:

- T-DNA enters plant cell as a single stranded structure which is immediately converted into double stranded form.
- Vir E2 also has nuclear localization sequence and is responsible for transfer of T DNA into plant cell nucleus
- Double stranded T-DNA integrate at random sites in the host plant genome.
- For integration 23-79 base pair deletion takes place at the integration or target site
- After integration of T-DNA into plant genome ,the genes for auxins, cytokinins and opines express themselves which result in uncontrolled growth in the form of tumor

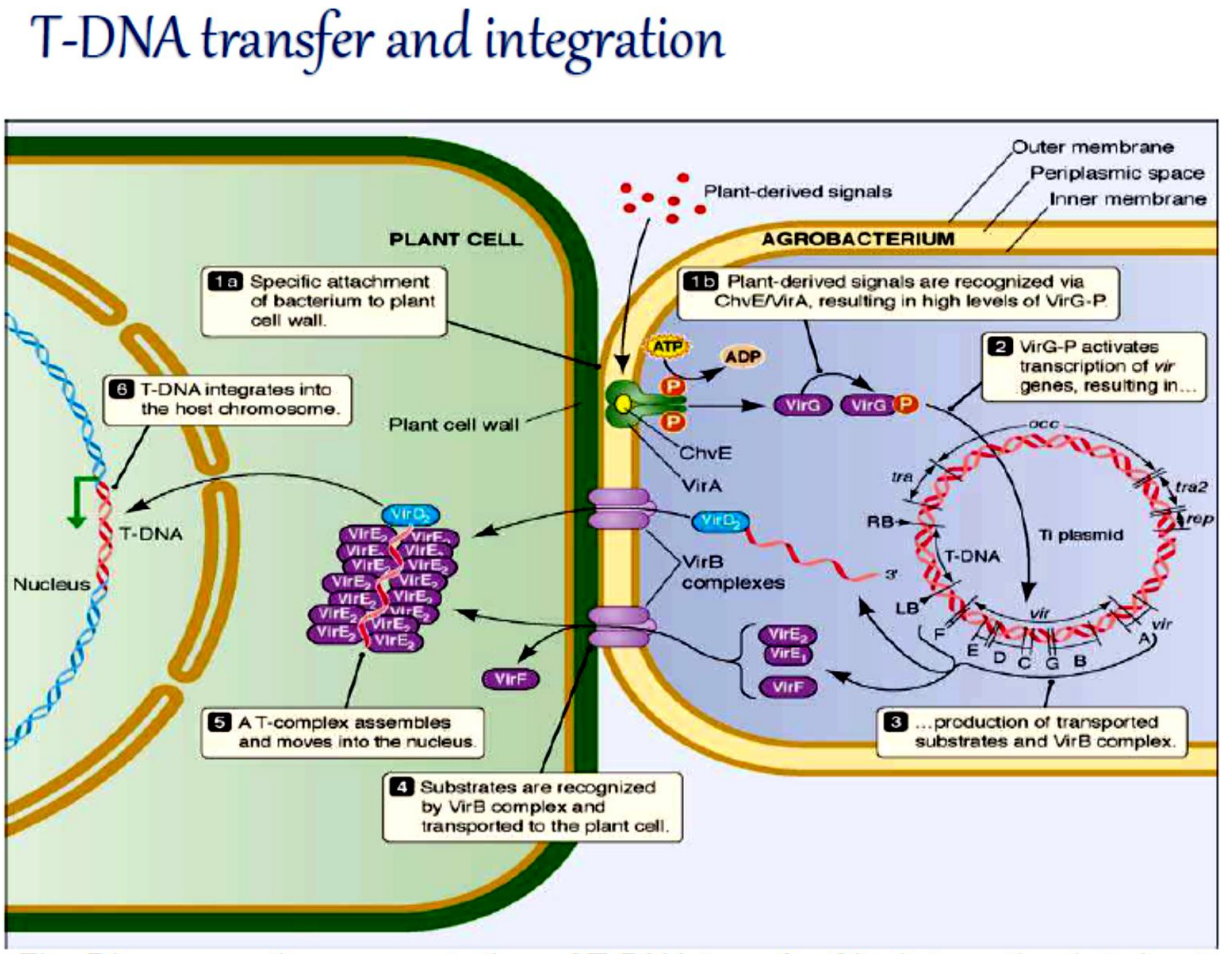


Fig: Diagrammatic representation of T-DNA transfer &its integration into host plant cell genome

Harnessing *agrobacterium* in transferring foreign gene into plant

Part I of this presentation has made it clear that *Agrobacterium* has the potentiality to transfer prokaryotic DNA to eukaryotic Genome as a Natural Genetic Engineer

In part two of this presentation discussion will be made on strategies involved for harnessing *Agrobacterium* for introducing new (desired) gene into plant cells

Agrobacterium's ability to introduce its DNA into plant genome with efficiency makes Ti plasmid an attractive vector for gene transfer into plants After knowing about how T-DNA is transferred and what modifications are needed , Genetic engineers employed different strategies and before actual transfer of desired gene Genetically engineered Agrobacterium were produced with disarmed T-DNA region in which oncogenes and opine biosynthesis genes were replaced by desired (Foreign) gene and a selectable marker gene. Along with this some other sequences as unique restriction sites were added. The natural Ti plasmids are unsuitable to be used directly as vectors for invitro manipulation due to following reasons

• Large size
• Tumor induction (Oncogenic) property
• Absence of unique restriction enzyme site

Now it is also well known that disarmed TDNA, left and right borders along with genes of Vir region are essential elements for designing of transformation vectors

Now let us see the strategies involved for harnessing *Agrobacterium* for introduction of new/desired/foreign genes into plants

Only useful attributes of Mega Ti plasmid (~200kb) have been exploited in designing plant transformation vector.

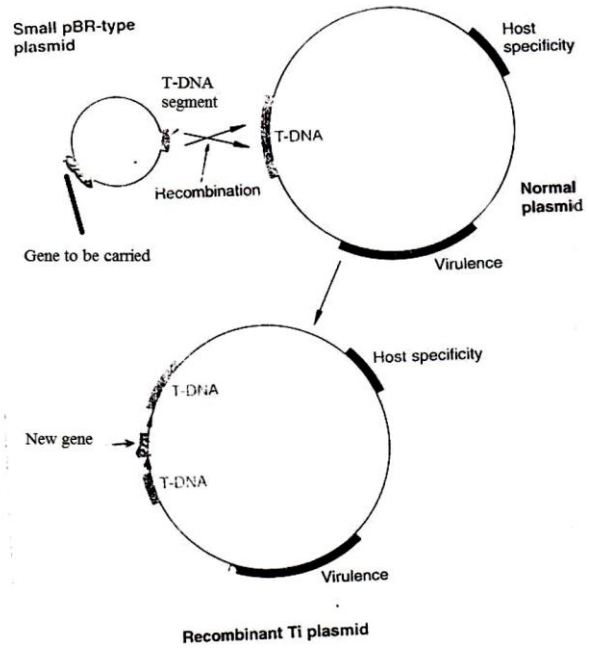
For using *Agrobacterium* for in vitro genetic engineering purposes following manipulation are to be made

Disarming:

- ✓ Ti plasmids are **disarmed** .Genes from T DNA responsible for growth hormones (Oncogenes) and Opine formation are deleted so that no disease will be produced and as opines will not be synthesized further growth of *Agrobacterium* inside the host tissue stops
- ✓ The space between lB and RB is used to insert desired gene which is to be integrated and expressed in host tissue for transformation. The foreign gene is inserted by RDT
- ✓ A selectable marker gene providing resistance against antibiotic like kanamycin is also inserted in the T-DNA region
- ✓ The oncogenic problem can be solved by use of disarmed T-DNA from which these gene are removed

The large size problem of Ti plasmid can be tackled by any of following two ways using manipulated *Agrobacterium* vectors

1. The Co- integrated vector For this purpose a suitably modified *E coli* plasmid or vector is integrated into disarmed Ti plasmid (*p*Ti), this gives rise to **co-integrate vector**. During disarming oncogenes of T-DNA are replaced by gene insert and other sequences of *E coli* plasmid. For this both plasmids are introduced into same *Agrobacterium* cell .Because of homology through recombination pBR plasmid is integrated into the T-DNA region. The gene to be cloned is therefore inserted into unique restriction site on the small pBR plasmid, introduced into *A tumifaciens* cell carrying a Ti plasmid and natural recombination process left to integrate the new gene into T-DNA.

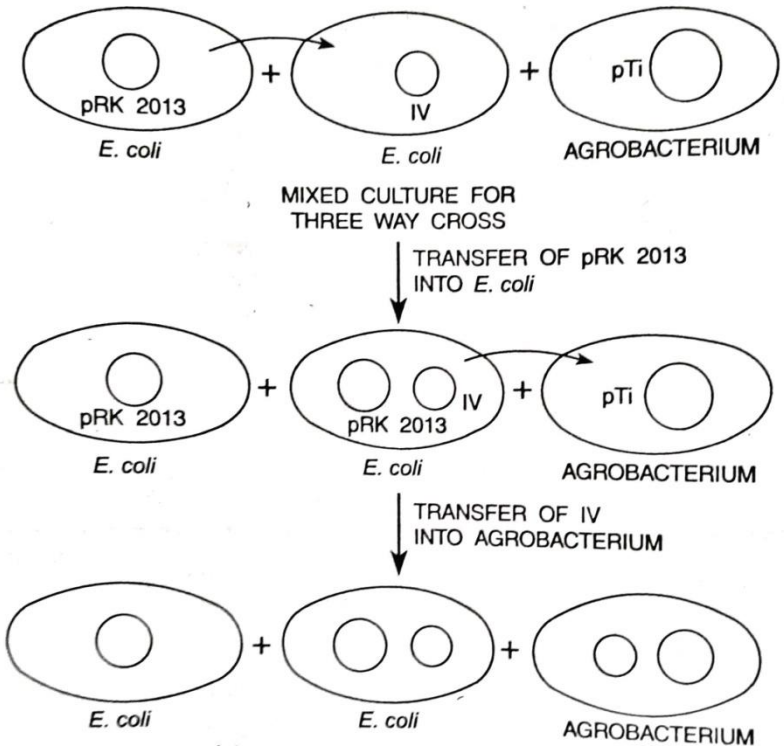


The cointegration strategy-Uses a entirely new plasmid based on pBR322 or a similar E coli vector but containing a small portion of T-DNA. The homology between the new molecule and the Ti plasmid means thai if both are present in the same Agrobacterium tumifaciens cell,then recombination can integrate the pBR plasmid into the T-DNA region. The gene to be cloned is therefore inserted into a unique restriction site on the small pBR plasmid introduced into A.tumifaciens cell carrying a Ti plasmid, and the natural recombination process left to integrate the new gene into the T-DNA. Infection of the plant leads to insertion of the new gene, along with the rest of the T-DNA into plant chromosome.

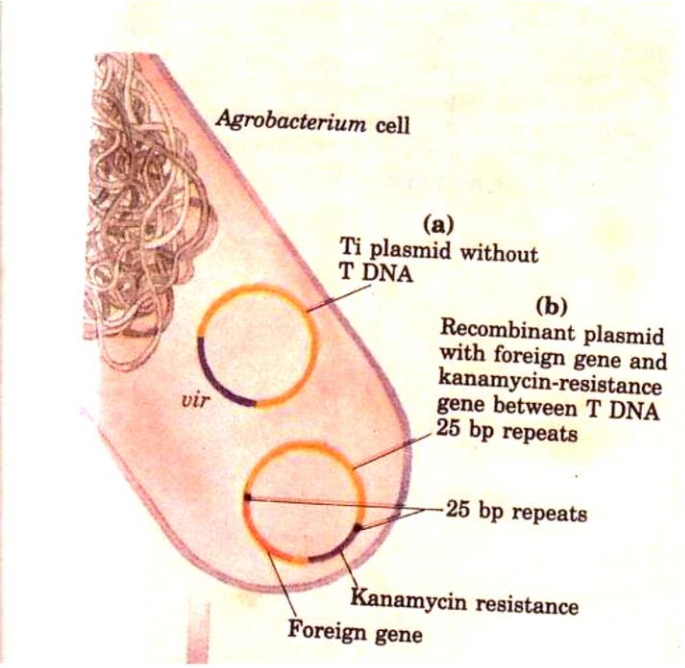
ii) Binary (Two) Vector strategy

The vir region of Ti plasmid need not be present in the same plasmid for an effcient transfer of T-DNA. The T-DNA present in one plasmid (which does not have the vir region) is readily transferred into plant cells in response to vir genes present in another plasmid contained in the same bacterial cell. This property has been exploited to construct binary vectors of pTi.

A **binary vector** consists of a pair of plasmids of which one plasmid contains disarmed T-DNA Sequences (at least the left and right borders of T-DNA must be present), while the other contains the Vir region, and ordinarily lacks the entire T-DNA including the border. The plasmid containing disarmed T-DNA is called **mini-Ti or micro-Ti** e.g., Bin19, and has the origins for replication in both *E. coli* and *Agrobacterium.* The DNA insert is integrated within the T-region of mini-Ti, and the recombinant mini-Ti is cloned in *E. coli*. Transfer of recombinant mini-Ti from *E. coli* into *Agrobacterium* is achieved either by a three-way cross (on the pattern depicted in Fig.) or by direct transformation of an *Agrobacterium* strain containing the helper Ti plasmid. Alternatively, the mini-Ti itself may be capable of conjugational transfer.



The **helper plasmid** is a Ti plasmid having a functional vir region but lacking the T-DNA region including the border sequences. pAL4404 helper Ti plasmid is derived from the wild type pTiAch5 by deletion of the entire T-region. The recombinant mini-Ti Bin19 is introduced into *Agrobacteriun* cells containing pAL4404 cither by conjugation or direct transformation. The vir genes present in the helper pAL4404 induce the transfer of T-DNA (containing the DNA insert) of the mini-Ti Bin19 into the plant cells. The transformed plant cells can be selected on kanamycin medium due to the gene neo present within the T-DNA. The binary system avoids the transfer into plant cells/genomes of unnecessary sequences, which Occurs in the case of cointegrate vectors. Although the maximum size of DNA insert acceptable by T-DNA is not definitely known, but it appears to be greater than 50 kb. But standard vectors generally do not afford transfer of larger than 30 kb inserts due to instability in the bacterial host. This problem is overcome by certain high capacity binary vectors that have been constructed on the pattern of artificial chromosome type vectors, viz., BAC (BAC2) and PAC (TAC).



Agrobacterium cell with Binary or two plasmids (a &b)

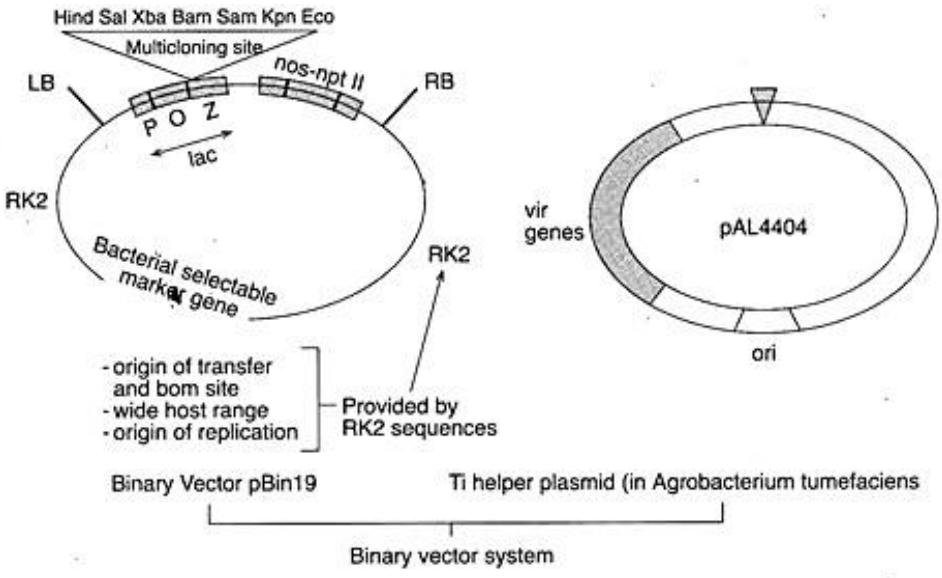


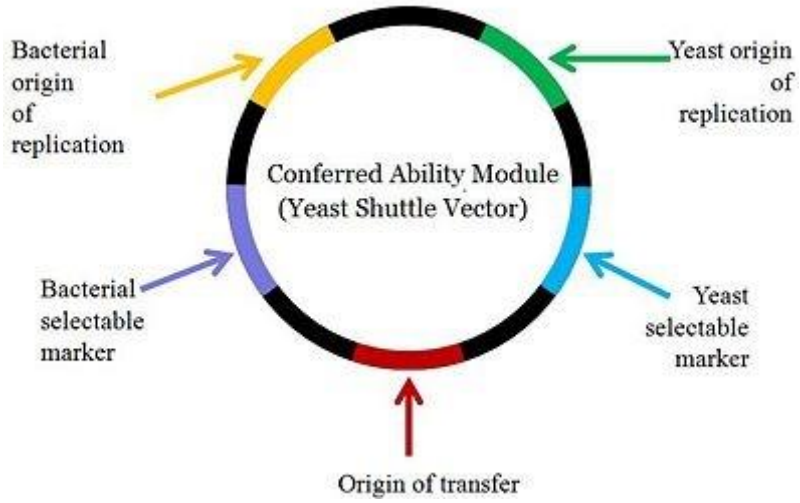
Fig. 18.22: A typical binary Ti vector system (from Chawla)

Shuttle Vector

A shuttle vector is a vector that can propagate in two different host species, hence, inserted DNA can be tested or manipulated in two different cell types. The main advantage of these vectors is that they can be manipulated in E. coli and then used in a system which is more difficult or slower to use.

Shuttle vectors can be used in both eukaryotes and prokaryotes. Shuttle vectors are frequently used to quickly make multiple copies of the gene in *E. coli* (amplification). They can also be used for in vitro

experiments and modifications such as mutagenesis and PCR. One of the most common types of shuttle vectors is the yeast shuttle vector that contains components allowing for the replication and selection in both E. coli cells and yeast cells. The E. coli component of a yeast shuttle vector includes an origin of replication and a selectable marker, such as an antibiotic resistance like beta lactamase. The yeast component of a yeast shuttle vector includes an autonomously replicating sequence (ARS), a yeast centromere (CEN), and a yeast selectable marker.



Genetic transformation

In molecular biology, **genetic transformation** is a process by which the genetic material carried by an individual cell is altered by the incorporation of foreign (exogenous) DNA into its genome. Genetic transformation discovered by **British bacteriologist Frederick Griffith in 1928** has revolutionized molecular biology, but it was not until the recombinant DNA was produced from *Escherichia coli* with the use of biochemical scissor called restriction enzymes that genetic transformation of cells started.

In plant cells, gene transfer to plant cells achieved using two different methods:

I. Indirect methods(Vector-mediated methods)
II. Direct methods (Vector less methods)

I. Indirect methods (Vector-mediated methods)

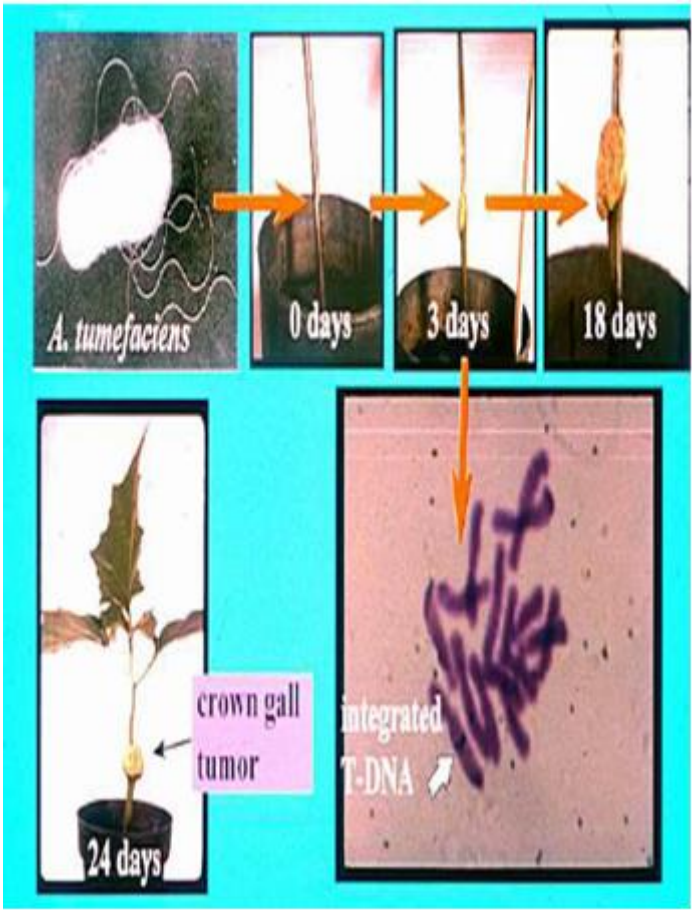
The vector mediated methods exploit the natural ability of certain bacteria (*Agrobacterium* species) and viruses to naturally transfer DNA to the genomes of infected plant cells.

***Agrobacterium* mediated gene transfer:**

Agrobacteria are natural plant parasites. Their natural ability to transfer genes provides another engineering method. To create a suitable environment for themselves, these *Agrobacteria* insert their genes into plant hosts, resulting in a proliferation of modified plant cells near the soil level (crown gall). The genetic information for tumor growth is encoded on a mobile, circular DNA fragment (plasmid). When *Agrobacterium* infects a plant, it transfers this T-DNA to a random site in the plant genome. When used in genetic engineering the bacterial T-DNA is removed from the bacterial plasmid and replaced with the desired foreign gene. The bacterium is a vector, enabling transportation of foreign genes into plants. This method works especially well for dicotyledonous plants like potatoes, tomatoes, and tobacco. *Agrobacteria* infection is less successful in crops like wheat and maize.

A.tumifaciens cause crown gall (tumor) and *A rhizogenes* hairy root disease in dicot plants by infecting through wounds on roots or stem at the soil surface. The bacterium contains Ti (Tumor

inducing) and Ri (Root inducing) plasmids. Both these plasmids can transfer part of their DNA (T-DNA) into plant cell chromosome by which Plant cells become transformed by expression of T-DNA gene which induce disease.



Description of Agrobacterium as Natural Genetic Engineer

Agrobacterium is a soil plant pathogenic bacterium. This bacterial cell possesses a Ti plasmid in addition to bacterial chromosome. The bacteria can infect plants specifically dicots through wounds near soil surface. During infection a segment (T-DNA) of Ti plasmid gets transferred and become integrated in the plant cell genome. This T-DNA carries genes for expression of Disease.

Transformation technique using *Agrobacterium*

- Some prerequisities for integration of foreign gene and production of transgenic plants are as follows
- The plant explants must produce acetosyringone to induce Vir genes for virulence
- Induced Agrobacterium should have access to cells that are competent for transformation or to take up DNA
- Cells must be meristematic, often transformed tissue or explants do not regenerate and are not totipotent, therefore transformation and regeneration competent cells should be taken as explants.
- The transformed explants cells should allow expression of transgene
- Disease causing genes should not be transferred and expressed, which is helped by disarmed T DNA

***Agrobacterium* gene transfer can be achieved in the following two ways**

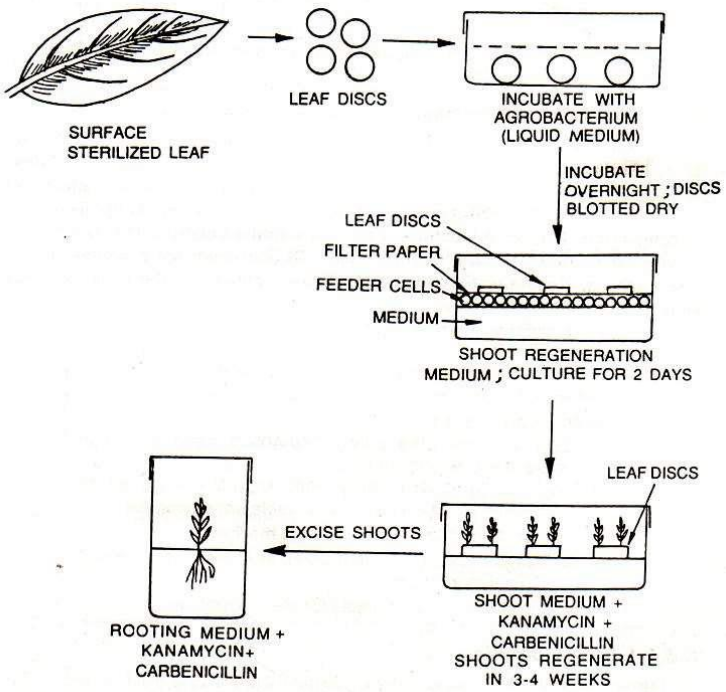
- Co culture with tissue explants
- In planta transfusion

Co- culture with tissue explants

For transformation explants like protoplast, callus , tissue slices, leaf disc, stem or floral tissue etc. can be co-cultured with genetically engineered. *Agrobacterium* with recombinant vectors for about 2 days. During co-culture acetosyringone(phenolic signal molecule) released from wounds (cut leaf disc) induce the Vir genes which bring about the transfer of recombinant T-DNA into many of the plant cells.

The explants can then be transferred to shoot inducing (regeneration medium) containing kanamycin and carbenicillin. Kanamycin allows only transformed plant cells to divide and regenerate shoots in about 3-4 weeks, while carbenicillin kills *Agrobacterium* cells.

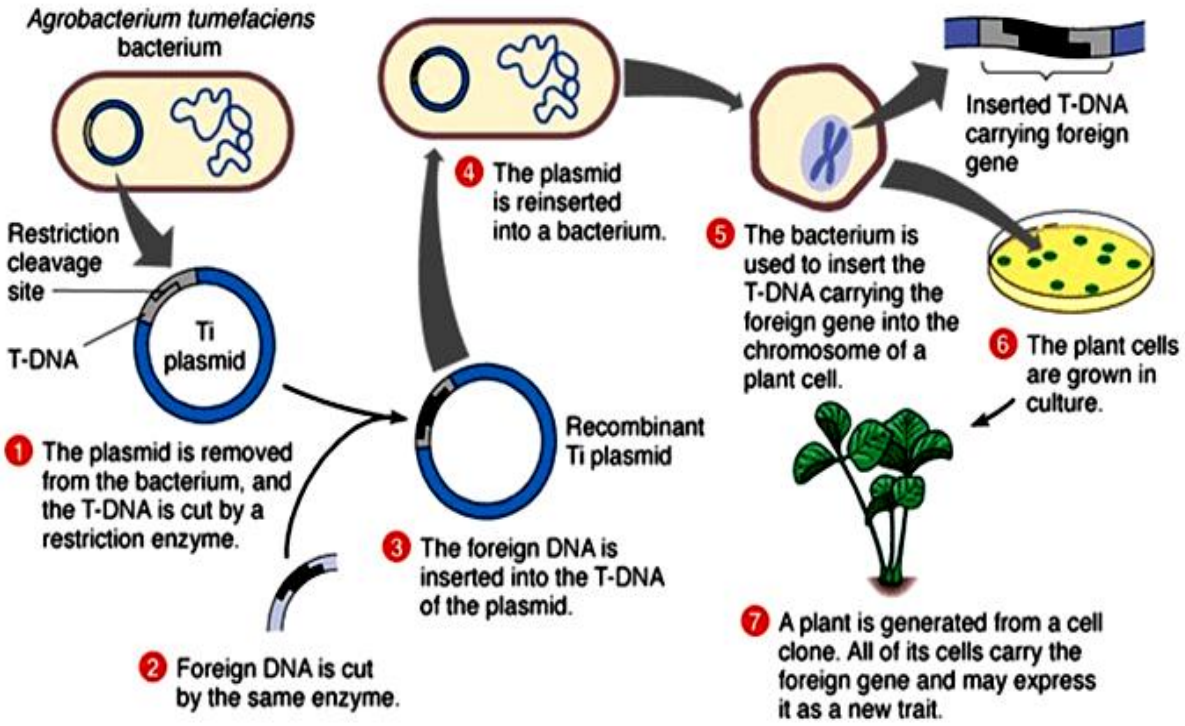
The shoots are separated and transferred to root inducing medium and finally after few weeks are transferred to soil.



By this co-culture method only dicot plants can be transformed. *Agrobacterium* mediated transformation of monocot plant (cereals) cells can be achieved by adding acetosyringone in the medium during co-culture

In planta transformation

Transformation can also be achieved by imbibition of seeds in fresh culturs of *Agrobacterium*. T-DNA becomes integrated into plant genome. It appears that *Agrobacterium* cells enter the seedling during germination, are retained within the plants, when flowers develop, the zygote or cells become transformed.



II. Direct Methods of Gene transfer (Vector less methods):

Vectorless methods use chemical or physical means to introduce DNA into plant cell. These methods are species and genotype independent in terms of DNA delivery.

Physical Methods

1. Electroporation:

Electroporation basically involves the use of high field strength electrical impulses to reversibly permeabilize the cell membranes for the uptake of DNA. This technique can be used for the delivery of DNA into intact plant cells and protoplasts.

The plant material is incubated in a buffer solution containing the desired foreign/target DNA, and subjected to high voltage electrical impulses. This results in the formation of pores in the plasma membrane through which DNA enters and gets integrated into the host cell genome.

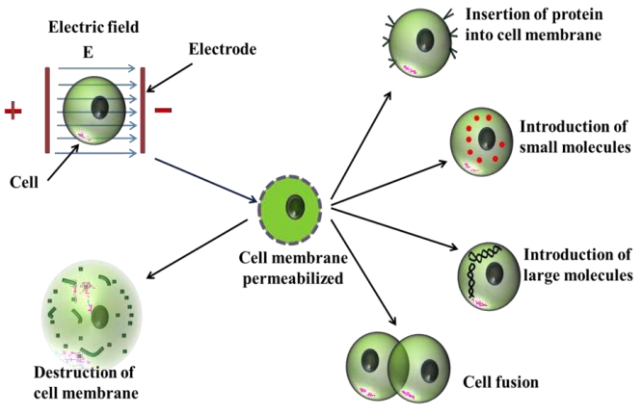
In the early years, only protoplasts were used for gene transfer by electroporation. Now a days, intact cells, callus cultures and immature embryos can be used with suitable pre- and post-electroporation treatments. Electroporation has been successfully used for the production of transgenic plants of many cereals e.g. rice, wheat, maize.

Advantages of electroporation:

i. This technique is simple, convenient and rapid, besides being cost-effective.

ii. The transformed cells are at the same physiological state after electroporation.

iii. Efficiency of transformation can be improved by optimising the electrical field strength, and addition of spermidine.



Limitations of electroporation:

i. Under normal conditions, the amount of DNA delivered into plant cells is very low.

ii. Efficiency of electroporation is highly variable depending on the plant material and the treatment conditions.

iii. Regeneration of plants is not very easy, particularly when protoplasts are used.

2. Particle Bombardment (Biolistics):

Particle (or micro projectile) bombardment is the most effective method for gene transfer, and creation of transgenic plants. This method is versatile due to the fact that it can be successfully used for the DNA transfer in mammalian cells and microorganisms.

The micro projectile bombardment method was initially named as biolistics by its inventor Sanford (1988). Biolistics is a combination of biological and ballistics. There are other names for this technique- particle gun, gene gun, bio blaster.

Micro carriers (micro projectiles), the tungsten or gold particles coated with DNA, are carried by macro carriers (macro projectiles). These macro-carriers are inserted into the apparatus and pushed downward by rupturing the disc.

The stopping plate does not permit the movement of macro carrier while the micro carriers (with DNA) are propelled at a high speed into the plant material. Here the DNA segments are released which enter the plant cells and integrate with the genome.

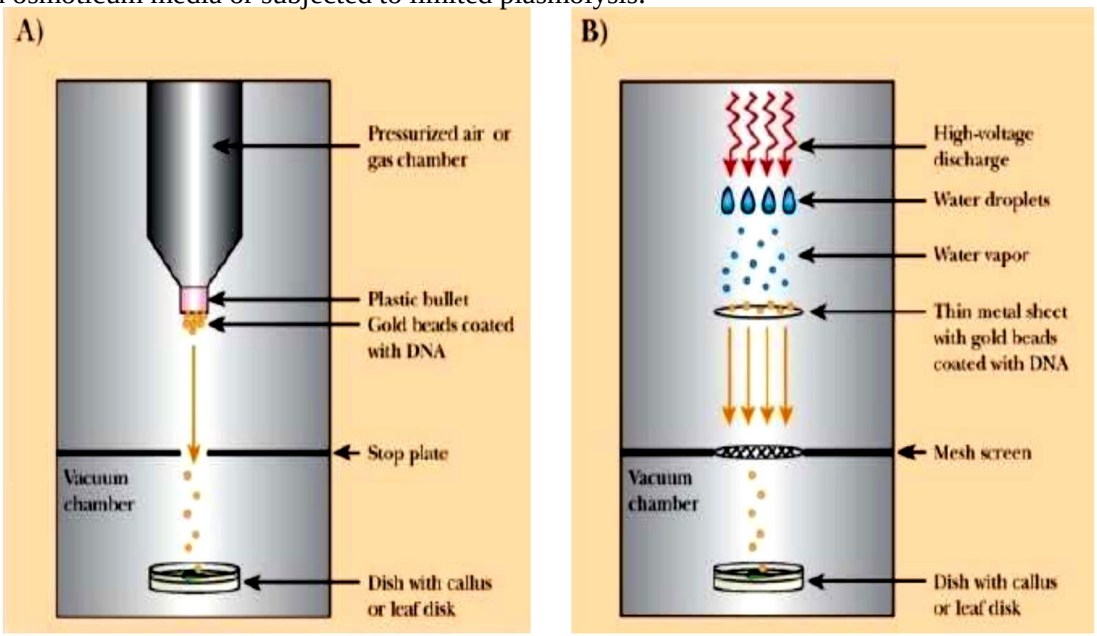
Plant material used in bombardment:

Two types of plant tissue are commonly used for particle bombardment:

1. Primary explants which can be subjected to bombardment that are subsequently induced to become embryo genic and regenerate.

2. Proliferating embryonic tissues that can be bombarded in cultures and then allowed to proliferate and regenerate.

In order to protect plant tissues from being damaged by bombardment, cultures are maintained on high osmoticum media or subjected to limited plasmolysis.



A diagrammatic representation of micro projectile bombardment system for the transfer of genes in plants

Transgene integration in bombardment:

It is believed (based on the gene transfer in rice by biolistics) that the gene transfer in particle bombardment is a two stage process.

1. In the pre-integration phase, the vector DNA molecules are spliced together. This results in fragments carrying multiple gene copies.
2. Integrative phase is characterized by the insertion of gene copies into the host plant genome.

The integrative phase facilitates further transgene integration which may occur at the same point or a point close to it. The net result is that particle bombardment is frequently associated with high copy number at a single locus. This type of single locus may be beneficial for regeneration of plants.

The success of bombardment:

The particle bombardment technique was first introduced in 1987. It has been successfully used for the transformation of many cereals, e.g. rice, wheat, maize. In fact, the first commercial genetically modified (CM) crops such as maize containing Bt-toxin gene were developed by this approach.

A selected list of the transgenic plants (developed by biolistics) along with the sources of the plant materials used is given in Table.

TABLE 49.2 A selected list of transgenic plants (along with cell sources) developed by microprojectile bombardment

Plant	*Cell source(s)*
Rice	Embryonic callus, immature zygotic embryos
Wheat	Immature zygotic embryos
Sorghum	Immature zygotic embryos
Corn	Embryonic cell suspension, immature zygotic embryos
Barley	Cell suspension, immature zygotic embryos
Banana	Embryonic cell suspension
Sweet potato	Callus cells
Cotton	Zygotic embryos
Grape	Embryonic callus
Peas	Zygotic embryos
Peanut	Embryonic callus
Tobacco	Pollen
Alfalfa	Embryonic callus

Factors affecting bombardment:

Several attempts are made to study the various factors, and optimize the system of particle bombardment for its most efficient use. Some of the important parameters are described.

Nature of micro particles:

Inert metals such as tungsten, gold and platinum are used as micro particles to carry DNA. These particles with relatively higher mass will have a better chance to move fast when bombarded and penetrate the tissues.

Nature of tissues/cells:

The target cells that are capable of undergoing division are suitable for transformation. Some more details on the choice of plant material used in bombardment are already given.

Amount of DNA:

The transformation may be low when too little DNA is used. On the other hand, too much DNA may result is high copy number and rearrangement of transgenes. Therefore, the quantity of DNA used should be balanced. Recently, some workers have started using the chemical aminosiloxane to coat the micro particles with low quantities of DNA adequate enough to achieve high efficiency of transformation.

Environmental parameters:

Many environmental variables are known to influence particle bombardment. These factors (temperature, humidity, photoperiod etc.) influence the physiology of the plant material, and consequently the gene transfer. It is also observed that some explants, after bombardment may require special regimes of light, humidity, temperature etc.

The technology of particle bombardment has been improved in recent years, particularly with regard to the use of equipment. A commercially produced particle bombardment apparatus namely PDS-1000/HC is widely used these days.

Advantages of particle bombardment:

i. Gene transfer can be efficiently done in organized tissues.

ii. Different species of plants can be used to develop transgenic plants.

Limitations of particle bombardment:

i. The major complication is the production of high transgene copy number. This may result in instability of transgene expression due to gene silencing.

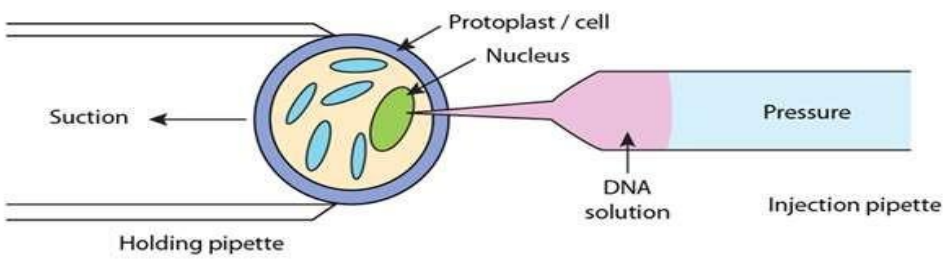
ii. The target tissue may often get damaged due to lack of control of bombardment velocity.

iii. Sometimes, undesirable chimeric plants may be regenerated.

3. Microinjection:

Microinjection is a direct physical method involving the mechanical insertion of the desirable DNA into a target cell. The target cell may be the one identified from intact cells, protoplasts, callus, embryos, meristems etc. Microinjection is used for the transfer of cellular organelles and for the manipulation of chromosomes.

The technique of microinjection involves the transfer of the gene through a micropipette (0.5-10.0 pm tip) into the cytoplasm/nucleus of a plant cell or protoplast. While the gene transfer is done, the recipient cells are kept immobilized in agarose embedding, and held by a suction holding pipette.



Advantages

- The amount of DNA delivered per cell is not limited by the technique and can be optimized. This improves the chance for integrative transformation.
- The delivery is precise, again increasing the chance of integrative transformation.
- The small structures can be injected containing only a few cells and with high regeneration potential.
- Since it is a direct physical approach, it is host-range independent.

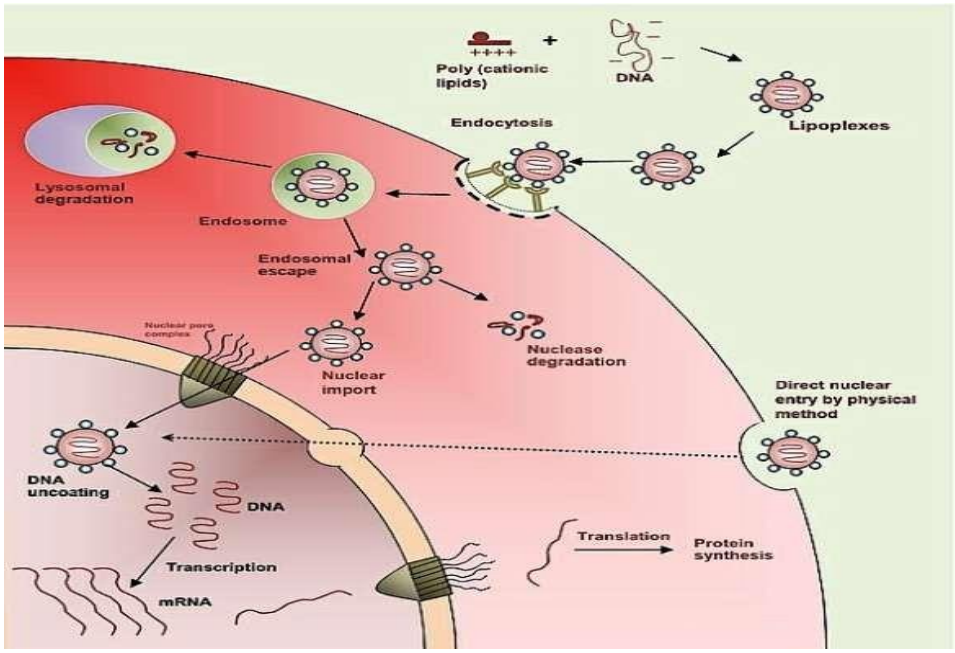
Disadvantages

- Injection can cause damage that affects embryonic survival and can result in quite high mortalities.
- Only one cell is targeted per injection.
- The handling requires specialized skill and instrumentation.
- Has low transformation rate.

4. Liposome mediated transformation

Liposomes are artificially created lipid vesicles containing a phospholipid membrane. They are successfully used in mammalian cells for the delivery of proteins, drugs etc. Liposomes carrying genes can be employed to fuse with protoplasts and transfer the genes.

The efficiency of transformation increases when the process is carried out in conjunction with polyethylene glycol (PEG). Liposome-mediated transformation involves adhesion of liposomes to the protoplast surface, its fusion at the site of attachment and release of plasmids inside the cell



Liposome mediated transformation

Advantages of liposome fusion:

i. Being present in an encapsulated form of liposomes, DNA is protected from environmental insults and damage.

ii. DNA is stable and can be stored for some time in liposomes prior to transfer.

iii. Applicable to a wide range of plant cells.

iv. There is good reproducibility in the technique.

Limitations of liposome fusion:

The major problem with liposome-mediated transformation is the difficulty associated with the regeneration of plants from transformed protoplasts.

Chemical gene mediated transfer:

1. Polyethylene glycol- mediated transformation

Polyethylene glycol (PEG), in the presence of divalent cations (using Ca^{2+}), destabilizes the plasma membrane of protoplasts and renders it permeable to naked DNA. In this way, the DNA enters nucleus of the protoplasts and gets integrated with the genome.

The procedure involves the isolation of protoplasts and their suspension, addition of plasmid DNA, followed by a slow addition of 40% PEG-4000 (w/v) dissolved in mannitol and calcium nitrate solution. As this mixture is incubated, protoplasts get transformed.

Advantages of PEG-mediated transformation:

i. A large number of protoplasts can be simultaneously transformed.

ii. This technique can be successfully used for a wide range of plant species.

Limitations of PEG-mediated transformation:

i. The DNA is susceptible for degradation and rearrangement.

ii. Random integration of foreign DNA into genome may result in undesirable traits.

iii. Regeneration of plants from transformed protoplasts is a difficult task.

2. Deae Dextran-Mediated transfer:

The desirable DNA can be complexed with a high molecular weight polymer diethyl amino ethyl (DEAE) dextran and transferred. The major limitation of this approach is that it does not yield stable trans-formants.

Selectable marker genes-

The selection is based on the survival of transformed cells when grown on a medium containing a toxic substance (antibiotic, herbicide, antimetabolite). This is due to the fact that the selectable marker gene confers resistance to toxicity in the transformed cells, while the non-transformed cells will get killed.

Some of them are given below:

Selective agent	**Marker gene/ Reporter gene**	**Source of gene**
Kanamycin (antibiotic)	NPT (neomycin phosphotransferase)	*E. coli*
Streptomycin(antibiotic)	SPT(streptomycin phosphotransferase)	streptomyces
Hygromycin(antibiotic)	HPT(hygromycin phosphotransferase)	*E. coli*
Phosphinothricin(herbicide)	BAR(phosphinothricin acetyltransferase)	streptomyces
B-glucuronidase	gus	*E.coli*
Glyphosate(herbicide)	EPSP synthase	Plant/micro-organism
Bleomycin	Ble	*E. coli*

Reporter genes- An assay for the reporter gene is carried out by estimating the quantity of the protein it produces or the final products formed. Reporter genes are often used as an indication of whether a certain gene has been taken up by or expressed in the cell or organism population.

Some of the important ones are given below:

Opine synthase (ocs), β-Glucuronidase (gus), Bacterial luciferase (luxA),

Firefly luciferase (luc).

Screenable marker gene:

The screenable markers presently used are mostly derived from bacterial genes coding for an enzymes that is readily detected by the use of chromogenic or other substances. A screenable marker gene is functional only if an enzyme with comparable activity is not present in non- transformed/non-transfected cells. Several reporter genes are used and the most commonly used examples of reporter genes are:

- **Green fluorescent protein** makes cells glow green under UV light. A specialized microscope is required to see individual cells. Yellow and red versions are also available, so scientists can look at multiple genes at once. It is commonly used to measure gene expression.[2]
- **GUS assay (using β-glucuronidase)** is an excellent method for detecting a single cell by staining it blue without using any complicated equipment. The drawback is that the cells are killed in the process. It is particularly common in plant science.
- **Blue white screen** is used in both bacteria and eukaryotic cells. The bacterial lacZ gene encodes a beta-galactosidase enzyme. When media containing certain galactosides (e.g. X-gal) is added, cells expressing the gene convert the X-gal to a blue product and can be seen with the naked eye.
- **Chloramphenical acetyl transferase (CAT)** encoding gene of E.coli: The CAT gene encodes the enzyme chloramphenicol acetyltransferase, which transfer acetyl groups from acetyl CoA onto the antibiotic chloramphenicol.
- **Luciferase** encoding gene of firefly, Photinus pyralis, catalyses the oxidation of luciferin with the emission of yellow-green light which can be detected easily even at low levels.

13. Cell Signaling: Intracellular and cell surface; receptor proteins: ion channel linked, G-protein linked and enzyme linked.

Cell signaling

Cell signaling is part of a complex system of communication that governs basic cellular activities and coordinates cell actions.All cells receive and respond to signals trom their surroundings. This is accomplished by a variety of signal molecules that are secreted or expressed on the surface of one cell and bind to receptors expressed by other cells, thereby integrating and coordinating the functions of the many individual cells that make up organisms. Each cell is programmed to respond to specific extracelular signal molecules. Extracellular signaling usually involves the following steps:

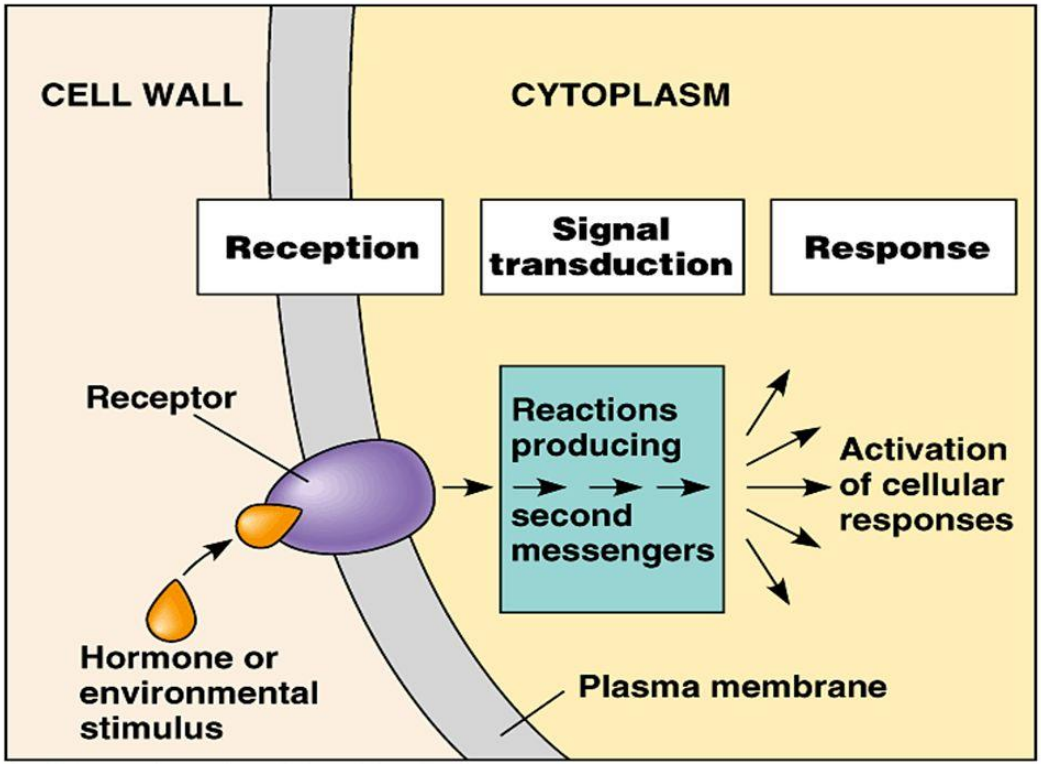
1. Synthesis and release of the signaling molecule by the signaling cell;
2. Transport of the signal to the target cell;
3. Binding of the signal by a specific receptor leading to its activation;
4. Initiation of signal-transduction pathways.

Cell signaling can be divided into 3 stages:

1**. Reception:** A cell detects a signaling molecule from the outside of the cell. A signal is detected when the ligand binds to a receptor protein on the surface of the cell or inside the cell.

2. **Transduction:** When the signaling molecule binds to the receptor, it changes the receptor protein. This change initiates the process of transduction. Each relay molecule in the signal transduction pathway changes the next molecule in the pathway.

3. **Response:** Finally, the signal triggers a specific cellular response as shown in Figure



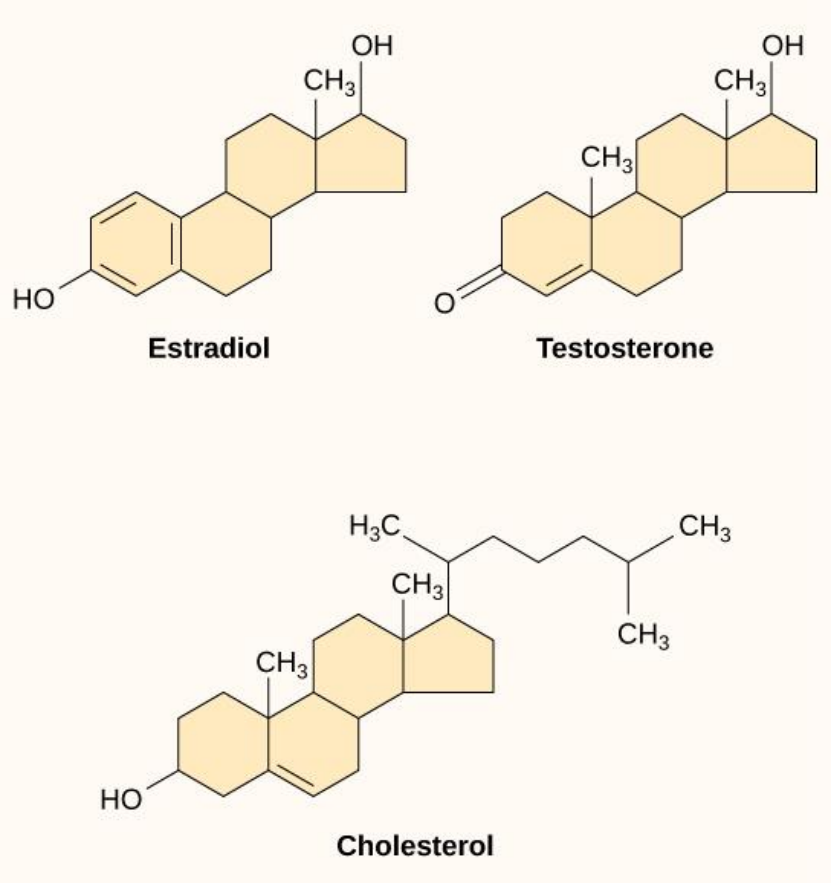
Signaling Molecules

Signaling molecules are necessary for the coordination of cellular responses by serving as ligands and binding to cell receptors.

Produced by signaling cells and the subsequent binding to receptors in target cells, ligands act as chemical signals that travel to the target cells to coordinate responses. The types of molecules that serve as ligands are incredibly varied and range from small proteins to small ions like calcium (Ca^{2+}).

Small Hydrophobic Ligands

Small hydrophobic ligands can directly diffuse through the plasma membrane and interact with internal receptors. Important members of this class of ligands are the steroid hormones. Steroids are lipids that have a hydrocarbon skeleton with four fused rings; different steroids have different functional groups attached to the carbon skeleton. Steroid hormones include the female sex hormone, estradiol, which is a type of estrogen; the male sex hormone, testosterone; and cholesterol, which is an important structural component of biological membranes and a precursor of steriod hormones. Other hydrophobic hormones include thyroid hormones and vitamin D. In order to be soluble in blood, hydrophobic ligands must bind to carrier proteins while they are being transported through the bloodstream.



Steroid Hormones: Steroid hormones have similar chemical structures to their precursor, cholesterol. Because these molecules are small and hydrophobic, they can diffuse directly across the plasma membrane into the cell, where they interact with internal receptors.

Water-Soluble Ligands

Water-soluble ligands are polar and, therefore, cannot pass through the plasma membrane unaided; sometimes, they are too large to pass through the membrane at all. Instead, most water-soluble ligands bind to the extracellular domain of cell-surface receptors. Cell-surface receptors include: ion-channel, G-protein, and enzyme-linked protein receptors. The binding of these ligands to these receptors results in a series of cellular changes. These water soluble ligands are quite diverse and include small molecules, peptides, and proteins.

Other Ligands

Nitric oxide (NO) is a gas that also acts as a ligand. It is able to diffuse directly across the plasma membrane; one of its roles is to interact with receptors in smooth muscle and induce relaxation of the tissue. NO has a very short half-life; therefore, it only functions over short distances. Nitroglycerin, a treatment for heart disease, acts by triggering the release of NO, which causes blood vessels to dilate (expand), thus restoring blood flow to the heart.

Receptors

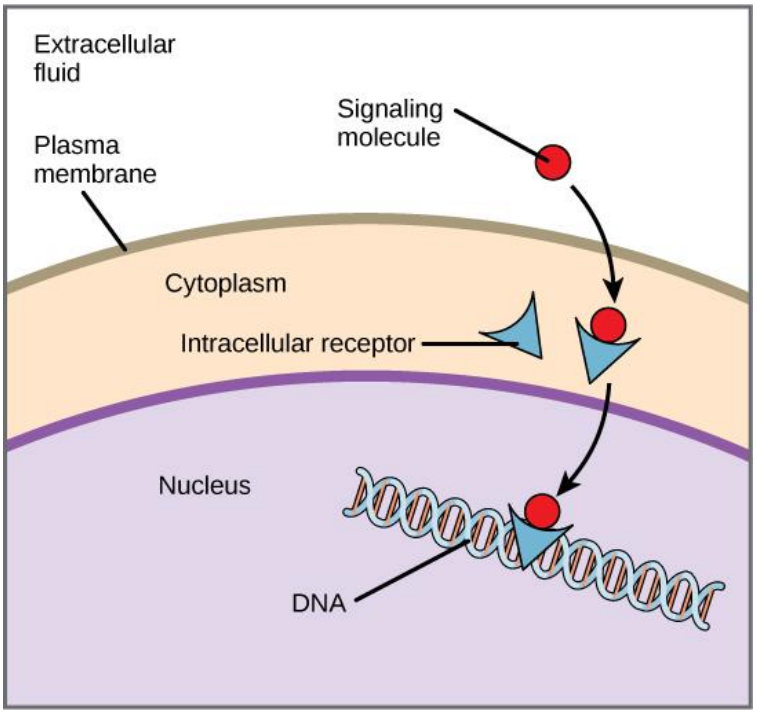
Cell surface receptors (membrane receptors, transmembrane receptors) are receptors that are embedded in the membranes of cells. They act in cell signaling by receiving (binding to) extracellular molecules. They are specialized integral membrane proteins that allow communication between the cell and the extracellular space. The extracellular molecules may be hormones, neurotransmitters, cytokines, growth factors, cell adhesion molecules, or nutrients; they react with the receptor to induce changes in the metabolism and activity of a cell. In the process of signal transduction, ligand binding affects a cascading chemical change through the cell membrane.

Types of Receptors

Receptors, either intracellular or cell-surface, bind to specific ligands, which activate numerous cellular processes.

Internal receptors

Internal receptors, also known as intracellular or cytoplasmic receptors, are found in the cytoplasm of the cell and respond to hydrophobic ligand molecules that are able to travel across the plasma membrane. Once inside the cell, many of these molecules bind to proteins that act as regulators of mRNA synthesis to mediate gene expression. Gene expression is the cellular process of transforming the information in a cell's DNA into a sequence of amino acids that ultimately forms a protein. When the ligand binds to the internal receptor, a conformational change exposes a DNA-binding site on the protein. The ligand-receptor complex moves into the nucleus, binds to specific regulatory regions of the chromosomal DNA, and promotes the initiation of transcription. Internal receptors can directly influence gene expression without having to pass the signal on to other receptors or messengers.



Intracellular Receptors: Hydrophobic signaling molecules typically diffuse across the plasma membrane and interact with intracellular receptors in the cytoplasm. Many intracellular receptors are transcription factors that interact with DNA in the nucleus and regulate gene expression.

Cell-Surface Receptors

Cell-surface receptors, also known as transmembrane receptors, are cell surface, membrane-anchored, or integral proteins that bind to external ligand molecules. This type of receptor spans the plasma membrane and performs signal transduction, converting an extracellular signal into an intracellular signal. Ligands that interact with cell-surface receptors do not have to enter the cell that they affect. Cell-surface receptors are also called cell-specific proteins or markers because they are specific to individual cell types.

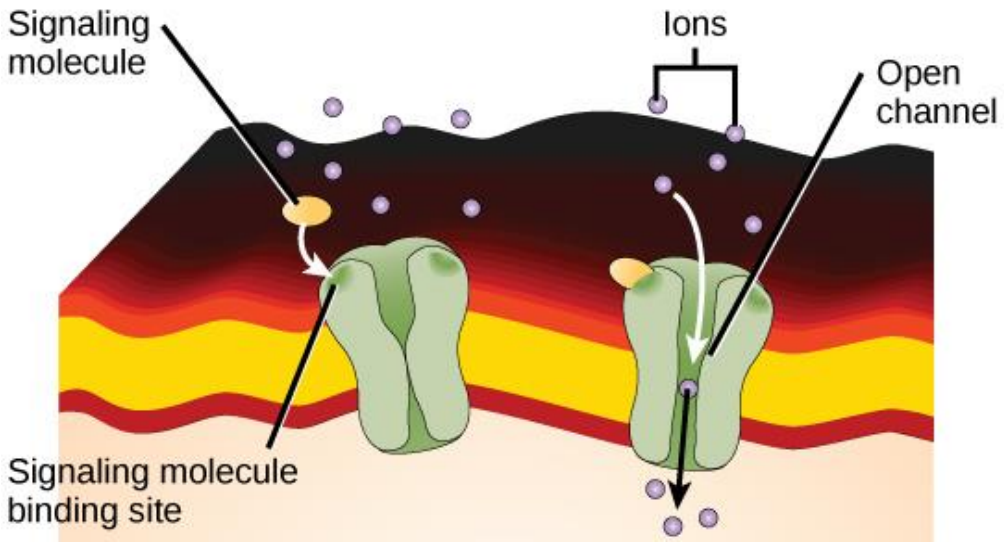
Each cell-surface receptor has three main components: an external ligand-binding domain (extracellular domain), a hydrophobic membrane-spanning region, and an intracellular domain inside the cell. The size and extent of each of these domains vary widely, depending on the type of receptor.

Cell-surface receptors are involved in most of the signaling in multicellular organisms. There are three general categories of cell-surface receptors: ion channel-linked receptors, G-protein-linked receptors, and enzyme-linked receptors.

Ion Channel-Linked Receptors

Ion channel-linked receptors bind a ligand and open a channel through the membrane that allows specific ions to pass through. To form a channel, this type of cell-surface receptor has an extensive membrane-spanning region. In order to interact with the phospholipid fatty acid tails that form the center of the plasma membrane, many of the amino acids in the membrane-spanning region are hydrophobic in nature. Conversely, the amino acids that line the inside of the channel are hydrophilic to allow for the passage of water or ions. When a ligand binds to the extracellular region of the channel, there is a conformational change in the protein's structure that allows ions such as sodium, calcium, magnesium, and hydrogen to pass through.

Acetylcholine receptor is a receptor linked to a cation channel. The protein consists of 4 subunits: α, β, γ, and δ subunits. There are two α subunits, with one acetylcholine binding site each. This receptor can exist in three conformations. The closed and unoccupied state is the native protein conformation. As two molecules of acetylcholine both bind to the binding sites on α subunits, the conformation of the receptor is altered and the gate is opened, allowing for the entry of many ions and small molecules. However, this open and occupied state only lasts for a minor duration and then the gate is closed, becoming the closed and occupied state. The two molecules of acetylcholine will soon dissociate from the receptor, returning it to the native closed and unoccupied state.



Gated-Ion Channels: Gated ion channels form a pore through the plasma membrane that opens when the signaling molecule binds. The open pore then allows ions to flow into or out of the cell.

G-Protein Linked Receptors

G-protein-linked receptors bind a ligand and activate a membrane protein called a G-protein. The activated G-protein then interacts with either an ion channel or an enzyme in the membrane. All G-protein-linked receptors have seven transmembrane domains, but each receptor has its own specific extracellular domain and G-protein-binding site.

Cell signaling using G-protein-linked receptors occurs as a cyclic series of events. Before the ligand binds, the inactive G-protein can bind to a newly-revealed site on the receptor specific for its binding. Once the G-protein binds to the receptor, the resultant shape change activates the G-protein, which releases GDP and picks up GTP. The subunits of the G-protein then split into the α subunit and the β subunit. One or both of these G-protein fragments may be able to activate other proteins as a result. Later, the GTP on the active α subunit of the G-protein is hydrolyzed to GDP and the β subunit is deactivated. The subunits reassociate to form the inactive G-protein, and the cycle starts over.

G-proteins: Heterotrimeric G proteins have three subunits: α, β, and γ. When a signaling molecule binds to a G-protein-coupled receptor in the plasma membrane, a GDP molecule associated with the α subunit is exchanged for GTP. The β and γ subunits dissociate from the α subunit, and a cellular response is triggered either by the α subunit or the dissociated β pair. Hydrolysis of GTP to GDP terminates the signal.

G protein can refer to two distinct families of proteins.

Heterotrimeric G proteins: sometimes also known as the large G proteins that are activated by G protein-coupled receptors and made up of alpha (α), beta (β), and gamma (γ) subunits.

Small G proteins: They are proteins of 20-25kDa that belong to the Ras superfamily of small GTPases. These proteins are homologous to the alpha (α) subunit found in heterotrimers, and are in fact monomeric. However, they also bind GTP and GDP and are involved in signal transduction.

Heterotrimeric G-protein

Heterotrimeric G proteins are more complex protein which were first characterized by Martin Rodbellare. It consists of three different subunits- α, β, and γ having molecular weight of these are 45, 37, and 9 kD respectively, among these α subunit binds to GDP in unactive state or GTP in active state, hence heterotrimeric G-protein is known as a member of the G protein superfamily. Heterotrimeric G proteins are held at the plasma membrane by lipid chains that are covalently attached to the α and γ subunits.

The alpha subunit has two domains - the transducing insertion domain fold whereas the other is a P-loop containing nucleoside triphosphate hydrolase fold. P-loop or a phosphate-binding loop is an ATP or GTP- binding site motif found in many nucleotide-binding proteins. It is a glycine-rich loop led by a beta sheet and followed by an alpha helix. It interacts with the nucleotide phosphate groups and with the Mg2+ ion that coordinates the β- and γ-phosphates in GTP. Upon nucleotide hydrolysis the P-loop does not significantly change conformation, but stays bound to the remaining phosphate groups. β- and γ- subunits are usually anchored to the membrane by covalently attached fatty acids. Gβγ can also directly participate in signal transduction. It activates a wide variety of signaling proteins including several isoforms of Adenylate Cyclase.

Small G protein

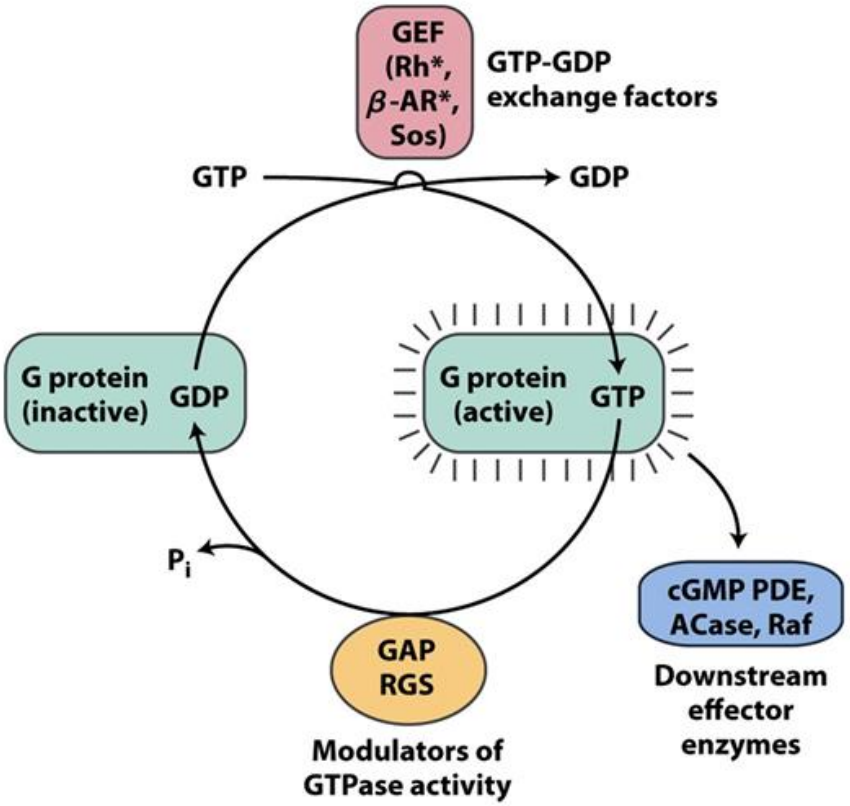
These proteins belong to a large superfamily referred to as small G proteins based on their low Mr of 20,000 to 35,000. The small G proteins, like the heterotrimeric G proteins, bind guanine nucleotides, possess intrinsic GTPase activity and cycle through GDP- and GTP-bound forms. One unifying feature of the various classes of G protein is that the binding of GTP versus GDP dramatically alters the affinity of the protein for some target molecule, apparently by inducing a large conformational

change. Small G proteins appear to function as molecular switches that control several cellular processes.

Examples of small G-protein: These are the following cellular actions which are performed by small G-protein.

Nature of G protein: G-Protein generally found in two states- *active form and unactive form.*

In unactivated state, the guanylyl nucleotide bound to the G-Protein is GDP. In this form, the G-protein exists as a heterotrimer consisting of α, β and γ subunits in which the α subunit (Gα) binds to nucleotide. The role of the hormone- bound receptor is to catalyze the exchange of GTP for GDP. Thus, inactive G-protein converted to active form and Gα subunit has no much affinity for Gβγ subunits, hence α-subunit dissociated from βγ- subunits. Now this is called activated state of G-protein. In activated state, Gα subunit stimulate effector protein such as adenylyl cyclase that lead to production of second messenger cAMP which may activate one or more signalling molecules. Other effectors are cGMP phosphodiesterase, phospholipase C-β. After dissociation from the Gα subunit, the βγ complex also has a signaling function and it can couple to at least four different types of effectors: PLC-β, K+ ion channels, adenylyl cyclase, and PI 3-kinase.



Regulation of G- protein activation and inactivation

G- protein couple receptor: GPCR comprise a large protein family of transmembrane receptors that sense molecules outside the cell and activate inside signal transduction pathways and ultimately, cellular responses. G protein-coupled receptors are found only in eukaryotes, including yeast, choanoflagellates and animals. The ligands that bind and activate these receptors include light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters, and vary in size from small molecules to peptides to large proteins. G protein-coupled receptors are involved in many diseases, and are also the target of approximately 40% of all modern medicinal drugs.

Classification of GPCR: The exact size of the GPCR superfamily is unknown but nearly 800 different human genes (or ≈4% of the entire protein-coding genome) have been predicted from genome sequence analysis. Although numerous classification schemes have been proposed, the

superfamily is classically divided into three main classes (A, B, and C) with no detectable shared sequence homology between classes. The largest class so far is class A, which accounts for nearly 85% of the GPCR genes. Of class A GPCRs, over half of these are predicted to encode olfactory receptors while the remaining receptors are liganded by known endogenous compounds or are classified as orphan receptors. Despite the lack of sequence homology between classes, all GPCRs share a common structure and mechanism of signal transduction.

In general, GPCRs can be classified into 5 classes based on sequence homology and functional similarity:

Role of G- protein coupled receptor: GPCRs are involved in a wide variety of physiological processes. Some examples of their physiological roles are as follows:

1. The visual sense: Rhodopsine which is complex of opsins and chromophore 11-cis retinal, use a photoisomerization reaction to translate electromagnetic radiation into cellular signals due to the conversion of *11-cis*-retinal to *all-trans*-retinal.
2. The sense of smell: receptors of the olfactory epithelium bind odorants (olfactory receptors) and pheromones (vomeronasal receptors)
3. Behavioral and mood regulation: receptors in the mammalian brain bind several different neurotransmitters, including serotonin, dopamine, GABA, and glutamate
4. Regulation of immune system activity and inflammation: Chemokine receptors bind ligands that mediate intercellular communication between cells of the immune system; receptors such as histamine receptors bind inflammatory mediators and engage target cell types in the inflammatory response
5. Autonomic nervous system transmission: Both the sympathetic and parasympathetic nervous systems are regulated by GPCR pathways, responsible for control of many automatic functions of the body such as blood pressure, heart rate, and digestive processes
6. Cell density sensing: A novel GPCR role in regulating cell density sensing.
7. Homeostasis modulation (water balance).

Mechanism of action: When a ligand binds to the GPCR it causes a conformational change in the GPCR, which allows it to act as a guanine nucleotide exchange factor (GEF). The GPCR then activate an associated G-protein by exchanging its bound GDP for a GTP. The G-protein's α subunit, together with the bound GTP then dissociate from the β and γ subunits to further affect intracellular signaling proteins or target functional proteins directly depending on the α subunit type (Gαs, Gαi/o, Gαq/11, Gα12/13).

There are two principal signal transduction pathways followed by the G protein-coupled receptors:

- the cAMP signal pathway
- the Phosphatidylinositol signal pathway.

Adenylate Cyclase regulated by Gs and Gi type of Gα

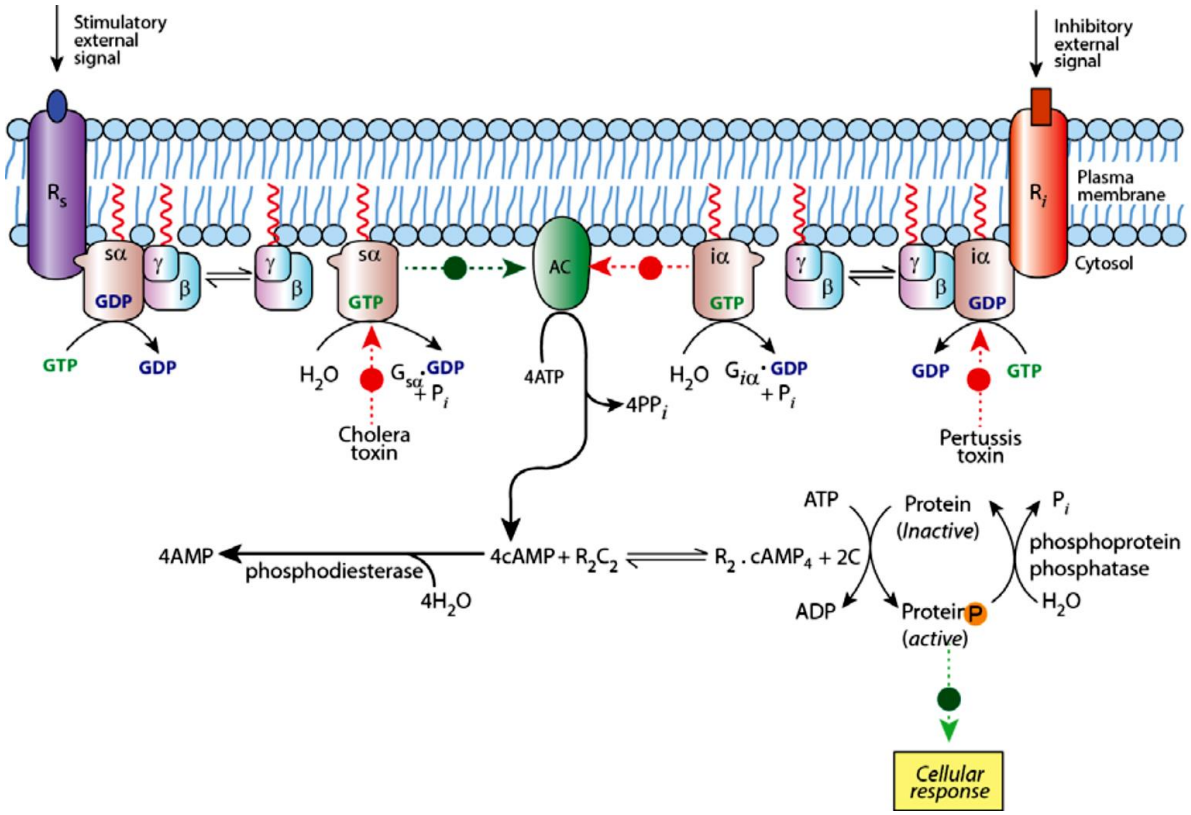
The adenylate cyclase pathway regulated by both stimulatory and inhibitory subunits of G protein Gs and Gi respectively.

In case of stimulatory subunit, Gα have intrinsic GTPase activity, which is used to hydrolyze bound GTP to GDP and Pi. This hydrolysis reaction is slow, however, requiring from second to minutes. Thus the GTP form of Gα is able to activate downstream components of signal transduction pathway before GTP hydrolysis that deactivates the subunit. In essence, the bound GTP acts as a built in clock that spontaneously resets the Gα subunit after a short time period. After GTP hydrolysis and release of Pi, the GDP –bound form of Gα then reassociates with Gβγ to re-form the inactive heterotrimeric protein. Since Gα hydrolyzes its bound GTP at a characteristic rate, it functions as a molecular clock that limits the length of time that both Gα. GTP and Gβγ can interact with their effectors.

On the other hands, Gβγ can also directly participate in signal transduction by activation of wide variety of signaling proteins including several isoforms of AC, certain Na+, K+ and Ca2+-specific ion

channels, various protein tyrosine kinases and phospholipase C- β (PLC-β; a component of the phospho-inositide signaling system; Section. Gβγ thereby provides an important source of cross talk between signaling systems.

"Several types of ligand–GPCR complexes may activate the same G protein.This occurs, for example, in liver cells in response to the binding of the corresponding hormones to glucagon receptors and to β-adrenergic receptors. In such cases, the amount of cAMP produced is the sum of that induced by the individual hormones."



Mechanism of receptor-mediated activation/inhibition of Adenylate Cyclase

In case of inhibitory type of α-subunit, some ligand–GPCR complexes inhibit rather than activate AC. These include the *α2-adrenergic receptor* and receptors for *somatostatin and opioids*. The inhibitory effect is mediated by "inhibitory" G protein, Gi, which may have the same β and γ subunits as does "stimulatory" G protein, Gs, but has a different α subunit,Giα (41 kD). Gi acts analogously to Gs in that on binding to its corresponding ligand–GPCR complex, its Giα subunit exchanges bound GDP for GTP and dissociates from G. However, Giαinhibits rather than activates AC, through direct interactions and possibly because the liberated Gβγ binds to and sequesters Gsα. The latter mechanism is supported by the observation that liver cell membranes contain far more Gi than Gs. The activation of Gi in such cells would therefore release enough Giα to bind than available Gs.

Phosphatidylinositol signal pathway:

One of the most widespread pathways of intracellular signaling is based on the use of second messengers derived from the membrane phospholipid **phosphatidylinositol 4,5-bisphosphate (PIP_2).** PIP_2 is a minor component of the plasma membrane, localized to the inner leaflet of the phospholipid bilayer. A variety of hormones and growth factors stimulate the hydrolysis of PIP2 by phospholipase C—a reaction that produces two distinct second messengers, diacylglycerol and **inositol 1,4,5-trisphosphate (IP3)**. Diacylglycerol and IP_3 stimulate distinct downstream signaling pathways

(protein kinase C and Ca^{2+} mobilization, respectively), so PIP_2 hydrolysis triggers a two-armed cascade of intracellular signaling.

It is noteworthy that the hydrolysis of PIP_2 is activated downstream of both G protein-coupled receptors and protein-tyrosine kinases. This occurs because one form of phospholipase C (PLC-β) is stimulated by G proteins, whereas a second (PLC-γ) contains SH_2 domains that mediate its association with activated receptor protein-tyrosine kinases. This interaction localizes PLC-γ to the plasma membrane as well as leading to its tyrosine phosphorylation, which increases its catalytic activity.

The diacylglycerol produced by hydrolysis of PIP_2 activates protein-serine/threonine kinases belonging to the protein kinase C family, many of which play important roles in the control of cell growth and differentiation. A good illustration of this role of protein kinase C is provided by the action of phorbol esters , which have been studied extensively because they promote the growth of tumors in animals. This tumor-promoting activity of the phorbol esters is based on their ability to stimulate protein kinase C by acting as analogs of diacylglycerol. Protein kinase C then activates other intracellular targets, including a cascade of protein kinases known as the MAP kinase pathway (discussed in detail in the next section), leading to transcription factor phosphorylation, changes in gene expression, and stimulation of cell proliferation.

Whereas diacylglycerol remains associated with the plasma membrane, the other second messenger produced by PIP_2 cleavage, IP_3, is a small polar molecule that is released into the cytosol, where it acts to signal the release of Ca^{2+} from intracellular stores. The cytosolic concentration of Ca^{2+} is maintained at an extremely low level (about 0.1 μM) as a result of Ca^{2+}pumps that actively export Ca^{2+} from the cell. Ca^{2+} is pumped not only across the plasma membrane, but also into the endoplasmic reticulum, which therefore serves as an intracellular Ca^{2+} store. IP_3 acts to release Ca^{2+} from the endoplasmic reticulum by binding to receptors that are ligand-gated Ca^{2+} channels. As a result, cytosolic Ca^{2+} levels increase to about 1 μM, which affects the activities of a variety of target proteins, including protein kinases and phosphatases. For example, some members of the protein kinase C family require Ca^{2+} as well as diacylglycerol for their activation, so these protein kinases are regulated jointly by both arms of the PIP_2 signaling pathway.

Many of the effects of Ca^{2+} are mediated by the Ca^{2+}-binding protein calmodulin, which is activated by Ca^{2+} binding when the concentration of cytosolic Ca^{2+} increases to about 0.5 μM Ca^{2+}/calmodulin then binds to a variety of target proteins, including protein kinases. One example of such a Ca^{2+}/calmodulin-dependent protein kinase is myosin light-chain kinase, which signals actin-myosin contraction by phosphorylating one of the myosin light chains. Other protein kinases that are activated by Ca^{2+}/calmodulin include members of the CaM kinase family, which phosphorylate a number of different proteins, including metabolic enzymes, ion channels, and transcription factors. One form of CaM kinase is particularly abundant in the nervous system, where it regulates the synthesis and release of neurotransmitters. In addition, CaM kinases can regulate gene expression by phosphorylating transcription factors. Interestingly, one of the transcription factors phosphorylated by CaM kinase is CREB, which is phosphorylated at the same site by protein kinase A. This phosphorylation of CREB illustrates one of many intersections between the Ca2+ and cAMP signaling pathways. Other examples include the regulation of adenylyl cyclases and phosphodiesterases by Ca^{2+}/calmodulin, the regulation of Ca^{2+} channels by cAMP, and the phosphorylation of a number of target proteins by both protein kinase A and Ca^{2+}/calmodulin-dependent protein kinases. The cAMP and Ca^{2+} signaling pathways thus function coordinately to regulate many cellular responses.

Ca^{2+} is an extremely common second messenger, and it is important to note that IP_3-mediated release of Ca^{2+} from the endoplasmic reticulum is not the only mechanism by which the intracellular concentration of Ca^{2+}+ can be increased. One alternative pathway involves the entry of extracellular

Ca^{2+} through Ca^{2+} channels in the plasma membrane. In many cells, the transient increase in intracellular Ca^{2+} resulting from production of IP_3 is followed by a more sustained increase resulting from extracellular Ca^{2+} entry. The entry of extracellular Ca^{2+} is particularly important in the electrically excitable cells of nerve and muscle, in which voltage-gated Ca2+ channels in the plasma membrane are opened by membrane depolarization. The resulting increases in intracellular Ca^{2+} then trigger the further release of Ca^{2+} from intracellular stores by activating distinct Ca^{2+} channels known as ryanodine receptors. One effect of increases in intracellular Ca^{2+} in neurons is to trigger the release of neurotransmitters, so Ca^{2+} plays a critical role in converting electric to chemical signals in the nervous system. Cells thus utilize a variety of mechanisms to regulate intracellular Ca^{2+} levels, making Ca^{2+} a versatile second messenger that controls a wide range of cellular processes.

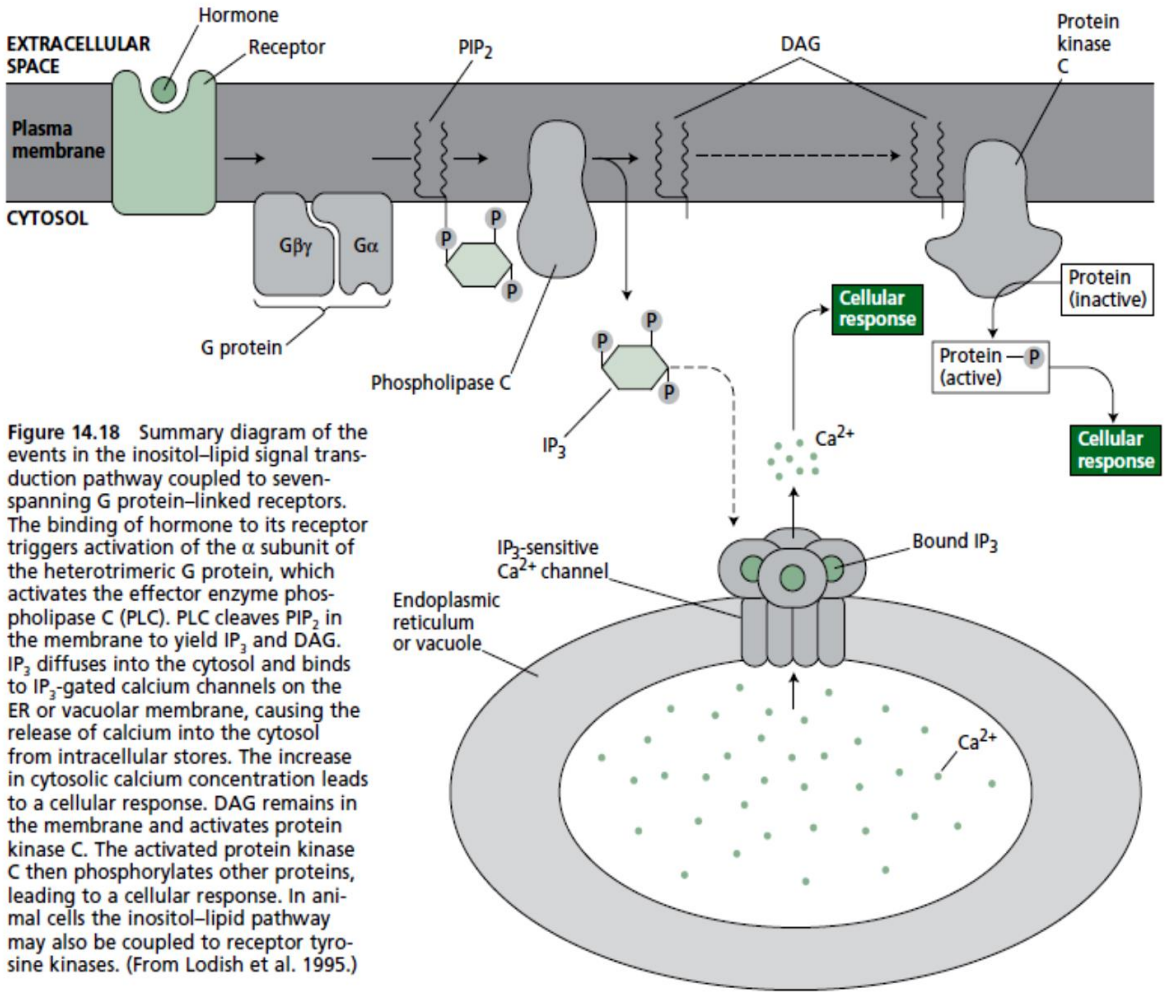


Figure 14.18 Summary diagram of the events in the inositol–lipid signal transduction pathway coupled to seven-spanning G protein–linked receptors. The binding of hormone to its receptor triggers activation of the α subunit of the heterotrimeric G protein, which activates the effector enzyme phospholipase C (PLC). PLC cleaves PIP_2 in the membrane to yield IP_3 and DAG. IP_3 diffuses into the cytosol and binds to IP_3-gated calcium channels on the ER or vacuolar membrane, causing the release of calcium into the cytosol from intracellular stores. The increase in cytosolic calcium concentration leads to a cellular response. DAG remains in the membrane and activates protein kinase C. The activated protein kinase C then phosphorylates other proteins, leading to a cellular response. In animal cells the inositol–lipid pathway may also be coupled to receptor tyrosine kinases. (From Lodish et al. 1995.)

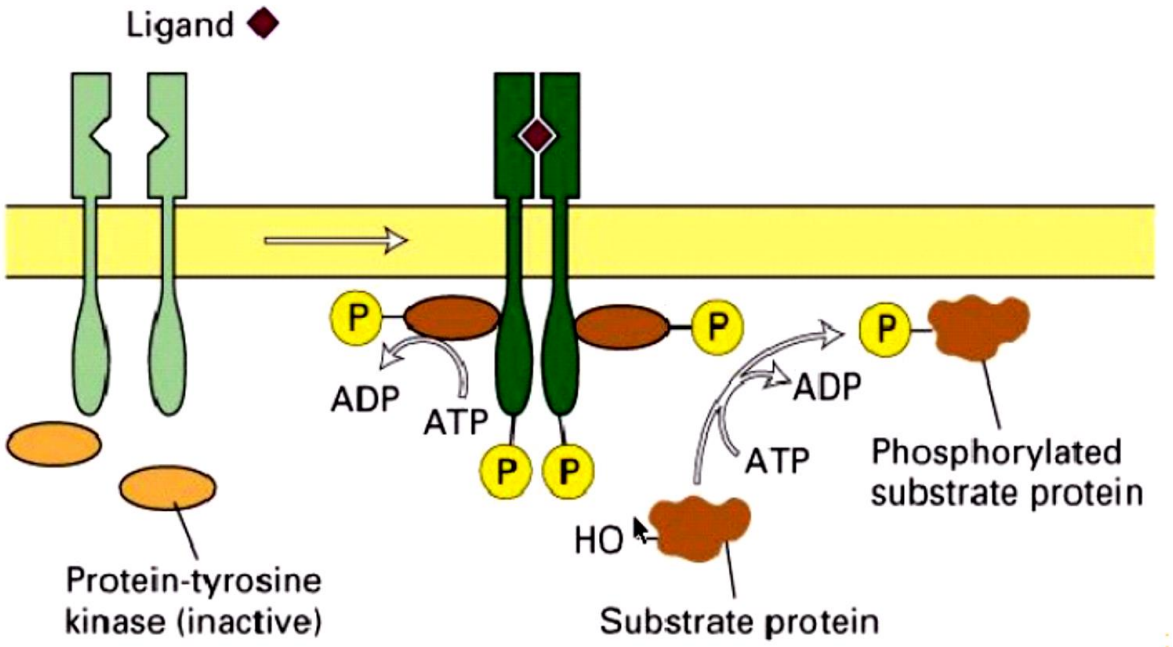
Enzyme-Linked Receptors

Enzyme-linked receptors are cell-surface receptors with intracellular domains that are associated with an enzyme. In some cases, the intracellular domain of the receptor itself is an enzyme or the enzyme-linked receptor has an intracellular domain that interacts directly with an enzyme. The enzyme-linked receptors normally have large extracellular and intracellular domains, but the membrane-spanning region consists of a single alpha-helical region of the peptide strand. When a ligand binds to the extracellular domain, a signal is transferred through the membrane and activates the enzyme, which sets off a chain of events within the cell that eventually leads to a response. An example of this type of enzyme-linked receptor is the tyrosine kinase receptor. The tyrosine kinase receptor transfers

phosphate groups to tyrosine molecules. Signaling molecules bind to the extracellular domain of two nearby tyrosine kinase receptors, which then dimerize. Phosphates are then added to tyrosine residues on the intracellular domain of the receptors and can then transmit the signal to the next messenger within the cytoplasm.

Receptor Tyrosine Kinases (RTK) are receptors that contains intrinsic tyrosine kinase activity. The simplest receptor tyrosine kinases (RTKs) have three domains: a ligand binding domain outside the cell, a single membrane-spanning domain, and a tyrosine kinase domain inside the cell. The ligands are usually diffusible peptides or small proteins produced elsewhere in the organism, and are typically growth factors, cytokines and hormones. In the absence of a ligand the receptor is inactive.

Ligand binding results in receptor dimerization with an adjacent receptor, causing the tyrosine kinase domain on the receptor to become active. The receptors can then phosphorylate each other on multiple tyrosine sites; they may then bind with one or more other proteins (called SH2 proteins) that specifically recognize the phosphorylated tyrosines. Some SH2 proteins are themselves enzymes, while others are adapter molecules that in turn attract and bind other enzymes. Often these enzymes are inactive until they join the receptor complex, because their substrates are found only in the membrane. The products of these enzymes may act on yet other molecules, thus continuing the signaling cascade, or they may be used in cell metabolism for growth or other responses.



14. Genomics: Structural genomics, molecular markers and mapping of genome using - RFLP, RAPD, AFLP, ESTS and micro-satellite markers; chromosome walking; Functional genomics: DNA microarray and chip technology; a brief idea on Human Genome Project.

Genomics

Genomics: The term genome was introduced by H. Winkler (1920) to denote the complete set of chromosomal and extra chromosomal genes present in an organism, including a virus.

The term genomics coined by T. H. Roderick (1987) mean mapping and sequencing to analyse the structure and organisation of genomes. But presently genomics includes sequencing of genomes, determination of the complete set of proteins encoded by an organism, and the functioning of genes and metabolic pathways in an organism.

Whole Genome Sequence Data:

Complete nucleotide sequences of nuclear, mito-chondrial and chloroplast genomes have already been worked out in large number of prokaryotes and several eukaryotes. By the year 2005, among prokaryotes, approx. 1400 viral genomes, 250 bacterial genomes (230 eubacteria and 20 archaea), 500 mitochondr-ial genomes, 35 chloroplast genomes have been fully sequenced.

Among the eukaryotes namely the whole genome of *Saccharomyces cerevisiae* (yeast), *Coenorhabditis elegans* (nematode), fruitfly (*Drosophila melanogaster*), Human (*Homo sapiens*), Crucifer weed (*Arabidopsis thaliana*) and rice (*Oryza sativa*) have been sequenced already and data available for annotation studies.

The sequence data of eukaryotic nuclear genome is an important source of identi-fication, discovery and isolation of important genes. This data is very much helpful in variety of application relevant to animal, plant and microbial biotechnology.

Types of Genomics:

The discipline of genomics consists of two parts, viz. structural genomics and functional genomics. These are defined as under:

i. Structural Genomics:

It deals with the study of the structure of entire genome of a living organism. In other words, it deals with the study of the genetic structure of each chromosome of the genome. It determines the size of the genome of a species in mega-bases [Mb] and also the genes present in the entire genome of a species.

ii. Functional Genomics:

The study of function of all genes present in the entire genome is known as functional genomics. It deals with transcriptome and proteome. The transcriptome refers to complete set of RNAs transcribed from a genome and proteome refers to complete set of proteins encoded by a genome.

iii. Comparative Genomics:

It deals with the study of multiple whole genomes for understanding the differences and similarities between all the genes of the multiple species. For example in the phylogenic tree analysis two specialized concepts are the rooted and unrooted trees.

Phylogenetic trees are designed to reveal evolutionary relationship among DNA or protein sequences. In unrooted trees, when distantly related sequence for comparison has not included when an unrooted tree is required.

Epigenomics

Epigenomics is the study of the complete set of epigenetic modifications on the genetic material of a cell, known as the epigenome. Epigenetic modifications are reversible modifications on a cell's DNA or histones that affect gene expression without altering the DNA sequence. Two of the most characterized epigenetic modifications are DNA methylation and histone modification. Epigenetic modifications play an important role in gene expression and regulation, and are involved in numerous cellular processes such as in differentiation/development and tumorigenesis. The study of epigenetics on a global level has been made possible only recently through the adaptation of genomic high-throughput assays.

Metagenomics

Environmental Shotgun Sequencing (ESS) is a key technique in metagenomics. (A) Sampling from habitat; (B) filtering particles, typically by size; (C) Lysis and DNA extraction; (D) cloning and library construction; (E) sequencing the clones; (F) sequence assembly into contigs and scaffolds.

Metagenomics is the study of metagenomes, genetic material recovered directly from environmental samples. The broad field may also be referred to as environmental genomics, ecogenomics or community genomics. While traditional microbiology and microbial genome sequencing rely upon cultivated clonal cultures, early environmental gene sequencing cloned specific genes (often the 16S rRNA gene) to produce a profile of diversity in a natural sample. Such work revealed that the vast majority of microbial biodiversity had been missed by cultivation-based methods. Recent studies use "shotgun" Sanger sequencing or massively parallel pyrosequencing to get largely unbiased samples of all genes from all the members of the sampled communities. Because of its power to reveal the previously hidden diversity of microscopic life, metagenomics offers a powerful lens for viewing the microbial world that has the potential to revolutionize understanding of the entire living world.

Role of Genomics in Crop Plants:

The discipline of genomics is of recent origin. The genome mapping was first completed in a free living bacteria *Haemophillus influenza* in 1995. Later on genome sequencing work was intensified both in prokaryotes and eukaryotes. In plants, genome sequencing was first completed in Arabidopsis thaliana (a weedy relative of mustard followed by rice (*Oriza sativa*).

S.No.	Species	Botanical Name	Genome Size [Mb]	Gene No.
		A. FIELD CROPS		
1.	Arabidopsis	*Arabidopsis thaliana*	120	27416
2.	Rice	*Oryza sativa*	370	40577
3.	Corn	*Zea mays*	2500	>32000
4.	Sorghum	*Sorghum bicolor*	700	34496
5.	Cucumber	*Cucumis sativa*	243.5	26682
6.	Soybean	*Glycine max*	950	46430
7.	Caster bean	*Ricinus communis*	320	31237
8.	Pigeon pea	*Cajanus cajan*	833	48680
9.	Potato	*Solanum tuberosum*	844	39031
10.	Cannabis	*Cannabis sativa*	534	30,000

Now genome sequencing work has been completed in more than 40 crop plants. The list of some field crops, fruit crops and other plant species in which genome sequencing work has been completed is presented below.

In plants, the smallest genome size has been reported in *Arabidopsis thaliana* (120 Mb) and the largest in corn (2500). Thus, in plants the genome size investigated so far varies from 120 Mb to 2500 Mb. After gene sequencing, function is assigned to individual gene through various molecular techniques.

Genes to be mapped:

In genome research, both types of genes, viz., major genes (oligogenes) and minor genes (polygenes) can be easily mapped. The mapping of polygenic traits is possible by genome mapping techniques which is not possible by conventional gene mapping techniques such recombination mapping and deletion mapping.

The genome mapping is done for morphological, productivity, resistance, quality, agronomic and some special traits as discussed below.

i. Morphological Characters:

It includes highly heritable traits such as shape, size and color of leaf, flower, calyx, corolla, etc. It also includes surface of leaf and stem (hairiness and smoothness).

ii. Productivity traits:

Such characters differ from species to species.

iii. Resistance Traits:

Such characters include resistance to diseases, insects, drought, soil salinity, soil alkalinity, soil acidity, heat, frost, water logging, cold, etc.

iv. Quality Traits:

Such traits include nutritional quality, market quality and keeping quality.

v. Agronomic Traits:

Such traits include earliness, plant height, plant type, etc.

vi. Special Characters:

Such characters include genes controlling male sterility, self-incompatibility, photo and thermo insensitivity, toxic substances, apomixes, adaptation, etc.

Genome Mapping Laboratories:

The genome mapping work is carried out by collaborative efforts of various International and National Research Laboratories of public domain. The list of some organizations where genome sequencing and mapping work on different crop species is carried out is presented in Table.

TABLE 35.2. List of some Genome Sequencing Organization

S.No.	Name of Crop	Scientific name	Name of Genome Project	In Collaboration with
1.	Arabidopsis	*Arabiddopsis thaliana*	Arabidopsis Genome Initiative	USA, China and Japan.
2.	Brassica	*Brassica* Spp.	Brassica Genome Gate way	UK, USA, France, Germany, Australia, South Korea, Poland and China.
3.	Wheat	*Tricum aestivum*	International Wheat genome	UK Scientists from Uni. of Liverpool, Bristol and John Innes.
4.	Rice	*Oriza* Spp.	International Rice Genome Sequencing Project	Mexico, USA, China and Japan
5.	Cotton	*Gossypium* Spp.	International Cotton Genome Initiative Project	USA, France, Australia
6.	Soybean	*Glycine max*	Uni. Purdue, North Carolina Uni, and 18 other centres	United States of America
7.	Potato	*Solanum tuberosum*	Potato Genome Sequencing Consortium	USA, UK, Russia, China, India, Poland, Chile,New Zealand, Nether land, Peru, etc.
8.	Tomato	*Lycopersicum esculentum*	Tomato Genome Consortium	China, japan, Germany,Korea, Spain, UK, USA, Israel, Netherland & Others
9.	Pigeon pea	*Cajanus cajan*	International Initiative for Pigeonpea Genomics	India (ICRISAT), China, USA, Mexico, etc.
10.	Apple	*Malus domesticus*	Apple Genome sequencing Initiative	Washington State Uni. USA and NRI COMPETITIVE GRANT

Genome Mapping in India:

In India, the functional genome research projects are looked after by the Depart of Biotechnology [DBT] and ICAR. The DBT has initiated such work on several crops such as rice, wheat, maize, chick

pea, banana, tomato, *Brassica*, etc. The ICAR has created genome mapping facilities for rice at NRCPB, IARI, New Delhi. In India, the genome mapping work is carried out at the following centres.

i. National Research Centre for Plant Biotechnology, IARI, New Delhi.

ii. International Centre for Genetic Engineering and Biotechnology, New Delhi.

iii. Jawahar Lal Nehru University, New Delhi.

iv. National Botanical Research Institute, Lucknow.

Role of Genomics in Crop Improvement:

Genomics has several practical applications in crop improvement. Genome mapping is useful in several ways. It is useful or provides information about genome size, gene number, gene mapping, gene sequencing, evolution of crop plants, gene cloning, identification of DNA markers, marker assisted selection, transgenic breeding, construction of linkage maps and QTL mapping.

All these aspects are briefly discussed as follows:

i. Genome Size:

Genome mapping is a very useful technique for determining the genome size in various plant species. In the plant species studied so far, the largest genome size has been reported in maize (2500 Mb) and the smallest in *Arabidopsis thaliana* (120 Mb).

ii. Gene Number:

Genome mapping provide information about gene number in a species. In crop plants studied so far, the maximum number of genes has been reported in rice (56,000).

iii. Gene Mapping:

Genome research is very much useful in mapping/tagging of genes on the different chromosomes of a genome. In other words, it helps in large scale discovery of new gene in a genome.

iv. Gene Sequencing:

Genome mapping helps in determining the order of genes on the chromosomes. The order of genes is determined on each chromosome of a genome.

v. Evolution:

Genome mapping provide information about the evolution of different species. It measures the association between different genomes and thus provides information about the relatedness or evolutionary biology of crop plants.

vi. Gene Cloning:

Genome research is very much useful in making multiple copies of a gene and transfer of the same from one genotype to another. Thus, it aids in specific gene transfer.

vii. Identification of DNA Markers:

The genome mapping techniques are useful in identification of DNA markers which can be used in molecular breeding i.e. marker assisted selection. The mapping populations developed from inter-specific crosses have high polymorphism for DNA markers than those mapping populations derived from intra-specific crosses.

viii. Marker Assisted Selection:

Marker assisted selection refers to indirect selection for a desired phenotype based on band pattern of linked DNA markers. The improvement of crop plants using such selection is cal molecular breeding. Various DNA markers used for such purpose include RFLP, AFLP, ISSR, etc.

The effect of DNA marker is correlated with morphological markers and then selection is made for particular trait. The selection based on DNA markers is more reliable because DNA markers are not influenced by environmental factors.

ix. Transgenic Breeding:

Genome mapping is useful in gene cloning. The gene of interest can be cloned and used in developing transgenic plants (genetically engineered plants). Transgenic breeding permits direct gene transfer bypassing sexual process.

x. Construction of Linkage maps:

Genome mapping helps in construction of linkage groups. The linkage groups can be constructed from the information of gene mapping and gene sequencing.

xi. QTL Mapping:

The genome mapping techniques is widely used for mapping of quantitative trait loci (QTL). The mapping of QTL or polygenic traits is not possible by conventional methods, viz., recombination mapping and deletion mapping techniques.

The study of genomics is divided into the following two domains:

1. Structural genomics deals with the determination of the complete sequence of genomes or the complete set of proteins produced by an organism. The various steps involved are: (i) construction of high resolution genetic and physical maps, (ii) Sequencing of the genome, and (iii) determination of the complete set of proteins in an organism. It also includes the determination of the three-dimensional structures of the concerned proteins.

Sequencing of Genomes:

Sequencing of genomes is a highly sophisticated and technically demanding process. At one go, a fragment of 500-600 bp can be sequenced. In contrast, genomes are extremely large, e.g., 4.2 x 106 for E. coli and 3.2 x 109 bp for humans. Therefore, the sequence of genoxne has to obtained in an extremely large number of small pieces, these pieces are then assembled into a sequence for the genome.

The pieces used for sequencing are generated by breaking the genomic DNA into fragments at random points. As a result, the location of fragment in the genome has to be experimentally determined. All the fragments obtained from genomic DNA of an organism are cloned in a suitable vector this generates a genomic library of the organism. The two approaches to sequencing of genomes are: (a) clone-by- clone sequencing and (b) shot-gun sequencing.

(a) Clone-by Clone Sequencing:

In this method, the fragments are first aligned into contigs also called as directed sequencing of BAC contigs. A contig consists of a series of clones that contain overlapping pieces of DNA converting a specific region of a chromosome or even the entire chromosome. They are usually constructed using BAC (bacterial artificial chromosome) and cosmid clones.

The general approach in creation of contigs is to identify the clones that have adjacent DNA segments from the chromosome, e.g., chromosome walking, chromosome jumping, etc. Thus the members of a contig must contain same overlapping region to allow the precise determination of their location-in the conting. The ultimate goal of physical mapping procedures is to obtain a complete contig for each chromosome of the genome.

The cloned DNA fragments of a contig can be correlated with locations along a chromosome obtained from linkage or cytogenetic mapping. This can be achieved by identifying members of the contig that contain inserts having such genes that have already been mapped by linkage or cytological methods. This would permit the alignment of the other members of the contig along the chromosome. Alternatively, RFLP (restriction fragment length polymorphism) and other DNA markers may be used to correlate the locations in a linkage map with the members of a contig.

(b) Shot-Gun Sequencing:

In this approach, randomly selected clones are sequenced until all clones in the genomic library are analyzed. Assembler software organises the nucleotide sequence information so obtained into a

genome sequence. This strategy works very well with prokaryotic genomes which have little repetitive DNA. But eukaryotic genomes have lots of repeated sequences which create confusion in the alignment of sequence. These problems are resolved by using enormous computing powers, specialized software and avoiding such regions that are rich in repetitive DNA (e.g., centromeric and telomeric regions).

Genome Sequence Compilation:

Genome sequencing projects necessitated the development of high throughput technologies that generate data at a very fast rate. This necessitated the use of computers to manage this flood of information and has given birth to a new discipline called bioinformatics. Bioinformatics deals with storage, analysis, interpretation and utilization of the information about biological systems (activities such as compilation of genome sequences, identification of genes, assigning functions to the identified genes, preparation of databases, etc.).

In order to ensure that the nucleotide sequence of a genome is complete and error free, the genome is sequenced more than once. Once the genome of an organism is sequenced, compiled and proofread (correcting the errors) the next stage of genomics, viz., annotation, begins.

Gene Prediction and Counting:

After a genome sequence has been obtained and checked for accuracy, the next task is to find all the genes that encode proteins. This is the first step in annotation. Annotation is a process that identifies genes, their regulatory sequences and their function(s). It also identifies non-protein coding genes including those that code for r-RNA, t-RNA and small nuclear RNAs. In addition, mobile genetic elements and repetitive sequence families are identified and characterized.

Locating protein-coding genes is done by inspecting the sequence, using a computer software or by eye. Protein-coding genes are identified by open-reading frames (ORFs). An ORF has a series a codons that specify an amino-acid sequence, it begins with an initiation codon (usually ATG) and end with a termination codon (TAA) TAG or TGA). ORFs are identified usually by a computer and is an effective method for bacterial genomes.

Genes in eukaryotic genomes (including the human genome) have several features that makes direct searching less useful. Firstly, most eukaryotic genes have a pattern of exons (coding regions) alternated with introns (non-coding regions). As a result, these genes are not organized as continuous ORFs. Secondly, genes in humans and other eukaryotes are often widely spaced thus increasing the chances of finding false genes. But newer versions of ORF scanning software for eukaryotic genomes make scanning more efficient.

After a genomic sequence is analyzed and genes are predicted, each gene is examined one at a time to identify the function of the encoded gene product and classified into functional groups. This analysis involves several programmes. For example, one may search databases such as Gene Bank, to find similar genes isolated from other organisms. The predicted ORFs may be compared with those from known, well-characterized bacterial genes. Finally, one may look for such nucleotide sequences for function motifs that encode protein domains involved with specific functions.

Thus, the objective of genome analysis is to determine the functions of all the genes and to understand how these genes interact in the development and function of the organism.

Molecular marker and genome mapping:

A **bio-marker, or biological marker** is a measurable indicator of some biological state or condition.

Markers are of four types, viz: (i) Morphological, (ii) Biochemical, (iii) Cytological, and (iv) Molecular or DNA markers.

These are briefly discussed as follows:

i. Morphological:

In plant breeding, markers that are related to variation in shape, size, colour and surface of various plant parts are called morphological markers. Such markers refer to available gene loci that have obvious impact on morphology of plant. Genes that affect form, coloration, male sterility or resistance among others have been analyzed in many plant species.

In rice, examples of this type of marker may include the presence or absence of awn, leaf sheath coloration, height, grain color, aroma etc. In well-characterized crops like maize, tomato, pea, barley or wheat, tens or even hundreds of such genes have been assigned to different chromosomes.

There are several demerits of morphological markers as given below:

a. They generally express late into the development of an organism. Hence their detection is dependent on the development stage of the organism.

b. They usually exhibit dominance.

c. Sometimes they exhibit deleterious effects.

d. They exhibit pleiotropy.

e. They exhibit epistasis.

f. They exhibit less polymorphism.

g. They are highly influenced by the environmental factors.

ii. Biochemical:

Markers that are related to variation in proteins and amino acid banding pattern are known as biochemical markers. A gene encodes a protein that can be extracted and observed; for example, isozymes and storage proteins.

iii. Cytological:

Markers that are related to variation in chromosome number, shape, size and banding pattern are referred to as cytological markers. In other words, it refers to the chromosomal banding produced by different stains; for example, G banding.

iv. Molecular Markers:

A molecular marker is a DNA sequence in the genome which can be located and identified. As a result of genetic alterations (mutations, insertions, deletions), the base composition at a particular location of the genome may be different in different plants.

These differences, collectively called as polymorphisms can be mapped and identified. Plant breeders always prefer to detect the gene as the molecular marker, although this is not always possible. The alternative is to have markers which are closely associated with genes and inherited together.

The molecular markers are highly reliable and advantageous in plant breeding programmes:

- **Polymorphism:**
  Markers should exhibit high level of polymorphism. In other words, there should be variability in the markers. It should demonstrate measurable differences in expression between trait types and/or gene of interest.
- **Co-Dominant:**
  Marker should be co-dominant. It means, there should be absence of intra-locus interaction. It helps in identification of heterozygotes from homozygotes.
- Even distribution across the whole genome (not clustered in certain regions)
- Clear distinct allelic features (so that the different alleles can be easily identified)
- Single copy and no pleiotropic effect
- Low cost to use (or cost-efficient marker development and genotyping)
- Easy assay/detection and automation
- High availability (un-restricted use) and suitability to be duplicated/multiplexed (so that the data can be accumulated and shared between laboratories)
- Genome-specific in nature (especially with polyploids)

- No detrimental effect on phenotype

DNA based markers can be classified as hybridization-bascd markers and polymerase chain reaction (PCR)-based markers. In hybridization based markers a labeled probe is used to visualize the DNA profile of restricted DNA vz. RFLP. Whereas, PCR based markers involve in vitro amplification of particular DNA sequences or loC1, with the heip ot specilically or arbitranly chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplitied fragments (amplicons) are separated clectrophoretically and banding pallens are detected by dilterent methods such as staining and autoradiagraphy, as in the case of RAPD, microsatellites, STMS and EST.

Types and deseription of DNA markers:

Markers Based On DNA Hybridization:

The DNA piece can be cloned, and allowed to hybridize with the genomic DNA which can be detected. Marker-based DNA hybridization is widely used. The major limitation of this approach is that it requires large quantities of DNA and the use of radioactivity (labeled probes).

Restriction fragment length polymorphism (RFLP):

RFLP markers were used for the first time in the construction of genetic maps by Botstein et al. in 1980. RFLP are codominant and show Mendelian inherilance pattern. The polymorphism in restricted fragments due to DNA rearrangements that occur due to evolutionary processes, point mutations with in the restriction enzyme recognition site sequences, insertions or deletions within the fragments, and uncqual crossing over. In RFLP, DNA polymorphism is detected by hybridizing a chemically labeled DNA probe to a Southern blot of DNA digested by restriction endonucleases, resulting in differential DNA fragment profile. Labelling of the probe may be performed with a radioactive isotope or with alternative nonradioactive stains, such as digoxigenin or fluorescein. Probes are generated through the construction of genomic of complementary DNA (cDNA) libraries and hence may be composed of specific sequence of unknown identity (genomic DNA) or part of the sequence of a functional gene (exons only, cDNA). The hybridization results can be visualized by autoradiography (if the probes are radioactively labelled), or using chemiluminesence (if nonradioactive, enzyme-linked methods are used for probe labeling and detection). RFLPs correspond to DNA fragments, usually within the range of 2-10 kb, that have resulted from the digestion of genomic DNA with restriction enzymes. The differential profile is generated due to nucleotide substitutions or DNA rearrangements like insertion or deletion or single nucleotide polymorphisms. The RFLPs markers are relatively highly polymorphic, codominanltly inherited and highly reproducible. RFLPs are applied in diversity and phylogenetic studies ranging from individuals within populations or species, to closely related species. RFLPs have been widely used in gene mapping studies because of their high genomic abundance due to the ample availability of different restriction enzymes and random distribution throughout the genome.

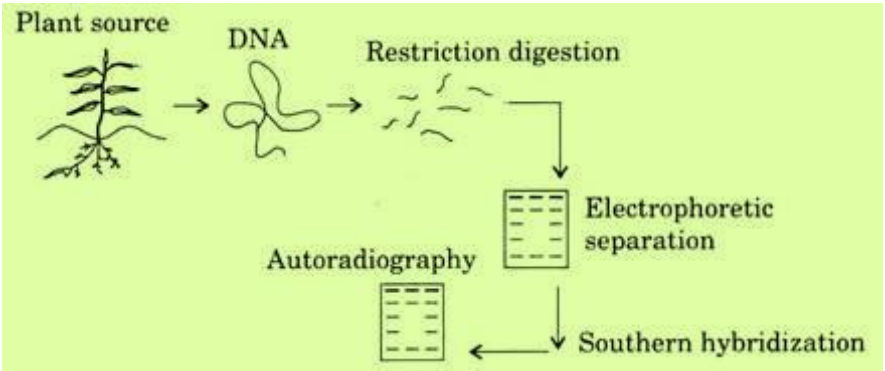
Steps:

Following are the steps involved in a typical RFLP assay

(1) Restriction digestion of genomic DNA using restriction endonuclease

(2) Resolving restriction genomic fragments through gel electrophoresis

(3) Transfer of resolved fragments from gel to nitrocellulose membrane using southern blotting



(4) Membrane containing DNA fragments hybridized with labeled probe using southern hybridization

(5) Detection of polymorphism through autoradiography or chemiluminescent technique.

Based on the presence of restriction sites, DNA fragments of different lengths can be generated by using different restriction enzymes. In the Fig., two DNA molecules from two plants (A and B) are shown. In plant A, a mutations has occurred leading to the loss of restriction site that can be digested by EcoRI.

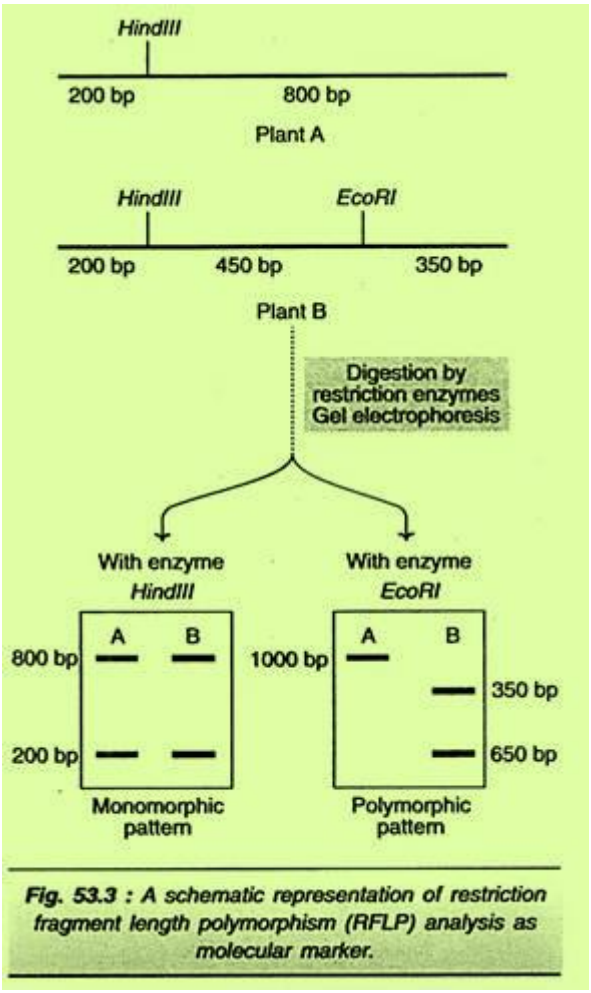


Fig. 53.3 : A schematic representation of restriction fragment length polymorphism (RFLP) analysis as molecular marker.

The result is that when the DNA molecules are digested by the enzyme Hindlll, there is no difference in the DNA fragments separated. However, with the enzyme EcoRI, plant A DNA molecule is not digested while plant B DNA molecule is digested. This results in a polymorphic pattern of separation.

Advantages of RFLP:

a. Present everywhere,
b. Mendelian inheritance,
c. Co-dominant expression,
d. No pleiotropic effects,
e. Independent of the environment,
f. Present at each developmental stage,
g. Long stability of cDNA probes,
h. Different loci may be identified by one probe,
i. Heterologous genes may be used as probes,
j. Any number of probes can be produced,

k. Probes are producible for coding and silent sequences,
l. Probes show the variability of flanking sequences,
m. Several characters can be screened in the same sample.

Disadvantages:

Developing sets of RFLP probes and markers is labour intensive. This technique requires large amount of high quality DNA. The multiplex ratio is low, typically one per gel. The genotyping throughput is low. It involves use of radioactive chemicals. RFLP finger prints for multi-gene families are often complex and difficult to score. RFLP probes cannot be shared between laboratories.

Uses:

They can be used in determining paternity cases. In criminal cases, they can be used in determining source of DNA sample. They can be used to determine the disease status of an individual. They are useful in gene mapping, germplasm characterization and marker assisted selection. They are useful in detection of pathogen in plants even if it is in latent stage.

Randomly Amplified Polymorphic DNA (RAPD):

Randomly amplified polymorphie DNA markers (RAPD): In 1991, Welsh and McClelland developed a new PCR-based genetic assay namely randomly amplified polymorphic DNA (RAPD) This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer an arbitrary nucleotide (8-12 bp) sequence. The primer anneals to complimentary sequence in template DNA in forward or reverse direction at multitude location of genonme. The amplification occurs between forward and reverse annealing generally 150-4000 bp apart, by resolving the resulting amplicons, profile with multiple bands can be secen. No knowledge of the DNA sequence for the targetes gene is required, as the primers will bind somewherc in the sequence, but it is not certain exactly where. This marker show lack of reproducibility and the assay is sensitive variation in DNA concentration. They are dominant markers and hence have limtaions in their use as markers for mappng, which can be overcome to some extent by selecting those markers that are linked in coupling.

RAPD assay has been used by several groups as efficient tools for identication of markers linked to agronomicaly important traits, whch are introgressed during the development of near isogenic lines (NILs).

Steps:

Important steps of RAPD can be summarized as follows.

Extraction of DNA

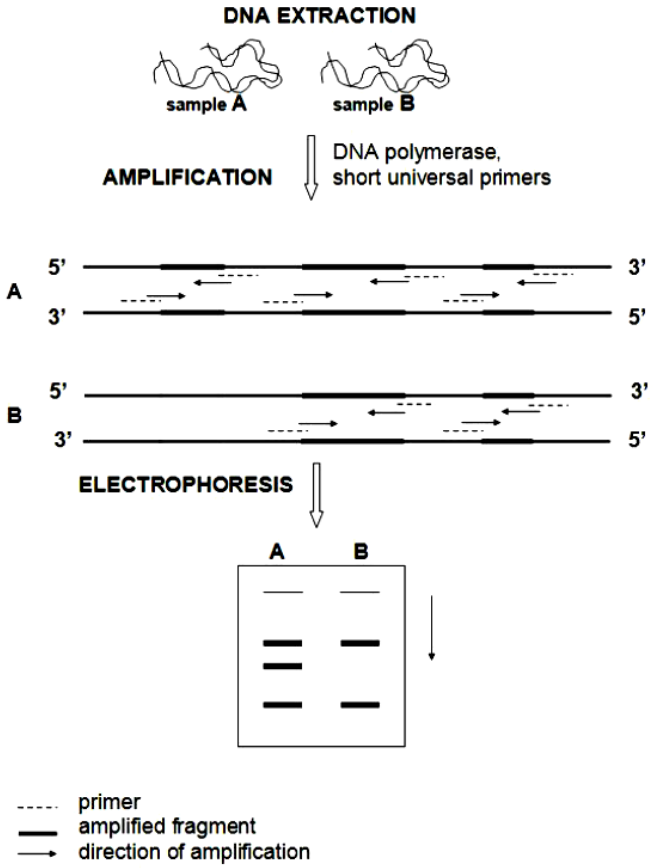
DNA is extracted by various methods. It also depends upon the species of plant under study but the basic procedure remains the same. Genomic DNA is basically extracted and purified from plant cell using proteinase K digestion and standard phenol: Chloroform extraction as per the standard protocol.

Selection of Primers

Random Amplified Polymorphic DNA (RAPD) is a multiplex marker system that conventionally uses single primer PCR to amplify random DNA fragments. Because of its multiplex nature, it is frequently used in Bulked Segregant Analysis (BSA). In view of the very large numbers of markers BSA often requires the use of mixtures of primers as a method of increasing the number of markers available. Theoretically, if a singleprimer reaction produces x bands on average, an unrestrained PCR process using a primers should produce xa2 bands. A total of 40 random oligonucleotide primers were used for amplification. All the random primers were 10 bp long and with high GC content and were custom synthesized from M/s Bangalore Genei, Bangalore, India a Yadav and Yadav (2007). The standard RAPD technology utilises short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR.

PCR Amplification

The polymerase Chain Reaction (PCR) is a relatively simple but powerful technique that amplifies a DNA template to produce multiple copies of specific DNA fragment in vitro. The bases (complementary to the template) are coupled to the primer on the 3' end (the polymerase adds dNTP's from 5'to 3', reading the template from 3'to 5' side; bases are added complementary to the template). 1. Using fresh clean tips, all the reagents are added to an autoclaved microfuge tube placed on ice. The PCR machine is programmed for the specific reaction conditions desired. After completion of the PCR reaction, the tubes are removed from the temperature block. The reaction products are separated according to size by agarose gel electrophoresis and visualized after staining the gel with ethidium bromide.



Agarose Gel Electrophoresis of PCR

After completion of the PCR programme, the products are checked in 2% agarose for the amplification. Before loading into the wells, gel loading dye (bromophenol blue in glycerol) is added to the sample and the samples are run under constant voltage condition (80 V) till the two dyes get separated. Amplified products appear as sharp orange color bands under UV Transilluminator due to the intercalation of ethidium bromide. To ensure that the amplified DNA bands originated from genomic DNA and not primer artifacts, negative control are carried out for each primer/breed combination (Galli and Satti, 2009) No amplification is detected in control reactions. All amplification products are found to be reproducible when reactions are repeated using the same reaction conditions.

Advantages:

RAPD primers are readily available being universal. They provide moderately high genotyping throughput. This technique is simple PCR assay (no blotting and no radioactivity). It does not require special equipment. Only PCR is needed. The start-up cost is low.

RAPD marker assays can be performed using very small DNA samples (5 to 25 ng per sample). RAPD primers are universal and can be commercially purchased. RAPD markers can be easily shared between laboratories. Locus-specific, co-dominant PCR-based markers can be developed from RAPD markers. It provides more polymorphism than RFLPs.

Disadvantages:

The detection of polymorphism is limited. The maximum polymorphic information content for any bi-allelic marker is 0.5. This technique only detects dominant markers. The reproducibility of RAPD assays across laboratories is often low. The homology of fragments across genotypes cannot be ascertained without mapping. It is not applicable in marker assisted breeding programme.

Uses:

This technique can be used in various ways such as for varietal identification, DNA fingerprinting, gene tagging and construction of linkage maps. It can also be used to study phylogenetic relationship among species and sub-species and assessment of variability in breeding populations.

Amplified Fragment Length Polymorphisms (AFLP)

To overcome the limitation of reproducibility associated with RAPD, AFLP technology (Vos et al., 1995) was developed. It combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA and selective PCR amplification of restriction fragments using a limited set of primers. The DNA is cut with two restriction enzymes, one being a frequent cutter and the other an infrequent cutter. This is followed by ligation of adapters, including restriction motifs followed by a two-step PCR amplification of selected fragments. The selective amplification uses primers composed of the adapters and 1 to 3 selected nucleotides at the 3' end. It limits the number of fragments to a resolvable range. The PCR-amplified fragments can then be separated by gel electrophoresis and banding patterns visualized. A range of enzymes and primers are available to manipulate the complexity of AFLP fingerprints to suit application. The AFLP banding profiles are the result of variations in the restriction sites or in the intervening region.

The AFLP technique simultaneously generates fragments from many genomic sites (usually 50-100 fragments per reaction) that are separated by polyacrylamide gel electrophoresis and that are generally scored as dominant markers. However, by use of automatic gel scanner heterozygote may be distinguished from homozygote based on band intensity differences, which facilitates the scoring of many AFLPs as codominant markers. The AFLP technique generates fingerprints of any DNA regardless of its source, and without any prior knowledge of DNA sequence. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping.

Steps

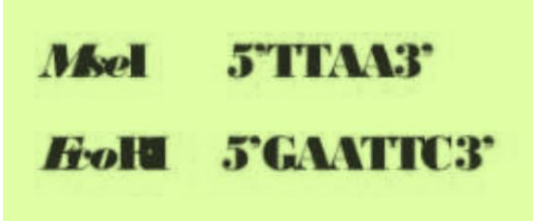
Step 1: DNA Extraction:

In the first step of AFLP clean and high molecular weight DNA is extracted using CTAB procedure.

Step 2: Restriction Digestion:

Restriction fragments of the genomic DNA are produced by using two different restriction enzymes: a frequent cutter (the four-base restriction enzyme Msel) and a rare cutter (the six-base restriction enzyme EcoRI).

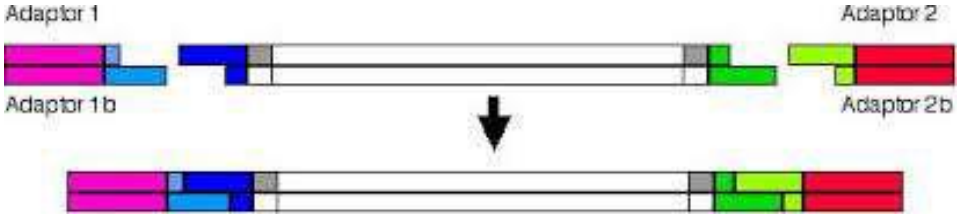
The frequent cutter serves to generate small fragments, which amplify well and which have the optimal size range for separation on a sequence gel, whereas the rare cutter limits the number of fragments to be amplified.



Step 3: Ligation of Oligonucleotide Adapters:

Double-stranded adapters consist of a core sequence and an enzyme-specific sequence. Therefore, adapters are specific for either the EcoRI site or the MseI site. Usually restriction and ligation take place in a single reaction.

Ligation of the adapter to the restricted DNA alters the restriction site in order to prevent a second restriction from taking place after ligation has occurred. The core sequence of the adapters consists of a known DNA sequence of 20 nucleotides, which will be used later as primer in the PCR.



Step 4: Pre-Amplification:

This step is a normal PCR where the adapters are used as primers. This first PCR, called pre-amplification, allows a first selection of fragments by only amplifying the DNA restriction fragments that have ligated an adaptor to both extremities.

Step 5: Amplification:

The aim of this step is to restrict the level of polymorphism and to label the DNA. For this second amplification, we added three more nucleotides at the 3′ end of the primer sequence used for the pre-amplification (adaptors sequence + 3 nucleotides). These two additional nucleotides make the amplification more selective and will decrease the number of restriction fragments amplified (polymorphism).

Step 6: Electrophoresis:

The PCR products are denaturized and run on acrylamide gel.

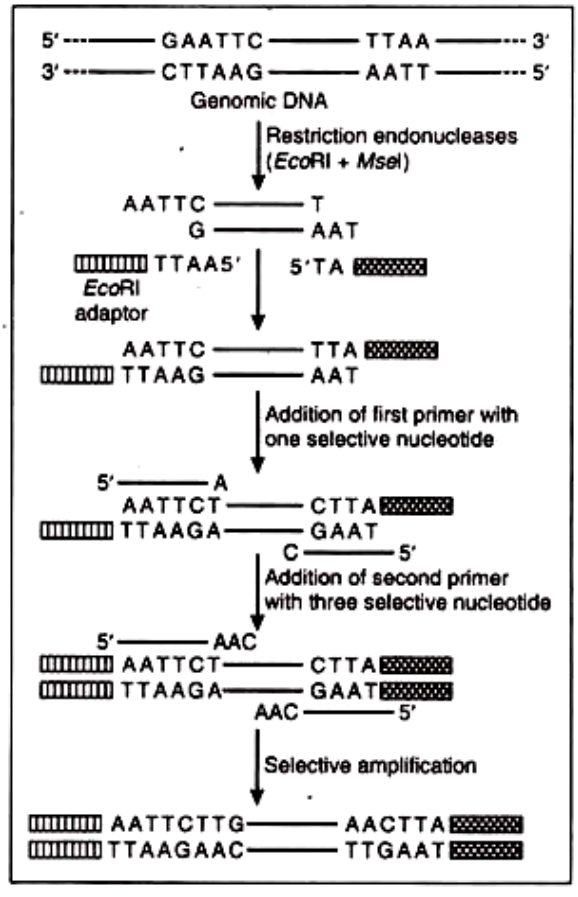


Fig. 8.4: Steps involved in AFLP

Advantages:

It provides very high multiplex ratio and genotyping throughput. These are highly reproducible across laboratories. No marker development work is needed; however, AFLP primer screening is often necessary to identify optimal primer specificities and combinations.

No special instrumentation is needed for performing AFLP assays; however, special instrumentation is needed for co-dominant scoring.

Start-up costs are moderately low. AFLP assays can be performed using very small DNA samples (typically 0.2 to 2.5 pg per individual). The technology can be applied to virtually any organism with minimal initial development.

Disadvantages:

The maximum polymorphic information content for any bi-allelic marker is 0.5. High quality DNA is needed to ensure complete restriction enzyme digestion. DNA quality may or may not be a weakness depending on the species. Rapid methods for isolating DNA may not produce sufficiently clean template DNA for AFLP analysis.

Proprietary technology is needed to score heterozygotes and ++ homozygotes. Otherwise, AFLPs must be dominantly scored. Dominance may or may not be a weakness depending on the application.

The homology of a restriction fragment cannot be unequivocally ascertained across genotypes or mapping populations. Developing locus specific markers from individual fragments can be difficult and does not seem to be widely done. The switch to non-radioactive assays has not been rapid. Chemiluminescent AFLP fingerprinting methods have been developed and seem to work well.

The fingerprints produced by fluorescent AFLP assay methods are often difficult to interpret and score and thus do not seem to be widely used. AFLP markers often densely cluster in centromeric regions in species with large genomes, e.g., barley (*Hordeum vulgare* L.) and sunflower (*Helianthus annuus* L.)

Uses:

This technique has been widely used in the construction of genetic maps containing high densities of DNA marker. In plant breeding and genetics, AFLP markers are used in varietal identification, germplasm characterization, gene tagging and marker assisted selection.

Single Nucleotide Polymorphism (SNP):

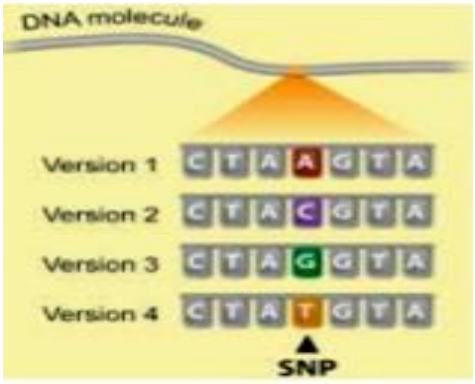
The variations which are found at a single nucleotide position are known as single nucleotide polymorphisms or SNP. Such variation results due to substitution, deletion or insertion. This type of polymorphisms has two alleles and also called bialleleic loci. This is the most common class of DNA polymorphism. It is found both in natural lines and after induced mutagenesis. Main features of SNP markers are given below.

1. SNP markers are highly polymorphic and mostly bialleleic.
2. The genotyping throughput is very high.
3. SNP markers are locus specific.
4. Such variation results due to substitution, deletion or insertion.
5. SNP markers are excellent long term investment.
6. SNP markers can be used to pinpoint functional polymorphism.
7. This technique requires small amount of DNA.

SNPs are found in

- coding and (mostly) noncoding regions.
- Occur with a very high frequency
- about 1 in 1000 bases to 1 in 100 to 300 bases.
- The abundance of SNPs and the ease with which they can be measured make these genetic variations significant.

- SNPs close to particular gene acts as a marker for that gene.
- SNPs in coding regions may alter the protein structure made by that coding region.



SNPs Discovery

1. Sequence databases searches
2. Target specific SNP discovery and development

-Conformation-based mutation scanning

-Direct DNA sequencing

Identification of Target Specific SNPs

Steps:

1. Amplify the genes of interests with PCR
2. Scan for mutation with various methods

-Conformation-based mutation scanning

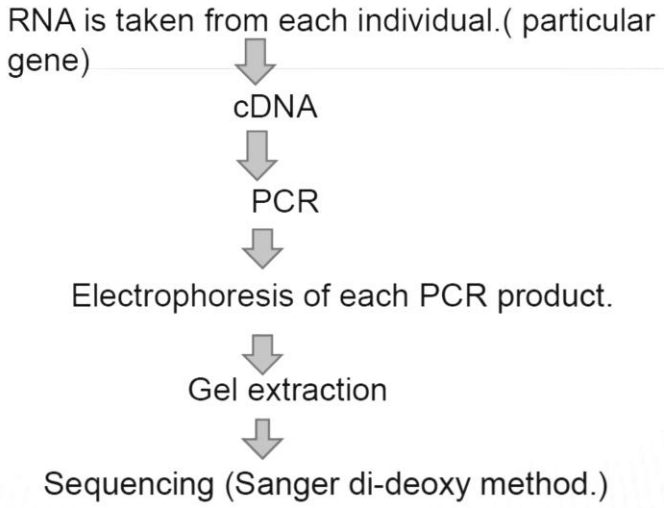
- Single -strand conformation polymorphism analysis
- Gel electrophoresis
- Chemical and enzymatic mismatch cleavage detection
- Denaturing gradient gel electrophoresis
- Denaturing HPLC

4. Align sequences from different sources to find SNPs
3. Sequence positive PCR products

-Sequence multiple individuals

-Sequence heterozygotes

Development of SNP (direct sequencing Method)



Technologies for Detecting Known SNPs

Gel-Based Methods

-PCR-restriction fragment length polymorphism analysis

-PCR-based allelic specific amplification

-Oligonucleotide ligation assay genotyping
-Minisequencing(10~20base)

Non-Gel-Based High Through Genotyping Technologies

-Solution hybridization using fluorescence dyes
-Allelic specific ligation
-Allelic specific nucleotide incorporation
1. High resolution separation
2. Chemical color reaction
-DNA microarray genotyping

Allele-Specific Codominant PCR Strategy

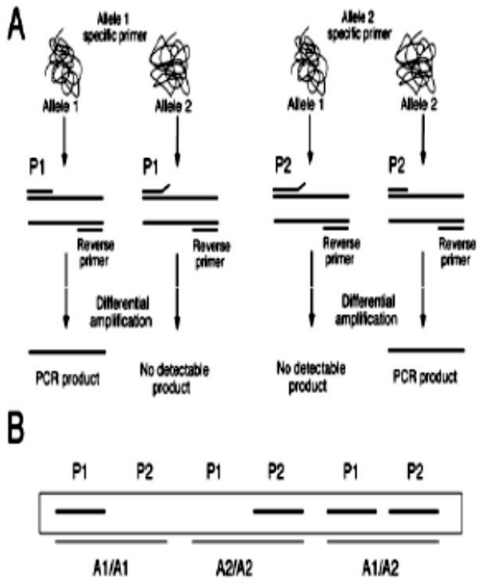


Figure. Schematic representation of the allele-specific codominant PCR strategy. Oligonucleotide primers with 3' nucleotides that correspond to an SNP site are used to preferentially amplify specific alleles. **A,** Primer P1 forms a perfect match with allele 1 but forms a mismatch at the 3' terminus with the DNA sequence of allele 2. Primer P2 similarly forms a perfect match with allele 2 and a 3' terminus mismatch with allele 1. **B,** Schematic of agarose gel analysis showing the expected outcome for the amplification of organisms homozygous and heterozygous for both alleles using primers P1 and P2. P1, Primer 1; P2, primer 2; A1, allele 1; A2, allele 2.

Eliana Drenkard et al. 2000 Plant Physiol 124: 1483-1492

Advantages:

SNP markers are useful in gene mapping. SNPs help in detection of mutations at molecular level. SNP markers are useful in positional cloning of a mutant locus. SNP markers are useful in detection of disease causing genes.

Disadvantages:

Most of the SNPs are bialleleic and less informative than SSRs. Multiplexing is not possible for all loci. Some SNP assay techniques are costly. Development of SNP markers is labour oriented. More (three times) SNPs are required in preparing genetic maps than SSR markers.

Uses:

SNPs are useful in preparing genetic maps. They have been used in preparing human genetic maps. In plant breeding, SNPs have been used to lesser extent.

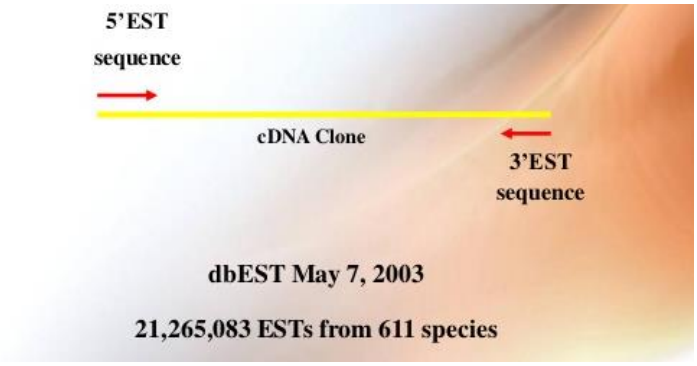
Expressed Sequence Tags (EST):

Expressed Sequence Tags (ESTs) are small pieces of DNA and their location and sequence on the chromosome are known. The variations which are found at a single nucleotide position are known.

The term Expressed Sequence Tags (ESTs) was first used by Venter and his colleagues in 1991. Main features of EST markers are given below.

1. ESTs are short DNA sequences (200-500 nucleotide long).
2. They are a type of sequence tagged sites (STS).
3. ESTs consist of exons only.

Single-pass sequencing reads from randomly selected cDNA clone



Steps for ESTs

•cDNA libraries (containing many of the expressed genes of an organism)
•pick cDNA clones randomly
•rapidly determine some of the sequence of nucleotides from the end of each clone.
•These ESTs could then be compared to all known sequences using a program called BLAST.

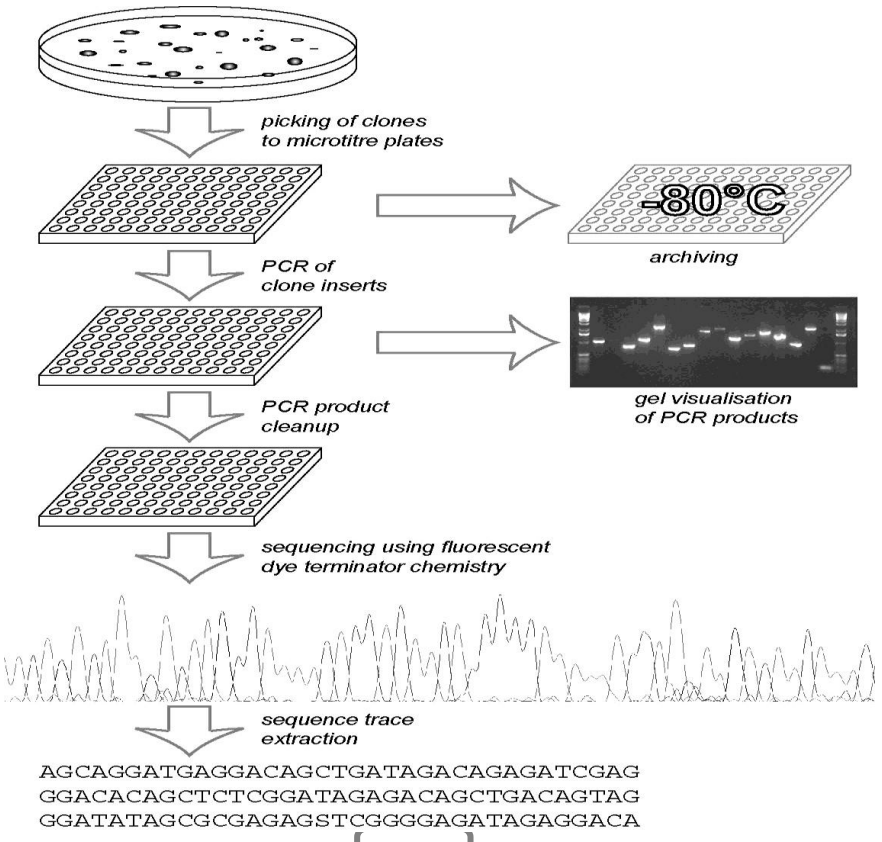
An exact match to a sequenced gene means that the gene encoding that EST is already known.

If the match was close but not exact one could conclude that the EST is derived from a gene with a function similar to that of the known gene.

The EST sequences with their putative identification are then deposited in the GenBank and the clones from which they were derived are kept in a freezer for later use.

Overview of the EST sequencing process

Clones are picked from petriedishes into microtitre plates, and archived for later use. All subsequent manipulations (PCR, clean up and sequencing) are carried out in microtitre plates to yield medium-throughput.



Advantages:

It is a rapid and inexpensive technique of locating a gene. ESTs are useful in discovering new genes related to genetic diseases. They can be used for tissue specific gene expression.

Disadvantages:

ESTs have lack of prime specificity. It is a time consuming and labour oriented technique. The precision is lesser than other techniques. It is difficult to obtain large (> 6kb) transcripts. Multiplexing is not possible for all loci.

Uses:

ESTs are commonly used to map genes of known function. They are also used for phylogenetic studies and generating DNA arrays.

Sequence Tagged Sites (STS):

In genomics, a sequence tagged site (STS) is a short DNA sequence that has a single copy in a genome and whose location and base sequence are known. Main features of STS markers are given below.

1. STSs are short DNA sequences (200-500 nucleotide long).
2. STSs occur only once in the genome.
3. STS are detected by PCR in the presence of all other genomic sequences.
4. STSs are derived from cDNAs.

Advantages:

STSs are useful in physical mapping of genes. This technique permits sharing of data across the laboratories. It is a rapid and most specific technique than DNA hybridization techniques. It has high degree of accuracy. It can be automated.

Disadvantages:

Development of STS is a difficult task. It is time consuming and labour oriented technique. It require high technical skill.

Uses:

STS is the most powerful physical mapping technique. It can be used to identify any locus on the chromosome. STSs are used as standard markers to find out gene in any region of the genome. It is used for constructing detailed maps of large genomes.

Microsatellites and mini-satellites

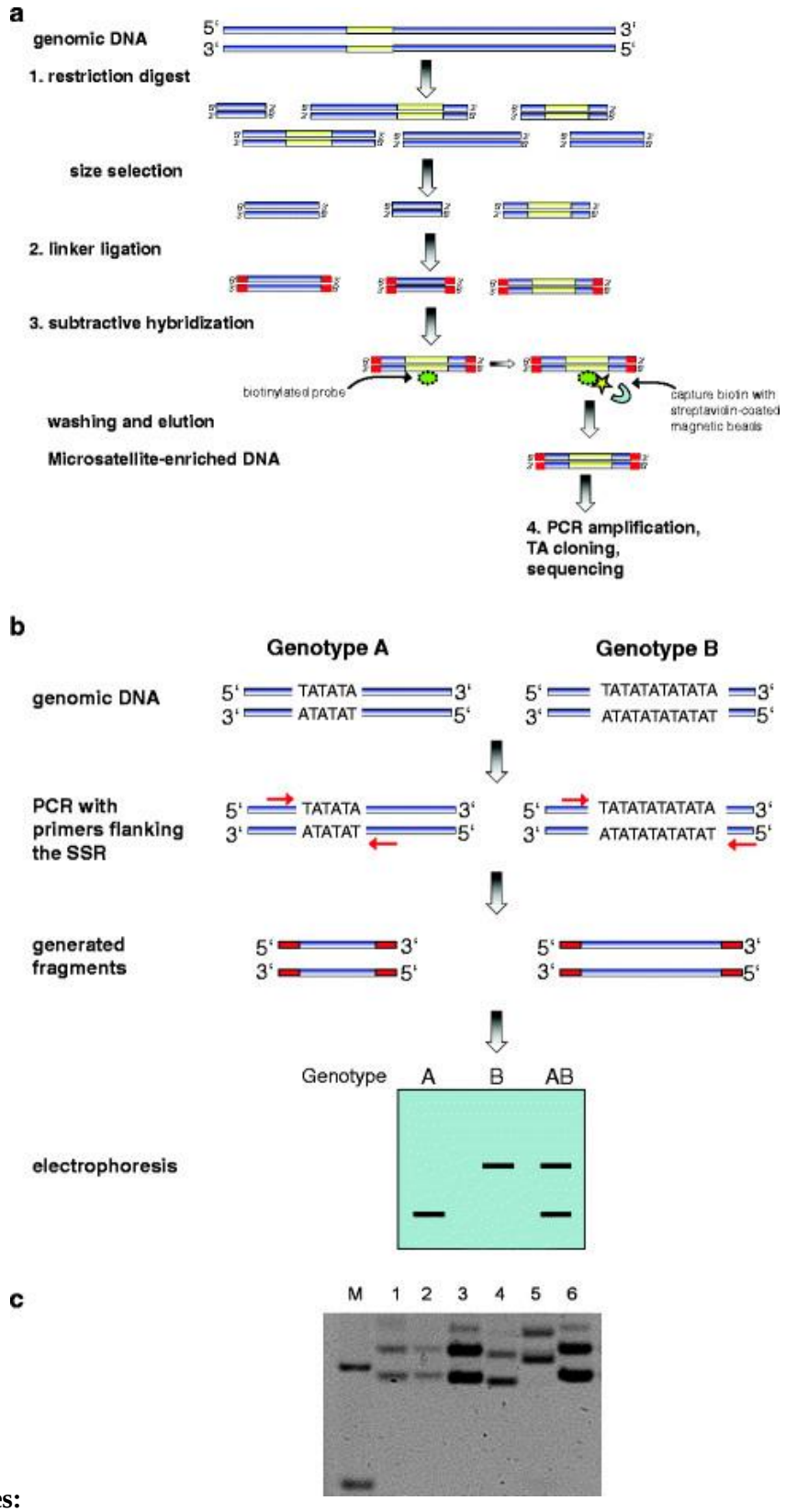
The tem microsatellites were coincd by Litt and Lutty (1989), while the term mini-satellites were used by Alec Jeffrey (1985). Both are multi-locus probes creating complex banding patterns. They essentially belong to the repetitive DNA family. Fingerprints generated by these probes are also known as oligo-nucleotide fingerprnts.

Mini-satellites are tandem repeats of DNA sequence with 10-100 bp repeat motifs whereas, microsatellites are tandem repeat of DNA sequence with 2-6 bp repeat motifs.These are also referred to as Variable Number o 1andem Repeats (VNTRS) and this is one of the basis of polymorphism at a locus. Many alleles exist in a population, the level of heterozygosity is high and they follow Mendelian inheritance.

Simple Sequence Repeats (SSRs):

Simple sequence repeats (SSRs) or microsatellites are tandemly repeated mono-, di-, tri-, tetra-, penta-, and hexanucleotide motifs. SSR length polymorphisms are caused by differences in the number of repeats. SSR loci are individually amplified by PCR using pairs of oligonucleotide primers specific to unique DNA sequences flanking the SSR sequence.

Jeffreys (1985) showed that some restriction fragment length polymorphisms are caused by VNTRs. The name **"mini satellite"** was coined because of the similarity of VNTRs to larger satellite DNA repeats.



Advantages:

SSR markers tend to be highly polymorphic. The genotyping throughput is high. This is a simple PCR assay. Many SSR markers are multi-allelic and highly polymorphic. SSR markers can be multiplexed, either functionally by pooling independent PCR products or by true multiplex- PCR. Semi-automated SSR genotyping methods have been developed. Most SSRs are co-dominant and locus specific.

No special equipment is needed for performing SSRs assays; however, special equipment is needed for some assay methods, e.g., semi-automated fluorescent assays performed on a DNA sequences. Start-up costs are low for manual assay methods (once the markers are developed). SSR assays can be performed using very small DNA samples (~100 ng per individual). SSR markers are easily shared between laboratories.

Disadvantages:

The development of SSRs is labor intensive. SSR marker development costs are very high. SSR markers are taxa specific. Start-up costs are high for automated SSR assay methods. Developing PCR multiplexes is difficult and expensive. Some markers may not multiplex.

Uses:

SSR markers are used for mapping of genes in eukaryotes.

Variable Number Tandem Repeat (VNTR):

A Variable Number Tandem Re-peat (VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and of-ten show variations in length between individuals. Each variant acts as an in-herited allele. Due to this reason VNTR can be used for personal or parental identification.

Use of VNTRs in Genetic Analysis:

VNTRs are frequently used in the develop-ment of linkage maps. Now that many ge-nomes have been sequenced, VNTRs have be-come essential to forensic crime investiga-tions, via DNA fingerprinting. When removed from surrounding DNA by the PCR or RFLP methods, and their size determined by gel electrophoresis or Southern blotting, they produce a pattern of bands unique to each individual.

When tested with a group of inde-pendent VNTR markers, the likelihood of two unrelated individuals having the same allelic pattern is extremely improbable. In the ex-ample considered in the diagram below locus A is a tandem repeat of the motif GC: there are four alleles, with two, three, four, or five repeats (A2, A3, A4, and A5, respectively).

Locus B is a tandem repeat of the motif AGCT: there are only two alleles, with two or three repeats (B2 and B3, respectively). Individual 1 is heterozygous at Locus A (A2/A5) and ho-mozygous at Locus 2 (B2/B2) which gives a single-banded phenotype in the fingerprint.

Individual 2 is heterozygous at both loci (A4/ A3 and B3/B2). The two individuals are dis-tinguishable at either locus. Typical finger-prints include a dozen or more VNTR loci. VNTR analysis is also being used to study ge-netic diversity and breeding patterns in populations of wild or domesticated animals.

Inter-Simple Sequence Repeats (ISSR)

The generation of ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite repeated sequence and in some cases primer also contains 1-4 base anchor at either 3′ or 5′ or at both ends, which target a subset of 'simple sequence repeats' (SSRs) and amplify the region between two closely spaced and oppositely oriented SSRs (Fang et al., 1997; Fang and Roose, 1997; Moreno et al., 1998). **I**SSR technique permits the detection of polymorphisms in microsatellites and inter-microsatellites loci without previous knowledge of the DNA sequence (Moreno et al., 1998). Some other microsatellites based on the same principle include the following:

(i) Randomly Amplified Microsatellite Polymorphism (RAMP): This is a micro satellite – based marker which shows a high degree of allellic polymorphism, but they are labor-intensive (Agarwal and Shrivastava, 2008). On the other hand RAPD markers are inexpensive but exhibit a low degree of polymorphism. To compensate for the weaknesses of these approaches, a technique termed as RAMP was developed (Wu et al, 1994). The technique involves a radiolabeled primer consisting of a 51 anchor and 31 repeats which is used to amplify genomic DNA in the presence or absence of RAPD primers. (Agarwal and Shrivastava, 2008).

(ii) The Sequence Characterized Amplified Region (SCAR): The SCARS are PCR-based markers that represent genomic DNA fragments at genetically defined loci that are identified by PCR amplification using sequence specific oligonucleotide primer (McDermoth et al, 1994).

(iii) Simple Primer Amplification Reaction (SPAR): SPAR uses the single SSR oligonucleotide principles.

(iv) Sequence – Related Amplified Polymorphism (SRAP): The aim of SRAP technique (Li and Quiros, 2001) is the amplification of open reading frames (ORFs). It is base on two-primer amplification using the AT- or GC- rich cores to amplify intragenic fragment for polymorphism detection (Agarwal and Shirvastava, 2008).

(v) Target region amplification polymorphism (TRAP): The TRAP technique (Hu and Vick, 2003) is a rapid and efficient PCR-based technique, which utilizes bioinformatics tools and expressed sequence tag (EST) database information to generate polymorphic markers, around targeted candidate gene sequences.

Type of DNA markers

	RFLP	PCR-based				
		RAPD	AFLP	SSR	STS	SNP
Principle	Restriction enzyme	Random priming	Selective PCR	SSR repeats	InDel length	DNA chip/ Hybridization
DNA required (μg)	10	0.02	0.5~1.0	0.02	0.02	0.02
Gel-based	Yes	Yes	Yes	Yes	Yes	Yes/No
Genomic abundance	High	Very high	Very high	Very high	Very high	Very high
Marker type	Codom.	Dom.	Dom.	Codom.	Codom.	Codom.
Reproducibility	Very high	Fair	Very high	Very high	Very high	Very high
Sequence informat. required	No	No	No	Yes	Yes	Yes
Ease of use	Labor intensive	Easy	Relat. difficult	Easy	Easy	Easy

Applcation of nmolecular marker in plant genomic analysis and breeding:

Molecular markers have evolved as potential tool lor a large number of applications ranging irom localization of a gene to improvement of plant varieties by marker assisted-Selection. With the advancement in the technology in the field of molecular marker our understanding in genetic analysis, and genomic has got signilicant impetus.

Fingerprinting of crop plants:

DNA fingerprinting refers to identify an individual unambiguously using multilocus DNA profiling. It can be done using hybridization markers, PCR based marker either locus specific amplification or by using random primers and sequencing. Huge number of scientific literature is available in various crops in the context of DNA fingerprinting. Alec Jeffery and his associates were the first to develop method of DNA fingerprinting through simulteneous detection of highly variable DNA fragments by hybridizing multilocus probes with electrophoretically seperated restriction fragments.

DNA fingerprinting has remarkable importance in plant vanety protection (PVP). And the utilites include identilication of cultivars and genotypes; true to type plants at juveline stage (DUS testing) for Seed purily mutants and chimeras; nucellar and zygotic embryos; somatic hybrids in fusion experiments and somaclonal variants etc.

Mapping and tagging of genes: Tools for MAS

Plant breeding is the science that aims at crop improvement, using the available variability. The outcome of crop improvement is selection of right kind of plant with right combination of genes/alleles. Conventional breeding take lot of time for evolution, identification and introgression of novel genes. Molecular markers have accelerated conventional plant breeding. It is a powerful tool for identification of diverse line, mapping and tagging of genes. With the use of molecular markers it is now a routine to trace valuable alleles in a segregating population. These markers once mapped enable dissection of the complex traits into component genetic units more precisely, thus providing breeder with a new tool to manage this complex unit more etficiently in breeding programme.

There are several examples of gene mapping and utility in marker assisted-selection in vaious crops using various molecular markers. The very first genome map in plants was reported in Maize, followed by rice, *Arabidopsis* etc. using RFLP markers.Maps have since then been constructed for several other crops like potato, barley, banana,members of Brassicaceae, etc.

Phylogeny and evolution:

Molecular markers are powerful tools in phylogenetic and evolutionary studies.These studies strengthened the eariler studies made based on morphological and cytological evidences for establishing relationship between the wild relatives of species and their cultivated species. The comprenensive studies on genetic structure using molecular markers have revaled evolutionary forces that led the wild relatives to the present cultivable fom of species. RFLP, DNA scquencing, and a number of PCR-based markers are being used exiensively for reconstructing phylogenies of various species. The tecniques are speculated to provide path-breaking information regarding the fine time scale on which closely related species have divergcd and what sort of genetic variations are associated with species formation. Furthermore, these studies hold a great promise for revealing more about the patterm of genetic variation within the species. In connection to plant breeding they are very much helpful in understanding the crop evolution from wild progenitor and to clasify them to appropiate groups.This would help in introgression of useful genes from wild progenitors into cultivated high yielding variaties ot crop species.

Diversity Analysis:

One of the important utility of molecular markers is diversity analysis. Lines with similar morphological characters may substantially diverse from each other at DNA level and vice-versa. Diversity analysis can be done based on pedigree data, biochemical data, and more recently molecular (DNA-based) data. DNA based markers can unambiguously distinguish two different lines The revealed through molecular markersb can be used to deduce genetic distance among the germplasm, breeding lines and population. There arc several utilities of diversity analysis like (1) selection of parents for developing hybrids (2) selection of parents for developing mapping population (3) to study genetic inheritance of a traít (4) combining ability studies (5) understanding the enviormental affect on geographically diverse lines (6) population genetic studics (7) identification of regions specifie fixed allelesin landraces.

The commonly used measures of genetic distance or genetic similarity (GS) using binary data are (1) Nei and Li's (1979) coefficient (GDNu), (2) Jaccard's (1908) (3) Sokal and Michener's (1958) simple matching coefficient(GDsm), and (4) Modified Rogers distance, (1972) (GDuR).

Functional Genomics:

It may be defined as the determination of the function of all the gene products encoded by the genome of an organism. It includes the following parameters: (1) when and where particular genes are expressed (expression profiling), (ii) the functions of specific genes by selectively mutating the desired genes, and (iii) the interactions that take place among proteins and between protein and other molecules. Functional genomics attempts to examine all the genes present in the genome in one go.

Therefore, the techniques used in functional genomics enable high throughput analysis that enable a very rapid data accumulation.

(i) Expression Profiling:

Determination of the cell types/tissues in which a gene is expressed as well as when the gene is expressed is called expression profiling. The aim of functional genomics is to study the expression pattern of all the genes present in the genome at the same time; this is called global expression profiling. This can be done either at the RNA level or at the protein level. At the RNA level, one could either use direct sequence sampling or DNA arrays.

At the protein level, one may use either two dimensional electrophoresis, followed by mass spectrometry or protein arrays. Global expression profiling provides insights into complex biological phenomena, including differentiation, response to stress, onset of a disease, etc. It also provides a new way to define cellular phenotypes.

(ii) Gene Function Determination:

An important aspect of functional genomics is to determine the function of specific genes/anonymous sequences. A potent way is to achieve this is to clone the gene, mutate it in vitro and reintroduce the mutated gene into the host organism and analyse its effect. Genome under mutant libraries have been developed in several model organisms like bacteria, yeast, plants and mammals. This is sometimes referred as mutational genomics. Such a library can be generated in one of the following three ways:

(a) Systematic mutation of every single gene one at time which will generate a bank of specific mutant strains.

(b) In the random approach, genes are mutated indiscriminately individual mutations are then characterized and catalogued.

(c) In this approach, a group of techniques is used to prevent the expression of specific/groups of genes.

(iii) Protein Interactions:

Gene function reflects the behaviour of proteins encoded by them. This behaviour may be seen as a series of interactions among various proteins, and between proteins and other molecules. Protein interactions are studied using high throughput techniques. A number of library-based protein interaction mapping methods allows hundreds or thousands of proteins to be screened at a time. These interactions may be assayed in vitro or in vivo. Protein interaction data from various sources are assimilated in databases.

Chromosome Walking

This technique is used for characterising large regions of chromosomes. Uenerally. a cosmid library is used for chromosome walking; each clone in such a library may be expected to have an insert, on an average of 50 kb.

In chromosome walking, one begins with a DNA fragment that contains a known gene/genetic marker. The sequence located at one end of this fragment is used to identily a clone that has such DNA Insert, which partly overlaps the first fragment. Now the other (nonoverlapping) end or his new DNA fragment is used as probe. In this way, one continues to move step-by-step toward a gene ol interest located close to the known gene/genetic marker. This technique, therefore, is called chromosome walking because each clone takes the researcher one step closer to the gene of interest. A generalised procedure for chromosome walking is given below.

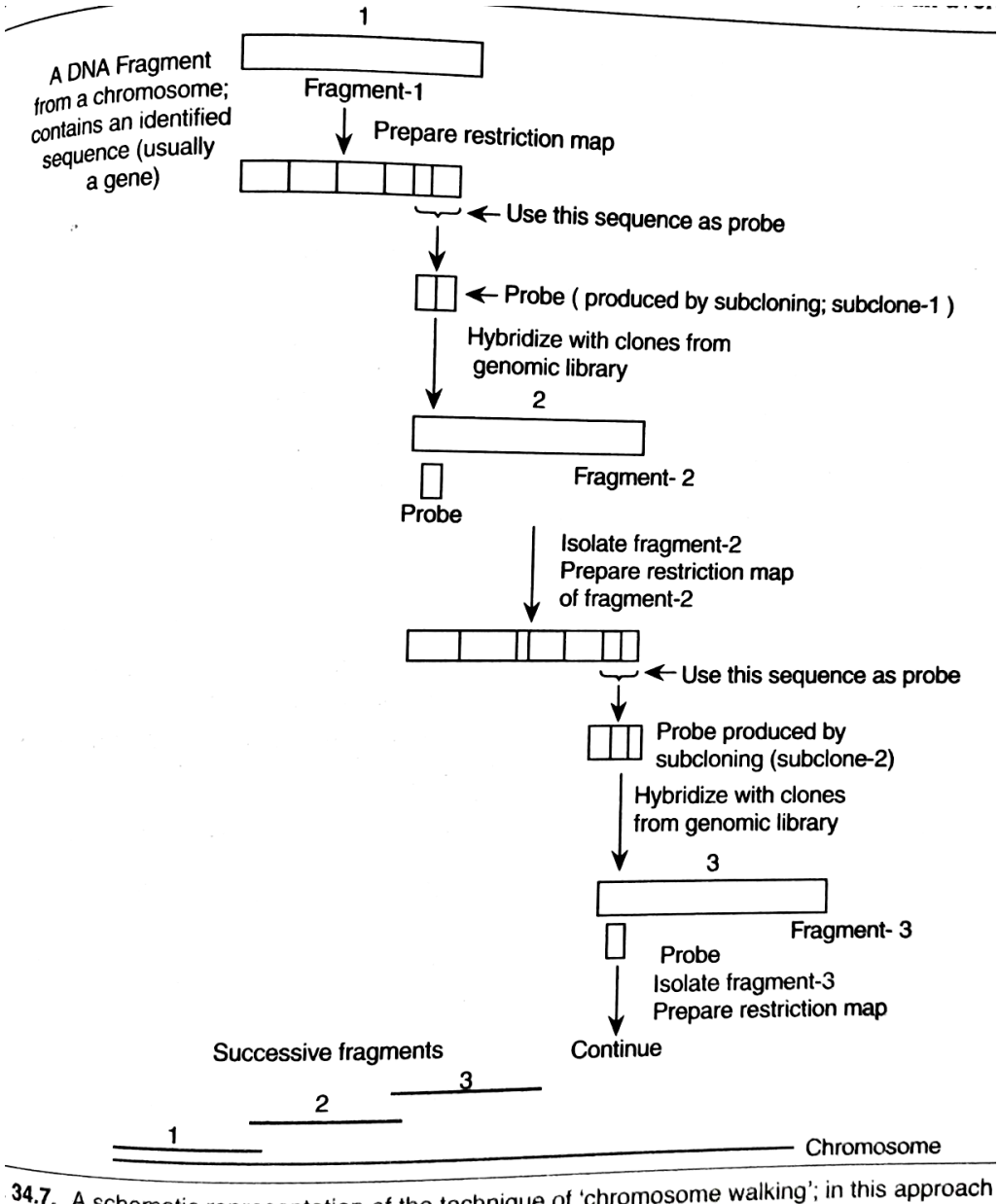
1. The first step in "walking" a chromosome consists of the isolation of a DNA fragment (fragment 1) containing a known gene/marker located near some region of interest in the given chromosome. This fragment provides the starting point for the 'chromosome walk; it also provides a point of reference in the genetic map.

2. A restriction map of this fragment is prepared. A small segment representing one end (or a region Close to one end) of this original fragment (fragment 1) is isolated and cloned: this is called **subcloning.** This subclone is now used as a probe for the identification of such clone(s) in the genomic library that overlap fragment.

3. The clone(s) identified in this way (by probing with the subclone of fragment 1) will contain such a DNA fragment that overlaps fragment-1, preferably at one end; the new fragment may be referred to as fragment 2. A restriction map of fragment 2 is prepared, and the sequence at the other end of this fragment is now used as a probe to identify clones having DNA fragments overlapping with fragment 2.

4. The DNA fragments obtained from such clones will be overlapping with fragment 2 preferably at one end; the new fragment may be called fragment 3. The process of step 3 is repeated till we reach one end or the chromosome.

We may also use the other end of fragment 1 as probe to identify overlapping clones: his permits 'walking' the chromsome in the opposite direction till the other end of chromosome is reached. Ideally, the subcloned sequence used as probe to isolate the next fragment should be complementary to one end of the new fragment.



34.7. A schematic representation of the technique of 'chromosome walking'; in this approach the sequence at one end of a fragment is used as probe to identify the next

Application:

- This technique can be used for the analysis of genetically transmitted diseases, to look for mutations.
- Chromosome Walking is used in the discovery of single-nucleotide polymorphism of different organisms.

Disadvantages

- There is a **limitation** to **the speed of chromosome walking** because of the small size of the fragments that are to be cloned.
- Another limitation is the difficulty of walking through the **repeated sequence** that are scattered through the gene.
- If the **markers were too far away**, it simply was not a viable option.
- Additionally, chromosome walking could easily be stopped by **unclonable sections of DNA**.
- A solution to this problem was achieved with the advent of **chromosome jumping** (Marx, 1989), which allows the skipping of unclonable sections of DNA.

Microarray:

Microarray technologies as a whole provide new tools that transform the way scientific experiments are carried out. The principle advantage of microarray technologies compared with traditional methods is one of scale. In place of conducting experiments based on results from one or a few genes, microarrays allow for the simultaneous interrogation of hundreds or thousands of genes.

Microarrays are microscope slides that contain an ordered series of samples (DNA, RNA, protein, tissue). The type of microarray depends upon the material placed onto the slide: DNA, DNA microarray; RNA, RNA microarray; protein, protein microarray; tissue, tissue microarray.

Since the samples are arranged in an ordered fashion, data obtained from the microarray can be traced back to any of the samples. This means that genes on the microarray are addressable. The number of ordered samples on a microarray can number into the hundred of thousands. The typical microarray contains several thousands of addressable genes.

Types of Microarrays:

1. DNA Microarray:

Basic Principle:

Complementary sequences of nucleotides stick to, or "hybridize" to, one another. For example, a DNA molecule with the sequence -A-T-T- G-C- will hybridize to another with the sequence -TA-A-C-G- to form double-stranded DNA.

Types of DNA Microarray:

There are two major types of DNA microarrays. Each type of microarray is manufactured differently.

(a) cDNA Microarray:

This type contains cDNA fragments 600 to 2400 nucleotides in length. When making a cDNA microarray, each of the different probes must be chosen independently and made by PCR or traditional cloning. Then all the DNA probes are spotted onto the slide.

(b) Oligonucleotide Microarray:

This type uses oligonucleotides of 20 to 50 nucleotides in length. When making an oligonucleotide array, the oligonucleotide is synthesized directly on the slide.

A. cDNA Microarrays:

Steps in cDNA Microarray:

1. Extracting and labelling the RNA Sample:

A typical workflow of the microarray experiment has been summarized in Figure below. Once microarrays have been made and obtained, the next stage is to obtain samples for labelling and hybridization.

Labelling RNA for expression analysis generally involves three steps:

(a) Isolation of RNA.

(b) Labelling the RNA by a reverse transcription procedure with fluorescent markers.

(c) Purification of the labelled products.

(a) Isolation:

RNA can be extracted from tissue or cell samples by common organic extraction procedures used in most molecular biology labs. Both total RNA and mRNA can be used for labelling, but the contaminating genomic DNA must be removed by DNase treatment.

The amount of total RNA necessary for a single labelling reaction is about 20 µg while the amount of mRNA necessary is about 0.5 µg. Lesser amounts are known to work, but require extreme purity and well developed protocols.

It is generally a good idea to check the RNA samples before using them in microarray experiments. In fact, for many core facilities it is a requirement. This can be done by assaying the absorption ratio 260/280 lambda and/or running a sample on an ethidium bromide stained agarose gel.

(b) Labelling the RNA by Reverse Transcription:

Direct Labelling:

Direct labelling of the RNA is achieved by producing cDNA from the RNA by using the enzyme reverse transcriptase and then incorporating the fluorescent labels, most commonly Cy3 and Cy5. Other fluorophores are available (e.g., Cy3.5, TAMRO, Texas red) but have not yet found widespread use.

Indirect Labelling:

In the indirect procedure, a reactive group, usually a primary amine, is incorporated into the cDNA first, and the Cy3 or Cy5 is then coupled to the cDNA in a separate reaction. The advantage of the indirect method is a higher labelling efficiency due to the incorporation of a smaller molecule during the reverse transcription step.

(c) Purification of the Labelled Products:

Once fluorescently labelled probes are made, the free unincorporated nucleotides must be removed. This is typically done by column chromatography using convenient spin-columns or by ethanol precipitation of the sample.

Some protocols' perform both purification steps. As small a side, radioactivity is still around and may even make a comeback in microarrays. Incorporation of 33P- or 35S-labelled nucleotides into cDNAs have high rates and provide more sensitivity than fluorescently labelled probes.

2. Hybridization:

Conditions for hybridizing fluorescently labelled DNAs onto microarrays are remarkably similar to hybridizations for other molecular biology applications. Generally the hybridization solution contains salt in the form of buffered standard sodium citrate (SSC), a detergent such as sodium dodecyl sulphate (SDS), and nonspecific DNA such as yeast tRNA, salmon sperm DNA, and/or repetitive DNA such as human Cot-1. Other nonspecific blocking reagents used in hybridization reactions include bovine serum albumin or Denhardt's reagent. Lastly, the hybridization solution should contain the labelled cDNAs produced from the different RNA populations.

Hybridization temperatures vary depending upon the buffers used, but generally are performed at approximately 15-20°C below the melting temperature, which is 42-45°C for PCR products in 4X SSC and 42-50°C for long oligos.

Hybridization volumes vary widely from 20 µ1 to several mL. For small hybridization volumes, hydrophobic cover slips are used. For larger volumes, hybridization chambers can be used. Hybridization chambers are necessary to keep the temperature constant and resist the hybridization solution from evaporating.

In small volumes, the hybridization kinetics are rapid, so a few hours can yield reproducible results, although overnight hybridizations are more common.

3. Scanning:

Following hybridization, microarrays are washed for several minutes in decreasing salt buffers and finally dried, either by centrifugation of the slide, or a rinse in isopropanol followed by quick drying with nitrogen gas or filtered air. Fluorescently labelled microarrays can then be **"read"** with commercially available scanners.

Most microarray scanners are basically scanning confocal microscopes with lasers exciting at wavelengths specifically for Cy3 and Cy5, the typical dyes being used in experiments. The scanner excites the fluorescent dyes present at each spot on the microarray and the dye then emits at a characteristic wavelength that is captured in a photomultiplier tube.

The amount of signal emitted is directly in proportion to the amount of dye at the spot on the microarray and these values are obtained and quantitated on the scanner.

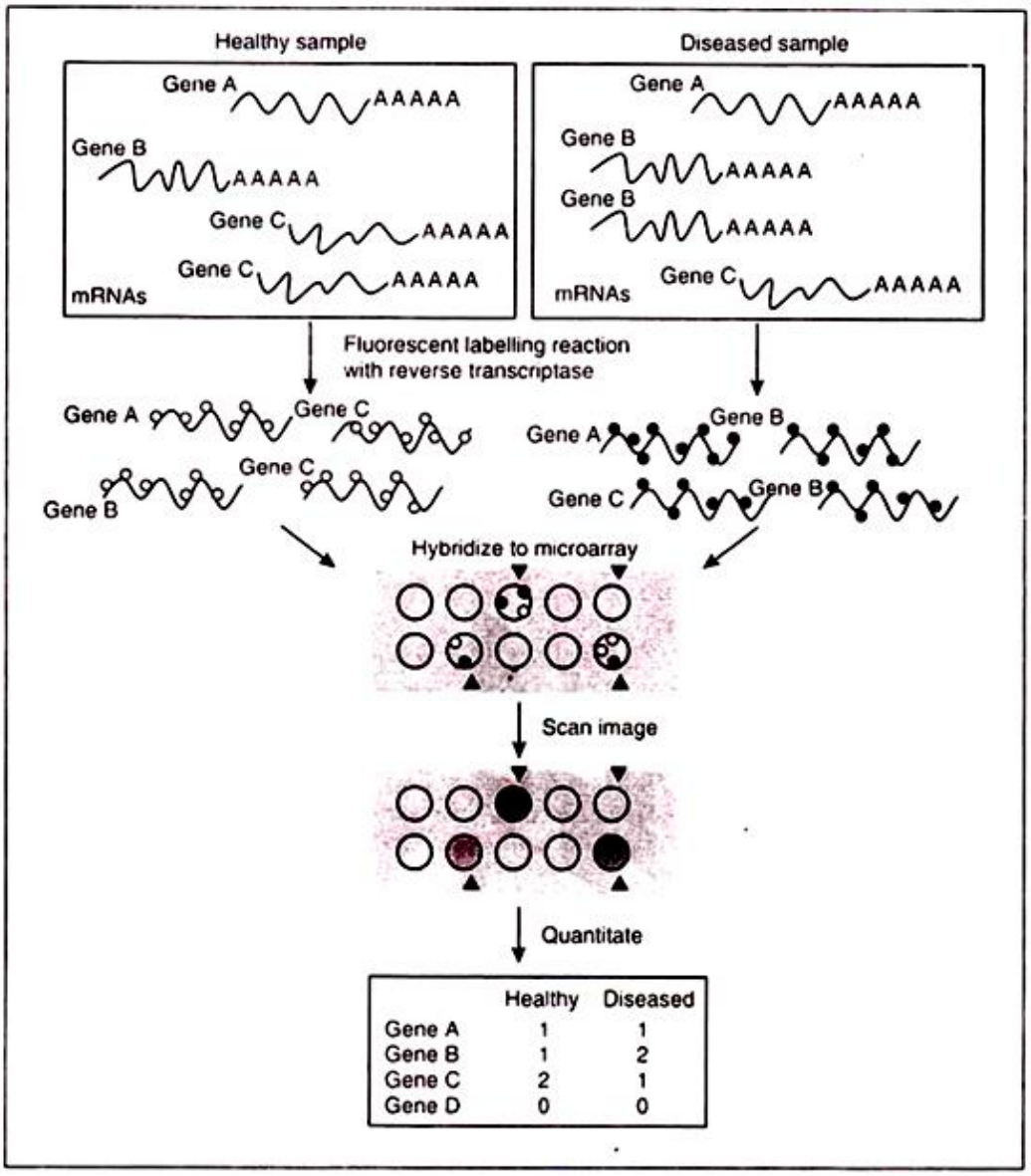


Fig. 9.1: Steps involved in a general microarray experiment

A reconstruction of the signals from each location on the microarray is then produced. For cDNA microarrays one intensity value is generated for the Cy3 and another for the Cy5. Hence, cDNA microarrays produce two-colour data.

Affymetrix chips produce one-colour data, because only one mRNA sample is hybridized to every chip. When both dyes are reconstructed together, a composite image is generated. This image produces the typical microarray picture.

Modern cDNA Technology:

Newer technology has been developed to decrease the variation in size of spotted samples in microarrays. In newer cDNA microarrays, the samples are spotted onto a glass slide using inkjet printer technology. The cDNA samples are sucked into separate chambers of the inkjet printer head, and then spotted onto the glass slide as much as ink is spotted onto paper in a printer.

Inkjet technology prevents variations in size and quantity of cDNA in the sample spots. Special adaptors have been developed to prevent the inkjet sample channels from mixing, thus preventing cross-contamination.

B. Oligonucleotide Microarray:

Oligonucleotides are traditionally synthesized chemically on beads of controlled pore glass (CPG). Therefore, it is not too much of a logical leap to synthesize many different oligonucleotides side-by-side on a glass slide.

The main differences between synthesizing single nucleotides on beads versus making arrays on glass slides is that the array has thousands of different oligonucleotides, and each must be synthesized in its proper location with a unique sequence. To accomplish this, photolithography and solid-phase DNA synthesis are combined.

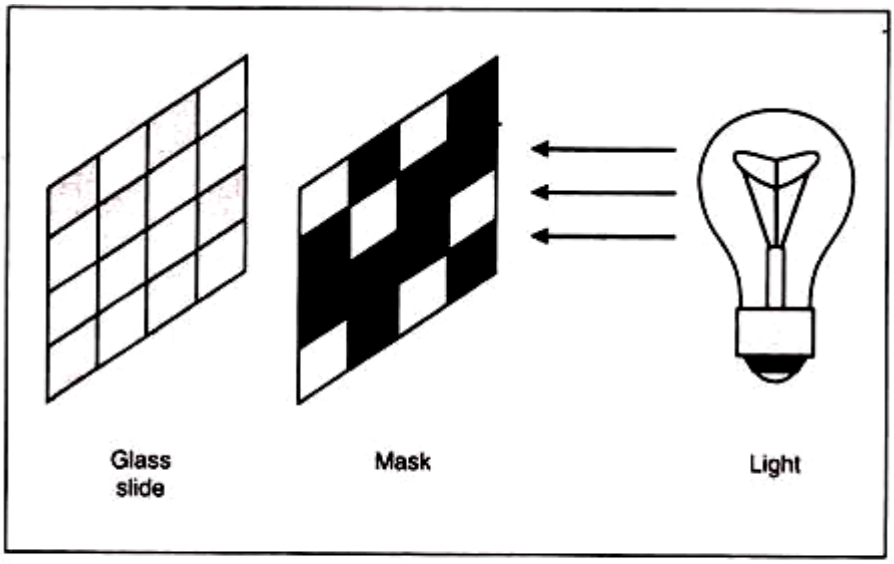


Fig. 9.2: Photolithography: Light passing through the mask makes a particular pattern on the glass slide. If the slide is coated with a light activated substance, only the regions that are illuminated will be activated for the addition of another nucleotide

Photolithography is a process used in making integrated circuits, where a mask makes a specific pattern of light on a solid surface. The light activates the surface it reaches, while the remaining surface remains inactivated.

A glass slide is first covered with a spacer that ends in a reactive group. This is then covered with a photosensitive blocking group that can be removed by light. In each synthetic cycle, those sites where

a particular nucleotide will be attached are illuminated to remove the blocking group. Each of the four nucleotides is added in turn.

At each addition, a mask is aligned with the glass slide. Light passes through holes in the mask and activates the ends of those growing oligonucleotide chains that it illuminates. Much as in traditional chemical synthesis, each nucleotide has its 52-OH protected. Thus after each addition, the end of the growing chain is blocked again.

These protective groups are light activated, so at each step, a new mask is aligned with the slide, and light deportments the appropriate nucleotides. The entire process continues for each nucleotide at each position on the glass slide. Making the masks is the key to this technology.

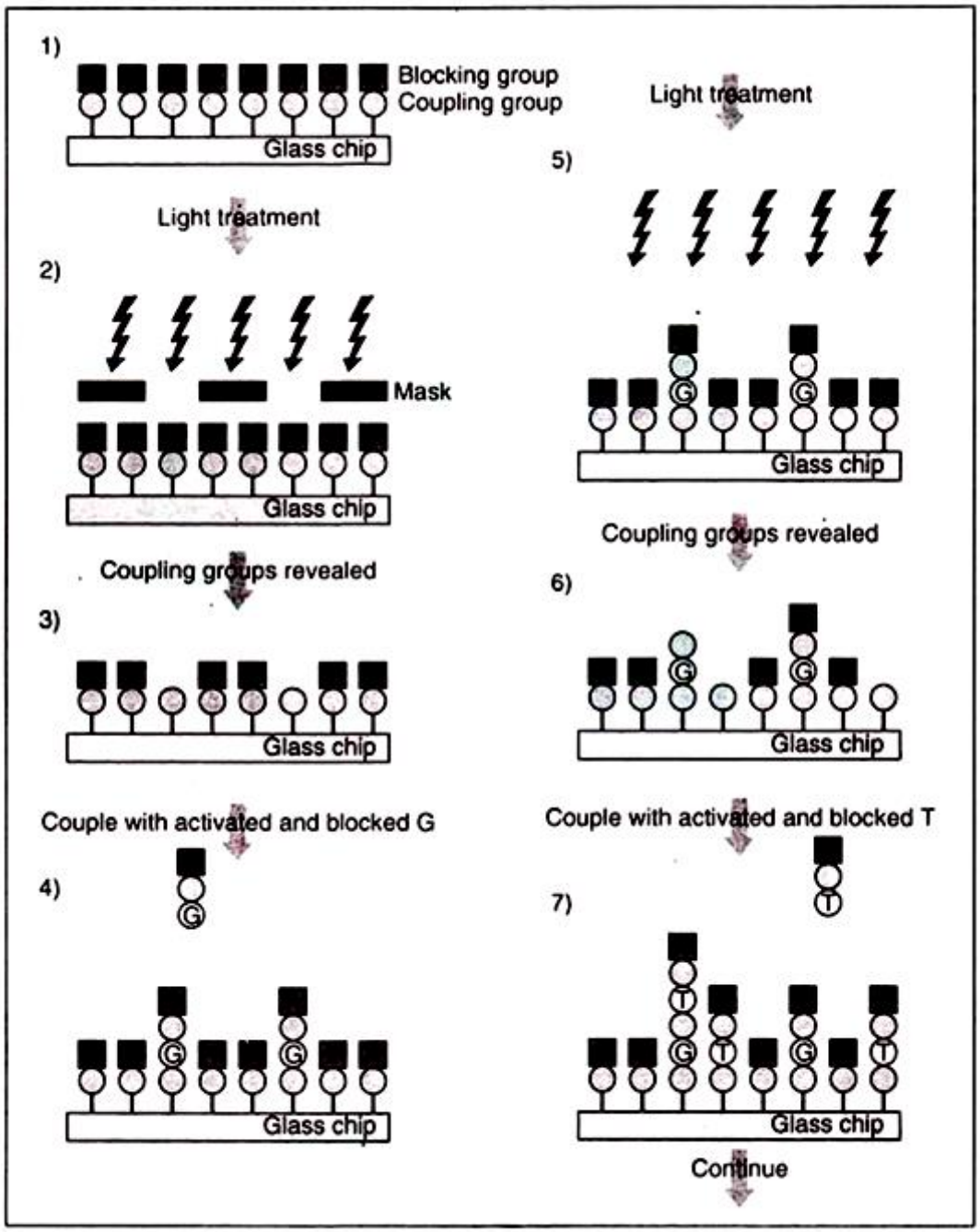


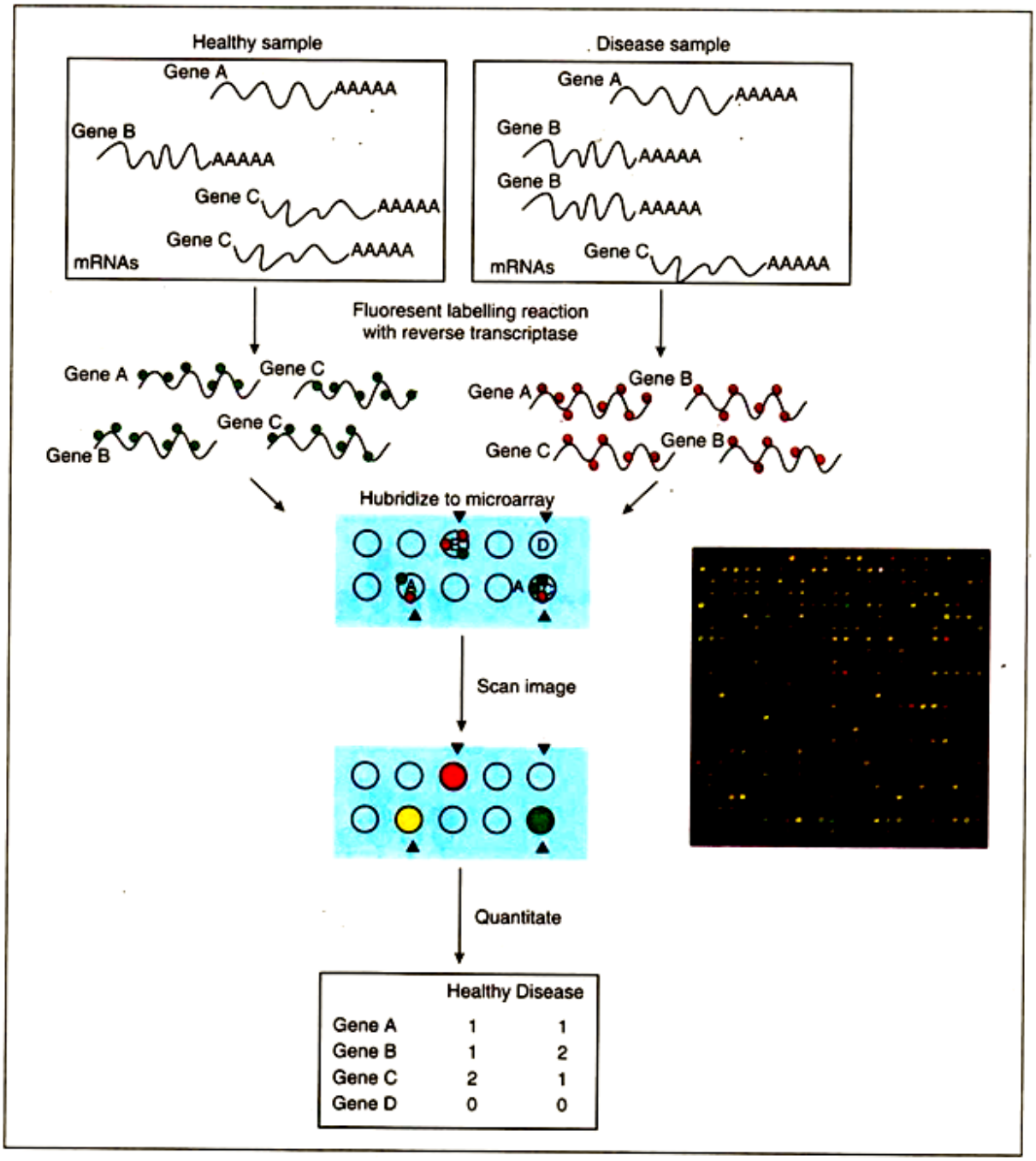
Fig. 9.3: On-Chip Synthesis of Oligonucleotides

On Chip Synthesis of Oligonucleotides:

Arrays may be created by chemically synthesizing oligonucleotides directly on the chip. First, spacers with reactive groups are linked to the glass chip and blocked. Then each of the four nucleotides is added in turn (in this example, G is added first, then T).

A mask covers the areas that should not be activated during any particular reaction. Light activates all the groups not covered with the mask, and a nucleotide is added to these. The cycle is repeated with the next nucleotide.

COLOUR PLATE



Work flow of a typical microarray experiment

Hybridization on DNA Microarrays:

Hybridization on a microarray is similar to the hybridization of DNA during other hybridization experiments, such as Southern blots, Northern blots, or dot blots. All these techniques rely on the complementary nature of double-stranded DNA.

When two complementary strands of DNA are near each other, the bases match with their complement, that is, thymine with adenine, and guanine with cy- to sine. On a DNA microarray, hybridization is affected by the same parameters as in these other techniques. How the DNA is attached to the slide can affect how well the probe DNA and target DNA hybridize, especially for oligonucleotide microarrays.

The short length of oligonucleotides requires that the entire piece be accessible to hybridize. The length of the spacer between the oligonucleotides and the glass slide optimizes hybridization. An oligonucleotide attached with a short spacer has many of its initial nucleotides too close to the glass and inaccessible to incoming RNA or DNA.

Oligonucleotides with longer spacers may fold back and tangle up; therefore, again the sequence is inaccessible for hybridization. Oligonucleotides attached with medium-sized spacers are far enough from the glass, but not so far as to get tangled. Thus medium-sized spacers give the best accessibility for hybridization.

Hybridization of two lengths of DNA (or RNA with DNA) requires certain sequence features. One important property is the relative number of A:T base pairs versus G:C base pairs. Because G:C base pairs have three hydrogen bonds holding them together, it takes more energy to dissolve the bonds. A:T base pairs have only two hydrogen bonds and require less energy.

Thus more GC base pairs give stronger hybridization. If the sequence has many A:T base pairs, the duplex may form slowly and be less stable. Another important consideration is secondary structure. If the target, the duplex may not form efficiently.

All these issues must be addressed when making an oligonucleotide microarray. Computer programs are available that identify suitable regions of genes with sequences that will produce effective probes.

Application of DNA Microarrays:

DNA microarrays are used to determine:

1. The expression levels of genes in a sample, commonly termed expression profiling.
2. The sequence of genes in a sample, commonly termed mini-sequencing for short nucleotide reads, and mutation or SNP analysis for single nucleotide reads.

2. Protein Microarrays:

A different but conceptually similar approach is being applied directly to proteins. Scientists have developed microarrays that can be used either to identify and quantify thousands of different proteins at once or to find associations between different kinds of proteins and between proteins and other molecules. These types of arrays are collectively referred to as protein arrays.

Protein-detecting arrays may be divided into those that use antibodies and those based on using tags. In the ELISA assay, antibodies to specific proteins are attached to a solid support, such as a micro-titre plate or glass slide. The protein sample is then added and if the target protein is present, it binds its complementary antibody. Bound proteins are detected by adding a labelled second antibody.

Another antibody-based protein-detecting array is the antigen capture immunoassay. Much like the ELISA, this method uses antibodies to various proteins bound to a solid surface. The experimental protein sample is isolated and labelled with a fluorescent dye. If two conditions are being compared, proteins from sample 1 can be labelled with Cy3, which fluoresces green, and proteins from sample 2 can be labelled with Cy5, which fluoresces red.

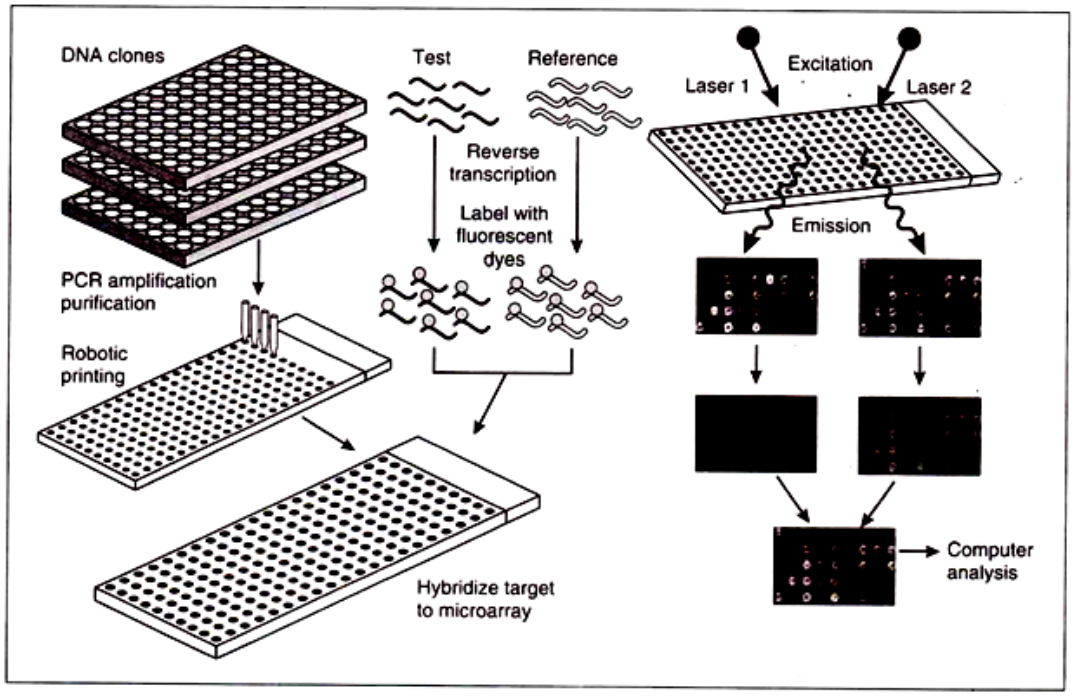


Fig. 9.5: Steps involved in DNA microarray

The samples are added to the antibody array, and complementary proteins bind to their cognate antibodies. If both sample 1 and 2 have identical proteins that bind the same antibody, the spot will fluoresce yellow.

If sample 1 has a protein that is missing in samples 2, then the spot will be green. Conversely, if sample 2 has a protein missing from sample 1, the spot will be red. This method is good for comparing protein expression profiles for two different conditions.

In the third method, the direct immunoassay or reverse-phase array, the proteins of the experimental sample are bound to the solid support. The proteins are then probed with a specific labelled antibody. Both presence and amount of protein can be monitored.

For example, proteins from different patients with prostate cancer can be isolated and spotted onto glass slides. Each sample can be examined for specific protein markers or the presence of different cancer proteins. The levels of certain proteins may be related to the stages of prostate cancer. This immunoassay helps researchers to decipher these correlations.

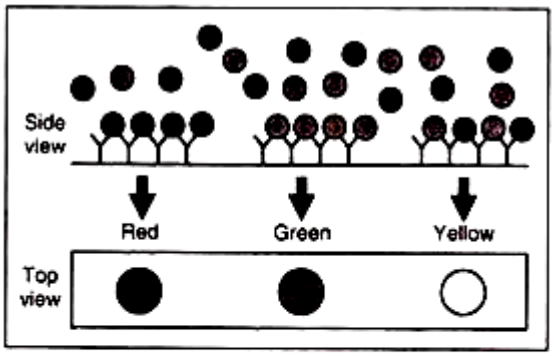


Fig. 9.6: **Ideal Results for Antigen Capture Immunoassay**: A variety of different antibodies are fused to different regions of a solid surface. Each spot has a different antibody. If the antibody recognizes only proteins labelled with Cy5, the region will fluoresce red (left). If the antibody recognizes only proteins labelled with Cy3, the region will fluoresce green (middle). If the antibody recognizes proteins in both conditions, the spot will fluoresce yellow (right)

Problem with Immune Based Arrays:

The main problem with immuno-based arrays is the antibody. Many antibodies cross-react .with other cellular proteins, which generates false positives. In addition, binding proteins to solid supports may not be truly representative of intracellular conditions. The proteins are not purified or separated; therefore, samples contain very diverse proteins.

Some proteins will bind faster and better than others. Also, proteins of low abundance may not compete for binding sites. Another problem is that many proteins are found in complexes, so other proteins in the complex may mask the antibody binding site.

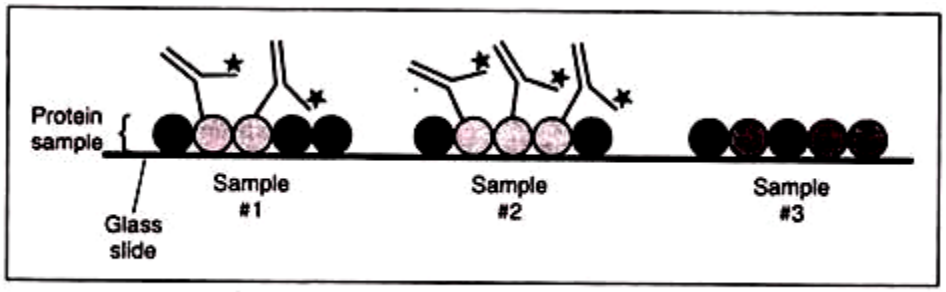


Fig. 9.7: Direct Immunoassay

Protein Interaction Arrays:

Rather than using antibodies, protein interaction arrays use a fusion tag to bind the protein to a solid support. The use of protein arrays to determine protein interactions and protein function is a natural extension of yeast two-hybrid assays and co-immunoprecipitation.

Protein arrays can assess thousands of proteins at one time, making this a powerful technique for studying the proteome. Protein arrays are often used in yeast because its proteome contains only about 6000 proteins. Libraries have been constructed in which each protein is fused to a His 6 or GST tag.

The proteins are then attached by the tags to a solid support such as a glass slide coated with nickel or glutathione. To build the array, each protein is isolated individually and spotted onto the glass slide. The tagged proteins bind to the slide and other cellular components are washed away. Each spot has only one unique tagged protein. Once the array is assembled, the proteins can be assessed for a particular function.

The direct immunoassay binds the protein samples to different regions on a solid support. Each spot has a different protein sample. Next, an antibody labelled with a detection system is added. The antibody binds only to its target protein. In this example, the antibody recognizes only a protein in patient samples 1 and 2.

In the laboratory of Michael Snyder at Yale University, the yeast proteome has been screened for proteins that bind calmodulin (a small Ca^{2+} binding protein) or phospholipids. Both calmodulin and phospholipid were tagged with biotin and incubated with a slide coated with each of the yeast proteins bound to the slide via His 6- nickel interactions.

The biotin-labelled calmodulin or phospholipid was then visualized by incubating the slide with Cy3-labelled streptavidin. (Streptavidin binds very strongly to biotin). The results identified 39 different calmodulin binding proteins (only six had been identified previously), and 150 different phospholipid binding proteins.

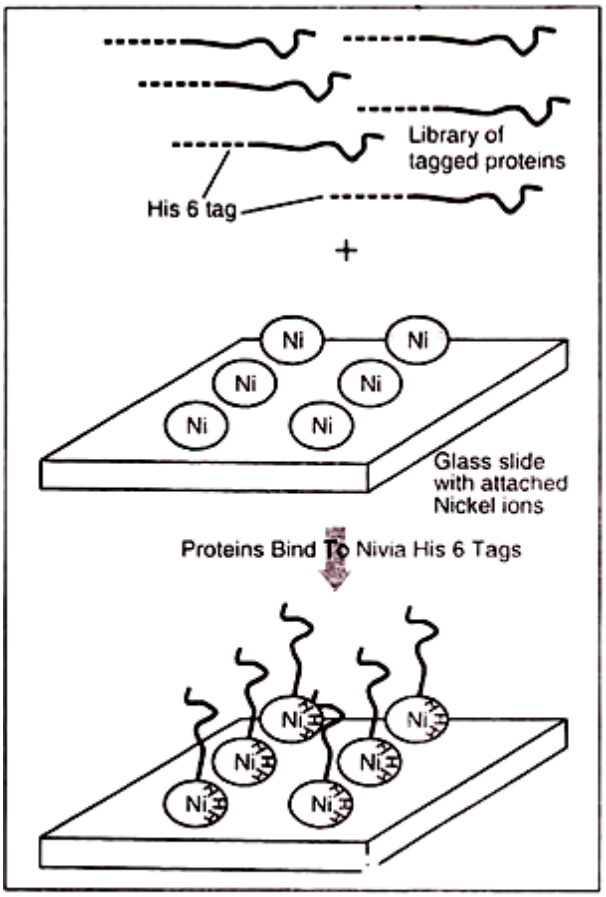


Fig. 9.8: **Protein Interaction Microarray—Principle**: To assemble a protein microarray, a library of His6-tagged proteins is incubated with a nickel-coated glass slide. The proteins adhere to the slide wherever nickel ions are present

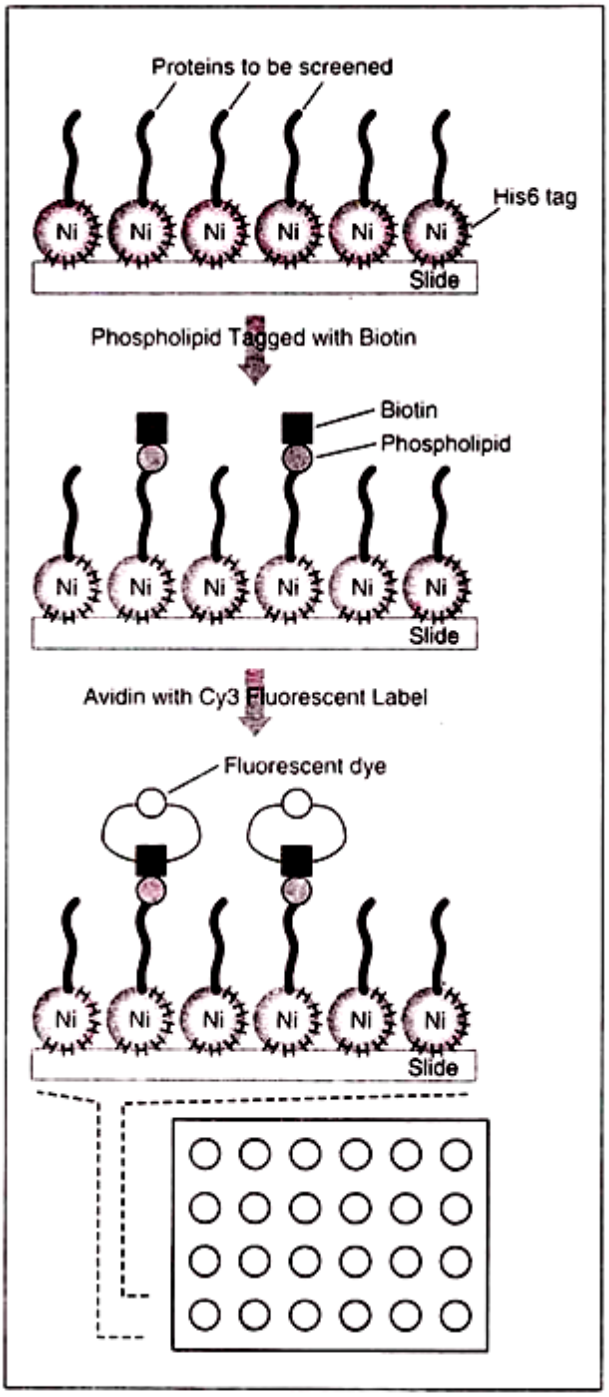


Fig. 9.9: **Screening Protein Arrays Using Biotin/Streptavidin**: Protein microarrays can be screened to find proteins that bind to phospholipids. The protein microarray is incubated with phospholipid bound to biotin. Then the bound phospholipid is visualized by adding streptavidin conjugated to a fluorescent dye. Spots that fluoresce represent specific proteins bind phospholipids

Application of Protein Microarrays:

Protein microarrays are measurement devices used in biomedical applications to determine the presence and/or amount (referred to as relative quantitation) of proteins in biological samples, such as blood. They have the potential to be an important tool for proteomics research.

Gene Discovery:

DNA Microarray technology helps in the identification of new genes, know about their functioning and expression levels under different conditions.

Disease Diagnosis:

DNA Microarray technology helps researchers learn more about different diseases such as heart diseases, mental illness, infectious disease and especially the study of cancer. Until recently, different types of cancer have been classified on the basis of the organs in which the tumors develop. Now, with the evolution of microarray technology, it will be possible for the researchers to further classify the types of cancer on the basis of the patterns of gene activity in the tumor cells. This will tremendously help the pharmaceutical community to develop more effective drugs as the treatment strategies will be targeted directly to the specific type of cancer.

Drug Discovery:

Microarray technology has extensive application in Pharmacogenomics. Pharmacogenomics is the study of correlations between therapeutic responses to drugs and the genetic profiles of the patients. Comparative analysis of the genes from a diseased and a normal cell will help the identification of the biochemical constitution of the proteins synthesized by the diseased genes. The researchers can use this information to synthesize drugs which combat with these proteins and reduce their effect.

Toxicological Research:

Microarray technology provides a robust platform for the research of the impact of toxins on the cells and their passing on to the progeny. Toxicogenomics establishes correlation between responses to toxicants and the changes in the genetic profiles of the cells exposed to such toxicants.

GEO

In the recent past, microarray technology has been extensively used by the scientific community. Consequently, over the years, there has been a lot of generation of data related to gene expression. This data is scattered and is not easily available for public use. For easing the accessibility to this data, the National Center for Biotechnology Information (NCBI) has formulated the Gene Expression Omnibus or GEO. It is a data repository facility which includes data on gene expression from varied sources.

Human Genome Project:

Human Genome Project is the most ambitious and exciting scientific undertaking by human being. Human genome project is administered by National Institute of Health and US Deptt. of Energy. In U.S.A. work started on this project in 1990 with determination to map and sequence the complete set of chromosomes in 15 years.

'Acquiring complete knowledge of the organization, structure and function of human genome – the master blueprint of each of us – is the broad aim of Human Genome Project.' With the target of three billion base pairs in human genome to be sequenced and by 1990- 100,000 had been analysed.

Many sequences in genome are highly repetitive and have no obvious function. This is about 50% of total DNA. As seen above, it is not a small job and labours and cost wise frustrating too. It is an ambitious project.

History:

In U.S., the HGP was carried out by the Department of Energy (Human Genome Program) directed by Ari Patrinos, and National Institute of Health (Human Genome Research Institute) directed by Francis Collins. In 2001, Craig Venter, CEO of Celera Genomics, co-announced the completion (90%) of sequencing of the human genome (draft sequence).

The full sequence was completed and published in 2003 (finished sequence). More refined sequence is available in 2006 and correction of minor errors (1 less in 10000 DNA subunits) requires some time to come.

Aims of Human Genome Project:

The project was aimed for the benefits of humankind, generation of biologists and researchers have been provided with detailed DNA information that will be key to understand the structure, organization and function of DNA in chromosomes.

Human Genome Size:

A genome is an organism's complete set of deoxyribonucleic acid (DNA), a chemical compound that contains the genetic instructions needed to develop and direct the activities of an organism. The human genome contains approx. Three billion base pairs which reside in 23 pairs of chromosomes.

Each chromosome contains hundreds and thou-sands of genes, and ranges in size from about 50000000 to 300000000 base pairs. The total number of genes is 30000 (approx.) and accounts for only 25% of the DNA; the rest is extra-genic DNA.

Human Genome Project Mapping:

Before begin-ning a sequencing project of the human genome, it was first necessary to produce a good frame-work map. Two general methods were devel-oped for mapping human genome — standard method and whole genome short-gun method.

The standard method involves finding a seg-ment of the genome and locating where it belongs. Genetic maps based on recombination frequencies between markers are useful in orde-ring genes. Molecular markers like RFLP, VNTRs (Microsatellites), STSs, SNPs have been used in mapping human genome.

The whole genome shotgun sequencing method involves shearing of genomic DNA followed by cloning, to produce a genomic library.

This is followed by sequencing of cloned DNA fragments at random, followed by shotgun assembly, i.e., the assembly of the frag-ment sequences into larger units on the basis of their overlaps. Groups of cloned DNA segments that can be aligned in an overlapping fashion to cover a region of the human genome are referred as contigs.

Yeast Artificial Chromosomes (YACs) were initially used as cloning agents when prima-ry task was mapping. However, as the emphasis of the project shifted to sequencing. Bacterial Artificial Chromosomes (BACs) were used.

Human Genome Project Sequence:

Sequencing means determining the exact order of the base pairs in a segment of DNA. The primary method used by the HGP to produce the finished version of the human genetic code is map-based or BAC- based sequencing. The human DNA is fragmen-ted into pieces that are relatively large, cloned in the bacteria, stored for replication as required.

A collection of BAG clones containing the entire human genome is called a BAC-library. In this method, each BAC clone is mapped to determine the location of that fragment in human chromo-some and then the DNA letters are sequenced from each clone and their spatial relation to sequenced human DNA in other BAC clones.

For sequencing, each BAC clone is cut into still smaller fragments that are about 2000 bases in length. These pieces are called "sub-clones". A "sequencing reaction" is carried out on these sub-clones. With the help of a computer then the short sequences are assembled into contiguous stretches of sequence of the clones.

In a short the whole process can be sum-marized:

i. Chromosomes, which range in size from 50 million to 250 million bases, must first be broken into much shorter pieces (sub-cloning step).

ii. Each short piece is used as a template to generate a set of fragments that differ in length from each other by a single base that will be identified in a later step (template preparation and sequencing step).
iii. The fragments in a set are separated by gel electrophoresis (separation step).
iv. The final base at the end of each frag-ment is identified (base-calling step). This process recreates the original sequence of As, Ts, Cs and Gs for each short piece generated in the first step.

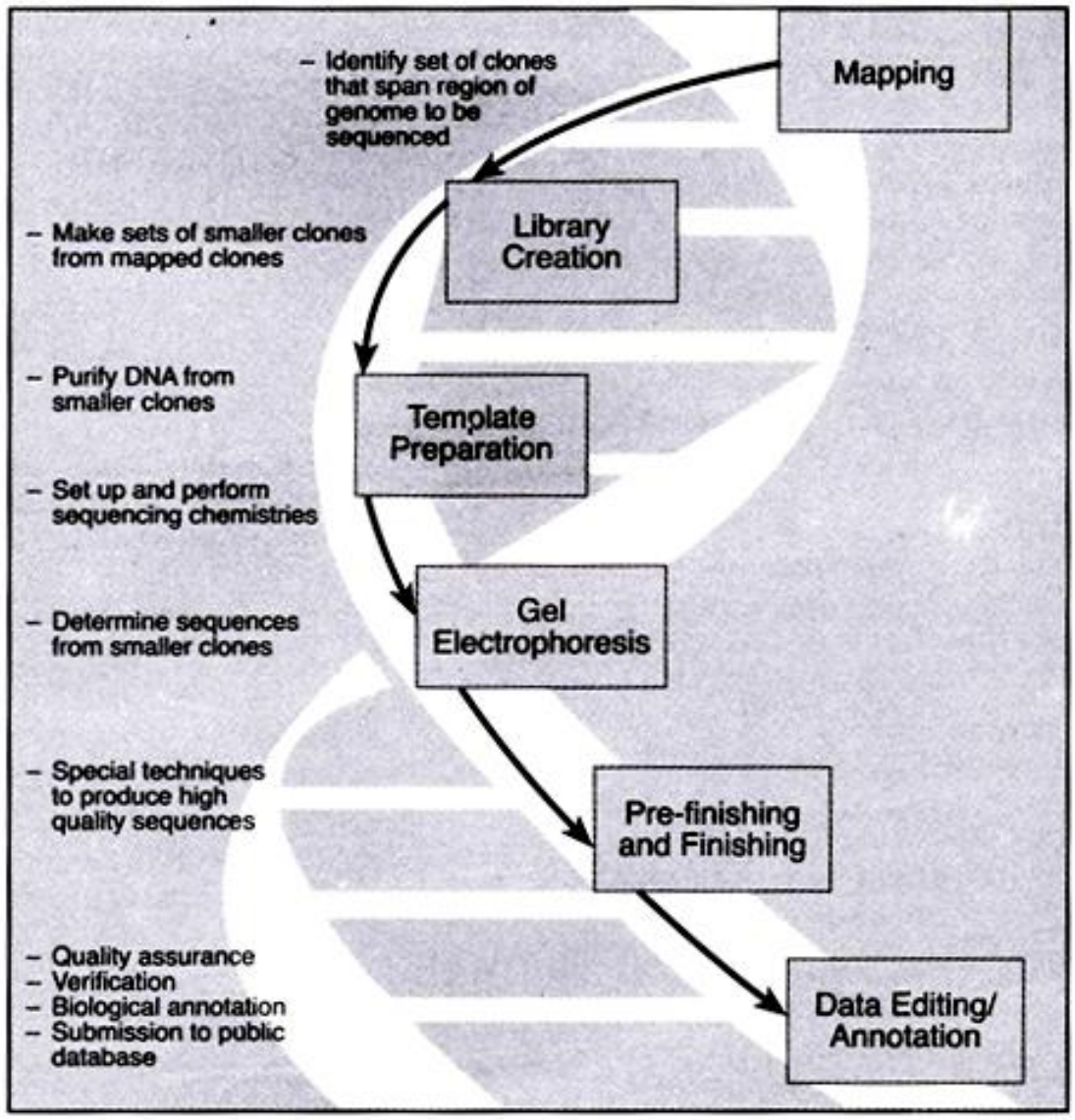


Fig. 18.18: DNA sequencing process

v. After the bases are 'read', computers are used to assemble the short sequences (in blocks of about 500 bases each called the read length) into long conti-nuous stretches that are analysed for errors, gene coding regions, and other characteristics.
vi. Finished sequence is submitted to major public sequence databases, making Human Genome Project sequence data thus freely available to anyone around the world.
The human genome reference sequence do not represent any one person's genome. Rather the knowledge obtained is applicable to every-one because all humans share the same basic set of genes and genomic regulatory regions that control the development.

Researchers collected blood (female) or sperm (male) samples from different races like European, African, American (North, Central, South) and Asian ancestry and a few samples were processed as DNA resources.

Few interesting features of human genome are:

(i) There are more than 3.2 billion base pairs.

(ii) Human genome is estimated to have 20,000 to 25,000 genes and to determine the sequences of 3 billion chemical base pairs that make up human DNA.

(iii) Within the human population are millions of single base differences called single nucleotide polymorphisms (SNPs). Each human differs from the next by about 1 bp in every 1,000 bp.

Fig. 6.16. Human genome project (HGP) goals and completion dates.

S. No.	Field of Work	HGP Goal	Standard Achieved	Date Achieved
1.	Genetic map	2- to 5-cM resolution map (600 - 1,500 markers)	1-cM resolution map (3,000 markers)	September 1994
2.	Physical map	30,000 STSs	52,000 STSs	October 1998
3.	DNA sequence	95% of gene-containing part of human sequence finished to 99.99% accuracy	99% of gene-containing part of human sequence finished to 99.99% accuracy	April 2003
4.	Capacity and cost of finished sequence	Sequence 500 Mb/year at< $0.25 per finished base	Sequence>1,400 Mb/year at <$0.09 per finished base	November 2002
5.	Human sequence variation	100,000 mapped human SNPs	3.7 million mapped human SNPs	February 2003
6.	Gene identification	Full-length human cDNAs	15,000 full-length human cDNAs	March 2003
7.	Model organisms	Complete	Finished genome	April 2003

(iv) The human genome bears gene rich areas separated by gene poor areas called gene deserts.

(v) About 45% of our genome is made up of transposons.

(vi) Only 2 percent of genome codes the proteins.

(vii) To improve tools for data analysis.

(viii) Use of concerned technology to sectors like industries.

(ix) Address the ethical, legal and social issues (ELSI) which may appear due to HGP.

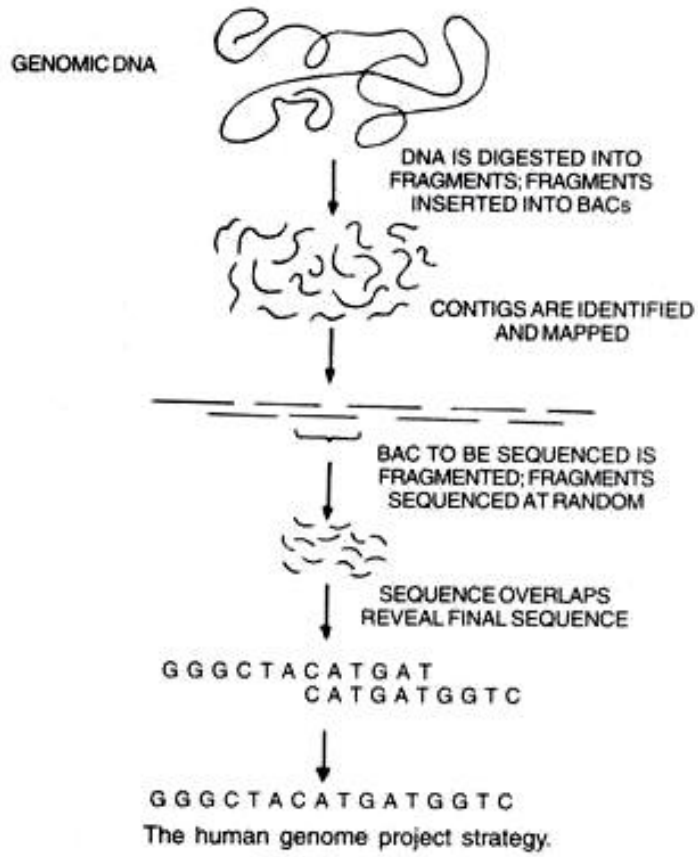
(x) The human genome contains 3164.7 million nucleoti

(x) The human genome contains 3164.7 million nucleotide bases. Largest known human gene is dystrophin which bears 2.4 million bases, with average gene having about 3000 bases. Functions of 50 percent of discovered genes are yet to be found.

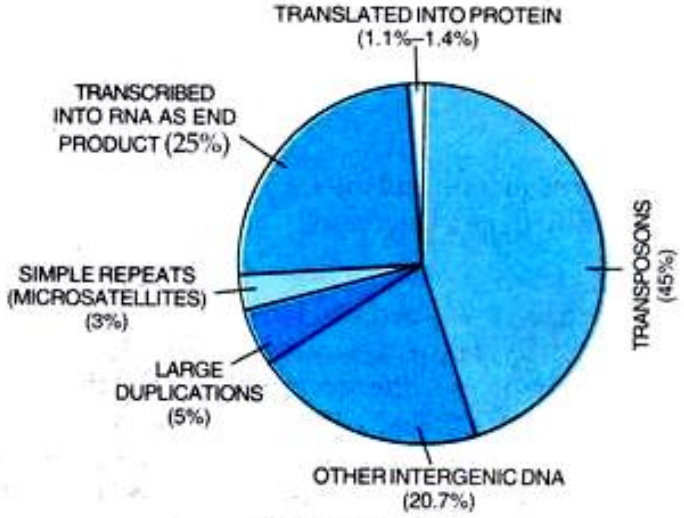
(xi) About 99.9 percent of nucleotide bases are exactly similar in all human beings. Previously total number of genes estimated was 80,000 to 1, 40,000 but now number is said to be 30,000.

(xii) Large amount of human genome is constituted of repeated sequences.

(xiii) Chromosome 1 bears maximum genes (2968), with Y chromosome having lowest (231).



The human genome project strategy.



Snapshot of human genome.

15. Proteomics: Concept of proteome; basic principles of 2-DE; advantages and limitations of 2-DE; gel free proteomics; mass spectrometry.

Proteome:

The proteome is the entire set of proteins that is, or can be, expressed by a genome, cell, tissue, or organism at a certain time. It is the set of expressed proteins in a given type of cell or organism, at a given time, under defined conditions. Proteomics is the study of the proteome.

Proteomics:

The entire protein component of a given organism is called 'proteome', the term coined by Wasinger in 1995. A proteome is a quantitatively expressed protein of a genome that provides information on the gene products that are translated, amount of products and any post translational modifications.

Proteomics is an emerging area of research in the post-genomic era, which involves identifying the structures and functions of all proteins of a proteome. It is sometimes also treated as structural based functional genomics.

Types of Proteomics:

i. Structural Proteomics:

One of the main targets of proteomics investigation is to map the structure of protein complexes or the proteins present in a specific cellular organelle known as cell map or structural proteins. Structural proteomics attempt to identify all the proteins within a protein complex and characterization all protein-protein interactions. Isolation of specific protein complex by purification can simplify the proteomic analysis.

ii. Functional Proteomics:

It mainly includes isolation of protein complexes or the use of protein ligands to isolate specific types of proteins. It allows selected groups of proteins to be studied its chracteristics which can provide important information about protein signalling and disease mechanism etc.

Expression proteomics

Expression proteomics includes the analysis of protein expression at larger scale. It helps identify main proteins in a particular sample, and those proteins differentially expressed in related samples—such as diseased vs. healthy tissue. If a protein is found only in a diseased sample then it can be a useful drug target or diagnostic marker. Proteins with same or similar expression profiles may also be functionally related. There are technologies such as 2D- PAGE and mass spectrometry that are used in expression proteomics.

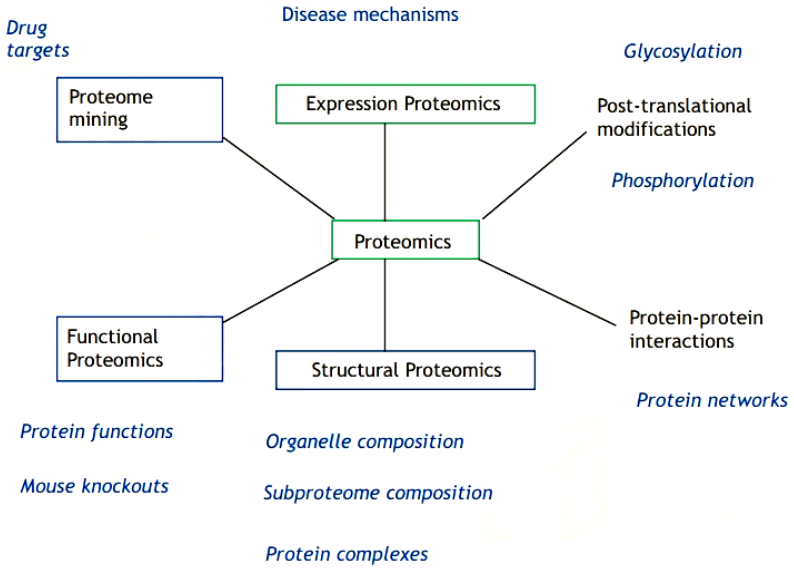


Fig. 5. Types of proteomics and the scientific knowledge that can be gained from them.

Basic Techniques in Protein Analysis

Some of the very basic analytical techniques are used as major proteomic tools for studying the proteome of an organism. We shall study most of these techniques as we progress in the course. The initial step in all proteomic studies is the separation of a mixture of proteins. This can be carried out using Two Dimensional Gel Electrophoresis technique in which proteins are first of all separated based on their individual charges in 1D. The gel is then turned 90 degrees from its initial position to separate proteins based on the difference in their size. This separation occurs in 2 nd dimension hence the name 2D. The spots obtained in 2D electrophoresis are excised and further subjected to mass spectrometric analysis of each protein present in the mixture.

The ultimate goal of proteomics is to identify or compare the proteins expressed in a given genome under specific conditions, study the interactions between the proteins, and use the information to predict cell behavior or develop drug targets. Just as the genome is analyzed using the basic technique of DNA sequencing, proteomics requires techniques for protein analysis. The basic technique for protein analysis, analogous to DNA sequencing, is mass spectrometry.

Mass Spectrometer: Matrix-Assisted Laser Desorbtion Ionisation – Time Of Flight (MALDI-TOF) Mass Spectrometer. Mass spectrometry can be used in protein analysis.

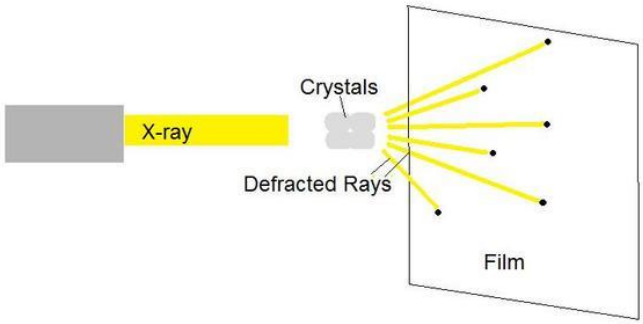
Mass Spectrometry

Mass spectrometry is used to identify and determine the characteristics of a molecule. It is a technique in which gas phase molecules are ionized and their mass-to-charge ratio is measured by observing acceleration differences of ions when an electric field is applied. Lighter ions will accelerate faster and be detected first. If the mass is measured with precision, then the composition of the molecule can be identified. In the case of proteins, the sequence can be identified. The challenge of techniques used for proteomic analyses is the difficulty in detecting small quantities of proteins, but advances in spectrometry have allowed researchers to analyze very small samples of protein. Variations in protein

expression in diseased states, however, can be difficult to discern. Proteins are naturally-unstable molecules, which makes proteomic analysis much more difficult than genomic analysis.

X-ray crystallography and Nuclear Magnetic Resonance

X-ray crystallography enables scientists to determine the three-dimensional structure of a protein crystal at atomic resolution. Crystallographers aim high-powered X-rays at a tiny crystal containing trillions of identical molecules. The crystal scatters the X-rays onto an electronic detector that is the same type used to capture images in a digital camera. After each blast of X-rays, lasting from a few seconds to several hours, the researchers precisely rotate the crystal by entering its desired orientation into the computer that controls the X-ray apparatus. This enables the scientists to capture in three dimensions how the crystal scatters, or diffracts, X-rays. The intensity of each diffracted ray is fed into a computer, which uses a mathematical equation to calculate the position of every atom in the crystallized molecule. The result is a three-dimensional digital image of the molecule.

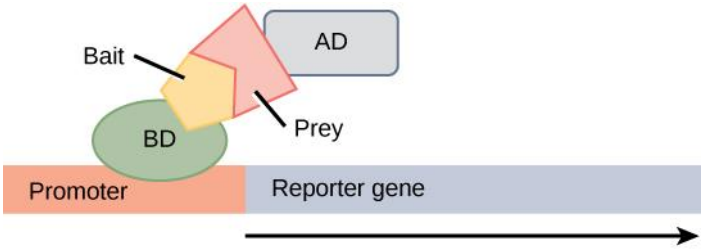


X-ray crystallography: X-rays that hit atomic nuclei are diffracted onto a detector.

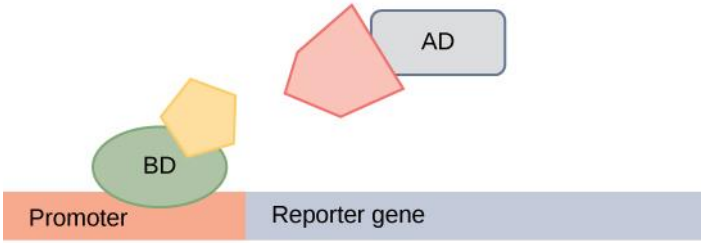
Another protein imaging technique, nuclear magnetic resonance (NMR), uses the magnetic properties of atoms to determine the three-dimensional structure of proteins. NMR spectroscopy is unique in being able to reveal the atomic structure of macromolecules in solution, provided that highly-concentrated solution can be obtained. This technique depends on the fact that certain atomic nuclei are intrinsically magnetic. The chemical shift of nuclei depends on their local environment. The spins of neighboring nuclei interact with each other in ways that provide definitive structural information that can be used to determine complete three-dimensional structures of proteins.

Protein Microarrays and Two- Hybrid Screening

Protein microarrays have also been used to study interactions between proteins. These are large-scale adaptations of the basic two-hybrid screen. The premise behind the two-hybrid screen is that most



If the bait protein interacts with the prey protein, the transcription factor's activator domain binds to the binding domain, and transcription occurs.



If the prey doesn't catch the bait, no transcription occurs.

eukaryotic transcription factors have modular activating and binding domains that can still activate transcription even when split into two separate fragments, as long as the fragments are brought within close proximity to each other. Generally, the transcription factor is split into a DNA-binding domain (BD) and an activation domain (AD). One protein of interest is genetically fused to the BD and another protein is fused to the AD. If the two proteins of interest bind each other, then the BD and AD will also come together and activate a reporter gene that signals interaction of the two hybrid proteins.

Two-hybrid screening: Two-hybrid screening is used to determine whether two proteins interact. In this method, a transcription factor is split into a DNA-binding domain (BD) and an activation domain (AD). The binding domain is able to bind the promoter in the absence of the activator domain, but it does not turn on transcription. A protein called the bait is attached to the BD, and a protein called the prey is attached to the AD. Transcription occurs only if the prey "catches" the bait.

Western Blot

The western blot, or protein immunoblot, is a technique that combines protein electrophoresis and antibodies to detect proteins in a sample. A western blot is fairly quick and simple compared to the above techniques and, thus, can serve as an assay to validate results from other experiments. The protein sample is first separated by gel electrophoresis, then transferred to a nitrocellulose or other type of membrane, and finally stained with a primary antibody that specifically binds the protein of interest. A fluorescent or radioactive-labeled secondary antibody binds to the primary antibody and provides a means of detection via either photography or x-ray film, respectively.

Steps in Proteomic Analysis

The following steps are involved in analysis of proteome of an organism as shown in.

1. **Purification of proteins:** This step involves extraction of protein samples from whole cell, tissue or sub cellular organelles followed by purification using density gradient centrifugation, chromatographic techniques (exclusion, affinity etc.)

2. Separation of proteins: 2D gel electrophoresis is applied for separation of proteins on the basis of their isoelectric points in one dimension and molecular weight on the other. Spots are detected using fluorescent dyes or radioactive probes.

3. Identification of proteins: The separated protein spots on gel are excised and digested in gel by a protease (e.g. trypsin). The eluted peptides are identified using mass spectrometry.

Analysis of protein molecules is usually carried out by MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) based peptide mass fingerprinting.

Determined amino acid sequence is finally compared with available database to validate the proteins. Several online tools are available for proteomic analysis such as Mascot, Aldente, Popitam, Quickmod, Peptide cutter etc.

Application of Proteomics:

1. Protein Expression Profiling:

The largest application of proteomics continues to be protein expression profiling. The expression levels of a protein sample could be measured by 2-DE or other novel technique such as isotope coded affinity tag (ICAT). Using these approaches the varying levels of expression of two different protein samples can also be analyzed.

This application of proteomics would be helpful in identifying the signaling mechanisms as well as disease specific proteins. With the help of 2-DE several proteins have been identified that are responsible for heart diseases and cancer (Celis et al. 1999). Proteomics helps in identifying the cancer cells from the non-cancerous cells due to the presence of differentially expressed proteins.

The technique of Isotope Coded Affinity Tag has developed new horizons in the field of proteomics. This involves the labeling of two different proteins from two different sources with two chemically

identical reagents that differ in their masses due to isotope composition (Gygi et al. 1999). The biggest advantage of this technique is the elimination of protein quantitation by 2-DE. Therefore, high amount of protein sample can be used to enrich low abundance proteins.

Different methods have been used to probe genomic sets of proteins for biochemical activity. One method is called a biochemical genomics approach, which uses parallel biochemical analysis of a proteome comprised of pools of purified proteins in order to identify proteins and the corresponding ORFs responsible for a biochemical activity.

The second approach for analyzing genomic sets of proteins is the use of functional protein microarrays, in which individually purified proteins are separately spotted on a surface such as a glass slide and then analyzed for activity. This approach has huge potential for rapid high-throughput analysis of proteomes and other large collections of proteins, and promises to transform the field of biochemical analysis.

Post-Translational Modifications:

Proteomics studies involve certain unique features as the ability to analyze post- translational modifications of proteins. These modifications can be phosphorylation, glycosylation and sulphation as well as some other modifications involved in the maintenance of the structure of a protein.

These modifications are very important for the activity, solubility and localization of proteins in the cell. Determination of protein modification is much more difficult rather than the identification of proteins. As for identification purpose, only few peptides are required for protease cleavages followed by database alignment of a known sequence of a peptide. But for determination of modification in a protein, much more material is needed as all the peptides do not have the expected molecular mass need to be analyzed further.

For example, during protein phosphorylation events, phosphopeptides are 80 Da heavier than their unmodified counterparts. Therefore, it gives, rise to a specific fragment (PO3- mass 79) bind to metal resins, get recognized by specific antibodies and later phosphate group can be removed by phosphatases (Clauser et al. 1999; Colledge and Scott, 1999). So protein of interest (post-translationally modified protein) can be detected by Western blotting with the help of antibodies or 32P-labelling that recognize only the active state of molecules. Later, these spots can be identified by mass spectrometry.

Protein-Protein Interactions:

The major attribution of proteomics towards the development of protein interactions map of a cell is of immense value to understand the biology of a cell. The knowledge about the time of expression of a particular protein, its level of expression, and, finally, its interaction with another protein to form an intermediate for the performance of a specific biological function is currently available.

These intermediates can be exploited for therapeutic purposes also. An attractive way to study the protein-protein interactions is to purify the entire multi-protein complex by affinity based methods using GST-fusion proteins, antibodies, peptides etc.

The yeast two-hybrid system has emerged as a powerful tool to study protein-protein interactions (Haynes and Yates, 2000). According to Pandey and Mann (2000) it is a genetic method based on the modular structure of transcription factors in the close proximity of DNA binding domain to the activation domain induces increased transcription of a set of genes.

The yeast hybrid system uses ORFs fused to the DNA binding or activation domain of GAL4 such that increased transcription of a reporter gene results when the proteins encoded by two ORFs interact in the nucleus of the yeast cell. One of the main consequences of this is that once a positive interaction is detected, simply sequencing the relevant clones identifies the ORF. For this reason it is a generic method that is simple and amenable to high throughput screening of protein-protein interactions.

Phage display is a method where bacteriophage particles are made to express either a peptide or protein of interest fused to a capsid or coat protein. It can be used to screen for peptide epitopes, peptide ligands, enzyme substrate or single chain antibody fragments.

Another important method to detect protein-protein interactions involves the use of fluorescence resonance energy transfer (FRET) between fluorescent tags on interacting proteins. FRET is a non-radioactive process whereby energy from an excited donor fluorophore is transferred to an acceptor fluorophore. After excitation of the first fluorophore, FRET is detected either by emission from the second fluorophore using appropriate filters or by alteration of the fluorescence lifetime of the donor.

A proteomics strategy of increasing importance involves the localization of proteins in cells as a necessary first step towards understanding protein function in complex cellular networks. The discovery of GFP (green fluorescent protein) and the development of its spectral variants has opened the door to analysis of proteins in living cells by use of the light microscope.

Large-scale approaches of localizing GFP-tagged proteins in cells have been performed in the genetically amenable yeast S. pombe (Ding et al. 2000) and in Drosophila (Morin et al. 2001). To localize proteins in mammalian cells, a strategy was developed that enables the systematic GFP tagging of ORFs from novel full-length cDNAs that are identified in genome projects.

Proteome mining:

Proteome mining is a functional proteomic approach used to extract information from the analysis of specific sub-proteomics. In principle, it is based on the assumption. In principle, it is based oil the assumption that all drug like molecule selectively compete with a natural cellular ligand for a binding site on a protein target.

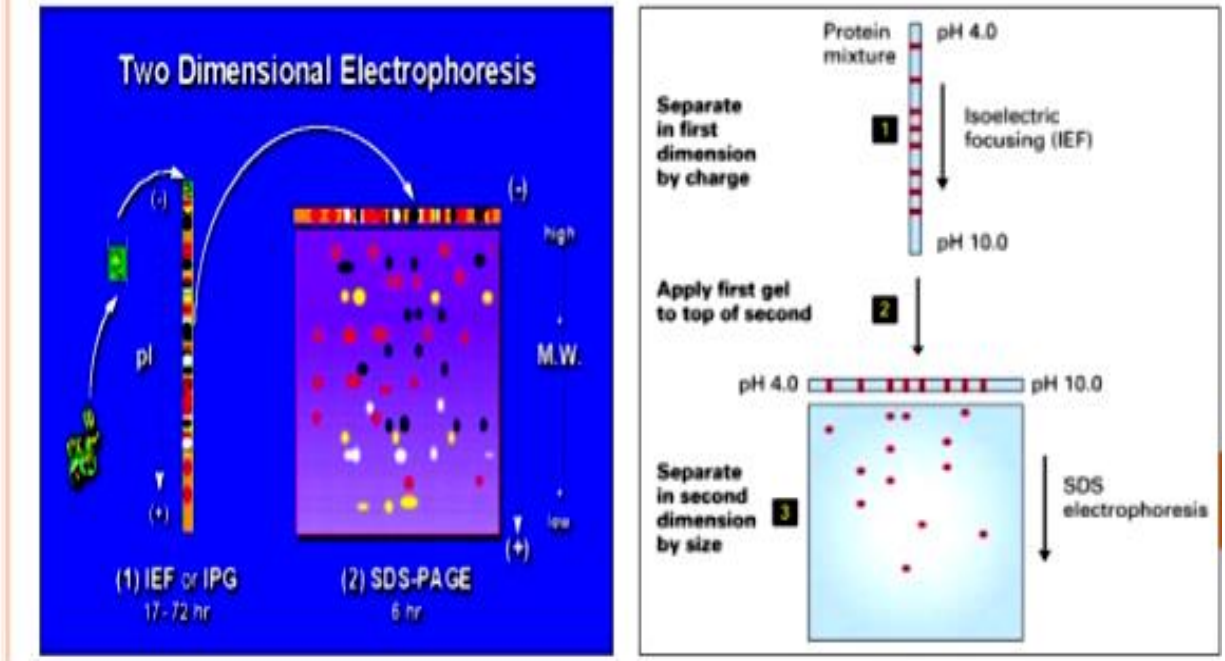
Two-Dimensional Gel Electrophoresis:

Two-dimensional electrophoresis (2-D electrophoresis) is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique sorts proteins according to two independent properties in two discrete steps: the first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pi); the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (Mr, relative molecular weight).

Each spot on the resulting two-dimensional array corresponds to a single protein species in the sample. Thousands of different proteins can thus be separated, and information such as the protein pi, the apparent molecular weight, and the amount of each protein are obtained.

Two-dimensional electrophoresis was first introduced by P. H. O’ Farrell and J. Klose in 1975. In the original technique, the first-dimension separation was performed in carrier ampholytecontaining polyacrylamide gels cast in narrow tubes. A. Gorg and colleagues developed the currently employed 2-D technique, where carrier ampholyte-generated pH gradients have been replaced with immobilized pH gradients and tube gels replaced with gels supported by a plastic backing.

A large and growing application of 2-D electrophoresis is “proteome analysis.” The analysis involves the systematic separation, identification, and quantification of many proteins simultaneously from a single sample. Two-dimensional electrophoresis is used in this technique due to its unparalleled ability to separate thousands of proteins simultaneously. Two-dimensional electrophoresis is also unique in its ability to detect post- and co-translational modifications, which cannot be predicted from the genome sequence. Applications of 2-D electrophoresis include proteome analysis, cell differentiation, and detection of disease markers, monitoring therapies, drug discovery, cancer research, purity checks, and micro-scale protein purification.



Principle:

In 2D GE proteins are separated as per isoelectric point and protein mass.

Separation of the proteins by isoelectric point is called isoelectric focusing (IEF). When a gradient of pH is applied to a gel and an electric potential is applied across the gel, making one end more positive than the other.

At all pH values other than their isoelectric point, proteins will be charged. If they are positively charged, they will be pulled towards the negative end of the gel and if they are negatively charged they will be pulled to the positive end of the gel. The proteins applied in the first dimension will move along the gel and will accumulate at their isoelectric point; that is, the point at which the overall charge on the protein is 0 (a neutral charge).

In separating the proteins by mass, the gel treated with sodium dodecyl sulfate (SDS) along with other reagents (SDS-PAGE in 1-D). This denatures the proteins (that is, it unfolds them into long, straight molecules) and binds a number of SDS molecules roughly proportional to the protein's length. Because a protein's length (when unfolded) is roughly proportional to its mass, Since the SDS molecules are negatively charged, the result of this is that all of the proteins will have approximately the same mass-to-charge ratio as each other.

In addition, proteins will not migrate when they have no charge (a result of the isoelectric focusing step) therefore the coating of the protein in SDS (negatively charged) allows migration of the proteins in the second dimension.

In the second dimension, an electric potential is again applied, but at a 90 degree angle from the first field. The proteins will be attracted tothe more positive side of the gel (because SDS is negatively charged) proportionally to their mass-to-charge ratio.

The gel therefore acts like a molecular sieve when the current is applied, separating the proteins on the basis of their molecular weight with larger proteins being retained higher in the gel and smaller proteins being able to pass through the sieve and reach lower regions of the gel.

Steps of 2-DE:

1. Sample preparation
2. Isoelectric focusing (first dimension)
3. SDS-PAGE (second dimension)
4. Visualization of proteins spots
5. Identification of protein spots

1. Sample preparation

- ❖ Must break all non-covalent protein-protein, protein-DNA, proteinlipid interactions, disrupt S-S bonds
- ❖ Must prevent proteolysis, accidental phosphorylation, oxidation, cleavage, deamidation
- ❖ Must remove substances that might interfere with separation process such as salts, polar detergents (SDS), lipids, polysaccharides, nucleic acids
- ❖ Must try to keep proteins soluble during both phases of electrophoresis process
- ❖ Protein Solubilization
  - 8 M Urea (neutral chaotrope)
  - 4% CHAPS (zwitterionic detergent)
  - 2-20 mM Tris base (for buffering)
  - 5-20 mM DTT (to reduce disulfides)
  - Carrier ampholytes or IPG buffer (up to 2% v/v) to enhance protein solubility and reduce charge-charge interactions
- ❖ Protease inhibitors
  - PMSF(PhenylmethaneSulfonyl Flouride), Pefabloc, EDTA,,
- ❖ leupeptin, Aproteinin, Pepstatin
- ❖ Contaminant removal
  - Filtration, Centrifugation, Chromatography, Solvent Extraction

1st dimension: Isoelectric Focusing

In a pH gradient and under the influence of an electric field, a **protein will move to the position in the gradient where its net charge is zero**. A protein with a net **positive charge will migrate toward the cathode**, becoming progressively less positively charged as it moves through the pH gradient until **it reaches its pI**. A protein with a net **negative charge will migrate toward the anode**, becoming less negatively charged until it also **reaches zero net charge**. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This is the focusing effect which allows proteins to be separated on the basis of very small charge differences. The resolution is determined by the slope of the pH gradient and the electric field strength so, IEF is therefore performed at high voltages (typically in excess of 1000 V). When the proteins have reached their final positions in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current. The original method for first-dimension IEF depended on ampholytegenerated pH gradients in cylindrical polyacrylamide gels cast in glass rods or tubes. Now it is replaced by DryStrip gels.

Advantages of DryStrip gels include:

- ❖ The first-dimension separation is more reproducible because the covalently fixed gradient cannot drift.
- ❖ Plastic-backed DryStrip gels are easy to handle. They can be picked up at either end with forceps or gloved fingers.
- ❖ The plastic support film prevents the gels from stretching or breaking.
- ❖ More acidic and basic proteins can be separated.
- ❖ The sample can be introduced into the DryStrip gel during rehydration.
- ❖ DryStrip gels eliminate the need to handle toxic acrylamide monomers.
- ❖ Immobilized pH gradients and precise lengths ensure high reproducibility and reliable gel-to-gel comparisons.

Isoelectric Point (pI):

– pH at which a protein has a neutral charge; loss or gain of protons

H+ in a pH gradient (In a pH below their pI, proteins carry a net

positive charge and in a pH above their pI, they carry a net negative
charge)
Requires very high voltages (10000V)
Requires a long period of time (10h)
Degree of resolution determined by slope of pH gradient and electric field strength
Uses ampholytes to establish pH gradient
IPG strips:
– An immobilized pH gradient (IPG) is made by covalently integrating acrylamide and variable pH ampholytes at time of gel casting,
Stable gradients

Components of rehydration solution

- The choice of the rehydration solution for the sample will depend on its specific protein solubility requirements.
- A typical solution generally contains urea, nonionic or zwitterionic detergent, DeStreak Reagent or DTT, the appropriate Pharmalyte or IPG Buffer and a tracking dye.
- **Urea solubilizes and denatures proteins, unfolding them to expose internal ionizable amino acids. Commonly,** 8 M urea is used, but the concentration can be increased to 9 or 9.8 M
- **Thiourea, in addition to urea, can be used to further improve protein solubilization, particularly for hydrophobic** proteins . When using both, the recommended concentration of urea is 7 M and that of thiourea 2 M.
- **Detergent solubilizes hydrophobic proteins and minimizes protein aggregation. The detergent must have zero net charge**—use only nonionic or zwitterionic detergents. CHAPS, Triton X-100, or NP-40 in the range of 0.5 to 4% are most commonly used.

2nd Dimension (SDS-PAGE)

SDS-PAGE is an electrophoretic method for separating polypeptides according to their molecular weights. The technique is performed in polyacrylamide gels containing sodium dodecyl sulfate (SDS). SDS is an anionic detergent. SDS masks the charge of the proteins themselves net negative charge per unit mass. Besides SDS, a reducing agent such as DTT is also added to break any disulfide bonds present in the proteins. When proteins are treated with both SDS and a reducing agent, the degree of electrophoretic separation within a polyacrylamide gel depends largely on the molecular weight of the protein. In fact, there is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide complex.

Separation of proteins on basis of MW, not pI

• Requires modest voltages (200V)
• Requires a shorter period of time (2h)
• Presence of SDS is critical to disrupting structure and making mobility ~ 1/MW
• Degree of resolution determined by %acrylamide & electric field strength

Steps of SDS PAGE

1) Preparing the system for second-dimension electrophoresis
2) Equilibrating the gel(s) in SDS equilibration buffer
3) Placing the equilibrated gel on the SDS gel
4) Electrophoresis

Equilibrating the gels:

• It is important to proceed immediately to gel equilibration, unless the IPG strip is being frozen (at -60 °C or below) for future analysis.

• Equilibration is always performed immediately prior to the seconddimension run, never before storage of the DryStrip gels.
• The second-dimension gel itself should be prepared and ready to accept the DryStrip gel before beginning the equilibration protocol.

Equilibration solution components

The equilibration step saturates the gel with the SDS buffer system required for the second dimension separation.

The equilibration solution contains buffer, urea, glycerol, reductant, SDS, and dye. An additional equilibration step replaces the reductant with iodoacetamide.

Equilibration buffer (75 mM Tris-HCl, pH 8.8) maintains the DryStrip gel in a pH range appropriate for electrophoresis.

Urea (6 M) together with glycerol reduces the effects of electroendosmosis by increasing the viscosity of the buffer.

Glycerol (30%) together with urea reduces electroendosmosis and improves transfer of proteins from the first to the second dimension.

Dithiothreitol (DTT) preserves the fully reduced state of denatured, unalkylated proteins.

Sodium dodecyl sulfate (SDS) denatures proteins and forms negatively charged protein-SDS complexes.

Iodoacetamide alkylates thiol groups on proteins, preventing their reoxidation during electrophoresis.

iodoacetamide also alkylates residual DTT to prevent point streaking and other silver-staining artifacts .

Tracking dye (bromophenol blue) allows monitoring of the progress of electrophoresis.

The most commonly used buffer system for second-dimension SDSPAGE is the Tris-glycine system described by Laemmli.

This buffer system separates proteins at high pH, which confers the advantage of **minimal protein aggregation and clean separation even at relatively heavy protein loads**.

Procedure:

Equilibration

1. Place 2 mL of Equilibration buffer I on each thawed strip in gel tray. (This step reduces the proteins.)
2. Place the lid on the gel tray. Place tray on shaker and shake gently for 10 minutes.
3. Remove equilibration buffer I from the strips and drain into nonchlorinated waste under the hood. Hold the flat side (not slanted side) down to discard buffer and do not touch the strips.
4. Repeat process with equilibration buffer II. (This step alkylates proteins.)

SDS Running Buffer

1. Rinse out the graduated cylinder specified for SDS running buffer with MilliQ H2O.
2. Fill to 100 mL with SDS running buffer (10x) and dilute to 1000 mL with MilliQ H2O.
3. Remove bubbles from top of beaker.

Preparation of Gels

1. While tray is shaking, get out the gels. The liquid within the packaging has sodium azide, so handle with caution. Remove the white strip and green comb from the gels.
2. Rinse gels with MilliQ H2O from bottle located in gel room. Be sure to thoroughly rinse the well and the molecular weight marker well.
3. Blot with filter paper squares to remove excess water (Do not touch surface of gel inside well). Be careful not to disturb molecular weight marker well. You do not want water in area where strip will be placed.

Loading of Gels

1. Loosen the lid and heat the overlay agarose in microwave until melted. Heat in 10 second (or less) increments. ~30 seconds total.
2. Pick the strip up with tweezers on the "+" side of the strip. Slide the gel strip onto the edge of the gel tray.
3. Dip the strip in SDS running buffer 5 times and then allow excess buffer to drain off onto a paper towel.
4. Lay down gel and place strip gel side up onto the top of the gel casette with the "+" end toward the molecular weight marker well. Leave the strip at the top aligned above well.
5. With the gel box upright (lean it against a large tip box), add 1 mL of overlay agarose to well and MW well. Make sure that no bubbles form. If they do, remove them with the end of a pipette tip.
6. Push the strip down into the overlay agarose. Push one side down 1st and slowly push other side down at an angle to prevent formation of bubbles. Make sure the strip lays flat against the gel.
7. Add 2 µL of appropriate molecular weight marker to the molecular weight well. Add marker as deep as possible in the well to prevent the molecular weight marker from spilling over into the other area of the gel.
8. Place gel in gel box. Be careful to hold the gel level while placing it in the gel box.
9. Once agarose is solidified, fill gel box with the SDS running buffer to the fill line (don't pour directly on agarose). You want to fill the cassette chambers with running buffer as well.

Gel Box Run

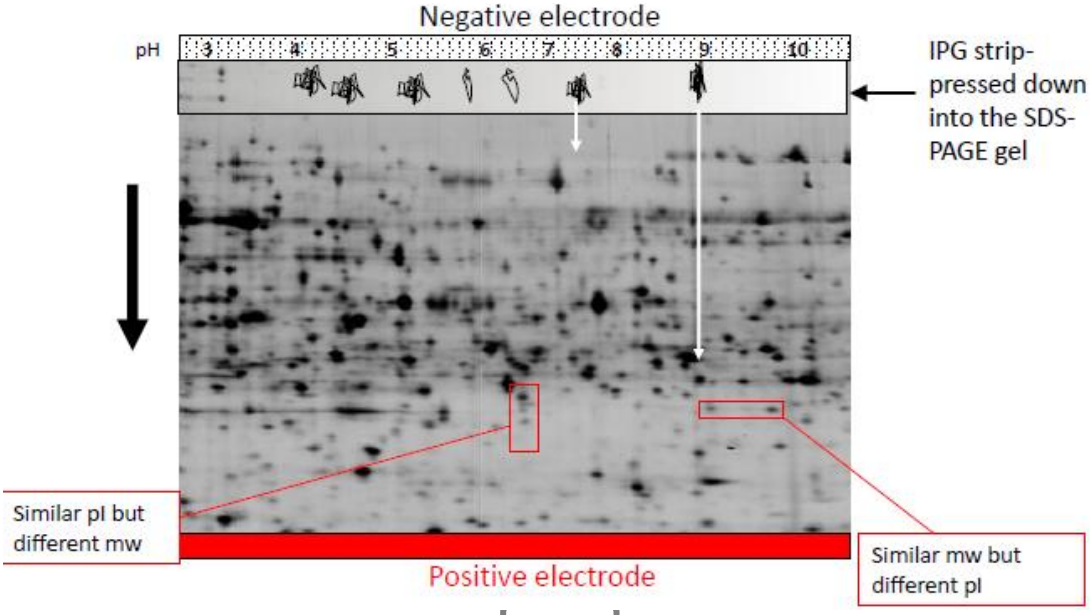
1. Place the lid on the gel box (red to red; black to black).
2. Plug the gel box into the voltage box (red to red; black to blue).
3. Turn on the voltage to 60 Volts for 15 minutes.
4. Turn the voltage up to 200 Volts for the remainder of the run (45-60 minutes).

After the Gel Run

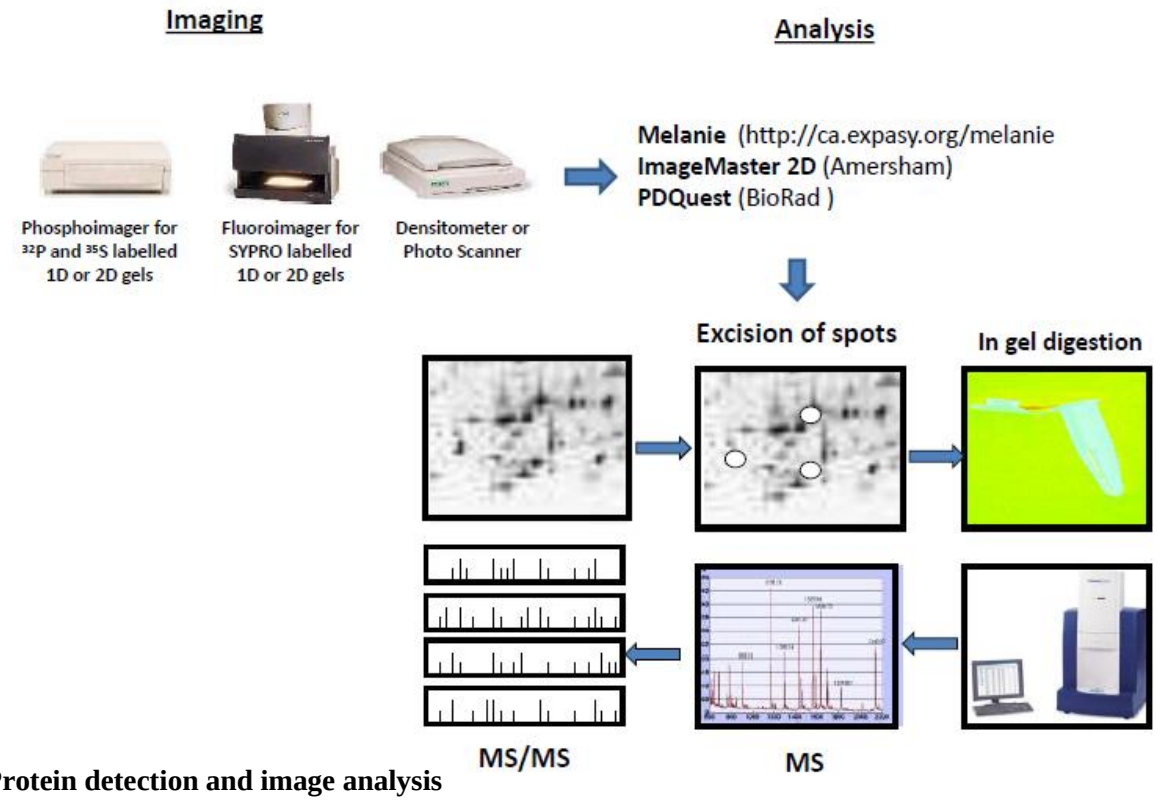
1. Unplug gel box from voltage box.
2. Remove cassette from gel box, drain SDS running buffer.
3. Crack all 4 joints of the cassette with a green comb.
4. Fill plastic bottom of gel container with MilliQ H2O.
5. Place gel in tray by turning cassette upside down and placing gently into water.
6. Follow the instructions on the wall of the gel room for the appropriate stain.

4. Detection/Visualization

• Coomassie Stain (100 ng to 10 mg protein)
• Silver Stain (1 ng to 1 mg protein)
• Fluorescent (Sypro Ruby) Stain (1 ng & up)



5. Protein Identification



Protein detection and image analysis

This step plays a crucial role, as i) only what is detected can be further analyzed and ii) quantitative variations observed at this stage are the basis to select the few spots of interest, in comparative studies, that will be the only ones processed for further analysis with mass spectrometry.

Detection with organic dyes can be summarized in one single process, colloidal Coomassie Blue staining, which has really become a reference standard.

Although the sensitivity is moderate and homogeneity are good and compatibility with mass spectrometry is excellent.

Silver staining is much more sensitive but less homogeneous, because of its delicate mechanism, and its compatibility with mass spectrometry is problematic.

The consequence of the presence of formaldehyde at the image development step, **formaldehyde-free silver staining protocols have been recently proposed.**

Protein detection by fluorescence give good sensitivity and also good compatibility with mass spectrometry.

Other modes of detection are environment-sensitive probes, noncovalent binding and covalent binding.

The use of chemically related, reactive fluorescent probes differing mainly by their excitation and emission wavelengths allows performing multiplexing of samples on 2D gels.

This multiplexing process solves in turn two difficult problems in the comparative analysis of gel images, namely the assignment of small positional differences and taking into account moderate quantitative changes.

Steps in proteome informatics for 2-DE is:

Image acquisition: This prepares each raw acquisition for subsequent comparative analysis. After scanning, the images are pre-processed by cropping (manual delineation), noise suppression, and background subtraction (e.g., with mathematical morphology or smooth polynomial surface fitting).

An image capture device is required, for which there are three main categories:

Flatbed scanner: This mechanically sweeps a standard charge-coupled device (CCD) under the gel and can be used to obtain 12–16 bits of greyscale or colour densitometry from visible light stains. Noise can be an issue due to size and cooling restrictions. Calibration is often required to provide linearity. Flatbed scanners are typically the least expensive offerings. Examples: ImageScanner (GE Healthcare), ProteomeScan (Syngene) and GS-800 (Biorad).

CCD camera: Since the sensor is fixed, its greater size and cooling provides a dramatic improvement in noise. Different filters and transillumination options allow a wide range of stains to be imaged, including visible light, fluorescent, reverse, chemiluminescent, and radioactive signals. However, the fixed sensor limits image resolution. Examples: LAS (Fuji Photo Film), ImageQuant (GE Healthcare), Dyversity (Syngene), BioSpectrum2D (UVP) and VersaDoc (Biorad).

Laser scanner: Photomultiplier detectors are combined with laser light and optical or mechanical scanning to pass an excitation beam over each target pixel. While slower than CCD cameras, spatial resolution is excellent. FLA (Fuji Photo Film), Typhoon (GE Healthcare) and Pharos FX (Biorad).

Conventional analysis (Spot Detection ≫ Spot Matching): Each protein spot is delineated and its volume quantified. The spots are segmented first by the watershed transform, where spots are slowly immersed in water. Point pattern matching is then employed to match the spots between gels, which finds the closest spot correspondence between a point pattern (source spot list) and a target point set (reference spot list).

Image-based analysis (Gel alignment ≫ Consensus Spot Modelling):

With current techniques, a "reference" gel is chosen and the other "source" gels are aligned to it in pair-wise fashion. A similarity measure which quantifies the quality of alignment between the warped source gel and the reference gel. The aim is to automatically find the optimal transformation that maximizes the similarity measure. Spot detection is then performed on an image, which is then propagated to each individual gel for spot quantification.

2D gel analysis software

In quantitative proteomics, these tools primarily analyze bio-markers by quantifying individual proteins, and showing the separation between one or more protein "spots" on a scanned image of a 2-DE gel. Additionally, these tools match spots between gels of similar samples to show, for example, proteomic differences between early and advanced stages of an illness. Software packages include Delta2D, ImageMaster, Melanie, PDQuest, Progenesis and REDFIN – among others.[citation needed] While this technology is widely utilized, the intelligence has not been perfected. For example, while PDQuest and Progenesis tend to agree on the quantification and analysis of well-defined well-separated protein spots, they deliver different results and analysis tendencies with less-defined less-separated spots.

Challenges for automatic software-based analysis include incompletely separated (overlapping) spots (less-defined and/or separated), weak spots / noise (e.g., "ghost spots"), running differences between gels (e.g., protein migrates to different positions on different gels), unmatched/undetected spots, leading to missing values, mismatched spots , errors in quantification (several distinct spots may be erroneously detected as a single spot by the software and/or parts of a spot may be excluded from quantification), and differences in software algorithms and therefore analysis tendencies.

Applications

Proteomics analysis of cellular response to oxidative stress

The proteomics analysis reported here shows that a major cellular response to oxidative stress is the modification of several peroxiredoxins. An acidic form of the peroxiredoxins appeared to be systematically increased under oxidative stress conditions due to post transcriptional modifications. **Peroxiredoxin 2 and 3 spots in Jurkat** cells were separated by twodimensional electrophoresis. The peroxiredoxin spots were identified by mass spectrometry. The cells were either control cells (*A) or*

cells treated with 75 M BHP for 1 h (*B).* increase in the acidic peroxiredoxin spots under oxidative stress, and the corresponding decrease in the basic spot under BHP treatment

Protein Identification by Mass Profile Fingerprinting

Due to the high resolution of 2D gels, very simple and cheap MS process can be used to identify a protein from a 2D gel. For example, the old peptide mass fingerprinting method, which is fairly cheap, fast, and can be carried out on low-price TOF MS, works only with 2D gel-separated proteins, and will never work with any other technique of less resolving power. We can identify proteins at the sub-microgram level without sequence determination by chemical degradation. The protein, usually isolated by one- or two-dimensional gel electrophoresis, is digested by enzymatic or chemical means and the masses of the resulting peptides are determined by mass spectrometry. The resulting mass profile, i.e., the list of the molecular masses of peptides produced by the digestion, serves as a fingerprint which uniquely defines a particular protein. This fingerprint may be used to search the database of known sequences to find proteins with a similar profile. This provides a rapid and sensitive link between genomic sequences and 2D gel electrophoresis mapping of cellular proteins.

2D gel-based proteomics in toxicology

2-DE is used to find an association between decreased calciumbinding protein (calbindin-D 28 kDa), urinary calcium wasting and intratubular corticomedullary calcifications in rat kidney . They show that in dogs and monkeys, which are generally devoid of cyclosporine A (CsA)-mediated nephrotoxicity b/c renal calbindin levels not affected by the CsA treatment whereas in CsA-treated human kidney-transplant recipients with renal vascular or tubular toxicity, a marked decrease in renal calbindin-D 28 kDa protein level was found in most of the kidney biopsy sections. It suggest that calbindin is a marker for CsA-nephrotoxicity. The discovery of calbindin-D 28 kDa being involved in CsA toxicity has evolved from the application of 2-DE and has not been reported previously, proving that proteomics can provide essential information in mechanistic toxicology.

2D gel-based proteomics in bacterial proteomics

2D gel-based proteomics is also widely used in bacterial proteomics, when the complexity of the sample is low enough.

(A) Theoretical proteome of *B. subtilis showing the* distribution of all 4100 predicted proteins according to their isolelectric points and molecular weights.

(B) *B. subtilis master* 2-D gel for cytoplasmic proteins which are separated in the standard pH range 4–7 and in the alkaline pH range 7–12. In the master 2-D gel 519 proteins are labeled that were identified in the pH range 4–7. In addition, 174 proteins were identified in the narrow range pH gradients (pH 4–5, 4.5–5.5, 5–6, and 5.5– 6.7) and 52 proteins in the alkaline pH range 7–12. Cytoplasmic proteins were harvested from *B. subtilis wild type cells grown in* Belitsky minimal medium at an OD500 of 0.4 and separated by 2-D GE.

Use in immunoproteomics

2 D GE is also used in Immunoproteomics, where it is the immune response of patients that is probed at a proteomic level. 2D GE maps of proteins from Chlamydia trachomatis were probed with sera from 17 seropositive patients with genital inflammatory disease. Immunoblot patterns (comprising 28 to 2 spots, average 14.8) were different for each patient; however, antibodies against a spot-cluster due to the chlamydia-specific antigen outer membrane protein-2 (OMP2) were observed in all sera. The next most frequent group of antibodies (15/17; 88%) recognized the hsp60 like protein, described as immunopathogenic in chlamydial infections. A novel outer membrane protein (OmpB) and, interestingly, five conserved bacterial proteins: RNA polymerase alpha-subunit, ribosomal protein S1, protein elongation factor EF-Tu, putative stress-induced protease of the HtrA family, and ribosomal protein L7/L12. These proteins were shown to confer protective immunity in other bacterial infections.

2D gels in post-translational modifications

2D gels are also very appropriate when post-translational modifications are studied. Many post translational modifications do alter the pI and/or the MW of the proteins and thus induce position shifts in 2D gels. This is true for example for phosphorylation, glycosylation, but also more delicate modifications such as glutathionylation, or more forgotten modifications such as protein cleavage.

Limitations of 2-DE

Low reproducibility

In the traditional method of the first dimension IEF, the carrier ampholytes is utilized to build pH gradient. The carrier ampholytes-based pH gradient made from soft unsupported tube gels (typically 4% acrylamide) is not stable. There is batch to batch variability and prone to cathodic drift (a progressive loss of basic proteins during long running of electro-focusing under electric field), leading to low reproducibility and requiring careful monitoring of electric field. Replacement of carrier ampholyte-based pH gradient in tube gel with the immobilized pH gradients (IPG) was the key development in increasing the reproducibility of 2-DE.

Difficulty in separating hydrophobic and extremely acidic or basic proteins

Different types of proteins can always be missing, due to the difficulty in separating membrane-bound (hydrophobic) and extreme proteins . Notably, highly acidic or basic proteins are neither easily extracted nor solubilized. This difficulty in extraction relies mainly on the solubilization power of the buffer used in the IEF step. Many efforts have been performed for better solubilization of membrane proteins using different chaotropes or detergents. For instant, Triton X- 114 and CHAPS showed a powerful recovering power towards hydrophobic proteins on 2-DE when tested immunologically. Another study reported the use of cationic detergent benzyldimethyl-n-hexadecylammonium chloride to improve resolving hydrophobic proteins with GRAVY index as low as 0.56. Similar improvement was reported when using detergents such as DHP and 1,4-dithioethanol. Although solubilization of wide range of proteins could be achieved using denaturating solution (Urea and Thiourea) and zwitterionic detergents (SB 3–10), resistance of certain proteins still remains a built-in problem.

Narrow dynamic range of 2-DE

Low dynamic range of proteins is one of the challenging problems encountered during 2-DE. Highly abundant peptides mask low abundant ones, which may be reflective of low abundant proteins. Moreover, the visualization of faint protein spots (low abundant) separated on 2-DE gels is also governed by staining sensitivity. For example, classic coomassie has a narrow dynamic range with detection limit of only about 100 ng. Colloidal coomassie is relatively higher in sensitivity with detection limit 10 ng. The dynamic range could be increased to a detection level below 1 ng with the availability of highly sensitive silver-staining method and a diversity of fluorescence dyes such as SYPRO-Ruby and Deep purple flurophore dyes. Therefore, using sensitive stain increases protein sample dynamic range, leads to successful gel imaging, and finally leads to successful mass spectrometric identification and immunological validation. Alternatively, depletion of highly abundant proteins such as albumin and hemoglobin significantly improves the dynamic range of 2-DE by allowing better focusing and mass spectrometric picking (see Prefractionation, enrichment, and depletion prior to 2-DE section). Therefore, the limited detection sensitivity of 2-DE does not cope with the actual dynamic range of protein concentration in cell and tissue extracts, or biological fluids. Other solution to overcome the dynamic range problem is to load more protein sample and using a giant 2-DE gel (24 cm). However, this approach could result in production of overcrowded images with non well-separated spots.

Low throughput and labor- intensiveness

2-DE is labor-intensive and has a relative low throughput. The throughput of 2-DE is adequate for many small-scale basic research studies, but it may present a serious obstacle for projects that involve

screening of a large number of clinical samples. Furthermore, 2-DE requires skills and experience to counteract any possible trouble shooting due to the procedural artifacts. Much care should be considered to minimize the variations during sample processing that lasts up to 3 days in larger gels.

Gel Free Proteomics

By means of recently developed bottom-up (shotgun) proteomics techniques, hydrophobic proteins as well as peptides can be analyzed. Instead of using 2D-PAGE, these approaches use multi-dimensional capillary liquid chromatography coupled to tandem mass spectrometry to separate and identify the peptides obtained from the enzymatic digest of an entire protein extract. In short, it is important to realize that in this aproach it is not the protein itselve, which is separated and identified. Instead, proteins are cleaved into peptides using proteolytic enzymes and, subsequently, these peptides are separated and subjected to tandem mass spectrometric analysis. Mass spectrometric identification of these peptides allows the determination of the protein content of the initial sample. Since peptides can be more easily separated by liquid chromatography than proteins, a peptide based proteomic analysis can be performed much faster and cheaper than a complete gel-based analysis. Several gel-free based proteomics technologies have now been described.

and

Some examples of gel-free approaches are liquid chromatography [LC], capillary electrophoresis.

Liquid chromatography (LC) is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase, the different solutes will interact with the other phase to differing degrees due to differences in adsorption, ion-exchange, partitioning, or size.

Liquid chromatography–mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). Coupled chromatography - MS systems are popular in chemical analysis because the individual capabilities of each technique are enhanced synergistically. While liquid chromatography separates mixtures with multiple components, mass spectrometry provides structural identity of the individual components with high molecular specificity and detection sensitivity. This tandem technique can be used to analyze biochemical, organic, and inorganic compounds commonly found in complex samples of environmental and biological origin. Therefore, LC-MS may be applied in a wide range of sectors including biotechnology, environment monitoring, food processing, and pharmaceutical, agrochemical, and cosmetic industries.

Capillary electrophoresis is an analytical technique that separates ions based on their electrophoretic mobility with the use of an applied voltage. The electrophoretic mobility is dependent upon the charge of the molecule, the viscosity, and the atom's radius.

Mass spectrophotometry:

Mass spectrometry (MS) is an analytical technique that measures the **mass-to-charge ratio of ions**. The results are typically presented as a mass spectrum, a plot of intensity as a function of the mass-to-charge ratio. Mass spectrometry is used in many different fields and is applied to pure samples as well as complex mixtures.

A mass spectrum is a plot of the ion signal as a function of the mass-to-charge ratio. These spectra are used to determine the elemental or isotopic signature of a sample, the masses of particles and of molecules, and to elucidate the chemical identity or structure of molecules and other chemical compounds.

In a typical MS procedure, a sample, which may be solid, liquid, or gaseous, is ionized, for example by bombarding it with electrons. This may cause some of the sample's molecules to break into charged fragments or simply become charged without fragmenting. These ions are then separated

according to their mass-to-charge ratio, for example by accelerating them and subjecting them to an electric or magnetic field: ions of the same mass-to-charge ratio will undergo the same amount of deflection. The ions are detected by a mechanism capable of detecting charged particles, such as an electron multiplier. Results are displayed as spectra of the signal intensity of detected ions as a function of the mass-to-charge ratio. The atoms or molecules in the sample can be identified by correlating known masses (e.g. an entire molecule) to the identified masses or through a characteristic fragmentation pattern.

Basic Principle

A mass spectrometer generates multiple ions from the sample under investigation; it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type.

The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound.

Components

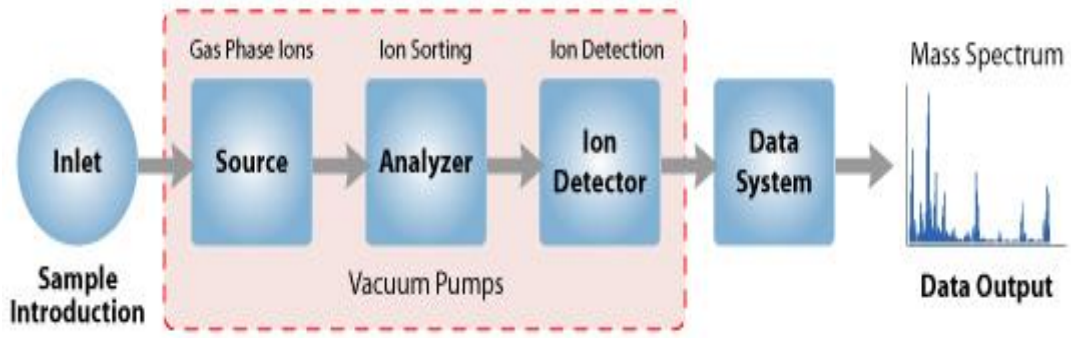
The instrument consists of three major components:

Ion Source: For producing gaseous ions from the substance being studied.

Analyzer: For resolving the ions into their characteristics mass components according to their mass-to-charge ratio.

Detector System: For detecting the ions and recording the relative abundance of each of the resolved ionic species.

In addition, a sample introduction system is necessary to admit the samples to be studied to the ion source while maintaining the high vacuum requirements (~10^{-6} to 10^{-8} mm of mercury) of the technique; and a computer is required to control the instrument, acquire and manipulate data, and compare spectra to reference libraries.



Components of a Mass Spectrometer

With all the above components, a mass spectrometer should always perform the following processes:

- Produce ions from the sample in the ionization source.
- Separate these ions according to their mass-to-charge ratio in the mass analyzer.
- Eventually, fragment the selected ions and analyze the fragments in a second analyzer.

- Detect the ions emerging from the last analyzer and measure their abundance with the detector that converts the ions into electrical signals.
- Process the signals from the detector that are transmitted to the computer and control the instrument using feedback.

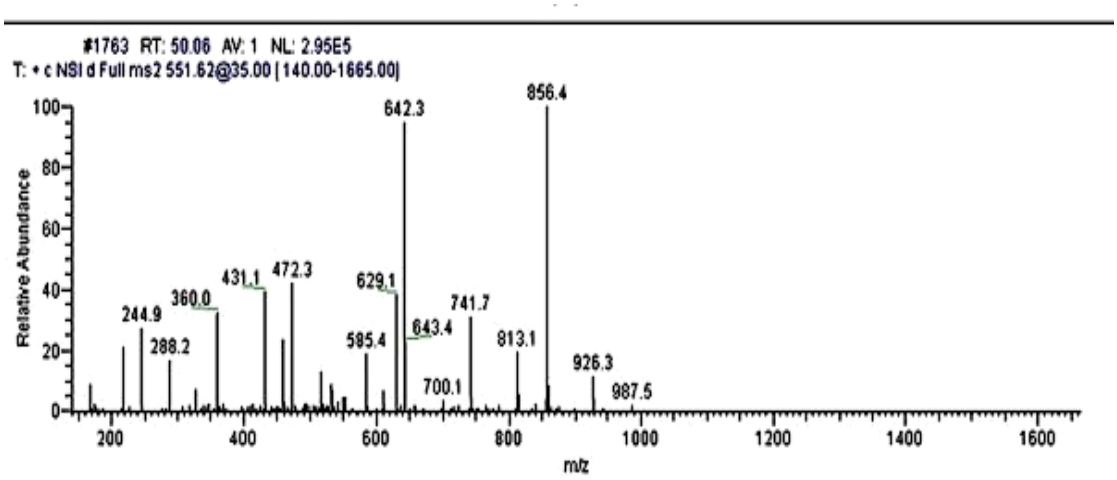
Calculations and Deductions:

Given a sample of a single element, the relative atomic mass can be calculated by looking at the peaks and performing a simple mean calculation.

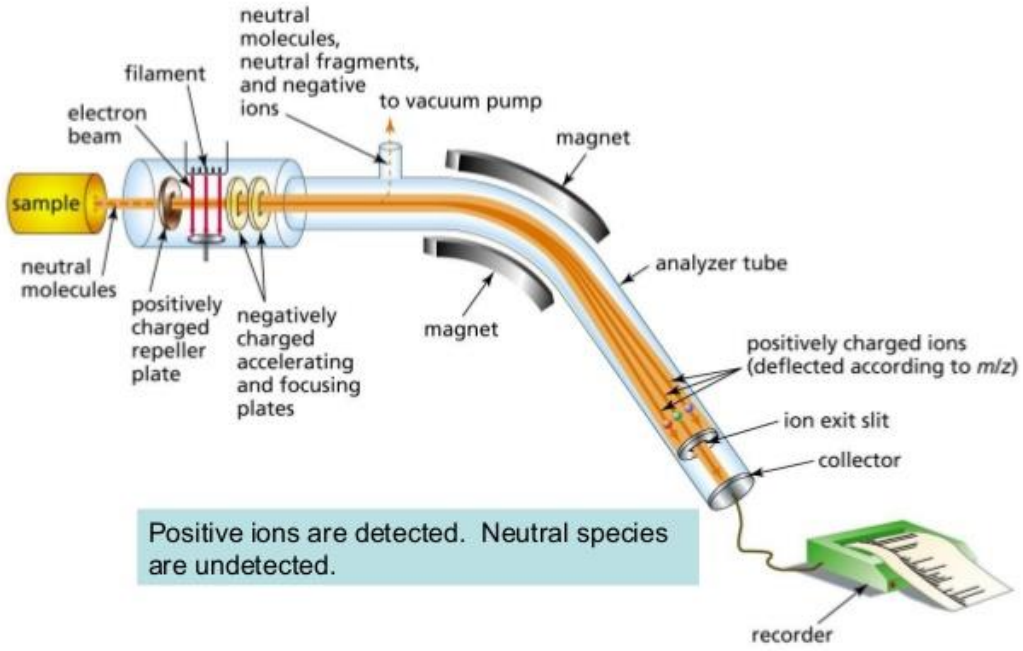
The relative molecular mass can be deduced by looking for the Molecular Ion peak, since this will be the peak caused by the whole molecule.

The structure of a molecule can be deduced by looking at the smaller peaks and inferring the structure of those, given the likely combinations of atoms present that could produce that mass. This is because these peaks will be cause by fragments of the whole molecule. The stronger the bond between atoms, the less likely it is to break, and so the lower the abundance of the fragments that would be formed by the breaking of that bond.

In reality, peaks will not be perfectly clear because of the varying mass of individual atoms. However, smaller peaks like this can help to determine the structure of the larger ones.



Mass Spectrometer



This tube is curved, and the ions are deflected by a strong magnetic field. Ions of different mass to charge (m/z) ratios are deflected to a different extent, resulting in a 'sorting' of ions by mass (virtually all ions have charges of z = +1, so sorting by the mass to charge ratio is the same thing as sorting by mass). A detector at the end of the curved flight tube records and quantifies the sorted ions.

Mass spectrometry of proteins - applications in proteomics

Protein mass spectrometry refers to the application of mass spectrometry to the study of proteins. Mass spectrometry is an important method for the accurate mass determination and characterization of proteins, and a variety of methods and instrumentations have been developed for its many uses. Its applications include the identification of proteins and their post-translational modifications, the elucidation of protein complexes, their subunits and functional interactions, as well as the global measurement of proteins in proteomics. It can also be used to localize proteins to the various organelles, and determine the interactions between different proteins as well as with membrane lipids.

The two primary methods used for the ionization of protein in mass spectrometry are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). These ionization techniques are used in conjunction with mass analyzers such as tandem mass spectrometry. In general, the protein are analyzed either in a "top-down" approach in which proteins are analyzed intact, or a "bottom-up" approach in which protein are first digested into fragments. An intermediate "middle-down" approach in which larger peptide fragments are analyzed may also sometimes be used.

Techniques

Mass spectrometry of proteins requires that the proteins in solution or solid state be turned into an ionized form in the gas phase before they are injected and accelerated in an electric or magnetic field for analysis. The two primary methods for ionization of proteins are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). In electrospray, the ions are created from proteins in solution, and it allows fragile molecules to be ionized intact, sometimes preserving non-covalent interactions. In MALDI, the proteins are embedded within a matrix normally in a solid form, and ions are created by pulses of laser light. Electrospray produces more multiply-charged ions than MALDI, allowing for measurement of high mass protein and better fragmentation for identification, while MALDI is fast and less likely to be affected by contaminants, buffers and additives.[8]

Whole-protein mass analysis is primarily conducted using either time-of-flight (TOF) MS, or Fourier transform ion cyclotron resonance (FT-ICR). These two types of instrument are preferable here because of their wide mass range, and in the case of FT-ICR, its high mass accuracy. Electrospray ionization of a protein often results in generation of multiple charged species of $800 < m/z < 2000$ and the resultant spectrum can be deconvoluted to determine the protein's average mass to within 50 ppm or better using TOF or ion-trap instruments.

Mass analysis of proteolytic peptides is a popular method of protein characterization, as cheaper instrument designs can be used for characterization. Additionally, sample preparation is easier once whole proteins have been digested into smaller peptide fragments. The most widely used instrument for peptide mass analysis are the MALDI-TOF instruments as they permit the acquisition of peptide mass fingerprints (PMFs) at high pace (1 PMF can be analyzed in approx. 10 sec). Multiple stage quadrupole-time-of-flight and the quadrupole ion trap also find use in this application.

Tandem mass spectrometry (MS/MS) is used to measure fragmentation spectra and identify proteins at high speed and accuracy. Collision-induced dissociation is used in mainstream applications to generate a set of fragments from a specific peptide ion. The fragmentation process primarily gives rise to cleavage products that break along peptide bonds. Because of this simplicity in fragmentation, it is possible to use the observed fragment masses to match with a database of predicted masses for one of many given peptide sequences. Tandem MS of whole protein ions has been investigated recently

using electron capture dissociation and has demonstrated extensive sequence information in principle but is not in common practice.

Approaches

In keeping with the performance and mass range of available mass spectrometers, two approaches are used for characterizing proteins. In the first, intact proteins are ionized by either of the two techniques described above, and then introduced to a mass analyzer. This approach is referred to as "top-down" strategy of protein analysis as it involves starting with the whole mass and then pulling it apart. The top-down approach however is mostly limited to low-throughput single-protein studies due to issues involved in handling whole proteins, their heterogeneity and the complexity of their analyses.

In the second approach, referred to as the "bottom-up" MS, proteins are enzymatically digested into smaller peptides using a protease such as trypsin. Subsequently, these peptides are introduced into the mass spectrometer and identified by peptide mass fingerprinting or tandem mass spectrometry. Hence, this approach uses identification at the peptide level to infer the existence of proteins pieced back together with de novo repeat detection. The smaller and more uniform fragments are easier to analyze than intact proteins and can be also determined with high accuracy, this "bottom-up" approach is therefore the preferred method of studies in proteomics. A further approach that is beginning to be useful is the intermediate "middle-down" approach in which proteolytic peptides larger than the typical tryptic peptides are analyzed.

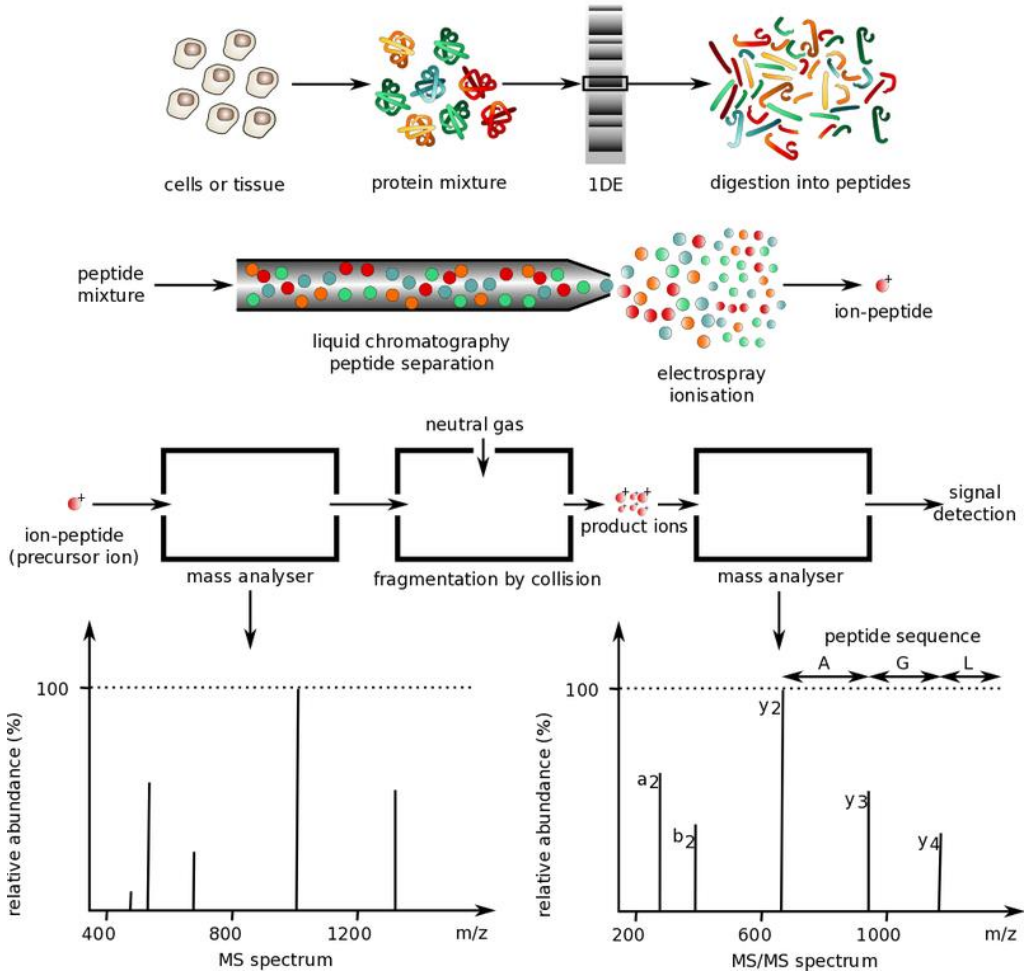
Protein and peptide fractionation

Proteins of interest are usually part of a complex mixture of multiple proteins and molecules, which co-exist in the biological medium. This presents two significant problems. First, the two ionization techniques used for large molecules only work well when the mixture contains roughly equal amounts of material, while in biological samples, different proteins tend to be present in widely differing amounts. If such a mixture is ionized using electrospray or MALDI, the more abundant species have a tendency to "drown" or suppress signals from less abundant ones. Second, mass spectrum from a complex mixture is very difficult to interpret due to the overwhelming number of mixture components. This is exacerbated by the fact that enzymatic digestion of a protein gives rise to a large number of peptide products.

In light of these problems, the methods of one- and two-dimensional gel electrophoresis and high performance liquid chromatography are widely used for separation of proteins. The first method fractionates whole proteins via two-dimensional gel electrophoresis. The first-dimension of 2D gel is isoelectric focusing (IEF). In this dimension, the protein is separated by its isoelectric point (pI) and the second-dimension is SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This dimension separates the protein according to its molecular weight. Once this step is completed in-gel digestion occurs. In some situations, it may be necessary to combine both of these techniques. Gel spots identified on a 2D Gel are usually attributable to one protein. If the identity of the protein is desired, usually the method of in-gel digestion is applied, where the protein spot of interest is excised, and digested proteolytically. The peptide masses resulting from the digestion can be determined by mass spectrometry using peptide mass fingerprinting. If this information does not allow unequivocal identification of the protein, its peptides can be subject to tandem mass spectrometry for de novo sequencing. Small changes in mass and charge can be detected with 2D-PAGE. The disadvantages with this technique are its small dynamic range compared to other methods, some proteins are still difficult to separate due to their acidity, basicity, hydrophobicity, and size (too large or too small).

The second method, high performance liquid chromatography is used to fractionate peptides after enzymatic digestion. Characterization of protein mixtures using HPLC/MS is also called shotgun proteomics and MuDPIT (Multi-Dimensional Protein Identification Technology). A peptide mixture that results from digestion of a protein mixture is fractionated by one or two steps of liquid

chromatography. The eluent from the chromatography stage can be either directly introduced to the mass spectrometer through electrospray ionization, or laid down on a series of small spots for later mass analysis using MALDI.



16. Let's sum up

- A restriction enzyme or restriction endonuclease is an enzyme that cleaves DNA into fragments at or near specific recognition sites within the molecule known as restriction sites. Restriction enzymes are commonly classified into five types, which differ in their structure and whether they cut their DNA substrate at their recognition site, or if the recognition and cleavage sites are separate from one another.
- A cloning vector is a small piece of DNA, taken from a virus, a plasmid, or the cell of a higher organism, that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted for cloning purposes.
- There are many types of cloning vectors, but the most commonly used ones are genetically engineered plasmids. Cloning is generally first performed using Escherichia coli, and cloning vectors in E. coli include plasmids, bacteriophages (such as phage λ), cosmids, and bacterial artificial chromosomes (BACs).
- Polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence, developed in 1983 by Kary Mullis.
- DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine—in a strand of DNA.
- Agarose gel electrophoresis is a method of gel electrophoresis used in biochemistry, molecular biology, genetics, and clinical chemistry to separate a mixed population of macromolecules such as DNA or proteins in a matrix of agarose, one of the two main components of agar. The proteins may be separated by charge and/or size
- Blotting is technique in which nucleic acids i.e., RNA and DNA or proteins are transferred onto a specific membrane
- A cDNA library is a combination of cloned cDNA (complementary DNA) fragments inserted into a collection of host cells, which together constitute some portion of the transcriptome of the organism and are stored as a "library".
- DNA footprinting is a method of investigating the sequence specificity of DNA-binding proteins in vitro. This technique can be used to study protein-DNA interactions both outside and within cells.
- The ultimate goal of transgenic (involving introduction, integration, and expression of foreign genes) is to improve the crops, with the desired traits (i) resistance to biotic stresses (ii) resistance to abiotic stresses (iii) improvement of crop yield, and quality (iv) for the manufacture of commercial products e.g. proteins, vaccines, and biodegradable plastics.
- A new era in the studies of chromosome pairing was ushered in with the introduction of electron microscope to biological research. A tripartite ribbon at the site of synapsis; this structure is called synaptonemal complex or synaptinemal complex.This complex occupies the space between the paired homologous chromosomes and appears to be a advice to bring together and allign the DNA molecules from paired homologous chromosomes which facilitates crossing over between them.

- RecBCD is an enzyme of the *E. coli* bacterium that initiates recombinational repair from potentially lethal double strand breaks in DNA which may result from ionizing radiation, replication errors, endonucleases, oxidative damage, and a host of other factors.

- Site specific recombination alters the relative position of nucleotide sequences in chromosome. The base pairing reaction depends on protein mediated recognition of the two DNA sequences that will combine. Very long homologous sequence is not required.

- A genetic disease is the result of changes, or mutations, in an individual's DNA. A mutation is a change in the letters that make up a gene. This is sometimes referred to as a "spelling" mistake. Genes code for proteins, the molecules that carry out most of the work, perform most life functions, and make up the majority of cellular structures. When a gene is mutated so that its protein product can no longer carry out its normal function, a disorder can result.

- Pedigrees are diagrams that show the relationships among the members of a family. It is customary to represent males as squares and females as circles. A horizontal line connecting a circle and a square represents a mating.

- A gene knockout is a genetic technique in which one of an organism's genes is made inoperative. However, KO can also refer to the gene that is knocked out or the organism that carries the gene knockout. Knockout organisms or simply knockouts are used to study gene function, usually by investigating the effect of gene loss.

- Gene expression is more complicated in eukaryotes than it is in prokaryotes because eukaryotic cells are compartmentalized by an elaborate system of membranes. Regulation can occur in the nucleus at either the DNA or RNA level, or in the cytoplasm at either the RNA or polypeptide level. Eukaryotic gene expression involves many steps, and almost all of them can be regulated.

- Females have two copies of every X-linked gene and males have only one copy, the amount of gene product encoded by X-linked genes would differ in the two sexes: females would produce twice as much gene product as that produced by males. This difference could be highly detrimental because protein concentration plays a critical role in development. Animals overcome this potential problem through dosage compensation**,** which equalizes the amount of protein produced by X-linked genes in the two sexes.
- Normally, the genetic information is not altered in the mRNA intermediary. However, the discovery of RNA editing has shown that exceptions do occur. RNA editing processes alter the information content of gene transcripts in two ways: (1) by changing the structures of individual bases and (2) by inserting or deleting uridine monophosphate residues.

- Antisense RNA is a single stranded RNA that is complementary to a protein coding messenger RNA (mRNA) with which it hybridizes, and thereby blocks its translation into protein. asRNAs have been found in both prokaryotes and eukaryotes.

- RNA interference (RNAi) is a mechanism where the presence of certain fragments of dsRNA interferes with the expression of a particular gene which shares a homologous sequence with this dsRNA.
- The Cancer is a group of disease characterized by uncontrolled cell growth and division.Cancers arise when critical genes are mutated. These mutations can cause biochemical processes to go awry and lead to the unregulated proliferation of cells.Without regulation, cancer cells divide ceaselessly, piling up on top of each other toform tumors. When cells detach from a tumor and invade the surrounding tissues, thetumor is malignant.

- Proto-oncogenes can be converted to oncogenes by several mechanisms, such as, (i) mutational change in the protein, (ii) constitutive activation, (iii) gene amplification resulting

in over expression of gene, (iv) reciprocal translocation and position effect (v) mutation in tumour suppressor gene.

- The transfer DNA is the transferred DNA of the tumor-inducing (Ti) plasmid of some species of bacteria such as *Agrobacterium tumefaciens* and The T-DNA is transferred from bacterium into the host plant's nuclear DNA genome. The capability of this specialized tumor-inducing (Ti) plasmid is attributed to two essential regions required for DNA transfer to the host cell.

- Cell surface receptors (membrane receptors, transmembrane receptors) are receptors that are embedded in the membranes of cells. They act in cell signaling by receiving (binding to) extracellular molecules. They are specialized integral membrane proteins that allow communication between the cell and the extracellular space. The extracellular molecules may be hormones, neurotransmitters, cytokines, growth factors, cell adhesion molecules, or nutrients; they react with the receptor to induce changes in the metabolism and activity of a cell.

- Genomics is an interdisciplinary field of biology focusing on the structure, function, evolution, mapping, and editing of genomes. A genome is an organism's complete set of DNA, including all of its genes. Molecular markers are effective because they identify an abundance of genetic linkage between identifiable locations within a chromosome and are able to be repeated for verification. They can identify small changes within the mapping population enabling distinction between a mapping species, allowing for segregation of traits and identity.

- A DNA microarray is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome.

- The term megadiverse country refers to any one of a group of nations that harbour the majority of Earth's species and high numbers of endemic species. Conservation International identified 17 megadiverse countries in 1998. A biodiversity hotspot is a biogeographic region with a significant reservoir of biodiversity that is under threat from humans.

- Proteomics is the large-scale study of proteins. Proteins are vital parts of living organisms, with many functions. The term proteomics was coined in 1997 in analogy with genomics, the study of the genome.

- Two-dimensional gel electrophoresis, abbreviated as 2-DE or 2-D electrophoresis, is a form of gel electrophoresis commonly used to analyze proteins. Mixtures of proteins are separated by two properties in two dimensions on 2D gels.

- Two methods are widely used for protein identification by mass spectrometry- MALDI-TOF based protein fingerprinting and LC-MS/MS based peptide sequencing. In MALDI-TOF based protein fingerprinting method, a sample is digested with certain proteolytic enzyme (usually trypsin) and one MS spectrum is acquired which generates the masses of all peptides, and these masses are used as a fingerprint to search proteins in a database.

17. Suggested Reading

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18. Assignment

1. Is cancer genetically inherited?
2. Discuss the role of P^{53} in regulation of cancer.
3. How protooncogenes are converted into oncogenes?
4. Distinguish beteen gene knockout and gene knockin.
5. What is guide RNA?
6. What do you mean by gene conversion?
7. Explain the molecular mechanism of Rec BCD pathway.
8. Differentiate between siRNA and miRNA.
9. Define molecular farming. Cite some examples.
10. How is the antisense RNA technology used for production of transgenic plant varieties
11. What is Golden rice?
12. What are the organizations of Vir genes?
13. What is Glyphosate? What are the different mechanisms of glyphosate function? Briefly describe mechanism of t-DNA transfer and integration.
14. What is pyro-sequencing?
15. Mention the role of alkaline phosphatase in gen cloning strategies.
16. What is LC-MS?
17. Write the characters of autosomal recessive inheritance.
18. Why secondary antibody is needed in western blotting?
19. Define meta genomics.
20. What is meant by disarming?
21. Mention the basic principles of 2-DE. Enumerate different steps of 2-DE.
22. What is RT-PCR?

23. Name two stain used in DNA gel electrophoresis.
24. What is SCAR?
25. How bt toxin helps to production of insecticide resistance plant.
26. What is meant by binary vector strategy?
27. What is electroporation?
28. Write a short note on direct method of gene transfer.
29. What is reporter genes?
30. How does a miRISC function in posttrancriptional gene silencing?
31. What is DNA methylation?
32. Write down the evolutionary significance of RNA editing.
33. Briefly describe four examples of gene regulation at the level of transcription.
34. What do you mean by genomic imprinting?
35. What is alternative splicing?
36. What are the types of gene therapy?
37. Mention the role of counsellor in gene counselling.
38. Explain G-protein linked receptor of cell signalling.
39. Name two PCR based markers used in genome mapping.
40. Give an explanatory note on Human genome project.
41. How does mass spectrometry helps in the study of protein structure?
42. What is protein mining?
43. Give two examples of enzyme linked receptors of cell signalling.
44. How SNPs markers help in genome mapping?
45. Discuss RNA processing in eukaryotes to produce mRNA from pre mRNA
46. What are different types of restriction enzymes?
47. What is isoschizomers?
48. Write a short note on DNA microarray and chip technology.
49. Define chromosome walking.
50. Enumerate the steps of recombinant DNA construction.
51. What is cosmid?
52. Differentiate between BAC abd YAC.
53. What is shuttle vector?
54. What are the principles of Southern blotting? Explain the procedure of its.
55. How c-DNA library is constructed?
56. Enumerate the steps of DNA fingerprinting. Mention its application.
57. What is meant by expression proteomics?
58. Write a short note on genomics.
59. Give a basic outline of genome annotationpipeline and delivery system.
60. Name two bioinformatics institute of worlds. Differentiate between genomics and proteomics.

All the materials are self writing and collected from ebook, journals and websites.

BOTANY

POST GRADUATE DEGREE PROGRAMME
(CBCS CURRICULUM)

SEMESTER: IV

PAPER: BOET 4.2

Plant Breeding & Biometry

Directorate of Open and Distance Learning
UNIVERSITY OF KALYANI
Kalyani, Nadia
West Bengal

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Website : www.klyuniv.ac.in

POST GRADUATE DEGREE PROGRAMME (CBCS)
IN
BOTANY

SEMESTER - IV

Course: BOET 4.2

(Plant Breeding & Biometry)

Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI
KALYANI – 741 235,
WEST BENGAL

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks are also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof. Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

SYLLABUS
COURSE – BOET 4.2
Plant Breeding & Biometry
(Full Marks – 100)

Course	Group	Details Contents Structure		Study hour
BOET 4.2	**Plant Breeding & Biometry**	**Unit 1. Male Sterility-I**	1. Male Sterility: Introduction and type	1
		Unit 2. . Male Sterility-II	2. Male Sterility: Characterization and application.	1
		Unit 3 Self incompatibility-I	3. Self incompatibility: Types, significance; overcoming self incompatibility.	1
		Unit 3 Self incompatibility-II	4. Self incompatibility: genetic and molecular basis	1
		Unit 5. Polyploidy in angiosperms	5. Polyploidy in angiosperms - genetic insight to the phenomenon.	1
		Unit 6. Distant hybridization	6. Distant hybridization: Barriers and achievements. Bridge species.	1
		Unit 7. Back cross method of breeding	7. Back cross method of breeding: Significance and limitations; multiline concept.	1
		Unit 8. Molecular marker assisted breeding-I	8. Molecular marker assisted breeding. Molecular markers in genome.	1
		Unit 9. Molecular marker assisted breeding-II	9. Molecular marker assisted breeding. QTL analyses.	1
		Unit 10. Breeding approaches to improve nutritional quality of food crops	10. Breeding approaches to improve nutritional quality of food crops.	1
		Unit 11. Biotechnological approaches to improve nutritional quality of food crops	11. Biotechnological approaches to improve nutritional quality of food crops	1
		Unit 12. Biotechnological approaches for improving abiotic stress tolerance in plants.	12. Biotechnological approaches to improve nutritional quality of food crops	1

Course	Group	Details Contents Structure		Study hour
BOET4.2	**Plant Breeding & Biometry**	**Unit 13. Genetics of disease resistance in crop plants & Concept of heritability**	13. Genetics of disease resistance in crop plants. 14. Concept of heritability.	1
		Unit 14. Use of biometrical tests in genetics and breeding with special reference to path-coefficient analysis-I	15. Use of biometrical tests in genetics and breeding with special reference to path-coefficient analysis-Mean, mode, median, measures of dispersion and chi-square test	1
		Unit 15. Use of biometrical tests in genetics and breeding with special reference to path-coefficient analysis-II	16. Use of biometrical tests in genetics and breeding with special reference to path-coefficient analysis- t-test, probability and simple and multiple correlation and regression	1
		Unit 16. Use of biometrical tests in genetics and breeding with special reference to path-coefficient analysis-III	17. Use of biometrical tests in genetics and breeding with special reference to path-coefficient analysis-ANOVA and path-coefficient analysis	1

Content

COURSE – BOET 4.2

(Plant Breeding & Biometry)

Hard Core Theory Paper **Credit: 4**

Content Structure

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1. Introduction
2. Course Objectives
3. Male Sterility: Introduction, characterization and application.
4. Self incompatibility: Types, significance; genetic and molecular basis; overcoming self incompatibility.
5. Polyploidy in angiosperms - genetic insight to the phenomenon.
6. Distant hybridization: Barriers and achievements. Bridge species.
7. Back cross method of breeding: Significance and limitations; multiline concept.
8. Molecular marker assisted breeding. Molecular markers in genome and QTL analyses.
9. Breeding and biotechnological approaches to improve nutritional quality of food crops.
10. Biotechnological approaches for improving abiotic stress tolerance in plants.
11. Genetics of disease resistance in crop plants.
12. Concept of heritability.
13. Use of biometrical tests in genetics and breeding with special reference to path-coefficient analysis.
14. Let's Sum Up
15. Suggested Reading
16. Assignment

1. Introduction

Plant breeding is an art and science, which tells us ways and means to change the genetic architecture of plants so as to attain a particular objective. Plant breeding can be accomplished through many different techniques ranging from simply selecting plants with desirable characteristics for propagation, to more complex molecular techniques Plant breeding has been practiced for thousands of years, since near the beginning of human civilization. It is now practiced worldwide by individuals such as gardeners and farmers, or by professional plant breeders employed by organizations such as government institutions, universities, crop-specific industry associations or research centers. International development agencies believe that breeding new crops is important for ensuring food security by developing new varieties that are higher-yielding, resistant to pests and diseases, drought-resistant or regionally adapted to different environments and growing conditions.

From this course, you will get the detail knowledge about various breeding approaches and biostatistical experiments relating to estimation and hypothesis testing.

2. Course Objectives

You should gather knowledge after studying the course:

1. How to improve the yield of "economic produce on economic part"
2. To know various achievements about crossing
3. Details explanation about Mass Selection Breeding
4. Gathering knowledge about disease resistance of plant

In this course you will learn how to effectively collect data, describe data, and use data to make inferences and conclusions about real world phenomena. After finishing this course, you should be able to:

1. Recognize the importance of data collection and its role in determining scope of inference.
2. Demonstrate a solid understanding of interval estimation and hypothesis testing.
3. Choose and apply appropriate statistical methods for analyzing one or two variables.
4. Use technology to perform descriptive and inferential data analysis for one or two variables.
5. Interpret statistical results correctly, effectively, and in context.
6. Understand and critique data-based claims.
7. Appreciate the power of data.

3. Male Sterility: Introduction, characterization and application

Introduction:

Male sterility is defined as an absence or non-function of pollen grain in plant or incapability of plants to produce or release functional pollen grains as a result of failure of formation or development of functional stamens, microspores or gametes. Main reason is mutation. The use of male sterility in hybrid seed production has a great importance as it eliminate the process of mechanical emasculation.

Morphological features of male sterility

The male sterility may be due to mutation, chromosomal aberrations, cytoplasmic factors or interaction of cytoplasmic and genetic factors. Because of any of the above reasons the following morphological changes may occur in male sterile plants.

- Viable pollen grains are not formed. The sterile pollen grains will be transparent and rarely takes up stain faintly.
- Non-dehiscence of anthers, even though viable pollens are enclosed within. This may be due to hard outer layer, which restrict the release of pollen grains.
- Androecium may abort before the pollen grains are formed.
- Androecium may be malformed, thus there is no possibility of pollen grain formation.

Manifestations of Male Sterility:

- Absence or malformation of male organs
- Failure to develop normal microsporogenous tissue- anther
- Abnormal microsporogenesis (deformed or inviable pollen)
- Abnormal pollen maturation
- Non dehiscent anthers but viable pollen, sporophytic control
- Barriers other than incompatibility preventing pollen from reaching ovule

History of Male Sterility:

- J.K. Koelreuter (1763) observed anther abortion within species & species hybrids
- Genic male sterility has been reported in cabbage (Rundfeldt 1960), cauliflower (Nieuwhof 1961)
- Male sterility systems have been also developed through genetic engineering (Williams et al. 1997) and protoplast fusion (Pelletier et al. 1995)
- Male sterility were artificially induced through mutagenesis¬Kaul 1988)

Why Male Sterility?

- Reduced the cost of hybrid seed production.
- Production of large scale of F1 seeds.
- Avoids enormous manual work of emasculation
- and pollination.
- Speed up the hybridization programme.
- Commercial exploitation of hybrid vigour.

Classification of Male Sterility:

Kaul (1988) Classified Male Sterility in three major groups

1. Phenotypic Male Sterility (Morphological)
   - Structural or Staminal Male Sterility
   - Pollen Male Sterility
   - Functional Male Sterility
2. Genotypic Male Sterility
   - Genetic Male Sterility (GMS)
     - Environmental Sensitive (EGMS)
       a) Thermo sensitive genetic male sterility (TGMS)
       b) Photoperiod sensitive genetic male sterility (PGMS)
     - Environmental non-sensitive
   - Cytoplasmic Male Sterility (CMS)
   - Cytoplasmic Genetic Male Sterility (CGMS)
   - Transgenic Male Sterility (TMS)
3. Chemically Induced Male Sterility (CHA)

Phenotypic Male Sterility:

Pollen sterility: In which male sterile individuals differ from normal only in the absence or extreme scarcity of functional pollen grains (the most common and the only one that has played a major role in plant breeding).

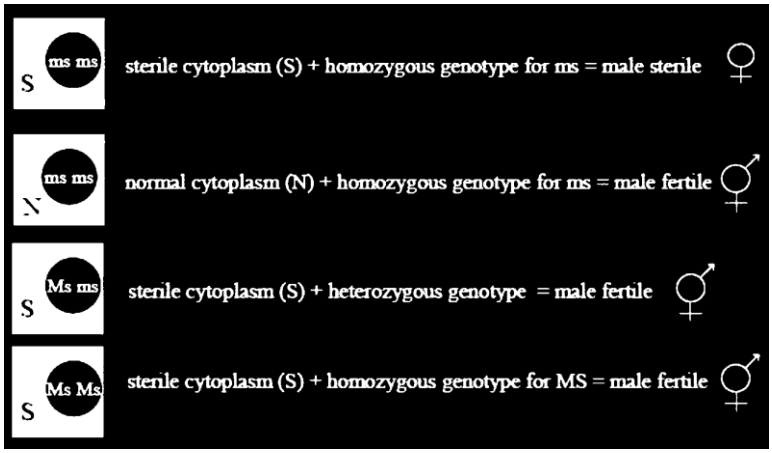
Structural or staminal male sterility: In which male flowers or stamen are malformed and non functional or completely absent.

Functional male sterility: in which perfectly good and viable pollen is trapped in indehiscent anther and thus prevented from functioning

Cytoplasmic Male Sterility (CMS):

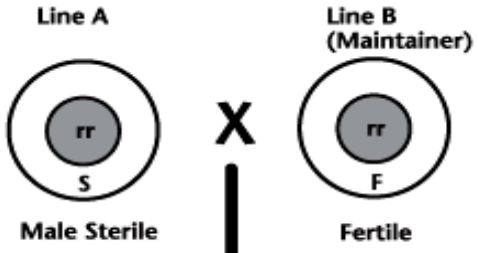
This type is governed by the cytoplasm (mitochondrial or chloroplast genes). Result of mutation in mitochondrial genome (mtDNA)-Mitochondrial dysfunction. Progenies would always be male sterile since the cytoplasm comes primarily from female gamete only. Nuclear genotype of male sterile line is almost identical to that of the recurrent pollinator strain. Male fertile line (maintainer line or B line) is used to maintain the male sterile line (A line). CMS is not influenced by environmental factors (temperature) so is stable.

Utilization of CMS in Plant Breeding: It may be used for hybrid seed production of certain ornamental species or in species where a vegetative part is of economic value. But not for crop plants where seed is the economic part because the hybrid progeny would be male sterile. This type of male sterility found in onion, fodder jowar, cabbage etc.

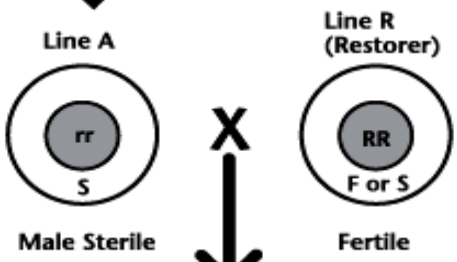


Use of CMS lines:

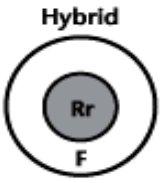
Step 1: Foundation seed crossing block to produce more Line A seed



Step 2: Hybrid seed crossing block to produce actual hybrid seed for commercial crops



Step 3: Commercial canola production



Fertile
(Self-pollinates to produce seed)

S = sterile cytoplasm
F = fertile cytoplasm
rr = homozygous for maintainer gene
RR = homozygous for dominant restorer gene
Rr = heterozygous for restorer gene

Transfer of CMS to new strains:

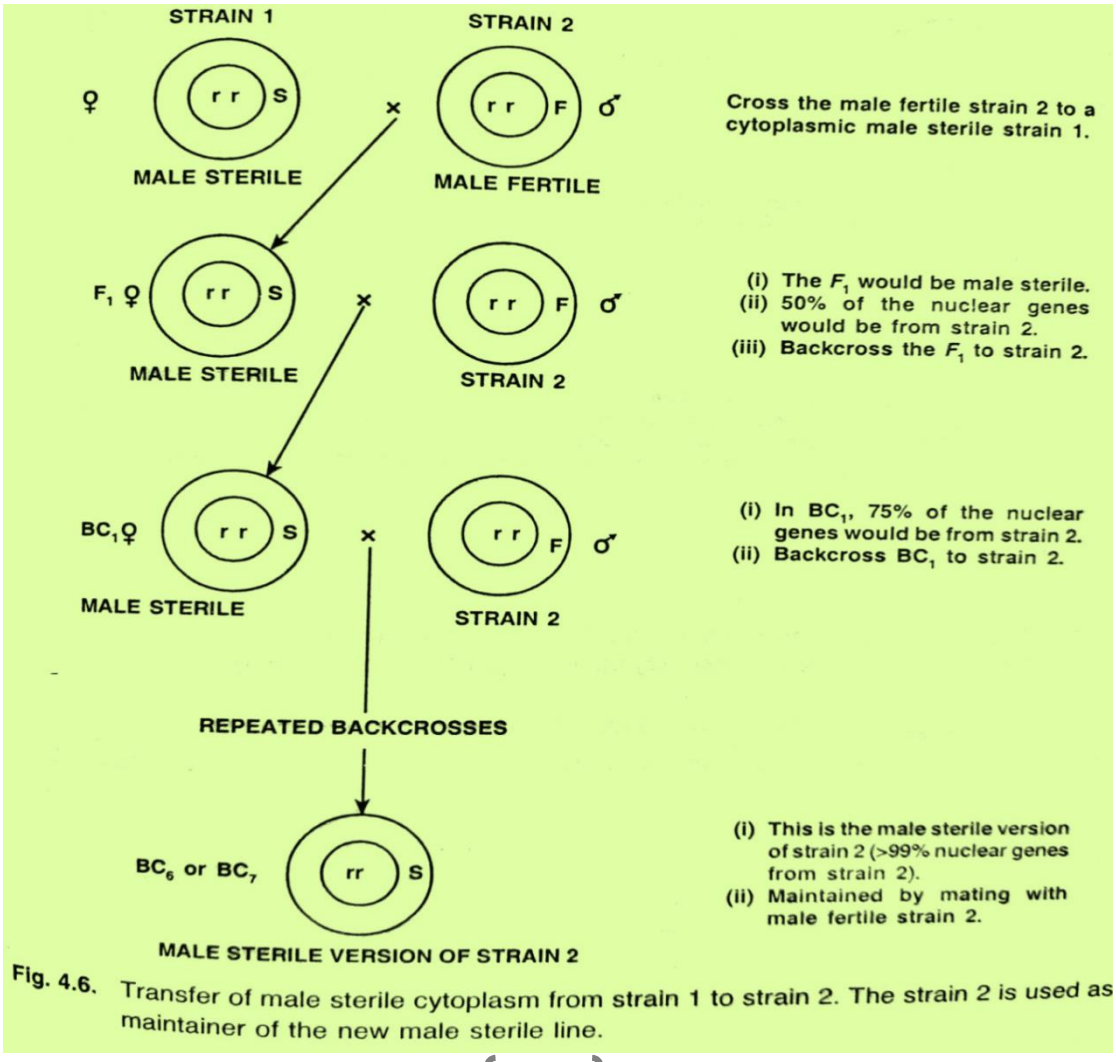


Fig. 4.6. Transfer of male sterile cytoplasm from strain 1 to strain 2. The strain 2 is used as maintainer of the new male sterile line.

Genetic male sterility:

Genetic male sterility (GMS) is governed by nuclear genes. It is wide occurrence in plants. Male sterility geness are generally recessive (*ms ms*) but dominant gene governing male sterility are also occur in safflower., and arise through spontaneous mutation or may be induced mutagen treatments. In rice, 25 ms genes are known. *'ms'* alleles may affect staminal initiation, stamen or anther sac development, PMC formation, meiosis, pollen formation, maturation and dehiscence.

Inheritance & Maintenance Of male sterile line:

When a male sterile plant (*ms ms*) is crossed with a male fertile one (*Ms Ms*), the F_1 (*Ms ms*) is male fertile. In F_2, a 3 male fertile: 1 male sterile ratio is obtained.

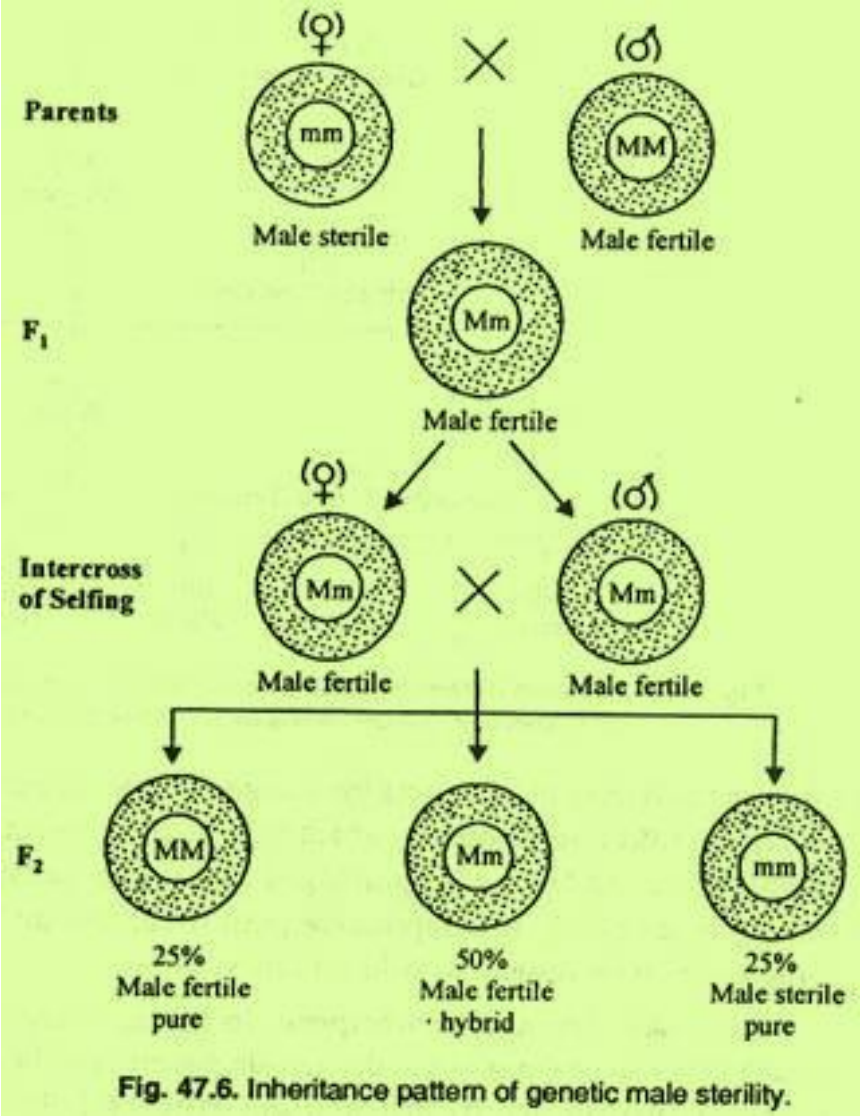
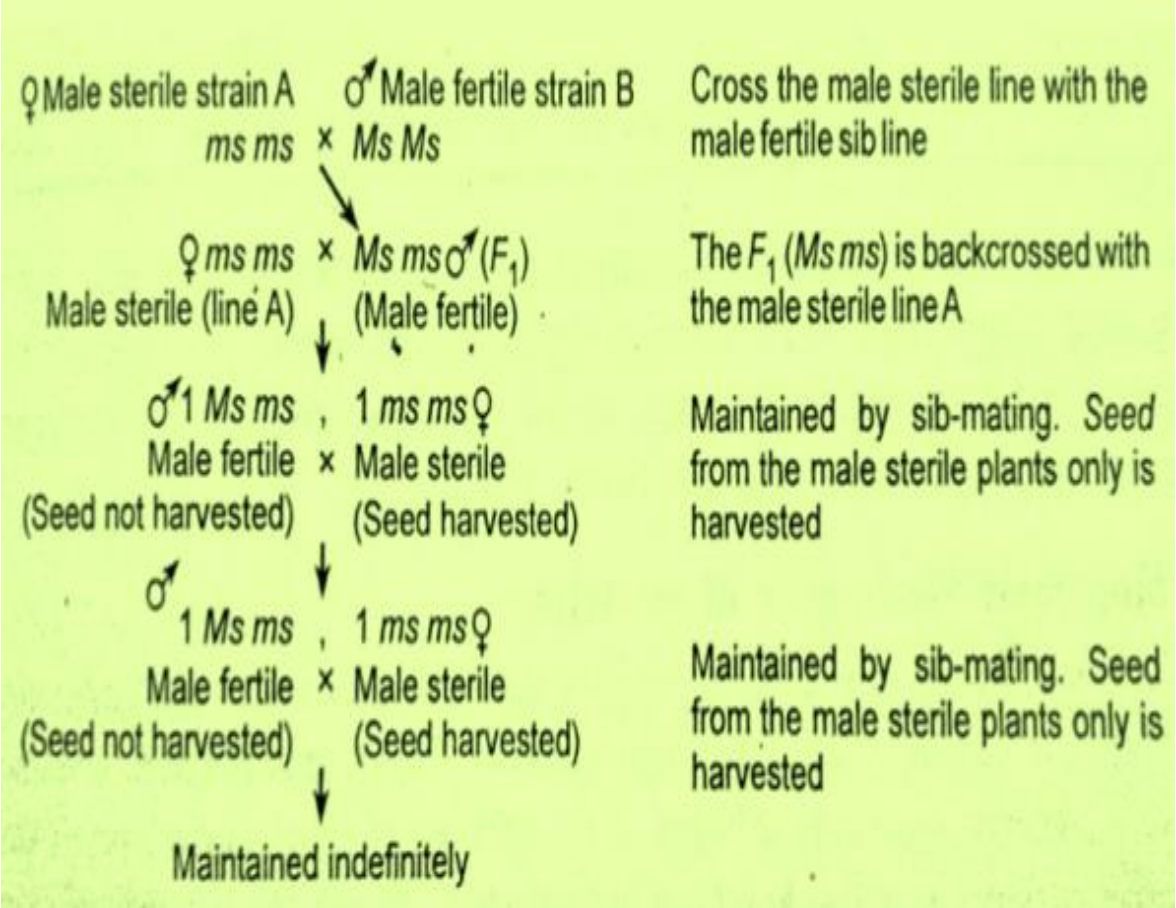


Fig. 47.6. Inheritance pattern of genetic male sterility.

A male sterile line (*ms ms*) is maintained by crossing it with a heterozygous male fertile (*Ms ms*) line. This is simply achived by harvesting the seeds produced only on the male sterile plants present in a segregating generation like F_2. The plants derived from such seeds will be of two types: some of will be male sterile (*ms ms*) and the rest will be male fertile (*Ms ms*). In this subsequent generations, seeds are hervested only from the male sterile plants; these seeds will give to 1 male sterile: 1 male fertile plants.



Difficulties in use of Gms

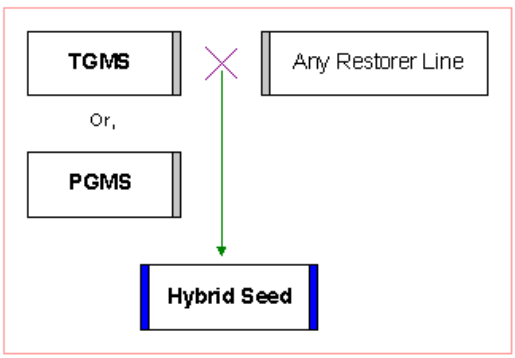
1. Maintenance of GMS requires skilled labour to identify fertile and sterile line. Labelling is time consuming and costly.
2. In hybrid seed production plot identification of fertile line and removing them is costly.
3. Use of double the seed rate of GMS line is costly
4. In crops like castor high temperature leads to break down of male sterility.

Types of GMS

- **Environment insensitive GMS:** *ms* gene expression is much less affected by the environment.
- **Environment sensitive GMS:** ms gene expression occurs within a specified range of temperature and /or photoperiod regimes (Rice, Tomato, Wheat etc.).

1. TGMS: sterility is at particular temperature e.g. In rice TGMS line (Pei- Ai645) at 23.30C (China).

- TGMS at high temperature is due to failure of pairing of two chromosomes at metaphase was evident.
- This abnormality led to abnormal meiosis, abnormal or sterile pollens.
- Anthers were shriveled and non-dehiscence-Male sterile.



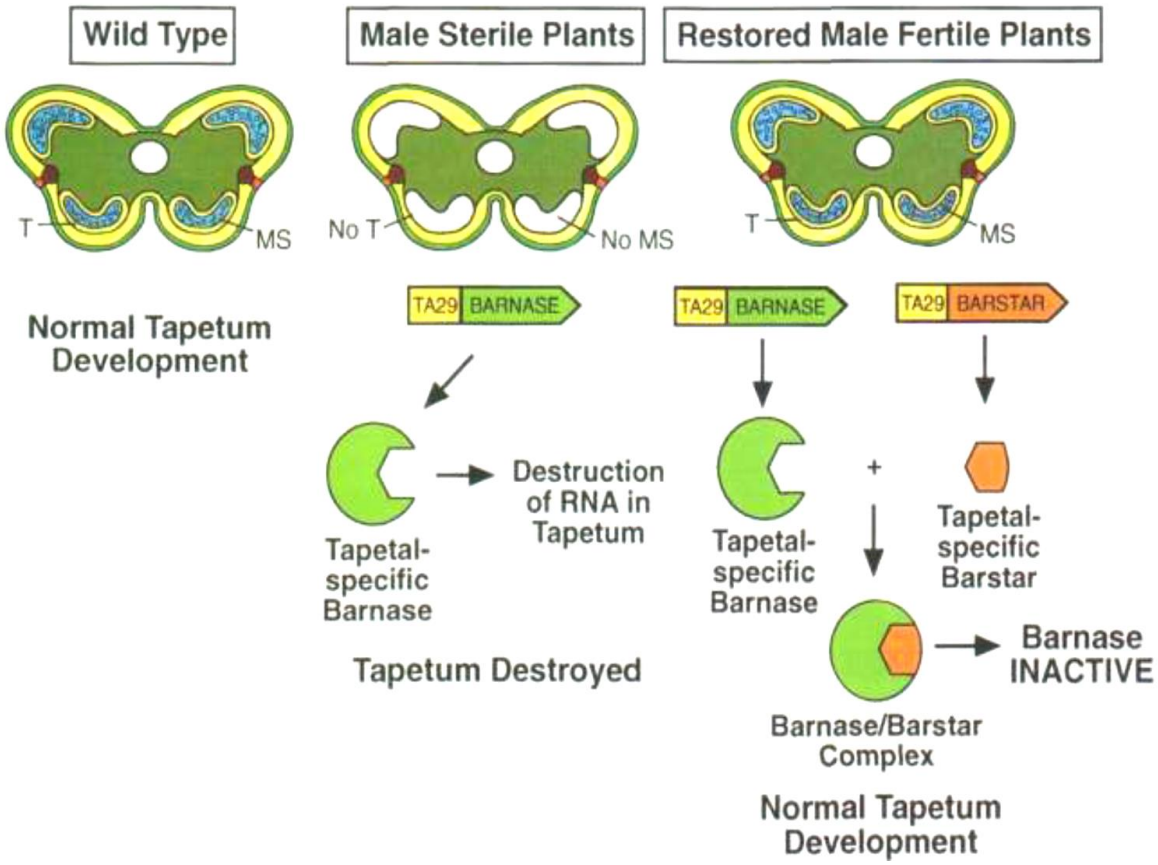
- However, these lines produced normal fertile pollen at low temp.
  Sensitive period: PMC formation to Meiosis

2. PGMS: Governed by 2 recessive genes.

- Sterility is obtained in long day conditions while in short days, normal fertile plant.
- **Rice:-** Sterile under Long day conditions (13 hr. 45 min + Temp. 23-290°C) but fertile under short day conditions.
- **Sensitive period:** Differentiation of secondary rachis branches to PMC formation

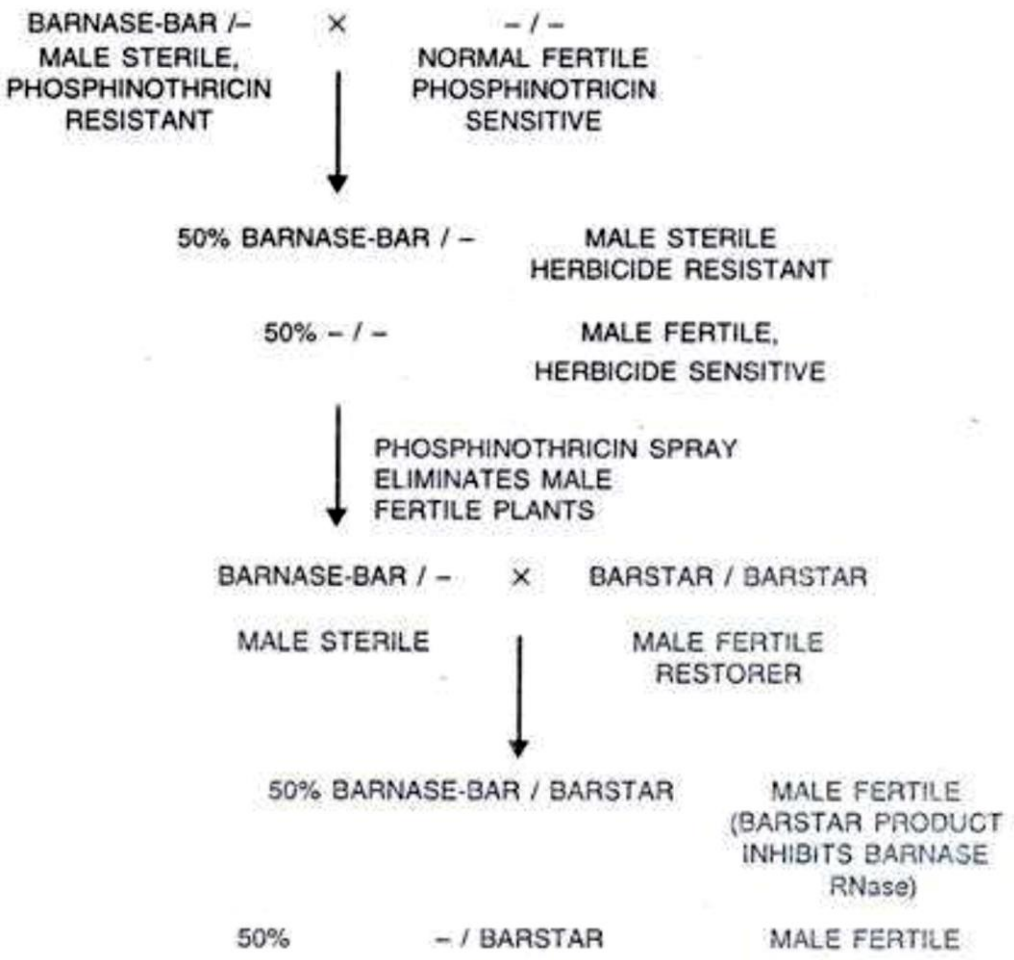
3. Transgenic Genetic Male Sterility:

A gene introduced into the genome of an organism by recombinant DNA technology or genetic engineering is called transgene. Many transgenes have been shown to produce genetic male sterility, which is dominant to fertility. Consequently, it is essential to develop effective fertility restoration system if these are to be utilized for hybrid seed production. An effective restoration system is available in at least one case called Barnase or Barstar system.



The Barnase gene of *Bacillus amyloliquefaciens* encodes RNAs. When Barnase gene is driven by TA 29 promoter, it is expressed only in tapetum cells causing their degeneration. Transgenic tobacco and *Brassica napus* plants expressing Barnase were completely male sterile. Another gene, Barstar, from the same bacterium encodes a protein, which is a highly specific inhibitor of Barnase RNAse. Therefore, transgenic plants expressing both Barstar and Barnase are fully male fertile.

The Barnase gene has been tagged with bar gene, which specifies resistance to the herbaicde phosphinothricin. This male sterile line is maintained by crossing with a male fertile line. The progeny so obtained contain 1 male sterile : 1 male fertile plants; the latter are easily eliminated at seedling stage by a phosphinothricin spray. The male sterile plants are crossed with the Barstar line to obtain male fertile hybrid progeny. This system of male sterility is yet to be commercially used.



Hybrid seed production using Barnase/Barstar system

Utilization of GMS in plant breeding:

This system (GMS) of male sterility has been used for hybrid seed production in castor (U.S.A), tomato (to a limited extent), and pigeonpea (India). Proposal for use in other crops like barley, wheat, cotton etc. have been made, but they are yet to be realized. Some of serious limitations of the system are briefly describe below.

1. The line used as female parent in hybrid seed production is the progeny from *ms ms* × *Ms ms* cross. Therefore, the female parent has 50% male fertile (*Ms ms*) plants, which have to be removed before flowering. This is usually quite difficult and costly to achive. This problem is not faced when TGMS or PGMS are used. These male sterile lines are maintained by growing them at temperatures/ under photoperiods, which produce male fertility in the male sterile lines. The selfted progeny of such lines therefore are 100% male sterile when grown under appropriate conditions.
2. Pollen dispersal from the male parent is generally poor.

Cytoplasmic Geneic Male Sterility

In this system, male sterility is determined by the cytoplasm, and the plasmagenes producing male sterility are ordinarily located in mtDNA just as is the case of CMS. But a nuclear gene, called restorer gene, restores male fertility and, thereby, eliminates the effects of male sterile cytoplasm. The

restorer gene is generally dominant, and is found in some varieties of the same species or in a related species. CGMS is known in several crops, and in many cases it is being commercially used. Repeated back cross are used for the transfer of male sterile cytoplasm, and the male sterile line is maintained in the same way as in the case of CMS. But the transfer of restorer genes using a backcross programme is a little more involved.

Plants having the male sterile cytoplasm will be male sterile only if their nuclear genotype is *rr* (*r* is the recessive allele of the fertility restorer gene *R* and is incapable of fertility restoration). But if the nuclear genotype is either *Rr* or *RR*, they will be male fertile.

A line or ms line: This tem represents a male sterile line belonging to anyone of the above categories. The A line is always used as a female parent in hybrid seed production.

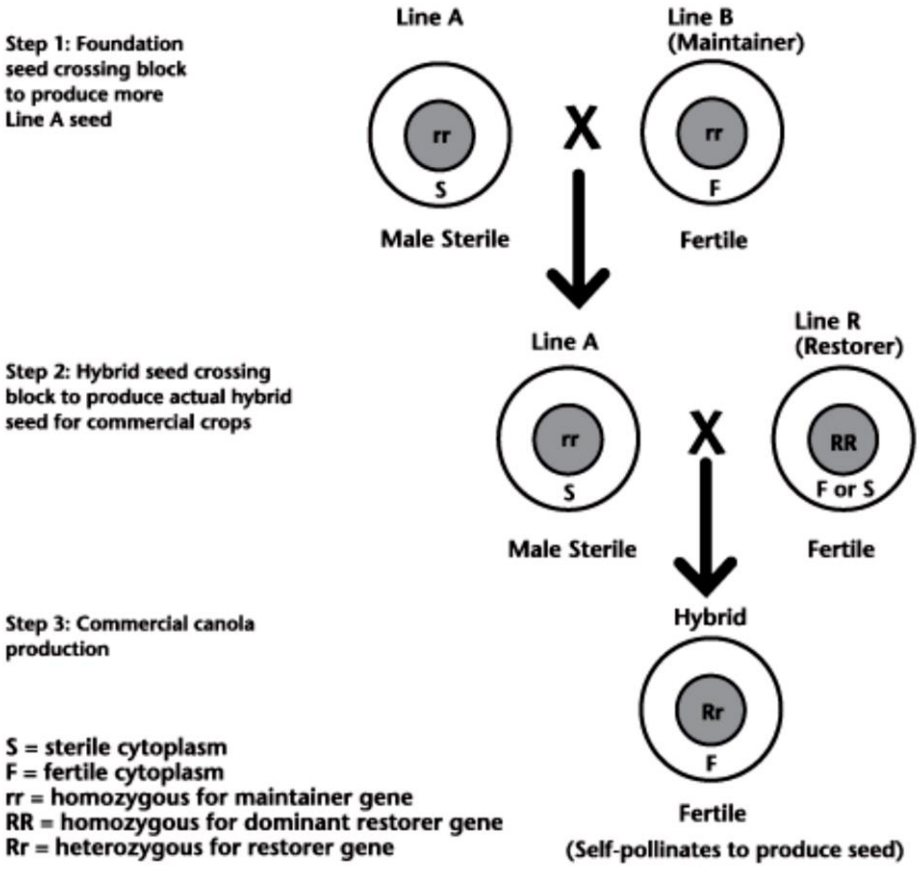
B line or maintainer line: This line is used to maintain the sterility of A line. The B line is isogenic line which is identical for all traits except for fertility status.

R line and restoration of fertility: It is other wise known as Restorer line which restores fertility in the A line. The crossing between A x R lines results in F 1 fertile hybrid seeds which is of commercial value.

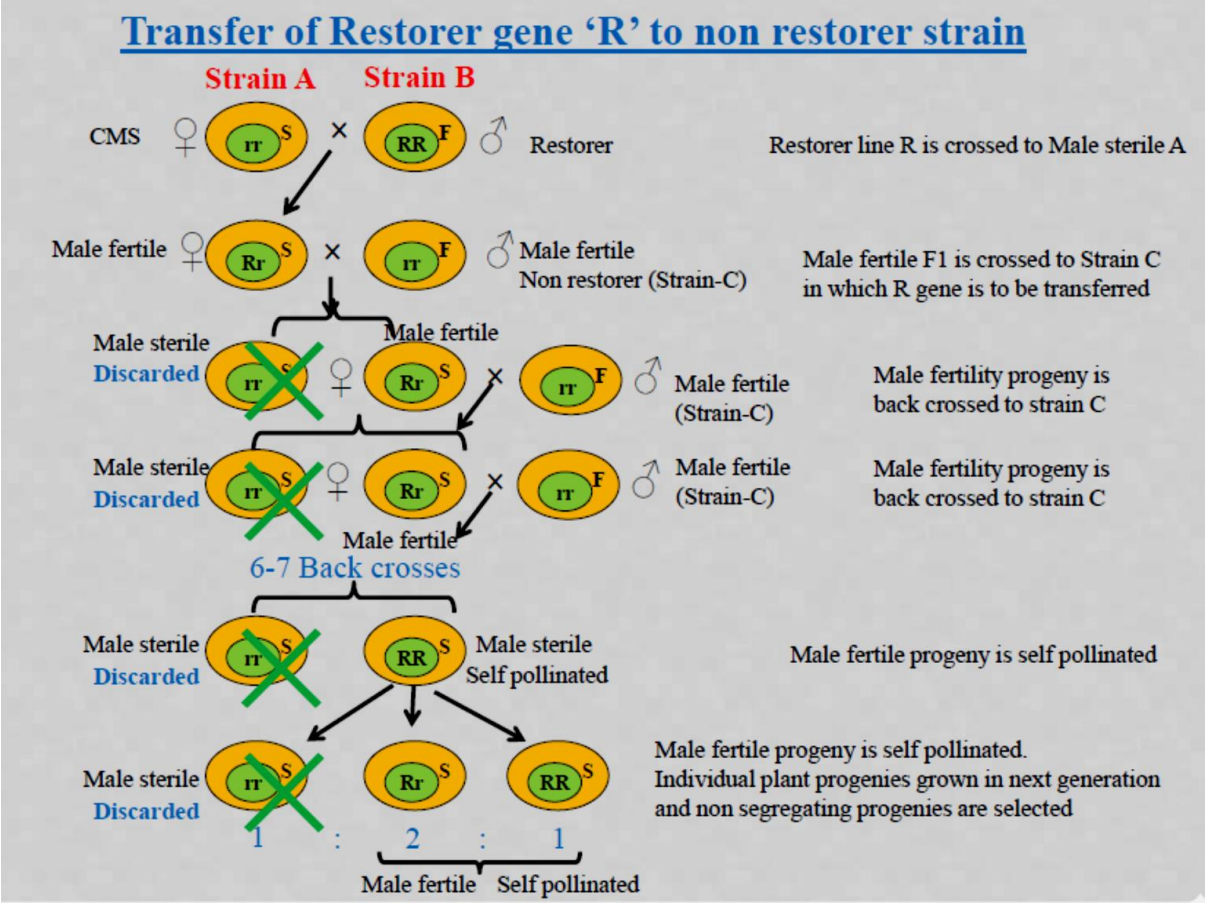
Utilization of CGMS in plant breeding:

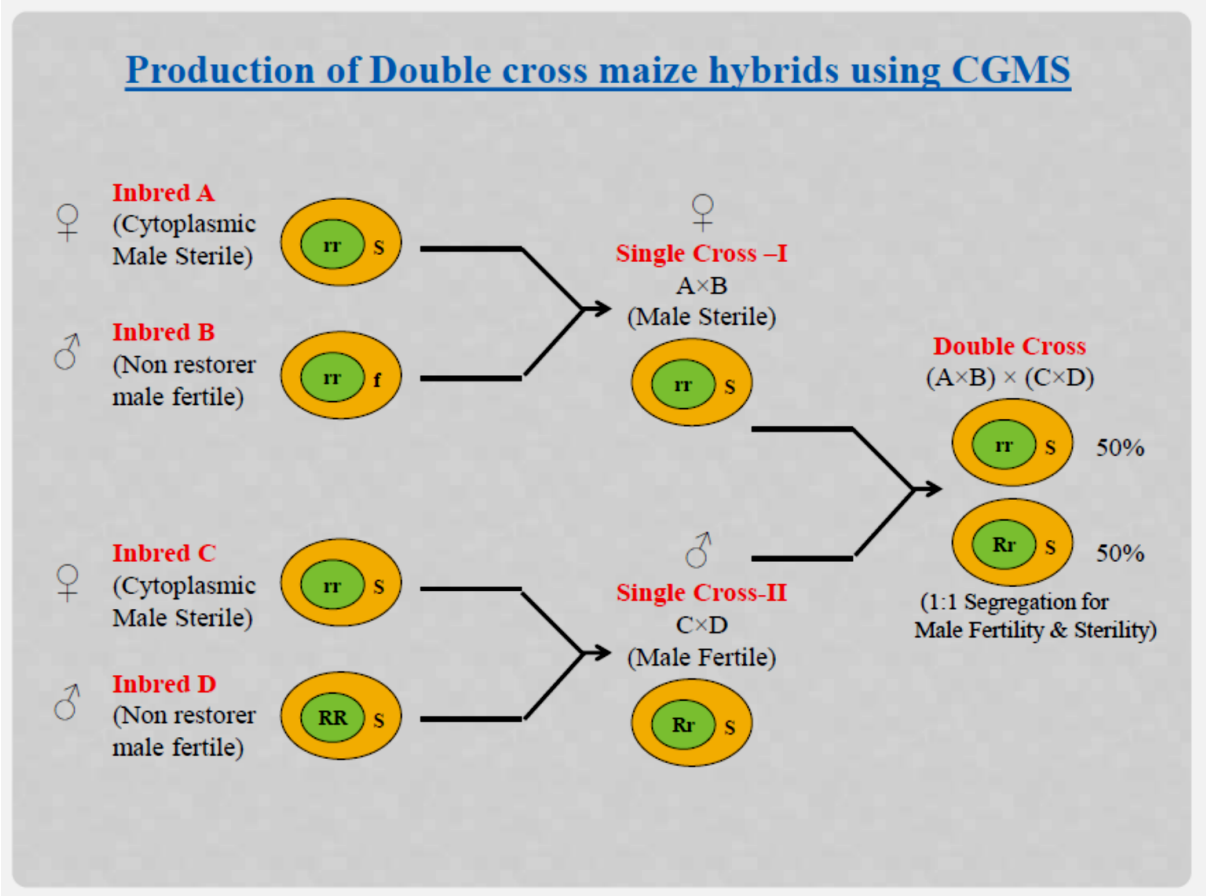
CGMS is being commercially used to prduce hybrid seed in bajra, jowar, maize, rice, wheat and many other crops. The male sterile line is maintained by crossing with a maintainer line; this line has *rr* nuclear genotype. For hybrid seed production, the male sterile line is crossed with a restorer line so that the F_1 plants are fertile. In addition to the restorefr line must combine well with the male sterile line to produce a high yielding F_1 hybrid. CGMS is, at present, commercially the most extensively used MS system.

Hybrid seed production using CGMS system:



Maintenance:





Sources of CMS & Restorer genes in some Crops

Crop species	Cytoplasm	Restorer Genes
Rice	CMS-CW	*O. spontanea*
	CMS-bo	*O. Sativa* boroII (single dominant)
	CMS-WA	*O. Spontanea* (WA, four genes)
	CMS-W18	*O. rufipogon*
Wheat (*T.aestivum)*	*T. timopheevi*	*Rf1 and rf2*
	A. caudata	-
T. Durum	*Aegilops ovata*	-
Maize	CMS-C	*Rf4*
	CMS-S	*Rf3*
	CMS-T	*Rf1 and Rf2*
Tobacco	*N. Debneyi*	-
	N. Megalosiphon	-
	N. bigelovii	-
Cotton	*G. Anomalum*	-
	G. Arboreaum	-
	G. harknesii	-
Sunflower	PET-1 *(H. petalaris)*	2 polymorphic genes (*Rf1, Rf2*)
Jowar	Milo or A1	Msc from kafir race
Bajra	Tift-23A	-

Limitations of Cytoplasmic-Genetic Male Sterility:

- Male sterile cytoplasms generally produce undesirable side effects. For example, CMS-T in maize causes a 2-4% reduction in yield and makes plants susceptible to *Helminthosporium* leaf blight.
- In many case, fertility restoration is not satisfactory.
- In many case, pollination during hybrid seed production is not satisfactory, e. g., in *Capsicum* sp. This is par ticularly so is self-pollinated crops.
- Spontaneous reversion
- Modifying genes (nuclear) may reduce effectiveness of CGMS
- Contribution of cytoplasm by male gamete
- Male sterility may not be complete under certain environments, e. g., in maize, bajra, jowar, etc.m
- Non availability of a suitable restorer line

Chemical Induced Male Sterility:

This type of male sterility is induced by treatmentwith certain chemicals and is, therefore confined to the generation of chemical treatment (i.e., it is not heritable). These chemicals are called male gametocides. It is also called male sterilants, selective male sterilants, pollen suppressants, pollenocide, androcide etc. The first report was given by Moore and Naylor (1950), they induced male sterility in Maize using maleic hydrazide (MH). They are applied during certain developmental stages sensitive to the treatment, and cause pollen abortion. Generally, repeated applications of chemical are required. It is being used to developrice hybrids in China; three hybrids have so far been released for cultivation.

Chemicals inducing male sterility are ethrel, GA_3, maleic hydrazide, naphthalene acetic acid, and sodium/zinc methyl arsenate. Unfortunately, none of them is an ideal male gametocide. For example, they often produce incomplete male sterility, repeated applications are needed, female fertility is also

reduced and often undiserable side effects are produced.But they have the unique advantage of application with any line/ varietyof a crop whenever desired.

Properties of an Ideal CHA:

- ❖ Must be highly male or female selective.
- ❖ Should be easily applicable and economic in use.
- ❖ Time of application should be flexible.
- ❖ Must not be mutagenic.
- ❖ Must not be carried over in F1 seeds.
- ❖ Must consistently produce >95% male sterility.
- ❖ Must cause minimum reduction in seed set.
- ❖ Should not affect out crossing.
- ❖ Should not be hazardous to the environment.

Some important CHAs

S.No.	CHAs	Critical stage	Crop species
1.	Zink Methyl Arsenate Sodium Methyl Arsenate	5 days before heading	Rice
2.	Ethephon/ Ethrel	Depends on crop	Barley , oat, bajra, rice
3.	Mendok	Depends on crop	Cotton, sugarbeet
4.	Gibberellic acid	1-3 days before meiosis	Maize, Barley, Wheat, Rice, Sunflower
5.	Maleic Hydrazide	Early microsporogenesis	Maize, wheat, cotton, onion

Hybrid Seed Production based on CHAs:

Conditions required:-

- ➢ Proper environmental conditions (Rain, Sunshine, temp, RH etc.)
- ➢ Synchronisation of flowering of Male & Female parents.
- ➢ Effective chemical emasculation and cross pollination
  - ❖ CHA at precise stage and with recommended dose
  - ❖ GA3 spray to promote stigma exertion.
  - ❖ Supplementary pollination to maximise seed set
  - ❖ Avoid CHA spray on pollinator row.

Advantages of CHAs:

- ➢ Any line can be used as female parent.
- ➢ Choice of parents is flexible.
- ➢ Rapid method of developing male sterile line.
- ➢ No need of maintaining A,B&R lines.
- ➢ Hybrid seed production is based on only 2 line system.

- Maintenance of parental line is possible by self pollination.
- CHA based F2 hybrids are fully fertile as compared to few sterile hybrids in case of CMS or GMS.

Limitations of CHAs

- Expression and duration of CHA is stage specific.
- Sensitive to environmental conditions.
- Incomplete male sterility produce selfed seeds.
- Many CHAs are toxic to plants and animals.
- Possess carryover residual effects in F1 seeds.
- Interfere with cell division.
- Affect human health.
- Genotype, dose application stage specific.

Significance of male Sterility in Plant Breeding:

- Male sterility a primary tool to avoid emasculation in hybridization.
- Hybrid production requires a female plant in which no viable pollens are borne. Inefficient emasculation may produce some self fertile progenies.
- GMS is being exploited (Eg.USA-Castor, India-Arhar).
- CMS/ CGMS are routinely used in Hybrid seed production in corn, sorghum, sunflower and sugarbeet, ornamental plants.
- Saves lot of time, money and labour.

Limitations in using Male Sterile line:

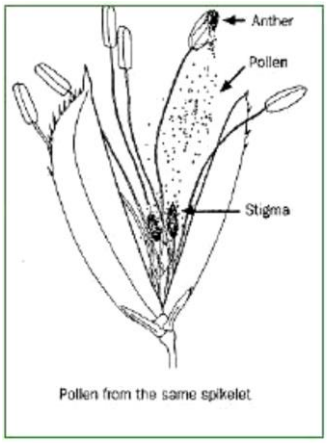
- Existence and maintenance of A, B & R Lines is laborious and difficult.
- If exotic lines are not suitable to our conditions, the native/adaptive lines have to be converted into MS lines.
- Adequate cross pollination should be there between A and R lines for good seed set.
- Synchronization of flowering should be there between A & R lines.
- Fertility restoration should be complete otherwise the F1 seed will be sterile Isolation is needed for maintenance of parental lines and for producing hybrid seed.

Application of male sterility in hybrid seed production:

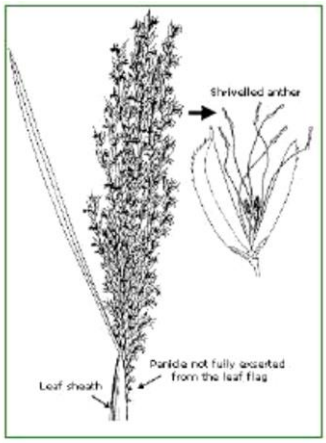
Male sterility system in Rice hybrid seed production

In rice following types of male sterility systems are used

- Cytoplasmic Male Sterility (Three line breeding)
- Genetic Male Sterility (Two line breeding).
- Male sterility induced by Chemical
- Hybridizing Agents



Normal Rice Spikelet
(self pollinated crop)



Sterile Rice Spikelet
(Male Sterility)

Cytoplasmic Male Sterility in rice

Male Sterility is governed by cytoplasmic genes or plasmagenes.

Source of Male Sterility

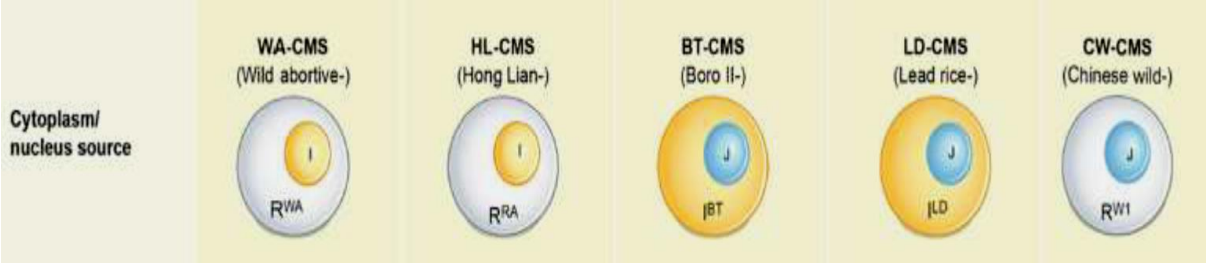


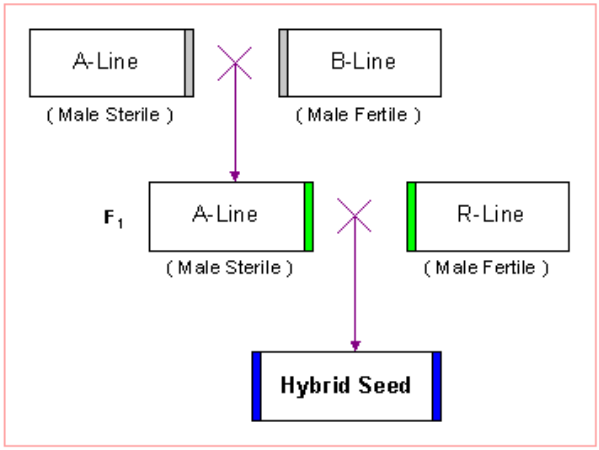
Figure 1 A schematic presentation of the five well-studied rice CMS types. Abbreviations for cytoplasm sources are R^{WA} for wild-abortive *Oryza rufipogon*, R^{RA} for red-awned *O. rufipogon*, and R^{W1} for Chinese wild rice (*O. rufipogon*) accession W1; I^{BT} and I^{LD} for *indica* Boro-II type and Lead rice, respectively. Nucleus sources are either *indica* (I) or *japonica* (J).

WA-CMS lines

- WA-CMS lines are the most widely deployed lines in hybrid rice production.
- Pollen abortion in WA-CMS occurs relatively early during microspore development, mainly at the uninucleate stage (Luo et al.2013), resulting in amorphous aborted pollen grains.
- The pollen abortion is determined by the genotype of sporophytic tissues, not by the genotype of the pollen itself.
- Efforts are made to transfer this WA cytosterility into genetic background of elite breeding lines.

CMS Line	Developed At	Elite line converted	Origin of elite line
IR 46826 A	IRRI	IR 10154-23-3-3	IRRI
IR 46827 A	IRRI	IR 10176-24-6-2	IRRI
IR 46828 A	IRRI	IR 10179-2-3-1	IRRI
IR 46829 A	IRRI	IR 19792-15-2-3-3	IRRI
IR 46831 A	IRRI	Jikkoku Seranai 52-37	India
IR 46883 A	IRRI	MR-365	India
IR 46885 A	IRRI	PAU 269-1-8-4-1-1-1	India
Madhu A	CRRI ,India	Madhu	India
HR 7017 A	Iri Korea	Samkangbyeo	Korea

Procedure of utilization of CMS line in Seed production



Hybrids developed using CMS in India

Hybrid	Parentage
APRH- 1	IR 58025 A x Vajram
APRH-2	IR 62829 A x MTU 9992
MGR- 1	IR 62829 A x 10198-66-2R
KRH-1	IR 58025 A x IR 9761-10-IR
CNRH-3	IR 62829 A x Ajaya R
DRRH-1	IR 58025 A x IR 40750-82-2-2-3R
KRH-2	IR 58025 A x KMR 3
Pant Shankar Dhan 1	IR 58025 A x UPRI 192-133R
CORH 2	IR 58025 A x C 20 R
ADTRH 1	IR 58025 A x IR66
Sahyadri	IR 58025 A x BR 827-35-3-1-1-R
Narendra Sankar Dhan 2	IR 58025 A x NDR 3026-3-I-R

Exploitation of GMS in Rice

- Male sterility is governed by nuclear genes.
- In GMS, the GMS line produce 50% of male fertile plants and 50% male sterile plants.
- So it's very much necessary to identify and eliminate male fertile plants before pollen shed .
- Thus this drawback of GMS is overcome by using
- Photoperiod Sensitive GMS.
- Temperature sensitive GMS.

List of PGMS line in Rice

Genetic Control	lines	Origin	Expression of MS
	PGMS lines		
Controlled by two recessive genes	Nongken 58S	Spontaneous mutation	Day length shorter than 13.75hr
	X88	Hybridization	Day length shorter than 13hr
	MG 201	EMS Mutagenesis	Day length 12hr

List of TGMS line in Rice

Genetic Control	lines	Origin	Expression of MS
Controlled by single recessive gene	Annong- 1s	Spontaneous mutation	Temperature of 27 °C
	Hennong S	Hybridization	Temperature < 29°C
	5460S	Irradiation	Temperature >30°C
	SM-38	Spontaneous mutation	Temperature < 22°C
	SM-5	Spontaneous mutation	Temperature < 22°C
	JP-2	Spontaneous mutation	Temperature < 26°C
	JP-38S	Spontaneous mutation	Temperature > 30°C

Hybrids developed using PGMS in Rice

Hybrid	Parentage
Hunan	Pei ai 64s x Tequing
Anhui	7001s x Xiusui 04
Hubei	7001s x 1514
Guangdong	Pei ai 64s x Shuanging11
Sichuan	Pei ai 64s x Shuangingyou 1

Exploitation of Male Sterility by CHA's in Rice

- Application of chemicals at specific growth stage of crop results in chemically induced male sterility and chemical components used are called as Chemical Hybridizing agents.
- Two arsenical CHA's are used in Rice
  - MG1(based on zinc methyl arsenate)
  - MG2 (based on sodium methyl arsenate)

CHA's in Hybrid seed Production in Rice

- Male sterility is artificially induced by spraying gametocides to cause stamen sterility without harming the pistil.
- The chemical which makes sterile the stamen, plant can be used as female parent for producing hybrid seed.
- Two selected lines are planted in alternate strips and one is utilized as female (chemically sterilized) and is pollinated by the other line, for production of hybrid seed.

Rice hybrids based on CHA's in rice

- Qing – Hua- Fu- Gwi.
- Gang – Hua- Qing – Lan.
- Gang – Hua- 2.
- You- Za-1.
- Ya- You-2.
- •hese are produced in china using arsenical CHA's.

Male sterility system in Maize hybrid seed production

Different ways of inducing male sterility in maize

I. Manual/mechanical emasculation (detasselling)

II. Genic male sterility

III. Cytoplasmic genetic male sterility

IV. Gametocides

1. Genetic Male sterility:

Male sterility determined by single recessive gene 40 loci involved have been identified (*ms1* to *ms52*) ms5 –cloned

Problem: impossible to maintain male sterile inbred detasselling required

2. Cytoplasmic Male sterility

i. CMS-T (Texas) (Rogers and Edwardson, 1952)

- ❖ Highly stable under all environmental conditions
- ❖ Characterized by failure of anther exertion and pollen abortion
- ❖ Susceptible to race T of the southern corn leaf blight - (*Cochliobolus heterostrophus* = Bipolaris maydis)
- ❖ Widespread use of T-cytoplasm for hybrid corn production led to epidemic in 1970 with the widespread rise of Race T.
- ❖ Toxin produced by *C. heterostrophus* = T-toxin.
- ❖ Fertility restoration is **sporophytic**
- ❖ ***Rf1*** (chr. 3) & ***Rf2*** *(*chr.9) are responsible for fertility restoration

T-urf13 gene in T cytoplasm maize

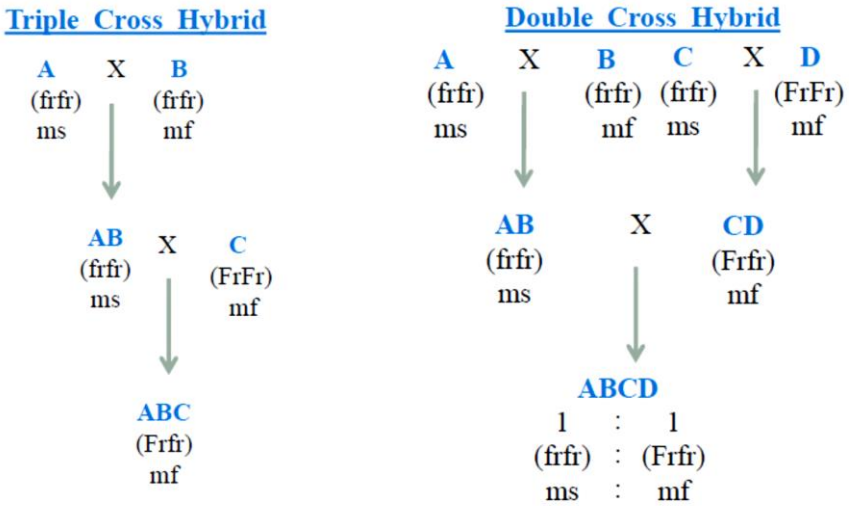
- ✓ Mitochondrial gene **T-urf13** is a unique chimeric sequence

Effect of URF13 protein-

- ▪ Degeneration of the tapetum during microsporogenesis
- ▪ Disruption of pollen development leading to male cell abortion

ii**. CMS-C** (Charrua) (Beckett, 1971)

- ❖ Mutations in three genes viz *atp*6, *atp* 9 and *cos*II- confer CMSphenotype
- ❖ Fertility restoration is **Sporophytic**
- ❖ ***Rf4, Rf5, Rf6*** are responsible for fertility restoration



iii. CMS-S (USDA) (Jones,1957)

- ❖ Sterility associated with *orf355-orf77* chimeric mt gene
- ❖ Fertility restoration is **Gametophytic**
- ❖ ***Rf3*** *(chr. 2) are responsible for fertility restoration*
- ❖ Plasmid like element **S1 & S2**

Reversion to fertility:

- ➢ The reversion of CMS strain to male fertility is based on genetic change
- ➢ Reversion can be spontaneous or mutagen induced

- S-cytoplasm revert rather frequently to male fertility (than T & C).

Male sterility system in *Brassica* hybrid seed production:

Cytoplasmic male-sterile:

Stamen (anther and filament) and pollen grains are affected. It is divided into:

a. Autoplasmic

Arisen within a species as a result of spontaneous mutational changes in the cytoplasm, most likely in the mitochondrial genome

Alloplasmic

Arisen from intergeneric, interpecific or occasionally intraspecific crosses and where the male sterility can be interpreted as being due to incompatibility or poor co-operation between nuclear genome of one species and the organellar genome. Another CMS can be a result of interspecific protoplast fusion

Various CMS systems

- Raphanus or *ogu* system
- Polima or *pol* system
- Shiga-Thompson or nap system
- Diplotaxis muralis or mur system
- Tournefortii (tour) system
- Moricandia arvensis or mori system
- Chinese juncea or jun system

17 systems are available, only difference is the use of male sterile cytoplasmic sources differs for each system

- **Nap system**– *B.napuus* cross b/w winter & spring var.
- **pol system** – *B.napus* var polima
- **mur system**--*Diplotaxis muralis* x *B.campestris* cv Yukina
- **tour system**– *B.juncea* collections

Ogu system:-

- First discovered in Japanese radish (*Raphanus sativus*) by Ogura, 1968
- *B.napus* genome was transferred into the back round of *R.sativus* (mst) through intergeneric crosses followed by back crossing with B.napus.
- CMS seedling under low temperature showed chlorosis , because chloroplast of *R.sativus* is sensitive to cold, it is governed by cp-DNA , but mst is governed by mt DNA.
- Protoplast fusion of *R.sativu*s with *B.napus* carried out to have normal green plants with ogu CMS characterisitics
- This system now has been used for developing alloplasmic male sterile line in *B.juncea* and *B.campestris.*

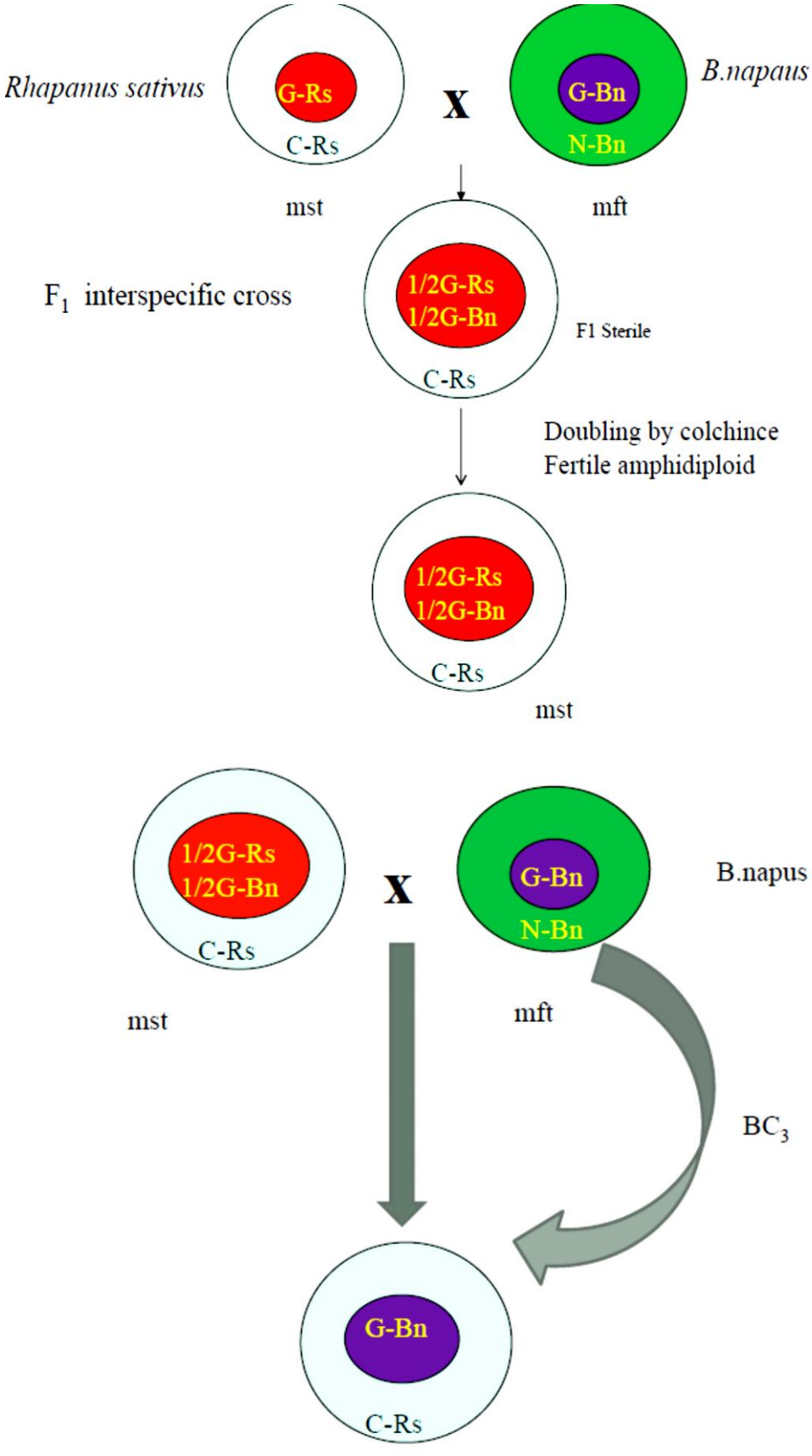
Genetic Male Sterility

GMS is governed by two genes either recessive or dominant genes(Kaul,1988). One more dominant gene is associated with development of male sterility in *B.napus* type by means of transgenic male sterility.

Chemical Male sterility

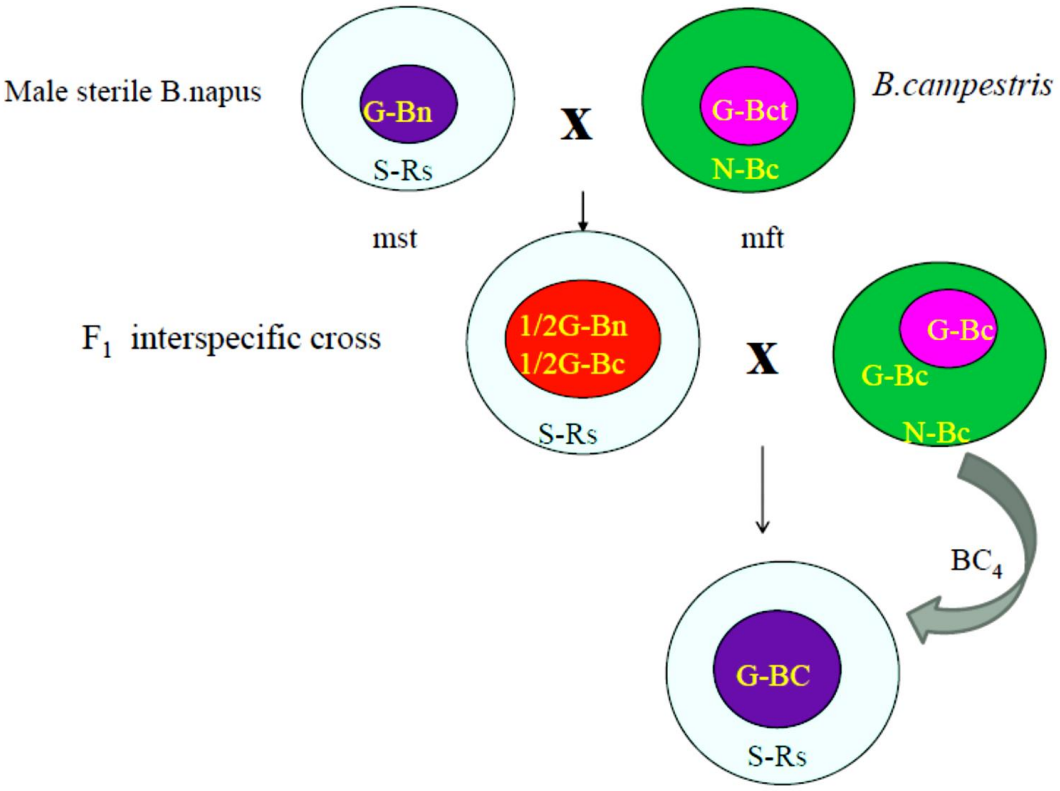
- **Enthrel** – *Brassica juncea*
- **Zinc methy arsenate**- *B.napus*
- **GA**- *B.oleracea var capitata*

Development of Male sterile *B. napus* from *R. sativus*



Male sterile B.napus

Development of Alloplasmic Male sterile *Brassica campestris*



Male sterility system in Safflower hybrid seed production:

Presently genetic male sterility (GMS), cytoplasmic male sterility (CMS) and thermo sensitive genetic male sterility (TGMS) lines are available in India.

Development of agronomically superior genetic male-sterile lines in safflower in India have resulted in the development and release of spiny safflower hybrids DSH-129 and MKH-11 in 1997 and NARIH- 15 in 2005, the first non-spiny hybrid safflower NARI-NH-1 in 2001.

Male sterility system in Sunflower hybrid seed production

Genetic Male sterility (GMS)

- **Complete male sterility**
  - *ms1-ms5* = male sterility in sunflower recessive gene
- **Two types of g-mst**
  - Type 1-gmst-Bloomington type
  - Type 2-gmst-Modern type
- Cultivated Sunflower variety Karlik-68(Dwarf 68)- two recessive genes *msi1,msi2* (Stable and complete male sterile)
- **Partial male sterility –p mst**

CGMS	
H.petiolaris × *H.annuus*	Repeated backcross of *H.annuus* results in cms_1 which is extensively used mst in hybrid seed production of sunflower all over the world
H.giganteus× *H.annuus*	Cms_3(S cytoplasm source)
H.*annuus* subspp *lenticularis* × H.*annuus* CV commander	Indiana 1

Male sterility system in Cotton hybrid seed production

All three types of male sterility occurs (g mst,c mst,gc mst) in cotton

- **Genetic Male Sterility (GMS):**
  - Reported in upland, Egyptian and arboreum cottons.
  - In tetraploid cotton, male sterility is governed by both recessive and dominant genes.
  - However, male sterility governed by recessive genes is used in practical plant breeding
  - Sixteen different genes in tetraploid cottons (13 in *G. hirsutum* and 3 in *G. barbadense*) and two in *G. arboreum* have been identified for genetic male sterility.
  - Sterility is conditioned by dominant alleles at five loci viz, MS4, MS7, MS10, MS11 and MS12 by recessive allele at other loci viz. msl, ms2, ms3, ms13, ms14 (Dong A), ms15 (Lang A) and ms16 (81 A).
  - *G. hirsutum* line Gregg (MS 399) from USA is the basic source of GMS possessing ms5 ms6 gene for male sterility.

Genetic Male Sterility

Gene	Species	Identified By
ms_1	*G. hirsutum*	Justus and Leinweber, 1960
ms_2	*G. hirsutum*	Richmond and Kohel, 1961
ms_3	*G. hirsutum*	Justus et al., 1963
MS_4	*G. hirsutum*	Allison and Fisher, 1964
ms_5ms_6	*G. hirsutum*	Weaver, 1968
MS_7	*G. hirsutum*	Weaver and Ashley, 1971
ms_8ms_9	*G. hirsutum*	Rhyne, 1971
MS_{10}	*G. hirsutum*	Bowman and Weaver, 1979
MS_{11}	*G. barbadense*	Turcotte and Feaster, 1979
MS_{12}	*G. barbadense*	Turcotte and Feaster, 1985
ms_{13}	*G. barbadense*	Percy and Turcotte, 1991

Table-2: Genotypes converted into CMS and GMS background

Male Sterility System	Genotypes
GMS (4x)	LRA 5166, SRT 1, DGMS 1, HGMS 2, GAK 32A, SHGMS-9, DGMS2, SHGMS-5
CMS	Germplasm - G 67, DMS A-8, RCMS A-2. GSCMS-15, 34, CAK32A, C1412, C 1998, CAK 1234, LCMS 6, JK 119, DMSA 15, IC 1547
	Varieties - Rajat, LH 900, Supriya, G. Cot l0, Laxmi, Abadhita, BN, K2, LRA 5166, H 777, G. Cot 14, Ganganagar Ageti, F 414, Bhagya, Kh3, Narmada, Deviraj
GMS (2x)	GMS 4, GMS 2, GAK 20A, GAK 09, SGMS 2, SGMS 4, RGMS A-2, RGMS 3,SGMS 13, GMS 4-1, GAK 15A, GAK 26A, Sujay, GAK 423, GAK 8615
R line	NH 258, AKH 545, GSR 22, AKH 39R, LR 29, AKH 26R, AKH 1167, GSR 6, DR 6, DR 1, AKH-01-143, LR 104

CMS System

In case of CMS, the originally discovered CMS sources involving *G. arboreum* and *G. anomalum* cytoplasmic systems having interaction with ms3 locus were not found effective or stable under different environments.

The only stable and dependable CMS source under varied environment was developed through the utilization of *G. harknessii*. The complete genome of *G.hirsutum* was transferred into the *G. harknessii* cytoplasm.

A single dominant gene 'Rf' from G.harknessii is essential for fertility restoration. Fertility enhancer factor 'E' for this CMS restorer system was obtained from a *G.barbadense* stock. The harknessii system is reported to contribute to good agronomic properties and attraction to honey bees.

Sources of Male sterility in Cotton

Source of ms cytoplasm	**Nuclear genome**
G. anomalum, G. arboreum, G. harknessii	G. hirsutum
G. anomalum, G. arboreum	Heat sensitive , less stable
G. harknessii × G. hirsutum	Stable cms all over the environment
New sources of CMS	
G. aridum Skovt. × *G. hirsutum*	(D4)
G. trilobum × *G. hirsutum*	CMS 8 (D-8)
G. sturtianum × *G. hirsutum*	CMS-C1
New sources of CGMS	
G. anomalum x G. thurberi	Cg-mst

Mutation

G. arboreum, the first spontaneous male sterility mutant was identified in variety DS-5

Chemical based male sterility

- FW 450(Sodium B-Dichloro-iso-butyrate)
- MH-30 (Maleic hydrazide)
- Ethidium bromide

Male sterility based hybrid Production

- GMS system. CPH2 (Suguna), First hybrid based on GMS released at CICR, RS, Coimbatore
- *G. harknessii* based cms with fertility restoration gene sources were used in developing the hybrid CAHH 468 (PKV Hy-3).

4. Self incompatibility: Types, significance; genetic and molecular basis; overcoming self incompatibility

Meaning of Self-Incompatibility:

Self-incompatibility or intraspecific incompatibility is a well-designed genetic mechanism by which certain plants recognize and reject their own pollen thus forcing outbreeding. It is defined as **"inability of the plant producing functional gametes to set seed upon self-pollination"**, Lundqvist (1964) defined it as "the inability of fertile hermaphrodite seed plant to produce zygotes after self-pollination".

Its genetic system is based on a single locus, the sterility (S) locus, with multiple alleles. Pollen germination or pollen tube growth is blocked when the pollen grain and the stigma upon which it lands have the same allele at the same locus. Besides the genetic factors, intraspecific incompatibility is also associated with different lengths of stamens and style in flowers on same plant.

This self- incompatibility is acquired nearly one or two days before anthesis as well as in open flowers. Nearly two-thirds of the families of angiosperms exhibit self- incompatibility. The significance of SI in the evolutionary context cannot be overstated, since its possession leads to obligate outbreeding and the maintenance of heterozygosity within a species.

In the crop and ornamental plants, most of the perennial grasses, forage, legumes, and members of Brassicaceae, Asteraceae, Rosaceae, and Solanaceae have SI mechanism of varying kinds and degrees of effectiveness.

General features of Self-incompatibility

- Prevents selfing and promotes out-breeding so increases the probability of new gene combinations.
- Causes may be morphological, physiological, genetical or biochemical.
- Normal seed set on cross pollination.
- May operate at any stage between pollination and fertilization.
- Reduces homozygosity.
- In plants, self-incompatibility is often inherited by a single gene (S) with different alleles (e.g. S1, S2, S3 etc.) in the species population

Types of self incompatibility:

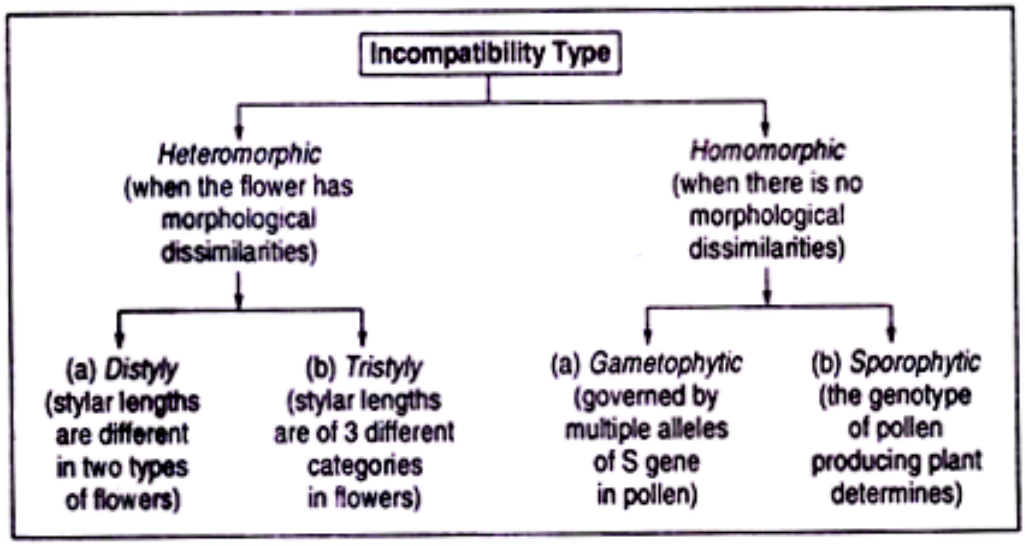
1) Single-locus self-incompatibility
   - Gametophytic self-incompatibility (GSI)
   - Sporophytic self-incompatibility (SSI)
2) 2-locus gametophytic self-incompatibility.
3) Heteromorphic self-incompatibility
4) Cryptic self-incompatibility (CSI)
5) Late-acting self-incompatibility (LSI)

Cause of Self incompatibility:

- Self incompatible pollen grain may fail to germinate on the stigmatic surface.
- Some may germinate but fails to penetrate the stigmatic surface.
- Some pollen grains may produce pollen tube, which enters through stigmatic surface, but its growth will be too slow. By the time the pollen tube enters the ovule the flower will drop.
- Sometime fertilziation is effected but embryo degenerates early.

Types of self incompatibility:

According to Lewis (1954) the self incompatibility is classified as follow



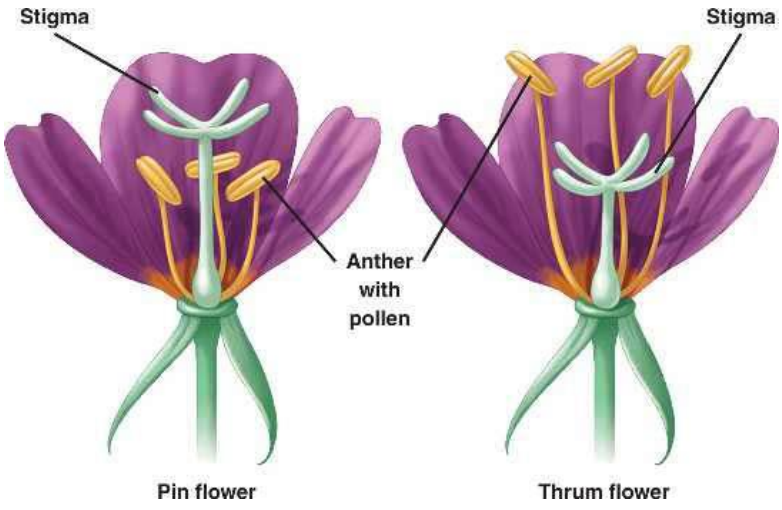
(a) Heteromorphic System:

When the species has two or three-different kinds of arrangement of floral part each type is self-incompatible but compatible with others. Here the genes or alleles associated with incompatibility are also linked with length of style and filament.

(i) Distyly: In *Primula,* there are two types of flowers:

'Pin type' – long style, short filament, large stigmatic cell, small pollen;

'Thrum type' – short style, long filament, small stigmatic cell, large pollen;

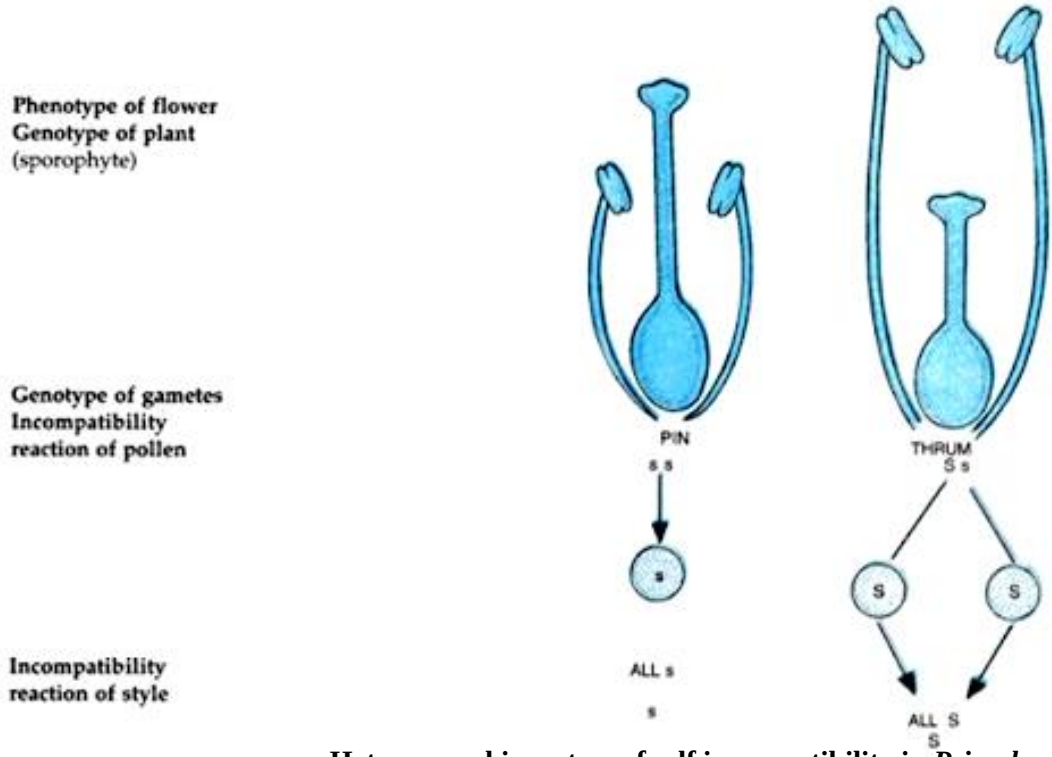


Pin flowers belong to one compatibility group, while thrum flowers form the other group. Pin and thrum flowers are produced on different plants. Flowers belonging to a single incompatibility group do not set seed when pin flowers are cross-pollinated with each other. Therefore, sedds sre produced only when pin flowers are pollinated by thrum flowers and vice versa. This system is of limited occurrence in crop plants, e.g., in sweet pea, buck wheat, *Primula*.

Both flower morphology and self incompatibility are governed by a single locus *S* having two alleles (*S* and *s*); the control of SI reaction is sporophytic. The 'thrum' is governed by Ss and 'pin' by ss. As the reaction of pollen is controlled by the gene of the sporophyte, the pollen of the 'thrum' type will behave like S. The s gametes are incompatible with s type but compatible with S type and the vice-versa. Mating between pin and thrum flowers produce thrum (*Ss*) and pin (*ss*) progeny in 1:1 ratio. It is now known that the locu *S* is a complex locus and it has at least six functional genes (*g*, *s*, l_1, l_2, *p* and *a*); pin flowers are homozygous recessive for all of them, while thrum flowers are heterozygous for all of them.

Heteromorphic system of self incompatibility in *Primula*

Mating		Progeny	
Phenotype	Genotype	Genotype	Phenotype
Pin x Pin	ss x ss	Incompatible mating	
Pin x Thrum	ss x Ss	1 Ss : 1 ss	1 Thrum : 1 Pin
Thrum x Pin	Ss x ss	1 Ss : 1 ss	1 Thrum : 1 Pin
Thrum x Thrum	Ss x Ss	Incompatible mating	



Heteromorphic system of self incompatibility in *Primula*

(ii) Tristyly:

In *Lythrum*, three types of flowers with different stylar length exist. Here the stylar length is governed by two independent loci M and S. Plants with S have short style irrespective of the nature of other allele. The three different morphological types are self-incompatible but cross compatible.

	Long style	Medium style	Short style
Genotype	mmss	Mmss / MMss	MmSs / mmSs/ mmSS / MMSS/ MMSs / MmSS

(b) Homomorphic System:

In this case there is no morphological distinction between the self-incompatible flowers and the incompatibility is governed by multiple alleles.

This system can be of two types:

1. Pollen tube growth is controlled by genotype of sporophyte; and
2. Pollen tube growth is governed by genotype of the pollen.

(i) Sporophytic Incompatibility:

This system was first reported by Hughes and Babcock in 1950 in *Crepis foetida* and by Gerstel in *Parthenium argentatum* (in the same year).It occurs in radish, diploid *Brassica* sp., sunflower,

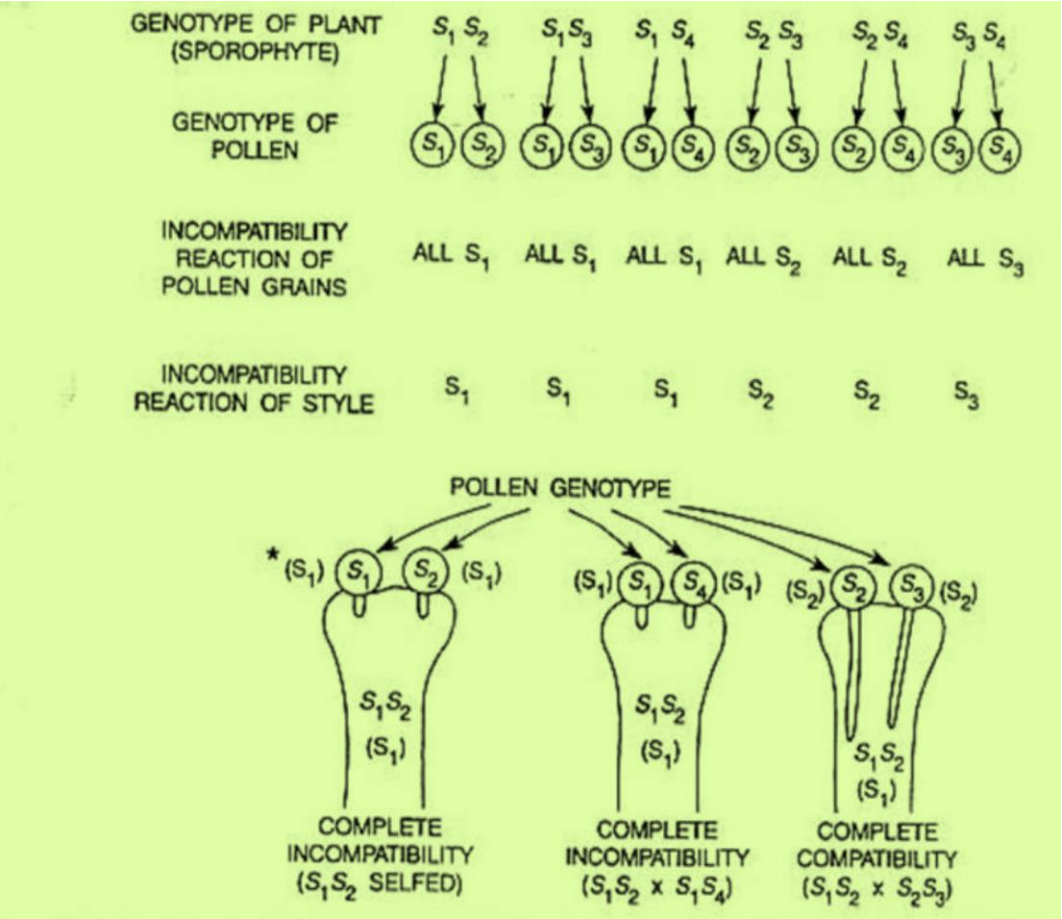
Sinapis, etc. Here the incompatibility is governed by multiple alleles but has the dominant-recessive reaction. Here the pollen tube growth is not controlled by the genotype of pollen but by the genotype of the plant on which it is being produced. Actually the behaviour of gametophyte is controlled by dominant allele of the genotype of sporophyte.

In this system, SI reaction of a pollen grain is determined by the genotype at the *S* locus of the plant, which produced it. The SI reaction is governed by a single *S* locus with multiple S_1, S_2, S_3, S_4; S_1 is dominant over others, S_2 is dominant over S_3 and S_4, S_3 is dominant over S_4, etc. Irrespective of the gametophytic genotype, the reaction will be of dominant type of the sporophyte.

S_1S_2 will produce all S, type gametophyte;

S_3S_4 will produce all S_3 type gametophyte;

S_2S_3 will produce all S_2 type gametophyte;



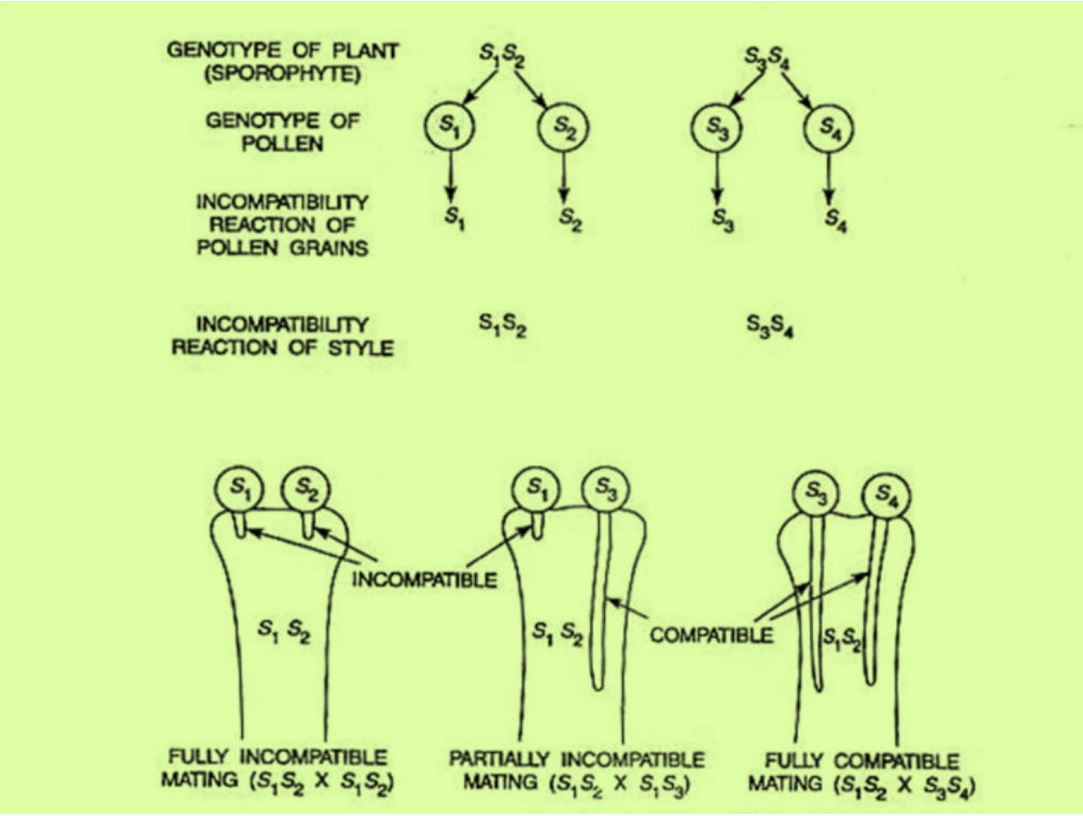
Sporophytic system of self incompatibility

(ii) Gametophytic Incompatibility:

This SI system was first decribed in 1925 by East and Mangelsdrof in *Nicotiana sanderae*. It is found in rye, sugar beet, pineapple, diploid clover, alfalfa and several wild species. In this system (1) the SI reaction of pollen grains is determined by their own genotypes. This is because the substance involved in SI reaction of pollen is produced after meiosis. (2) In style, the two *S* alleles show co-dominance. (3) In many species like *Nicotiana* sp., *Lycopersicon* sp., *Solanum* sp., *Petunia* sp., etc., the SI reaction is specified by a single gene *S* with multiples S_1, S_2, S_3, etc. In such cases, polyploidy may eliminate SI. (4) In some species, e.g., *Phalaris*, *Physalis*, etc., two loci (*S* and *Z*) govern SI, while in some others like sugar beet, *Papaver*, etc., three loci are invoved. In these cases, polyploidy has no

effect on SI. (5) A mutant allele *Sf* of the *S* locus allows the pollen grains carrying it to effect fertilization of flowers with any *S* genotype.

If a pollen grain lands on a stigma, which has the same *S* allele as the pollen (*e.g.*, S_1 pollen on S_1S_2, S_1S_3, S_1S_4, etc. stigma), it germinates normally. But the pollen tube growth is so slow that fertilization does not take place. However, if the stigma does not have the same *S* allele that is present in the pollen, pollen tube grows normally and fertilization is effected. Therefore, depending on the *S* locus genotype, matings between different plants are of the following three types: (1) fully compatible (e.g., $S_1S_2 \times S_3S_4$), (2) partially compatible (e.g., $S_1S_2 \times S_1S_3$) and (3) fully incompatible (e.g., $S_1S_2 \times S_1S_2$).



Gametophytic system of self incompatibility

Cryptic self-incompatibility (CSI):

In this mechanism, the simultaneous presence of cross and **self** pollen on the same stigma, results in higher seed set from cross pollen, relative to **self** pollen.

It exists in a limited number of taxa (for example, there is evidence for CSI in

- Bladder Campion-*Silene vulgaris* (Caryophyllaceae),
- Viper's Bugloss or Blueweed –*Echium vulgare* (Boraginaceae),
- Waterwillow or swamp loosestrife –*Decodon verticillatus* (Lythraceae)

However, as opposed to 'complete' or 'absolute' SI, in CSI, self-pollination without the presence of competing cross pollen, results in successive fertilization and seed set; in this way, reproduction is assured, even in the absence of cross-pollination. CSI acts, at least in some species, at the stage of pollen tube elongation, and leads to faster elongation of cross pollen tubes, relative to self pollen tubes. The cellular and molecular mechanisms of CSI have not been described.

The strength of a CSI response can be defined, as the ratio of crossed to selfed ovules, formed when equal amounts of cross and self pollen, are placed upon the stigma; in the taxa described up to this day, this ratio ranges between 3.2 and 11.5.

Late-acting self-incompatibility (LSI):

It is also termed **ovarian self-incompatibility (OSI)**. In this mechanism, self pollen germinates and reaches the ovules, but no fruit is set. LSI can be pre-zygotic (e.g. deterioration of the embryo sac prior to pollen tube entry, as in *Narcissus triandrus*) or post-zygotic (malformation of the zygote or embryo, as in certain species of *Asclepias* and in *Spathodea campanulata*).

Other types of Self Incompatibility:

Criteria	Types
Genes involved (number)	Monoallelic (governed by single gene)
	Diallelic (governed by two genes)
	Polyallelic (governed by many genes)
Cytology of pollen	Binucleate (pollens with two nuclei)
	Trinucleate (pollens with three nuclei)
Expression site	Ovarian (expression site is ovary)
	Stylar (expression site is style)
	Stigmatic (expression site is stigma)

Mechanism of Self Incompatibility

This is quite complex and is poorly understood. The various phenomena observed in Self incompatibility is grouped into three categories.

1. Pollen – Stigma interaction
2. Pollen tube – Style interaction
3. Pollen tube – Ovule interaction

1. Pollen – Stigma interaction

This occurs just after the pollen grains reach the stigma and generally prevents pollen from germination. Previously it was thought that binucleate condition of pollen in gametophytic system and trinucleate condition in sporophyutic system was the reason for self incompatibility. But later on it was observed that they are not the reason for SI. Under homomorphic system of incompatibility there are differences in the stigmatic surface which prevents pollen germination. In gametophytic system the stigma surface is plumose having elongated receptive cells which are commonly known as wet stigma. The pollen grain germinates on reaching the stigma and incompatibility reaction occurs at a later stage.

In the sporophytic system the stigma is papillate and dry and covered with hydrated layer of protein known as pellicle. This pellicle is involved in incompatibility reaction. With in few minutes of reaching the stigmatic surface the pollen releases exine exudates which are either protein or glycerol protein. This reacts with pellicle and induces callose formation, which further prevents the growth of pollen tube.

2. Pollen Tube – Style interaction

Pollen grains germinate and pollen tube penetrates the stigmatic surface. But in incompatible combinations the growth of pollen tube is retarded with in the style as in *Petunia, Lycopersicon, &*

Lilium. The protein and poly saccharine synthesis in the pollen tube stops resulting in bursting up of pollen tube and leading to death of nuclei.

3. Pollen tube – Ovule interaction

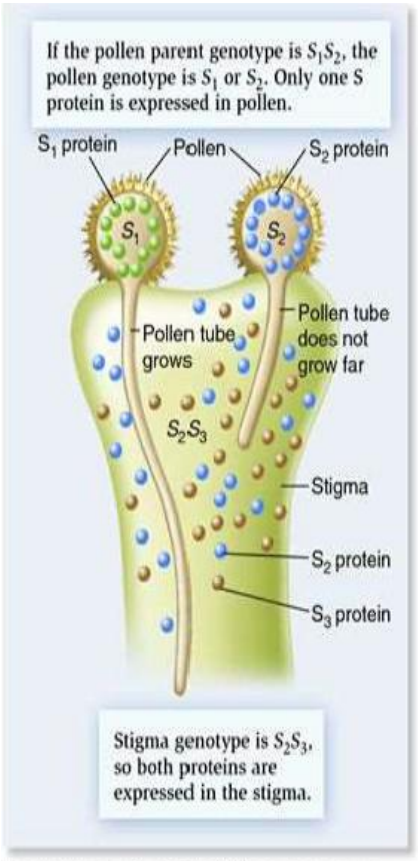
In *Theobroma cacao* pollen tube reaches the ovule and fertilization occurs but the embryo degenerates later due to some biochemical reaction.

Molecular mechanisms of single-locus self-incompatibility

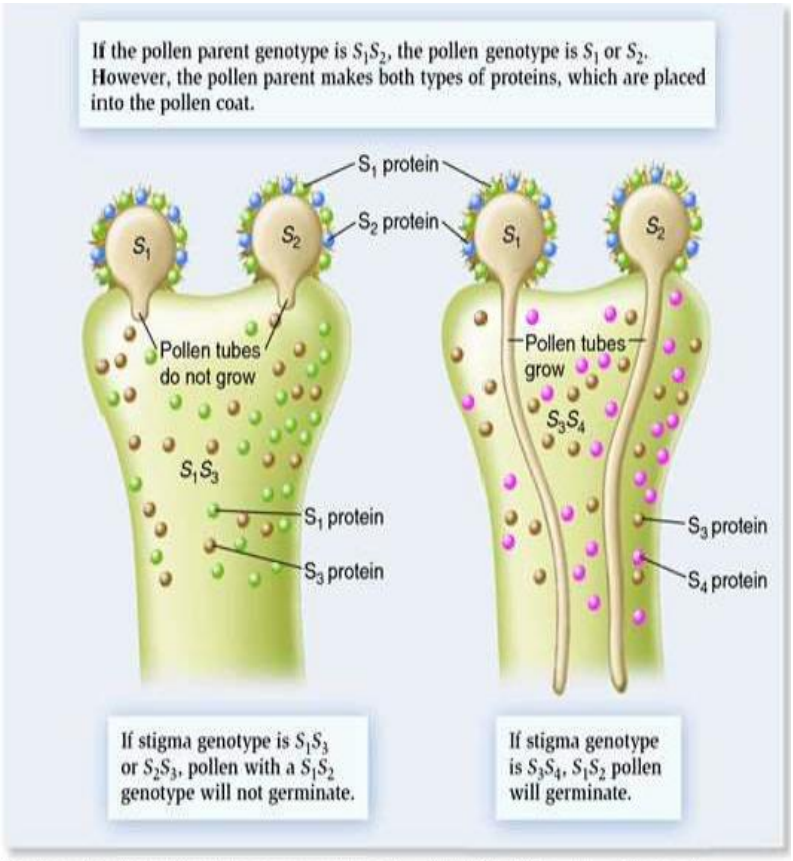
Mechanisms of single-locus self-incompatibility

The best studied mechanisms of SI act by inhibiting the germination of pollen on stigmas, or the elongation of the pollen tube in the styles. These mechanisms are based on protein-protein interactions, and the best-understood mechanisms are controlled by a single locus termed S, which has many different alleles in the species population. Despite their similar morphological and genetic manifestations, these mechanisms have evolved independently, and are based on different cellular components; therefore, each mechanism has its own, unique S-genes.

The S-locus contains two basic protein coding regions - one expressed in the pistil, and the other in the anther and/or pollen (referred to as the female and male determinants, respectively). Because of their physical proximity, these are genetically linked, and are inherited as a unit. The units are called S-haplotypes. The translation products of the two regions of the S-locus are two proteins which, by interacting with one another, lead to the arrest of pollen germination and/or pollen tube elongation, and thereby generate an SI response, preventing fertilization. However, when a female determinant interacts with a male determinant of a different haplotype, no SI is created, and fertilization ensues. This is a simplistic description of the general mechanism of SI, which is more complicated, and in some species the S-haplotype contains more than two protein coding regions.



(a) Gametophytic SI: If pollen *S* allele does not match either stigma allele, pollen will germinate.



(b) Sporophytic SI: If pollen coat S proteins do not match either stigma S protein, pollen tubes will grow.

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Gametophytic self-incompatibility (GSI)

In gametophytic self-incompatibility (GSI), the SI phenotype of the pollen is determined by its own gametophytic haploid genotype. This is the more common type of SI. Two different mechanisms of GSI have been described in detail at the molecular level, and their description follows.

The RNase mechanism

The female component of GSI in the Solanaceae was found in 1989. Proteins in the same family were subsequently discovered in the Rosaceae and Plantaginaceae. Despite some early doubts about the common ancestry of GSI in these distantly related families, phylogenetic studies and the finding of shared male determinants (F-box proteins) clearly established homology. Consequently, this mechanism arose approximately 90 million years ago, and is the inferred ancestral state for approximately 50% of all plants.

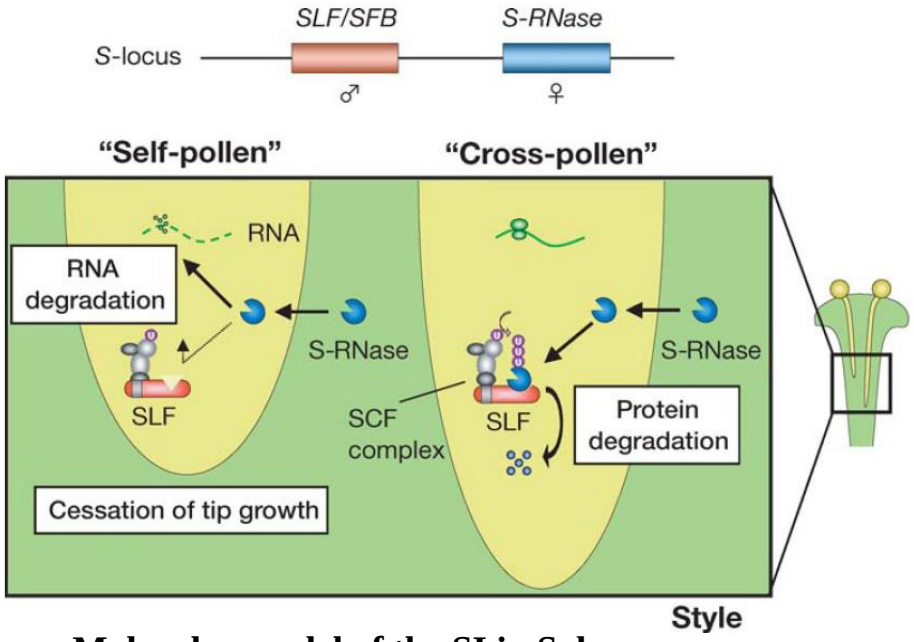
In this mechanism, pollen tube elongation is halted when it has preceded approximately one third of the way through the style. The female component ribonuclease, termed S-RNase probably causes degradation of the ribosomal RNA (rRNA) inside the pollen tube, in the case of identical male and female S alleles, and consequently pollen tube elongation is arrested, and the pollen grain dies.

The male component was only recently putatively identified as a member of the "F-box" protein family. Despite some fairly convincing evidence that it may be the male component, several features also make it an unlikely candidate.

The block within incompatible pollen tubes is created by an S-locus-encoded **ribonuclease** (SRNase), which is

- synthesized within the **style**;
- enters the pollen tube and
- destroys its RNA molecules
- halting pollen tube growth.

The RNase molecules contain a **hypervariable region**, each encoded by a different allele, which establishes each S specificity (S1, S2, S3, etc.). The pollen tube expresses a protein designated SLF(**Slocus F**-box **protein**) that binds S-RNase. SLF also exists in different S specificities (S1, S2, S3, etc.). In **compatible** ("nonself") tubes, the SLF or SCF(Skp1–Cul1–F-box-**protein** ubiquitin ligase) triggers the degradation (in proteasomes) of the S-RNase thus permitting RNAs in the pollen tube to survive and growth to continue. In **incompatible** ("self") tubes the interaction of, for example, the S1 SCF with the S1 S-RNase blocks its degradation so the RNAs of the pollen tube are destroyed and growth is halted.



Molecular model of the SI in Solanaceae

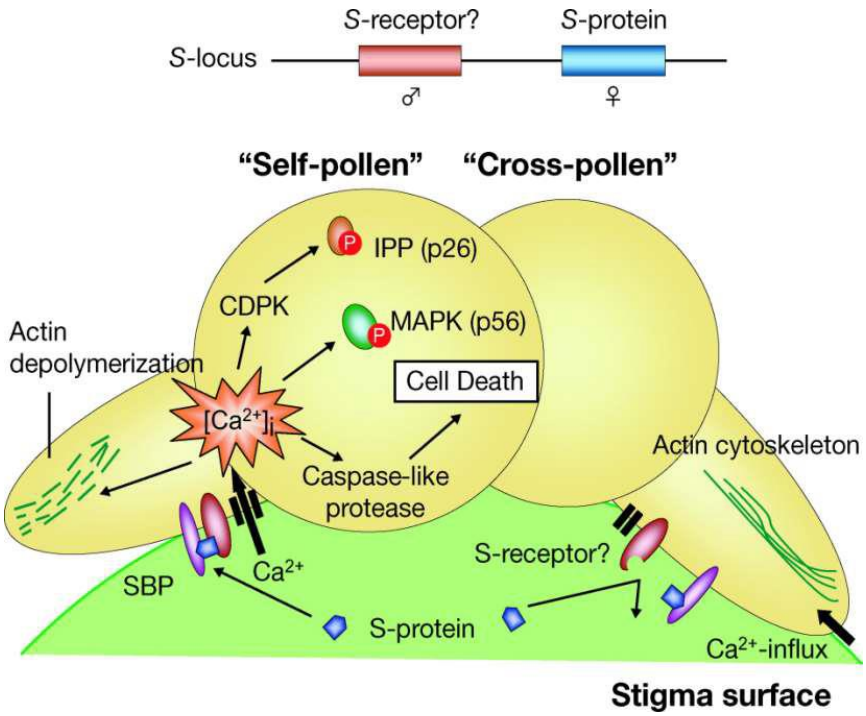
The S-glycoprotein mechanism

The following mechanism was described in detail in *Papaver rhoeas*. In this mechanism, pollen growth is inhibited within minutes of its placement on the stigma.

The female determinant is a small, extracellular molecule, expressed in the stigma; the identity of the male determinant remains elusive, but it is probably some cell membrane receptor. The interaction between male and female determinants transmits a cellular signal into the pollen tube, resulting in strong influx of calcium cations; this interferes with the intracellular concentration gradient of calcium ions which exists inside the pollen tube, essential for its elongation. The influx of calcium ions arrests tube elongation within 1–2 minutes. At this stage, pollen inhibition is still reversible, and elongation can be resumed by applying certain manipulations, resulting in ovule fertilization.

Subsequently, the cytosolic protein p26, a pyrophosphatase, is inhibited by phosphorylation, possibly resulting in arrest of synthesis of molecular building blocks, required for tube elongation. There is depolymerization and reorganization of actin filaments, within the pollen cytoskeleton. Within 10 minutes from the placement on the stigma, the pollen is committed to a process which ends in its death. At 3–4 hours past pollination, fragmentation of pollen DNA begins, and finally (at 10–14 hours), the cell dies apoptotically.

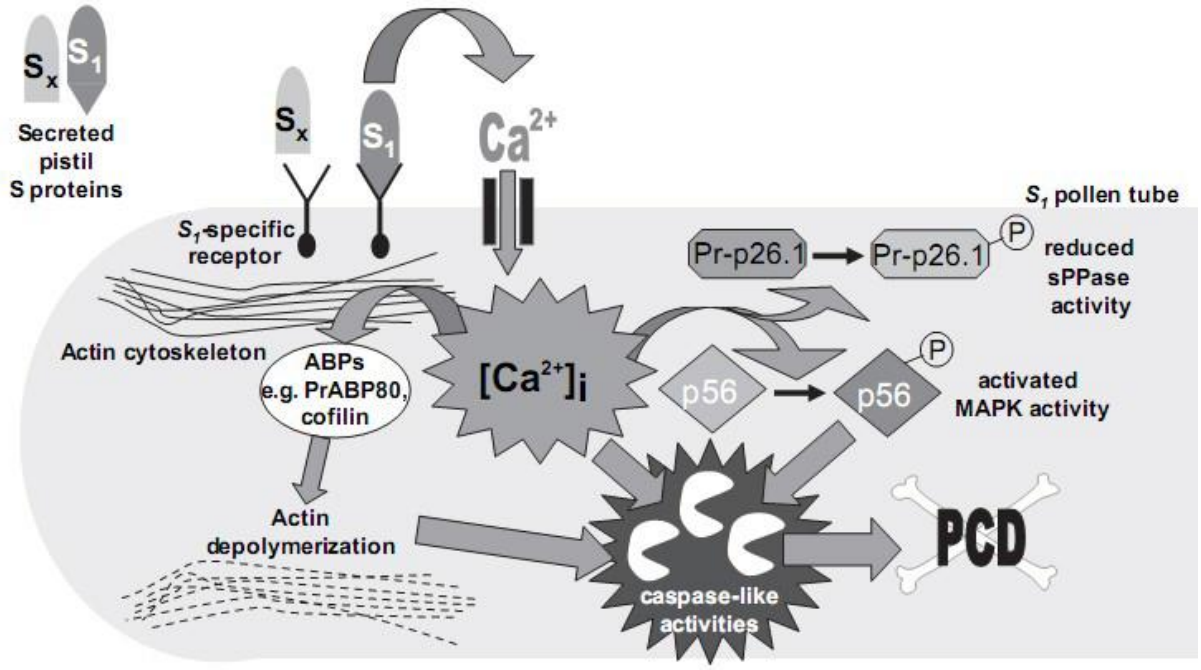
- Stigmatic S-proteins isolated through in vitro assays of pollen tube inhibition •the male determinant is believed to be a receptor located at the pollen plasma membrane
- SBP (S protein binding protein) specifically binds S-proteins, but without haplotype specificity
- Interaction of self-pollen with the stigma induces a calcium-dependent signaling cascade leading to programmed cell death in the pollen (Thomas & Franklin-Tong 2004)



Molecular model of the SI in Papavaraceae

Incompatible pollen undergoes an *S* haplotype-specific interaction. Secreted stigmatic *S*-proteins interact with the pollen S receptor. An haplotype-specific interaction such as binding S1 protein to S1 pollen results in triggering an intracellular Ca^{2+} signalling cascade(s), involving large-scale Ca^{2+} influx

and increases in [Ca^{2+}]i. A series of events then occur in the incompatible pollen. Within 1 min there is a dissipation of the tip-focused calcium gradient that is required for continued pollen growth and the activation of calciumdependent protein kinase (CDPK). The CDPK phosphorylates Pr-p26.1, a soluble inorganic pyrophosphatase (sPPase). Both calcium and phosphorylation inhibit sPPase activity, resulting in a reduction in the biosynthetic capability of the pollen, thereby inhibiting growth. Dramatic changes to
pollen cytoskeleton organization are apparent within 1 min, with extensive depolymerization of the Factin accompanying this, also predicted to cause rapid arrest of tip growth. p56-MAPK is activated and may signal to PCD. PCD is triggered, involving key features of PCD including caspase-like activity, cytochrome c leakage and DNA fragmentation. This ensures that incompatible pollen does not start to grow again.



Molecular model of the SI in Papavaraceae

Sporophytic self-incompatibility (SSI)

In sporophytic self-incompatibility (SSI), the SI phenotype of the pollen is determined by the diploid genotype of the anther (the sporophyte) in which it was created. This form of SI was identified in the families: Brassicaceae, Asteraceae, Convolvulaceae, Betulaceae, Caryophyllaceae, Sterculiaceae and Polemoniaceae. Up to this day, only one mechanism of SSI has been described in detail at the molecular level, in *Brassica* (Brassicaceae).

Since SSI is determined by a diploid genotype, the pollen and pistil each express the translation products of two different alleles, i.e. two male and two female determinants. Dominance relationships often exist between pairs of alleles, resulting in complicated patterns of compatibility/self-incompatibility. These dominance relationships also allow the generation of individuals homozygous for a recessive S allele.

Compared to a population in which all S alleles are co-dominant, the presence of dominance relationships in the population, raises the chances of compatible mating between individuals. The frequency ratio between recessive and dominant S alleles, reflects a dynamic balance between

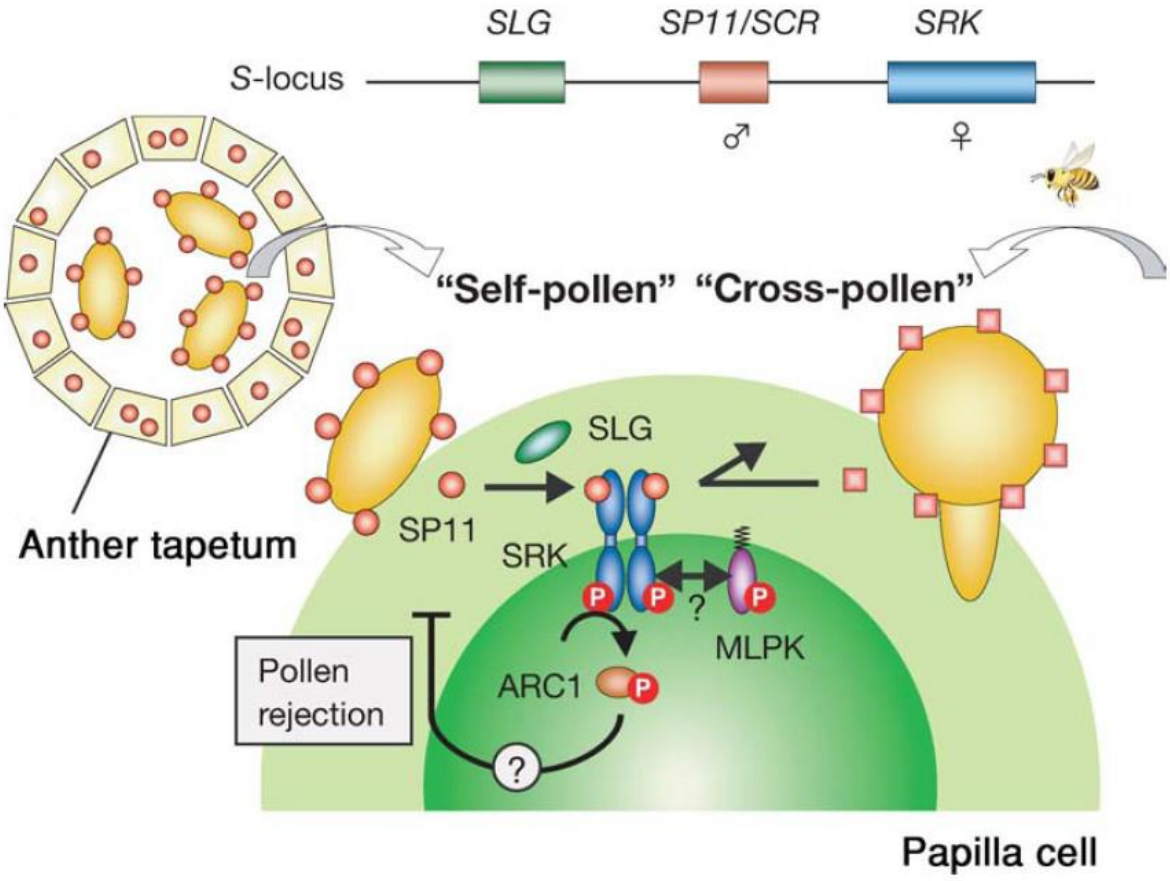
reproduction assurance (favoured by recessive alleles) and avoidance of selfing (favoured by dominant alleles).

The SI mechanism in *Brassica*

As previously mentioned, the SI phenotype of the pollen is determined by the diploid genotype of the anther. In *Brassica*, the pollen coat, derived from the anther's tapetum tissue, carries the translation products of the two S alleles. These are small, cysteine-rich proteins. The male determinant is termed SCR or SP11, and is expressed in the anther tapetum as well as in the microspore and pollen (i.e. sporophytically). There are possibly up to 100 polymorphs of the S-haplotype in *Brassica*, and within these there is a dominance hierarchy.

The female determinant of the SI response in *Brassica*, is a transmembrane protein termed SRK, which has an intracellular kinase domain, and a variable extracellular domain. SRK is expressed in the stigma, and probably functions as a receptor for the SCR/SP11 protein in the pollen coat. Another stigmatic protein, termed SLG, is highly similar in sequence to the SRK protein, and seems to function as a co-receptor for the male determinant, amplifying the SI response.

SP11 is predominantly expressed in anther tapetum and accumulates in the pollen coat during pollen maturation. On pollination, SP11 penetrates the papilla cell wall and binds to the receptor complex consisting of SRK and SLG (or its relatives). This binding induces the autophosphorylation of SRK, which triggers the signalling cascade that results in the rejection of self-pollen. Another protein essential for the SI response is MLPK, a serine-threonine kinase, which is anchored to the plasma membrane from its intracellular side.



Molecular model of the SI in Brassicaceae

Methods to Overcome Self-Incompatibility in Plants

The following methods have been used to overcome self-incompatibility in plants.

Method 1. Bud Pollination:

It is the most successful method in both the gametophytic and sporophytic systems. The most effective stage for the buds to overcome self-incompatibility is 2-7 days before anthesis. In *Petunia* inhibition is nullified if the buds are self- pollinated two days before anthesis. At the bud stage, the stigma lacks exudates, which appear only during anthesis. Thus, if the stigma is self-pollinated at bud stage, when the factors (present in the exudates) responsible for the self-incompatibility are not appeared, the pollen tubes will grow normally and effect fertilization.

Method 2. Mixed Pollination:

In this method the stigma is camouflaged from recognizing the incompatible pollen. This is achieved by pollinating the stigma with a mixture of compatible and incompatible pollens.It is presumed that when a stigma is pollinated with compatible pollen along with incompatible pollen, there the proteins released from the compatible pollen cover-up the inhibition reaction for the incompatible pollen at the surface of the stigma. By this method self-incompatibility can be successfully overcome in Cosmos (sporophytic self-incompatibility) and *Petunia hybrida* (gametophytic self- incompatibility).

Method 3. Deferred Pollination:

It has been observed that if pollination is deferred for few days, incompatible pollen tubes pass through the style. In *Brassica* and *Lilium* delayed pollination has been successful in overcoming self-incompatibility.

Method 4. Test Tube Pollination:

Such a method to overcome self- incompatibility was first reported by Kanta (1962) in *Papaver somniferum* and later success also achieved in *Argemone mexicana*, *Nicotiana rustica* and *N. tabacum* of Solanaceae.

In this method, stigmatic, stylar, and ovary wall tissues are completely removed from the path of pollen tube. The bare ovules are directly dusted with pollen grains. Successfully pollinated ovules are cultured in a nutrient medium that supports germination as well as development of fertilized ovules into seeds.

Method 5. Stub Pollination:

Those incompatibilities that are restricted to the stigma or to the length of the style that is larger than the maximum length attained by the pollen tubes have been overcome by removing the stigma and part of the style. The stigmatic surface of *Ipomoea trichocarpa* is the primary site of incompatibility and if the stigmatic lobe is removed and the cut surface pollinated then the pollen tube grows uninhibited in to the ovule.

Method 6. Intra-Ovarian Pollination:

In cases where the zone of incompatibility lies in the stigma or in the style, there pollen suspension can be applied directly in the ovary to overcome incompatibility. Viable seeds have been obtained by this method in, *Argemone mexicana* by Kanta and Maheshwari (1963).

In this method the ovary is at first surface sterilized, followed by injecting the aqueous pollen suspension (with or without specific substance for germination) by a hypodermic syringe followed by sealing the holes with petroleum jelly. The introduced pollen grains germinate and achieve fertilization. The method has also been successful in other members of Papaveraceae, like *Papaver rhoeas and P. somniferum.*

Method 7. In Vitro Pollination:

This method was developed by Kanta (1962) in *Papaver somniferum* to overcome pre-zygotic barriers to fertility. The exposed ovules, achieved by removing the stigmatic, stylar, and ovary wall tissues

were directly dusted with pollen grains and then cultured in a suitable nutrient medium that supported both the germination of pollen as well as the development of fertilized ovules.

Method 8. Elevated Temperature Treatment:

Incompatibility reactions are affected by high temperature treatment. Hot water treatment of *Lilium longiflorum* detached or intact styles at 50 °C for 6 minutes before pollination help to overcome self-incompatibility.

Method 9. Irradiation:

X-ray irradiation of flower buds at pollen mother cell stage helps to overcome self- incompatibility. In fact irradiation damages the physiological mechanism of self- incompatibility in the style, thus allowing the pollen tube to pass through the style.

Method 10. Surgical Method:

The stigma becomes the zone of inhibition, in plants with sporophytic type of incompatibility. Thus in such cases removal of such barrier is effective in overcoming self- incompatibility. Defacement or decapitation of the stigma before pollination or deposition of pollen grains directly into the stylar tissue through a slit has helped in overcoming self- incompatibility.

Method 11. Application of Chemicals:

Different chemicals including growth hormones have been recorded to be effective in overcoming self-incompatibility. Olivomycin and cycloheximide, the inhibitors of RNA and protein synthesis could overcome self- incompatibility in *Petunia hybrida*, when injected into the flower buds just 2-3 days before anthesis. The treatment of *Brassica oleracea* stigma before pollination with hexane was found to be effective in fruit set.

Method 12. Protoplast Fusion:

In cases where sexual incompatibility does not permit to raise hybrids by the conventional methods, there the fusion of isolated protoplasts have achieved great success. Since it involves the fusion of somatic protoplast, the method is described as parasexual hybridization.

The technique involves three basic steps, viz., isolation of protoplasts, fusion of the isolated protoplasts, and culture of hybrid protoplast to regenerate whole plants.

Significance of Self-Incompatibility in Plant Breeding:

Self-incompatibility effectively prevents self-pollination; as a result, it has a profound effect on plant breeding approaches and objectives.

1) In self incompatible fruit trees, it is necessary to plant two cross-compatible varieties to ensure fruitfulness.
2) Self-incompatibility may be used in hybrid seed production. For that, two self-incompatible but crosscompatible lines are to be interpolated; seeds obtained from both the lines would be hybrid seed.
3) Self incompatibility provides a way for hybrid seed production without emasculation and without resorting to genetic or cytoplasmic male sterility.
4) Self incompatibility system permits combining of desirable genes in a single genotype from two or more different sources through natural cross pollination which is not possible in self compatible species.
5) In case of pineapple, commercial clones are selfincompatible. As a result, their fruits develop parthenocarpically & are seedless.

Limitations

1) It is very difficult to produce homozygous inbred lines in a self incompatible species.
2) Bud pollination has to be made to maintain the parental lines.
3) Self incompatibility is affected by environmental factors such as temperature and humidity. Incompatibility is reduced or broken down at high temperature and humidity.
4) There is a limited use of self-incompatibility due to problems associated with the maintenance of inbred lines through hand pollination as it is tedious and costly.

5. Polyploidy in angiosperms - genetic insight to the phenomenon

Polyploids (three or more genomes):

The organisms having more than two sets of chromosomes by the addition of another chromosome is called polyploidy. It may arise as a result of abnormal mitosis where chromosomes divide by cytoplasm fails to divide during cytokinesis. The basic set of chromosomes undergoes multiplications. For example, in *Chrysanthemum* basic set is x = 9. Its species and hybrids show multiple of 9, such as 18, 27, 36, 45. In *Nicotiana* and *Solanum* basic set is x = 12 and multiple of somatic chromosome numbers are 24, 48 and 72 and in *Triticum* it is x = 7 and multiples are 14, 21, 42.

Origin of Polyploidy:

i) Polyploids are originated by failure of normal mitotic division in somatic cells.
ii) Polyploids may originate due to abnormal reduction divisions resulting
iii) It may naturally occur due to fertilization of egg by more than one male gamete.
iv) It may originate by artificial induction using colchicines or may be originated by cross hybridization between haploid and diploids.

Types of Polyploidy:

There are mainly four different types of Polyploidy, namely:
i) Auto-Polyploidy,
ii) Allopolyploids,
iii) Segmental allopolyploids and
iv) Auto-allopolyploids.

(i) Auto-Polyploidy:

When more than two genomes developed by the multiplication of chromosome number of some individual is called Autopolyploids. On the other hand autopolyploids are the individuals of which body cells contains more than two identical set of chromosomes derived by self-duplication. It arises due to failure of anaphase.

Examples:

Autotriploids (3n) – *Oenothera, Datura, Dahlia,* Rose etc. autotriploids are more vigorous, shows more perenation of the organs of vegetative propagation and are highly sterile.

Autotetraploids: (4n) – it contain four identical genomes and arise by fusion of two diploid gametes. It may results from duplication of somatic chromosomes. Autotetraploids show great adaptability, disease resistant, larger seeds, high vitamin-C content and low fertility. These are found in Apples, Grapes, Marigolds.

Meiosis in an Autopolyploid:

Meiotic behaviour in an autopolyploid such as autotetraploid is different than in a diploid. This is due to the presence of four homologous chromosomes of each kind.

Assuming that the primary material is a diploid species with 14 chromosomes (AA), these will form seven pairs (bivalents) at meiosis . In the tetraploid (AAAA) there will be four chromosomes of each type, and at meiosis, these seven groups of four chromosomes may form seven quadrivalents.

A quadrivalent is an association of four homologous chromosomes. Quadrivalents may be of different appearances. Sometimes, the homologous chro-mosomes are represented by an association of three chromosomes, called a trivalent and a univalent or by two bivalents.

As a rule, the average number of quadrivalents per cell is, therefore, lower than the medium possible num-ber. Autotetraploids of different species behave differently in this respect. Some of them have a very high frequency of quadrivalents as in *A. tuberosum*, in some cases bivalents are formed.

The occurrence of trivaients and univalents at meiosis in an autotetraploid leads to distur-bances in chromosome distribution and to the formation of gametes with deviating chromo-some numbers. This is the principal cause for the high degree of sterility in an autotetraploid.

Segregation of Genes in Autopolyploids:

The number of alleles of each gene is represented according to the ploidy level of the Polyploidy individual and gametes containing more than one allele of each gene (homo- or heterozygotic) may be produced.

According to the number of dominant and recessive alleles at a particular locus, the genotype of an autotetraploid may be quadriplex (AAAA or A_4), triplex (AAAa or A_3a), duplex (AAaa or A_2a_2), monoplex or simplex (Aaaa or Aa_3) and nulliplex (aaaa or a_4).

Auto-Polyploidy such as tetraploids show the so called tetrasomic inheritance. The segregation of genes in auto-Polyploidy is affected by factors which play no essential role in diploid.

Among such factors are the number and position of chiasmata in the multivalents, the distance between particular locus and centromere, the behaviour of homologues in multivalent associations during anaphase I and the presence of univalents.

In auto-tetraploids, if it is assumed that the four homologous chromosomes are distributed to poles in 2:2 during anaphase I, theoretical seg-regation ratios for various autotetraploid genotypes of a locus may be calculated.

Table 11.3: Frequencies of the gamete types and zygote types of autotetraploid genotypes

Parent	Gametes				Zygotes					
Genotype	AA	Aa	aa	divisor	A^4	A^3a	A^2a^2	Aa^3	a^4	divisor
Quadriplex (AAAA)	1	–	–	1	1	–	–	–	–	1
Triplex (AAAa)	1	1	–	2	1	2	1	–	–	4
Duplex (AAaa)	1	4	1	6	1	8	18	8	1	36
Monoplex (Aaaa)	–	1	1	2	–	–	1	2	1	4
Nulliplex (aaaa)	–	–	1	1	–	–	–	–	1	1

Applications of Autopolyploidy in Crop Improvement

Autopolyploidy has found some valuable applications in crop improvement. These are briefly summarized below:

Triploids –

Triploids are produced by hybridization between tetraploid and diploid strains. They are generally highly sterile, except in a few cases. This feature is useful in the production of seedless watermelons. In certain species, they may be more vigorous than the normal diploids, e.g., in sugarbeets. These two examples are described in some detail.

Seedless watermelons are grown commercially in Japan. They are produced by crossing tetraploid (4x, used as female) and diploid (2x, used as male) lines, since the reciprocal cross (2x x 4x) is not successful. The triploid plants do not produce true seeds; almost all the seeds are small, white rudimentary structures like cucumber (*Cucumis sativus*) seeds. But a few normal sized seeds may occur, which are empty. For good fruit setting, pollination is essential. For this purpose, diploid lines are planted in the ratio 1 diploid: 5 triploid plants. There are several, problems, viz., genetic instability of 4x lines, irregular fruit shape, a tendency towards hollowness of fruits, production of empty seeds and the labour involved in triploid seed production (by hand-pollination). Recently, some diploid hybrids of watermelon ('ice-box type') have been developed that produce seedless fruits (all their seeds are like cucumber seeds).

Triploid sugarbeet produce larger roots and more sugar per unit area than do diploids, while tetraploids produce smaller roots and lower yields than diploids. Apparently, 3x is the optimum level of ploidy in sugarbeets.

Tetraploids –

Autotetraploids have been produced in a large number of crop species and have been extensively studied in several cases. Tetraploids may be useful in one of the following ways: (1) in breeding, (2) improving quality, (3) overcoming self-incompatibility, (4) making distant crosses and (5) used directly as varieties.

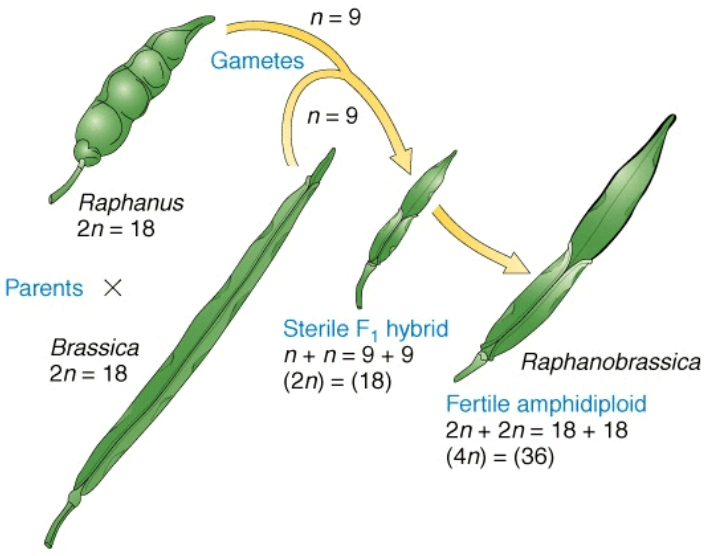
In banana (*M. sapientum*), autotetraploids are inferior to triploids in that they have weaker leaves and increased fertility. But they offer the only available chance of adding disease resistance to commercially successful varieties. In banana, autoteraploids are produced by chance fertilization of an unreduced triploid egg (AAA) by a haploid pollen from a disease resistant diploid parent. A large number of such tetraploids have been produced, but they have not yet gained any commercial success. This is an unusual case where auto tetraploidy is the only practical approach to breeding an otherwise successful triploid crop species.

Autotetraploidy is able to overcome self-incompatibility in certain cases, e.g., some genotypes of tobacco and white clover (*Trifolium repens*), *Petunia*, etc. Certain distant crosses are not successful at the diploid level, but are relatively successful at the autotetraploid level, e.g., 4x *Brassica oleracea* x *B. chinensis* is successful, but when *B. oleracea* is diploid it is unsuccessful. Similarly, autotetraploids of certain *Solanum* species produce hybrids with *S. tuberosum*, while the diploids do not.

Autotetraploids are larger in size and are more vigorous than diploids. Autotetraploid varieties of forage crops have been considerably successful. The most successful examples are, tetraploid red clover (*Trifolium pratense*) and ryegrass (*Lolium perenne*). Other examples are tetraploids of alsika clover (*Trifolium hybridum*, Variety Tetra) and berseem (*Trifolium alexandrium*, variety Pusa Giant Berseem).

(ii) Allopolyploids:

Polyploidy may also result from doubling of chromosome number in hybrid which is derived from two or more distinctly different species. This brings two (or more) different sets of chromosome in hybrid. The doubling of chromo-somes in the hybrid, which gives rise to a Polyploidy, is called an allopolyploid.



An allopolyploid in which a sterile hybrid (AB) originating out of the combination of two different species, undergoes duplication of chromosome set, is known as amphidiploid (AABB).

Raphanobrassica is a classical example of amphidiploidy. In 1927, Karpechenko, a Russian scientist, reported a cross between *Raphanus sativus* (2n = 18) and *Brassica oleracea* (2n = 18) to produce F2 hybrids which were completely sterile.

This sterility was due to lack of chromosome pairing, since there is no homology between genomes from *Raphanus sativus* and *Brassica oleracea*. Among these ste-rile hybrids certain fertile plants were found. On cytological examination, these fertile plants were found to have 2n = 36 chromosomes, which showed normal pairing into 18 bivalents.

Thus in allopolyploids the paring is of autosyndesis type (paternal-paternal or maternal- maternal pairing) in contrast to allosyndesis (paternal-maternal pairing) in diploids and autopolyploid.

Of the allopolyploids, amphidiploid hybrids containing two sets of each species are of special importance because they are usually fertile, occur rather widely among angiosperms in nature, afford clues to the relationship of certain species, and open a new path to the improvement of cultivated plants.

Triticale is the most successful synthetic allopolyploid produced by crossing wheat (tetraploid or hexaploid) with rye. *Triticales* derived from tetraploid wheats have been the most successful, but those from hexaploid wheats may also become a successful crop species. The breeding strategy involves (l) production of a large number of triticale strains using different combinations (varieties as well as species) of wheat and, rye, (2) hybridization of these triticale strains among themselves, and (3) improvement of the defects of the triticale through selection.

One of the earliest known amphidiploid hybrids was the fertile *Primula kewensis*, with 36 somatic chromosomes. A cross between *P. floribunda* (2n = 18) and *P. verticillata* (2n = 18) had yielded the sterile diploid *P. kewensis* (2n = 18) with one genome from each parent species.

Amphidiploids sometimes arise in ways other than by somatic chromosome doubling. Diploid spores, and, therefore, diploid gametes may appear on failure of meiosis and union of two diploid gametes gives rise to tetraploid. Although the chance of obtaining such plants in this manner seems to be relatively small.

(iii) Segmental Allopolyploids:

In some allopolyploids the different genomes that are present are not quite different from one another, i.e.,, having partial homology with each other (616,8282). Consequently, in these Polyploidy, chromosomes from different genomes do pair together to some extent and multivalents are formed. This means that segments of chromosomes and not the whole chromosome is homologous.

Such allopolyploids are called segmental allopolyploids (Stebbins). These chromosomes which are partially homologous and not completely homologous with each other are sometimes also described as homologous chromosomes. It is also believed that most of the naturally occurring Polyploidy are neither true auto-Polyploidy nor true allopolyploids.

Solanum tuberosum is the best example of segmental allopolyploid.

(iv) Auto-Allopolyploids:

When autopolyploidy is combined with allopolyploidy, autoallopolyploids are produced (AAAA6B). Polyploidy of this type are possible from hexaploid level upward as observed in *Nicotiana tabacum* and *Solanum nigrum.* Autoallopolyploids have importance in the evolution of certain plant species.

Role of Polyploidy:

Some of the important roles played by polyploidy are described below:

i. Role of Polyploidy in Plant Breeding:

When the techniques for artificial chromosome doubling became established, investigations on the origin of many of our economic plants were resumed. Many important crop plants like wheat, oat, sugarcane, cotton, tobacco as well as many fruits and vegetables are the Polyploidy of various degrees.

One of the important effects of polyploidy is the changes in the blooming season of the induced Polyploidy. As such, interspecific hybrids can be obtained of such species which otherwise remain isolated by seasonal isolation and different blooming season.

By artificial polyploidy induction, disease resistance and other desirable characters have been incorporated into some commercial crop plants. For example, *Nicotiana tabacum* is susceptible to TMV whereas *N. glutinosa* appears to be resistant.

The two tobacco species when crossed, the hybrids were found to be resistant but totally sterile. When the chromosomes were doubled it was possible to secure a fertile Polyploidy resistant to the virus. Many Polyploidy are selected and culti-vated because of their larger size, vigour and ornamental values. Several varieties of apples, pears and grapes have produced giant fruits which are of much economic value.

ii. Role of Polyploidy in Evolution:

Polyploidy combined with interspecific hybridization provides a mechanism by which new species may arise in nature and play a role in evolution. Allopolyploidy can produce new species by combining new characters and stable in evolution. It has already been discussed under amphidiploidy how different types of new species may be evolved.

Among the inter-specific hybridization, the most important are *Primula kewensis* (n = 18) obtained by crossing *P. floribunda* (n = 9) and *P. verticillata* (n = 9), *Digitalis mertqnensis* (n = 56) obtained by crossing *D. pur*purea (n = 28) and *D. ambigua* (n = 28) and *Spartina townsendii* (n = 63) obtained from cross of *S. stricta* (n = 28) and *S. alterniflora* (n = 35).

Origin of some of the economically important plants like rice, wheat, cotton, tobacco is important in this aspect. The chromosome number of rice (*Oryza sativa*) is 2n = 24. It is an example of typical secondary allopolyploids with basic chromosome number x = 5.

The present cultivated variety of rice is actually produced by hybridization followed by aneuploidy and euploidy. The origin of wheat, cotton, Mustard, etc. are given below:

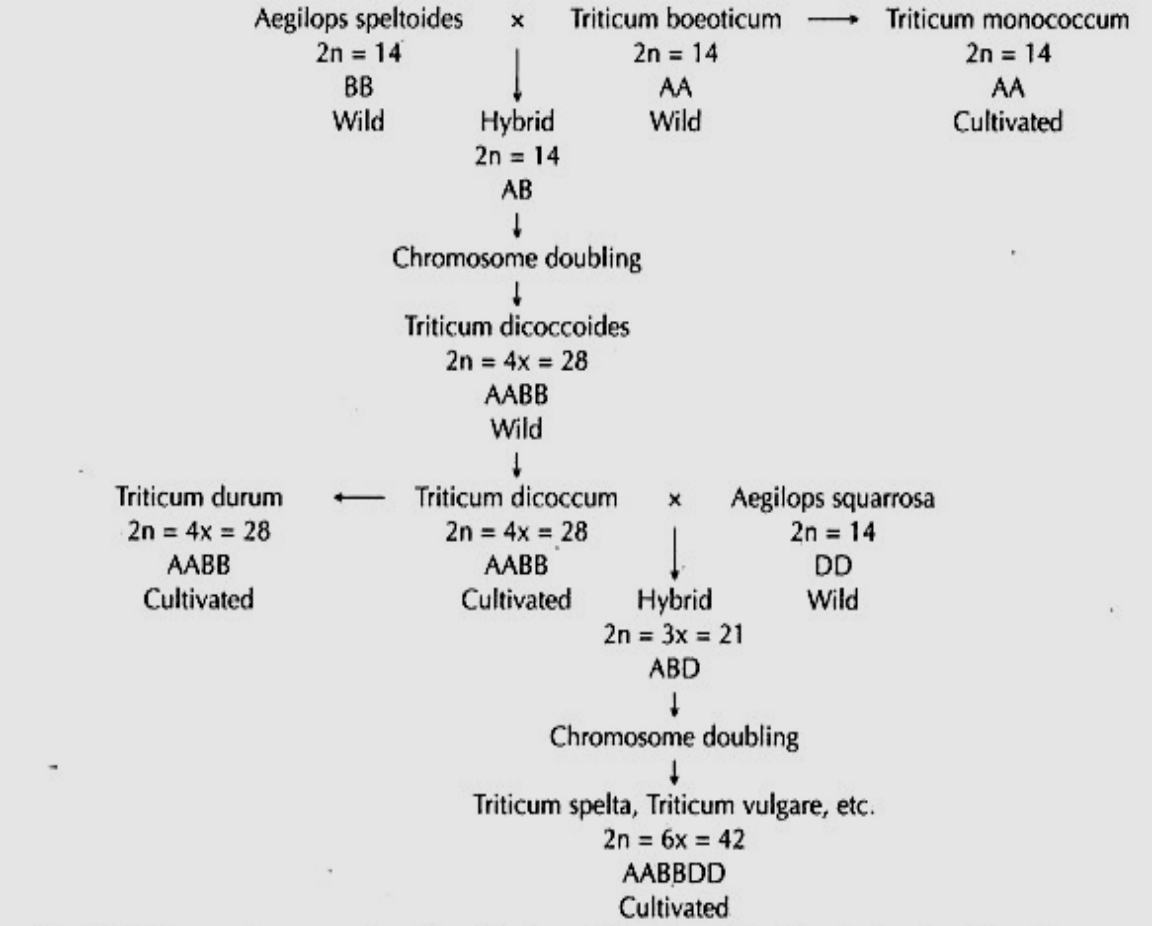


Fig. 11.12: Diagrammatic representation of the origin of tetraploid and hexaploid cultivated wheat from their wild ancestors

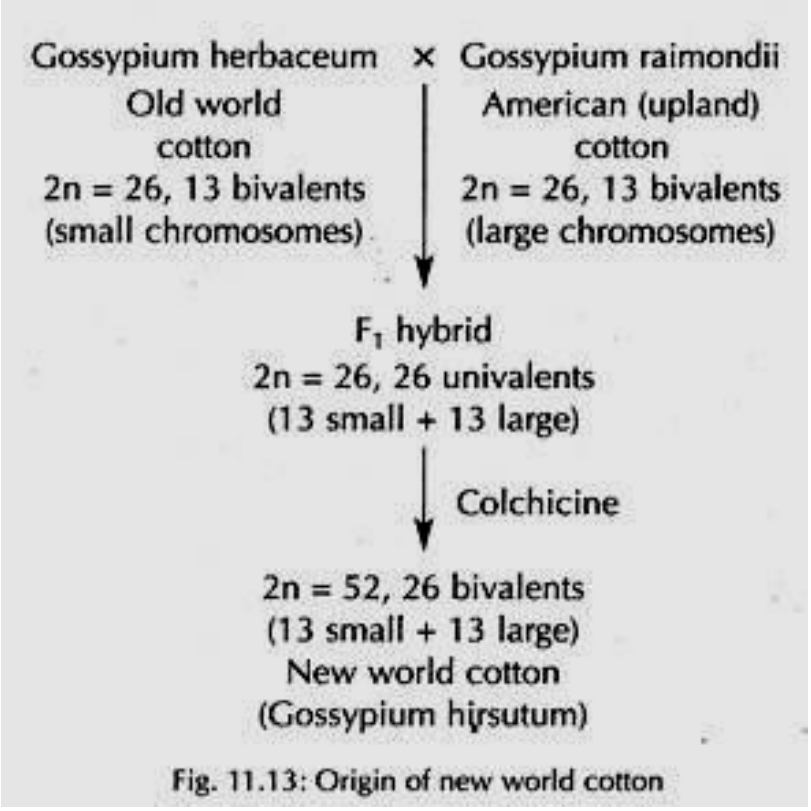


Fig. 11.13: Origin of new world cotton

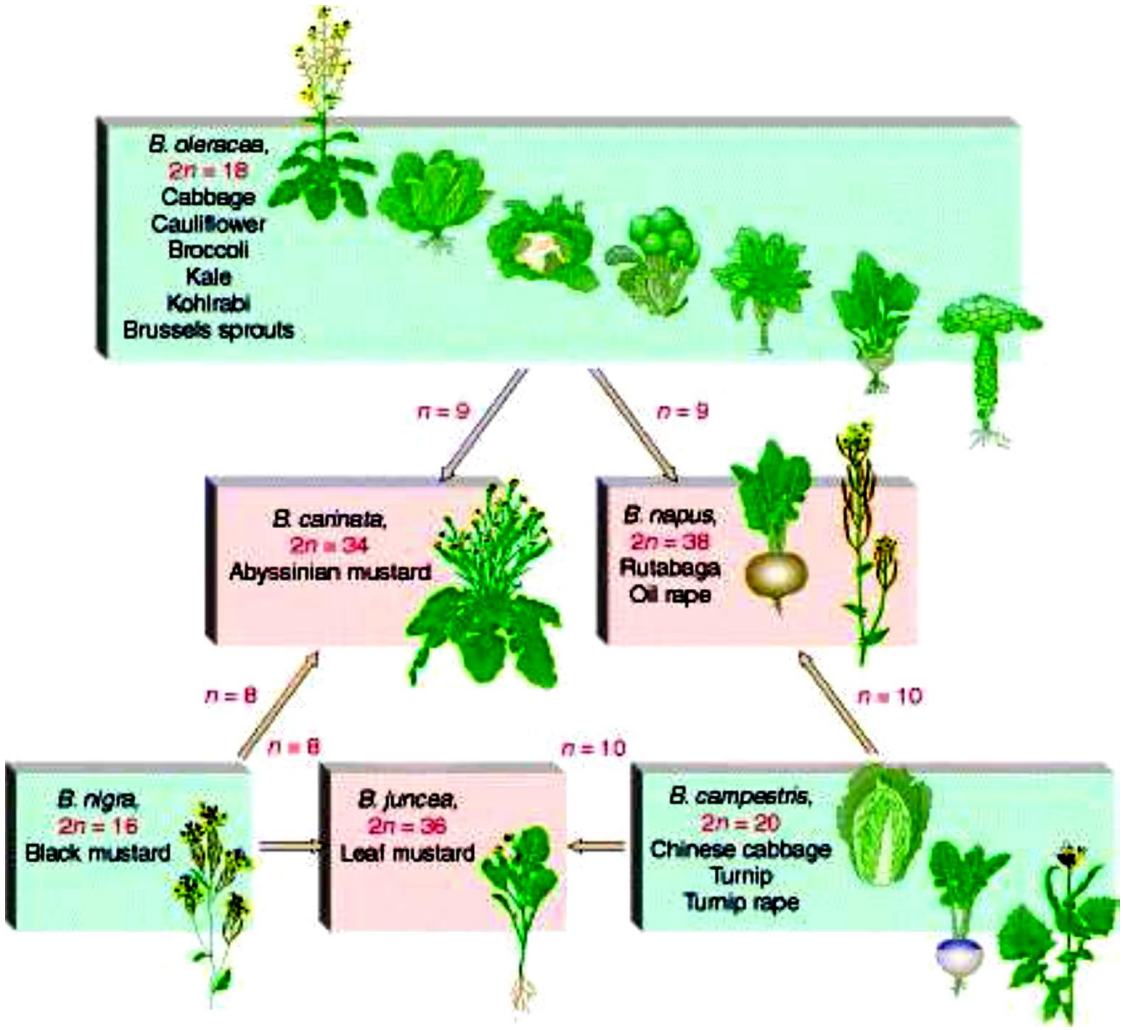


Fig. Origin of *Brassica* (triangle)

iii. Media of Conservation of Characters:

Polyploidy plays an important role in conserving the characters. A recessive mutation in order to be expressed in an autotetraploid, all four genes must be in recessive condition which is a time requiring process. Thus the characters in a Polyploidy plant could be conserved.

iv. Polyploidy and Geographical Distribution:

The Polyploidy plants can cope with diverse geographical areas than a diploid. Hence, the geographical distributions of Polyploidy plants are greater than diploids. Auto-polyploidy cannot produce new species, but they can colonize a new environment easily. As allopolyploids contain different genomes, they can withstand different environmental condition.

Both these power of colonization and coping with a diverse environment of the Polyploidy plants, help their wide geographical distribution.

Limitation of Polyploidy:

Polyploidy has several limitations. Some important limitations of polyploidy in crop improvement are briefly presented below:

1. Limited use: The single species polyploidy has limited applications. It is generally useful in those crop species which propagate asexually like banana, potato, sugarcane, grapes etc.

2. Difficulty in maintenance: The maintenance of monoploids and triploids is not possible in case of sexually propagating crop species.

3. Undesirable characters: In bispecies or multispecies polyploids characters are contributed by each of the parental species. These characters may be sometimes undesirable as in case of *Raphanobrassica*.

4. Some other defects: Induced polyploids have several defects such as low fertility, genetic instability, low growth rate, late maturity, etc.

5. Chances of developing new species through allopolyploidy are extremely low.

Current Concept of polyploidy: A genetic insight:

The phenomenon of polyploidy has been reported to encompass significant impacts on biodiversity as well as ecosystem functioning and act as a major driving force of plant evolution. Polyploidy is known to produce significant effects on genome structure and gene expression. Polyphyletic origin of polyploid species: Provides source for intra and intergenomic admixture following hybridisation event resulting in (1) higher level of heterozygosity, (2) less inbreeding depression, (3) higher genetic diversity, (4) genome rearrangement and (5) occurrence of large region of homologous and homeologous sequences in DNA.

Polyploid research in flowering plants has been a major focus of interest in plant science for long as it provides significant path to evolutionary biology. In recent past several important articles are documented highlighting the phenomenon in different aspects but still it seems to be an enigma. The present review article is documented on angiosperm members taking into account the significant findings on polyploidy considering genome doubling and its significance, genome restructuring and gene silencing, genome size dynamics, meiotic pairing behaviour and adaptive significance with an objective to describe the phenomenon in a more comprehensive manner. The review article may provide genetic insight for further exploration in polyploidy research unravelling the mysteries still associated to it.

Genome Doubling and Hybridisation

whole genome duplications has been long recognised as an important evolutionary force in plant species. Genome doubling resulting in polyploids is reported to be significant due to (1) masking of deleterious recessive mutation, (2) transgressive performance of allopolyploid and heterozygous autopolyploid offsprings, (3) functional modification of duplicated genes including silencing,

activation, DNA loss and epigenetic changes resulting in increase of adaptive potential of the plant species and (4) possible elimination of self-incompatibility barrier. In the context several difficulties exist as (1) pairing behaviour and segregation patterns of chromosome, (2) cellular architecture and (3) cytotype disadvantages.

Investigation on *Cleome* transcriptome by Barker et al. (2009) contributes towards understanding the dynamics of genome duplication and evolution of Rosid clade. Wang et al. (2006) used microarrays to measure the transcriptomic changes in neoallopolyploids in comparison to autopolyploid parents and suggested that several genes to be differentially regulated between parent and offspring. The same study was performed in autotetraploid which underwent WGD but not through hybridisation event documented less remarkable changes between polyploids and diploid progenitors. The experiments lead to a controversy to come to a conclusion whether the hybridisation event acts as major driving force for transcriptomic changes in allopolyploids or not? Madlung (2013) from his experiments on autopolyploid Arabidopsis partially supported the view that hybridisation results in greater genomic change than genome doubling. Pignatta et al. (2010) reported that tissue specific green fluorescent genes activated by endogenous enhancer elements are responsible for genetic variation between diploid and autotetraploid Arabidopsis; while, Yu et al. (2010) opined that ecotypes may possess a role in differential transcriptomic changes in response to genome doubling. Flagel et al. (2008) reported that about 24 % of genes were with a significantly biased regulation in synthetic F1 hybrid and natural allopolyploid *Gossypium*, thereby suggesting that altered gene expression is due to genome merging.

Genome Restructuring

Genomic rearrangements through intra and intergenomic translocations and transposable elements (TEs) have played significant role in genome restructuring. Gill and Friebe (2013) reported centromeric and non-centromeric break point intimated by reciprocal chromosomal translocation as most common source of polymorphism in polyploidy lineage of *Triticum turgidum* (2n = 4x = 28; AABB) and *T. timopheevii* (2n = 4x = 28; AAGG) as compared to donor diploid. Geographically isolated polyploidy wheat population experienced translocations, few\ restricted to specific chromosomal region, with repeated hybridisation event. Shoemaker et al. (1996) following genome mapping suggested that tetraploid *Glycine max* reveals significant intrachromosomal rearrangements, where interchromosomal translocation plays a minor role.

Genome Size Dynamics

Genome size dynamics in polyploids is rather variable but plays significant role in evolutionary processes. Genetic drift, hybridisation events, genome introgression and environmental changes are reported to influence genome size variation and species phylogeny. Pellicer et al. (2013) opined that change in repetitive DNA content and chromosome rearrangements within and between genera of the order Nymphaeales possibly reflects distinctiveness in the pattern of genome diversity. Recurrent polyploidization events in angiosperms over evolutionary time and accumulation of TEs are also reported to be possible causes for genome size expansion in polyploids. The transposition of the retrotransposons and their ubiquitous dispersion in plant species might explain the positive correlation between the genome size of plants and prevalence of retrotransposons as well as genome restructuring after polyploidization.

Altered Gene Expression and Gene Silencing

Altered gene expression as the consequence of polyploidization is well documented. Genes in large numbers, result of recurrent duplication event, show biasness in their retention or loss. Wang et al. (2004) reported that following duplications of rice genome, there has been largescale chromosomal rearrangements and loss of 32–65 % duplicated genes leading to diploidization. Preferential retention

of duplicated gene encoding proteins involved in transcription or signal transduction and selective silencing of DNA repair or defence mediating genes are reported. Partitioning of duplicated gene expression encoding serine threonine kinase during period of few generations following polyploidy has been studied in Arabidopsis. Gene (encoding mono-oxygenase) silencing in synthetic allopolyploid cotton is reported to be plastic as well as stochastic. The possible causes for altered gene expression followed by gene silencing are

(1) change in chromosome number,
(2) change in cytosine methylation; histone modification like deacetylation, methylation and positional effect arising out of the consequences of higher order changes in chromatin structure,
(3) epigenetic change at transcriptional as well as translational level,
(4) occurrence of repeats including long terminal repeats of retroelements,
(5) small nuclear RNAs (snRNA) and RNAi,
(6) antisense transcript generated by read out transcription of a retrotransposon exemplified in synthetic wheat polyploid among others.

Meiotic Pairing Behavior

Ph1 gene located in 5B chromosome in wheat is reported to enforce bivalent pairing by unknown genetic mechanism. It is reported it acts by reducing non-homologous centromere associations promoting true homologous association induced to associate. *Ph1* gene also induced chromosome pairing in autotetraploid rye.

Genetic Insight to the phenomenon of polyploidy

1) Is there any selective force(s) that control variable number of intra and interspecific hybridisation as it is a prerequisite for polyploid formation?
2) Why geographical isolation of species is not a hindrance for genome merging through hybridisation?
3) How did the duplication event(s) occur? Is it genetically programmed as the number of duplication cycle varies from one to many among the plant species?
4) Which of the key event among hybridisation and genome duplication was earlier or both were concomitant?
5) How normal meiotic pairing and fertility are restored in polyploids immediately after hybridisation as well as after duplication of genomes as artificially induced polyploids in sexually propagated plants show high degree of multivalent formation and sterility?
6) Is complete chromosome or chromosome segment elimination played any significant role in meiotic stability of polyploids?
7) Why gene silencing is preferential? Is it genetically controlled for adaptation?
8) How do the transposable elements accumulate within the genome in course of evolution regulating gene function?
9) Why no correlation exists between mean DNA amount/content and ploidy level of a species?

Hypothetical Thought Process

Upon considering that divergence of angiosperms has taken place from a common stock which possibly encompasses complex genome with multiple repeats in which natural selection force(s) including gene silencing, chromosome elimination/fusion, cryptic deletion, inter and intra chromosomal translocations, accumulation of transposable elements among others may have been operative in genome restructuring producing novel genotypes as well as phenotypes adaptive to environment. Such genotypes are ‘diploids’ possessing two sets of genome contributed by either of

the parents forming normal homologues. However, experimental evidences are required to validate the presumed concept.

Studies performed on recent polyploids reveal interference of environmental factor(s) onWGD events making the scenario rather complicated. However, whole genome sequencing analysis in association with comparative studies among different taxa (polyploids and respective diploid progenitor species) in angiosperm may provide new insight on polyploidy in an explicit dimension as it is extremely important for understanding evolutionary biology. Further, current research on ancient DNA (aDNA), still inadequately explored may unravel some significant aspects of polyploid research in future.

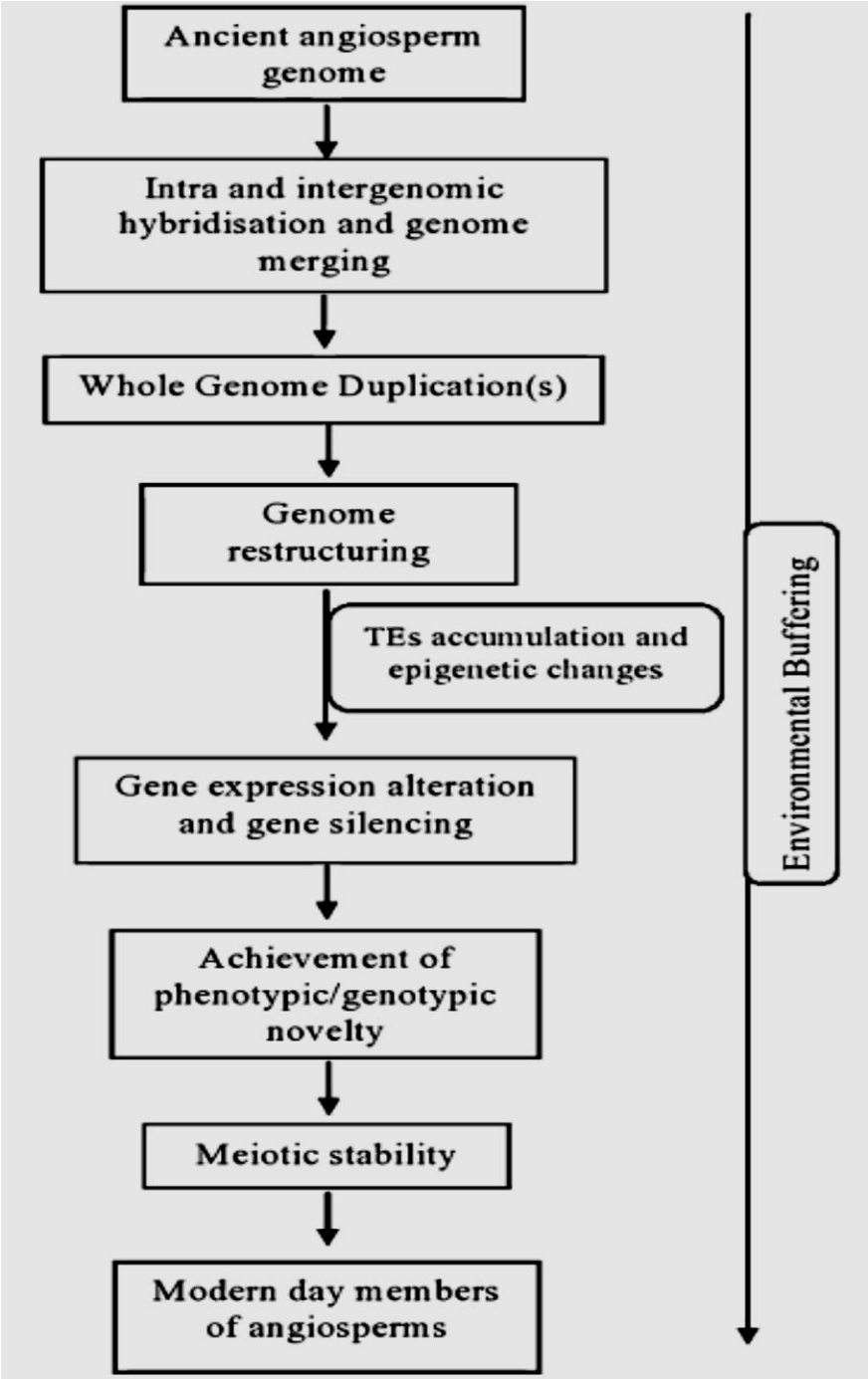


Fig. 1 Diagrammatic representation of different events related to polyploidization

6. Distant hybridization: Barriers and achievements. Bridge species.

When crosses are made between two different species or between two different genera, they are generally termed as distant hybridization (or) wide hybridization.

Thomas Fairchild 1717 was the first man to do distant hybridization. He produced a hybrid between two species of *Dianthus Dianthusc aryophyllus* (Carnation) x *D. barbatus* (Sweet william)

Inter generic hybrid produced by Karpechenko, a Russian Scientist in 1928. ***Raphanobrassica*** is the amphidiploid from a cross between Radish (*Raphanussativus*) and cabbage (*Brassicaoleraceae*). *Triticale* was produced by Rimpau in 1890 itself. ***Triticale*** is an amphidiploid obtained from cross between wheat and rye. Another example is *Saccharumnobilisation* involving three species.

It is an effective means of transferring desirable genes into cultivated plants from related species and genera. Distant crosses are more successful in more closely related species or genera than in less closely related species or genera.

Types of Distant Hybridization:

Distant hybridization is of two types, viz: (1) Interspecific hybridization, and (2) Intergeneric hybridization.

I. Interspecific Hybridization:

Crossing or mating between two different species of the same genus is referred to as interspecific hybridization. Because interspecific hybridization involves two species of the same genus, it is also termed as intrageneric hybridization.

Main features of interspecific hybridization are given below:

1. It is used when the desirable character is not found within the species of a crop.

2. It is an effective method of transferring desirable genes into cultivated plants from their related cultivated or wild species.

3. Interspecific hybridization is more successful in vegetatively propagated species like sugarcane and potato than in seed propagated species.

4. Interspecific hybridization leads to introgression which refers to transfer of some genes from one species into the genome of another species.

5. Interspecific hybridization gives rise to three types of crosses, viz.

(a) Fully fertile,

(b) Partially fertile, and

(c) Fully sterile in different crop species.

a. Fully Fertile Crosses:

Interspecific crosses are fully fertile between those species that have complete chromosomal homology. Chromosomes in such hybrids have normal pairing at meiosis and as a result the F_1 plants are fully fertile.

Fully fertile interspecific crosses have been observed between some species in cotton, wheat, oats and soybean as given below:

i. Cotton:

There are four cultivated species of cotton viz. *Gossypiumhirsutum*, *G. barbadense*, *G. arboreum* and *G. herbaceum*. The first two New World species belong to tetraploid group (2n = 52) and the last two Old World species to the diploid group (2n – 26). Crosses between tetraploid species *G. hirsutum* and *G. barbadense* and between diploid species *G. arboreum* and *G. herbaceum* are fully fertile.

G. hirsutum (2n = 52) x *G. barbadense* (In = 52) → F_1 plants are fully fertile.

G. arboreum (2n = 26) x *G. herbaceum* (2n = 26) → F_1 plants are fully fertile.

ii. Wheat:

The hexaploid wheat (2n = 42) has several species. Interspecific crosses between common wheat (*Triticumaestivum*) and club wheat (*T. compactum*) are fully fertile.

Triticumaestivum (2n = 42) x *T. compactum* (2n = 42) → F_1 plants are fully fertile.

ii. Oats:

There are two cultivated species of oat, viz. white oat (*Avenasativa*) and red oat (*Avenabyzantiana*). Both these species are hexaploid (2n = 42). Crosses between these two species are fully fertile.

Avenasativa (2n = 42) x *A. byzantiana* (2n = 42) → F_1 plants are fully fertile.

iv. Soybean:

The cultivated soybean (*Glycine max*) is believed to have originated from wild species *G. soja*. Both these species are annual diploid (2n = 40). The other wild species are perennials. The crosses between *G. max* and *G. soja* are fully fertile.

Glycine max (2n = 40) x *G. soja* (2n = 40) → F_1 plants are fully fertile.

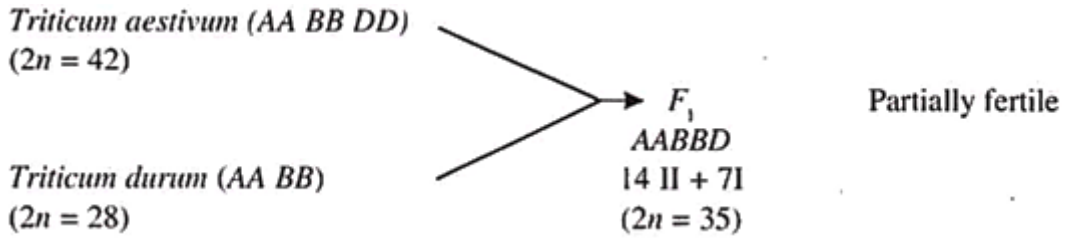
b. Partially Fertile Crosses:

Interspecific crosses are partially fertile between those species which differ in chromosome number but have some chromosomes in common. In such situations, the F_1 plants are partially fertile and partially sterile.

Partially fertile interspecific crosses have been reported in wheat, cotton and tobacco as given below:

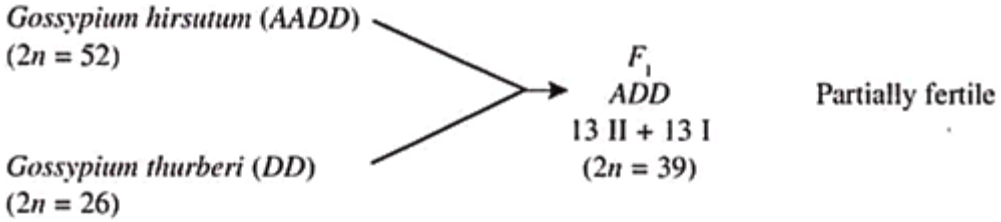
i. Wheat:

In wheat, there are three types of species, viz. diploid (2n = 14), tetraploid (2n = 28) and hexaploid (2n = 42). The cross between common wheat (*Triticumaestivum*, 2n = 42) and durum wheat (T. durum, 2n = 28) are partially fertile. In both these species chromosomes of A and B genomes are common and as a result the F_1 hybrids are partially fertile. In F_1 there are 14 bivalents and 7 univalents during meiosis. There is occasional seed set in this cross.



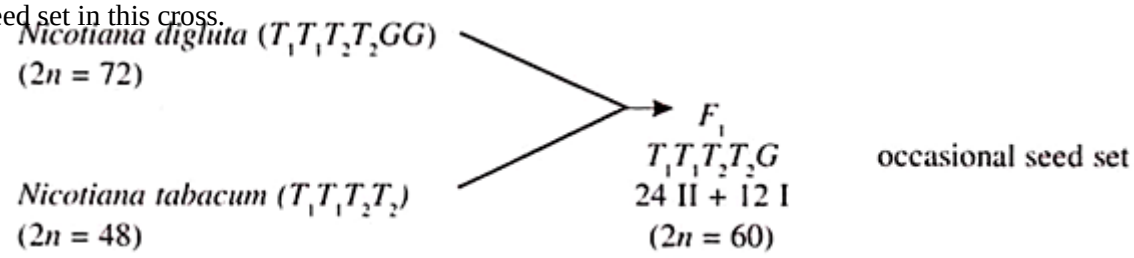
ii. Cotton:

In cotton, there are two types of species, viz. diploid (2n = 26) and tetraploid (2n = 52). The cross between American cultivated cotton (*G. hirsutum*, 2n = 52) and American wild diploid (*G. thurberi*) are partially fertile, because these two species have chromosomes of D genome in common. Meiosis in F_1 leads to formation of 13 bivalents and 13 univalents. There is occasional seed set in this cross.



iii. Tobacco:

In tobacco, there are three types of species, viz. diploid (2n = 24), tetraploid (2n = 48) and hexaploid (2n = 72). The cross between hexaploid wild tobacco (*Nicotianadigluta*) and common tetraploid tobacco (*N. tabacum*) shows partial fertility due to common chromosomes of T_1 and T_2 genomes in these two species. Meiosis in F_1 leads to formation of 24 bivalents and 12 univalents. There is rarely seed set in this cross.



c. Fully Sterile Crosses:

Interspecific crosses are fully sterile between those species which do not have chromosomal homology. In such species, chromosome number may or may not be similar. The lack of chromosomal homology does not permit pairing between the chromosomes of two species during meiosis.

As a result, the F_1 plants are fully self-sterile. Such hybrids can be made self-fertile by doubling of chromosomes through colchicine treatment. Fully sterile F hybrids have been reported in tobacco, wheat, cotton, *Brassica*, *Vigna* and several other crops.

i. Tobacco:

Clausen and Goodspeed (1928) made a cross between two wild diploid species of tobacco, viz. *Nicotianasylvestris* (2n = 24) and *N. tomentosa* (2n = 24). The F_1 hybrid was sterile. When the F_1 plants were treated with colchicine, a fully fertile tetraploid (2n = 48) was obtained which resembled cultivated species (*N. tabacum*).

They made another cross between another two wild diploid species of tobacco, namely *N. paniculata* (2n = 24) and *N. undulata* (2n = 24). Again the F_1 was sterile. Treatment of F_1 with colchicine resulted in the production of fertile amphidiploid (2n = 48) which was similar to cultivated species *N. rustica*.

ii. Cotton:

Harland (1940) made a cross between Asian cultivated diploid (*Gossypiumarboreum*, 2n = 26) and American wild diploid (*G. thurberi*, 2n = 26). The F_1 was sterile. Treatment of F_1 plants with colchicine resulted in the production of fertile amphidiploid (2n = 52) which was similar to upland cotton (*G. hirsutum*).

iii. Brassica:

Interspecific crosses in the genus Brassica were made by several workers. Three crosses were made among three species namely cabbage (*Brassica oleracea*), rapeseed (*B. campestris*) and black mustard (*B. nigra*). The F_1 hybrids were sterile in all the three crosses.

The treatment of F_1 plants with colchicine resulted in the production of fertile amphidiploid in each cross as given below:

Crosses		F_1	New species	Remarks
B. nigra × *B. oleracea*		Sterile, Colchicine	*B. carinata*	*Fertile*
2*n* = 16	2*n* = 18	2*n* = 17 treatment	2*n* = 34	*BBCC*
(*BB*)	(*CC*)	(*BC*)		
B. nigra × *B. campestris*		*Sterile, Colchicine* treatment	*B. juncea*	*Fertile*
2*n* = 16	2*n* = 20	2*n* = 16	2*n* = 32	*AA BB*
(*BB*)	(*AA*)	(*AB*)		
B. oleracea × *B. campestris*		*Sterile, Colchicine treatment*	*B. napus*	*Fertile*
2*n* = 18	2*n* = 20	2*n* = 19	2*n* = 38	*AACC*
(*CC*)	(*AA*)	(*AC*)		

II. Intergeneric Hybridization:

Intergeneric hybridization refers to crossing between two different genera of the same family. Such crosses are rarely used in crop improvement because of various problems associated with them.

The main features of intergeneric crosses are given below:

1. Intergeneric hybridization is used when the desirable genes are not found in different species of the same genus.

2. This method is rarely used in crop improvement programmes and that too for transfer of some specific characters into cultivated species from allied genera.

3. Intergeneric hybridization has been generally used in asexually propagated species.

4. F_1 hybrids between two genera are always sterile. The fertility has to be restored by doubling of chromosomes through colchicine treatment.

5. Intergeneric hybridization was used by some workers to develop new crop species.

Some examples of intergeneric hybridization are given below:

i. Wheat-Rye Cross:

The first intergeneric cross was made in the family Gramineae between bread wheat (*Triticumaestivum*, 2n = 42) and rye (*Secalecereale* 2n = 14) by Rimpau around 1890 in Sweden. The F_1 was sterile which was made fertile through colchicine treatment. The amphidiploid (2n = 56) was named as ***Triticale***.

This combines yield potential and grain quality of wheat and hardiness of rye. *Triticale* is the best example of the practical achievements of intergeneric hybridization. Now *Triticale* is commercially grown in countries like Canada and Argentina. Several improved varieties of *Triticale* have been released for commercial cultivation. Research work on Triticale is in progress at CYMMIT, Mexico.

ii. Radish Cabbage Cross:

Intergeneric cross between radish (*Raphanussativus*) and caage (*Brassica oleracea*) of the family Cruciferae was made by Karpechenko in 1928 in Russia. The main objective was to combine root of radish with leaves of cabbage. The F_1 was sterile. e doubling of chromosome number by colchicine treatment resulted in development of fertile amphidiploid which was named as ***Raphanobrassica*** by Karpechenko. But the new species thus developed had roots like cabbage and leaves like radish, which was a useless combination.

iii. Intergeneric Crosses in Sugarcane:

Several intergeneric crosses have been made in sugarcane. There are eight genera in which intergeneric hybrids have been made with sugarcane (*Saccharum*). These genera *include Eccoilopus, Erianthus, Miscanthidium, Miscanthus, Narenga, Rapidum, Sclerostachya*and *Sorghum (*sweet sorghums*).*

Many of the intergeneric hybrids are easily made with the help of male sterility. However, intergeneric hybrids with sugarcane have made little contribution to the development of modern commercial cultivars. Intergeneric hybrids have great potential for the improvement of germplasm.

iv. Maize-*Tripsacum* Crosses:

Intergeneric crosses between maize and *Tripsacum* were also attempted. The basic chromosome number is 10 in maize and 9 in *Tripsacum*. Crosses are successful in both directions, but hybrids can be more easily produced when *Tripsacum* is used as the female parent, because the maize pollens are able to produce long pollen tube to reach the ovule.

On the other hand, *Tripsacum* pollen are unable to produce long pollen tube to reach the ovule of maize. Hence when reciprocal cross is made, the maize styles (ear silks) have to be reduced in length by cutting so that the *Tripsacum* pollen tube can reach the ovule. Now hybrid derivatives of *Tripsacum* x diploid maize are being utilized in commercial crop improvement programmes.

Differences between Interspecific and Intergeneric hybridization

Particulars	*Interspecific Hybridization*	*Intergeneric Hybridization*
Parents Involved	Involves two different species of the same genes.	Involves two different genera of the same family.
Fertility	Such hybrids vary from completely fertile to completely sterile.	Hybrids are always sterile.
Seed setting	More than intergeneric crosses.	Low
Use in crop Improvement	More than intergeneric crosses.	Less than interspecific crosses.
Release of Hybrid varieties	Possible in some crops.	Not possible.
Evolution of new crops	Not possible, but evolution of new species is sometimes possible.	Sometimes possible, example is *Triticale*.

Barriers of distant hybridization:

The main barriers to the use of distant hybridization include:

i. Cross Incompatibility:

Inability of the functional pollens of one species or genera to effect fertilization of the female gametes of another species or genera is referred to as cross incompatibility. In another words, failure of male and female gametes to unite to form zygote in interspecific and intergeneric hybrids is known as cross incompatibility.

This is a major problem in distant hybridization. There are three main reasons of cross incompatibility, viz. lack of pollen germination, insufficient growth of pollen tube to reach ovule and inability of male gamete to unite with egg cell. These barriers are known as pre-fertilization barriers.

ii. Hybrid Inviability:

In some wide crosses, fertilization occurs and zygote formation also takes place. But the zygote does not grow. This inability of a hybrid zygote to grow into a normal embryo under the usual conditions of development is referred to as hybrid inviability.

This may result due to three main factors:

(i) Unfavourable interaction between chromosomes of two species,

(ii) Disharmony between cytoplasm and nuclear genes and,

(iii) Unfavourable interaction among embryo, endosperm and maternal tissues.

The following techniques may be useful to overcome the problem of hybrid inviability:

Proper choice of parents, making reciprocal crosses and application of growth hormones increase favourable conditions for the development of zygote into viable seed. If the growth of embryo is inhibited by the endosperm the embryo can be removed and transferred to the culture medium.

The new plants can be regenerated from the embryoids in the culture medium. The embryo cultures have been identified and developed for various plant species. Thus embryo culture technique is an effective way of overcoming the problem of hybrid zygote development.

iii. Hybrid Sterility:

In most of the wide crosses, hybrid sterility is the major problem. The hybrid sterility refers to the inability of a hybrid to produce viable offspring. The problem of hybrid sterility is more acute in intergeneric crosses than in interspecific crosses. The interspecific crosses vary from complete fertility to complete sterility.

But intergeneric crosses are always sterile. The main cause of hybrid sterility is lack of structural homology between the chromosomes of two species.

This leads to non-pairing or reduced pairing of chromosomes resulting in following meiotic abnormalities:

i. Scattering of chromosomes throughout spindles during metaphase I.

ii. Extension of chromosomes into cytoplasm.

iii. Lagging of chromosomes during anaphase.

iv. Formation of Anaphase Bridge.

v. Presence of ring and chain configurations.

vi. Irregular and unequal anaphase separation of chromosomes.

All these meiotic irregularities lead to structural chromosomal changes, viz. deletions, duplications, translocations and inversions which cause absence of pollen formation or formation of non-functional or abortive pollens. In some cases, sterility has been found to be associated with completely normal pairing of chromosomes (genie sterility).

Sometimes, the sterility is due to small structural changes in chromosomes which is not detectable during meiosis. Stebbins termed it as criptic structural hybridity.

The sterility caused by structural differences between the chromosomes of two species can be overcome by doubling the chromosome number of the hybrid through colchicine treatment. After chromosome doubling, each chromosome will have a pairing partner at meiosis. This will lead to normal chromosome pairing and production of viable gametes.

iv. Hybrid Breakdown:

Hybrid breakdown is a major problem in interspecific crosses. When F_1 plants of an interspecific cross are vigorous and fertile but their F_2 progeny is week and sterile, it is known as hybrid breakdown. Hybrid breakdown hinders the progress of interspecific gene transfer.

There are two main causes of hybrid breakdown, viz.:

(i) Gene combination, and

(ii) Structural differences.

(i) Gene Combination:

Sometimes, homozygous dominant alleles on several loci prefer to remain in one species and homozygous recessive alleles at the same loci in another species. The F_1 cross between such species would be heterozygous and vigorous. In F_2, the favourable combination of dominant and recessive genes is broken due to segregation and recombination. The plants which do not have a dominant allele at each locus or which are not homozygous for all recessive alleles would be weak and sterile.

(ii) Structural Differences:

There may exist some small structural differences in the chromosomes of two species, which do not affect chromosome pairing in F_1. In such hybrids, recombination between chromosome segments during meiosis may lead to production of gametes with deletions or duplications. The gametes with deletions and duplications result in hybrid breakdown.

Techniques to make wide crosses successful:

1. Selection of plants

The most compatible parents available should be selected for the crosses.

2. Reciprocal crosses

Reciprocal cross may be attempted when one parental combination fails.

e.g. Mung x udid- cross compatible and Udid x mung-cross incompatible

3. Manipulation of ploidy

Diploidization of solitary genomes to make them paired will be helpful to make the cross fertile.

4. Bridge crosses

When two parents are incompatible, a third parent that is compatible with both the parents can be used for bridge crosses and thus it becomes possible to perform cross between the original parents.

e.g. Tobacco, -*Nicotiana repanda* x *N.tabaccum*– cross incompatible

-*Nicotiana repanda* x *N.sylvestris*- cross compatible

-*Nicotiana syivestris* x *N.tabaccum*- cross compatiblexi. Grafting

Role of Distant Hybridization in Crop Improvement:

Wild species or wild genetic resources are the potential sources of desirable genes for various characters of crop plants. Wide crossing is an effective method of exploiting desirable characters from wild species for the improvement of cultivated crop plants. Thus the significance of wild species and distant hybridization are interlinked.

i. Character Improvement:

(i) Disease and Insect Resistance:

Distant hybridization has been instrumental in transferring disease resistance from wild species into cultivated ones. For example, resistance to rust and black arm in cotton; mosaic virus, wild fire, black-fire, blue mould, black root rot, and Fusarium wilt diseases in tobacco; sereh disease in sugarcane; late blight, leaf roll and virus x in potato; rust and eye spot in wheat; and yellow mosaic virus in okra have been transferred from wild species of these crops into cultivated species (Table 28.2).

In tomato, resistance to bacterial cankar, bacterial wilt, Fusarium wilt, grey leaf spot, leaf moulds Verticillium wilt, curly top virus, mosaic virus has been transferred from wild species to the commercial cultivars. Use of wild root stocks, in commonly grafted crops such as citrus, rubber, grape, pistachio and peach has eliminated many insect pests and diseases of these horticultural crops.

Less progress has been made on insect resistance. Resistance to jassids and boll weevil in cotton, leaf chewing insects in peanut, and aphids in strawberry has been transferred from their wild species to cultivars.

Resistance to diseases and insects transferred through interspecific hybridization in different crops

Crop	*Character transferred*	*Species transferred from*	*Species transferred to*
Cotton	Jasssid resistance	*Gossypium tomentosum*	*G. hirsutum*
	Smoothness for boll weevil resistance	*G. armourianum*	*G. hirsutum*
	Rust resistance	*G. raimondii*	*G. hirsutum*
	Blackarm resistance	*G. arboreum*	*G. barbadense*
Tobacco	Resistance to mosaic virus	*Nicotiana glutinosa*	*N. tabacum*
		N. repanda	*N. tabacum*
	Resistance to wild fire and blackfire diseases.	*N. longiflora*	*N. tabacum*
	Resistance to blue mould black root rot, wild fire and *Fusarium* wilt.	*N. debneyi*	*N. tabacum*
Sugarcane	Sereh disease resistance	*Saccharum spontaneum*	*S. officinarum*
Potato	Resistance to late blight, leaf roll and virus X.	*Solanum denissum*	*S. tuberosum*
Wheat	Resistance to eyespot	*Aegilops ventricosa*	*Triticum aestivum*
	Rust resistance	*Agropyron*	*T. aestivum*
Peanut	Resistance to leaf chewing insects	*Arachis monticola*	*A. hypogaea*
Strawberry	Red stele and aphid resistance	*Fragaria chiloensis*	Cultivated strawberry
Okra	Resistance to yellow mosaic virus	*Abelmoschus manihot*	*A. esculenta*

ii) Improvement in Quality:

In some crops, wild species have been used to improve the quality of cultivated ones. For example, protein content in rice, oats and rye; fibre length in cotton; oil quality in oil palm; carotenoid content in tomato; starch content in potato; leaf quality in tobacco; and oil per cent in oats have been improved through the use of their wild species in the hybridization programme.

Teosinte has been used to improve maize for silage. Wild Sorghum has been used to improve green fodder in cultivated species. Wild tobacco has been utilised to reduce nicotin content in cultivated species and flavour of cultivated tea has been improved through the use of wild tea.

Improvement in quality and other characters through use of wild species and distant hybridization in some crop plants

Crop	*Character*	*Species*	
		Transferred from	*Transferred to*
Cotton	Fibre length	*Gossypium thurberi* and *G. raimondii*	*G. hirsutum*
Palm	Oil quality	Wild species	Cultivated species
Rice, Oats and Rye	Seed protein	Wild species	Cultivated species
Tomato	Carotenoid Content	*Lycopersicon hirsutum*	*L. esculentum*
Tabacco	Leaf quality	*Nicotiana debneyi*	*N. tabacum*
Potato	Starch content	Wild species	Cultivated species
Oat	High oil content	Avena sterilis	*A. sativa*
Other characters			
Cotton	Male sterility	*Gossypium harknessii*	*G. hirsutum*
Potato	Frost resistance	*Solanum acaule*	*S. tuberosum*

(iii) Alien addition lines:

These lines carries one chromosome pair from a different species in addition to the normal somatic chromosome complement of the parent species. When only one chromosome from another species is present, it is known as alien addition monosome. Alien addition have also been done in rice, sugar beet, cotton, *brassica*. The main purpose of alien addition is the transfer of disease resistance from related wild species. e.g. Transfer of mosaic resistance from *Nicotiana glutinosa* to *N. Tabacum*. The alien addition lines have been developed in case of wheat, oats, tobacco and several other species. Alien addition lines are of little agricultural importance since the alien chromosome generally carries many undesirable genes. e.g. Reduced growth and short, broad leaves in addition to mosaic resistance.

(iv) Alien substitution lines:

This line has one chromosome pair from a different species in place of the chromosome pair of the recipient species. When a single chromosome (not a pair) from different species in place of a single chromosome of the recipient species, known as alien-substitution monosome. Alien –substitution line have been developed in wheat, cotton, tobacco, oats, etc. In case of tobacco, mosaic resistance gene N was transferred from the *N. Glutinosa* to *N. tabacum* line had 23 pairs of *N. tabacum* chromosomes and one pair (chromosome H)of *N. glutinosa* chromosomes. The alien substitution show more undesirable effects than alien additions and as a consequence is of no direct use in agriculture.

(v) Improvement in Adaptation:

Adaptation to various environmental conditions has been improved through the use of wild species. For example, tolerance to cold in rye, wheat, onion, potato, tomato, grapes, strawberry and peppermints etc. has been transferred from wild species of these crops in Russia. In wheat, increased winter hardiness has been transferred from Agropyron.

In grape, hardier vines have been developed through the use of wild species *Vitisamurensis* in the breeding programme. In sugarcane, cold tolerance has been transferred from wild species in USA. Drought tolerance in peas and wheat, salt tolerance in tomato, tolerance to calcareous soils and photo insensitivity in *Pennisetum* have been achieved through the use of their wild species in the breeding programmes.

(vi) Improvement in Yield:

Improvement in yield has also been achieved through the use of wild species in some crops. For example, in oat yield increase of 25-30% over the recurrent parent was obtained from a cross between *Avenasativa* x *A. sterilis*. High yielding transgressive segregants were obtained after 4 backcrosses.

Increase in yield has been reported in several crops such as *Vigna, Zea, Ribes, vanilla, Arachis*, potato and tobacco through interspecific hybridization. In tobacco, yields were increased by the use of wild species *Nicotiana cidebneyi*. Yields of sugarcane and octaploid strawberries have been increased by the use of their wild species.

(vii) Mode of Reproduction:

Use of wild species in the hybridization programmes sometimes leads to alteration in the mode of reproduction. The male sterility is the most common alteration in the mode of reproduction which results from interspecific hybridization. Cytoplasmic male sterility (CMS) is an economic device for hybrid seed production.

CMS has been discovered in crosses between wild and cultivated species in wheat, cotton, barley, tobacco, potato, sunflower and ryegrass. The CMS has been transferred to cultivated species of these crops. Apomictic genes have been transferred from maize — *Tripsacum* cross to maize and from wild species of Beta to cultivated species. The cleistogamy and self-fertility traits of wild *Secale* have been transferred to cultivated rye (*Secale cereale*).

(viii) Other Characters:

There are several other desirable characters which have been transferred from wild species to cultivated plants. For example, wild species have been used to transfer dark green colour and excellent leaf texture in lettuce and bright red thin flesh in red peppers. Semi-dwarf wheat has obtained from *Triticum* x *Agropyron* hybrid derivatives. Short statured oil palms resulted from interspecific hybrids. Earliness has been achieved from use of wild species in soybean.

ii. Hybrid Varieties:

Improved hybrid cultivars have been developed through the use of wild species mainly in sugarcane, potato and some forage crops. Most of the modem cultivars of sugarcane and potato are the derivatives of interspecific hybridization. In cotton, commercial interspecific hybrids have been developed both at tetraploid and diploid levels but between cultivated species only.

Some of the varieties of upland cotton (MCU 2, MCU 5, Deviraj, Devitej, G 67, Khandwa 1, Khandwa 2, Badnawar 1 PKV081, Rajat and Arogya) are derivatives of interspecific hybridization. A hybrid between Pearl-millet and napier grass has been developed which has become very popular by virtue of its high fodder yield potential and superior fodder quality.

iii. New Crop Species:

Sometimes, distant hybridization and polyploidy lead to creation of new crop species.*Nicotianadigluta* has been synthesized from a cross between *N. tabacum* and *N. glutinosa*. *Triticale* is the example of new crop which has evolved from an intergeneric cross between *Triticumaestivum* and *Secalesereale* and combines good characters of both the species.

Achievements of Distant Hybridization:

There are three main achievements of distant hybridization:

(1) Transfer of various characters from wild species to the cultivated species,

(2) Development of interspecific hybrids in some crops, and

(3) Creation of new crop plants

Practical Achievements of wide hybridization

S.N.	Crop and Achievements	Varieties/species Developed
1.	Cotton : Development of Varieties Cotton	MCU 2, MCU 5,, Deviraj, Devitej, Khandwa 1, Khandwa 2 and Badnawar 1, Gujarat 67, PKV 081, Rajat, AKA 8401, Arogya, etc.
	Sugarcane	Many verieties
	Potato	Many varieties
2.	Cotton : Development of Hybrids	Varalaxmi, Jayalaxmi, DHB 105, DDH 2, DH 7, DH 9, NHB 12, TCHB 213, HB 224, etc.
	Napier Grass	Hybrid Napier
3.	Evolution of New Crop species	
	Wheat-Rye	Triticale
	Tobacco	Nicotiana digluta
	Radish-Cabbage	Raphanobrassica

Various characters such as disease and insect resistance, improved quality and adaptation, earliness, dwarfness, tolerance to frost, drought and salinity have been transferred from wild species to the cultivated species through interspecific and intergeneric hybridization. Resistance to various diseases has been achieved in several crops like wheat, cotton, tobacco, sugarcane, potato, strawberry, okra etc. through distant hybridization.

Cytoplasmic male sterility has been transferred from wild species to cultivated ones in wheat, barley, cotton, tobacco, ryegrass and several other crops. Resistance to boll weevil and jassids in cotton and leaf chewing insects in peanut has been incorporated from wild species. Quality has also been improved in several crop plants.

Interspecific hybrids have been developed for commercial cultivation in sugarcane. Several modern cultivars of sugarcane have been developed from crosses of *Saccharumofficinarum* with *S. spontaneum* or *S. barberi*. These crosses combine high sugar content of *S. officinarum*with the disease

resistance, cold tolerance and vigour of *S. spontaneum* and *S. barberi*. Similarly, most of the modern cultivars of potato are derivatives of interspecific hybrids.

In India, interspecific hybrids have been developed for commercial cultivation in cotton. Interspecific hybrids have been developed between cultivated tetraploid species viz. *Gossypiumhirsutum* and *G. barbadense*, and cultivated diploid species, viz. *G. arboreum*and *G. herbacium*.

The important tetraploid hybrids include Varalaxmi, JKHY 11, CBS 156, Savitri, DCH 32, HB 224, NHB 12, TCHB 213 DHB 105 and Sruthi. These hybrids are grown in south and central cotton growing zones. Four hybrids have been developed between G. arboreum and G. herbaceum (DH 7, DH 9, Pha 46 and DDH 2). The first two are grown in Gujarat State. DDH 2 in Karnataka and Pha 46 in Maharashtra.

There are two examples of new crops which have evolved through distant hybridization. The first is the *Triticale* which has evolved from intergeneric cross between *Triticumaestivum*and*Secalecereale*. Another example is garden strawberry which has evolved from a natural interspecific cross between American octaploids*Fragariacliloensis*and *F. virgineana* in a botanical garden. The resulting hybrid combines desirable character of both the parents. *Triticale* also combines good characters of both the parents, viz., grain quality and yield potential of wheat and winter hardiness of rye. Both these new species are grown for commercial cultivation.

Limitations of Distant Hybridization:

Though distant hybridization has several useful applications in crop improvement, it has some limitations which have restricted its extensive use in crop improvement.

Some of the limitations are briefly discussed below:

1. Distant crosses are associated with problems of cross incompatibility, hybrid inviability, hybrid sterility and hybrid breakdown. These problems pose several difficulties in interspecific or intergeneric gene transfer.

2. Several special techniques, viz. ploidy manipulation pistil manipulation, chemical (growth regulator) treatment, bridge crossing, grafting, embryo culture etc. have to be adopted to make distant hybrids successful in some cases. Thus this is a combursome task.

3. Desirable characters are generally linked with some undesirable characters which pose difficulties in the use of desirable genes from wild species through distant hybridization. Several chromosome addition and substitution lines have been developed in wheat but- none of them could be used for commercial cultivation due to presence of some undesirable genes.

4. Sometimes, distant hybrids have several undesirable characters such as non-flowering, late maturity and seed dormancy and useless combinations like *Raphanobrassica*.

5. Transfer of characters controlled by recessive genes is very difficult in interspecific crosses.

6. In distant hybridization transfer of characters is not as simple as in intervarietal crosses.

7. Back cross method of breeding: Significance and limitations; multiline concept

Back cross:

Back-cross is the event of crossing of F_1 with either of the parents, but here in the back-cross method of breeding the hybrid is to be crossed with the superior parent whose genotype is to be transferred to the local variety. For example, in a particular area there is a well-adapted high yielding local variety but susceptible to a particular disease, this variety will be considered as recipient parent.

The disease resistant variety will be the donor parent. The recipient parent will be used in this method of breeding repeatedly to get all the genes to be transferred which is also known as recurrent parent and the donor parent is known as non-recurrent parent.

Pre-requisite for back cross breeding

- A suitable recurrent parent must be available which lacks in one or two characteristics.
- A suitable donor parent must be available
- The character to be transferred must have high heritability and preferably it should be determined by one or two genes.
- A sufficient number of back crosses should be made so that the genotype of recurrent parent is recovered in full.

Application of back cross method

This method is commonly used to transfer disease resistance from one variety to another. But it is also useful for transfer of other characteristics.

1. Intervarietal transfer of simply inherited characters

E.g. Disease resistance, seed coat colour

2. Intervarietal transfer of quantitative characters.

E.g. Plant height, Seed size, Seed shape.

3. Interspecific transfer of simply inherited characters

E.g. Transfer of disease resistance from related species to cultivated species.

E.g. Resistance to black arm disease in cotton from wild tetraploid species into *G.hirsutum*

4. Transfer of cytoplasm

This is employed to transfer male sterility. The female parent will be having the sterile cytoplasm and recurrent parent will be used as male parent.

5. Transgressive segregation

Back cross method may be modified to produce transgressive segregants. The F_1 is backcrossed to recurrent parent for 2 to 3 times for getting transgressive segregants.

6. Production of isogenic lines

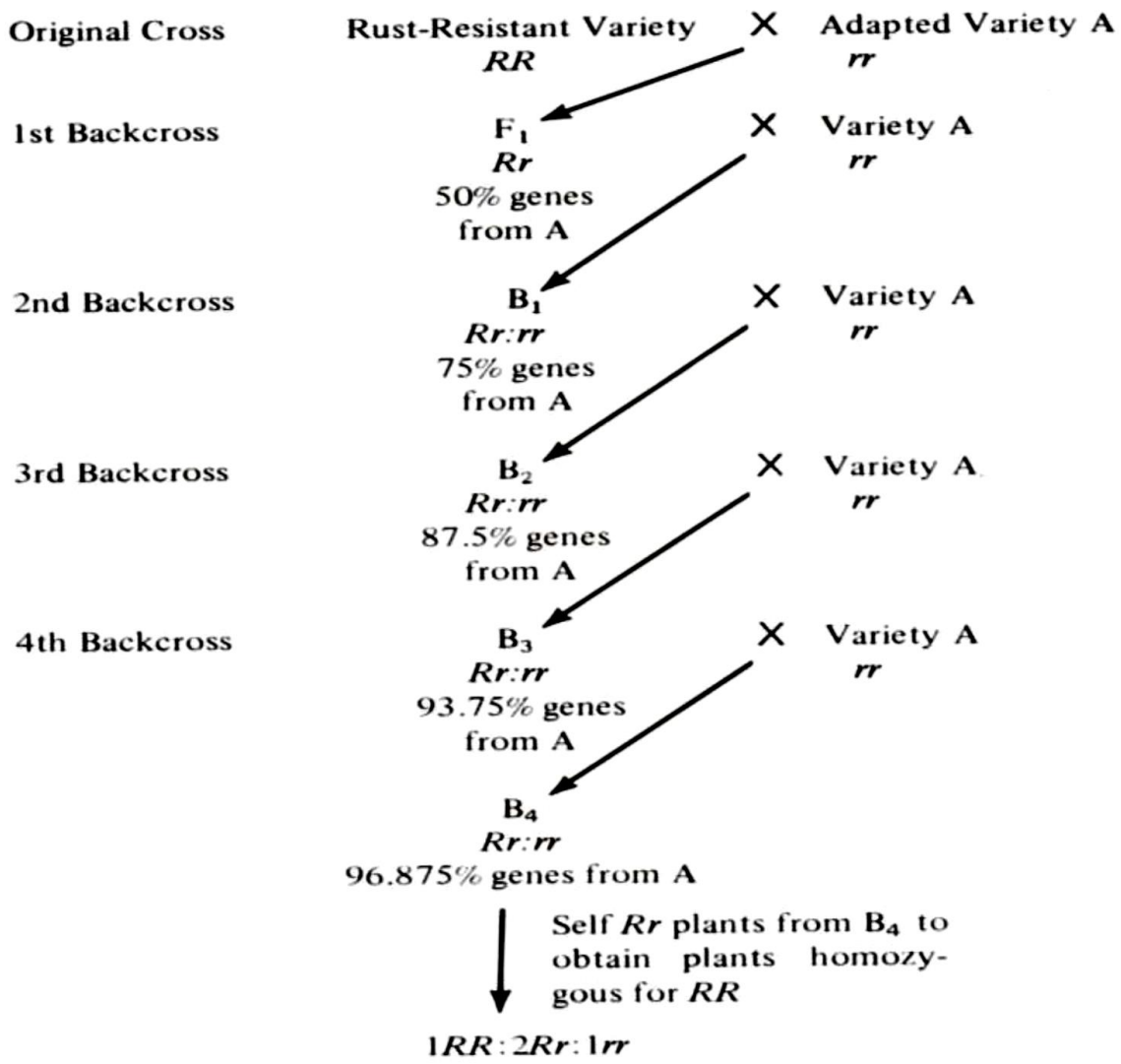
7. Germplasm conversion

E.g. Production of photo insensitive line from photo Sensitive germplasm through back crossing. This was done in the case of sorghum. Popularly known as conversion programme.

Genetic consequences of Repeated Backcrossing:

- Reduction in heterozygosity
- Increased similarity with the recurrent parent
- Selection for gene under transfer
- Extended opportunity for breaking undesirable linkages

❖ Selection for Recurrent Parent type



Selection of parents:

Backcross method of breeding change the genotype of recurrent parent only for the gene(s) under a transfer to correct the specific defect of the recurrent parent. But some unexpected changes in one or more character may also occur due to gene tightly linked with the gene being transferred. Therefore, the recurrent parent must be the most popular variety of the area, which has high yielding ability, desirable quality and high adaptability. In each crop, one or two varieties dominant, and they are vary popular with the farmers. Such a variety may have one or two defects, e.g.,susceptibility to disease or undesirable seed size or colour, which may be removed by this method. The nonrecurrnt parent is selected for high intensity of the character that is to be improved in the recurrent parent, and yielding ability and other feature of the parent are not important. The intensity of the character should preferably be more than that desired in the recurrent parent because the intensity may decline during the transfer and in the new genetic background of the recurrent parent.

Transfer of a Dominant Gene –

❖ Suppose that a high yielding variety and widely adapted wheat variety A is susceptible to stem rust. Another variety B is resistant to stem rust and this resistance is dominant to susceptibility.

- Therefore, variety A is taken as Recurrent parent and variety B is used as Non-recurrent parent.
- Procedure is simple for dominant gene transfer than recessive gene transfer.

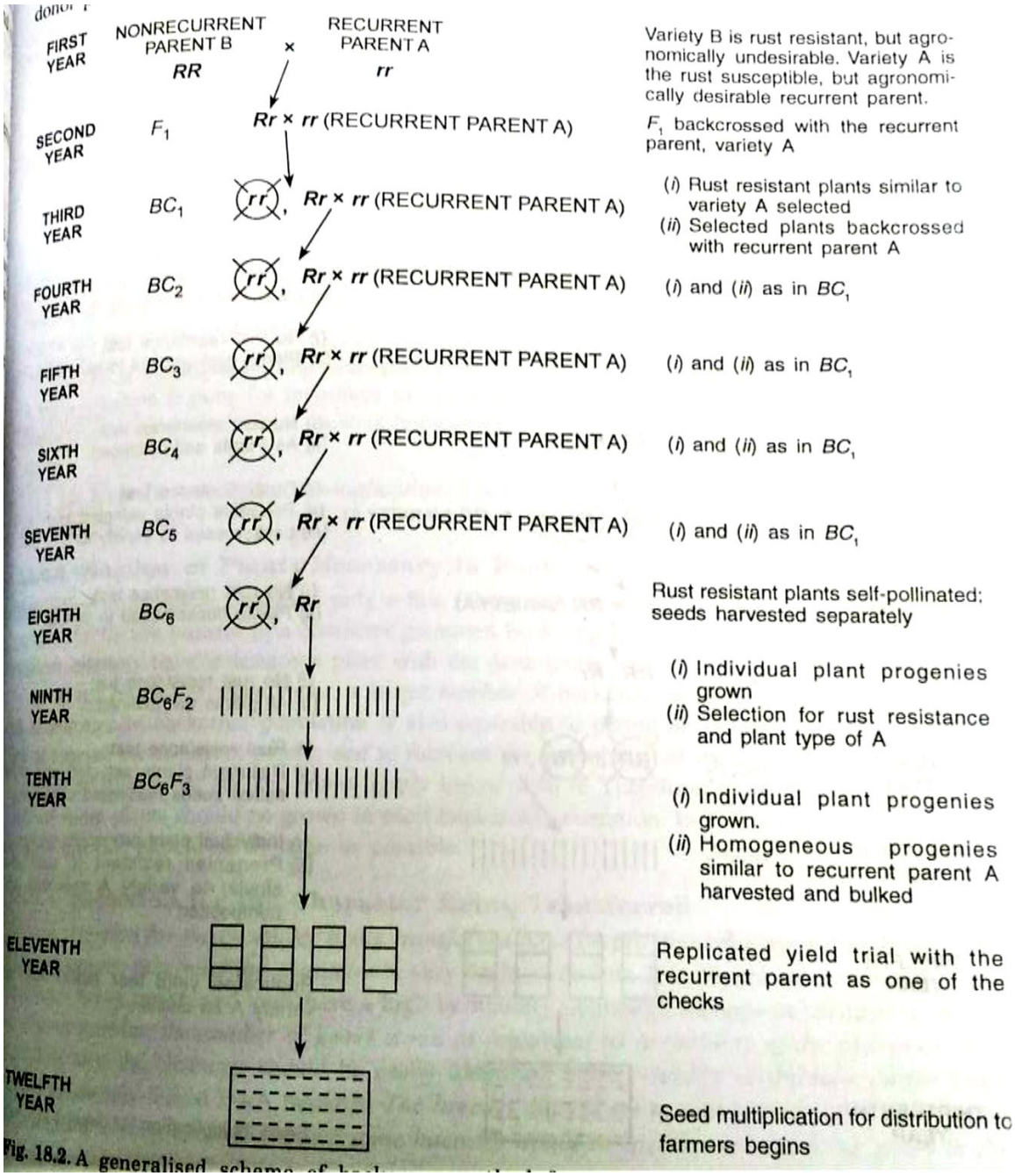
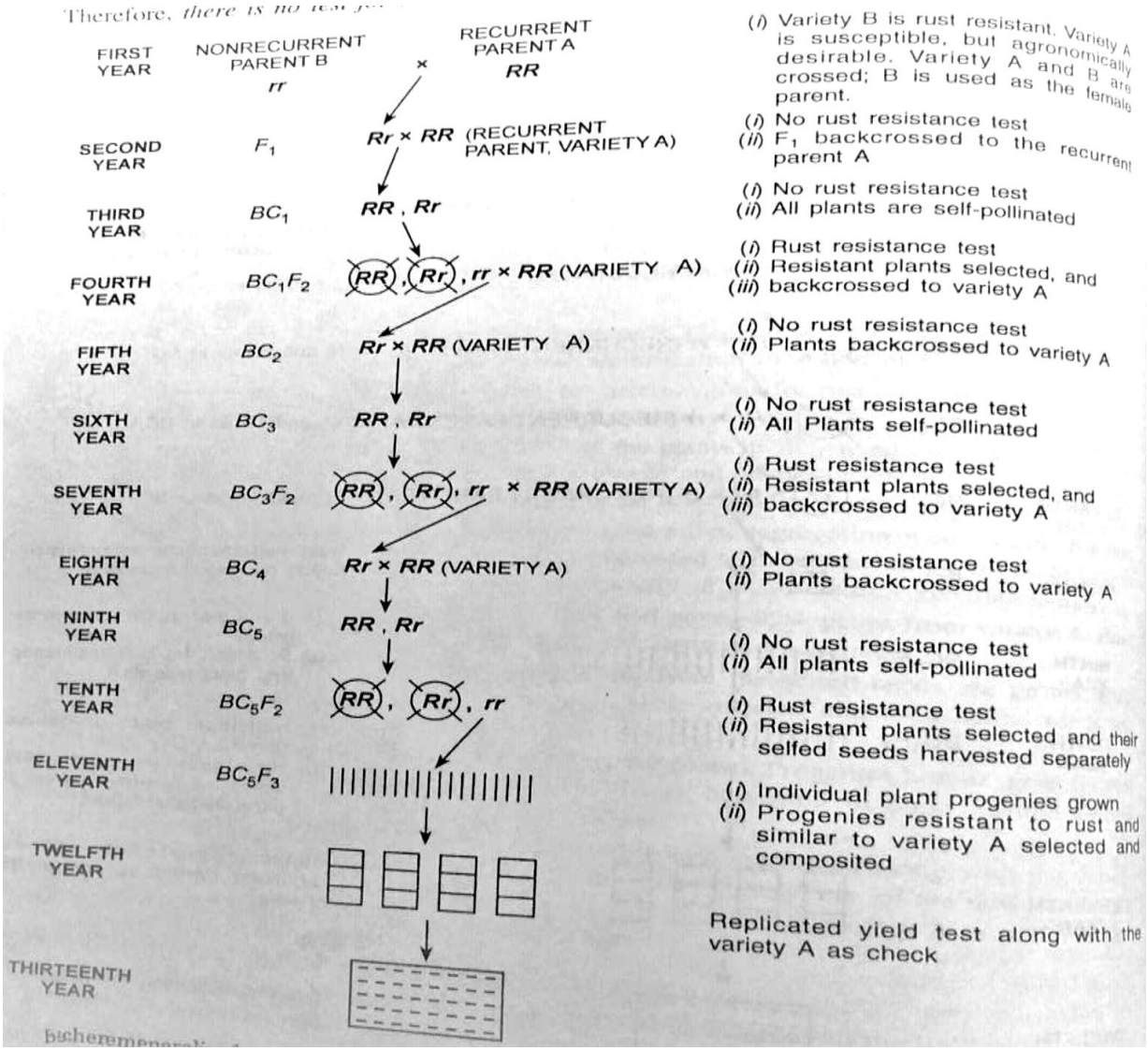


Fig. 18.2. A generalised scheme of

Transfer of Recessive Gene –

- When rust resistance is due to recessive gene, all the backcross cannot be made one after the other. After the first backcross, and after every two subsequent backcrosses, F_2 generations must be grown to identify rust resistant plants.
- The F_1 and the backcross progenies are not inoculated with rust because they will be uniformly susceptible so, only the F_2 populations are tested for rust resistance.
- Here, Variety B is rust resistance (rr) and it is Nonrecurrent parent, whereas Variety A is agronomically desirable and it is as Recurrent parent.



Merits of Backcross Method –

- The genotype of new variety is nearly identical with that of the recurrent parent, except for the gene transfer. Thus the outcome of a backcross programme is khown before hand and it can be reproduced any time in future.
- Much smaller population is needed in this method than in pedigree method.
- It is not necessary to test the variety developed by the backcross method in extensive yield tests because the performance of recurrent parent is already known. This may save up to 3-4 years time and considerable expense.
- The backcross programme is not dependent on the environment, except for the needed for selection of the character under transfer.
- This is the only method for inter-specific gene transfers, and for the transfer of cytoplasm.
- Defect like susceptibility to the disease of a well adapted variety can be removed without affecting its performance and adaptability.

Demerits of the Backcross Method

- The new variety generally cannot be superior to the recurrent parent, except for the character that is transferred.
- Linkage drag (close linkage between a desirable and undesirable gene.)

- Hybridization required for each backcross; which is often difficult, time taking and costly.
- By the time backcross programme improves it, recurrent parent may have been replaced by other varieties superior in yielding ability and other characteristics.

Achievements:

- Rust resistance has been transferred to Kalyan Sona from several diverse sources, eg. Robin, HS19, Bluebird etc.
- Tift23A was used in backcross programme with resistant lines from India and Africa to develop downy mildew resistant male sterile lines, such as MS521, MS541, etc
- *G. herbaceum* varieties Vijapla, Vijay, Digvijay and Kalyan are some of the cotton varieties developed by this method.

Multiline Varieties

Generally, pureline varieties are highly adapted to a limited area, but poorly adapted to wider regions. Further, their performance is not stable from year to year because of changes in weather and other environmental factors. Purelines often have only one or a few major genes for disease resistance, such as, rust resistance, which make them resistant to some races of the pathogen. New races are continuously produced in many pathogens, which may overcome the resistance present in the pureline varieties. For example, Kalyan Sona wheat *(T.aestivum)* originally resistant to brown rust (leaf rust), soon became susceptible to new races of the pathogen.

To overcome these limitations, particularly the breakdown of resistance to disease, it was suggested to develop multiline varieties. Multiline varieties are mixtures of several purelines of similar height, flowering and maturity dates, seed colour and agronomic characteristics, but having different genes for disease resistance. The purelines constituting a multiline variety must be compatible, i.e., they should not reduce the yielding ability of each other when grown in mixture.

In 1954, Borlaug suggested that several purelines with different resistance genes should be developed through back cross programmes using one recurrent parent. This is done by transferring disease resistance genes from several donor parents carrying different resistant genes to a single recurrent parent. Each donor parent is used in a separate backcross programme so that each line has different resistant gene or genes. Five to ten of these lines may be mixed depending upon the races of the pathogen prevalent in the area. If a line or lines become susceptible, they would be replaced by resistant lines. New lines would be developed when new sources of resistance become available. The breeder should keep several resistant lines in store for future use in the replacement of susceptible lines of multiline verities.

Merits of Multiline varieties

1. All the lines are almost identical to the recurrent parent in agronomic characteristics, quality etc. Therefore, the disadvantages of the pureline mixtures are not present in the
2. Only one or a few lines of the mixture would become susceptible of the pathogen in anyone season. Therefore, the loss to the cultivator would be relatively low.
3. The susceptible line would constitute only a small proportion of the plants in the field. Therefore, only a small proportion of the plants would be infected by the pathogen. Consequently the disease would spread more slowly than when the entire population was susceptible. This would reduce the damage to the susceptible line as well.

Demerits of Multiline Varieties

1. The fanner has to change the seed of multiline varieties every few years depending upon the change in the races of the pathogen.
2. There is a possibility that a new race may attack all lines of a multiline variety.

Achievements

Multiline variety appears to be a useful approach to control diseases like rusts where new races are continuously produced. In India, three multiline varieties have been released in wheat *(T.aestivum).* Kalyan Sona, one of the most popular varieties in the late sixties, was used as the recurrent parent to produce these varieties. Variety 'KSML 3' consists of 8 lines having rust resistance genes from Robin, Ghanate, Kl, Rend, Gabato, Blue Brid, Tobari etc. Multiline 'MLKS 11' is also a mixture of 8 lines; the resistance is derived from E 6254, E 6056, E 5868, Frecor, HS 19, E 4894 etc. The third variety, KML 7406 has 9 lines deriving rust resistance from different sources.

Dirty Multiline

This term is used when a multiline is having one or two susceptible lines also. The idea of including susceptible lines is to prevent race formation.

7. Molecular marker assisted breeding. Molecular markers in genome and QTL analyses

A **bio-marker, or biological marker** is a measurable indicator of some biological state or condition. Markers are of four types, viz: (i) Morphological, (ii) Biochemical, (iii) Cytological, and (iv) Molecular or DNA markers.

These are briefly discussed as follows:

i. Morphological:

In plant breeding, markers that are related to variation in shape, size, colour and surface of various plant parts are called morphological markers. Such markers refer to available gene loci that have obvious impact on morphology of plant. Genes that affect form, coloration, male sterility or resistance among others have been analyzed in many plant species.

In rice, examples of this type of marker may include the presence or absence of awn, leaf sheath coloration, height, grain color, aroma etc. In well-characterized crops like maize, tomato, pea, barley or wheat, tens or even hundreds of such genes have been assigned to different chromosomes.

There are several demerits of morphological markers as given below:

a. They generally express late into the development of an organism. Hence their detection is dependent on the development stage of the organism.

b. They usually exhibit dominance.

c. Sometimes they exhibit deleterious effects.

d. They exhibit pleiotropy.

e. They exhibit epistasis.

f. They exhibit less polymorphism.

g. They are highly influenced by the environmental factors.

ii. Biochemical:

Markers that are related to variation in proteins and amino acid banding pattern are known as biochemical markers. A gene encodes a protein that can be extracted and observed; for example, isozymes and storage proteins.

iii. Cytological:

Markers that are related to variation in chromosome number, shape, size and banding pattern are referred to as cytological markers. In other words, it refers to the chromosomal banding produced by different stains; for example, G banding.

iv. Molecular Markers:

A molecular marker is a DNA sequence in the genome which can be located and identified. As a result of genetic alterations (mutations, insertions, deletions), the base composition at a particular location of the genome may be different in different plants.

These differences, collectively called as polymorphisms can be mapped and identified. Plant breeders always prefer to detect the gene as the molecular marker, although this is not always possible. The alternative is to have markers which are closely associated with genes and inherited together.

The molecular markers are highly reliable and advantageous in plant breeding programmes:

- **Polymorphism:**
  Markers should exhibit high level of polymorphism. In other words, there should be variability in the markers. It should demonstrate measurable differences in expression between trait types and/or gene of interest.
- **Co-Dominant:**
  Marker should be co-dominant. It means, there should be absence of intra-locus interaction. It helps in identification of heterozygotes from homozygotes.

- Even distribution across the whole genome (not clustered in certain regions)
- Clear distinct allelic features (so that the different alleles can be easily identified)
- Single copy and no pleiotropic effect
- Low cost to use (or cost-efficient marker development and genotyping)
- Easy assay/detection and automation
- High availability (un-restricted use) and suitability to be duplicated/multiplexed (so that the data can be accumulated and shared between laboratories)
- Genome-specific in nature (especially with polyploids)
- No detrimental effect on phenotype

DNA based markers can be classified as hybridization-bascd markers and polymerase chain reaction (PCR)-based markers. In hybridization based markers a labeled probe is used to visualize the DNA profile of restricted DNA vz. RFLP. Whereas, PCR based markers involve in vitro amplification of particular DNA sequences or loC1, with the heip ot specilically or arbitranly chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. The amplitied fragments (amplicons) are separated clectrophoretically and banding pallens are detected by dilterent methods such as staining and autoradiagraphy, as in the case of RAPD, microsatellites, STMS and EST.

Types and deseription of DNA markers:

Markers Based On DNA Hybridization:

The DNA piece can be cloned, and allowed to hybridize with the genomic DNA which can be detected. Marker-based DNA hybridization is widely used. The major limitation of this approach is that it requires large quantities of DNA and the use of radioactivity (labeled probes).

Restriction fragment length polymorphism (RFLP):

RFLP markers were used for the first time in the construction of genetic maps by Botstein et al. in 1980. RFLP are codominant and show Mendelian inherilance pattern. The polymorphism in restricted fragments due to DNA rearrangements that occur due to evolutionary processes, point mutations with in the restriction enzyme recognition site sequences, insertions or deletions within the fragments, and uncqual crossing over. In RFLP, DNA polymorphism is detected by hybridizing a chemically labeled DNA probe to a Southern blot of DNA digested by restriction endonucleases, resulting in differential DNA fragment profile. Labelling of the probe may be performed with a radioactive isotope or with alternative nonradioactive stains, such as digoxigenin or fluorescein. Probes are generated through the construction of genomic of complementary DNA (cDNA) libraries and hence may be composed of specific sequence of unknown identity (genomic DNA) or part of the sequence of a functional gene (exons only, cDNA). The hybridization results can be visualized by autoradiography (if the probes are radioactively labelled), or using chemiluminesence (if nonradioactive, enzyme-linked methods are used for probe labeling and detection). RFLPs correspond to DNA fragments, usually within the range of 2-10 kb, that have resulted from the digestion of genomic DNA with restriction enzymes. The differential profile is generated due to nucleotide substitutions or DNA rearrangements like insertion or deletion or single nucleotide polymorphisms. The RFLPs markers are relatively highly polymorphic, codominanltly inherited and highly reproducible. RFLPs are applied in diversity and phylogenetic studies ranging from individuals within populations or species, to closely related species. RFLPs have been widely used in gene mapping studies because of their high genomic abundance due to the ample availability of different restriction enzymes and random distribution throughout the genome.

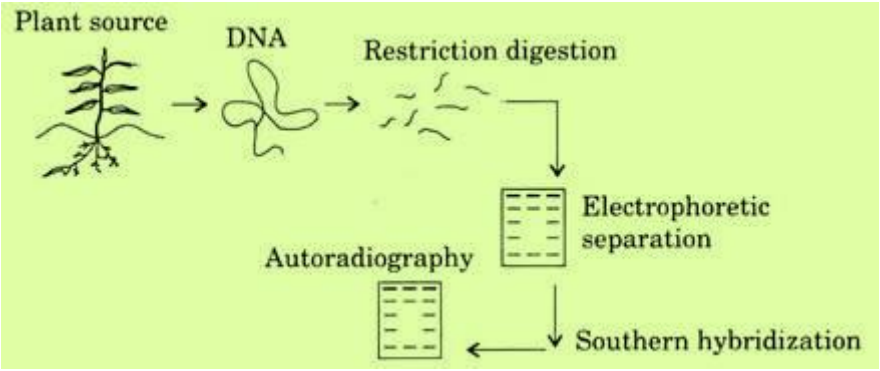
Steps:

Following are the steps involved in a typical RFLP assay

(1) Restriction digestion of genomic DNA using restriction endonuclease

(2) Resolving restriction genomic fragments through gel electrophoresis

(3) Transfer of resolved fragments from gel to nitrocellulose membrane using southern blotting
(4) Membrane containing DNA fragments hybridized with labeled probe using southerm hybridization
(5) Detection of polymorphism through autoradiography or chemiluminescent technique.



Based on the presence of restriction sites, DNA fragments of different lengths can be generated by using different restriction enzymes. In the Fig., two DNA molecules from two plants (A and B) are shown. In plant A, a mutations has occurred leading to the loss of restriction site that can be digested by EcoRI.

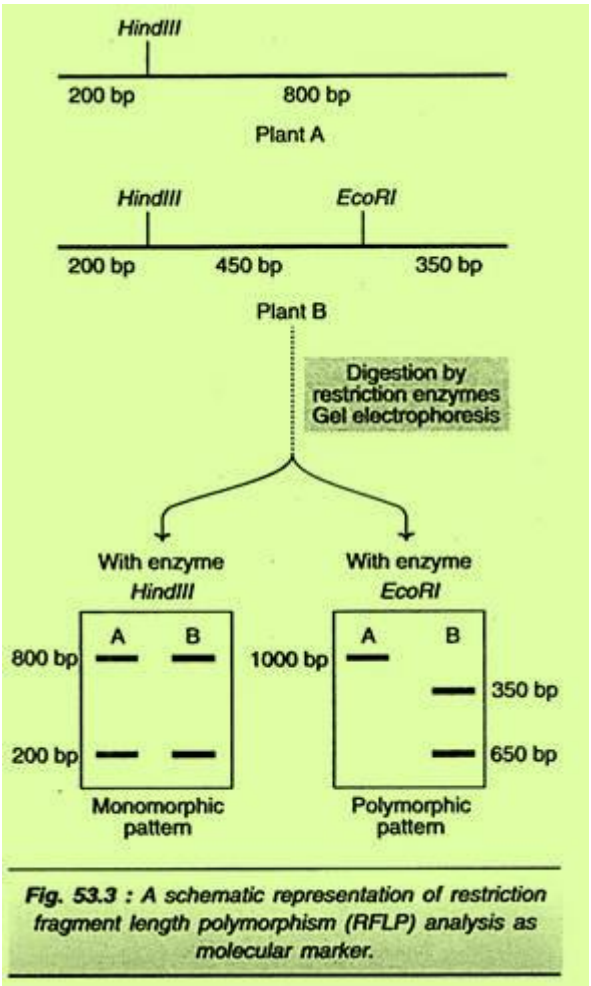


Fig. 53.3 : A schematic representation of restriction fragment length polymorphism (RFLP) analysis as molecular marker.

The result is that when the DNA molecules are digested by the enzyme Hindlll, there is no difference in the DNA fragments separated. However, with the enzyme EcoRI, plant A DNA molecule is not digested while plant B DNA molecule is digested. This results in a polymorphic pattern of separation.

Advantages of RFLP:

a. Present everywhere,
b. Mendelian inheritance,
c. Co-dominant expression,
d. No pleiotropic effects,
e. Independent of the environment,
f. Present at each developmental stage,
g. Long stability of cDNA probes,
h. Different loci may be identified by one probe,
i. Heterologous genes may be used as probes,
j. Any number of probes can be produced,
k. Probes are producible for coding and silent sequences,
l. Probes show the variability of flanking sequences,
m. Several characters can be screened in the same sample.

Disadvantages:

Developing sets of RFLP probes and markers is labour intensive. This technique requires large amount of high quality DNA. The multiplex ratio is low, typically one per gel. The genotyping throughput is low. It involves use of radioactive chemicals. RFLP finger prints for multi-gene families are often complex and difficult to score. RFLP probes cannot be shared between laboratories.

Uses:

They can be used in determining paternity cases. In criminal cases, they can be used in determining source of DNA sample. They can be used to determine the disease status of an individual. They are useful in gene mapping, germplasm characterization and marker assisted selection. They are useful in detection of pathogen in plants even if it is in latent stage.

Randomly Amplified Polymorphic DNA (RAPD):

Randomly amplified polymorphie DNA markers (RAPD): In 1991, Welsh and McClelland developed a new PCR-based genetic assay namely randomly amplified polymorphic DNA (RAPD) This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer an arbitrary nucleotide (8-12 bp) sequence. The primer anneals to complimentary sequence in template DNA in forward or reverse direction at multitude location of genonme. The amplification occurs between forward and reverse annealing generally 150-4000 bp apart, by resolving the resulting amplicons, profile with multiple bands can be secen. No knowledge of the DNA sequence for the targetes gene is required, as the primers will bind somewherc in the sequence, but it is not certain exactly where. This marker show lack of reproducibility and the assay is sensitive variation in DNA concentration. They are dominant markers and hence have limtaions in their use as markers for mappng, which can be overcome to some extent by selecting those markers that are linked in coupling.

RAPD assay has been used by several groups as efficient tools for identication of markers linked to agronomicaly important traits, whch are introgressed during the development of near isogenic lines (NILs).

Steps:

Important steps of RAPD can be summarized as follows.

Extraction of DNA

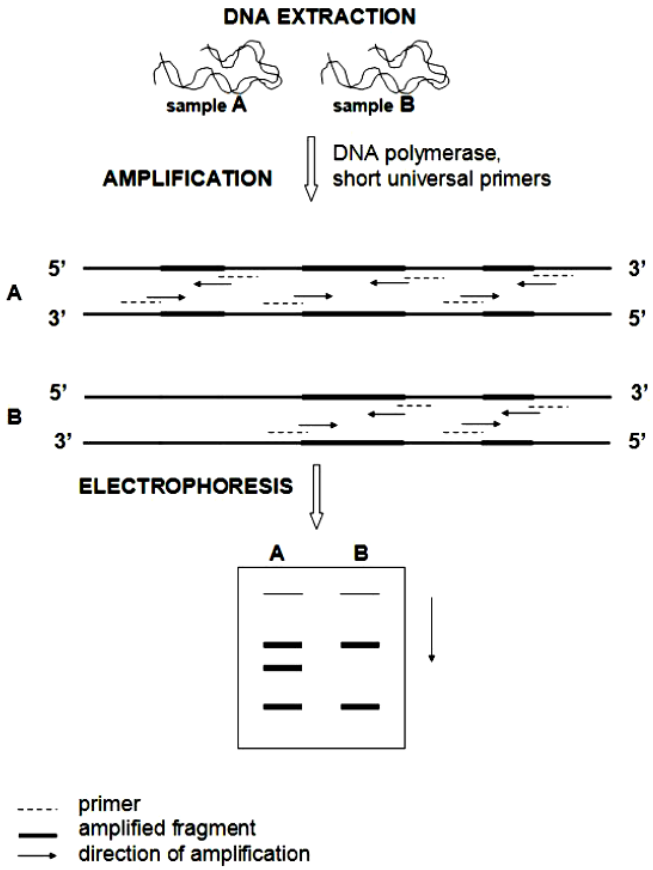
DNA is extracted by various methods. It also depends upon the species of plant under study but the basic procedure remains the same. Genomic DNA is basically extracted and purified from plant cell using proteinase K digestion and standard phenol: Chloroform extraction as per the standard protocol.

Selection of Primers

Random Amplified Polymorphic DNA (RAPD) is a multiplex marker system that conventionally uses single primer PCR to amplify random DNA fragments. Because of its multiplex nature, it is frequently used in Bulked Segregant Analysis (BSA). In view of the very large numbers of markers BSA often requires the use of mixtures of primers as a method of increasing the number of markers available. Theoretically, if a singleprimer reaction produces x bands on average, an unrestrained PCR process using a primers should produce xa2 bands. A total of 40 random oligonucleotide primers were used for amplification. All the random primers were 10 bp long and with high GC content and were custom synthesized from M/s Bangalore Genei, Bangalore, India a Yadav and Yadav (2007). The standard RAPD technology utilises short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR.

PCR Amplification

The polymerase Chain Reaction (PCR) is a relatively simple but powerful technique that amplifies a DNA template to produce multiple copies of specific DNA fragment in vitro. The bases (complementary to the template) are coupled to the primer on the 3' end (the polymerase adds dNTP's from 5'to 3', reading the template from 3'to 5' side; bases are added complementary to the template). 1. Using fresh clean tips, all the reagents are added to an autoclaved microfuge tube placed on ice. The PCR machine is programmed for the specific reaction conditions desired. After completion of the PCR reaction, the tubes are removed from the temperature block. The reaction products are separated according to size by agarose gel electrophoresis and visualized after staining the gel with ethidium bromide.



Agarose Gel Electrophoresis of PCR

After completion of the PCR programme, the products are checked in 2% agarose for the amplification. Before loading into the wells, gel loading dye (bromophenol blue in glycerol) is added

to the sample and the samples are run under constant voltage condition (80 V) till the two dyes get separated. Amplified products appear as sharp orange color bands under UV Transilluminator due to the intercalation of ethidium bromide. To ensure that the amplified DNA bands originated from genomic DNA and not primer artifacts, negative control are carried out for each primer/breed combination (Galli and Satti, 2009) No amplification is detected in control reactions. All amplification products are found to be reproducible when reactions are repeated using the same reaction conditions.

Advantages:

RAPD primers are readily available being universal. They provide moderately high genotyping throughput. This technique is simple PCR assay (no blotting and no radioactivity). It does not require special equipment. Only PCR is needed. The start-up cost is low.

RAPD marker assays can be performed using very small DNA samples (5 to 25 ng per sample). RAPD primers are universal and can be commercially purchased. RAPD markers can be easily shared between laboratories. Locus-specific, co-dominant PCR-based markers can be developed from RAPD markers. It provides more polymorphism than RFLPs.

Disadvantages:

The detection of polymorphism is limited. The maximum polymorphic information content for any bi-allelic marker is 0.5. This technique only detects dominant markers. The reproducibility of RAPD assays across laboratories is often low. The homology of fragments across genotypes cannot be ascertained without mapping. It is not applicable in marker assisted breeding programme.

Uses:

This technique can be used in various ways such as for varietal identification, DNA fingerprinting, gene tagging and construction of linkage maps. It can also be used to study phylogenetic relationship among species and sub-species and assessment of variability in breeding populations.

Amplified Fragment Length Polymorphisms (AFLP)

To overcome the limitation of reproducibility associated with RAPD, AFLP technology (Vos et al., 1995) was developed. It combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA and selective PCR amplification of restriction fragments using a limited set of primers. The DNA is cut with two restriction enzymes, one being a frequent cutter and the other an infrequent cutter. This is followed by ligation of adapters, including restriction motifs followed by a two-step PCR amplification of selected fragments. The selective amplification uses primers composed of the adapters and 1 to 3 selected nucleotides at the 3' end. It limits the number of fragments to a resolvable range. The PCR-amplified fragments can then be separated by gel electrophoresis and banding patterns visualized. A range of enzymes and primers are available to manipulate the complexity of AFLP fingerprints to suit application. The AFLP banding profiles are the result of variations in the restriction sites or in the intervening region.

The AFLP technique simultaneously generates fragments from many genomic sites (usually 50-100 fragments per reaction) that are separated by polyacrylamide gel electrophoresis and that are generally scored as dominant markers. However, by use of automatic gel scanner heterozygote may be distinguished from homozygote based on band intensity differences, which facilitates the scoring of many AFLPs as codominant markers. The AFLP technique generates fingerprints of any DNA regardless of its source, and without any prior knowledge of DNA sequence. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping.

Steps

Step 1:

DNA Extraction:

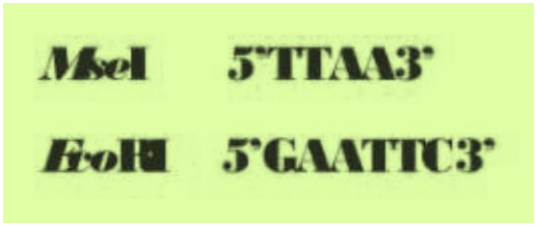
In the first step of AFLP clean and high molecular weight DNA is extracted using CTAB procedure.

Step 2:

Restriction Digestion:

Restriction fragments of the genomic DNA are produced by using two different restriction enzymes: a frequent cutter (the four-base restriction enzyme Msel) and a rare cutter (the six-base restriction enzyme EcoRI).

The frequent cutter serves to generate small fragments, which amplify well and which have the optimal size range for separation on a sequence gel, whereas the rare cutter limits the number of fragments to be amplified.

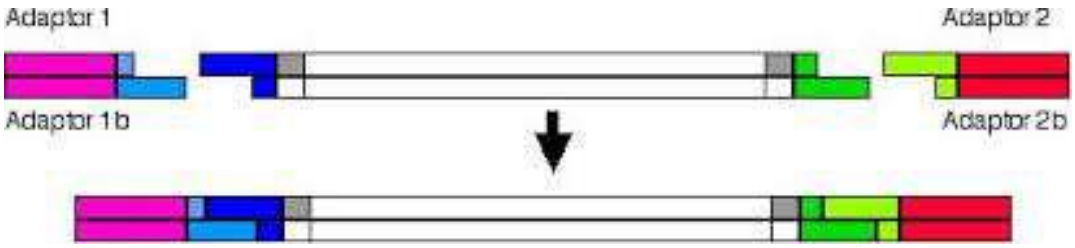


Step 3:

Ligation of Oligonucleotide Adapters:

Double-stranded adapters consist of a core sequence and an enzyme-specific sequence. Therefore, adapters are specific for either the EcoRI site or the Msel site. Usually restriction and ligation take place in a single reaction.

Ligation of the adapter to the restricted DNA alters the restriction site in order to prevent a second restriction from taking place after ligation has occurred. The core sequence of the adapters consists of a known DNA sequence of 20 nucleotides, which will be used later as primer in the PCR.



Step 4:

Pre-Amplification:

This step is a normal PCR where the adapters are used as primers. This first PCR, called pre-amplification, allows a first selection of fragments by only amplifying the DNA restriction fragments that have ligated an adaptor to both extremities.

Step 5:

Amplification:

The aim of this step is to restrict the level of polymorphism and to label the DNA. For this second amplification, we added three more nucleotides at the 3′ end of the primer sequence used for the pre-amplification (adaptors sequence + 3 nucleotides). These two additional nucleotides make the amplification more selective and will decrease the number of restriction fragments amplified (polymorphism).

Step 6:

Electrophoresis:

The PCR products are denaturized and run on acrylamide gel.

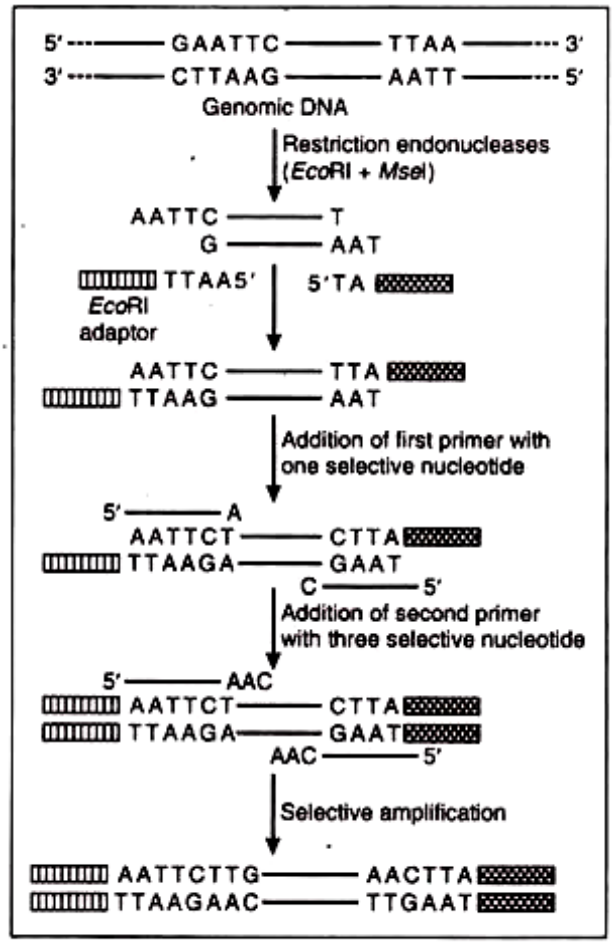


Fig. 8.4: Steps involved in AFLP

Advantages:

It provides very high multiplex ratio and genotyping throughput. These are highly reproducible across laboratories. No marker development work is needed; however, AFLP primer screening is often necessary to identify optimal primer specificities and combinations.

No special instrumentation is needed for performing AFLP assays; however, special instrumentation is needed for co-dominant scoring.

Start-up costs are moderately low. AFLP assays can be performed using very small DNA samples (typically 0.2 to 2.5 pg per individual). The technology can be applied to virtually any organism with minimal initial development.

Disadvantages:

The maximum polymorphic information content for any bi-allelic marker is 0.5. High quality DNA is needed to ensure complete restriction enzyme digestion. DNA quality may or may not be a weakness depending on the species. Rapid methods for isolating DNA may not produce sufficiently clean template DNA for AFLP analysis.

Proprietary technology is needed to score heterozygotes and ++ homozygotes. Otherwise, AFLPs must be dominantly scored. Dominance may or may not be a weakness depending on the application.

The homology of a restriction fragment cannot be unequivocally ascertained across genotypes or mapping populations. Developing locus specific markers from individual fragments can be difficult and does not seem to be widely done.

The switch to non-radioactive assays has not been rapid. Chemiluminescent AFLP fingerprinting methods have been developed and seem to work well.

The fingerprints produced by fluorescent AFLP assay methods are often difficult to interpret and score and thus do not seem to be widely used. AFLP markers often densely cluster in centromeric regions in species with large genomes, e.g., barley (*Hordeum vulgare* L.) and sunflower (*Helianthus annuus* L.)

Uses:

This technique has been widely used in the construction of genetic maps containing high densities of DNA marker. In plant breeding and genetics, AFLP markers are used in varietal identification, germplasm characterization, gene tagging and marker assisted selection.

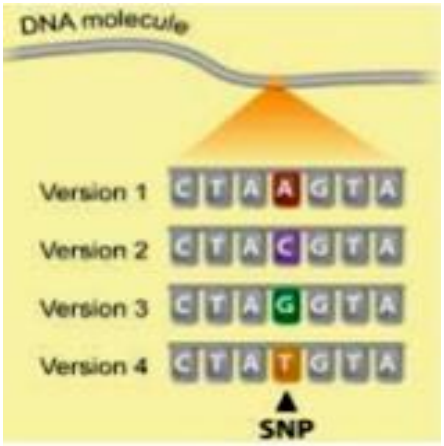
Single Nucleotide Polymorphism (SNP):

The variations which are found at a single nucleotide position are known as single nucleotide polymorphisms or SNP. Such variation results due to substitution, deletion or insertion. This type of polymorphisms has two alleles and also called bialleleic loci. This is the most common class of DNA polymorphism. It is found both in natural lines and after induced mutagenesis. Main features of SNP markers are given below.

1. SNP markers are highly polymorphic and mostly bialleleic.
2. The genotyping throughput is very high.
3. SNP markers are locus specific.
4. Such variation results due to substitution, deletion or insertion.
5. SNP markers are excellent long term investment.
6. SNP markers can be used to pinpoint functional polymorphism.
7. This technique requires small amount of DNA.

SNPs are found in

- coding and (mostly) noncoding regions.
- Occur with a very high frequency
- about 1 in 1000 bases to 1 in 100 to 300 bases.
- The abundance of SNPs and the ease with which they can be measured make these genetic variations significant.
- SNPs close to particular gene acts as a marker for that gene.
- SNPs in coding regions may alter the protein structure made by that coding region.



SNPs Discovery

1. Sequence databases searches
2. Target specific SNP discovery and development

-Conformation-based mutation scanning

-Direct DNA sequencing

Identification of Target Specific SNPs

Steps:

1. Amplify the genes of interests with PCR
2. Scan for mutation with various methods

-Conformation-based mutation scanning

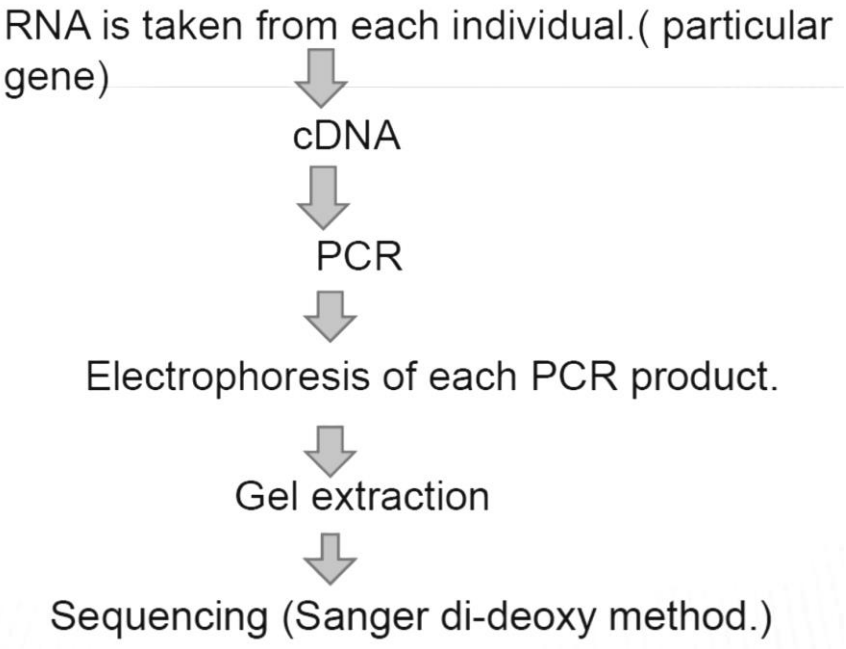
- Single -strand conformation polymorphism analysis
- Gel electrophoresis
- Chemical and enzymatic mismatch cleavage detection
- Denaturing gradient gel electrophoresis
- Denaturing HPLC

4. Align sequences from different sources to find SNPs
3. Sequence positive PCR products

-Sequence multiple individuals

-Sequence heterozygotes

Development of SNP (direct sequencing Method)



Technologies for Detecting Known SNPs

Gel-Based Methods

-PCR-restriction fragment length polymorphism analysis

-PCR-based allelic specific amplification

-Oligonucleotide ligation assay genotyping

-Minisequencing(10~20base)

Non-Gel-Based High Through Genotyping Technologies

-Solution hybridization using fluorescence dyes

-Allelic specific ligation

-Allelic specific nucleotide incorporation

1. High resolution separation
2. Chemical color reaction

-DNA microarray genotyping

Allele-Specific Codominant PCR Strategy

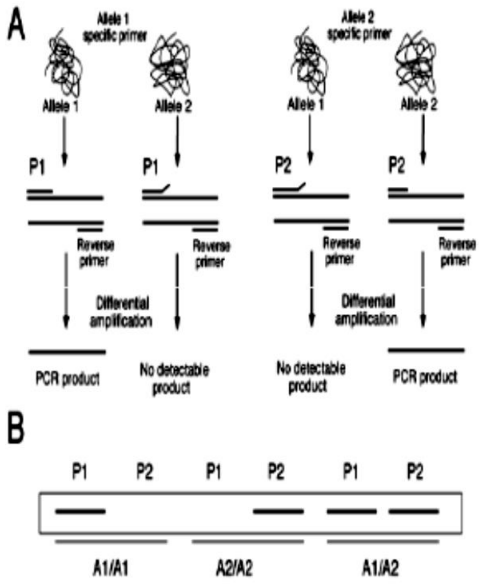


Figure. Schematic representation of the allele-specific codominant PCR strategy. Oligonucleotide primers with 3' nucleotides that correspond to an SNP site are used to preferentially amplify specific alleles.
A, Primer P1 forms a perfect match with allele 1 but forms a mismatch at the 3' terminus with the DNA sequence of allele 2. Primer P2 similarly forms a perfect match with allele 2 and a 3' terminus mismatch with allele 1.
B, Schematic of agarose gel analysis showing the expected outcome for the amplification of organisms homozygous and heterozygous for both alleles using primers P1 and P2. P1, Primer 1; P2, primer 2; A1, allele 1; A2, allele 2.

Eliana Drenkard et al. 2000 Plant Physiol 124: 1483-1492

Advantages:

SNP markers are useful in gene mapping. SNPs help in detection of mutations at molecular level. SNP markers are useful in positional cloning of a mutant locus. SNP markers are useful in detection of disease causing genes.

Disadvantages:

Most of the SNPs are bialleleic and less informative than SSRs. Multiplexing is not possible for all loci. Some SNP assay techniques are costly. Development of SNP markers is labour oriented. More (three times) SNPs are required in preparing genetic maps than SSR markers.

Uses:

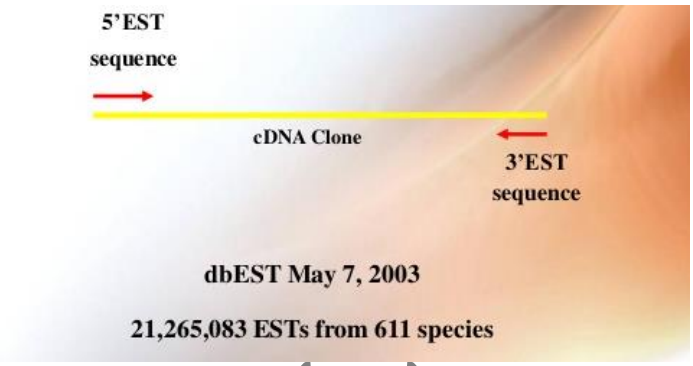
SNPs are useful in preparing genetic maps. They have been used in preparing human genetic maps. In plant breeding, SNPs have been used to lesser extent.

Expressed Sequence Tags (EST):

Expressed Sequence Tags (ESTs) are small pieces of DNA and their location and sequence on the chromosome are known. The variations which are found at a single nucleotide position are known. The term Expressed Sequence Tags (ESTs) was first used by Venter and his colleagues in 1991. Main features of EST markers are given below.

1. ESTs are short DNA sequences (200-500 nucleotide long).
2. They are a type of sequence tagged sites (STS).
3. ESTs consist of exons only.

Single-pass sequencing reads from randomly selected cDNA clone



Steps for ESTs

•cDNA libraries (containing many of the expressed genes of an organism)

•pick cDNA clones randomly

•rapidly determine some of the sequence of nucleotides from the end of each clone.

•These ESTs could then be compared to all known sequences using a program called BLAST.

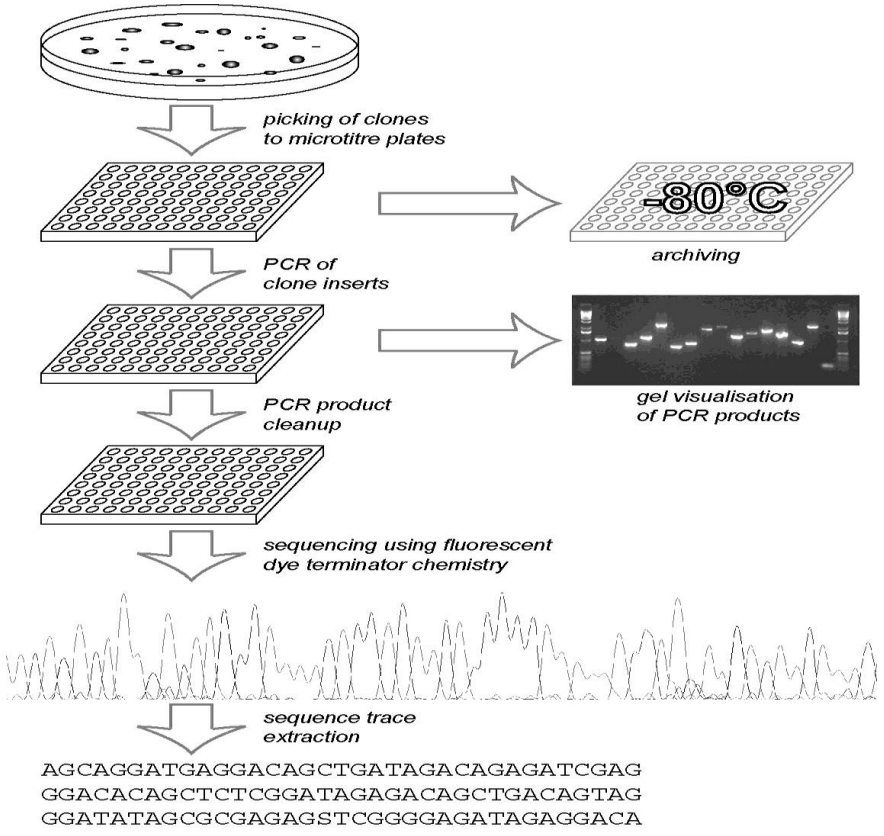
An exact match to a sequenced gene means that the gene encoding that EST is already known.

If the match was close but not exact one could conclude that the EST is derived from a gene with a function similar to that of the known gene.

The EST sequences with their putative identification are then deposited in the GenBank and the clones from which they were derived are kept in a freezer for later use.

Overview of the EST sequencing process

Clones are picked from petriedishes into microtitre plates, and archived for later use. All subsequent manipulations (PCR, clean up and sequencing) are carried out in microtitre plates to yield medium-throughput.



Advantages:

It is a rapid and inexpensive technique of locating a gene. ESTs are useful in discovering new genes related to genetic diseases. They can be used for tissue specific gene expression.

Disadvantages:

ESTs have lack of prime specificity. It is a time consuming and labour oriented technique. The precision is lesser than other techniques. It is difficult to obtain large (> 6kb) transcripts. Multiplexing is not possible for all loci.

Uses:

ESTs are commonly used to map genes of known function. They are also used for phylogenetic studies and generating DNA arrays.

Sequence Tagged Sites (STS):

In genomics, a sequence tagged site (STS) is a short DNA sequence that has a single copy in a genome and whose location and base sequence are known. Main features of STS markers are given below.

1. STSs are short DNA sequences (200-500 nucleotide long).
2. STSs occur only once in the genome.
3. STS are detected by PCR in the presence of all other genomic sequences.
4. STSs are derived from cDNAs.

Advantages:

STSs are useful in physical mapping of genes. This technique permits sharing of data across the laboratories. It is a rapid and most specific technique than DNA hybridization techniques. It has high degree of accuracy. It can be automated.

Disadvantages:

Development of STS is a difficult task. It is time consuming and labour oriented technique. It require high technical skill.

Uses:

STS is the most powerful physical mapping technique. It can be used to identify any locus on the chromosome. STSs are used as standard markers to find out gene in any region of the genome. It is used for constructing detailed maps of large genomes.

Microsatellites and mini-satellites

The tem microsatellites were coincd by Litt and Lutty (1989), while the term mini-satellites were used by Alec Jeffrey (1985). Both are multi-locus probes creating complex banding patterns. They essentially belong to the repetitive DNA family. Fingerprints generated by these probes are also known as oligo-nucleotide fingerprnts.

Mini-satellites are tandem repeats of DNA sequence with 10-100 bp repeat motifs whereas, microsatellites are tandem repeat of DNA sequence with 2-6 bp repeat motifs.These are also referred to as Variable Number o 1andem Repeats (VNTRS) and this is one of the basis of polymorphism at a locus. Many alleles exist in a population, the level of heterozygosity is high and they follow Mendelian inheritance.

Simple Sequence Repeats (SSRs):

Simple sequence repeats (SSRs) or microsatellites are tandemly repeated mono-, di-, tri-, tetra-, penta-, and hexanucleotide motifs. SSR length polymorphisms are caused by differences in the number of repeats. SSR loci are individually amplified by PCR using pairs of oligonucleotide primers specific to unique DNA sequences flanking the SSR sequence.

Jeffreys (1985) showed that some restriction fragment length polymorphisms are caused by VNTRs. The name **"mini satellite"** was coined because of the similarity of VNTRs to larger satellite DNA repeats.

Advantages:

SSR markers tend to be highly polymorphic. The genotyping throughput is high. This is a simple PCR assay. Many SSR markers are multi-allelic and highly polymorphic. SSR markers can be multiplexed, either functionally by pooling independent PCR products or by true multiplex- PCR. Semi-automated SSR genotyping methods have been developed. Most SSRs are co-dominant and locus specific.

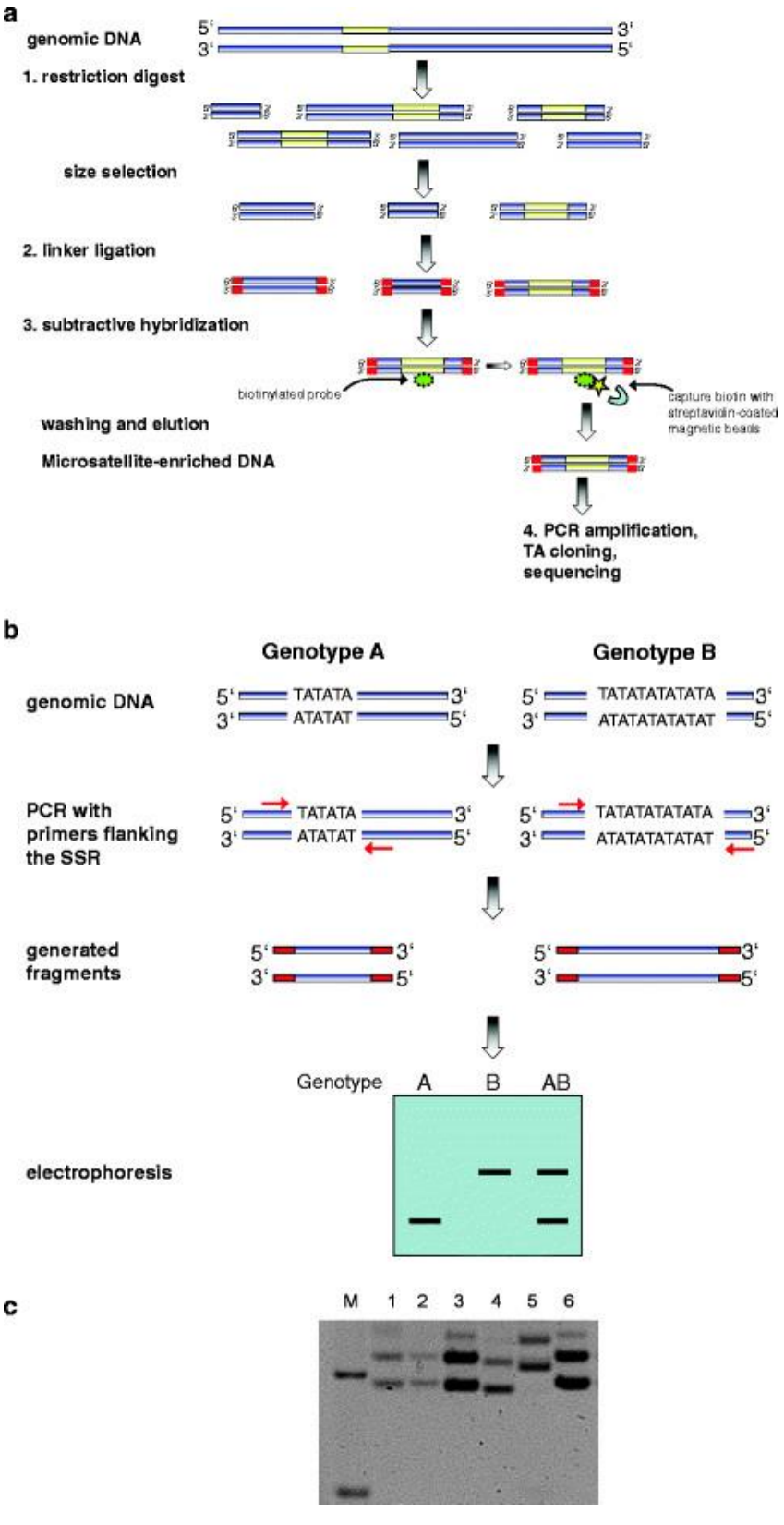
No special equipment is needed for performing SSRs assays; however, special equipment is needed for some assay methods, e.g., semi-automated fluorescent assays performed on a DNA sequences. Start-up costs are low for manual assay methods (once the markers are developed). SSR assays can be performed using very small DNA samples (~100 ng per individual). SSR markers are easily shared between laboratories.

Disadvantages:

The development of SSRs is labor intensive. SSR marker development costs are very high. SSR markers are taxa specific. Start-up costs are high for automated SSR assay methods. Developing PCR multiplexes is difficult and expensive. Some markers may not multiplex.

Uses:

SSR markers are used for mapping of genes in eukaryotes.



Variable Number Tandem Repeat (VNTR):

A Variable Number Tandem Re-peat (VNTR) is a location in a genome where a short nucleotide sequence is organized as a tandem repeat. These can be found on many chromosomes, and of-ten show variations in length between individuals. Each variant acts as an in-herited allele. Due to this reason VNTR can be used for personal or parental identification.

Use of VNTRs in Genetic Analysis:

VNTRs are frequently used in the develop-ment of linkage maps. Now that many ge-nomes have been sequenced, VNTRs have be-come essential to forensic crime investiga-tions, via DNA fingerprinting. When removed from surrounding DNA by the PCR or RFLP methods, and their size determined by gel electrophoresis or Southern blotting, they produce a pattern of bands unique to each individual.

When tested with a group of inde-pendent VNTR markers, the likelihood of two unrelated individuals having the same allelic pattern is extremely improbable. In the ex-ample considered in the diagram below locus A is a tandem repeat of the motif GC: there are four alleles, with two, three, four, or five repeats (A2, A3, A4, and A5, respectively).

Locus B is a tandem repeat of the motif AGCT: there are only two alleles, with two or three repeats (B2 and B3, respectively). Individual 1 is heterozygous at Locus A (A2/A5) and ho-mozygous at Locus 2 (B2/B2) which gives a single-banded phenotype in the fingerprint.

Individual 2 is heterozygous at both loci (A4/ A3 and B3/B2). The two individuals are dis-tinguishable at either locus. Typical finger-prints include a dozen or more VNTR loci. VNTR analysis is also being used to study ge-netic diversity and breeding patterns in populations of wild or domesticated animals.

Inter-Simple Sequence Repeats (ISSR)

The generation of ISSR markers involve PCR amplification of DNA using a single primer composed of a microsatellite repeated sequence and in some cases primer also contains 1-4 base anchor at either 3′ or 5′ or at both ends, which target a subset of 'simple sequence repeats' (SSRs) and amplify the region between two closely spaced and oppositely oriented SSRs (Fang et al., 1997; Fang and Roose, 1997; Moreno et al., 1998). **I**SSR technique permits the detection of polymorphisms in microsatellites and inter-microsatellites loci without previous knowledge of the DNA sequence (Moreno et al., 1998). Some other microsatellites based on the same principle include the following:

(i) Randomly Amplified Microsatellite Polymorphism (RAMP): This is a micro satellite – based marker which shows a high degree of allellic polymorphism, but they are labor-intensive (Agarwal and Shrivastava, 2008). On the other hand RAPD markers are inexpensive but exhibit a low degree of polymorphism. To compensate for the weaknesses of these approaches, a technique termed as RAMP was developed (Wu et al, 1994). The technique involves a radiolabeled primer consisting of a 51 anchor and 31 repeats which is used to amplify genomic DNA in the presence or absence of RAPD primers. (Agarwal and Shrivastava, 2008).

(ii) The Sequence Characterized Amplified Region (SCAR): The SCARS are PCR-based markers that represent genomic DNA fragments at genetically defined loci that are identified by PCR amplification using sequence specific oligonucleotide primer (McDermoth et al, 1994).

(iii) Simple Primer Amplification Reaction (SPAR): SPAR uses the single SSR oligonucleotide principles.

(iv) Sequence – Related Amplified Polymorphism (SRAP): The aim of SRAP technique (Li and Quiros, 2001) is the amplification of open reading frames (ORFs). It is base on two-primer amplification using the AT- or GC- rich cores to amplify intragenic fragment for polymorphism detection (Agarwal and Shirvastava, 2008).

(v) Target region amplification polymorphism (TRAP): The TRAP technique (Hu and Vick, 2003) is a rapid and efficient PCR-based technique, which utilizes bioinformatics tools and expressed

sequence tag (EST) database information to generate polymorphic markers, around targeted candidate gene sequences.

Type of DNA markers

	RFLP	PCR-based				
		RAPD	AFLP	SSR	STS	SNP
Principle	Restriction enzyme	Random priming	Selective PCR	SSR repeats	InDel length	DNA chip/ Hybridization
DNA required (μg)	10	0.02	0.5~1.0	0.02	0.02	0.02
Gel-based	Yes	Yes	Yes	Yes	Yes	Yes/No
Genomic abundance	High	Very high	Very high	Very high	Very high	Very high
Marker type	Codom.	Dom.	Dom.	Codom.	Codom.	Codom.
Reproducibility	Very high	Fair	Very high	Very high	Very high	Very high
Sequence informat. required	No	No	No	Yes	Yes	Yes
Ease of use	Labor intensive	Easy	Relat. difficult	Easy	Easy	Easy

Applcation of nmolecular marker in plant genomic analysis and breeding:

Molecular markers have evolved as potential tool lor a large number of applications ranging irom localization of a gene to improvement of plant varieties by marker assisted-Selection. With the advancement in the technology in the field of molecular marker our understanding in genetic analysis, and genomic has got signilicant impetus.

Fingerprinting of crop plants:

DNA fingerprinting refers to identify an individual unambiguously using multilocus DNA profiling. It can be done using hybridization markers, PCR based marker either locus specific amplification or by using random primers and sequencing. Huge number of scientific literature is available in various crops in the context of DNA fingerprinting. Alec Jeffery and his associates were the first to develop method of DNA fingerprinting through simulteneous detection of highly variable DNA fragments by hybridizing multilocus probes with electrophoretically seperated restriction fragments.

DNA fingerprinting has remarkable importance in plant vanety protection (PVP). And the utilites include identilication of cultivars and genotypes; true to type plants at juveline stage (DUS testing) for Seed purily mutants and chimeras; nucellar and zygotic embryos; somatic hybrids in fusion experiments and somaclonal variants etc.

Mapping and tagging of genes: Tools for MAS

Plant breeding is the science that aims at crop improvement, using the available variability. The outcome of crop improvement is selection of right kind of plant with right combination of genes/alleles. Conventional breeding take lot of time for evolution, identification and introgression of novel genes. Molecular markers have accelerated conventional plant breeding. It is a powerful tool for identification of diverse line, mapping and tagging of genes. With the use of molecular markers it is now a routine to trace valuable alleles in a segregating population. These markers once mapped enable dissection of the complex traits into component genetic units more precisely, thus providing breeder with a new tool to manage this complex unit more etficiently in breeding programme.

There are several examples of gene mapping and utility in marker assisted-selection in vaious crops using various molecular markers. The very first genome map in plants was reported in Maize, followed by rice, *Arabidopsis* etc. using RFLP markers.Maps have since then been constructed for several other crops like potato, barley, banana,members of Brassicaceae, etc.

Phylogeny and evolution:

Molecular markers are powerful tools in phylogenetic and evolutionary studies.These studies strengthened the eariler studies made based on morphological and cytological evidences for establishing relationship between the wild relatives of species and their cultivated species. The comprenensive studies on genetic structure using molecular markers have revaled evolutionary forces that led the wild relatives to the present cultivable fom of species. RFLP, DNA scquencing, and a number of PCR-based markers are being used exiensively for reconstructing phylogenies of various species. The tecniques are speculated to provide path-breaking information regarding the fine time scale on which closely related species have divergcd and what sort of genetic variations are associated with species formation. Furthermore, these studies hold a great promise for revealing more about the patterm of genetic variation within the species. In connection to plant breeding they are very much helpful in understanding the crop evolution from wild progenitor and to clasify them to appropiate groups.This would help in introgression of useful genes from wild progenitors into cultivated high yielding variaties ot crop species.

Diversity Analysis:

One of the important utility of molecular markers is diversity analysis. Lines with similar morphological characters may substantially diverse from each other at DNA level and vice-versa. Diversity analysis can be done based on pedigree data, biochemical data, and more recently molecular (DNA-based) data. DNA based markers can unambiguously distinguish two different lines The revealed through molecular markersb can be used to deduce genetic distance among the germplasm, breeding lines and population. There arc several utilities of diversity analysis like (1) selection of parents for developing hybrids (2) selection of parents for developing mapping population (3) to study genetic inheritance of a traít (4) combining ability studies (5) understanding the enviormental affect on geographically diverse lines (6) population genetic studics (7) identification of regions specifie fixed allelesin landraces.

The commonly used measures of genetic distance or genetic similarity (GS) using binary data are (1) Nei and Li's (1979) coefficient (GDNu), (2) Jaccard's (1908) (3) Sokal and Michener's (1958) simple matching coefficient(GDsm), and (4) Modified Rogers distance, (1972) (GDuR).

Marker Assisted Breeding (MAS):

Marker assisted breeding is defined as the application of molecular markers in combination with linkage maps and genomics to alter and improve plant traits on the basis of genotypic assays. This term is used to describe several modern breeding strategies including marker-assisted selection (MAS). MAS are based on the concept that it is possible to infer the presence of a gene from the presence of a marker which is tightly linked to the gene of interest.

Features of Marker Assisted Selection (MAS):

1. Pre-Requisites:

There are two pre-requisites for marker assisted selection. These are: (i) a tight linkage between molecular marker and gene of interest, and (ii) high heritability of the gene of interest.

2. Markers Used:

MAS makes use of various types of molecular markers. The most commonly used molecular markers include amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats

(SSR) or micro satellites, single nucleotide polymorphisms (SNP), etc. The use of molecular markers differs from species to species also.

3. Efficiency:

The relative efficiency of MAS is greatest for characters with low heritability, if a large fraction of the additive genetic variance is associated with the marker loci. In other words, MAS is useful when the heritability of the trait is low. Moreover, MAS is more efficient than purely phenotypic selection in quite large populations.

It has been found by some workers that MAS may become less efficient than phenotypic selection in the long term. This is because the rate of fixation of unfavourable alleles at QTLs with small effects is higher under MAS than under phenotypic selection. It may be a consequence of the strong selection applied to QTLs with large effects under MAS in early generation. However, such problem comes after a long period.

4. Accuracy:

Molecular markers have very high accuracy. They are not affected by environmental conditions. MAS is a new breeding tool which is available to make more accurate and useful selections in breeding populations. MAS allows heritable traits to be linked to the DNA which is responsible for controlling that trait.

5. Speed of Progress:

MAS is a rapid method of crop improvement. For example, in conventional breeding when we transfer a recessive character through backcross, one selfing is required after every backcross for identification of recessive character. MAS permits identification of recessive alleles even in heterozygous condition and thus speeds up the progress of crop improvement work.

6. Traits Improved:

MAS can be used for improvement of both oligogenic and polygenic traits. In the past, MAS has been mostly used for the genetic improvement of oligogenic traits and little progress has been made with polygenic traits.

7. Material Developed:

MAS leads to development of non-transgenic genotypes or cultivars. In other words, MAS is used for development of non-transgenic cultivars. The transgenic cultivars face public resistance. On the other hand, cultivars developed by MAS are acceptable by consumers.

8. Cost:

MAS is very costly as compared to phenotypic selection. In MAS, the costly items include equipment's, consumables, infrastructure, labour and DNA extraction process. MAS requires sophisticated and well equipped laboratory.

9. Application:

MAS is applicable for genetic improvement of plants as well as animals. In plants, it is equally applicable in both self-pollinated and cross pollinated species.

Steps in Marker Assisted Selection (MAS):

In the marker aided selection, RFLP markers are widely used for genetic improvement of crop plants for various economic characters.

The marker aided selection consists of five important steps, viz:

(i) Selection of parents

(ii) Development of breeding population

(iii) Isolation of DNA from each plant

(iv) Scoring RFLPs

(v) Correlation with morphological traits

i) Selection of Parents:

Selection of suitable parents is an important step in marker aided selection. The parents should be such so that we can get usable level of polymorphism (variation) in the RFLP markers. In other words, parents with contrasting characters or divergent origin should be chosen. This will help in identification of DNA of both the parents and also their segments in F2 generation in various recombinations.

For selection of parents, we have to screen germplasm and select parents with distinct DNA. The parents that are used for MAS should be pure (homozygous). In self- pollinated species, plants are usually homozygous. In cross-pollinated species, inbred lines are used as parents.

ii) Development of Breeding Populations:

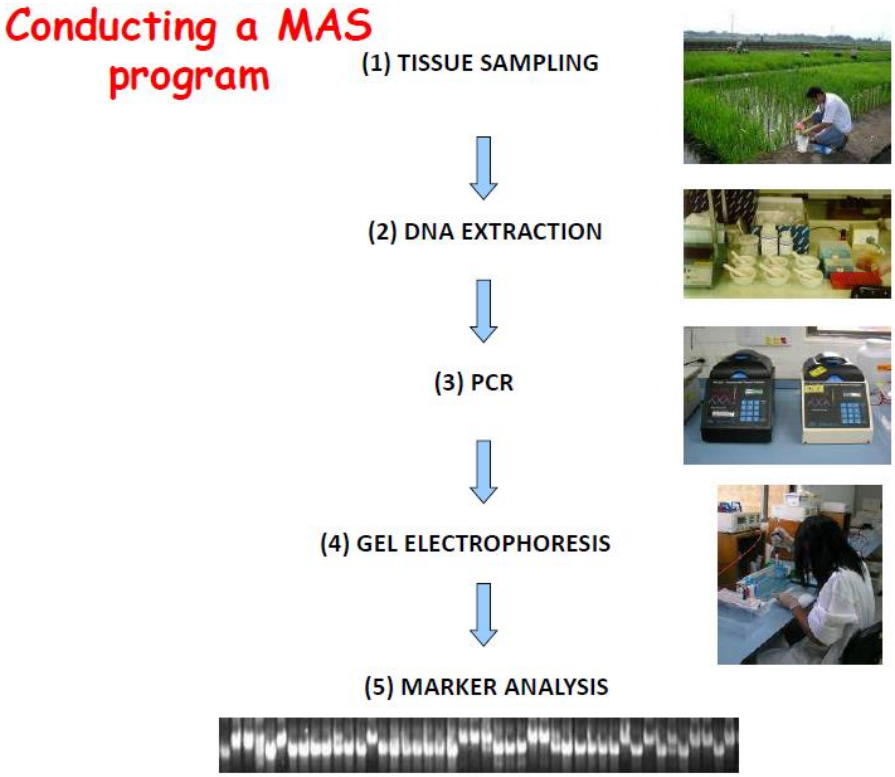
This is the second important step for application of marker aided selection. The selected parents are crossed to obtain F1 plants. F1plants between two pure-lines or inbred lines are homogeneous (alike phenotypically) but are heterozygous for all the RFLPs of two parents involved in the F1. The F2 progeny is required for the study of segregation pattern of RFLPs. Generally 50-100 F2 plants are sufficient for the study of segregation of RFLP markers.

iii) Isolation of DNA:

The third important step is isolation of DNA from breeding population. The main advantage of MAS is that DNA can be isolated even from the seedlings and we need not to wait for flowering or seed development stage. The DNA is isolated from each plant of F2population. Standard procedures are available for DNA isolation.

The isolated DNA is digested with specific restriction enzyme to obtain fragments of DNA. The DNA fragments of different sizes are separated by subjecting the digested DNA to agarose gel electrophoresis. The gel is stained with ethidium bromide and the variation in DNA fragments can be viewed in the ultraviolet light.

The DNA of chloroplasts, when digested with specific enzyme, produces about 40 fragments of different sizes. The nuclear DNA of higher plants, when digested with specific restriction enzymes, produces millions of fragments in a continuous range of sizes. It is a tedious job to identify individual DNA fragment in such cases.



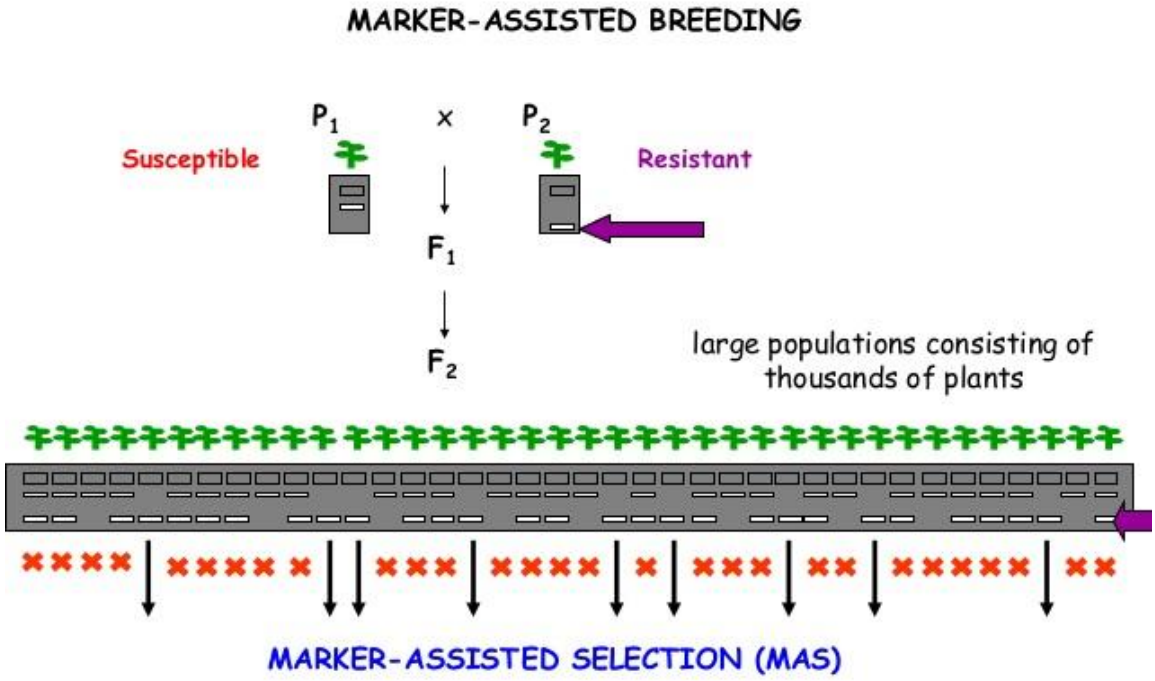
iv) Scoring RFLPs:

The polymorphism in RFLPs between the parents and their involvement in the recombinants in F2 population is determined by using DNA probes. The labelled probes are used to find out the fragments having similarity.

The probe will hybridize only with those segments which are complementary in nature. Generally 32P is used for radioactive labelling of DNA probe. Now non-radioactive probe labelling techniques are also available. In this way RFLPs are determined.

v) Correlation with Morphological Traits:

The DNA marker (say RFLPs) are correlated with morphological markers and the indirect selection through molecular markers is confirmed. Once the correlation of molecular markers is established with morphological markers, MAS can be effectively used for genetic improvement of various economic traits.



Method whereby phenotypic selection is based on DNA markers

What is the importance of QTL mapping for MAS?

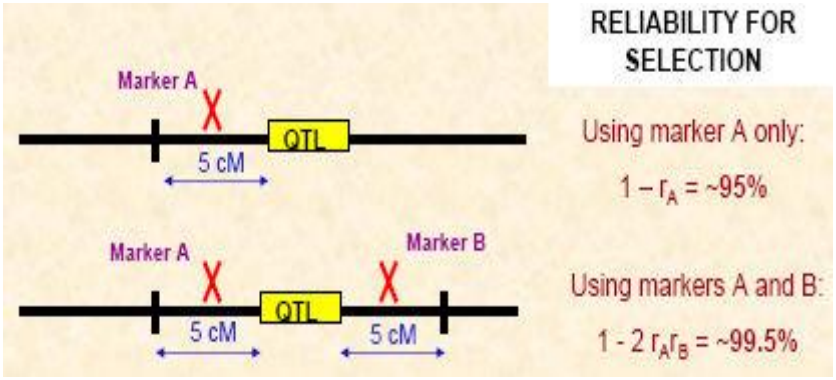
The identification of genes and quantitative trait loci (QTLs) and DNA markers that are linked to them is accomplished via QTL mapping experiments. QTL mapping thus represents the foundation of the development of markers for MAS.

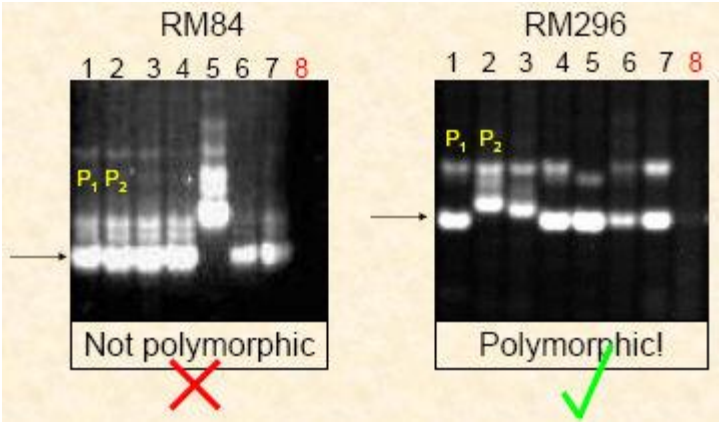
Previously, it was generally assumed that markers could be directly used in MAS. However, there are many factors that influence the accuracy of QTL mapping such as population size and type, level of replication of phenotypic data, environmental effects and genotyping errors. These factors are particularly important for more complex quantitative traits with many QTLs each with relatively small effects (e.g. drought tolerance, yield).

Therefore, in recent years it has become widely-accepted that QTL confirmation, validation and/or additional marker testing steps may be required after QTL mapping and prior to MAS.

These steps may include:

- Marker conversion - may be required such that the marker genotyping method is technically simpler for MAS or so that the reliability is improved**.**
- QTL confirmation - testing the accuracy of results from the primary QTL mapping study
- QTL validation - generally refers to the verification that a QTL is effective in different genetic backgrounds
- Marker validation - testing the level of polymorphism of most tightly-linked markers within a narrow window (say 5 - 10 cM) spanning a target locus and also testing the reliability of markers to predict phenotype.

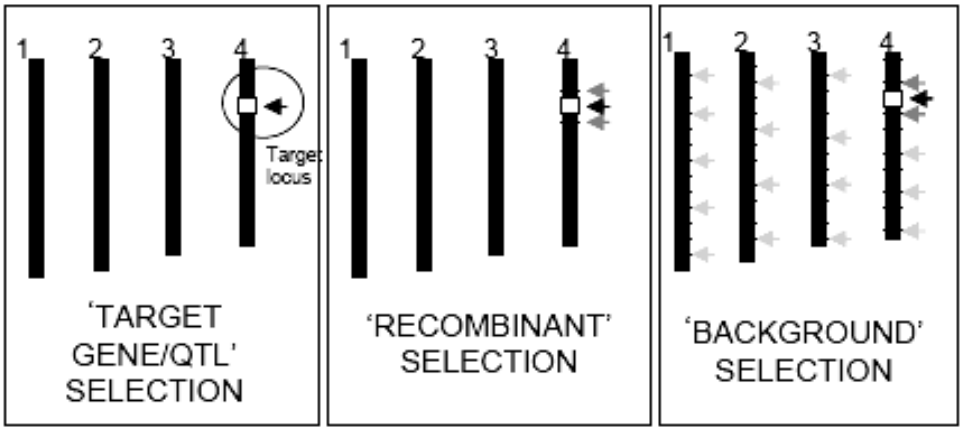




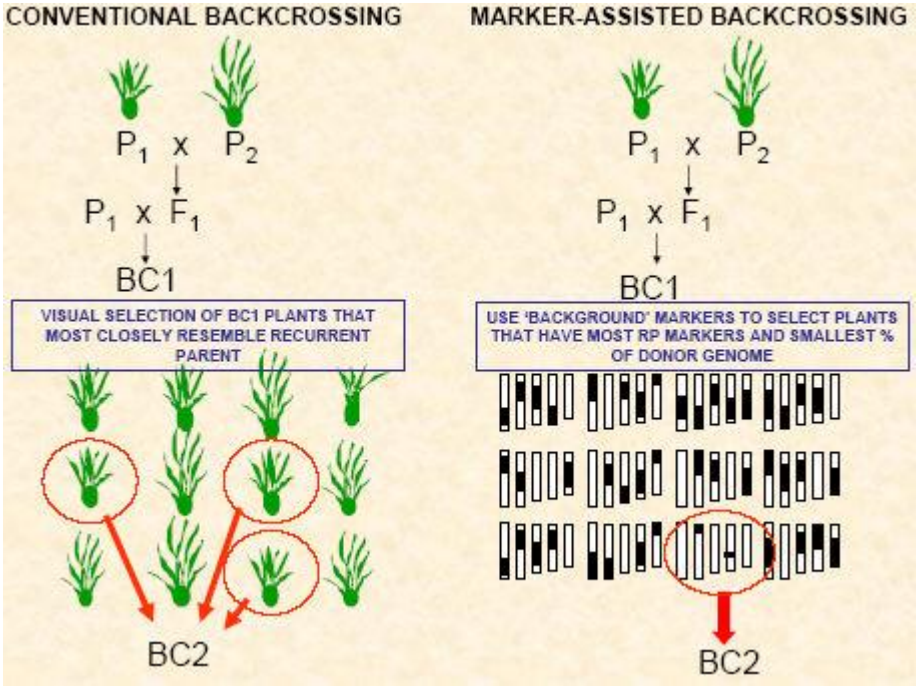
MAS schemes in plant breeding

Marker assisted backcrossing

There are three levels of selection in which markers may be applied in backcross breeding. In the first level, markers may be used to screen for the target trait, which may be useful for traits that have laborious phenotypic screening procedures or recessive alleles. The second level of selection involves selecting backcross progeny with the target gene and tightly-linked flanking markers in order to minimize linkage drag. We refer to this as recombinant selection. The third level of MAB involves selecting backcross progeny (that have already been selected for the target trait) with background markers. In other words, markers can be used to select against the donor genome, which may accelerate the recovery of the recurrent parent genome.



With conventional backcrossing, it takes a minimum of five to six generations to recover the recurrent parent. Data from simulation studies suggests that at least two but possibly three or even four backcross generations can be saved by using markers.



Backcross inbred lines (BILs) for introgression of wild genes

Exploiting the rich source of genetic diversity in exotic germplasm is met with a host of challenges, not the least of which are those that are genetic in origin. Wild domesticated crosses have problems such as F_1 hybrid sterility, infertility of the segregating generations, linkage drag, and suppressed recombination between chromosomes of the two species. Tanksley and Nelson developed a procedure, advanced backcross breeding, for the simultaneous discovery and transfer of desirable QTL from unadapted germplasm into elite lines. Basically, this procedure postpones QTL mapping until the BC_2 or BC_3, applying negative selection during these generations to reduce the occurrence of undesirable alleles from the donor (unadapted genotype). The advantage of this strategy is that BC_2/BC_3 provides adequate statistical power for QTL identification, while at the same time being sufficiently similar to the recurrent parent to allow selection for QTL-NIL (near isogenic lines) in a short time (1–2 years). The QTL discovered can be verified and the NILs used directly as improved cultivars or as parents for hybrid breeding.

Backcross inbred lines (BILs) are populations of plants derived from the repeated backcrossing of a recombinant line with the wild type, using phenotypic or molecular marker selection techniques to generate introgression lines. This is conceptually the same as the advanced backcross strategy. BILs are immortal lines and, hence, can be used in replicated experimentation at multiple locations and years. Being near-isogenic, BILs have high genetic and morphological similarity with the recurrent parent to allow precise estimates of traits. Genes that are not detected in an F_2 analysis are more likely to be picked up in BIL studies. Single gene detection is more favored in this strategy. BIL studies tend to reveal QTL that are not involved in interactions, thus making the introgression of the wild trait into commercial cultivars straightforward. However, they are laborious and costly to generate, taking six generations to develop BILs to cover the entire genome (F_1, BC_1, BC_2, BC_3, BC_4 and BC_4S_1). They are superior to an F_2 population for introgression of quantitative traits, except when a single dominant gene conditions the trait or when the trait is expressed through epistatic interactions of a few genes.

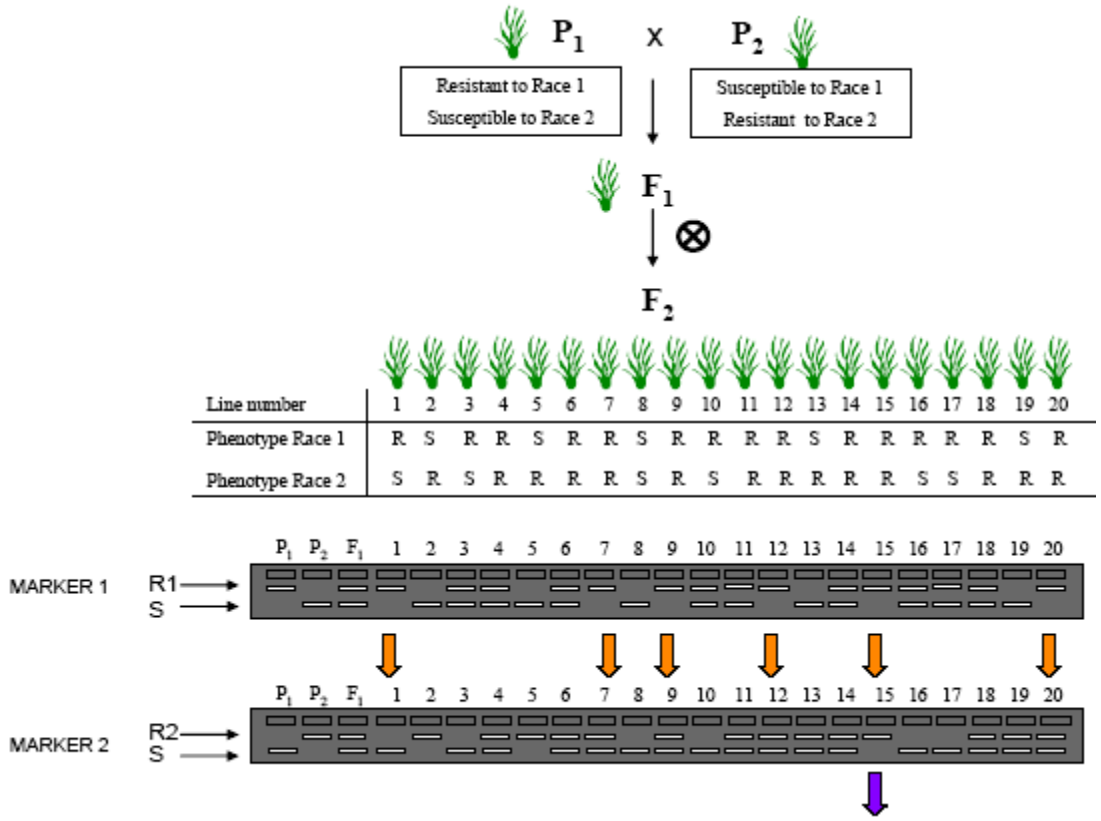
Marker assisted "forward selection"

In spite of the advantages discussed, MAS for backcrossingrepresents a very limited application of molecular markers in plant improvement, because the breeding method is extremely conservative,

improving the current cultivar only one or a few genes at a time. On the other hand, "forward selection" programs allow the breeder to recombine alleles throughout the genome to produce new allele combinations. To be cost effective and efficient, molecular markers used in forward breeding programs are tightly linked to a few loci that have large effects on traits that are challenging or expensive to phenotype. More importantly, the linkage between specific markers and target trait loci are stable or consistently diagnostic for target alleles across different populations, thus eliminating the need to re-establish such associations in every population. This is important because the parental stocks with these linkage phases are used in new combinations from time to time to create new breeding experimental populations for selection (i.e., the same set of markers are effective for use in future crosses).

Marker assisted pyramiding

Pyramiding is the process of simultaneously combining multiple genes/QTLs together into a single genotype. This is possible through conventional breeding but extremely difficult or impossible at early generations. Using conventional phenotypic selection, individual plants must be phenotypically screened for all traits tested. Therefore, it may be very difficult to assess plants from certain population types (e.g. F_2) or for traits with destructive bioassays. DNA markers may facilitate selection because DNA marker assays are non-destructive and markers for multiple specific genes/QTLs can be tested using a single DNA sample without phenotyping. The most widespread application for pyramiding has been for combining multiple disease resistance genes in order to develop durable disease resistance.

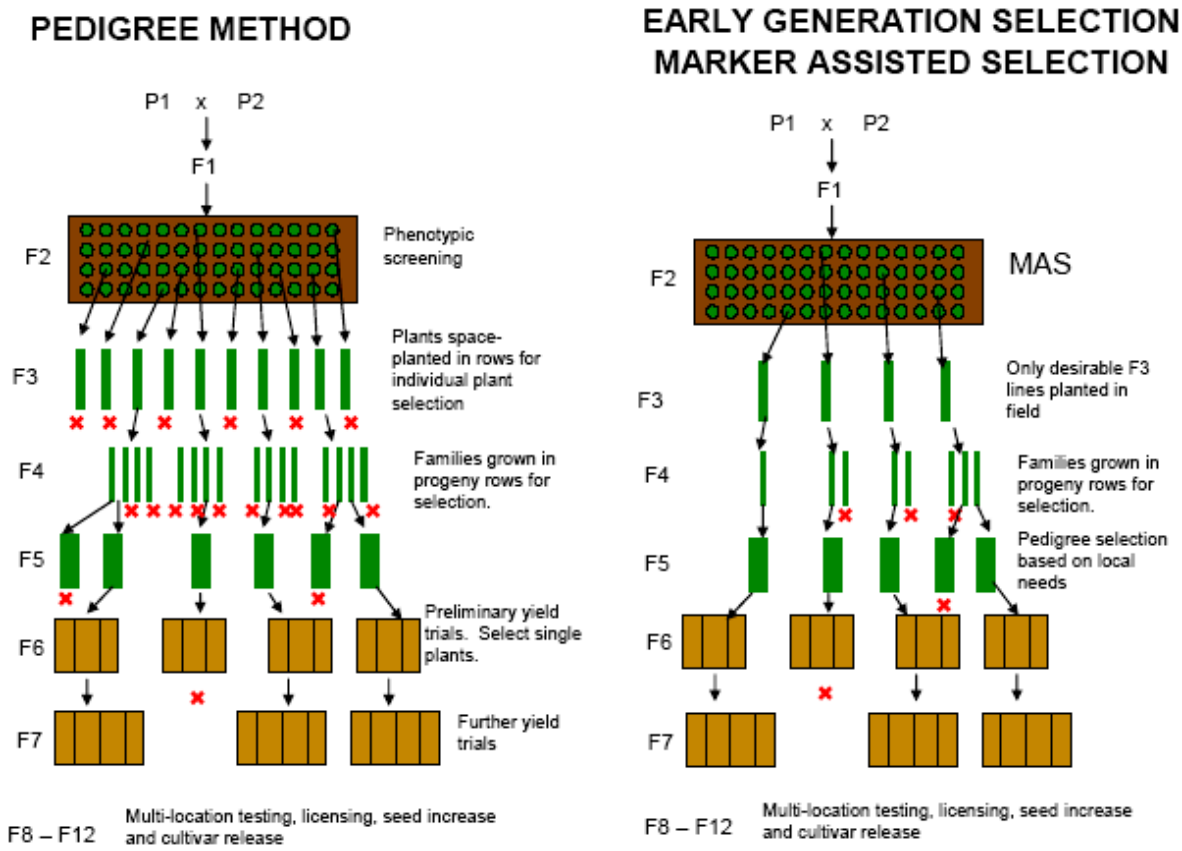


Marker assisted pyramiding of two disease resistance genes. Note that homozygotes can be selected from the F_2 population.

Early generation marker assisted selection

One of the most intuitive stages to use markers to select plants is at an early generation (especially F_2 or F_3). The main advantage is that many plants with unwanted gene combinations, especially those

that lack essential disease resistance traits and plant height, can be simply discarded. This has important consequences in the later stages of the breeding program because the evaluation for other traits can be more efficiently and cheaply designed for fewer breeding lines (especially in terms of field space).



Early generation selection scheme proposed by Ribaut & Betran (1999), Note that many lines can be discarded in an early generation which permits the evaluation of fewer lines in later generations.

Combined appoaches

In some cases, a combination of phenotypic screening and MAS approach may be useful

- To maximize genetic gain (when some QTLs have been unidentified from QTL mapping)
- Level of recombination between marker and QTL (in other words marker is not 100% accurate)
- To reduce population sizes for traits where marker genotyping is cheaper or easier than phenotypic screening

Applications of Marker Assisted Selection (MAS):

In crop improvement programmes MAS can be used in various ways. In other words, MAS has several useful applications in plant breeding.

Important applications of MAS in plant breeding are briefly presented below:

i. MAS is very effective, efficient and rapid method of transferring resistance to biotic and abiotic stresses in crop plants.

ii. It is useful in gene pyramiding for disease and insect resistance.

iii. It is being used for transfer of male sterility and photo period insensitivity into cultivated genotypes from different sources.

iv. MAS is being used for improvement of quality characters in different crops such as for protein quality in maize, fatty acid (linolenic acid) content in soybean and storage quality in vegetables and fruit crops.
v. MAS can be successfully used for transferring desirable transgene (such as Bt gene) from one cultivar to another.
vi. MAS is very effective in introgression of desirable genes from wild into cultivated genotypes.
vii. MAS is equally effective in genetic improvement of plants and animals.
viii. MAS is useful in genetic improvement of tree species where fruiting takes very long time (say 20 years) because for application of phenotypic selection we have to wait for such a long time.
ix. MAS has wide application for genetic improvement of oligogenic traits as compared to polygenic traits.

Achievements of Marker Assisted Selection (MAS):

MAS has been used for genetic improvement of different field crops such as maize, barley, rice, wheat, sorghum, soybean, chickpea, pea, sunflower, tomato, potato and some fruit crops for various economic characters. Some notable examples of the use of MAS:

i) Rice:

In rice MAS has been successfully used for developing cultivars resistant to bacterial blight and blast. For bacterial blight resistance four genes (Xa4, Xa5, Xa13 and Xa21) have been pyramided using STS (sequence tagged site) markers.

The pyramided lines showed higher level of resistance to bacterial blight pathogen. In Indonesia, two bacterial blight resistant varieties of rice viz Angke and Conde have been released through MAS. For blast resistance, three genes (Pil, Piz5 and Pita) have been pyramided in a susceptible rice variety Co 39 using RFLP and PCR based markers.

ii) Maize:

In maize, normal lines have been converted into quality protein maize (QPM) lines through MAS using opaque 2 recessive allele. This work has been done at CIMMYT (International centre for wheat and maize improvement, Mexico).

Three SSR markers (Umc 1066, Phi 057 and Phi 112) present within opaque 2 gene have been used for this purpose. The MAS used for conversion of normal maize lines into QPM is simple, rapid and accurate.

iii) Soybean:

In soybean cyst nematodes pose serious problem and most of the varieties are susceptible to this parasite. The resistant gene (rhg 1) is available. In soybean, nematode resistant lines have been developed through MAS using SSR marker (Sat 309).

Advantages of Marker Assisted Selection (MAS):

MAS has several advantages over phenotypic selection and other breeding techniques.

i) Accuracy:

The accuracy of MAS, is very high because molecular markers are not affected by environmental conditions. It is very effective even with the characters having low heritability.

ii) Rapid Method:

MAS is a rapid method of crop improvement. It takes 3-5 years for developing a new cultivar against 10-15 years taken by the conventional method of breeding.

iii) Non-transgenic Product:

MAS leads to development of non-transgenic cultivars which are acceptable to everybody. In other words, it does not involve transgene. Hence there is no question of gene silencing.

iv) Identification of Recessive Alleles:

MAS permits identification of recessive alleles even in heterozygous condition and thus speeds up the progress of crop improvement programmes. In other words, it is equally effective for the genetic improvement of recessive characters.

v) Early Detection of Traits:

MAS permits early detection of traits that are expressed late in the life of plant. For example characters such as grain or fruit quality, flower colour, male sterility, photoperiod sensitivity that express late in the life of a plant can be screened in the seedling stage. In other words, DNA tested at seedling stage can through light about the trait which are expressed later on.

vi) Screening of Difficult Traits:

MAS permits screening traits that are extremely difficult expressive and time consuming to score phenotypically. For example, screening for traits such as root morphology and resistance to biotic (insects and diseases) and abiotic stresses (drought, salinity, heat, frost etc.) is very easy through MAS.

vii) Gene Pyramiding:

MAS is very effective method in accumulating multiple genes for resistance to specific pathogens and pests within the same cultivar. This process is called gene pyramiding. Maker assisted backcrossing is routinely applied in breeding programmes for gene introgression. MAS can provide an effective and efficient breeding tool for detecting, tracking, retaining, combining and pyramiding genes for disease resistance.

viii) Small Sample for Testing:

MAS requires only a small amount of plant tissue for DNA testing. In other words, MAS can be carried out with small breeding populations. Moreover, MAS can be applied at any stage of plant growth.

ix) Permits QTL Mapping:

MAS permits mapping or tagging of quantitative trait loci (QTL) which is not possible by conventional method.

x) Highly Reproducible:

The MAS is based on DNA fingerprinting technique and the results of DNA fingerprinting pattern are highly reliable and reproducible.

Limitations of Marker Assisted Selection (MAS):

i. MAS is a costly method. It requires well equipped laboratory viz. expensive equipment's, glassware and chemicals.

ii. MAS requires well trained manpower for handling of sophisticated equipments, isolation of DNA molecule and study of DNA markers.

iii. The detection of various linked DNA markers (AFLP, RFLP, RAPD, SSR, SNP etc.) is a difficult, laborious and time consuming task.

iv. MAS sometimes involves use of radioactive isotopes in labelling of DNA, which may lead to serious health hazards. This is a major disadvantage of RFLP based markers. The PCR, markers are safe in this regard.

v. It has been reported that MAS may become less efficient than phenotypic selection in the long term.

vi. The use of MAS is more difficult for QTL because they have minor cumulative effects and are greatly influenced by environmental conditions and genetic background.

QTL mapping:

Many agriculturally important traits such as yield, quality and some forms of disease resistance are controlled by many genes and are known as **"quantitative traits** or **polygenic** or **multifactorial** or **complex traits".**

- These traits show continuous variation in a population.
- These traits **do not** fall into **discrete classes**.
- They are measurable.

A **quantitative trait locus (QTL)** is a locus (section of DNA) that correlates with variation of a quantitative trait in the phenotype of a population of organisms. QTLs are mapped by identifying which molecular markers (such as SNPs or AFLPs) correlate with an observed trait. This is often an early step in identifying and sequencing the actual genes that cause the trait variation.

- The loci controlling quantitative traits are called **quantitative trait loci** or **QTL**.
- Term first coined by **Gelderman** in 1975.
- It is the region of the genome that is associated with an effect on a quantitative trait.
- It can be a single gene or cluster of linked genes that affect the trait.

QTLs have the following characteristics

- These traits are controlled by multiple genes, each segregating according to Mendel's laws.
- These traits can also be affected by the environment to varying degrees.
- Many genes control any given trait and Allelic variations are fully functional.
- Individual gene effects is small &The genes involved can be dominant,or codominant.
- The genes involved can be subject to epistasis or pleiotrophic effect.

QTL Mapping

The process of constructing linkage maps and conducting QTL analysis i.e. to identify genomic regions associated with traits is known as **QTL mapping**. Identify the location of polygenes or **QTL** by use of DNA markers. It involves testing DNA markers throughout the genome for the likelihood that they are associated with a QTL.

Principles

Genes and markers segregate via chromosome recombination during meiosis, thus allowing their analysis in the progeny. It detects the association between phenotype and genotype of markers. QTL analysis depends on the linkage disequilibrium. QTL analysis is usually undertaken in segregating mapping populations.

Objectives

The basic objective is to detect QTL, while minimizing the occurrence of false positives (Type I errors that is declaring an association between a marker and QTL when in fact one does not exist).

- To identify the regions of the genome that affects the trait of interest.
- To analyze the effect of the QTL on the trait.
- How much of the variation for the trait is caused by a specific region?
- What is the gene action associated with the QTL – additive effect? Dominant effect?
- Which allele is associated with the favorable effect?

Prerequisites for QTL mapping

Availability of a good linkage map (this can be done at the same time the QTL mapping)

A segregating population derived from parents that differ for the trait(s) of interest, and which allow for replication of each segregant, so that phenotype can be measured with precision (such as RILs or DHs)

A good assay for the trait(s) of interest

Software available for analyses

Molecular Markers

Sophisticated Laboratory

Type of mapping population:

Backcross Populations

To analyze specific DNA fragments derived from parent A in the background of parent B, a hybrid F_1 plant is backcrossed to parent B. In this situation, parent A is the donor of DNA fragments and parent B is the recipient. The latter is also called the recurrent parent. During this process two goals are achieved: unlinked donor fragments are separated by segregation and linked donor fragments are minimized due to recombination with the recurrent parent. To reduce the number and size of donor fragments, backcrossing is repeated and, as a result, so-called advanced backcross lines are generated. With each round of backcrossing, the proportion of the donor genome is reduced by 50 %.

Advantages: It is easier to identify QTL as there are less epistatic and linkage drag effects; especially useful for crosses with wild species.

Disadvantages: Difficult or impossible in species that are highly heterozygous and outcrossing.

Use: best when inbred lines are available

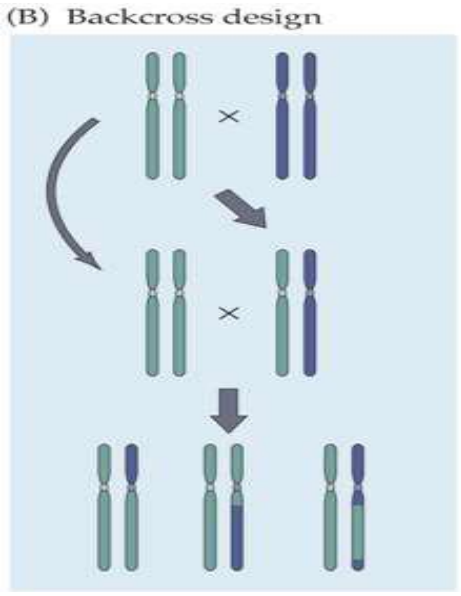
$$P_1(AA) \quad x \quad P_2(aa)$$

$$\downarrow$$

$$P_1(AA) \; x \; F_1(Aa)$$

$$\downarrow$$

$$BC_1$$



F_2 Populations

The simplest form of a mapping population is a collection of F_2 plants. This type of population was the basis for the Mendelian laws (1865) in which the foundations of classic genetics were laid. Two pure lines that result from natural or artificial inbreeding are selected as parents, parent 1 (P_1) and parent 2 (P_2). Alternatively, doubled haploid lines can be used to avoid any residual heterozygosity. If possible, the parental lines should be different in all traits to be studied. The degree of polymorphism can be assessed at the phenotypic level (e. g., morphology, disease resistance) or by molecular markers at the nucleic acid level. For inbreeding species such as soybean and the Brassicaceae, wide crosses between genetically distant parents help to increase polymorphism. However, it is required that the cross lead to fertile progeny. The progeny of such a cross is called the F_1 generation. If the parental lines are true homozygotes, all individuals of the F_1 generation will have the same genotype and have a similar phenotype. This is the content of Mendel's law of uniformity. An individual F_1 plant is then selfed to produce an F_2 population that segregates for the traits different between the parents. F_2 populations are the outcome of one meiosis, during which the genetic material is recombined. The expected segregation ratio for each codominant marker is 1:2:1 (homozygous like P_1:heterozygous:homozygous like P_2).

Advantage: Fast and easy to construct

Disadvantage: F_3 families are still very heterozygous, so the precision of the estimates can be low (because of the high standard error); can't be replicated

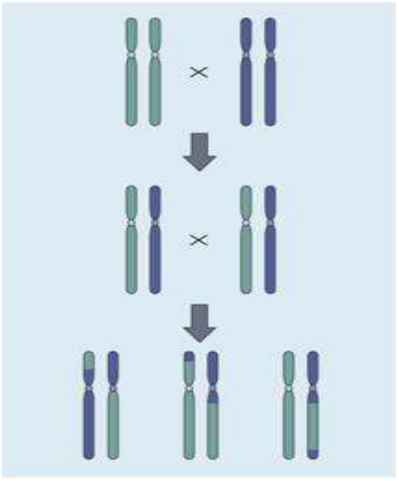
$$P_1(AA) \quad x \quad P_2(aa)$$

$$\downarrow$$

$$F_1(Aa) \quad x \quad F_1(Aa)$$

$$\downarrow$$

$$F_2(AA,Aa,aa)$$

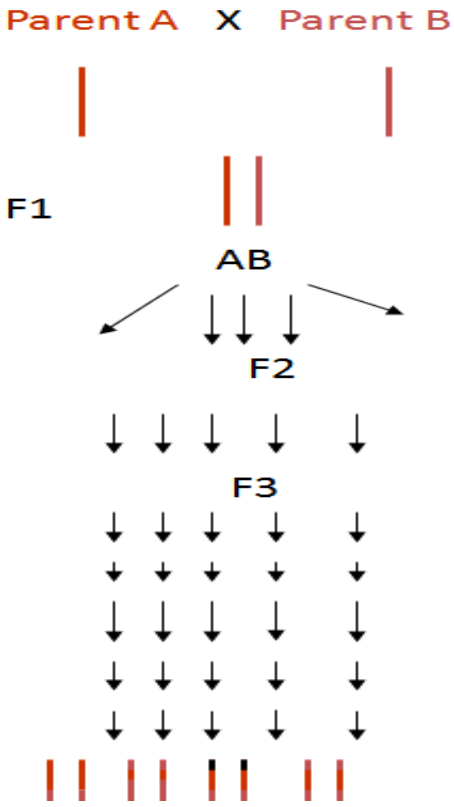


Recombinant Inbred Lines

Recombinant inbred lines (RILs) are the homozygous selfed or sib-mated progeny of the individuals of an F_2 population (Figures. The RIL concept for mapping genes was originally developed for mouse genetics. In animals, approximately 20 generations of sib mating are required to reach useful levels of homozygosity. In plants, RI lines are produced by selfing, unless the species is completely self-incompatible. Because in the selfing process one seed of each line is the source for the next generation, RILs are also called single-seed descent lines. Self-pollination allows the production of RILs in a relatively short number of generations. In fact, within six generations, almost complete homozygosity can be reached. Along each chromosome, blocks of alleles derived from either parent alternate. Because recombination can no longer change the genetic constitution of RILs, further segregation in the progeny of such lines is absent.

Advantages: fixed lines so can be replicated across many locations and/or years; can eliminate problem of background heterozygosity.

Disadvantages: Can take a long time to produce. (Some species are not amenable).



Doubled Haploid Lines

Doubled haploid lines contain two identical sets of chromosomes in each cell. They are completely homozygous, as only one allele is available for all genes. Doubled haploids can be produced from haploid lines. Haploid lines either occur spontaneously, as in the case of rape and maize, or are artificially induced. Haploid plants are smaller and less vital than diploids and are nearly sterile. It is possible to induce haploids by culturing immature anthers on special media. Haploid plants can later be regenerated from the haploid cells of the gametophyte. A second option is microspore culture. In cultivated barley it is possible to induce the generation of haploid embryos by using pollen from the wild species *Hordeum bulbosum*. During the first cell divisions of the embryo, the chromosomes of *H. bulbosum* are eliminated, leaving the haploid chromosomal set derived from the egg cell. Occasionally in haploid plants the chromosome number doubles spontaneously, leading to doubled haploid (DH) plants.

AA	aa	aa	AA	aa	AA
BB	bb	BB	bb	bb	BB
cc	CC	cc	cc	cc	CC
dd	DD	dd	dd	DD	dd
ee	ee	EE	EE	ee	ee
FF	ff	FF	FF	FF	ff

Advantages:

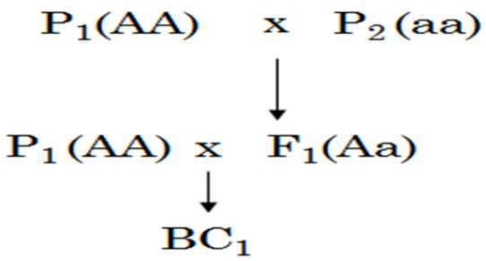
1) Spontaneous chromosome doubling of Haploid microspores in in vitro culture

2) Homozygosity achieved in a single step Plants.

Disadvantages: Less recombination between linked markers Not all systems are amenable to in vitro culture

Near Isogenic Lines (NILs):

NILs can be developed by repeated selfing or backcrossing of F1 with recurrent parent. Irrespective of dominant or codominant marker NILs segregate in 1:1 ratio.



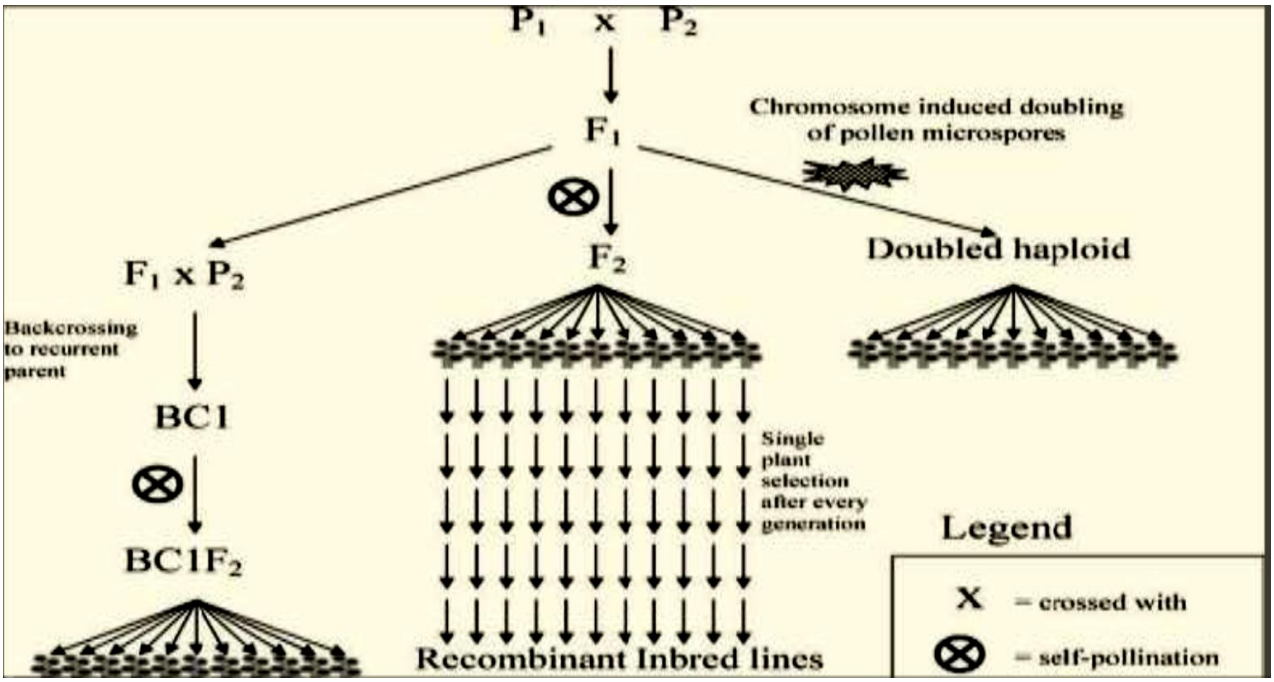
Advantage: Very precise and statistically strong, as background is constant; especially useful for validation experiments

Disadvantage: Can take time to construct; only useful for specific target QTL

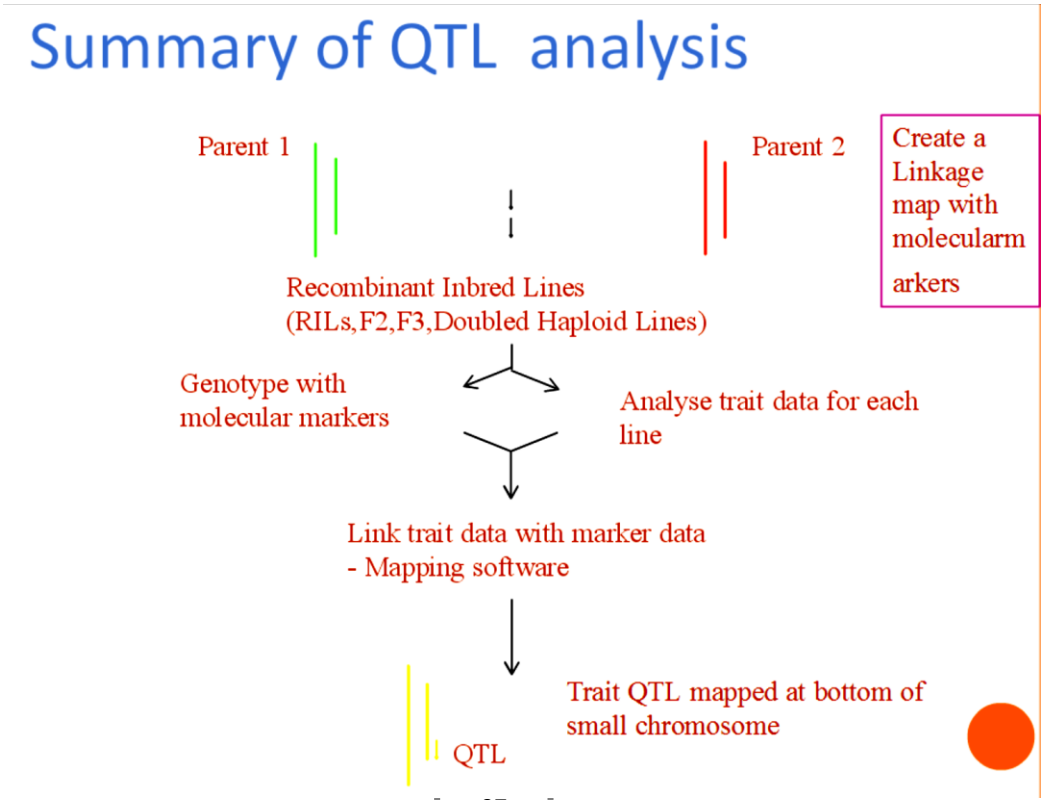
Steps involved in QTL Mapping:

- **Selection of parental lines**
  - Sufficient **polymorphism**
  - Parental lines are highly **contrasting phenotypically**
  - **Genetically divergent**
- **Selection of molecular markers (dominant/codominant)**
- **Making crosses**

- **Creation of mapping population**

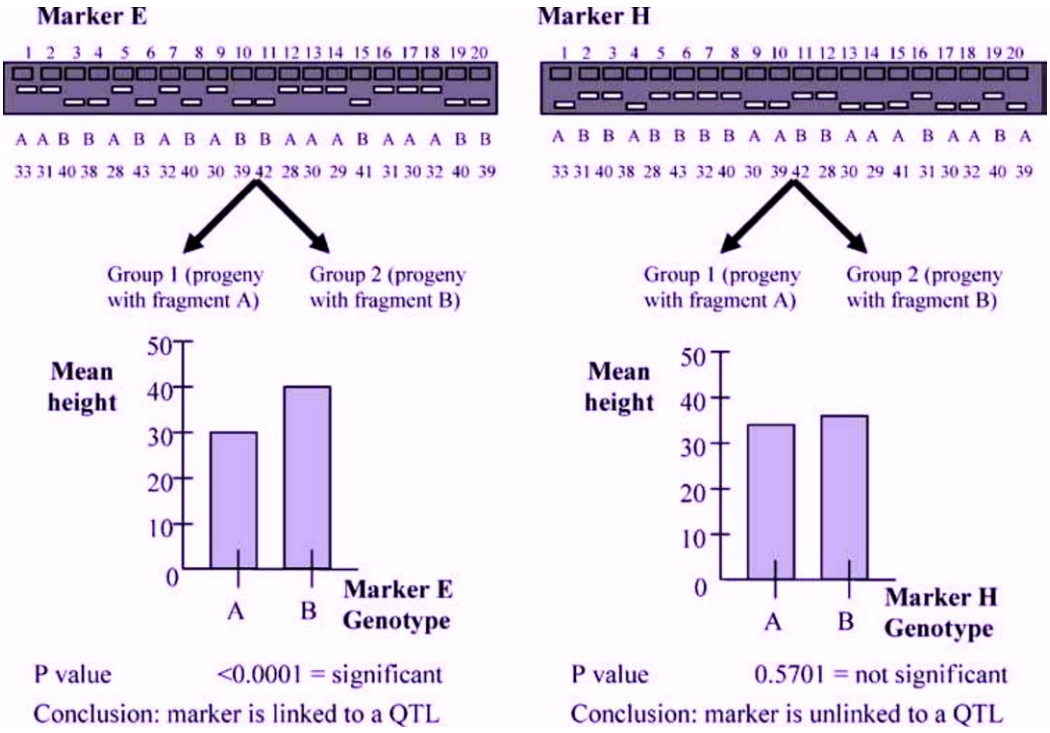


- **Phenotyping of the progenies**
- **Genotyping of the progenies**
- **Construction of linkage map**
  - Screening the mapping population using polymorphic molecular markers
  - Segregation patterns
  - Data is then analyzed using a statistical package such as **MAPMAKER** or **JOINMAP**
  - Assigning them to their linkage groups on the basis of **recombination** values
  - For practical purposes, in general recombination events considered to be less than 10 recombinations per 100 meiosis, or a map distance of less than 10 centiMorgans(cM).



QTL analysis

It is based on the principle of detecting an association between phenotype and the genotype of the markers. Markers are used to partition the mapping population into different genotypic groups based on the presence or absence of a particular marker locus and to determine whether significant differences exist between groups with respect to the trait being measured. A significant difference between phenotypic means of the groups, depending on the marker system and type of mapping population It is not easy to do this analysis manually and so with the help of a computer and a software it is done. The segregation data of both the phenotype and the genotype are collected and arranged in an excel sheet for QTL analysis using the appropriate software.

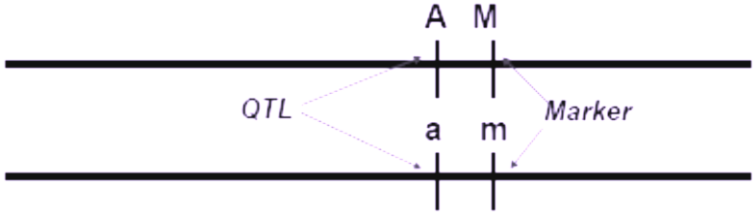


Methods to detect QTLs

- Single-marker analysis,
- Simple interval mapping and
- Composite interval mapping
- Multiple Interval Mapping
- Bayesian Interval Mapping

Single-Marker Analysis (SMA)

It is also known as single- point analysis. It is the simplest method for detecting QTLs associated with single markers.



This method does not require a complete linkage map and can be performed with basic statistical software programs. The statistical methods used for single-marker analysis **include t-tests, analysis of variance (ANOVA) and linear regression.** Linear regression is most commonly used because the coefficient of determination (R_2) from the marker explains the phenotypic variation arising from the QTL linked to the marker.

Limitations

- Likelihood of QTL detection significantly decreases as the distance between the marker and QTL increases
- It cannot determine whether the markers are associated with one or more markers QTLs
- The effects of QTL are likely to be underestimated because they are confounded with recombination frequencies.

To overcome these limitations **the use of large number of segregating DNA markers covering the entire genome may minimize these problems.** QGene and MapManager QTX are commonly used computer programs to perform singlemarker analysis.

Simple Interval Mapping (SIM)

It was first proposed by **Lander** and **Bolstein**. It takes full advantages of the linkage map. This method evaluates the target association between the trait values and the genotype of a hypothetical QTL (target QTL) at multiple analysis points between pair of adjacent marker loci (target interval). Presence of a putative QTL is estimated if the log of odds ratio exceeds a critical threshold. The use of linked markers for analysis compensates for recombination between the markers and the QTL, and is considered statistically more powerful compared to single-point analysis. MapMaker/QTL and QGene are used to conduct SIM. The principle behind interval mapping is to test a model for the presence of a QTL at many positions between two mapped loci.

Statistical methods used for SIM

Maximum Likelihood Approach

- It is assumed that a QTL is located between two markers, the two loci marker genotypes (i.e. AABB, AAbb, aaBB, aabb for DH progeny) each contain mixtures of QTL genotypes.
- Maximum likelihood involves searching for QTL parameters that give the best approximation for quantitative trait distribution that are observed for each marker class.
- Models are evaluated by comparing the likelihood of the observed distributions with and without finding QTL effect
- The map position of a QTL is determined as the maximum likelihood from the distribution of likelihood values.

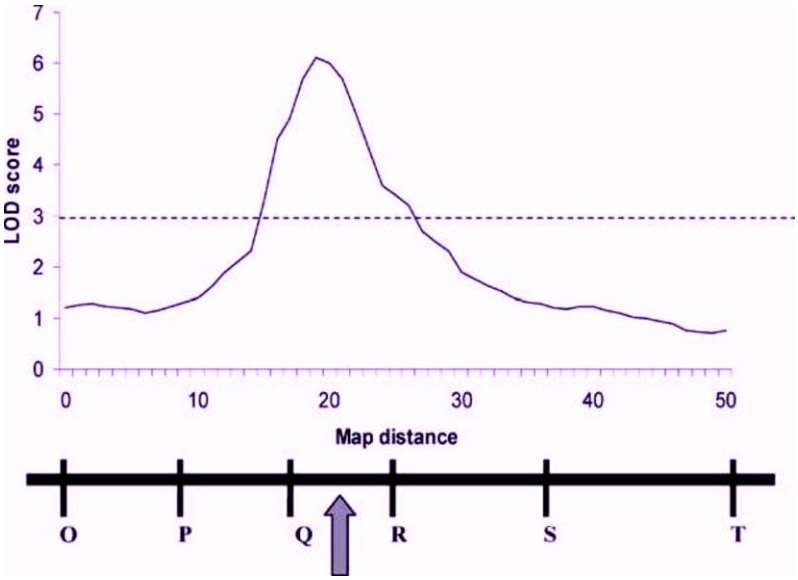
Logarithm of the odds ratio (LOD score):

- Linkage between markers is usually calculated using odds ratio.
- This ratio is more conveniently expressed as the logarithm of the ratio, and is called a logarithm of odds (LOD) value or LOD score.
- LOD values of >3 are typically used to construct linkage maps.

$$\text{Odds ratio} = \frac{\text{probability of the data occurring with a QTL}}{\text{probability of the data occurring with no QTL}}$$

- LOD of 2 means that it is 100 times more likely that a QTL exists in the interval than that there is no QTL.

- LOD of 3 between two markers indicates that linkage is 1000 times more likely (i.e. 1000:1) than no linkage.
- LOD values may be lowered in order to detect a greater level of linkage or to place additional markers within maps constructed at higher LOD values.
- **The LOD score is a measure of the strength of evidence for the presence of a QTL at a particular location.**



Hypothetical output showing a LOD profile for chromosome 4. The dotted line represents the significance threshold determined by permutation tests. The output indicates that the most likely position for the QTL is near marker Q (indicated by an arrow). The best flanking markers for this QTL would be Q and R.

Interval Mapping by Regression

It is essentially the same as the method of basic QTL analysis(regression on coded marker genotypes) except that phenotypes are regressed on QTL genotypes.

Since QTL genotypes are unknown they are replaced by probabilities estimated from the nearest flanking markers.

Softwares used: PLABQTL,QTL Cartographer, MapQTL

Composite Interval Mapping (CIM)

This method is developed by Jansen and Stam in 1994. It combines interval mapping for a single QTL in a given interval with multiple regression analysis on marker associated with other QTL. It is more precise and effective when linked QTLs are involved. It considers marker interval plus a few other well chosen single markers in each analysis, so that n-1 tests for interval – QTL associations are performed on a chromosome with n markers.

Advantages:

- Mapping of multiple QTLs can be accomplished by the search in one dimension.
- By using linked markers as cofactors, the test is not affected by QTL outside the region, thereby increasing the precision of QTL mapping.
- By eliminating much of the genetic variance by other QTL, the residual variance is reduced, thereby increasing the power of detection of QTL.

Problems

- The effects of additional QTL will contribute to sampling variation.
- If two QTL are linked their combined effects will cause biased estimates.

Multiple Interval Mapping (MIM)

It is also a modification of simple interval mapping. It utilizes multiple marker intervals simultaneously to fit multiple putative QTL directly in the model for mapping QTL. It provides information about number and position of QTL in the genome. It also determines interaction of significant QTLs and their contribution to the genetic variance. It is based on Cockerham's model for interpreting genetic parameters.

Bayesian Interval Mapping (BIM) (Satagopan *et al.* in 1996)

It provides a model for QTL mapping. It provides information about number and position of QTL and their effects. The BIM estimates should agree with MIM estimates and should be similar to CIM estimates. It provides information posterior estimates of multiple QTL in the intervals. It can estimate QTL effect and position separately.

Comparison of methods of QTL Mapping

Particulars	Interval mapping	Composite Interval Mapping	Multiple Interval Mapping	Bayesian Interval Mapping
1. Markers used	Two markers	Markers used as cofactors	Multiple markers	Two markers
2. Information obtained about	Number and position of QTL	Number and position of QTL and interaction of QTLs	Number and position of QTL	Number and position of QTL and their effects
3. Designated as	SIM	SIM	MIM	BIM
4. Precision	High	Very high	Very high	Very high

Merits of QTL Mapping

- Where mutant approaches fail to detect genes with phenotypic functions , QTL mapping can help
- Good alternative when mutant screening is laborious and expensive e.g circadium rhythm screens
- Can identify New functional alleles of known function genes e.g.Flowering time QTL,EDI was the CRY2 gene
- Natural variation studies provide insight into the origins of plant evolution
- Identification of novel genes

Limitations

- Mainly identifies loci with large effects.
- Less strong ones can be hard to pursue.
- No. of QTLs detected, their position and effects are subjected to statistical error.
- Small additive effects / epistatic loci are not detected and may require further analyses.

Future Prospects

- Constant improvements of Molecular platforms
- New Types of genetic materials (e.g. introgression lines: small effect QTLs can be detected)
- Advances in Bioinformatics.

9. Breeding and biotechnological approaches to improve nutritional quality of food crops

Ideotype concept:

Ideotype may be defined as a biological model, which is expected to perform or behave in a predictable manner within a defined environment. The idiotype concept was introduced by Donald in 1968. A crop ideotype performs best at crop densities (because it is a poor competitor), and when it is surrounded by plants of the same genotype. Such an ideotype is likely to be identified in yield tests based in homozygous and homogeneous progenies. Thus crop ideotype would differ markedly from the plant types that would perforrm best in competition (competition ideotype) or in isolation (isolation ideotype).

Development of Ideotypes:

Ideotypes are based on experimental findings and theoretical concepts related specifically to capacity for high grain/economic yield when grown as a crop. The capacity for high yield will depend on (i) yield per plant in the absence of competition, and (ii) response to competition from plants of the same genotype. The development of an ideotype involves the following considerations: (i) definition of the target environment where the ideotype is to be grown, (i) quality considerations, (iii) current agronomic practices, (iv) determining the desired traits in the ideotype, (v) determining the effects of changes in these traits on yield, (vi) use of simulation analysis for determining the effects, and (vii) other considerations.

The various groups of traits that limit growth and yield are as follows: (i) morphological and anatomical traits, (i) compositional traits, (iii) process rate traits and (iv) process control traits. The likely value of a trait in breeding may be assessed in the followng i) comparison of a series of varieties, (ii) correlation analyses, and (iii) construction of evaluation of experimental genotypes. This has been done in several crops and a number of cases of pleiotropic effects of important traits have been detected.

Choice of Traits for Ideotypes

The choice of traits for inclusion in an ideotype will depend on the following considerations: (i) importance of the trait in enhancing yield, (ii) ease, cost and speed in measurement of the trait, (iii) stability in the expression, (iv) presence of genetic variation, and (v) high heritability. A crop ideotype should have the following features: (i) it should be a weak competitor, (ii) should make minimum demand for producing each unit of dry matter, and (iii) should serve as an adequate sink for the photosynthates. Ideotype is a moving goal and is likely to involve concurrent modification of the environment. In addition, a crop ideotype should have (iv) high harvest index, (v) should be grown in a weed-free situation, and (vi) a planned selection has to be done for the features of crop ideotypes.

Ideotypes have been defined for a number ot crops, e.g., barley, wheat, maize, rice, cotton, *Brassica* spp. The ideotype of wheat is briefly described here. The main features of tnis ideotype are briefly described below.

1. Short strong stem: It reduces the risk of lodging, and would also reduce the amount of photosynthates invested in stem production. But the stem should not be excessively short as it may lead to mutual shading of leaves.

2. Erect Leaves: In a dense plant population, near vertical leaves will permit adequate illumination of a greater area of leaf suface than would long horizontal/drooping leaves. This would be applicable to all such species in which the leaf is nearly saturated for photosynthesis at a light intensity substantially below the prevailing light intensity, e.g. in rice, wheat, barley. Vertical leaves are associated with higher productivity, low competitive abilty, and greater tolerance for crowding.

3. **Few Small Leaves**: The number of leaves on the main shoot of wheat ranges from 7 to 20 or more; the axils of lower leaves give rise to tillers. In a monoculum plant, leaves serve mainly as photosynthetic, respiring and transpiring surfaces. Therefore, few small leaves per uniculum plant are desirable as the planting density will be high.
4. **Large Ear:** This would mean large number of fertile florets per ear, i.e. per unit of dry matter of above-ground parts. This is highly desirable since in wheat ear is normally limiting sink for photosynthates.
5**. Erect Ear:** This will ensure illumination of ears from all sides and, thereby, greater photosynthesis. This is a common trait in commercial varieties.
6. **Presence of Awns**: Awns provide substantial photosynthates to the developing grains more than 10% of grain dry weight), especially, under semiarid conditions. This is because awns are xerophytic structures.
7. **Single Culm:** In tillering genotypes, at least some of the tillers do not produce ears; this represents a waste of resources and additional useless competition. This problem will not be encountered in the uniculm genotypes. Nontillering mutants are available in barley and monoculm lines have been developed in wheat.
8. **Other Traits:** The parents used for developing the proposed ideotype must include high yielding and locally adapted cultivars; they should also include resistance to the diseases prevalent in the locality. Other desirable traits may be early flowering, longer grain-filling period and, possibly, heavy accumulation of sugar in the stem followed by the maximum translocation to the growing grain.
The above ideotype was proposed for an agronomic practice having the following main features: (1) suitably increased planting density, (2) heavy nutrient supply, (3) narrower rows, (4) effective weed control, and (5) assured moisture availability.

Ideotype Breeding

Ideotype breeding aims to enhance genetic yield potential by modifying individual's traits to their predefined optimum levels. It consists of the following four steps (i) development of an ideotype, (ii) creation of adequate genetic diversity for the concerned traits, (iii) selection of plants/lines with the desired phenotypes, and (iv) evaluation of the phenotype in several genetic and cultural backgrounds.

Limitations of or Limiting Factors in ldeotype Breeding

Progress of idegtype breeding may be hindered due to the following: (i) symmetry in size of plant parts, (ii) compensation among plant parts, (iii) pleiotropy and (iv) inferior genetic background. The main problems in ideotype breeding are as follows (i) use of physiological and biochemical traits is problematic, (ii) no trait enhances yield universally, (iin) need for teamwork, (iv) need for considerable breeding effort, etc.

Breeding for Quality:

A trait that defines some aspect of produce quality is called a **quality trait.** Each crop has a specific set of quality traits, which may be classified into the following groups: (i) morphological, (ii) organoleptic, (iii) nutritional, (iv) biological, and (v) others. For example, the important quality traits of wheat are grain size, grain colour, grain texture, protein content, gluten content, Polshenke value, sedimentation value, alveogram and mixogram

Nutritional Quality:

Nutritional quality of a produce determines the etfects on animal/human health of continued consumption of the produce. It has the following component traits: (i) protein content, (ii) protein quality, (ii) protein digestibility, (iv) oil content, (v) oil quality, (vi) vitamin content. (vii) mineral content and (vii) antinutritional factors.

Important quality traits of some selected crops

Crop	Trait	Preferred type	Remarks
Food Crops			
Rice	Hulling and milling recovery	High	Range, ~70-79% (total rice)
	Endosperm appearance	Transparent	Opaque appearance due to loose packing of starch; polygenic, low heritability
		Nonwaxy	A single dominant gene
	Length and shape of grain	Long slender (in India)	*Five groups: LB, LS, MS, ShB, ShS; polygenic, high H.
	Cooking quality		
	1. Amylose content	Intermediate	Groups: Low (up to 20%), intermediate (21-25%), high (>25%); single gene
	2. Grain elongation	Greater elongation	—
	3. Volume expansion	More expansion	—
	4. Water absorption	Intermediate absorption	—
	5. Gelatinization temperature	Intermediate	Range, 56-79°C
	6. Grain elongation on cooking	Preferred	Special trait of 'basmati' rices
	7. Disintegration in 1.7% KOH (milled rice grain)	Intermediate	Indicates gelatinization temperature
	Protein content	Deep diffused network of proteins	Range, 6-18%; polygenic; good amino acid balance; lysine content 3.8-4%
	Aroma	Presence of aroma	Scented types fetch higher price; oligogenic; recessive
Wheat	Grain size	Bold, plump	—
	Grain colour	Amber to white	—
	Grain texture	Hard, vitreous, lustrous	—
	Protein content	High	Atlas 66 and Nap Hal have over 19% protein
	Gluten content	High	C273 and UP302 have high gluten (~13%)
	**Polshenke value	Strong (>150 min)	Suitable for bread making
		Medium strong (81-150 min)	Suitable for *chapati* making
		Weak (<80 min)	Suitable for biscuits and confectionery
	Sedimentation value	20-40	All purpose wheats; lower the value, weaker the gluten
	Alveogram	Strong (>300), medium strong (200-300), weak (<200)	Measures gluten strength

	Mixogram	—	Indicates baking quality; also gluten strength
Cotton	Fibre colour	White	Colours: cream; light/medium/ dark brown, light green; oligogenic
	Fibre length		
	1. Halo length	Long	Length of fibre with attached seed
	2. Mean length	Long (24.5 to 26.0 mm)	Mean length of all the fibres present in a sample
	3. 2.5% span length	Long (28 to 31.5 mm)	Length of the longest 2.5% of fibres
	Fibre strength	Good to very good (43.1 to >47.5 g/tex)	Determines yarn strength
	Fibre fineness	Fine to very fine (<3 micronaire value)	Determines spinning capacity of cotton, uniformity and strength of yarn
	Fibre maturity	Good to very high maturity (maturity coefficient > 68)	Index of the degree of fibre development; depends on secondary wall deposition.
Tomato	**A. For fresh use**		
	Appearance		
	1. Size	Large	Polygenic
	2. Shape	Round	Oligogenic (allele *O*)
	3. Colour	Red	Allele og^c increases lycopene (red colour); *hp* increases carotenoids and gives excellent colour
	Texture and firmness	Firm (for transportation), soft and juicy for fresh consumption	—
	Flavour	—	Mainly due to acids and sugars, volatile compounds, etc.
	Nutritional value	Vitamin A Vitamin C	Affected by og^c and *hp* Some varieties have up to 50 mg/100 g fresh fruit
	B. Processing quality		
	Colour	Red	Determined by colorimeter
	Fruit pH	<4.5	Important for effective sterilization
	Titratable acidity	High	Expressed as per cent citric acid; polygenic
	Total soluble solids (TSS)	High	Mainly due to sugars; estimated as refractive index
	Viscosity	High	Esimated by passing juice through viscometer or as insoluble solids.

* L, Long; S, Slender; M, Medi...

Antinutritional Factors

A compound that produces adverse effects on animal/human health on continued consumption is called **antinutritional factor**. Several crops contain antinutritional factors, e.g., erucic acid and glucosinolates in *Brassica* spp., gossypol in cotton, haemagglutinin in faba beans, an oligopeptide causing goitre in soybean, phenolic glucoside causing goitre in groundnut seed skin, BOAA (β-N-oxalyl-α-β-diaminopropionic acid) in khesari, cyanogenic glucoside in forage legumes, phytic acid in pearlmillet and trypsin inhibitors in soybean.

Sources of Quality Traits

The quality traits may be governed by oligogenes, polygenes and some traits may even show maternal effects. The quality traits may be obtained from (i) a cultivated variety. (i) a germplasm line, (iii) a spontaneous or induced mutant, (iv) a somaclonal variant, (v) a wild relative and (vi) a transgenes. Transgenes provide a powerful tool for modification of quality traits; such modifications have been successfully done in the case of both protein and oil quality.

TABLE 23.5. Selected examples of antinutritional factors present in crop plants

Antinutritional factor	*Crop*	*Effect on health*	*Remarks*
BOAA (β-N-Oxalyl-α-β-diaminopropionic acid)	*Khesari (L. sativus)*	Lathyrism (paralysis of lower limbs)	Content reduced to 0.03-0.05% from the normal 0.55%
Erucic acid	*Brassica* spp.	Reduced body weight gain	'Zero' varieties developed
Glucosinolates	*Brassica* spp.	Goitre (in extreme cases)	'Zero' varieties developed
Gossypol	Cotton	Toxicity	Involved in insect resistance
Haemagglutinin*	Broad bean (*Vicia faba*)	Favism	Due to the consumption of raw broad beans
Oligopeptide (low molecular weight)	Soybean	Goitre	Destroyed by cooking/ heat treatment
Phenolic glycoside	Groundnut seed skin	Goitre	Destroyed by cooking/ heat treatment
Cyanogenic glycoside	Forage legumes (particularly)	HCN toxicity	Removed by breeding
Phytic acid	Pearlmillet (bajra)	Mineral deficiency	Occurs in seed coat and germ
Trypsin inhibitor*	Soybean	—	Destroyed by heat processing

Breeding for Protein Quality:

Human protein requirements are met mainly from plant sources. Protein quality concerns with the amino acid balance of proteins. Human body can not synthesize the following amino acids: Ile, Leu, Lys, Met, Phe, Thr, Trp and Val; these are called essential amino acids. Cereal proteins are deficient in Lys, Trp and Thr, those of pulses in Met and Trp, of nuts and oilseeds in Lys and those of green leafy vegetables in Met.

TABLE 23.6. A selected list of wild relatives from which quality traits have been/can be transferred

Crop	*Wild relative*	*Quality trait*
Cotton	*G. thurberi*	Fibre strength (latent trait)
	G. armourianum	Fibre strength (latent trait)
	G. anomalum	Fibre fineness, strength and maturity
	G. raimondii	Fibre strength and fineness
	G. tomentosum	Fibre strength and fineness
Tomato	*L. hirsutum*	Fruit colour, carotene content (latent trait)
	L. pimpinellifolium	Vitamin C content
	L. peruvianum	Vitamin C content
Pigeonpea	*Atylosia* spp.	High protein content
Soybean	*Glysine soja*	High protein (has 45% protein), reduced lipoxygenase activity
Oats	*Avena sterilis*	High protein (~27% protein)

TABLE 23.7. Essential amino acids (EAA) deficient in some selected vegetarian foods

Food	*Deficient EAA**	*Deficiency due to the protein fraction*
Cereals	Lys, Trp, Thr	Prolamines
Pulses	Met, Trp	
Nuts and oilseeds	Lys	
Green leafy vegetables	Met	

TABLE 23.8. The list of mutants affecting protein quality in cereals

Crop	Mutant	Origin	Features	Remarks
Maize	*Opaque-2* (o_2)	Spontaneous	High Lys, Trp	Reduced prolamine
	Opaque-7 (o_7)	Spontaneous	High Lys, Trp	Reduced prolamine
	Floury-2 (*fl2*)	Spontaneous	High Lys and S-amino acids	Reduced prolamine
	Brittle-2	Spontaneous	High Lys, Trp	Modified starch, reduced prolamine
Barley	Hiproly (*lys*)	Spontaneous	High protein, Lys	Also several minor genes
	Notch-1	EMS*	High protein, Lys	Reduced prolamine
	Notch-2	EMS	High protein, Lys	Reduced prolamine
	Riso 1580	EI**	High lysine	Reduced prolamine; qualitative change in proteins
Sorghum	IS11167 (*hl*)	Spontaneous	High lysine	Shrivelled endosperm, reduced prolamine
	IS11758 (*hl*)	Spontaneous	High lysine	Shrivelled endosperm, reduced prolamine
	P-721 opaque	DES†	High lysine	Reduced prolamine; monogenic; opaque endosperm; modifying genes make the endosperm vitreous
	P-721 vitreous††	DES	High lysine	Reduced prolamine

Approaches for Breeding for Protein Quality:

The breeding approaches for quality traits are as follows: (i) screening of germplasm, (ii) mutagenesis, (ii) hybridization, (iv) interspecific hybridization (e.g. fruit colour, fruit size, provitamin A and vitamin C contents in tomato), (v) somaclonal variation eg. Scarlet variety of sweet potato), and (vi) genetic engineering. Genetic engineering allows modiication of protein quality by following two strategies: (i) introduction of an appropriate transgene (e.g., 7s legume seed storage protein transferred in rice), and (ii) modification of endogenous genes. Antisense RNA technology has been used to suppress the function of endoganous genes to modity quality, e.g., delayed fruit softening in tomato (by suppressing polygalacturonase gene).

It is important that improved quality (i) should not increase produce cost, (ii) should be present in a variety having high and stable yield, (iii) breeding for quality should be integrated with breeding for yield, (iv) increased protein content is not associated with lower yields and (v) a close interaction between breeders and nutritionists will increase the rate of progress in nutritional quality.

Priorities in Quality Improvement:

The priorities in quality breeding are as follows: (i) consumer acceptance. (ii) yield can not be sacrificed for quality, (iii) elimination of antinutritional factors has a high priority, (v) improved protein quality should have a higher priority than increased protein content (v) in cereals like maize and barley, improving Lys content should have priority, (vi) in rice, improved distribution of proteins is of high priority to prevent protein loss during milling, (Vii) in wheat, high protein content is the only option (since high Lys lines are not available), and (vi) in pulses, breeding for yield and resistance to stresses should be the priority.

Selection Criteria for Protein Content

Selection criteria for protein content should be total protein yield per plant or per unit area. Increased protein per grain must not be associated with a reduction in either grain number per spike or in number of spikes per unit area.

Breeding for Oil Quality

Lipids are very rich in energy, and vegetable oils rank first in both national and international trade. The daily recommended intake of oils is 30g/day per person. Oils and fats are triglycerides (one glycerol molecules linked to 3 fatty acid molecules). Fatty acids are long chain aliphatic organic acids, may be saturated or unsaturated (1, 2 or more double bonds in the carbon Skeleton). The properties of fetty acids depend on the numbers of carbon atoms and double bonds. The physical properties and nutritional value of oils is determined by the types and proportions of the fatty acids in them. Fatty acids are synthesized using acetyl-CoA, which is first converted into malonyl-CoA; the latter is used for chain elongation by adding 2 carbons each time, e.g., 2 (acety1-CoA)→4→6→8→10→12→14→16→18→20→22, etc. This involves 7-8 distinct enzymes. Unsaturated fatty acids are produced from saturated fatty acids by the action of specific desturases, e.g., stearic acid (18C) gives unsaturated oleic 91 double bond), linoleic (2 double bonds) and linolenic acids (3 double bonds).

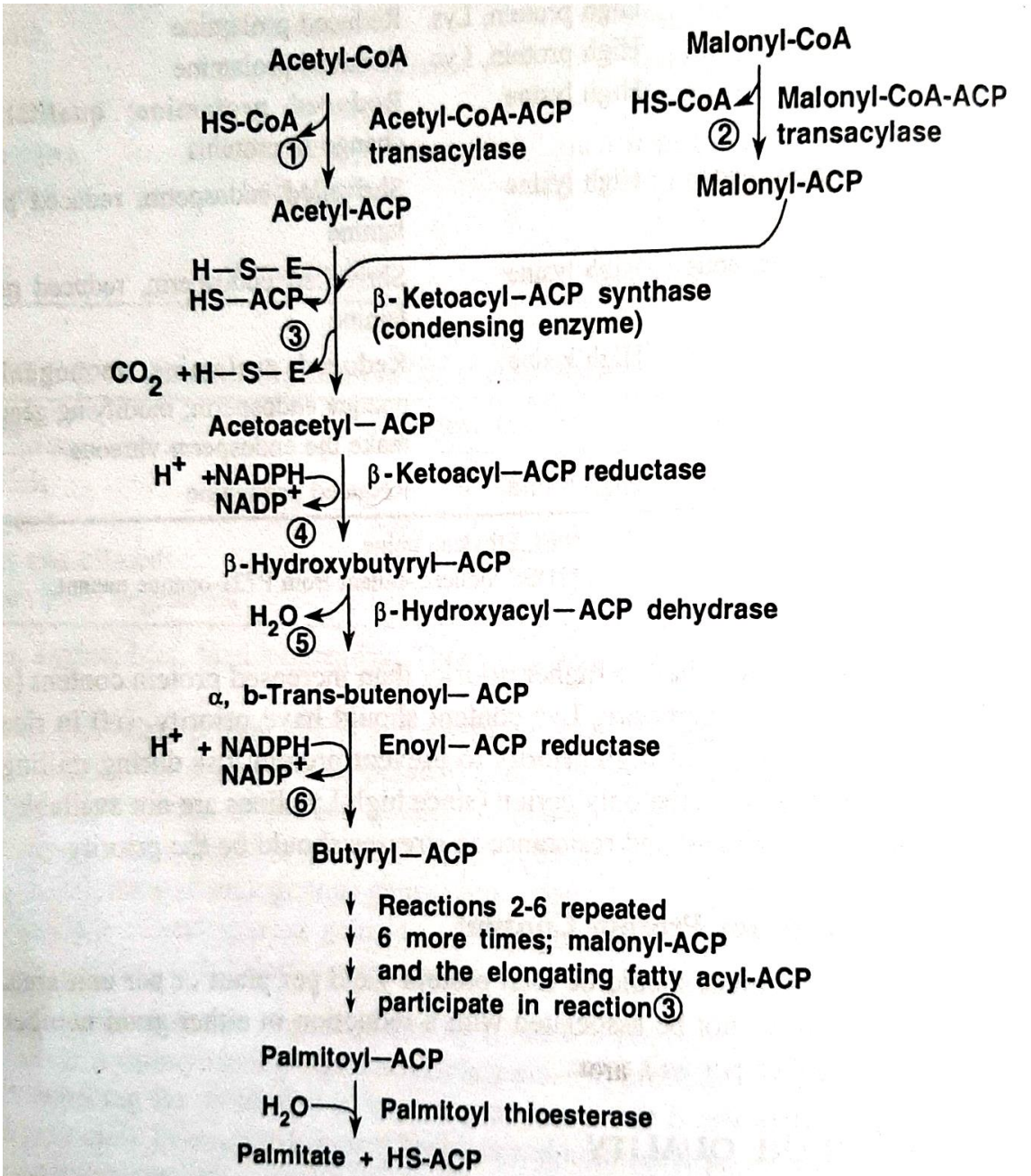


Fig. 23.1. A schematic representation of biosysthesis of palmitate (palmitic acid 16 : 0).

TABLE 23.9. Common fatty acids found in seed oils (based on Mitra and Bhatia, 1979)

Fatty acid	*Number of carbon atoms*	*Number of double bonds*	*Production value**
	Saturated fatty acids		
Lauric acid	12	0	0.361
Myristic acid	14	0	0.349
Palmitic acid	16	0	0.340
Stearic acid	18	0	0.333
Eicosanoic acid	20	0	0.328
Behemic acid	22	0	0.323
Lignoceric acid	24	0	0.320
	Unsaturated fatty acids		
Palmitoleic acid	16	1	0.327
Ricinoleic acid	18	1	0.333
Oleic acid	18	1	0.323
Linoleic acid	18	2	0.311
Linolenic acid	18	3	0.300
Eicosenoic acid	20	1	0.318
Erucic acid	22	1	0.313
Nervonic acid	24	1	0.309

Oilseed Crops

Oils are extracted from seeds of certain plant species called oilseed plants. Oil yields have been greatly enhanced by breeding, e.g, in case of sunflower from 30% to 54%. Oil quality may be defined as the types and the proportions of fatty acids present in a given oil; this Varies greatly from one plant species to the other. For example, our important oilseed crops like maize, groundnut, soybean, sunflower, safflower, sesame, cotton, etc. produce oil rich in oleic and linoleic acids. Oil quality is determined mainly by the proportion of oleic and linoleic acids. Linolenic acid is readily oxidized and gives a rancid off-flovour; therefore the content of this fatty acid should be less than 1% in edible oils. The genetic control of fatty acid composition varies from crop to crop. For example, in the case of soybean, it is affected by maternal genotype, shows low heritability and is affected by location and year. Similarly, in the case of groundnut, fatty acid composition varies due to variety and, even, location. Sesame oil does not have linolenic acid, it also has an antioxidant called sesamol.

TABLE 23.10. Fatty acid composition of oils from some important oilseed crops (based on Mitra and Bhatia, 1979 and Khanna and Singh, 1991)

Crop	*Oil content (%)*	*Fatty acid composition (g/100 g fatty acids)*									*Total unsaturated fatty acids (%)*
		12 : 0	*14 : 0*	*16 : 0*	*18 : 0*	*18 : 1*	*18 : 2*	*18 : 3*	*20 : 1*	*22 : 1*	
Major Oilseeds											
Coconut	—	50	15	9	2	7	1	—	—	—	
Cotton seed	25	—	—	23.4	2.5	17.9	54.2	—	—	—	
Groundnut	45	—	—	6.7	4.3	71.4	11.1	—	—	—	
Oilpalm (*E. guineensis*)	56+	—	1.1	44.0	4.5	39.2	10.1	—	—	—	
Rapeseed*	—	—	—	3	0.8	9.9	13.5	9.8	6.8	53.6	
Mustard	48	—	—	3.4	—	25.3	24.3	12.7	12.6	21.9	
Soybean	20	—	—	15	4	24	49	8	—	—	
Sunflower	29	—	—	5.6	6.5	19.1	67	—	—	—	
Others											
Maize	5	—	—	11.5	2.2	26.6	58.7	0.8	—	—	
Linseed**	38	—	—	6.3	2.5	19.5	24.1	47.4	—	—	90.5
Safflower	33	—	—	7.2	1.9	12.4	78.5	—	—	—	90.5
Sesame	54	—	—	8.5	4.2	47.3	39.3	—	—	—	

Canola Quality Oil:

The oils from *Brassica* spp. and other cruciferous plants, e.g., taramira (*Eruca sativa*), are rich in erucic acid (in some species, up to 6096), and contain glucosinolates; both of these are considered as antinutritional factors. Therefore, oils from such species should contain these compounds in very low, if possible zero, amounts. The first 'zero' erucic acid selection was obtained by Downey in 1964 in *B. campestris*. Zero' erucic acid lines have <3.0% erucic acid such lines have been obtained in *B. Campestris*, *B. juncea* and *B. Napus*. A 'zero' glucosiuolate line has <30 µmol g^{-1} glucosinolate in defatted oilseed cake. **'Canola'** quality oil has "zero' erucid acid ard glucosinolate contents ('double zero' lines). Fatty acid composition is mainly oligogenically controlled and is governed by the genotype of the embryo. In contrast, glucosinolate content is determined by the maternal genotype, and also by plasmagenes; it is affected by 6-8 genes with additive effects.

Linseed oil, on the other hand, has 35-53% linolenic acid, is unsuitable as edible oil, and has industrial uses. Fatty acid composition is governed by both major and minor genes, and by maternal genotype. Low linolenic acid lines have been isolated; they show a corresponding increase in linoleic acid content.

Breeding Approaches for Oil Quality

The various breeding approaches for oil quality are as follows: (i) domestication (e.g., of jojoba, *Simmondsia chinensis*), (ii) mutation (e.g., a mutant of *B. juncca* has low (3.6%) linolenic acid), (iii) selection (e.g "zero erucic acid lines of *B. campestris*), (iv) hybridization with selected lines/mutants (selected for oil quality; in most cases, fatty acid composition is govened by 1 to 3 additive genes with major effects, i.e., polymeric gene action), (v) interspecific hybridization, (vi) somaclonal variation (including variants from anther culture; some encouraging results), (vii) somatic hybridization (e.g., to obtain high erucic acid *B. napus* lines; erucic acid has many industrial applications), and (viii) genetic engineering (both exprssion of transgenes to produce new fatty acids, and suppression of endogenous gene to increase the content of a desired fatty acid). The *B. napus* variety "Laurical" has high lauric acid (12C, 0 double bond); it was produced by genetic engineering.

Biotechnological approach:

Genetic engineering has enabled the development of insect pest and/or disease resistant varieties in many crop species; many of varieties are already in cultivation. It has also become feasible to develop such varieties that are suited to specific consumer, industrial, etc. needs. Such varieties are popularly termed as '**designer crops**' since they possess such specific features as were specified before their development, i.e., they are specifically designed to meet certain specified requirements. The designer crops may have a specified protein, fat or starch quality; nutriuonal feature like vitamin content, elimination of an antinutritional factor, colour, flavour, taste, etc. of fruit/grain; keeping quality: etc., or may produce a specific novel biochemical of pharmaceutical/industrial value, or may have some other features that make them more useful to humankind.

It is interesting that the first transgenic variety to attain commercial status was one that was modified for a quality trait, viz., "FlavrSavr variety of tomato that showed delayed fruit softening; it was approved for commercial cultivation in 1994. Since then some other transgenic varieties have been developed that show modificatlion of one or the other quality trait or possess some novel features. In this chapler, we shall consider in some detail some of the important cases of designer crops mainly with a view to highlight their biological basis, technological innovation, and the associated limitations, if any. These considerations relate to (1) modifications of starch, (2) oil and (3) protein qualities, (4) suppression of endogenous genes, (5) male sterility, and (6) biochemical production.

Modification of Starch Quality

Starch, a polymer of glucose, is the chiel storage polysaccharide in plants; it is stored in photosynthetically inactive organs like roots, modified shoots and seeds. The starch grains contain amylose and amylopectin. Amylose is usually a linear molecule, while amylopectin, is highly branched, and is the major component of starch granules. The properties of starch molecules are mainly detemined by the following: (i) chain length, (ii) types of bonds linking the glucose units, and (iii) the degree of branching (including branch length). The important enzymes for starch biosynthesis are as follows: (i) AGPase. (ii) SSs. (iii) SBEs and (iv) SDBEs.

Modifications of Starch:

Starch is used in the diet of much of the world's population. It is used in food and beverage industries as a thickener and sweetener. Starch is also useful in the paper and textile industries. There are three basic strategies to modify starch quality: (i) genetic modification of crops (by breeding or biotechnology), (ii) food processing (using enzymes and chemicals), and (iii) food supplementation (modification of starch by employing microorganisms). The modified forms of starch have varied applications in food and other industries.

Starch Modification by Genetic Engineering:

Genctic modilication of crops to obtain starch with changed properties is desirable to chemical/enzymatic modification since it reduces cost, effort and the environmental damage. Genetic engineering offers possibilities for creating novel starch with new functional properties. The genes encoding the main enzymes of starch biosynthesis have been cloned and used to produce transgenic lines.

Granule-bound starch synthase I (GSS) is the main enzynme of amylose biosynthesis. Therefore, increased uctivity of GBSS I is expected to increase the amylose content of starch, while suppression of GBSS I should enhance the amylopectin content. Suppression of GBSS I activity in potato (by antisense RNA technology) yielded an amylose-free or "waxy" starch in potato. This was the first successful genetic modification of starch reported in 1991.

Soluble starch synthase (SSs) is the main enzyme of amylopectin biosynthesis; it has two isoforms called SSS I and SSS II, A freeze-thaw stable potato starch has been created by simultaneous down-regulation of all the three starch synthase, viz., GBSS I and SSS I and SSS II, genes, using antisense RNA technology. This manipulation yielded an amylose-free short chain amylopectin. This starch is extremely freeze-thaw stable, and shows no syneresis even after five freeze-thaw cycles. **Syneresis** is the separation of starch gel and water phases. The use of this starch has potential for environmental and consumer benefits as its production requires no chemical modification.

Starch branching enzyme (SBE) activity is required for amylopectin biosynthesis. An increased SBE actvity is expected to increase branching, while its suppression should reduce the amount of amylopectins. In potato, SBE activities were suppressed to <1% of the wild type by using antisense constructs of the genes concerned. This resulted in a very high amylose starch; this unique starch (high amylose, low amylopectin, and high phosphorous levels) has novel food and industrial applications.

Modification of Oil Quality:

Vegetable oils and fats constitute an important component in human diet. They are very rich source of energy, are an important source of certain vitamins and hormones (in animals) and form structural components of cells. Mammals (including humans) can not synthesize linoleic (18:2) and linolenic (18:3) fatty acids. Therefore, they must be supplied in their diet, and they are called **essential fatty acids**. Further, oils and fats have many industrial applications, including manufacture of soaps, detergents, paints, varnishes, lubricants, synthetic resins, plasticisers, etc.

Oil qualty may be defined as the types and proportions of different fatty acids present in the given oil, which determine the uses to which this oil can be put to. The properties of a fatty acid, in turn, depend on its chain length and the number of double bonds present in its carbon chain.

Modification of Oil Quality

Oil quality can be changed by genetic modification of crops or by chemical modification of the fatty acids. Conventional breeding has been remarkably successful in modifying Oil quality. Genetic engineering now offers unique opportunities to produce novel fatty acids. The various strategies used for modifying fatty acids are as follows: (i) introduction of a novel enzyme, e.g, an acyl transferase or an acyl-ACP thioesterase, acyl-ACP desaturase, etc., (i) suppression of an enzyme acuvity, e.g., a acyl-ACP desaturase), (ii) site-directed mutagenesis to alter the specificity, etc. of an enzyme, e.g., acyl-ACP desaturase, etc., and (iv) creation of hybrid genes to generate novel enzyme activities. Some of the interesting examples are summarised in Table, and are briefly described below.

TABLE 12.2. Some selected examples of fatty acid modification by genetic engineering

Target enzyme	*Source organism*	*Expressed in*	*Phenotypic effects/Remarks*
Lauroyl-ACP thioesterase	California bay (*Umbellularia californica*)	*Brassica napus*	Some lines accumulated upto 60% lauric acid in oil; released for cultivation as 'Laurical'
Δ^6 Fatty acid desaturase	Borage (*Borage officinalis*)	Tobacco (*N. tabacum*)	Accumulated ~13% γ-linoleic acid, and ~10% octadecateraenoic acid
Stearoyl-ACP desaturase (antisense construct)*	*Brassica* sp.	*B. napus*	A dramatic increase in stearate level in the oils
sn-2 acyl transferase	Yeast (*S. cerevisiae*)	*Arabidopsis, B. napus*	Increased (8-48% more) oil content of seeds; increased levels of very long chain fatty acids
Δ^6-Stearoyl-ACP desaturase	Coriander		Petroselinic acid accumulated upto 5% of the total fatty acids.

Perhaps the most successful example is the increased lauric acid content of *B. napus*; a transgenic line name 'Laurical' has been released for commercial cultivation. Lauric acid does not occur naturally in *Brassica* sp. oil. But seeds of undomesticated California bay (*Umbellularia californica*) accumulate laurate (12:0). The gene encoding lauroyl-ACP thioesterase was isolated from *U. californica* and transferred into *B. napus*. Some of the transgenic lines showed upto 60% lauric acid in their oils. There is some evidence that a part of the lauric acid in transgenic *B. napus* is subjected to β-oxidation.

Modification of Seed Protein Quality:

Cereal seed proteins are deficient in lysine, while those of pulses are deficient in sulphur containing amino acids, e.g., methionine, and in tryptophan. This limits their nutritional value for man since these amino acids are essentialfor man. Therefore, improvement of seed storage protein quality is an important and seemingly feasible objective. The various approaches to achieve this objective are, (1) introduction of an appropriate transgene, (2) modification of the endogenous protein encoding gene, (3) expressing a suitably designed synthetic gene, (4) over-expressing a homologous gene, and (5) suppression of genes encoding a less desirable protein fraction.

Introduction of an Appropriate Transgene

In this approach, a new gene encoding a storage protein, which is rich in the deficient anino acids, is introduced into the crop to correct its amino acid deticiency. The transgene is linked to a seed-specific or tissue-specific promoter to ensure its expression only in seeds or the tissue of choice. For example, a sunflower seed storage protein, sunflower albumin 8 (SFA8), contains 23% methionine plus cysteine. The gene coding for SFA8 has been cloned and transferred in forage species. It has not been

used in a food crop due to the possibility of it being allergenic although originally it was planned to be expressed in peas. The 25 seed protein of *Amaranthus* is rich in methionine. Gene amAI has been cloud and expressed in commercial cultivars of potato. Tubers of these transgenic potatoes have 600 more protein, improved amino acid balance, and moderate inerease in yield as well.

In a recent study, the 7S legume seed storage protein, β-phaseolin, gene (driven by rice storage protein gene gtI, glutelin 1) promoter was transferred in rice. Transgenic rice plants expressed the gene in their endosperm, and some plants showed up to 4% of their total proteins to be β -phaseolin. The 11S legumin protein gene driven by gtI promoter has also been transferred, and expressed in rice endosperm.

Modification of Endogenous Genes

This approach is based on the isolation and modification of the concerned protein encoding gene sequence either by (i) replacing one or few codons with the selected codons or, generally, by (ii) inserting one or few selected additional codons at appropriate sites. For example, prolamine storage proteins, e.g.. zein, of cereals are deficient in the essential amino acids lysine and tryptophan. The γ-zein of maize is rich in sulphur containing amino acids. The gene encoding γ-zein was modified by inserting (Pro-Lys), encoding sequences at appropriate sites. The modified gene was expressed in maize, but the altered γ-zein was not stable. It is important that the insertion of additional codons must not affect structure, stability and/or function of the encoded proteins. Some other genes like napin gene of *Brassica* spp., glycinin gene of soybean, etc. have been modified and expressed in the concerned crops.

Rice gtI gene encodes the major rice seed storage protein. It has been modified to encode higher levels of lysine, tryptophan and methionine. The modified gtI gene driven by its own promoter was transferred into rice protoplasts: the resulting transgenic rice plants expressed the modified gene in their developing endosperm. Similarly, a modified zein protein gene encodes a protein having improved methionine content. This gene was introduced into maize, rice and wheat by microinjection, microprojectile bombardment or electroporation. Transgenic plants expressing the modified zein gene showed upto 3.8% methionine in their seed proteins.

The major problems of this approach are as follows: (i) the maintenance of open reading frames in the genes and (i) stability of the resultant proteins. Since lysine is hydrophobic, its insertion within a polypeptide, particularly in a stretch of hyrophilic amino acids, may influence the folding pattern and the stability of the resultant protein.

Overexpression of Homologous Genes

The gene encoding a desirable protein fractuon may be overexpressed (by introducing additional copies of the gene driven by a strong promoter) to increase tne concentration of this fraction and, thereby, enhance seed protein quality. Maize seed protein 10 kDa zein is rich in methionine. The gene encoding this fraction was overexpressed by increasing mRNA stability; this resulted in increased methionine content of maize grains. Similarly, high molecular weight glutelin subunits encoding genes have been overexpressed in wheat with a view to improve dough qualty.

Suppression of Endogenous Genes

In this approach, the genes encoding the undesirable seed protein fractions are suppressed by RNAi or antisense RNA technology in order to increase the accumulation ot the desired fraction. For example, Cruciferin is the dominant protein fraction in *Brassica* spp. and is deficient in S-containing amino acids. Suppression of CruA gene encoding Crucitern by antisense RNA technology resulted in decreased level of Cruciferin and increased level of Napin, which has a better amino acid balance.

This resulted in 10, 8 and 32 per cent increase in Lys, Met and Cys levels, respectively. RNAi and antisense RNA technologies were used to suppress tne expression of 19 and/or 22 kDa zein encoding

genes; this resulted in decreased accumulation of the respective α-zeins and in increased Lys and Trp contents.

Expression of Synthetic Seed Storage Protein Genes

Synthetic storage protein genes can be designcd to provide any essential amino acid composition and to ensure 100% bioavailability of these amino acids. For example, Du Pont (USA) scientists have synthesized and patented a gene encoding a protein, called CP3-5, containing 35% lysine and 22% methiomine. The CP3-5 gene was coupled with secd-specific promoters and transferred into maize; the gene was expressed by tissue cuture cells. A transgenic line of sweet potato has been developed by expressing a synthetic gene, which encodes a protein that has high contents of the essential amino acids like lysine, sulphur containing amino acids, etc. Different transgenic lines showed a 2.5 to 5-fold increase in the protein contents of their storage roots. It is noteworthy that the root yield was not adversely aftected; in fact, there was a slight (4.9-11.4%) improvement in the yield. Animal feeding experiments showed that the group fed on high-protein sweet potatoes weighed 56% more than did the controls after 28 days; in addition, there was no evidence of any toxic effects.

Golden Rice:

Vitamin A-deficiency causes blindness among children and may even lead to their death. Vit. A deiciency often occurs where rice is the staple food, since rice grain does not contain provitamin A. i. e. β-carotene. Three transgenes providing phytoene synthase. phytoene desaturase, zeta-carotene desaturase and lycopene cyclase activities were transferred into rice by *Agrobacterium*- mediated transformation. All the transgenes were introduced together in a single co-transformation experiment. The resulting transgenic rice, popularly called "golden rice" contains good quantities of β -carotene, which gives the grains a golden colour. In one transgenic line, the β -carotene content was as high as 85% of the total carotenoids present in the grain.

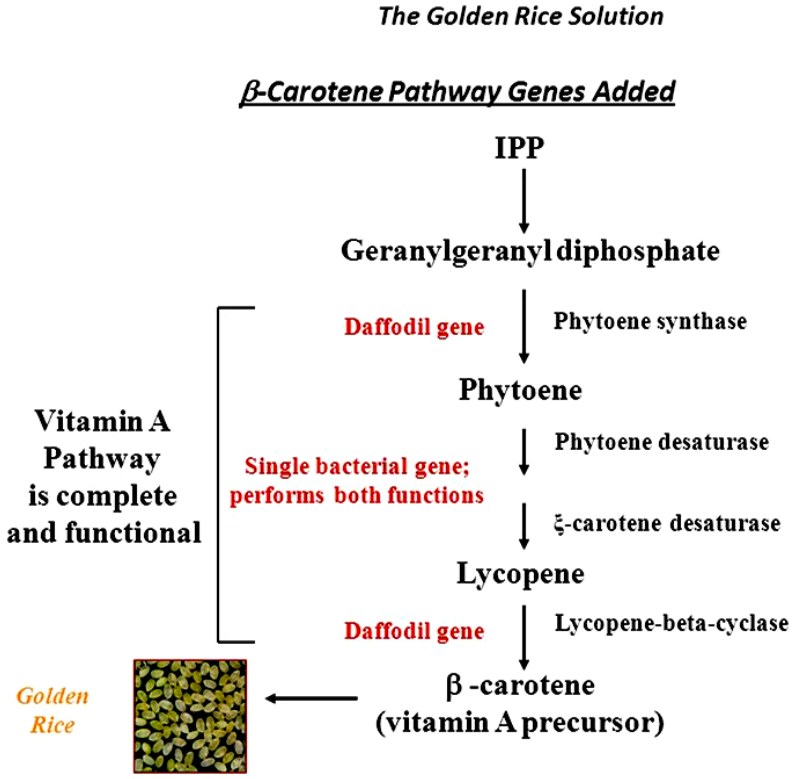
Iron-deficiency anaemia is the most common nutritional disorder in the world. Rice grain has the lowest iron content among crops; it also has phytate, which reduces iron absorption in human intestine by up to 98%. In addition, iron absorption from a vegetarian diet is rather poor. Phytate is used for phosphate storage in seeds, and it is used during seed germination. The high iron rice was produced expressing in rice three transgenes, viz., ferretin encoding gene, metallothionein gene and a thermostable phytase encoding gene.

A ‘high iron-high provitamin A’ rice line was produced by crossing the golden rice and "high iron' transgenic lines of rice. It is proposed to distribute this rice line free to subsistence farmers of the developing world with a view to alleviate vitamin A and iron deficicncies.

TABLE 12.3. Enzyme activities that must be provided by transgenes to enable the production of β-carotene, and the transgenes transferred to generate high iron rice

Enzyme activity	*Function*
Golden Rice	
Phytoene synthase (a plant gene)	Produces phytoene by condensation of two molecules of geranyl-geranyl pyrrophosphate, the last precursor of β carotene biosynthesis found in rice endosperm
*Phytoene desaturase	Converts phytoene into zeta-carotene
*ζ-carotene desaturase	Converts zeta-carotene into lycopene
Lycopene β-cyclase (a plant gene)	Converts lycopene into β-carotene, the cyclic C_{40} carotene, which is commonly known as provitamin A
High Iron Rice	
Ferritin gene from *Phaseolus*	Endosperm iron content increased by a factor of 2.5
A metallothionin-like gene from *Oryza*	Endosperm cystein content increased 7-fold; cystein enhances iron absorption in intestine
A mutant phytase gene from *Aspergillus fumigatus* encoding a thermostable enzyme	Hydrolyses phytate to inorganic phosphate and myoinositol. The mutant enzyme refolds to 80% activity after 20 min at 100°C, but the phytase expressed in rice, for some reason, failed to do so.

* In golden rice, both these activities were provided by the *Erwinia* phytoene desaturase encoded by gene *crtI*. The enzymes contained signal sequences for localization in plastids. Conversion of phytoene to lycopene involves a total of four desaturation steps.



Suppression of Endogenous Genes

In many crops, certain quality related traits can be improved by reducing/eliminating the expression of specific genes the level of endogenous gene expression can be reduced by the following four approaches: (1) antisense gene, (2) ribozyme, (3) gene disruption, and (4) overtranscription leading to co-suppression.

Antisense Gene Approach

Originally, the term 'antisense' was used to describe inhibition of mRNA translation by hybridization of an oligonucleotide to a selected region of the mRNA. Since mRNA represents the 'sense' sequence, the oligonuclecotide complementary to the mRNA was called 'antisense oligonucleotide. In any gene, the DNA strand oriented as 3'→5' in relation to its promoter is transcribed; this strand is called the **antisense strand**. The mRNA base sequence, therefore, is complementary to that of the antisense strand. The remaining DNA strand of the gene, called **sense strand**, is naturally complementary to the antisense strand or the gene. Theretore, the base sequence of sense strand of a gene is the same as that of the mRNA produced by it (except for T in the place of U). Hence, the hnRNA/mRNA produced by a gene in normal orientation is also known as **sense RNA**.

An **antisense gene** is produced by inverting, i.e., reversing the orientation of, the protein-encoding region of a gene in relation to its promoter. As a result, the natural sense strand of the gene becomes oriented in the 3'→5' direction with reference to its promoter, and is transcribed. (The normal antisense strand is not transcribed since now its orientation is 5'→3') The RNA produced by this gene has the same sequence as the antisense strand of the normal gene (except forT in DNA in the place of U in RNA), and is, therefore, known as **antisense RNA** or, sometimes, **as RNA**.

When an antisense gene is present in the same nucleus as the normal endogenous gene, transcription of the two genes yields antisense and sense RNA transcripts, respectively. Since the sense and the antisense RNAs are complementary to each other, they would pair to produce double-stranded RNA molecules. This event makes (1) the mRNA unavailable for translation. At the same time, (2) the double-stranded RNA molecules are altacked and degraded by double-stranded RNA specific RNases.

Finally, 3) these events somehow lead to the methylation of the promoter and coding regions of the normal gene resuling in silencng of the endogenous gene, The application of antisense RNA technology is explained using following examples.

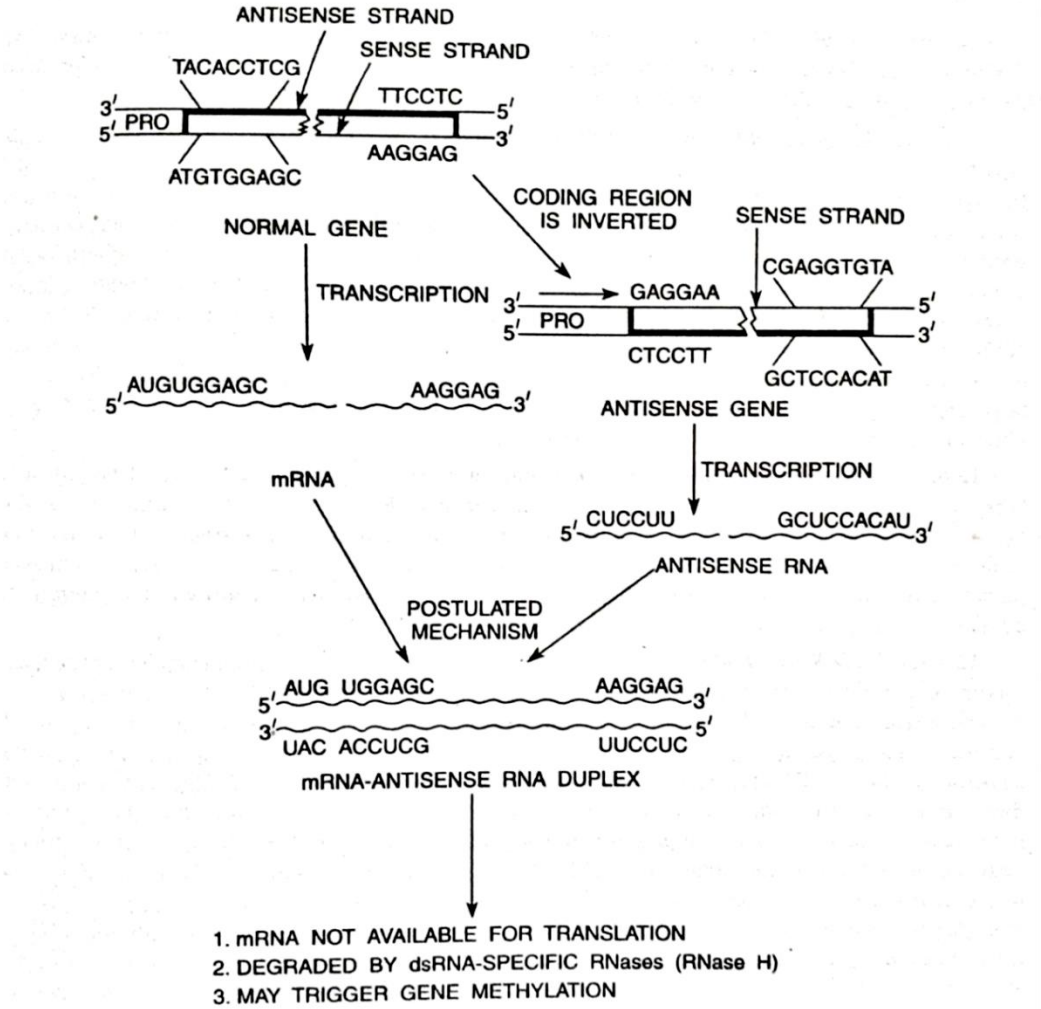


FIG. 12.2. A schematic representation of antisense RNA technology. The antisense gene is produced by inverting the normal endogenous gene (except its regulatory sequences). The mRNA produced by the normal gene is complementary to the antisense RNA produced by the antisense gene; as a result, both the RNAs pair to form a duplex, which effectively makes the mRNA unavailable for translation. Antisense strand, the strand of the normal gene that is transcribed; sense strand, the strand of the normal gene that is not transcribed (it has the same base sequence as the mRNA, except for T in the place of U); dsRNA, double-stranded RNA.

Slow Fruit Softening Tomato:

In tomato, enzyme polygalacturonase (PG) degrades pectin, which is the major component of fruit cell wall. This leads to the softening of fruits and deterioration in fruit quality. Transgenic tomatoes have ben produced, which contain antisense construct of the gene encoding PG. These transgenics show a drastically reduced expresion of PG and markedly slower fruit softening; these tomatoes have about 2 weeks longer shelf-life than normal tomatoes. Such tomatoes were approved for marketing in U.S.A. under the name "FlavrSavr'

The FlavrSavr tomatoes have improved flavour and total soluble solids (TSS), in addition to the enhanced shelf-life. These desirable features are the consequence of the fact that the fruits of FlavrSavr can be allowed to ripen on the plants. As a result, the fruits accumulate more sugars and organic acids that improve their taste and TSS content. In contrast, the conventional tomato fruits have to be picked green and hard, they are treated with ethylene in the warehouse to ripen them. But

the conventional tomato-picking and packaging equipment damages the soft, naturally-ripened fruits of FlavrSavr; this is the chief reason for the withdrawal of this variety from commercial cultivation. This highlights an important aspect of the commercial field: a perfectly sound, feasible and useful project may become a nonperformer simply due to some or other practical consideration, e.g. picking and packaging in the case of FlavrSavr tomato.

The antisense RNA technology has been used to suppress ethylene biosynthesis in tomato. The antisense construct of ACC synthase gene was transferred in tomato; this reduced ethylene production to <1% of the normal. The fruits remain green and hard on the plants, so that they accumulate more sugars and organic acids that make the fruits sweeter and richer in taste. After the harvest, the fruits are ripened in the warehouse by exposing them to ethylene. The new transgenic variety is called 'Endless Summer', the ripened fruits of this varicty stay plump and fresh for about four weeks, which is about two weeks more than is the case with nontransgenic tomatoes.

Changed Fatty Acid Composition of *Brassica* Oil:

The enzyme stearoyl-ACP desaturase (ACP = acyl carrier protein) catalyzes the conversion of stearoyl-ACP to oleoyl-ACP, which is the first desaturation step in seed fatty acid biosynthesis. Transgenie *Brassica rapa* and *B. napus* plants containing the antisense gene construct of *B. campestris* stearoyl-ACP desaturase encoding gene have been produced. The antisense construct was linked to a seed-specific promoter (napin or AC promoter) to ensure its expression only in seeds. The transgenic plants showed highly reduced stearoyl-ACP desaturase activity, a dramatic increase in the level of stearic acid (from < 2% to up to 40%), and an associated decline in oleic acid levels in their seeds. These findings demonstrate the potential of antisense RNA technology in modifying the fatty acid composition of vegetable oils by targetting selected enzymes of the fatty acid biosynthesis. These modifications are aimed at generating alternative sources of saturatcd fatty acids now obtained from cocoa butter.

Delayed Sensescence Carnation:

Flower senscence is promoted by ethylene. Ethylene biosynthesis involves two enzymes, viz, ACC synthase (encoded by acs) and ACC oxidase (encoded by aco). Gene aco was suppressed by using the antisense construct of its cDNA; such transgenic carnation flowers produce little detectable cthylene. The vase-life of flowers from such plants is, as a result, increased by 200%. This trnasgenic carnation was approved for commercial cultivation in 1997 in Australia.

Male Sterility:

Flavonoids are essential for nomal pollen development and function, and flavonoid deficiency prevents pollen maturation. Chalcone synthase (CHS) is a key enzyme of flavonoid biosynthesis. In *Petunia*, antisense construct of the gene encoding CHS has been transferred, and transgenic plants carrying this construct have been regenerated. These plants show negligible chalcone synthase (CHS) acivity, white flowers and nonfunctional pollen. However, these pollen grains become functional when certain flavonoids are either applied to the stigmas or mixed with the pollen. It has been proposed to apply flavonoid during pollination of the CHS antisense male sterile (MS) lines to obtain 100% male sterile progeny. This treatment, therefore, allows the maintenance of MS lines indefinitely, but this needs to be demonstrated on a large scale. In addition, this type of male sterility is dominant as a result ol which the F_1s from crosses with normal male fertile lines are all sterile. Therefore, an elfective restoration system has to be developed for this male sterility system before it is of any practical use. Antisense approach has also been used to restore ferulity in the case of MS induced by the rolC gene of *A. rhizogenes*.

Ribozyme Approach:

A ribozyme is an RNA molecule, which has enzymatic activity, usually, concerned with RNA degradation. e.g., the satellite RNA of tobacco ringspot virus (TobRV) has sequences that have

endoribonuclease acuvity. In the ribozyme approach, a DNA sequence specifying an enzymatic RNA sequence is fused with a sequence of the gene against which the ribozyme is aimed; the gene sequence is, as a rule, in the antisense orientation. Therefore, the RNA product of this gene construct has a sequence complementary to the sense RNA (mRNA) produced by the target gene (the ribozyme sequence is attached to a sequence of this gene). The complementary sequence of this RNA pairs with the sense RNA produced by the target gene, and the ribozyme sequence linked to it degrades the sense RNA. This strategy provides a highiy specific tool for abolishing the expression of selected endogenous genes. This approach has been tried, with limited success, to produce virus resistant transgenic plants, and is proposed to be used for restoration of fertility in some of the nuclear (=genetic) male sterility systems listed in Table.

TABLE 12.4. Some selected transgenes used for production of male sterility; the male sterility produced is dominant

Transgene	*Source*	*Transgenic plant*	*Female fertility affected*	*Restoration of male fertility demonstrated*
*rolC**	*Agrobacterium rhizogenes*	Tobacco, potato, *Arabidopsis*	Yes	Yes (antisense approach)
DTA (diptheria toxin A-chain)	Diptheria pathogen	Tobacco	n.d.**	No
barnase	*Bacillus amylo-liquefaciens*	Tobacco, *B. napus*, maize	No	Yes (inhibitor protein)
RNase T1	*Aspergillus oryzae*	Tobacco, oilseed rape	No	No
Chalcone synthase gene antisense construct	Petunia	Petunia, tabacco	No	No
Chalcone synthase-cDNA (co-suppression)	Petunia	Petunia	No	No
rolB	*A. rhizogenes*	Tobacco	n.d.**	No
Bcp1 (antisense construct)	*Brassica campestris*	*Arabidopsis*, *B. oleracea*+	—	Any line restores fertility
β-glucouronidase (*gus*)++	*E. coli*	—	—	Restoration not required
β-1, 3-glucanase†	—	Tobacco	No	No

Disruption of Endogenous Genes:

The feasibility of transgene integration into the plant genome by homologous recombination has been demonstrated. This has raised the possibility of targetted integration of transgenes into specified sites in plant genomes. Once the techniques for targetted gene transfer are refined, it should become feasible to abolish the function of a specified endogenous gene by gene disruption techniques used for the production of transgenic 'knockout' mice.

In contrast, random gene disruptions are readily produced by integration, within the genes, of either (1) a mobile genetic element like transposon or (2) *Agrobacterium* T-DNA, which occurs during infection/transformation by the bacterium. Such gene disruptions produce random gene mutations, and the resulting phenotypic changes in the various mutant lines give clues to the disrupted genes. The gene disrupted in a given mutant line is easily isolated from its genomic library by using as probe the specific transposon/T-DNA that was used to produce the mutant line. The sequences lying on eitner side of the transposon/T-DNA will represent the disrupted gene. This technique is called gene tagging, and is widely used for the identification and isolation of genes concerned with various metabolic, developmental, etc. functions.

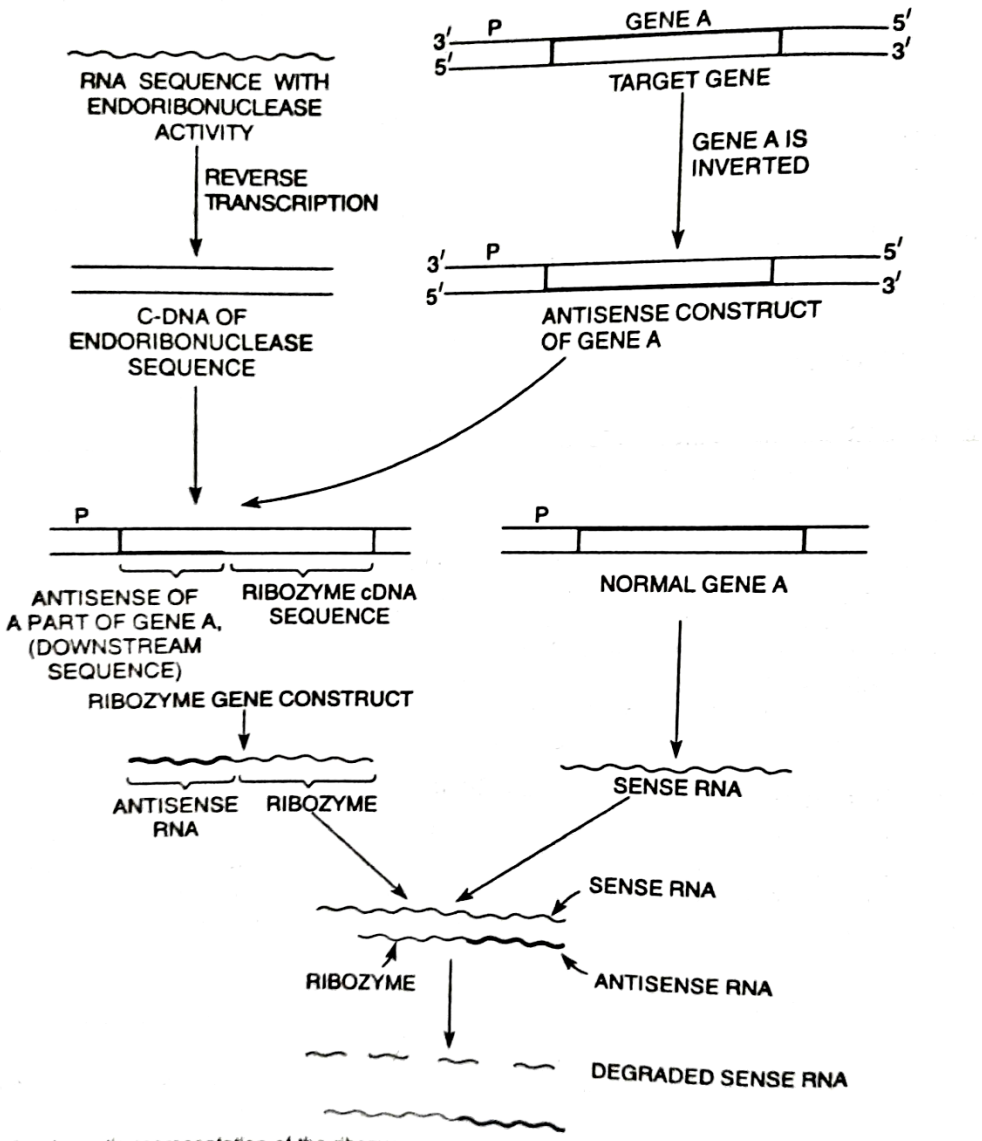


FIG. 12.3. A schematic representation of the ribozyme approach to prevent the expression of a hypothetical gene *A*. The ribozyme sequence is presumed to be from tobacco ringspot virus satellite RNA. Both the normal gene *A* and the ribozyme-antisense *A* gene construct are present and expressed in the same nucleus. *P*, promoter.

Co-suppression of Genes:

In case of many endogenous plant genes, an overexpression of the sense RNA or mRNA leads, surprisingly, to a drastic reduction in the level of expression of the genes concerned; this is called **co-suppression**. One way of achieving an overexpression of the mRNA is to introduce a homologous sense construct of the concerned gene so that it also produces sense RNA or mRNA (in addition to the endogenously present gene). Cosuppression often occurs only in a proportion of the independent transformants. For example, in *petunia*, upto 50% of the transgenic plants produced by independent transformation events for the chalcone synthase (CHS) gene sense construct produced white flowers or white flower sectors. The efficiency of cosuppression seems to vary among different plant genes.

Cosuppression has never been observed for the *petunia* chalcone isomerase gene, while tobacco glutamine synthetase nuclear gene is always cosupressed; CHS gene (*petunia*) represents the intermediate situation. In some cases, cosuppression is modulated or reset by, the stages of development.

The mechanism of cosuppression is not understood, but several models have been proposed. According to a **threshold model**, when RNA transcripts of a gene accumulate beyond a critical threshold level, they are selectively degraded by RNases. The specificity in RNA degradation may be due to (1) the presence in RNAs of target sequences for RNases, (2) lack in the RNAs of sites for endogenous activator molecules necessary for RNA stability, or (3) production of antisense RNAs.
Antisense RNAs Could be produced in one of the following two ways:

1. At the site of transgene integration, an endogenous promoter may be located at the 3'-end of the transgene. Transcription sponsored by this endogenous promoter will yield antisense RNA transcript of the transgene.
2. Aberrant sense RNA transcripts of the transgene may be produced due to one or the several reasons. e.g. accumulation of high levels of the transcript, delayed RNA processing, delay in the transcripton of the 3'-end of the transgene, etc. An accumulation of aberrant RNA transcripts of the transgene is proposed to activate RNA-dependent RNA polymerase of plant origin, which transeribes the RNA transcripts to produce antisense RNA.

The antisense RNA transcripts associate with the accumulated normal and aberrant RNA transcripts of the transgene as well as of the endogenous gene, thereby producing RNA duplexes, which present targets for double-stranded RNA-specific RNases like RNase H. Degradation of the RNA transcripts of a gene either directly or after antisense RNA formation is postulated to somehow lead to a hypermethylaion of the DNA sequences homologous to the degraded RNA sequences. This often leads to a drastic reduction in the level of expression of the transgene in question and also of homologous endogenous gene(s), if any: this is called **gene silencing**. Thus gene silencing is not specific to transgenes only.
Ethylene is an important phytohormone and is involved, among other things, in fruit ripening. In banana, tomato, avocado, etc. leaf abscission and flower senescence. It is produced from amino acid methionine, the terminal two reactions of ethylene biosynthesis being as follows. A reduced ethylene production results in delayed petal senescence in carnation and slow ripening of tonato fruits. Drastically reduced ethylene production has becn achieved in one of the following ways: (i) expression of antisense constructs of ACC synthase or ACC oxidase, (ii) co-supression of either of these enzymes, and (ii) expression of such enzymes that metabolize S-adenosyl methionine (SAM), eg, SAM hydrolase gene from bacteriophage T3 (expressed in tomato) or ACC, e.g. ACC deaminase gene from *Pseudomonas* (overexpression in tomato).

FIG. 12.4. The terminal reactions in ethylene biosynthesis; ethylene is produced from the amino acid methionine.

A carnation variety with longer vase-1ife has its ACC synthase gene cosuppressed. This delayed senescence variety was to be released in 1996. A similar cosuppression approach has been used to biock the onset of fruit ripening in tomatoes. The phenomenon of cosuppression has been exploited to produce male sterile petunias by suppressing the expresion of CHS gene. This was achieved by transferring into petunia the cDNA of CHS gene driven by CaMV 35s constitutive promoter (gene construct: p35S-CHS cDNA: p = promoter). The presence of an additional copy of the CHS gene (as DNA) drastically reduced the expression of this gene due to cosuppression, and resulted in male sterility.
Cosuppression is also involved in the strain-specific resistance of certain transgenic plants to the different members of potyviruses. These plants contained in their genome untranslatable constructs of ethier viral coat protein (CP) gene or RNA polymerase gene of potyviruses. The virus resistance of

transgenic plants was associated with the production of very low levels of transgenic RNA, and inactivalion of homologous transgenes, indicating that cosuppression was involved in the virus resistance of these plants.

RNA-Mediated Interference (RNAi)

Silencing of homologous gene expression triggered by double-stranded RNA (dsRNA) is called **RNA- mediated interference or RNA interference (RNAi)**. Introduction of long double-stranded RNA into the cells of plants, invertebrates as well as mammals leads to a sequcnce-specific degradation of the homologous gene transcripts. The long dsRNA molecules are cleaved by an RNase III enzyme called Dicer, this generates small 21-23 nucleotide long dsRNA molecules called **small interfering RNAs (siRNAs)**.

There are multiple mechanisms by which short synthetic oligonucleotides can be used to modulate gene expression in mammalian cells. Two of the common mechanisms are as follows: (1) RNase H-dependent antisense mechanism, and (2) si-RNA mechanism. The antisense oligodeoxynucleotides pair with the homologous sequence of the larget RNA molecules. Ihis creates the substrate for RNase H action; as a result, the target RNA is cleavcd and the expression or the concerned gene is suppressed.

The siRNA molecules bind to a protein complex called **RNA-induced silencing complex**; this complex contains a helicase activity that unwinds the two strands of RNA molecules. The antisense RNA strands so generated pair with the target RNA molecules, and an endonuclease activity then hydrolyzes the target RNA at the site where the antisense strand is bound.

The two strands of an siRNA molecule are either synthesized chemically in vitro and then anncaled to produce the double-stranded molecules, or the two RNA strands/the duplex form is produced in vivo by transcription. It may be pointed out that chemically synthesized siRNA molecules have to be introduced into the cells, and they inhibit target gene expression for only 4-6 days. But the DNA vector construct once transfected into the cels will keep on generating siRNA molecules and, thereby, cause a permanent inhibition of the gene.

The RNAi is a recent but potent technology and is rapidly gaining wide acceptance. The main applications of RNAi are as follows: (1) RNAi serves as an antiviral defence mechanism. (2) RNAi is becoming a powerlful and widely used tool for the analysis of gene function in invertebrates, plants, and mammals. (3) DNA vector-based srategy allows the suppression of endogenous genes and to produce transgenic lines with suitably modilied traits. RNAi has been used to produce low caffeine coffee, and *Meloidogyne incognita* resistant tobacco. *M. incognita* resistance was produced by transferring a construct containing two genes of *M. incognita,* the transcript of these genes formed a double-stranded RNA molecules that were cleaved by plant enzymes to produce siRNA.

The RNAi strategy suffers from sone important limitations, e. g. (1) RNAi based on exogenous siRNAs is rather short-lived, e.g., for 4-6 days only, (2) longer dsRNA molecules (longer than 30 base pairs) trigger antiviral response and a general suppression of gene expression, and (3) exogenous siRNAs must be delivered into the cells, which is not highly efficient.

Male Sterility:

Male sterility (dominant genetic) can be produced by transferring certain genes from other species (as against endogenous genes, e.g., rolB and rolC genes from *Agrobacterium rhirogenes*, barnase gene from *Bacilus amyloliquefaciens* etc., Gene barnase is the first transgene that was used to produce male sterility by Mariani and coworkers in 1990; it has an effective fertility restoration system in barstar (another gene from *B. amyloliquefaciens*) and is one of the systems in which crop plants like maize and oilseed rape have performed satisfactorily.

Barnase-barstar System:

Gene barnase encodes an RNase, which kills the cells in which it is expressed by degrading RNA. The expression of barnase was confined to tapetal cells by fusing it with the promoter of tobacco tapetum Specific gene TA29 (gene construct: pTA29-barnase; p = promoter). When the chimaeric gene construct pTA29-barnase was transferred and expressed in tobacco and oilseed rape, the tapetal cells of anthers were destroyed, and there was no pollen development. However, there was no effect on female fertility. Since the male sterility due to barnase is dominant, the male sterile plants are always heterozygous (barnase/-; the- sign indicates the absence of barnase gene in the homologous chromosome), and they have to be maintained by crossing to any normal, non-transformed male fertile line (-/-; barnase gene absent).

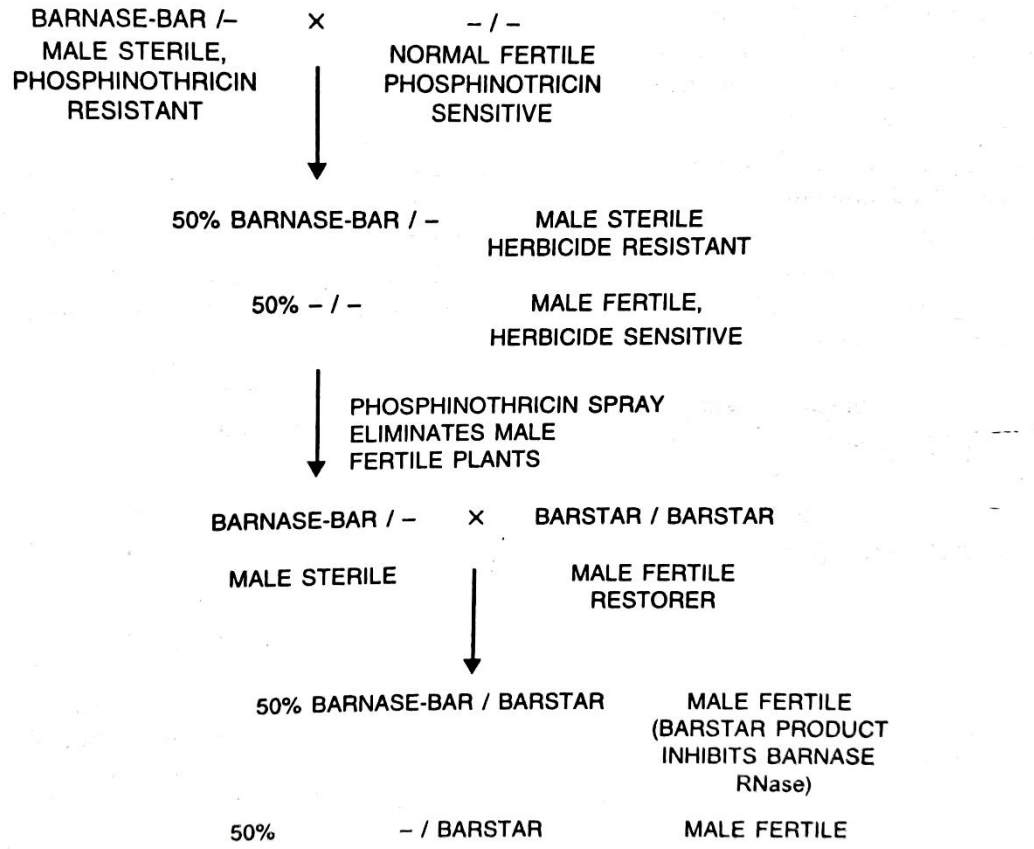


FIG. 12.5. Male sterility due to the bacterial gene *barnase* and its restoration by another bacterial gene *barstar*. Gene *barnase* produces an RNase, which kills the cells expressing this gene, while *barstar* encodes a specific inhibitor of this RNase. Herbicide resistance gene *bar* is linked with *barnase* to eliminate the male fertile plants from the male sterile line.

Thus male sterile lines (barnase /-) will have to be crossed to be normal male fertile lines (-/-) in each generation, and only 50% of the progeny from such crosses will be male sterile (barnase/-) while the rest 50% will be male fertile (-/-). In a hybrid seed production programme, the male fertile plants present in the male sterile line must be readily identified and easily eliminated. This has been done by linking the barnase gene with the bar gene from *Streptomyces*; bar gene confers resistance to the herbicide phosphinothricin (gene construct: pTA29- barnase +p35S-bar, simply depicted as barnase-bar). When such male sterile (harnase-bar/-) plants are maintained by crossing with normal male ferile plants (-/-), all the male sterile progeny (bamase-bar /-) are resistant to the herbicide, while the entire male fertile plants (-/-) are herbicide susceptible. The male fertile plants are, therefore, easily eliminated by a herbicide spray at an early stage of plant growth.

The male ferility of barnase male steriles is restored by another gene, barstar, of the bacterium *B. amyloliquefaciens*. The gene barstar encodes a specific inhibitor of barnase encoded RNase. The barstar product (Barstar) forms a highly stable 1:1 noncovalently bound complex with the barnase RNase (Barnase); this reaction provides protection to the bacterial cells from their own RNase product. Transgenic plants expressing barstar are male fertile without any phenotypic effect, and are easily maintained in the homozygous state by self-pollination. When a homozygous barstar male fertile line is crossed with a barnase male sterile line, all the progeny plants are male fertile since barstar gene product effectively inhibits the barnase RNase in barnase-bar/barstar plants. This male sterility/fertility system has shown commercial promise in maize and oilseed rape; it is now available in cauliflower, chicory, tomato and wheat as well. In India, transgenic lines expressing barnase and barstar genes have been developed in *B. juncea*. These lines are stable and provide a complete and usable male sterility-restorer system; they are expected to promote hybrid development in *B. juncea*.

It may be pointed out that to be of commercial value, a dominant genetic male sterility system must have the following features: (i) efficient fertility restoration system, (ii) easy maintenance of male sterile lines, (iii) easy elimination of male fertile plants from the male sterile line, (iv) lack of adverse ettects on other traits, (v) stable male sterile phenotype over different environments and genetic backgrounds, and (vi) satisfactory performance of the F_1 hybrids. The barnase-bar/barstar appears to satisty these requirements.

Hormone-Inducible Male Sterily Based on Bcp1

The most attractive system of transgenie male sterility system generated thus far is based on the antisense construct of *B. campestris* gene Bcp 1 driven by Bcp 1 promoter linked with a hormone inducible enhancer sequence; this system is ready for use in hybrid seed production in *B. oleracea* The endogenous Bcp 1 is expressed both in tapetum and in microspores, and its expression in both these tissues is essential for normal pollen development. Pollen grains in which the expression of Bcp 1 is prevented by the presence ot an antisense construct of Bcp 1 driven by the pollen-specific LAT 52 promoter do not develop normally and become aborted. When the antisense construct of Bcp 1 driven by its own promoter was expressed in *Arabidopsis thaliana*, the transgenic plants were male sterile. It was discovercd that two copies of the Bcp 1 antisense construct per nucleus are essential for complete male sterility, while a single copy per nucleus leads to a leaky male sterile phenotype.

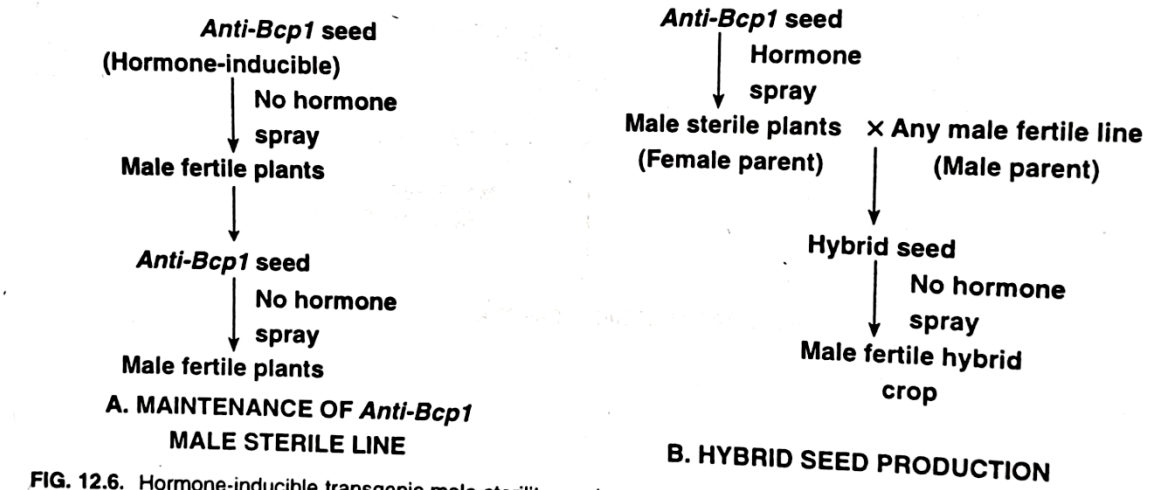


FIG. 12.6. Hormone-inducible transgenic male sterility produced by antisense construct of gene *Bcp-1*, and its use in hybrid seed production.

A Bcp 1 antisense construct has now been prepared that is driven by the Bcp 1 promoter linked to a hormone-inducible enhancer sequence. The presence of this enhancer sequence ensures the expression

of Bcp 1 antisense RNA only in response to the hormone treatment, while Bcp 1 promoter limits this expression to tapetum and microspores. The male sterile lines containing this construct remain male fertile and, as a result, self-maintainable so long as they are not sprayed with the hormone. For hybrid seed production, the hormone is sprayed at the critical developmental stage; this spray induces male sterility in the anti-Bcp 1 male sterile line. The hybrid seed produced on this line will be 100% male ferile unless the hormone is sprayed on the hybrid crop. As a result, ferility restoration is not required and any male fertule line can be used as the male parent or the hybrid. This male sterility system is more atttractive than the barnase-barstar system as it uses only 2 lines as compared to 3 lines in the case of latter.

Biochemical Production:

Plants are the chief source of carbohydrates, e.g., starch, sugar, etc., lipids, proteins, and a variety of unique biochemicals. Transgenes have been shown to introduce novel branches in the biosynthetic pathways of plants and. thereby, to generate valuable products or to produce new, valuable proteins. Virtually all the cases are promising and most of them are in developmental stages, except for the thrombin inbibitor protein hirudin, which is the first commercial example. Production of value-added products like, nutrition supplements, pharmaceuticals fuels, etc. using transgenic crops is popularly called **molecular farming**.

Hirudin, a Plypeptide

Recombinant hirudin is encoded by a synthetic gene, and is expressed in fusion with the oil-body protein olesin, which greatly facilitates the purification of heterologous (different from those endogenous to the plant species) polypeptides. The seed tissue expressing a heterologous protein fused with olesin is extracted with water and the extract is centrifuged. Oil bodies containing the olesin-fusion protein float on the surface, and are easily separated from the rest of seed proteins. The heterologous polypeptide is cleaved from olesin at a protease recognition site located at their junction. Thus hirudin provides a success ful example of transgene expression in plants for the isolatúon of a polypeptide of interest at a commercial scale. Recombinant hirudin is produced in transgenic *B. napus* at a commercial scale.

Phytase, an Enzyme

An example of a promising heterologous enzyme produced in plants is provided by phytase. Many plant tissues, especially secd tissucs, store phosphorus as phytate, which can not be utilized by many monogastric animals like chickens. Enzyme phytase hydrolyzes phytate to inorganic phosphate and inositol, thereby making the phosphorus readily available. Phytase is cncoded by a gene from the fungus *Aspergillus niger*. It enhances phosphorus utilization by chickens from their feed to the extent that phosphate supplement in feed becomes unnecessary. Transgenic tobacco seeds expressing the gene encoding phytase were fed to broiler chickens for a period of 4 weeks. This produced a gain in body weight that was comparable to those obtained with feed supplemented with phosphate or with *A. niger* phytase. This raises the feasibility of a direct use of proteins/enzymes (without purification) expressed in plants either as food/feed or for industrial applications.

Polyhydroxybutyrate, a Biodegradable Plastic Substrate

Polyhydroxyalkanoates (PHAs), e.g., polyhydroxybutyrate (PHB), are synthesized from acetyl-CoA used as precursor, and are used for the synthesis of biodegradable plastics with thermoplastic properties. At present, PHAs are produced by bacterial fermentation based on *Ralstonia eutropha*. ICl, U.K. markets this PHB as "Biopol' at around us s 8-10 per pound and the cost of biodegradable plastic is substantially higher than that of synthetic plastics. Attempts are being made to produce PHAs in transgenic plants to reduce tne cost. Genes encoding the two enzymes, aceto-acetyl-CoA reductase (phbB) and PHB synthase (phbC), and the gene phbA, which are involved in the PHB synthesis from the precursor acetyl-CoA, have been transferred from the bacterium *R. eutropha* and expressed in

Arabidopsis thaliana. When the enzymes were synthesized and located in the cytoplasm, a low level (100 µg PHB/g fresh wt., i.e., 0.14% of dry wt.) of PHB was produced. It was reasoned that since the biosynthesis of fatty acids occurs in plastids, acetyl-CoA (which is the precursor for fatty acids as well) will be available mainly in plastids, and its supply in the cytoplasm will be low and limiting to PHB biosynthesis.

TABLE 12.5. Some important transgenes, which modify starch/lipid, produce a novel non-protein compound in plants, or modify quality/yield traits of crop plants

Gene encoding the enzyme	Source	Expressed in	Consequences of expression
Starch			
Cyclodextrin glycosyltransferase	*Klebsiella pneumoniae*	Potato	1. α- and β-cyclodextrins produced 2. Only 0.001-0.01% of the total starch
Fructosyl transferase	*Bacillus subtilis*	Tobacco	Fructan upto 3-8% of leaf dry wt.
		Potato	1. Fructan upto 1-30% of leaf dry wt., and upto 1-7% of microtuber dry wt. 2. Total nonstructural carbohydrates in leaves upto 35% as compared to only 7% in the control
Manitol-1 phosphate dehydrogenase	*E. coli* (gene *mtlD*)	Tobacco	1. Manitol at >6 µmol/g fresh wt. of leaf and root 2. Increased tolerance to high salinity.
Biodegradable plastic			
Polyhydroxybutyeate (PHB)			
1. *phbA* 2. Acetyl-CoA reductase (*phbB*) 3. PHB synthase (*phbC*)	*Ralstonia eutropha*	*Arabidopsis thaliana*	1. Gene products targeted to chloroplasts 2. Polyhydrobutyrate accumulated in plastids; upto 14% of leaf dry mass
Quality/Yield trait			
Tryptophan-2-oxygenase (*iaaM*)[1]	*Pseudomona ssyringae* pv. *savastanoi*	Tobacco, brinjal (eggplant)	Seedless fruits due to parthenocarpic development even under environment that prevents fruit set[2]

TABLE 12.6. Some important transgenes encoding valuable polypeptides/enzymes expressed in plants

Polypeptide/Enzyme	Source	Expressed in	Applications	Remarks
Polypeptides				
Antibodies (IgG, IgM, chimaeric, single chain, V_H chains, etc.)	Mouse	—	Various applications	Expressed at levels of 2% of the extractable proteins
Hirudin	Synthetic	*B. napus*	Thrombin inhibitor	Expressed in fusion with olesin; ***first commercial example***
α-Trichosanthin	Chinese medicinal plant	—	Inhibition of HIV replication	High level of expression
Interferon	Human	—	Antiviral activity	—
Enzymes				
α-Amylase	*Bacillus licheniformis*	Tobacco	Liquefaction of starch	Industrial applications
(1-3, 1-4)-β- Glucanase	*Trichoderma reesei;* hybrid of two *Bacillus* species	Tobacco, barley	Brewing	Industrial applications
Phytase	*Aspergillus niger*	Alfalfa, tobacco	Increased phosphate utilization from feed	Upto 14% of soluble protein in leaves close to senescence
Research Reagents				
*Avidin	Chicken	Maize	Research reagent	Industrial applications 2-3% of seed dry mass; ***marketed by Sigma, U.S.A.***
β-Glucuronidase	Bacteria (*E. coli*)	Maize	Research reagent	0.4-0.7% of seed dry weight; ***marketed by Sigma, U.S.A.***

The enzymes were, therefore, targetted into the plastids, and there was a 100-fold increase in PHB production (about 14% on dry weight basis). PHB Production by transgenic plants provides an example of a novel compound synthesized in plants. Transgenic trees like poplar expressing phbB and phbC accumulate PHB in their leaves; the leaves can be collected and used for PHB extraction. It has been estimated that PHB concentration should be ~15% on dry weight basis to make extraction and processing economic. The present yields of PHB are merely around 3%.

Cyclodextrins from Starch

Attempts are being made to modify endogenous starch and lipid reserves of plants to obtain products of high commercial value. Cyclodextrin production from starch is a good example of this type. Cyclodextrins are cyclic oligosaccharides having 6 (α), 7 (β) or 8 (γ) α-1, 4-linked glucopyranose units. They have applications in drug delivery, flavour and odour enhancement, and removal of undesired compounds, e.g., caffeine, from foods since they form inclusion complexes with a wide variety of organic molecules. Currently, cyclodextrins are produced from starch as follows: starch is first hydrolyzated to increase its solubility for better enzyme access and action, and then treated with the enzyme cyclodextrin glucosyltransferase (CGT) from the bacterium *Klebsiella pneumoniae*. The cost of production has limited the use of cyclodextrins.

The bacterial gene encoding CGT was transferred into potato and expressed in tubers. The chimaeric gene construct consisted of the following: (i) patatin gene promoter (for tuber-specific expression), (ii) the sequence encoding the transit peptide of the small subunit of ribulose-bis-phosphate carboxylase (for ensuring the transport of the protein encoded by the chimaeric gene into the starch accumulating amyloplasts), (iii) the CGT gene, and (iv) the 3'-sequence of nopaline synthase (nos) gene of *Agrobacterium*. The expression of this gene construct resulted in the production of cyclodextrins upto 0.001 to O.01% of the total starch content of the tubers. This low level of production may be due to either a poor enzyme expression or a poor accessibility of enzyme to the substrate starch. In the commercial process Substrate accessibility is improved by prehydrolyzation of starch.

Research Reagents

Several proteins and enzymes are used as reagents in research activities. Such proteins/enzymes could be produced in plants on a commercial scale; there are already two commercial examples. The protein avidin is traditionally isolated from chicken egg. The avidin gene has been expressed in maize, where it accumulates in seeds upto 2-3% of seed dry mass. Avidin purified from transgenic maize seed is being marketed by Sigma, U.S.A. as a research reagent. Similarly, β-glucouronidase (GUS) enzyme is being produced from transgenic maize, and marketed by Sigma, U.S.A.

Plantibodies

The term 'plantibody' is derived from fusion of "plant' and 'antibody'. Plantibodies are produced by transgenic plants that express the animal antibody genes turansferred into them. The first plantibody was produced in 1989 by expressing mouse antibody gene in tobacco. Plants can produce full length antibodies, but it is far more convenient and succesful to express only the variable parts of the light and heavy chains fused with a linker peptide. Plantibodies are purified cheaply, and function in the same way as normal antibodies. However, glycosylation pattern in plants differs from that in animals. Therefore, it is feared that an injection of plantibodies may trigger an immune response.

So far, only sIgA (secretary IgA) has been produced in the plant systems. Plantibodies have two applications: (1) treatment of human and animal diseases and (2) protection of plants from nematodes and viruses. Plantibodies have been produced from transgenic rice, tobacco and maize. Several plantibodies are in clinical trials.

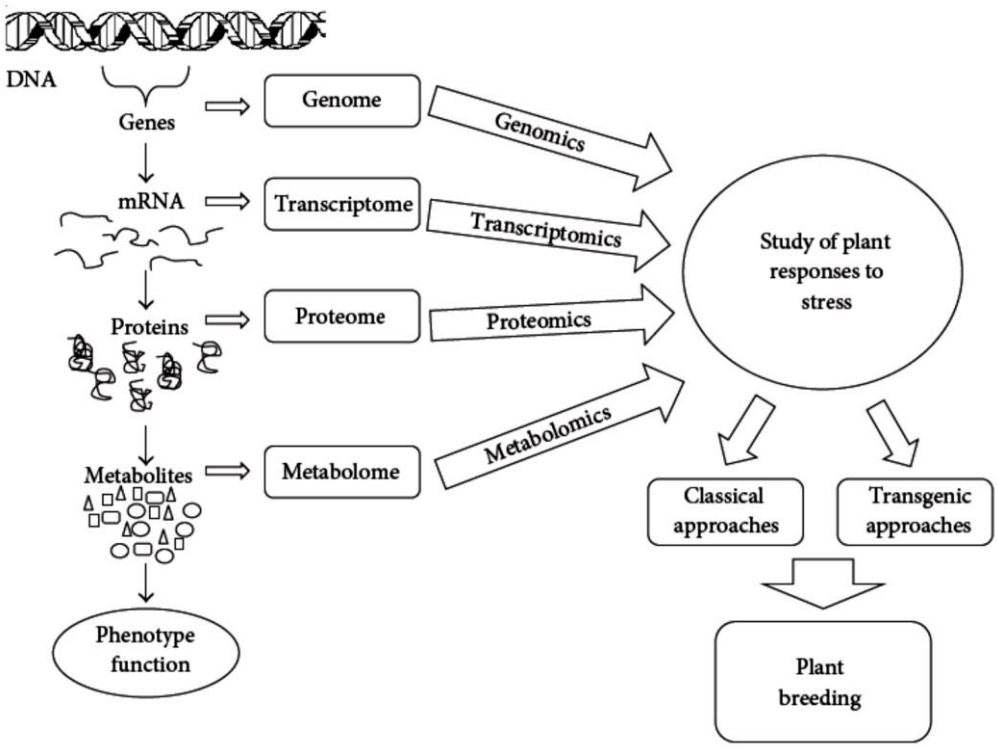
10. Biotechnological approaches for improving abiotic stress tolerance in plants.

Plants and animals share some response mechanisms to unfavorable environmental conditions; however, plants, being sessile organisms, have developed, in the course of their evolution, highly sophisticated and efficient strategies of response to cope with and adapt to different types of abiotic and biotic stress imposed by the frequently adverse environment.

Stress can be understood as a stimulus or influence that is outside the normal range of homeostatic control in a given organism: if a stress tolerance is exceeded, mechanisms are activated at molecular, biochemical, physiological, and morphological levels; once stress is controlled, a new physiological state is established, and homeostasis is reestablished. When the stress is retired, the plant may return to the original state or to a new physiological situation.

In the last years, and because of the great interest for both basic and applied research, there has been an important progress in the understanding of the mechanisms and processes underlying abiotic stress adaptation and defense in different plant species. The sensing of biotic or abiotic stress conditions induces signaling cascades that activate ion channels, kinase cascades, production of reactive oxygen species (ROS), and accumulation of hormones such as salicylic acid, ethylene, jasmonic acid, and abscisic acid. These signals ultimately induce expression of specific subsets of defense genes that lead to the assembly of the overall defense reaction

The emergence of the novel "omics" technologies, such as genomics, proteomics, and metabolomics, is now allowing researchers to identify the genetic behind plant stress responses. These omics technologies enable a direct and unbiased monitoring of the factors affecting plant growth and development and provide the data that can be directly used to investigate the complex interplay between the plant, its metabolism, and also the stress caused by the environment or the biological threats (insects, fungi, or other pathogens). Plant responses to stress are mediated via profound changes in gene expression which result in changes in composition of plant transcriptome, proteome, and metabolome.



Genomics

A gene by gene approach has been typically used to understand its function. In Table 1, some of the genes involved in plant responses to stress are listed. Functional genomics allows large-scale gene function analysis with high throughput technology and incorporates interaction of gene products at cellular and organism level. The information coming from sequencing programs is providing enormous input about genes to be analyzed. The availability of many plant genomes nowadays facilitates studying the function of genes on a genomewide scale. The lack of information from other plant genomes will also be compensated in part by the availability of large collection of expressed sequence tags (ESTs) and cDNA sequences. The basic interest behind these EST projects is to identify genes responsible for critical functions. ESTs, cDNA libraries, microarray, and serial analysis of gene expression (SAGE) are used to analyze global gene expression profiles in a functional genomics program. Large mutant collections are tools that complement large-scale expression studies. Gene identification through physical and chemical mutagens has become amenable for large-scale analysis with the availability of markers, but gene tagging is more promising for functional analysis on a wider scale. Moreover, the understanding of the complexity of stress signaling and plant adaptive processes would require the analysis of the function of numerous genes involved in stress response.

Numerous investigations show that plant defense response genes are transcriptionally activated by pathogens and also by different types of abiotic stress. It has been described that the induction of specific defense genes, in the response against certain pathogens, is dependent on specific environmental conditions, suggesting the existence of a complex signaling network that allows the plant to recognize and protect itself against pathogens and environmental stress. Similar induction patterns of members of the 14.3.3 gene family (GF14b and GF14c) by abiotic and biotic stresses such as salinity, drought, ABA, and fungal inoculation have been documented in rice. The rice GF14 genes contain cis-elements in their promoter regions that are responsive to abiotic stress and pathogen attack. The 14-3-3s family genes are also subject to the regulation by certain transcript factors.

Table 1: Genes involved in plant responses to stress.

	Stress
14.3.3 gene family (GF14b, GF14c)	Salinity, drought, fungal
MAPK	Abiotic and biotic stresses
MEKK1 and ANP1	Environmental stress
MPK3, MPK4 and MPK6	Abiotic stress (pathogens) and oxidative stress
CBF/DREB families (CBF1, CBF2, DREB2A)	Drought, cold, salinity
HVA1	Salinity and drought
Glycerol-3-phosphate acyltransferase gene	Cold
ICS	Pathogens, UV light
LOX	Wounding, drought, and pathogens
bZIPs family (e.g., ABF1, ABF2)	Drought, temperature, salinity
WRKY family (AtWRKY2, AtWRKY6, AtWRKY18)	Pathogens, wounding, salinity, temperature, drought, oxidative stress
ATAF	Wounding, drought, salinity, cold, pathogens

On the other hand, kinase cascades of the mitogen-activated protein kinase (MAPK) class play a remarkably important role in plant signaling of a variety of abiotic and biotic stresses, and it is an essential step in the establishment of resistance to pathogens. It has been described that in Arabidopsis MEKK1 and ANP1 act in the environmental stress response, and MPK3, MPK4, and MPK6, are activated by a diversity of stimuli including abiotic stresses, pathogens, and oxidative stress.

Elucidating the molecular mechanism that mediates the complex stress responses in plants system is an important step to develop improved variety of stress tolerant crops. Many crop traits are quantitative, complex, and controlled by multiple interacting genes. Recent progress in molecular biology provides the tools to study the genetical make-up of plants, which allows us to unravel the inheritance of all traits whether they are controlled by single genes or many genes acting together, known as the quantitative trait loci (QTL). The molecular marker technologies available since the 1980s allows dissecting the variation in traits. With the progress of QTL mapping, new breeding approaches such as marker-assisted selection and breeding by design have emerged.

Advances in plant genomics research have opened up new perspectives and opportunities for improving crop plants and their productivity. The genomics technologies have been found useful in deciphering the multigenicity of biotic and abiotic plant stress responses through genome sequences, stress-specific cell and tissue transcript collections, protein and metabolite profiles and their dynamic changes, protein interactions, and mutant screens.

Proteomics

The adaptation of plants to biotic or abiotic stress conditions is mediated through deep changes in gene expression which result in changes in composition of plant transcriptome, proteome, and metabolome. Since proteins are directly involved in plant stress response, proteomics studies can significantly contribute to elucidate the possible relationships between protein abundance and plant stress acclimation. Several studies have already proven that the changes in gene expression at transcript level do not often correspond with the changes at protein level. The investigation of changes in plant proteome is highly important since proteins, unlike transcripts, are direct effectors of plant stress response. Proteins not only include enzymes catalyzing changes in metabolite levels, but also include components of transcription and translation machinery.

In the last years, there has been an important progress in the knowledge of several families of plant transcription factors linked to plant stress responses, such as responses to ultraviolet light, wounding, anaerobic stress, and pathogens. The most important ones are as follows.

(i) The ethylene-responsive-element-binding factors (ERFs). This protein family has been linked to a wide range of stresses; the RNA levels of specific ERF genes are regulated by cold, drought, pathogen infection, wounding or treatment with ethylene, SA or JA. ERF proteins are shown to function as either activators or repressors of transcription, which is of great relevance in all processes related to plant development and its responses to adverse growing conditions due to both biotic and abiotic factors. It has been reported that ERF proteins from one plant species function in other plant species, enhancing their potential utility in increasing the stress tolerance of plants. However, constitutive overexpression of ERF genes generally causes deleterious effects. To overcome this problem, the use of stress-inducible promoters to control the expression of the ERF genes has been successfully used.

(ii) NAC proteins are plant-specific transcription factors having a variety of important functions not only in plant development but also in abiotic stress tolerance. NAC domain-containing proteins represent one of the largest TF families, firstly identified in model plants as Arabidopsis and rice but also recently characterized in woody fruit species.

(iii) Another important family of transcription factors is the called “basic-domain leucine-zipper (bZIP)” which are regulators of important plant processes such as organ and tissue differentiation, cell elongation, nitrogen/carbon balance control, pathogen defense, energy metabolism, unfolded protein response, hormone and sugar signaling, light response, osmotic control, and seed storage protein gene regulation. One class of bZIP proteins that is linked to stress responses comprises the TGA/octopine synthase (ocs)-element-binding factor (OBF) proteins. These bind to the activation sequence-1 (as-1)/ocs element, which regulates the

expression of some stress-responsive genes. A major advance was the discovery that TGA/OBF family members interact with nonexpressor of PR1 (NPR1), a key component in the SA defense signaling pathway.

(iv) (iv) WRKY proteins are a family of transcription factors that are unique to plants specific WRKY family members show enhanced expression and/or DNA-binding activity following induction by a range of pathogens, defense signals, and wounding. Significant progress has been made in the past years in identifying target genes for WRKY factors. WRKY proteins bind to the W box, which is found in the promoters of many plant defense genes. WRKY proteins also regulate the expression of regulatory genes such as receptor protein kinases. Positive and negative regulation of WRKY promoters by specific WRKY proteins has been observed, and the promoters of many of the pathogen- and/or SA-regulated AtWRK genes are rich in W boxes.

(v) MYB proteins are key factors in regulatory networks controlling development, metabolism, and responses to biotic and abiotic stresses. Since the Arabidopsis genome sequence was published, some years ago, an important amount of data has accumulated on the roles of MYB transcription factors in plants and some members of this family are involved in these responses. Therefore, AtMYB30 encodes an activator of the hypersensitive cell death program in response to pathogen attack; AtMYB96 acts through the ABA signaling cascade to regulate water stress and disease resistance. AtMYB33 and AtMYB101 are involved in ABA-mediated responses to environmental signals. AtMYB15 is also involved in cold stress tolerance. AtMYB108 in both biotic and abiotic stress responses. The elucidation of MYB protein function and regulation that is possible in Arabidopsis will allow predicting the contributions of MYB proteins to the responses to biotic and abiotic stress conditions in other plant species.

(vi) MYC proteins are involved in the response of plants to unfavorable environmental conditions. This transcription factor family plays a role in the induction of apoptosis, important in the hypersensitive cell death program in response to pathogen attack. Another putative MYC target is the ornithine decarboxylase gene, involved in polyamines synthesis. On the other hand, MYC proteins activate the major ABA-dependent stress response.

Therefore, studies of plant reaction upon stress conditions at protein level can significantly contribute to our understanding of physiological mechanisms underlying plant stress tolerance. Proteomics studies could thus lead to identification of potential protein markers whose changes in abundance can be associated with quantitative changes in some physiological parameters related to stress tolerance.

Table 2: Proteins and enzymes involved in plant responses to stress.

	Stress
ERF family	Cold, drought, pathogen infection, wounding, ET, SA, and JA
bZIPs family (e.g., ABF1, ABF2)	Drought, temperature, salt
WRKY	Pathogens, wounding, salinity, temperature, drought, oxidative stress
MYB family (AtMYB15, AtMYB30, AtMYB33 AtMYB60, AtMYB96, AtMYB101 AtMYB15, and AtMYB108)	Biotic and abiotic stress (pathogens, drought, cold)
ABF	Drought
NAC	Drought, salinity, cold
MYC	Environmental stresses
LEA family (PMA 80, PMA 1959)	Salinity and drought
Heat shock proteins	Temperatures
LOX family (e.g., LOX1)	Wounding, drought, and pathogens
Glutathione peroxidase, superoxide dismutase, ascorbate peroxidases, and glutathione reductases	Oxidative stress

Metabolomics

The possibility of monitoring a complete set of metabolites could largely improve the understanding of many physiological plant processes. This systematic study, defined as "metabolomics," is intended to provide an integrated view of the functional status of an organism. Besides its use as a breeding or selection tool, metabolomics techniques have also been used to evaluate stress responses in barley, Citrus, *Medicago truncatula*, and *Arabidopsis thaliana*.

Targeted analysis is the most developed analytical approach in metabolomics. It is used to measure the concentration of a limited number of known metabolites precisely, by using either gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR).

Other approaches using high throughput metabolite analysis focus on a subset of useful information while avoiding the difficulties of comprehensive metabolite characterization; metabolic fingerprinting uses signals from hundreds to thousands of metabolites for rapid sample classification via statistical analysis. In the last years, metabolite profiling attempts to identify and quantify a specific class or classes of chemically related metabolites that often share chemical properties that facilitate simultaneous analysis.

The metabolome represents the downstream result of gene expression and is closer to phenotype than transcript expression or proteins. Extensive knowledge on metabolic flows could allow assessment of genotypic or phenotypic differences between plant species or among genotypes exhibiting different tolerance to some biotic or abiotic stresses. In addition, target metabolites have been analyzed as nutritional and/or agronomical biomarkers to classify different crop cultivars or to optimize growth conditions.

In contrast to high throughput methodology for the analysis of DNA, RNA, and proteins, current strategies for metabolite characterization still face significant obstacles. These challenges are largely caused by the high degree of chemical diversity among metabolite pools as well as the complexity of spatial and temporal distribution within living tissues. Plant metabolomics methodology and instrumentation are being developed at a rapid pace to address these analytical challenges.

Like other functional genomics research, metabolomics generates large amounts of data. Handling, processing, and analyzing this data is a clear challenge for researchers and requires specialized mathematical, statistical, and bioinformatic tools. Further developments in this area require improvements in both analytical science and bioinformatics. Development of new analytical techniques is largely focused on increasing resolution and comprehensiveness, increasing speed and throughput of analytical assays and equipment miniaturization

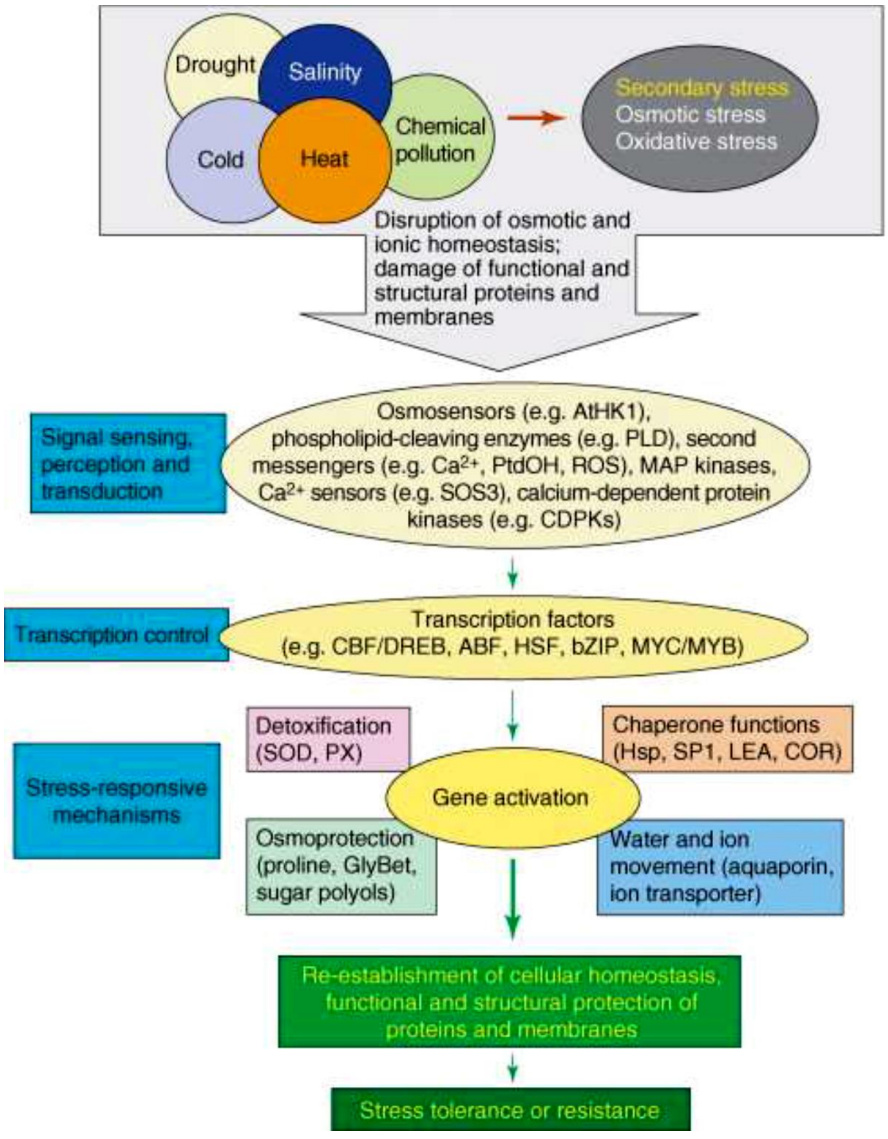
TABLE 3: Metabolites and hormones involved in plant responses to stress.

	Stress
Abscisic acid, jasmonic acid, salicylic acid, polyamines, and others	Drought, salinity, cold
Proline, glycine-betaine, and other compatible osmolytes	Environmental stresses: drought, salinity, osmotic
Phytoalexins	Microbial pathogens
Terpenes	Toxins and pathogens
Phenolic compounds (coumarin, lignin, flavonoids, tannins, isoflavonoids)	Pathogens, oxidative stress, UV light
Alkaloids	Pathogens (predators)
Unsaturated fatty acids	Environmental stresses
ROS, malondialdehyde	Biotic and abiotic stresses
Phytochelatins and metallothioneins	Heavy metal intoxication

Transgenic approach:

As we all know that Agriculture is totally dependent on climate. So a variety of Abiotic Stresses causing a serious crop loss of about >50 % on an average thus limiting the agricultural productivity world wide. By 2025, 30% of crop production will be at risk due to the declining water availability. World Bank projects that the climate change will depress crop yields by 20% or more by the year 2050. (Narendra Tuteja, 2012) Efforts have been made by Plant breeder in developing abiotic stress resistant crop plants but are not sufficient enough. Thus the role of Transgenic Approach in crop improvement has become of great importance in assuring worlds future food security.

Transgenic techniques are being used to introduce biotic and abiotic stress related genes in crop plants (Ashraf et al., 2008). Plant responses under abiotic stresses are complex involving number of genes with additive effects, hence prospects of improve stress tolerance are not very bright. In spite of it, researchers have exerted efforts during last decade to generate transgenic crops with improved tolerance against abiotic stresses. The major emphasis has been to introduce gene encoding compatible organic osmolytes, heat shock proteins, plant growth regulators, late embryogenesis abundant proteins and transcription factors responsible in activating gene expression (Ashraf et al., 2010). Many genes play an important role in the synthesis of osmoprotectants in stress tolerant plant like proline, glycinebetaine and polyamines, mannitol, trehalose and galactinol which accumulate during osmotic adjustment (Vincour and Altman, 2005).



Plant response to abiotic stress to develop tolerance or resistance

Approaches

1. Engineering of genes for **Osmolyte biosynthesis**
2. Engineering of genes encoding enzymes for **scavenging active oxygen**
3. Engineering of genes encoding **LEA Proteins**
4. Engineering of genes encoding heterologous enzymes with different **temperatures**
5. Genetic engineering of **molecular chaperones**
6. Engineering of genes encoding **transcription factors**
7. Genetic engineering of **cell membranes**

1. Engineering of Genes for Osmolyte biosynthesis

Tolerance to abiotic stresses has mainly been achieved at cellular levels of osmotically-active solutes

- Proline, glycine betaine
- Sugars such as sucrose, trehalose and fructans
- Sugar alcohols like sorbitol, mannitol, ononitol, pinitol and polyols

These osmolytes are uniformly neutral with respect to cellular functions

Accumulation of these molecules helps plants to –

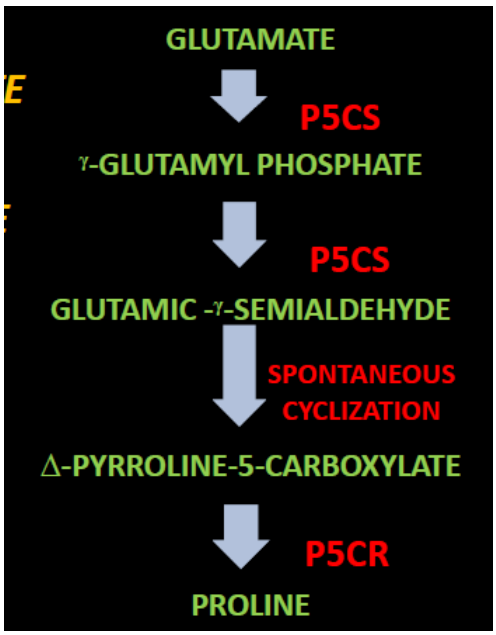
Retain water within cells and protects cellular compartments from injury caused by dehydration

Maintains turgor pressure during water stress

Stabilize the structure and function of certain macromolecules

Signalling functions or induction of adaptive pathways

Scavenge reactive oxygen species



Proline

Proline accumulates in many organisms in response to drought and salinity. Proline is encoded by a nuclear gene *Pyrroline-5-carboxylate synthetase (P5CS)*. Proline may serve as a hydroxyl radical scavenger reducing the acidity of the cell. It may also function as an osmolyte, molecular chaperone, protect protein integrity and enhance the activities of different enzymes. Glutamate can be achieved by using the *P5CS* gene from Moth bean (*Vigna aconitifolia*) and antisense proline dehydrogenase (*PDH*) in transgenic *Arabidopsis*. However, *P5CS*, limiting enzyme in proline biosynthesis, feedback inhibition by prolinee and removed this feedback inhibition by site-directed mutagenesis and the resulting gene ***P5CSF129A.***

Gene	Molecular function	Source	Transformed plant	Performance of transgenic plant	Additional note
P5CS	*D1-pyrroline-5-carboxylate synthase*	*V. aconitifolia*	*O. sativa*	Drought & salt tolerance	Increased biomass (higher fresh shoot & root weight)
P5CS F129 A	*Mutated P5CS*	*V. Aconitifolia*	*N. tabacum*	Oxidative & osmotic stress tolerance	Removal of feedback inhibition resulted in 2-fold high proline than the plants expressing P5CS
PDH	*Proline dehydrogenase*	*A. thaliana*	*G. max*	Drought & heat tolerance	Rapid increase in proline resulted in least water loss under drought stress

Gene	Molecular function	Source	Transformed plant	Performance of transgenic plant	Additional note
P5CSF129A	*Mutated P5CS*	*V. aconitifolia*	*O. sativa*	Salt stress tolerance	Transgenic plants with high proline level showed higher biomass & growth performance under salinity stress
P5CSF129A	*Mutated P5CS*	*V. aconitifolia*	*P. vulgaris*	Drought tolerance	Elevated free proline resulted in better adaptation to water stress
δ-OAT	*Ornithine-daminotransferasea*	*A. thaliana*	*N. Plumbaginifolia*	Osmotic stress tolerance	Transgenic plant showed higher germination and increased biomass

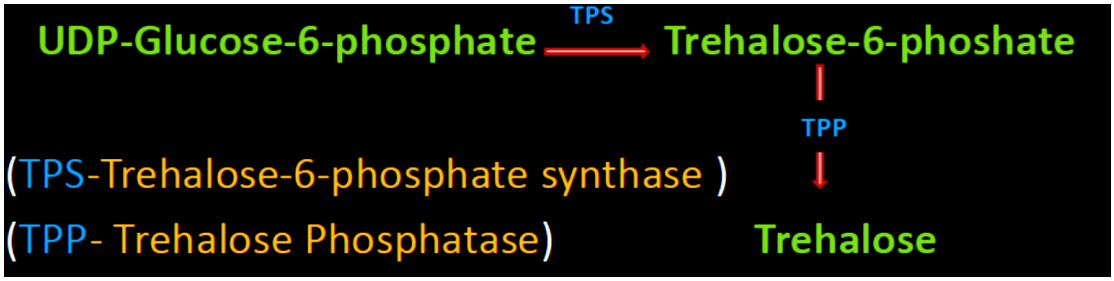
Glycine Betaine

GB accumulates in the **chloroplasts** and **plastids** and increases the tolerance of plants to various abiotic stresses (drought, salinity and freezing). The physiological role of GB is to **alleviating osmotic stress**. It can also **protects proteins** and **enzyme** activities under water deficits and **stabilize membranes** during freezing. It can help stabilize the protein tertiary structure and prevent or reverse disruption of the tertiary structure. Accumulation of GB is limited due to **choline** supply. Transgenic potato plants expressing a bacterial choline oxidase (*betA*) gene leads to high levels of GB under drought stress.

Gene	Molecular function	Source	crop	Function	Additional note
BADH	*Betaine aldehyde dehydrogenase*	*A. Hortensis*	*O. sativa*	Improved growth & salinity tolerance	10% transgenic plants set seed in 0.5% NaCl solution in greenhouse
BADH	*Betaine aldehyde dehydrogenase*	*H. Vulgare*	*N. tabacum*	Salt tolerance	Tolerance was judged by high biomass & photosynthetic
CHO	*Choline dehydrogenase*	*E. coli*	*O. sativa*	Drought & salt tolerance	Higher yield under stresses
betA	*Choline dehydrogenase*	*E. coli*	*Z. mays*	Drought tolerance at seedling stage	Grain yield was significantly higher compared with wild-type
betA	*Choline dehydrogenase*	*E. coli*	*G. hirsutum*	Drought tolerance	GB accumulation was positively correlated with drought stress
codA	*Choline oxidase*	*E. coli*	*S. tuberosum*	Oxidative, Drought & salt Stress tolerance	Stress-inducible GB production in non accumulators resulted in tolerance
CMO	*Choline monooxygenase*	*A. hortensis*	*G. hirsutum*	Salt tolerance	Seed cotton yield was significantly higher in transgenic plants

Trihelose biosynthesis:

Trehalose is a non-reducing disaccharide of glucose that functions as a protectant in the stabilization of biological structure and enhances the tolerance of organisms to abiotic stress. Trehalose protects the biological molecules in response to different stress conditions. It does not accumulate to high enough levels in most plants, probably because of the presence of trehalase activity. Trehalose synthesized in two steps from **glucose-6-phosphate** and **uridine diphosphoglucose**, *via* trehalose-6-phosphate. The first step is catalyzed by **trehalose phosphate synthase** (TPS), and the second by **trehalose-6-phosphatase** (TPP). TPS1 Gene from budding yeast have been cloned and used for engineering drought and salinity resistance in crop plants.



Gene	Molecular function	Source	Transformed plant	Function	Additional note
TPS1	Trehalose-6-phosphate synthase	*S. cerevisiae*	*N. tabacum*	Enhanced drought tolerance	A few transgenic plants showed phenotypic alteration along with stress tolerance
otsA & otsB	*TPS & TPP* fusion gene	*E. coli*	*O. sativa*	Drought, salt & cold tolerance	Sustained growth, less photo-xidative damage, enhanced photosynthesis under various stresses
TPP1	Trehalose-6-phosphate phosphatase	*O. sativa*	*O. sativa*	Enhanced salt & cold stress Tolerance	Analysis of transgenic plants suggested a possible role of TPP in transcriptional regulation pathways
TPS1	*Trehalose-6-phosphate synthase*	*S. cerevisiae*	*L. esculentum*	Drought, salt & oxidative stress tolerance	Sustained yield under all stress
TP	*Trehalose phosphorylase*	*P. sajor-caju*	*S. officinarum*	Drought tolerance	Transgenic plants did not withered even after 10 days of water deficit & also exhibited no pleiotropic effects
TPS1+ TPS2	*Bifunctional gene*	*S. cerevisiae*	*M. sativa*	Multiple abiotic stress tolerance	Improved growth with significant increase in biomass

Sugars and Sugar Alcohols

Accumulations of sugar-related compounds can response to osmotic stress. These compounds stabilize the membranes and proteins during dehydration. Sugars can replace the water molecules and stabilize the proteins or membranes in a similar of water molecules .They can form a glass phase in the dry state of high viscosity have capable of slowing down chemical reactions lead to long-term stability in a living system.

Manitol gene: Osmotically shocked cells synthesize and accumulate massive amount of osmoprotectory compounds. Such compounds possibly help the cells to lower their osmotic potential and to draw water from the outside medium. Manitol as an osmoprotectory compound is primarily found in microbes. By introducing manitol-1-phosphate dehydrogenease gene (mt1D) isolated from E. coli (Tarezynski et al., 1993) showed over-expression of manitol in tobacco plants. These transgenic plants showed tolerance to high NaCl levels (250 mM). Seeds of transgenic Arabidopsis transformed with mt1D gene under control of CaMV 35 promoter over produced manitol and germinated in a medium supplemented with high amount of NaCl (Thomas et al., 1995). Li et al. (2004) introduced mt1D gene into upland rice (Oryza sativa var. japonica) by microprojectile bombardment. Growth rate of transgenic plants was significantly higher than the control on MS medium containing 1% NaCl. Non-transgenic plants died after 35 days. They reported less membrane damage and low Na+/K+ ratio than the control under salt stress.

Gene	*Gene action*	*Species*	*Phenotypic expression*
mt1D	Mannitol-1-phosphate dehydro genase (mannitol synthesis)	Tobacco, *Arabidopsis*	Increased plant height and fresh weight under salinity stress Increased germination under salinity stress
m6pr	Mannose-6-phosphate reductase (mannitol synthesis)	*Arabidopsis*	Increased salt tolerance
IMT1	Myo-inositol O-methyl transferase (D-ononitol synthesis)	Tobacco	Performed better under drought and salinity stress

Fructans and other Carbohydrates

Fructan or polyfructose molecules serve as the main storage carbohydrate in many plant species. However, fructans are to be involved in abiotic stress tolerance by virtue of their presence in vacuoles**.** Fructans are also thought to protect plants against drought and cold stress evidence of this was derived from transgenic plants. It act as regulators or signal molecules, thus influencing plant metabolism, as scavengers of ROS**.** It promote the process of root branching, thus increasing the root surface and subsequent water uptake

Gene	Molecular function	Source	Transformed plant	Function
sacB	Levan sucrase, a fructosyl transferase	B. subtilis	N. tabacum, B. vulgaris	PEG-induced drought tolerance
Imt1	Myo-inositol-o methyl transferase	E. coli	N. tabacum	Drought & salt tolerance
mtlD	Mannitol-1-phosphate dehydrogenase	E. coli	S. melongena	Salt, drought & chilling tolerance
Ipk2β	Inositol polyphosphate 6-/3-kinase	A. thaliana	N. tabacum	Enhanced drought, salinity & freezing tolerance

Polyamine biosynthesis genes: In plants, polyamine, accumulate under several abiotic stress stimuli, including drought and salt. It has been suggested that this increase in polyamine concentration could be considered as an indicator of plant stress. With the availability of genes responsible for polyamine biosynthesis such as ADC (encodes for arginine decarboxylase), it is now possible to manipulate polyamine content using sense and antisense constructs of these genes in transgenic plants. Engineering of the plant polyamine biosynthesis pathway has concentrated mostly on two species, tobacco and rice (Kumar and Minocha, 1998; Capell and Christou, 2004). They suggested that further studies are required to understand the tolerance ability of these genes. The increase in putrescine levels in plants under stress might be the cause of stress-induced injury or alternatively a mean of protection against stress. Roy and Wu (2001) expressing oat adc cDNA in rice under control of an ABA-inducible promoter resulted in transgenic rice plants with increased biomass when grown under salt stress. Capell and Christou (2004) have generated a diverse rice germplasm with altered polyamine content. Transgenic rice plant expressing the SAMDC DNA accumulated spermidine and spermine in seed at 2-3 fold higher levels compared to wild type. In another set of experiment, they have obtained ten fold putrescine accumulation in transgenic rice plants laboring oat adc cDNA compared to wild type. Spermidine and spermine de-novo synthesis in transgenic plants under drought stress is corroborated by the activation of the rice SAMDC gene. Transcript levels for rice SAMDC reach their maximum levels at 6 days after stress induction. Such increase in the endogenous spermidine and spermine pools of transgenic plants not only regulates the putrescine response, but also exerts an anti-senescence effect at the whole plant level, resulting in phenotypically normal plants. Wild type plants, however, are not able to raise their spermidine and spermine levels after 6 days of drought stress and consequently exhibit the classical drought stress response (Capell and Christou, 2004).

Choline monooxygenase (CMO) catalyzes the committed step of glycinebetaine (GlyBet) biosynthesis in many flowering plants. Over-expression of AhCMO improved drought tolerance in transgenic tobacco when cultured in medium containing PEG6000 (Shen et al., 2002).

2. Engineering of genes encoding enzymes for scavenging active oxygen

A plant suffering from various stresses, which leads to overproduces active oxygen species in cells. To minimize the damaging effects of active oxygen, plants produce antioxidant to detoxifying harmful oxygen. Genes encoding enzymes with antioxidant capacity, such as ascorbate peroxidase, superoxide dismutase and glutathione reductase are involved in oxidative stress.

Example:

- Transgenic alfalfa overproducing **superoxide dismutase (SOD)** showed reduced injury from water deficit and freezing stresses in field conditions
- Engineering chloroplastic **superoxide dismutase (SOD)** in tobacco led to an increase of chilling tolerance of photosynthesis
- Tobacco plants that overproduced cytosolic **ascorbate peroxidase** showed increased tolerance to oxidative stress
- Over expression of **glutathione S-transferase** or **glutathione peroxidase** provided some protection against cold and salt stress in tobacco

Super oxide dismutase: Within a cell, the SODs constitute the first line of the defense against ROS. It is found in all subcellular locations, like mitochondria, chloroplast microsomes, glyoxysomes, peroxysomes, apoplast and the cytosol. SODs are metaloproteins found in various compartments of plant cells and contain Cu and Zn, Fe or Mn cofactors. Transgenic tobacco plants containing oxidative stress- related genes showed elevated levels of glutathione reductase, superoxide dismutase and ascorbate peroxidase, resulting in enhance drought tolerance (Van Rensburg and Kruger, 1994).

3. Engineering of genes encoding LEA Proteins

A genes encoding LEA (**Leaf Embryo Abundance**) protein is activated under osmotic stress. LEA proteins play a role in desiccation tolerance during seed development and in response to dehydration, salinity and cold stress.

- Maintenance of protein or membrane structure
- Sequestration of ions,
- Binding of water
- Operating as molecular chaperones

Transgenic rice carrying barley HVA1 gene had shown drought resistance (Xu et al., 1996). Gene HVA1 encodes for a group of three LEA proteins which get accumulated in vegetative organs during drought condition. Transgenic rice showed enhanced accumulation of the HVA1 protein, which led to higher growth rates, delayed stress-related damage systems and improved recovery from the removal of stress conditions (Xu et al., 1996). Transgenic wheat plants containing the HVA1 gene showed consecutive expression of the transgene resulting in improvement of growth characteristics under water deficient conditions, more biomass and more efficient water use (Sivamani et al., 2000). Rohila et al. (2002) transformed Pusa Basmati 1 with HVA1 to increase tolerance against abiotic stresses. They developed transgenic lines which showed increased stress tolerance in terms of cell integrity and growth after imposed salt-and water stresses. Their findings exhibited high levels of LEA3 accumulation in the leaves of transgenic Pusa Basmati 1 rice plants might have conferred the significant increase in tolerance against drought and salt stresses.

Osmotic stresses induce Late-embryogenesis-abundant (LEA) proteins in vegetative tissues of plants. The functions of LEA proteins are largely unknown. Nevertheless, their considerable synthesis during the late embryogenesis, their induction by stress and their structural characteristics permit the prediction of some of their functions. It has been suggested that LEA type proteins act as water binding molecules, in ion sequestration and membrane stabilization. LEA-proteins are encoded by Responsive to Dehydration (RD), Early Responsive to Dehydration (ERD), cold inducible (KIN), cold regulated (COR) and responsive to ABA (RAB) genes in different plant species. Xu et al. (1996) found that the hva1 gene, which encodes for a specific class of LEA proteins, when overexpressed in rice leads to increased salt tolerance. This study demonstrated that subcellular compartmentalization of the biosynthesis of glycinebetaine was a critical step in attaining enhancement of tolerance for salinity and water stress. Tansformation of Chinese cabagge (Brassica campestris ssp. pekinensis) by overexpression a B. napus Group 3 LEA gene enhanced tolerance to salinity and drought (Park et al., 2005).

It was found that the COR15am the mature COR15a polypeptide, acts directly as cryoprotective protein by inhibiting the formation of hexagonal II phase lipid, a major type of freeze-induced membrane lession in non-acclimated plants (Steponkus et al., 1998). The COR15a gene enhanced the freezing tolerance of chloroplasts in engineered plants by almost 2C, which was nearly one third of the increase seen due to cold acclimation. While, this might not appear as large increase, an improvement of freeze tolerance by 2°C could potentially benefit certain crop plants (Artus et al., 1996). COR85, a group-II LEA protein was shown to be involved in cryoprotection of freezing-sensitive enzymes (Kazuaka and Oeda, 1994).

Overexpression of atRZ-1a did not affect the expression of various cold-responsive genes such as COR6.6, COR15a, COR47, RD29A, RD29B and LTI29 (Kim and Hunseung, 2006). Proteome analyses revealed that overexpression of atRZ-1a modulated the expression of several stress-responsive genes and the transcript levels and RNA stability of these target genes were not affected by atRZ-1a. atRZ-1a successfully complements the cold sensitivity of Escherichia coli lacking four cold

shock proteins. These results strongly suggest that atRZ-1a plays a role as an RNA chaperone during the cold adaptation process.

4. Engineering of genes encoding heterologous enzymes with different temperature optima

Homologous proteins and their specific enzymatic activities from different plant species differ in terms of their temperature

– E.g: When a gene of **NADH-hydroxypyruvate reductase** from cucumber (higher optimum growth temperature) was transformed into tobacco (lower optimum growth temperature), the optimum temperature of transgenic tobacco was increased.

5. Genetic Engineering of molecular Chaperones

'Chaperone' : assist and maintain correct folding and trafficking of cell proteins, are crucial for plants to survive in abiotic stresses. E.g: An antisense inhibition of the *HSP70* family prevented Arabidopsis plants from acquiring thermotolerance. Heat shock proteins *HSP101* from soybean, corn, wheat and tobacco complemented a thermo tolerance defect in yeast caused by deletion of yeast *HSP104*.

Heat shock proteins: It is important to maintain protein in their functional conformations and preventing aggregation of non-native proteins under stress. Many stress responsive proteins, particularly Heat Shock Proteins (HSPs) have been shown to act as molecular chaperones, which are responsible for protein synthesis, targeting, maturation and degradation in a broad array of normal cellular process. Furthermore, molecular chaperones function in the stabilization of proteins and membranes and in assisting protein folding under stress conditions.

Ahn and Zimmerman (2006) fused DcHSP17.7 gene (a carrot heat shock protein gene encoding HSP17.1) to a 6XHistidine (His) tag to distinguish the engineered protein from endogenous potato proteins and it was introduced into the potato cultivar Desiree under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The integration was confirmed by Western Blot, which showed constitutive integration of DcHSP17.7 in transgenic potato lines before heat stress. They observed improved cellular membrane stability at high temperature, compared with wild type and vector controlled plants. Transgenic potato lines also exhibited enhanced tuberization in vitro.

Table 4: Gene encoding for molecular chaperones and transgenic development

Gene	Gene action	Species	Phenotypic expression
Hsp70	Heat-inducible anti-sense HSP70	*Arabidopsis thaliana*	Increased thermotolerance in transgenic plants
Hsp17.7	Heat shock protein	*Daccus carota* L.	Increased or decreased thermotolerance
P5CR	Inducible heat shock promoter (IHSP)	*Glycin max*	Increased proline accumulation
Hsp101	Heat shock protein	*Arabidopsis thaliana*	Decreased thermotolerance in Hsp101-deficient (hot1) mutant
Hsp101	Heat shock protein	*Arabidopsis thaliana*	Manipulated thermotolerance in transgenic plants
DcHSP17.7	Heat shock protein	*Solanum tubersum* L.	Improved cellular membrane stability and enhanced *in vitro* tuberization

Heat shock factors: Various studies showed that plant heat shock proteins are not only express in response to heat shock, but also under water, salt, oxidative stress and at low temperature. Li et al. (2003) suggested that Hsf (heat shock factor) gene may play a pivotal role in heat-shock-induced chilling tolerance and constitutive expression of the transcriopton regulated gene in chilling sensitive crops may be useful in improving tolerance against chilling stress. They transferred Arabidopsis thaliana Hsf1b (AtHsf1a) gene into tomato. The transgenic tomato plants harbouring this gene showed increased chilling tolerance.

6. Engineering of genes encoding Transcription Factors

Responses of plants to abiotic stresses are multigenic and a single gene is not likely to induce the whole cascade. Recently, transcription factors for abiotic stress-induced genes have been identified,

cloned and used in transgenic experiments. Simultaneous expression of downstream stress-inducible genes have been achieved with parallel increase in stress tolerance.

Dehydration responsive transcription factor: Plant genomes contain a large number of Transcription Factors (TFs). TFs are key regulatory proteins that enhance or repress the transcriptional rate of their target genes by binding to specific promoter regions upon activation or deactivation of upstream signaling cascade. Most of these transcriptional factors belong to a few large multigene families. Individual members of the same family often respond differently to various stress stimuli, on the other hand, some stress responsive may share the same transcriptional factor (Chen et al., 2002; Kreps et al., 2002). The Dehydration-responsive Element (DRE) and C-repeat binding factors (CBF) was identified as a cis-acting element regulating gene expression in response to dehydration (salt, drought and cold stresses) in Arabidopsis (Van Rensburg and Kruger, 1994). The dehydration-responsive transcription factors DREB and C-repeat binding factors (CBF) bind to DRE and CRT cis-acting elements that contain the same motif (CCGAC). Members of the CBF/DREB1 family, such as CBF1, CBF2 and CBF3 (or DREB1B, DREB1C and DREB1A, respectively) are themselves stress-inducible. DREB/CBF proteins are encoded by AP2/EREBP multigene families and mediate the transcription of several genes. DREB1A, a transcription factor that recognizes dehydration response elements, has been shown in Arbidopsis thaliana to play a crucial role in promoting the expression of drought tolerant genes (Pellegrineschi et al., 2003, 2004). They have transformed DREB1A gene into wheat. Plant expressing the gene demonstrated substantial resistant to water stress compared with the control under stress condition. Recently, overexpression of CBF4 from barley has been shown to confer salinity, drought and low temperature tolerance in transgenic rice (Oh et al., 2007).

The NAC gene family members encode one of the largest families of plant specific TFs and are expressed in various developmental stages, tissues and environmental factors. Hu et al. (2006) isolated and characterized NAC gene SNAC1. SNAC1 over-expressing transgenic rice plants showed significantly improved drought resistance under field conditions and strong tolerance to salt stress (Hu et al., 2006).

The phosphoinositide pathway and inositol-1,4,5-trisphosphate (InsP3) are implicated in plant responses to stress. To determine the downstream consequences of altered InsP3-mediated signaling, Perera et al. (2008) generated transgenic Arabidopsis thaliana plants expressing the mammalian type I inositol polyphosphate 5-phosphatase (InsP 5-ptase), which specifically hydrolyzes soluble inositol phosphates and terminates the signal. Rapid transient Ca2+ responses to a cold or salt stimulus were reduced by ~30% in these transgenic plants. Drought stress studies revealed, surprisingly, that the InsP 5-ptase plants lost less water and exhibited increased drought tolerance. The onset of the drought stress was delayed in the transgenic plants and abscisic acid (ABA) levels increased less than in the wild-type plants. Stomatal bioassays showed that transgenic guard cells were less responsive to the inhibition of opening by ABA but showed an increased sensitivity to ABA-induced closure. Transcript profiling revealed that the drought-inducible ABA-independent transcription factor DREB2A and a subset of DREB2A-regulated genes were basally upregulated in the InsP 5-ptase plants, suggesting that InsP3 is a negative regulator of these DREB2A-regulated genes. These results indicated that the drought tolerance of the InsP 5-ptase plants is mediated in part via a DREB2A-dependent pathway and that constitutive dampening of the InsP3 signal reveals unanticipated interconnections between signaling pathways (Perera et al., 2008).

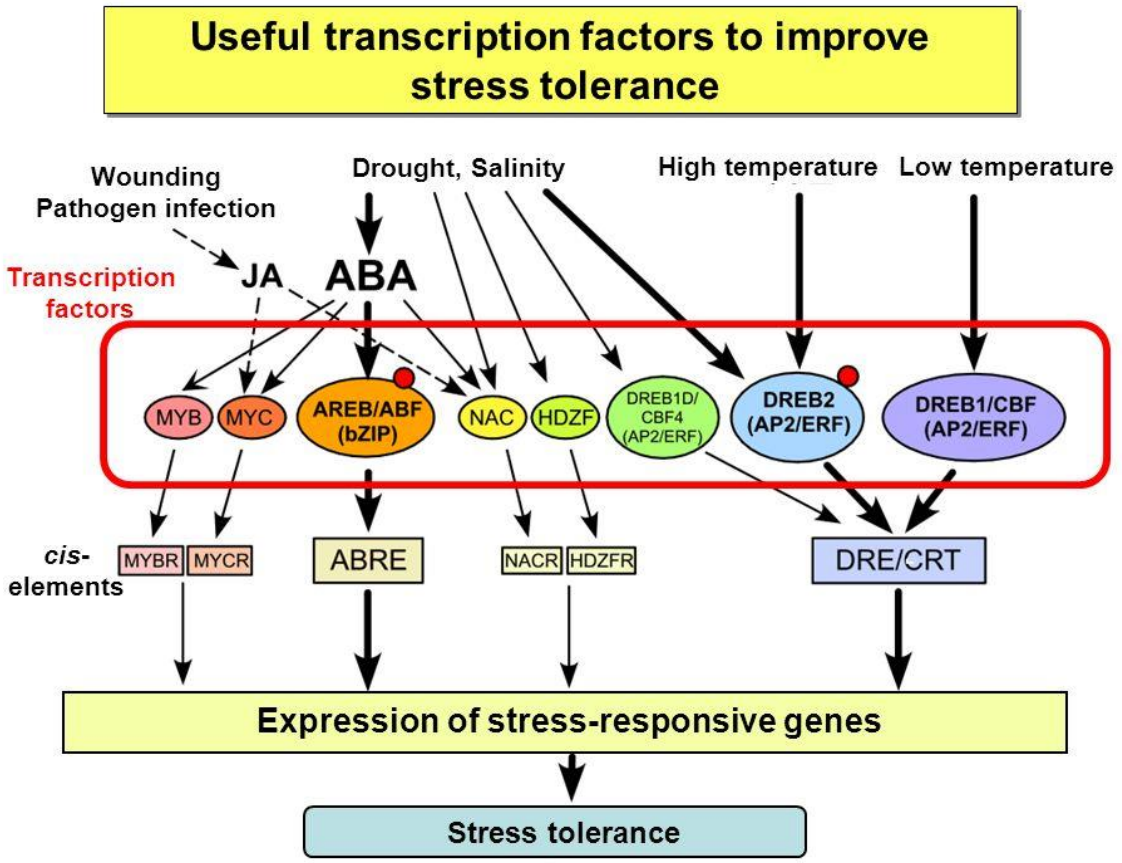
Mitogen-activated protein: Expression of mitogen-activated protein kinase gene (MAPK) genes activates an oxidative signal cascade and lead to the tolerance of freezing, drought, heat and salinity stressing transgenic tobacco. MAPKs perform their function as a part of kinase modules, which is mainly composed of MAPKs, MAPKKs and MAPKKKs. Shou et al. (2004) transformed maize with a

tobacco MAPKKK (NPK1). They found that the NPK1 expression enhanced drought tolerance in transgenic maize. Under drought stress, it maintained significantly higher photosynthesis rates than did the non-transgenic control, suggesting that protected photosynthesis machinery is protective from dehydration damage.

ABA Signaling: Protecting crop yield under drought stress is a major challenge for modern agriculture. One biotechnological target for improving plant drought tolerance is the genetic manipulation of the stress response to the hormone abscisic acid (ABA). In response of water stress, ABA levels in plant greatly increase resulting closure of stomata, thereby reducing the level of water loss through transpiration from leaves and activate response of genes. ERA1, a gene identified in Arabidopsis, encodes β-subunit of a farenesyl-transfarase and involves in ABA signaling. Wang et al. (2005) developed transgenic plants which performed significantly better under water stress, with consistently higher yield over conventional varieties of canola, whereas they observed no difference in performance of transgenic and control under sufficient water.

Dehydration responsive element: Many of the known cold regulated genes were under control of a primary master regulator, CBF/DREB1. Dehydration Response Element (DRE) plays an important role in the response to low temperature. The transcription factor DREB1A specifically interacts with DRE and induces the expression of stress tolerance genes in plant.

Over expression of DREB1A in transgenic Ar*a*bidopsis plants activated the expression of many of these stress tolerance genes and resulted in tolerance to freezing (Liu et al., 1998; Kasuga et al., 1999; Seki et al., 2001; Urao et al., 2000). Liu et al. (1998) and Jaglo-Ottosen et al. (1998) activated gene expression by overexpressing a homolog of CBF1, designated DREB1A. Their result indicated that the expression of the CRT/DRE regulation also increase drought tolerance. Liu et al. (1998) observed that the overexpression of DREB1A in transgenic Arabidosis resulted in dwarf phenotype. Fan et al. (2002) cloned an antifreeze gene from carrot and successfully transferred it into tobacco.



Ion transporter and Antiporter genes: A salt concentration of 200 mM is equivalent to 40% of the salt concentration of sea water and will inhibit growth of almost all crop plants.

Ion trans porters selectively transport ions and maintain them at physiologically relevant concentrations while Na+/H+ antiporters also play a crucial role in maintaining cellular ion homeostasis, thus permitting plant survival and growth under saline conditions. The Na+/H+ antiporters catalyze the exchange of Na+ for cytoplasmic pH, sodium levels and cell turgour (Serrano et al., 1998).

A construct containing the DtNHX gene, coding for a vacuolar Na+/H+ antiport from Arabidopsis thaliana, was introduced into the genome of Brassica napus cv. Westar by Zhang et al. (2001). Over expression of the vacuolar Na+/H+ antiport did not affect the growth of transgenic plants since similar growth was observed when wild type and transgenic plants were grown in the presence of 10 mM NaCl. While growth of wild type plants was severely affected by the presence of 200 mM NaCl in the growth solution, transgenic plants grew, flowered and produced seeds. They noted that the transgenic plants grown at 200 mM NaCl produced number of seeds similar to those of wild-type plants grown at low salinity. Moreover, qualitative and quantitative analysis of oil content showed no significant differences between seeds from wild type plants grown at low salinity and transgenic plants grown at high salinity. They also observed that the transgenic plants accumulated up to 6% Na without altering the yield and oil content. Transgenic Brassica juncia plants over expressing pgNHX1 withstand 300 mM salt stress till the seed setting stage and exhibited normal growth phenotype without much loss in seed yield (Rajgopal et al., 2007).

Glenn et al. (1999) have engineered transgenic Arabidopsis plants that overexpress AtNHX1, a vacuolar Na+/H+ antiport, which allowed the plants to grow in 200 mM NaCl. Zhang and Bhumwald (2001) reported the genetic modification of tomato plants to overexpress the Arabidopsis thaliana AtNHX1 antiport, which likewise allowed those plants to grow in the presence of 200 mM NaCl. Besides providing farmers with a cash crop for salted lands, such, plants may also pull salt out of soils, enabling other crops to thrive again. Wheat productivity is severely affected by soil salinity due to Na+ toxicity to plant cells. Xue et al. (2004) generated transgenic wheat expressing a vacuolar Na+/H+ antiport gene AtNHX1. The transgenic wheat lines exhibited improved biomass production. The field trial revealed that the transgenic wheat lines produced higher grain yield and heavier and larger grains in the field of saline soil. The transgenic rice accumulated a lower level of Na+ and higher level of K+ in the leaves than the non-transgenic plants under saline environment.

The *Escherchia coli* nahA gene encodes a Na+/H+ antiporter, which plays critical role in ion homeostasis has been transferred into rice (Oryza sativa L. sp. Japonica) by Wu et al. (2005). The transgenic plants showed better germination rate, growth and average yield per plant than control. They also reported higher sodium and proline content in transgenic lines, implying that nhaA over-expression enhance osmoregulation by activating the bio-synthesis of proline.

H+-pyrophosphatase (H+-Ppase) gene: An H+-Ppase gene named TsVP involved in basic biochemical and physiological mechanisms was cloned from Thellungiella halophila. Transgenic tobacco overexpressing TsVP had 60% greater dry weight than wild-type tobacco at 300 mM NaCl (Gao et al., 2006). Their findings suggested that over expression of H+-Ppase causes the accumulation of Na+ in vacuoles instead of in the cytoplasm and avoids the toxicity of excess Na+ in plant cells.

Wax production: The gene designated WXP1, is able to activate wax production and confer drought tolerance in alfalfa (Medicago sativa). Overexpression of WXP1 under the control of CaMV355 promoter led to a significant increase in cuticular wax loading on leaves of transgenic alfalfa (Zhang et al., 2005). WXP1 over expression induced a number of wax-related genes.

Transgenic leaves showed reduced water loss and chlorophyll leaching- transgenic alfalfa plants with increased cuticular wax showed enhanced drought tolerance demonstrated by delayed wilting after

watering has been ceased and quicker and better recovery when the dehydrated plants were re-watered.

A full length rDNA of dehydrin BcDh2 from *Boea crassifolia* and its antisense nucleotide sequence was transferred into tobacco, var. NC89 under the control of CMV promoter (Ye et al., 2004). Under progressive water stress, the photosynthetic rate, transpiration rate and stomatal conductance of sense and antisense plants decreased. However, those parameters increased after 24 h of watering and the enhanced was higher in sense and antisense plants than the control.

Table 5: Cold tolerance gens/transgenic plants

Genes	Gene action	Species	Phenotypic Expression
Sod	Cu/Zn-SOD	Tobacco	Retained 90% photosynthesis under chilling and heat stress
Socl	Mn-SOD	Alfalfa	Increased tolerance to freezing stress
Nt107	Glutathion Stransferase	Tobacco	Sustained growth under cold and salinity stress
Wx	Controls amylase synthesis	Rice	Increased amylose content at low temperatures
CBF1	Transcription factor	*Arabidopsis*	Increased cold tolerance
CodA	Choline oxised (Glycine betaine synthesis)	*Arabidopsis*	Seedling tolerant of salinity stress and increased germination under cold
COR15a	Cold induced gene	*Arabidopsis*	Increased freezing tolerance
DREB	Transcription factor	*Arabidopsis*	Increased tolerance to cold, drought and salinity
AB13	Transcription factor	*Arabidopsis*	Increased freezing tolerance
Gs2	Chloroplastic glutamine synthetase	Rice	Increased salinity resistance and chilling tolerance
OsCDPK7	Transcription factor	Rice	Increased tolerance to cold, salinity and drought

Osmotin: Sarad et al. (2004) developed transgenic tomato with osmotin gene. Their preliminary tests revealed that the transgenic plants are more tolerant to cold than wild types.

Dehydrin: To elucidate the contribution of dehydrins (DHNs) to freezing stress tolerance, Puhakainen et al. (2004) developed transgenic Arabidopsis plants over-expressing multiple DHN genes. The transgenic plants exhibited lower LT50 values and improved survival when express to freezing stress compared to control plants. Thus they concluded that dehydrines contribute to freezing stress tolerance in plants and this could be partially due to dehydrins protective effect on membrane.

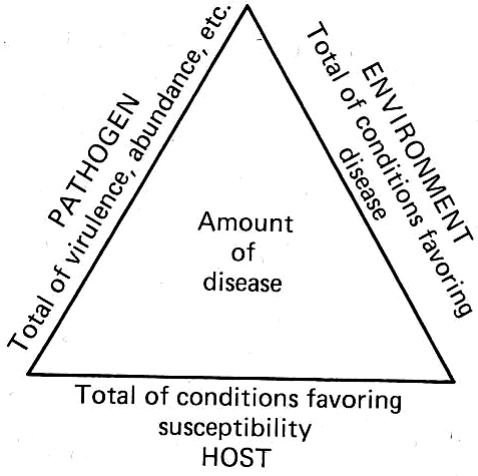
11. Genetics of disease resistance in crop plants.

Disease:

Disease is an abnormal condition in a plant produced by an organism or some other factor. Agrios (1970) has defined disease as follows: "Disease is a series of invisible and visible responses of plant cells and tissues to a pathogenic microorganism or an environmental factor that result in adverse changes in form, function or integrity of plant and may lead to partial impairment or death of the plant or it parts." The organism causing a desease is known as pathogen, while the plant affected by the disease is called host.

Disease Development

The events occurring from the time of landing of a pathogen on a host to the point of appearance of disease symptoms is called **disease development**. In case of fungal pathogens, when a spore comes in close contact with its host, it is termed as **contact**. The spore germinates and hypha enters into the host tissue either through a natural opening. a wound or with the help of hydrolytic enzymes; this is known as infection. During establishment phase, the pathogen proliferates and spreads within host tissue, but no disease symptoms appear. The next phase, development phase, is characterised by the appearance of typical symptoms of the disease. In addition, the pathogen produces spores, i.e., it multiplies.



Development of any disease is affected by (1) host and (2) pathogen genotypes, and (3) the environment. Both contact and infection stages are greatly affected by the environment; disease escape is the consequence of this effect. Host genotype greatly influences the establishment and development phases. Some cases of resistance prevent establishment, while others reduce symptom development and spore production. The pathogen genotype determines if it will be able to produce disease symptoms in a given host strain.

Disease Escape:

When plants/strains susceptible to a disease remain free from the disease, it is called **disease escape**. Disease escape occurs primarily by avoiding contact, i.e., failure of pathogen to land on the susceptible plant or a specific part of the plant. But often unfavourable weather conditions may prevent infection. In crop production, disease escape is a valuable tool. It can be achieved by early varieties, changed dates or sites of planting, use of resistant root stocks (for soil-born pathogens), a balanced application of NPK, and use of chemicals to control either the disease vector or the pathogen

itself. But in breeding for dise resistance, disease escape is a nuisance as it does not allow an efficient selecton for resistance.

Inheritance of Pathogenicity:

The ability of a pathogen to produce disease in its host plant is called **pathogeneicity or virulence**. In most pathogens, pathogenicity is governed by several genes. Each gene usually has two alleles. In general, the dominant allele of a gene specifies nonpathogeniciy or avirulence, while the recessive allele conditions virulence or pathogenicity. A study of inheritance oI pathogenicity is possible only when resistant varieties are available in the host, while isolate B is virulent on Wealthy only. The ascospores produced by the cross A x B are of four types: (1) avirulent on both, (2) virulent on both, (3) virulent on Wealthy only, and (4) virulent on McIntosh only. It is clear from the above that virulence in apple scab fungus is determined by at least two genes and each gene has two alleles. More genes for pathogenicity may be discovered if more isolates of the pathogen are evaluated on several different resistant strains of the host.

Pathogens have a wide range of flexible mechanisms for producing genetic variability, including that for pathogenicity. New genetic variability in pathogenicity may be created in one of the following ways: (i) mutation (it is the ultimate source of all genetic variability), (ii) segregation and recombination accompanying sexual reproduction, (iii) heterokaryosis in case of fungi; two genetically different nuclei are present in a single cell), and (iv) parasexual reproduction (in fungi). Heterokaryosis may produce a new race of a fungal pathogen. When it is coupled with nuclear fusion, followed by production of haploid nuclei from them due to mitotic irregularities (parasexual reproduction), new gene combinations are produced that may lead to formation of new races.

TABLE 21.1. Inheritance of pathogenicity in the fungus Venturia inaequalis, the causal agent of apple scab

Pathogen isolate	*Host variety*			*Remarks*
	Wealthy	*McIntosh*		
A	R*	S		
B	S	R		
Ascospores from A × B cross	R	R	(25% spores)	Pathogenicity due to 2 genes; each gene has two alleles.
	R	S	(25% spores)	
	S	R	(25% spores)	
	S	S	(25% spores)	

*R = resistant host response (disease symptoms absent), and S = susceptible host response (disease symptoms present).

Physiological Races and Pathotypes

Physiological races are strains of a single pathogen species, which differ from each other in their ability to attack different varieties of its host species. The varieties of a host species used to identity physiological races of a pathogen are called **host testers or differential hosts**. The genes for resistance present in the host testers are ordinarily not known.

But when pathogen isolates are classified on the basis of their virulence to host varieties each of which carries a single distinct gene for resistance, they are referred to as **pathotypes**. Pathotypes are identified by **ideal differential hosts**, cach of which carries a single and distinct gene for resistance.

A set of 12 ideal differentials is available for pathotype classification of the pathogen for late blight of potatoes (*Phytophthora infestans*). Pathotype differentiation is common in air-borne fungi and viruses. Some pathotype differentiation occurs in soil-born fungi, bacteria, nematodes and insects.

TABLE 21.2. Pathotype classification based on a set of 12 differential hosts, each carrying a single distinct resistance gene (R_1 - R_{12}) to Phytophthora infestans, the fungus causing potato late blight

Resistance gene present in the differential host	*Pathotype of potato late blight fungus*											
	$P_{(1)}$	$P_{(2)}$	$P_{(3)}$	$P_{(4)}$	$P_{(5)}$	$P_{(6)}$	$P_{(7)}$	$P_{(8)}$	$P_{(9)}$	$P_{(10)}$	$P_{(11)}$	$P_{(12)}$
R_1	S											
R_2		S										
R_3			S									
R_4				S								
R_5					S							
R_6						S						
R_7							S					
R_8								S				
R_9									S			
R_{10}										S		
R_{11}											S	
R_{12}												S

Note: Only the susceptible reactions are shown. Complex pathotypes carrying more than one virulence gene, *e.g.*, $P_{(1, 2)}$, $P_{(1, 5, 8, 12)}$, etc., are identified by their virulence against the respective host genes for resistance (*e.g.*, R_1, R_2, and R_1, R_5, R_8, R_{12}, etc., respectively). A susceptible reaction of the host indicates the virulence of pathotype, while a resistant reaction shows avirulence.

Disease Resistance

The response of different lines of a host to different strains of a pathogen may be classified as follows: (1) susceptible, (2) immune, (3) resistant and (4) tolerant.

Suseceptible Reaction: There is profuse disease development and spore production presumably unchecked by host genotype.

Immune Reaction: The host plants do not show any symptom of the disease. Generally, it is produced by a **hypersensitive reaction** by the host, i.e., a group of cells surrounding the point of infection dies and, usually, the pathogen also dies along with the host cells. This type of reaction is found in case of obligate parasites or biotrophic pathogens. In some cases, it may result from a prevention of the pathogen from reaching the appropriate part of its host.

Resistance: Disease symptom production and pathogen reproduction both occur. But both are much less in magnitude than those in the case of susceptible response. It is believed to involve nutritional factors and, in some cases, chemical growth inhibitors may be involved.

Tolerance: In this case, disease symptoms and pathogen reproduction are comparable to those in susceptible response. But the reduction in yield and/or biomass is significantly lower. Plant breeders always select jor tolerance whenever yield is the basis election

Genetics of Disease Resistance

Disease resistance shows the follwing three distinct modes of inhertance: (1) oligogenic, (2) polygenic and (3) cytoplasmic.

Oligogenic Resistance:

In such cases, one or few oligogenes/major genes govern resistance and resistance is generally dominant to the susceptible reaction. Oligogenes usually produce immune reaction, which is race-specific i.e., it is synonym to vertical resistance. More than 30 different resistance genes are known for stem rust of wheat.

Polygenic Resistance:

In polygenic inheritance, resistance is determined by several genes. Each gene produces a small effect, which is mainly additive in nature, but they also have non-additive effects. There is, in addition, a large environmental effect. As a result, host reaction (disease severity) shows a continuous variation. This type of resistance is race-nonspecific and synonymous to horizontal resistance. The mechanism of resistance involves resistance to infection, slow growth of pathogen, and reduced spore production. This type of resistance has never been shown to be eroded or broken down.

In some cases, a single oligogene, e.g, Lr34 in case of leaf rust of wheat, and 2 or other genes may act in additive manner to produce what is called **slow rusting or partial resistance**. A similar situation is known for wheat stem rust resistance gene Sr34 and some additional genes having additive effects.

Accurate measurement of polygenic resistance is based on an evaluation of disease severity. This assessment is not easy mainly because disease severity is affected by several factors, including inoculum pressure and interplant interference. Polygenic resistance be assessed as (i) percentage of tissue covered with the pathogen, (ii) area of tissue showing disease symptoms, e.g, discoloration, or (iii) the severity of symptoms, e.g., leaf rolling, moltling, etc., depending mainly on the host-pathogen system.

This type or disease reaction, i.e., severity of the disease, is thought to generally have the following components: infection frequency, latent and incubation periods, lesion size, spore production and infectious period. Many workers feel that selection for the component traits may be more desirable than that based on disease reaction itself.

Cytoplasmic Inheritance:

In a few cases, disease resistance is specified by plasmagenes located in the cytoplasm. The only good example of this type is the resistance in maize to *Helminthosporium* leaf blight; it is specified by mitochondrial DNA.

Vertical and Horizontal Resistance

These terms harizontal (HR) and vertical vesistance (VR) were introduced by Van der Plank and are widely used. A brief discussion of these terms is given below.

Vertical Resistane:

It is generally determined by major genes and is race-specific. Race-specificity means that a host variety carrying a resistance gene, says R_1, will be attacked by only that race, which has the matching virulence gene; all other races will be avirulent. Thus vertical resistance is overcome by the appropriate virulent race.

Horizontal Resistance:

This type of resistance is generally controlled by polygenes and is race-nonspecific. It does not produce immune response, but produces resistant response to all the races of the pathogen.

The relative usefulness of horizontal and vertical types of resistance depends mainly on the crop species and the disease in question. For example, in case of potato late blight, horizontal resistance is the goal of breeding, while in case of wheat stem rust vertical resistance remains the strategy of choice.

Comparison between Vertical and Horizontal Resistance:

VR and HR differ for various features like type of response to the pathogen, the nature of genetic control, the stage of host development when they are expressed, etc.; these are summarised in Table.

resistance

Feature	*Vertical resistance*	*Horizontal resistance*
Pathotype-specificity	Specific	Nonspecific
Nature of gene action	Oligogenic	Polygenic; rarely oligogenic
Response to pathogen	Usually, hypersensitive	Resistant response
Phenotypic expression	Qualitative	Quantitative
Stage of expression	Seedling to maturity	Expression increases as plant matures
Selection and evaluation	Relatively easy	Relatively difficult
Risk of 'boom and bust'	Present (rarely durable)	Absent (durable)
Suitable for :		
1. Host	1. Annuals but not perennials	Both annuals and perennials
2. Pathogen	2. Immobile pathogens, *e.g.*, soil pathogens, but not for mobile air-borne, pathogens	All pathogens
Need for specific deployment of resistant varieties	Critical for success with mobile pathogens	None
Need for other control measures	Likely	Much less likely
Host-pathogen interaction*	Present	Absent
Commonly used, but not strictly synonyms terms	Major gene, race-specific, seedling, monogenic, differential, specific, pathotype-specific resistance	Polygenic, race-nonspecific, pathotype-nonspecific, mature plant, adult plant, field, uniform resistance
Efficiency	Highly efficient against specific races	Variable, but operates against all races

*Detectable by analysis of variance of a suitable experiment.

Vertical resistance to specific races is generally governed by a single (monogenic) dominant gene or by a few dominant genes. Some of these genes may be multiple alleles as in leaf rust gene, Lr2 that accords resistance to *Puccinia* recondite tritici. In that locus, four genes designated as Lr2a, Lr2b, Lr2c and Lr2d are present and are tightly linked. Each of these genes accord resistance to a different spectrum of races and hence can be differentiaited from one another. Such multiple alleles exist on Sr9 locus of wheat for *P.graministritici* and gene Pi-k in rice for resistance to *Pyricularivagrisea*. The tight linkage between the multiple alleles permits an efficient transfer of all these genes in one attempt.

'Horizontal resistance' (HR) reduces the rate of disease spread and is evenly spread against all races of the pathogen. The low terminal disease severity in HR is assumed to result from polygenic resistance. Morphological features such as size of stomata, stomatal density per unit area, hairiness, waxiness and several others influence the degree of resistance expressed. Partial resistance, dilatory resistance, lasting resistance are some other terms coined for denoting horizontal resistance.

The phenomenon of slow rusting manifested as lesser number of pustules per unit leaf area, smaller size of uredosori and increased latent period in some wheat cultivars is a typical example of this type of resistance. Although it is preferable to use varieties that have both vertical and horizontal resistance, most of the resistant varieties carry only one or few (2 or 3) major genes of vertical resistance. If varieties are resisitant only to some of the races of pathogen and if the pathogen is airborne, then new races evolve easily, as happens with cereal rusts, the powdery mildlew and *Phytophthorainfestans*. Appearance of new races leads to breakdown of resistance of the popular, ruling genotype. As a result, varieties with vertical resistance need to be replaced at frequent intervals.

Boom and burst cycle

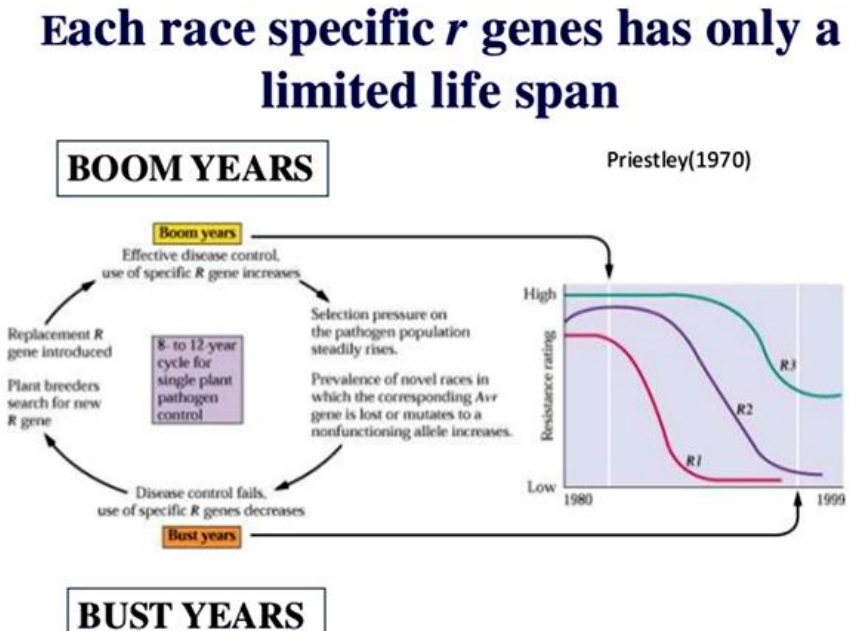
In varietial improvement programmes, it is easy to incorporate the monogenic vertical resistance genes. But the success of exploiting the monogenic host resistance invariably does not last long. Whenever a single gene-based resistant variety is widely adopted, the impact would be the arrival of new matching pathotypes.

These pathotypes soon build up in population to create epidemics and eventually the variety is withdrawn. This phenomenon is generally called " boom and burst". The avoid the implications of boom and burst phenomenon, use of durable host resistance is advocated in several crops. Durable resistance remains effective even though it may be widely grown ovoer a long period of time, in an environment that favours the disease. For example, oat variety, Red Rust Proof is still resistant against crown rust even after a hundred years. Wheat varieties, Thatcher and Lee have withstood stem rust for 55 and 30 years, respectively. Cappelle Desprez expresses at adult stage, a moderate resistance to yellow rust and this has been maintained for the last 20 years.

Two of the genes like Lr34 for resistance of leaf rust and Sr2 for resistance to stem rust have been recognized for durability. Wheat cultivars such as HD2189, HP1102, DL153-2, DL803-3 and DL802-2, which possess Lr34 with other gene combinations have a good degree of resistance and hae become popular with growers. So far, there is not precise way available to identify the genetic components that are associated with durable resistance. Nor does dissociation of genes for virulence totally explain the basis of varietal durability, though it is likely to be the most plausible reason Boom and burst cycle-a characteristic of vertical resistance Resistance to virus and virus vectors Resistane to plant pathogenic viruses is generally oligogenic in nature

For example, the host pathogen reaction to the barley yellow dwarf virus (BYDV) is controlled by detectable single gene. The discovery of Yd2 gene in Ethiopial barley further confirms that against some of the viral diseases, vertical resistance is very much functional. Antiobiotics is the most common phemomenon where the host plant metabolites interfere with the normal life and growth of the insects following feeding activity.

Invariably, the adult body weight, fecundity and various facets of multiplication of the insects are adversely affected. The number of life cycles completed in a given period of time is also less. Therefore, in plants that exhibit antibiosis towards crop maturity, there is marked reduction in the level of pest infestation (virus vector population) and host damage. Mechanism of disease resistance or Nature of disease resistance Disease resistance is governed by several in built mechanisms of the host, plants against infection by the pathogen. They are disease escape, disease endurance or tolerance and true resistance.



a. Disease escape

It is a prevention mechanism that causes the host to escape pathogenic infection. Early or late maturity of the crop may prevent physical contact of the pathogen with the host. Mechanical and anatomical barriers such as thick cuticle, waxy bloom on leaves and stem, stomatal regulation prevent penetration of spores. Ergot, a fungal disease of inflorescence in cereals caused by *Clavicepspurpurea* does not affect varieties of wheat and barley in which the flowers remain closed until pollination occurs. Erect leaves of barley avoid deposition of spores of *Erysiphegraminis* tritici in contrast to prostrate leaves. Early maturing varieties of groundnut escape early leaf spot infection (*Cercosporaarachidicola*) and early varieties of wheat escape rust and loose smut infection.

A change in planting season has also been successfully employed as a measure of securing escape, e.g., the leaf rust of sugarcane (Puccinia sacchari) in the canal areas of Bombay severely affects cane when planted in June, but is of minor importance or absent in crops sown in October. Disease escape confers pseudo-resistance.

b. Disease endurance

The host after being infected by the pathogen tolerates the infection and suffers less damage. It does not result in any substantial decrease in yield. This is brought about by influence of external factors. It is a well-known phenomenon that plants fertilized with phosphatic and potash manures are more tolerant to disease; this is the case in wheat against rust infection. Rice crops fertilized by silicates are "resistant" to blast (*Pyricularia oryzae*) in Japan. Wheat crops fertilized by potash and phosphatic manures are highly tolerant to mildew and rust infection. The fertilizers act indirectly to arrest vegetative growth and promote early maturity, better straw and strengthening tissues to protect the plant which form a bulwark against pathogenic invasion.

c. True resistance

It is the ability of the host plant to resist or withstand the attack of a pathogen. True resistance is inheritable and much less subject to environmental influence. It is specific in character. The basis of resistance may be morphological, functional, structural or protoplasmic. Functional nature of resistance is determined by opening of the stomata, time of opening of flowers and time of maturity, rate of cork formation and cambial activity.

Structural characters include the proportion of strengthening tissues, fibre content, nature of middle lamella, corky layers, number and structure of stomata and lenticels and their sizes. Protoplasmic factors controlling resistance are related to cell contents and include acids, tannins, anthocyanins, chemical constituents and their proportion, antibiotic activity and hypersensitivity present in the plant cells and in addition biological antagonism of the protoplasm of the host and the pathogen. True resistance, however, is of a specific character and is determined by the defence equipment and activities of the plant itself against the parasitic invasion and is therefore not subject to any appreciable modifications by external factors.

R genes and R proteins

Plants have evolved R genes (resistance genes) whose products mediate resistance to specific virus, bacteria, oomycete, fungus, nematode or insect strains. R gene products are proteins that allow recognition of specific pathogen effectors, either through direct binding or by recognition of the effector's alteration of a host protein. Many R genes encode NB-LRR proteins (proteins with nucleotide-binding and leucine-rich repeat domains, also known as NLR proteins or STAND proteins, among other names). Most plant immune systems carry a repertoire of 100-600 different R gene homologs. Individual R genes have been demonstrated to mediate resistance to specific virus, bacteria, oomycete, fungus, nematode or insect strains. R gene products control a broad set of disease resistance responses whose induction is often sufficient to stop further pathogen growth/spread.

Studied R genes usually confer specificity for particular strains of a pathogen species (those that express the recognized effector). As first noted by Harold Flor in his mid-20th century formulation of the gene-for-gene relationship, a plant R gene has specificity for a pathogen avirulence gene (Avr gene). Avirulence genes are now known to encode effectors. The pathogen Avr gene must have matched specificity with the R gene for that R gene to confer resistance, suggesting a receptor/ligand interaction for Avr and R genes. Alternatively, an effector can modify its host cellular target (or a molecular decoy of that target), and the R gene product (NLR protein) activates defenses when it detects the modified form of the host target or decoy.

Gene-for-Gene Relationship:

The gene-for-gene hypothesis of host and pathogen relationship was proposed, in 1955, by Flor on the basis of his work on flax rust resistance. So far, this hypothesis has been valid for almost all host-pathogen relationships, and it is universally accepted. According to this hypothesis, for every resistant gene present in the host, pathogen has a corresponding virulence gene. When pathogen has the virulence gene corresponding to the resistance gene present in the host, the pathogen produces disease, i.e., the host response is susceptible. But when the pathogen does not have the virulence gene corresponding to a resistance gene present in the host, disease is not produced, i.e., host response is resistant. Flor showed that the inheritance of both resistance in the host and parasite ability to cause disease is controlled by pairs of matching genes. One is a plant gene called the resistance (R) gene. The other is a parasite gene called the avirulence (Avr) gene. Plants producing a specific R gene product are resistant towards a pathogen that produces the corresponding Avr gene product. Gene-for-gene relationships are a widespread and very important aspect of plant disease resistance. An example can be seen with *Lactuca serriola*.

RR = homozygous resistant ; Rr = heterozygous resistant; rr = homozygous susceptible
AVR AVR = homozygous avirulent; AVR avr = heterozygous avirulent; avr avr = homozygous virulent

		HOST GENOTYPE	
		RR or Rr	rr
PATHOGEN GENOTYPE	AVR avr or AVR AVR	Disease-resistant	Susceptible to disease
	avr avr	Susceptible to disease	Susceptible to disease

Gene-for-gene hypothesis Multifactor Interactions

Avirulence/virulence	Resistance/susceptibility			
	R1 R2	r1 R2	R1r2	r1r2
A1A2	-	-	-	+
a1A2	-	-	+	+
A1a2	-	+	-	+
a1a2	+	+	+	+

where, – = Resistance
+ = Susceptible

(Agrios 2007)

Resistance genes

Classes of resistance gene

There are several different classes of R Genes. The major classes are the NBS-LRR genesand the cell surface pattern recognition receptors (PRR). The protein products of the NBS-LRR R genes contain a nucleotide binding site (NBS) and a leucine rich repeat (LRR). The protein products of the PRRs contain extracellular, juxtamembrane, transmembrane and intracellular non-RD kinase domains.

Within the NBS-LRR class of R genes are two subclasses:

- One subclass has an amino-terminal Toll/Interleukin 1 receptor homology region (TIR). This includes the *N* resistance gene of tobacco against tobacco mosaic virus (TMV).
- The other subclass does not contain a TIR and instead has a leucine zipper region at its amino terminal.

The protein products encoded by this class of resistance gene are located within the plant cell cytoplasm.

The PRR class of R genes includes the rice XA21 resistance gene that recognizes the ax21 peptide and the Arabidopsis FLS2 peptide that recognizes the flg22 peptide from flagellin

here are other classes of R genes, such as the extracellular LRR class of R genes; examples include rice Xa21D for resistance against *Xanthomonas* and the *cf* genes of tomato that confer resistance against *Cladosporium fulvum.*

The *Pseudomonas* tomato resistance gene (Pto) belongs to a class of its own. It encodes a Ser/Thr kinase but has no LRR. It requires the presence of a linked NBS-LRR gene, *prf,* for activity.

Specificity of resistance genes

R gene specificity (recognising certain Avr gene products) is believed to be conferred by the leucine rich repeats. LRRs are multiple, serial repeats of a motif of roughly 24 amino acids in length, with leucines or other hydrophobic residues at regular intervals. Some may also contain regularly spaced prolines and arginines.

LRRs are involved in protein-protein interactions, and the greatest variation amongst resistance genes occurs in the LRR domain. LRR swapping experiments between resistance genes in flax rust resulted in the specificity of the resistance gene for the avirulence gene changing.

Recessive resistance genes

Most resistance genes are autosomal dominant but there are some, most notably the *mlo* gene in barley, in which monogenic resistance is conferred by recessive alleles. *mlo*protects barley against nearly all pathovars of *powdery mildew*.

Avirulence genes

The term "avirulence gene" remains useful as a broad term that indicates a gene that encodes any determinant of the specificity of the interaction with the host. Thus, this term can encompass some conserved microbial signatures (also called pathogen or microbe associated molecular patterns (PAMPs or MAMPs)) and pathogen effectors (e.g. bacterial type III effectors and oomycete effectors) as well as any genes that control variation in the activity of those molecules.

There is no common structure between avirulence gene products. Because there would be no evolutionary advantage to a pathogen keeping a protein that only serves to have it recognised by the plant, it is believed that the products of Avr genes play an important role in virulence in genetically susceptible hosts.

Example: AvrPto is a small triple-helix protein that, like several other effectors, is targeted to the plasma membrane by N-myristoylation.AvrPto is an inhibitor of PRR kinase domains. PRRs signal plants to induce immunity when PAMPs are detected. The ability to target receptor kinases is required for the virulence function of AvrPto in plants. However, Pto is a resistant gene that can detect AvrPto and induce immunity as well. AvrPto is an ancient effector that is conserved in many *P.syringae*

strains, whereas Pto R gene is only found in a few wild tomato species.This suggests recent evolution of the Pto R gene and the pressure to evolve to target AvrPto, turning a virulence effector to an avirulence effector.

Unlike the MAMP or PAMP class of avr genes that are recognized by the host PRRs, the targets of bacterial effector avr proteins appear to be proteins involved in plant innate immunity signaling, as homologues of Avr genes in animal pathogens have been shown to do this. For example, the AvrBs3 family of proteins possess DNA binding domains, nuclear localisation signals and acidic activation domains and are believed to function by altering host cell transcription.

Biotrophy and gene for gene systems:

All the parasites in which gene for gene relationship has been proved are essentially biotrophic or biotrophs at least for some time after start of infection.

- (*Xanthomonascampestris* pv. *malvacearum*, *Phytophthorainfestans*, *Venturiainaequalis* (Vander Plank, 1978).
- The genes-for-gene systems thus involve biotrophy.
- But the converse is not necessarily true. For example, *Plamodiophorabrassicae* , the cause of club root of crucifers, is biotrophic but no evidence has yet been presented in the literature to suggest that host-pathogen interaction in them is based on a gene for gene systems.

According to Van der Plank (1978), specificity in gene for gene relationships lies in susceptibility.

 He explains it with the help of interactions of five host and five pathogens attacking them specifically.

 Suppose there are five host varieties with five different R genes; R1, R2, R3----------R5. A plant with resistance gene R1 is attacked by a pathogen having virulence gene v1 and not to pathogen without this particular resistance gene irrespective of how many the virulence genes it may have.

Table. The diagonal check for specificity in a gene-for gene relationship

Pathogen	**Plant**				
	$R_1R_1^b$	R_2R_2	R_3R_3	R_4R_4	R_5R_5
v_1v_1	S	R	R	R	R
v_2v_2	R	S	R	R	R
v_3v_3	R	R	S	R	R
v_4v_4	R	R	R	S	R
v_5v_5	R	R	R	R	S

a. Plant reaction when resistance gene R_1, R_2, R_3, R_4, R_5 at
interact with virulence genes v_1, v_2, v_3, v_4, v_5 at five loci
pathogen

b. Resistance is assumed to be dominant and RR can be r
by Rr. Virulence is assumed to be recessive. However, re
resistance and dominant virulence are also known.

R= resistant S= susceptible

Vander Plank (1978) elaborated protein for proteins hypothesis as a biochemical explanation of gene for gene interaction.

The protein for protein hypothesis states that in **gene for gene diseases the mutual recognition of host and pathogen is not by the genes themselves but by their coded proteins**.

Vander Plank (1978) hypothesized that in susceptibility the pathogen excretes a protein (virulence for product) into the host cell which copolymenizes with a complementary host protein (resistance gene product). This co-polymerization interferes with one auto regulation of the host gene that codes for the protein and by so doing turns the gene on to produce more protein.

In resistance, the protein specified by the gene for avirulence in the pathogen and excreted into the host does not polymerize with the protein coded for by the gene for resistance. It is not recognized by the host at all.

From a practical point of view, gene for gene relationship can be used to study the following:

1. The source of pathogenic variability in pathogens
2. The mutability of resistance and virulence genes
3. Why host resistance is expressed under one set of conditions and not others
4. Prediction of putative genotypes
5. Race nomenclature
6. Genetic dissection of complex loci
7. Cataloguing and storing of R genes in the form of plant seeds or cuttings and V genes in the form of pathogen strains
8. Management and deployment of resistance genes in space and tome
9. Detection of linkage and allelic relationship
10. Geographic distribution of R and V genes
11. Synthesis of multilines and multigene cultivars.

Vertifolia effect:

The vertifolia effect was discovered by Van der Plank (1963) who named it after a potato cultivar of this name, in which the effect was very pronounced. The vertifolia effect is a loss of horizontal resistance which occurs during breeding for vertical resistance. Its meaning was later extended to include the loss of horizontal resistance that occurs during breeding under the protection of pesticides.

The level of horizontal resistance can only be assessed by the level of parasitism. Clearly, if there is no parasitism because of a functioning vertical resistance, or a pesticide, the level of horizontal resistance cannot be assessed. Because individual plants with a high level of horizontal resistance are rather rare in a mixed screening population, the chances are that individuals with a relatively low level of horizontal resistance will then be selected on the basis of their other attributes. The loss is usually quite small in a single breeding cycle but, after many cycles, it can become very serious indeed.

The prime example of the vertifolia effect is the loss of horizontal resistance to potato blight (*Phytophthora infestans*) that has continued ever since both the discovery of Bordeaux mixture in the late nineteenth century, and the discovery of vertical resistance in the twentieth century. A loss of horizontal resistance to cotton pests has continued ever since the discovery of DDT in the 1940s.

The vertifolia effect is a very modern phenomenon. Its overall consequences are seen in the high levels of horizontal resistance in heritage cultivars, when they are compared to modern cultivars. This is the main reason why heritage cultivars are so valued by organic farmers.

One of the main objectives of most amateur plant breeders will be to restore the horizontal resistances that were lost to the vertifolia effect.

Systemic Acquired Resistance

Systemic acquired resistance (SAR) is a long lasting systemic resistance induced by pathogens that cause a necrotic reaction, and is often effective against viral, bacterial and fungal pathogens. SAR is

accompanied by elevated levels of salicylic acid, which may be required for transmission of the signal that mediates SAR. In general terms, pathogen intfection initiates specific defence responses, which spread to the non-infected tissues of the host, and lead to increased resistance to subsequent pathogen infections. There is some evidence that SAR may impose a yield penalty.

Development of SAR is associated with the activation of a set of genes often called SAR genes. Different SAR genes appear to be involved in SAR in monocots and dicots. In tobacco, SAR genes comprise a set of genes that encode pathogencsis related (PR) proteins. SAR genes play an active role in disease resistance, and they can be activated by certain chemicals, e.g., salicylic acid, 2, 6-dichlroisonicotinic acid (INA) and benzo (1,2, 3) thiadiazole- 7-carbothioic acid-S-methyl ester (BTH). These chemicals are effective in both monocots and dicots, and BTH is the most promising among them. For example, a single application of 30g/ha of BTH can protect wheat against powdery mildew in the field for the entire season.

Protein "Harpin' obtained from Erwinia amylovora induces SAR in crop plants.'Messenger is a biopesticide based on Harpin protein; it is repoted to protect plants from bacteria, fungi, viruses, insects, mites and nematodes. Harpin is being produced in *E. coli* by expressing the *E. amylovora* Harpin encoding gene.

Methods of breeding for disease resistance

The methods of breeding varieties resistant to diseases do not differ greatly from those adopted for other characters. The following methods are used:

1. Introduction,
2. Selection,
3. Hybridization followed by selection,
4. Back cross method,
5. Induced mutagenesis,
6. Development of multilines and
7. Tissue culture techniques

1. Introduction

It is a very simple and inexpensive method. Varieties resistant to a particular disease elsewhere may be thoroughly tested in the regions in which they are proposed to be introduced. Their yield performance and disease resistance should be confirmed by large scale cultivation. It is possible that a variety resistant in one region need not be resistant in another region due to variation in the physiological race of the pathogen or due to a much different agroclimatic condition in the new location.

Introductions have served as a useful method of disease control. For example, Ridley wheat introduced from Australia has been useful as a rust resistant variety. Manila, a rice variety introduced in Karnataka from the Philippines, has tolerance to blast, bacterial leaf blight and sheath blight. Intan, a Javanica type rice variety introduced in Karnataka from Indonesia is highly resistant to blast. Munal, a rice variety introduced in West Bengal from the U.S.A. is tolerant to blast, bacterial leaf blight and leaf folder (pest). Some of IRRI rice varieties such as IR 20, IR.24, IR.28, IR.34, IR.36 and IR .50 possess resistance to one or more diseases. Early varieties of groundnut introduced from U.S.A. havebeen resistant to laf spot (*Cercosoraarachidicola*).

Kalyan Sona and Sonalika wheat varieties originated from the segregating materials introduced from CIMMYT, Mexico and were rust resistant. Introduftions also serve as sources of resistance in breeding programmes. For example, African pearlmillet (*P.americanum*) introductions have been used for developing downy mildew resistant male sterile lines (Tift 23A cytoplams) for use in hybrid pearlmillet production. This is an important development in the hybrid pearlmillet programmes since the original male sterile lines Tift 23A and 23D2A were extremely susceptible to

downy mildew.The introduction of Co.475 variety of sugarcane in Mumbai has conquered red rot but brought in leaf rust and whip smut to the fore.

2. Selection

This is better method than introduction and has more chances of success in obtaining disease-resistant plants. The work of selection is carried out either in the naturally infected fields under field conditions or under artificially inoculated conditions. The resistance in such individuals will occur in nature by mutation. To ensure the resistant character of a plant, large population of crop plant may be exposed to the attack of pathogen under artificial conditions and the non-infected plants may be chosen. Suvarnamodan rice of Kerala is a pure line of ARC. 11775 and is highly tolerant to blast.

Sugandh of Bihar is a selection from Basmati rice of Orissa tolerant to bacterial leaf blight. Rice varieties Sudha (Bihar), Sabita, Nalini (West Bengal), Patel 85 (Madhya Pradesh), Janaki(Bihar), Improved White Ponni (Tamil Nadu), Ambika (Maharashtra), are some of rice selections resistant to one or more diseases. MCU 1 cotton, a selection from Co 4, is resistant to Kufri Red, a potato selection from Darjeeling Red Round is a disease resistant variety.

3. Hybridization

When selection of resistant varieties is not feasible, resistant varieties may be evolved by crossing the susceptible popular variety with resistant wild variety where in the resistant gene or genes transferred into the genetic make up of susceptible variety. Very often the F1 from crosses may be resistant but carry the other undesirable qualities of the resistant parent. The bad qualities are removed by several back crossing of F1 with the susceptible parent may ultimately yield a resistant progeny with good agronomic characteristics.

Under certain circumstances pedigree or bulk method of selection is followed to obtain a resistant variety. In this method, the crosses are made till F2 population is got. Selections are made in F2 generation for superior genetic traits including disease resistance. By continued selfing, selections are made through F3 to F5 or F6 generations and the best variety is selected. This method is suited for small grains and beans but unsuited to fruits and vegetables.

4. Back cross method

Back cross method is widely used to transfer disease resistance from wild species. Wild species are rice sources of disease resistance. Interspecific hybridization is made to transfer the gene or genes for resistance to the cultivated species. Resistance to grassy stunt virus from *Oryza nivara* to *O.sativa*, late blight resistance from *Solanumdemissum* to cultivated potato, rust resistance from durum to *aestivum* wheat are some of the examples involving interspecific hybridization. Depending upon the number of genes governing resistance and the nature of the gene, whether dominant or recessive, the procedure varies. The number of back crosses to the cultivated species may be five to six. Once the back cross progeny resemble the cultivated parent, then they are selfed and segregating progeny screened for disease resistance.

5. Induced mutagenesis

While following mutation breeding for disease resistance, a large number of mutation progeny should be produced and screened under artificial epiphytotic condition to select resistant plants. MCU10 cotton, a resistant variety to bacterial blight was evolved in Tamil Nadu by subjecting seeds of a susceptible variety CO4 to gamma rays followed by rigorous screening and selection 6. Development of multilines The concept of multilines was first suggested by Jensen(1952) and developed by Borlaug (1959) for evolving multiline varieties to resist stem rust in wheat. A multiline variety is a composite of genetically similar lines, except that each line possesses a different gene for resistance to the pathogen.

Lines that are genetically similar, except for one gene, are called isoline. It is assumed that gene for resistance in each isoline contributes resistance to a separate physiological race or group of races. Genes for disease resistance are transferred by backcrossing from donor varieties to a common disease susceptible, but agronomically superior, recurrent parent. Isolines are generated differing only in the gene for disease resistance. The isolines are composited to synthesize a multiline variety. The isolines are maintained for resynthesizing the multiline whenever needed. A multiline variety is composed of a mixture of resistant and susceptible genotypes and provides a buffering effect against rapid development of disease. It will provide resistance or tolerance to a broad spectrum of races of a pathogen. If new races of the pathogen are identified at a later stage, additional isolines resistant to the newly arisen races may be constituted and incorporated.

Care should be taken to see that there is uniformity for height, maturity and other features in the multiline. Though multilines provide stability of yield due to reduction of damage by pathogens, the limitations of multiline varieties are that the yield level of multiline varieties is limited to that of the recurrent parent, 4 to 5 years are required to stabilize isogenic lines and the pathogen may produce new races at a faster rate than the development of a multiline. Multiline varieties have been developed for resistance to stem rust and stripe rust of wheat and crown rust of oats. The first multiline variety in wheat, 'Miramar 60' was developed and released in Columbia to combat the attack of yellow rust. 'Miramar 63' and 'Miramar 65' were resistant to stem rust and stripe rust. 'Yoqui 50', 'Crew' and 'Tumult' are a few other wheat multilines. Kalyan sona and Sonalika-based multilines of wheat resistant to different races of rust have been developed in India.

7. Tissue culture technique

Tissue culture techniques to produce somaclonal variation for disease are developed in different crops. Somaclonal variations for disease resistance are reported in *Zeamays* for *Drechsleramaydis* race T-toxin resistance, in *Brassicanapus* for resistance/tolerance to Phoma lingam, early and late blight resistance in potato, *Pseudomonas* and *Alternaria* resistance in tobacco, besides smut and rust disease resistance in sugarcane.

12. Concept of heritability.

Heritability is a statistic used in the fields of breeding and genetics that estimates the degree of variation in a phenotypic trait in a population that is due to genetic variation between individuals in that population. Heritability is estimated by comparing individual phenotypic variation among related individuals in a population. Heritability is an important concept in quantitative genetics, particularly in selective breeding and behavior genetics.

Heritability, amount of phenotypic (observable) variation in a population that is attributable to individual genetic differences. Heritability, in a general sense, is the ratio of variation due to differences between genotypes to the total phenotypic variation for a character or trait in a population. The concept typically is applied in behaviour genetics and quantitative genetics, where heritability estimates are calculated by using either correlation and regression methods or analysis of variance (ANOVA) methods.

- Heritability is the ratio of genotypic variance to the phenotypic variance
- Heritability denotes the proportion of phenotypic variance that is due to genotype i.e., heritable .
- It is generally expressed in percent (%)
- It is a good index of transmission of characters from parents to their offspring

Heritability is expressed as

$H^2 = Vg/Vp$,

Where, H is the heritability estimate, Vg the variation in genotype, and Vp the variation in phenotype. Heritability estimates range in value from 0 to 1. If $H = 1$, then all variation in a population is due to differences or variation between genotypes (i.e., there is no environmentally caused variation). If $H = 0$, there is no genetic variation; in this case all variation in the population comes from differences in the environments experienced by individuals.

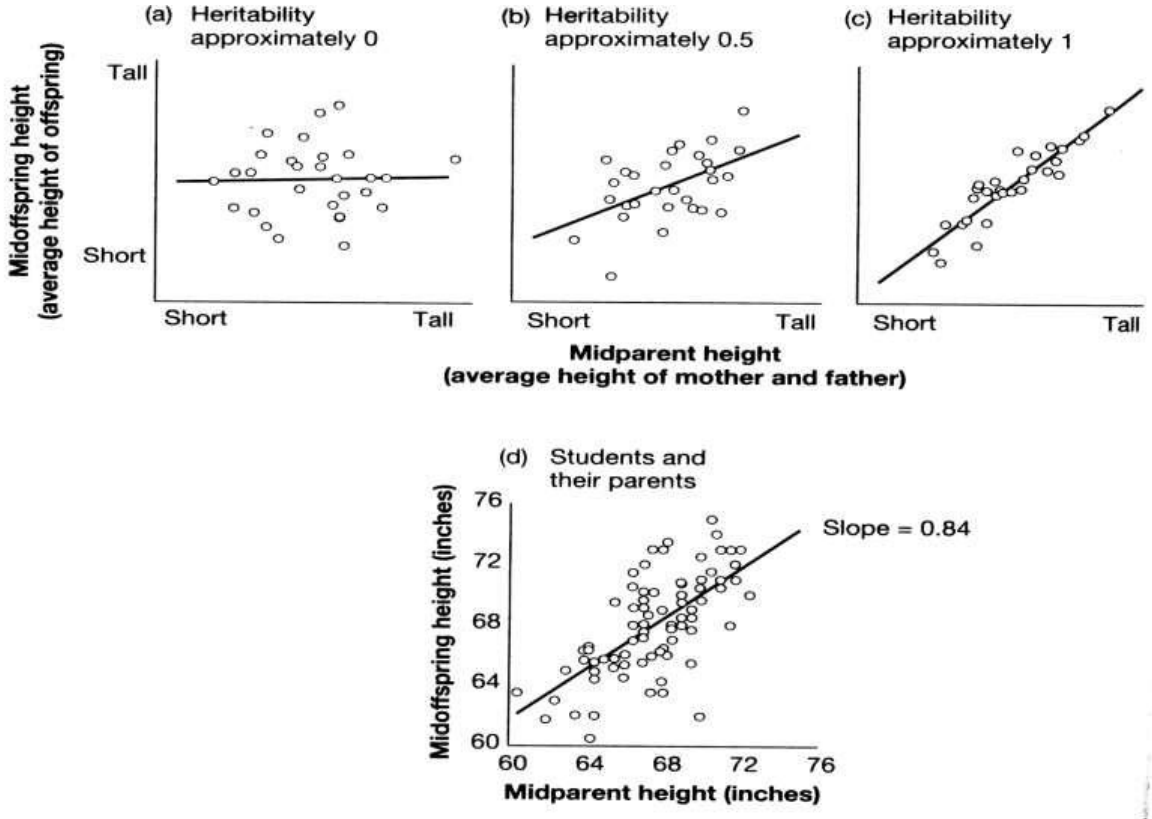


Fig: heritability in between parents & off springs height

Depending upon the components of variance used as numerator in the calculation ,there are 2 definitions of Heritability

1. Broad sense heritability

2. Narrow sense heritability

Broad sense heritability:

Broad sense heritability includes all components of genetic variance. Often it is informative to calculate the proportion of the total phenotypic variance that is due to genetic differences among individuals in a population. This proportion is called the broad-sense heritability, symbolized H^2. In terms of Fisher's variance components,

According to Falconer, broad sense heritability is the ratio of genotypic variance to total or phenotypic variance

It is calculated with the help of following formula

$$\text{Heritability } (h^2) = V_g / V_p \times 100 = V_g / V_g + V_e \times 100$$

The total phenotypic variance may be decomposed:

V_p = total phenotypic variance

V_g = total genetic variance

V_e = environmental variance

- Broad heritability (h2) separates genotypic from environmentally induced variance: h2 = Vg / Vp
- It can be estimated from both parental as well as segregating populations
- It express the extent to which the phenotype is determined by the genotype , so called degree of genetic determination
- It is most useful in clonal or highly selfing species in which genotypes are passed from parents to offspring more or less intact
- It is useful in selection of superior lines from homozygous lines

The symbol for the broad-sense heritability, H_2 , is written with the exponent 2 to remind us that this statistic is calculated from variances, which are squared quantities. Because of the way it is calculated, the broad-sense heritability must lie between 0 and 1. If it is close to 0, little of the observed variability in the population is attributable to genetic differences among individuals. If it is close to 1, most of the observed variability is attributable to genetic differences. The broad-sense heritability therefore summarizes the relative contributions of genetic and environmental factors to the observed variability in a population. However, it is important to note that this statistic is population-specific. For a given trait, different populations may have different values of the broad-sense heritability. Thus, the broad-sense heritability of one population cannot automatically be assumed to represent the broad-sense heritability of another population. In the F^2 wheat population, H^2= 11.98/14.26 = 0.84. This result tells us that in this population 84 percent of the observed variability in wheat maturation time is due to genetic differences among individuals. However, it does not tell us what these differences are. The genetic variance upon which the broad-sense heritability depends includes all the factors that cause genotypes to have different phenotypes: the effects of individual alleles, the dominance relationships between alleles, and the epistatic interactions among different genes. In Chapter 4 we saw how these factors influence phenotypes. In the next two sections, we will see that by breaking out these components of genetic variability and by focusing on the component that involves the effects of individual alleles, we can predict the phenotypes of offspring from the phenotypes of their parents.

Narrow sense heritability includes only the additive genetic variance and it is this form of heritability that usually of interest.

The ability to make predictions in quantitative genetics depends on the amount of genetic variation that is due to the effects of individual alleles. Genetic variation that is due to the effects of dominance and epistasis has little predictive power.

- It is the ratio of additive or fixable genetic variance to the total or phenotypic variance
- Also known as degree of genetic resemblance
- It plays an important role in the selection process in plant breeding
- For estimation of narrow sense heritability , crosses have to be made in a definite fashion
- It is estimated from additive genetic variance
- It is useful for plant breeding in selection of elite types from segregating populations

It is calculated with the help of following formula

where VA or D = additive genetic variance

VP

To see how dominance limits the ability to make predictions, consider the ABO blood types in humans. This trait is determined strictly by the genotype; environmental variation has essentially no effect on the phenotype.

However, because of dominance, two individuals with the same phenotype can have different genotypes. For example, a person with type A blood could be either I A I A or I A i. If two people with type A blood produce a child, we cannot predict precisely what phenotype the child will have. It could be either type A or type O, depending on the genotypes of the parents; however, we know that it will not have type B or type AB blood. Thus, although we can make some kind of prediction about the child's phenotype, dominance prevents us from making a precise prediction. Our ability to make predictions about an offspring's phenotype is improved in situations where the genotypes are not confused by dominance. Consider, for example, the inheritance of flower color in the snapdragon, *Antirrhinummajus*. Flowers in this plant are white, red, or pink, depending on the genotype. As with the ABO blood types, variation in flower color has essentially no environmental component; all the variance is the result of genetic differences. However, for the flower color trait, the genotype of an individual is not obscured by the complete dominance of one allele over the other. A plant with two w alleles has white flowers, a plant with one w allele and one W allele has pink flowers, and a plant with two W alleles has red flowers. In this system, the phenotype depends simply on the number of W alleles present; each W allele intensifies the color by a fixed amount. Thus, we can say that the color-determining alleles contribute to the phenotype in a strictly additive fashion. This kind of allele action improves our ability to make predictions in crosses between different plants. A mating between two red plants produces only red offspring; a mating between two white plants produces only white offspring; and a mating between red and white plants produces only pink offspring. The only uncertainty is in a cross involving heterozygotes, and in this case the uncertainty is due to Mendelian segregation, not to dominance.

Quantitative geneticists distinguish between genetic variance that is due to alleles that act additively (such as those in the flower color example just discussed) and genetic variance that is due to dominance. These different variance components are symbolized as:

V_a = additive genetic variance

V_d = dominance variance

In addition, geneticists define a third variance component that measures variation due to epistatic interactions between alleles of different genes:

V_i = epistatic variance

Epistatic interactions, like dominance, are of little help in predicting phenotypes. Altogether, these three variance components constitute the total genetic variance:

$V_g = V_a + V_d + V_i$

If we recall that $V_T = V_g + V_e$, we can express the total phenotypic variance as the sum of four components:

$V_T = Va + V_d + V_i + V_e$

Of these four variance components, only the additive genetic variance, V_a, is useful in predicting the phenotypes of offspring from the phenotypes of their parents. This variance, as a fraction of the total phenotypic variance, is called the narrow-sense heritability, symbolized h^2. Thus,

$h_2 = V_a/V_T$

Like the broad-sense heritability, h^2 lies between 0 and 1. The closer it is to one, the greater is the proportion of the total phenotypic variance that is additive genetic variance, and the greater is our ability to predict an offspring's phenotype. Human stature is highly heritable, but litter size in pigs is not. Thus, if we knew the parental phenotypes, we would be better able to predict the height of a human's offspring than the litter size of a pig's offspring.

It is also referred to as **resemblance between relatives**.

Because individual components of variance are not directly measurable, it is necessary to use comparative measurements of phenotype to determine the contribution of individual variance components.

For example:

- By measuring a specific trait such as height in individual organisms from several populations, one could determine the range of height measurements for that species.
- Individuals from different populations could then be grown in a common garden and measured at the same point of maturity as the original organisms. The common garden would eliminate the environmental variance experienced between the different populations.
- Therefore, the difference between the phenotype variance of the wild populations and that of the common garden would give an estimate of the total genetic variance.

Factor affecting heritability:

- **Type of genetic material:** the magnitude of heritability is largely governed by the amount of genetic variance present in a population for the character under study
- **Sample size:** Large sample is necessary for accurate estimates
- **Sampling methods**: 2 sampling methods , Random and Biased. The random sampling methods provide true estimates of genetic variance and hence of heritability
- **Layout or conduct of experiment:** Increasing the plot size and no. of replications we can reduce experimental error and get reliable estimates
- **Method of calculation :** heritability is estimated by several methods
- **Effect of linkage :** high frequency of coupling phase (AB/ab) causes upward bias in estimates of additive and dominance variances
- **Excess of repulsion phase linkage** (Ab/aB) leads to upward bias in dominance variance and downward bias in additive variances

Genetic advance:

Improvement in the mean genotypic value of selected plants over the parental population is known as genetic advance. It is the measure of genetic gain under selection. The success of genetic advance under selection depends upon three factors (Allard , 1960).

- Genetic variability : greater the amount of genetic variability in base populations higher the genetic advance

- Heritability : the G.A. is high with characters having high heritability
- Selection intensity : the proportion of individuals selected for the study is called selection intensity . high selection intensity gives better results

Selection differential:

It is the difference between the mean phenotypic value of selected population and mean phenotype of original population

This is the measure of the selection intensity and denoted by K.

K = Xs – Xo

where Xs = mean of phenotypic value of selected plants

Xo = mean of phenotypic value of parental population

Genetic gain:

The difference between the mean phenotypic value of the progeny of selected plants and the original parental population is known as genetic gain

It is denoted by R

R = Xp – Xo

where , Xp = mean phenotypic value of progeny of selected plants

Xo = mean of phenotypic value of base population

Merits of heritability:

- It is useful in predicting the effectiveness of selection.
- It is also helpful for deciding breeding methods to be followed, for effective selection.
- It gives an idea about the response of various characters to selection pressure.
- It is useful in predicting the performance under different degree of intensity of selection.
- It helps for construction of selection index.
- Estimates of heritability serve as a useful guide to the breeder, to appreciate the proportion of variation, which is due to genotypic or additive effects

The Limitations of Heritability

• Heritability does not indicate the degree to which a characteristic is genetically determined.

• Pure breed no polydactilly rabbits: still polydactilly can happen

• An individual does not have heritability.

• Narrow-sense heritability of 0.6 in population does not indicate that an individual's characteristic is 60% additive

• There is no universal heritability for a characteristic.

• Two populations will have different heritability due to environment

• Even when heritability is high, environmental factors may influence a characteristic.

• Human height

• Heritability indicates nothing about the nature of population differences in a characteristic.

13. Use of biometrical tests in genetics and breeding with special reference to path-coefficient analysis.

Biometry:

Statistics is a branch of science which deals with methods of collection, classification and analysis, i.e., drawing inferences from collected data; then testing of hypothesis and making comment on these. The statistics can be applied in all scientific branches like social, physical and bio-logical. The use of statistics in Biology is known as Biostatistics or Biometry. It deals with the application of different statistical methods and analysis of data collected from biological system.

Application of Biometry:

In biological system, it is very difficult to draw any concrete conclusion about any happening, as there is much difference among the individuals. Such as in the same plant, the leaves are all different; again in a field, all the same kind of plants are not identical; in the same place, the same crop does not respond equally in different plots. So it is very difficult to reach at a definite and reliable conclusion unless we take the help of statistical analysis of the observed data.

In case of plant breeding, the application of biometry has immense importance, as the statistical analysis only helps to conclude about a population of plant; such as:

(a) How the character, which is acquired by the population, is distributed, how the character is being inherited, etc.

(b) Whether two characters in a population are correlated or not, if correlated, how much they are correlated.

(c) In case of plant breeding whether Mendelian Genetics is being followed or not, can be tested by chi-square test.

(d) The application of fertilizer or irrigation or any kind of cultural practice can be effectively justified by application of test of significance.

(e) The laws of probability is of importance in genetics for forecasting the chance of obtaining certain result from a cross and elucidating the operation of ge-netic principle.

(f) The effects of two or more different fertilizer application on the same field can be analysed by analysis of variance.

(g) Study of alleles of genes in populations and the factors which maintain or change the frequencies of particular genotype in populations can be studied.

Limitation of Biometry:

(a) All the conclusions about statistical analysis depend on the availability of sample data. If the sampling is biased, the analysis will be eronotic.

(b) Statistics can be used and applied only on collective data, not on individual data.

(c) Statistical laws are always true in case of large population on the average, not on the small sample.Frequency distribution

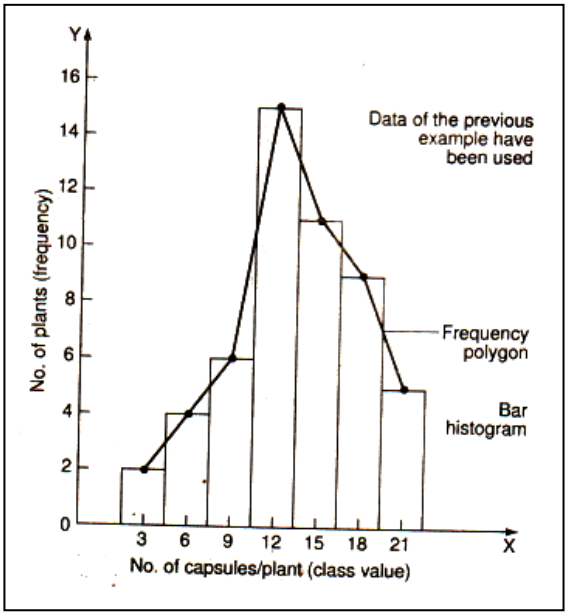
For any statistical analysis, the handling with raw data requires some treatment, i.e., the classification of data to organise the available values in a more compact way. The frequency distribution presents the data very concisely indicating how frequently a vari-able occurs in a group of study.

Frequency distribution

The diagrams commonly used to depict statistical data, given in the form of frequency distribution are: 1. Histogram, 2. Frequency polygon, 3. Ogive.

1. **Histogram**: It is the most common form of diagrammatic representation of a grouped frequency distribution. It consists of a set of adjoining rectangles drawn on a horizontal base line with area proportional to the class frequencies.

Uses: (a) give a visual representation of the relative size of the various groups; (b) the surface of the tops of rectangles gives an idea of nature of frequency curve for the population; (c) may be used to find out the mode graphically.



2. **Frequency polygon**: It is alternative to histogram and derived by joining the mid-points of the tops of consecutive rectangles.

3. **Ogive**: It is the graphical representation of cumulative frequency distribution and hence called cumulative frequency polygon.

Normal Distribution:

If we observe in any population any attribute is distributed mostly near the mean value and equally distributed to the higher and lesser value gradually in decreasing order then the distribution pattern is called normal distribution.

When this kind of normally distributed attribute is plotted graphically with the help of available data, the normal distribution pattern gives a bell shaped symmetrical curve which is called 'normal distribution curve'. In this curve the mean value lies in the peak of the curve.

Properties of Normal Distribution Curve:

1. It is a continuous bell shaped curve which is associated with continuous variable.

There is only one maximum peak (unimodal). The normal curve is symmetrical and asymptotic (touches at infinity).

3. The height of normal curve is maximum at its Mean. Mean, Median and Mode coincides in normal curve.

4. The peak divides the distribution in two equal halves.

5. Most of the observations are clustered around the Mean and there are rela-tively a few observations at the extremes.

6. The normal distribution curve has a fixed mathematical characteristic feature independent of the scale. (unit of measurement) of magnitude.

Skewness and Kurtosis:

In normal distribution, most of the cases fall in the middle but there are cases in which central tendency do not exhibit normal behaviour.

There are two types of divergence from normal distribution:

(i) Skewness and

(ii) Kurtosis

Skewness means that the curve is not symmetrical. In a skewed distribution, the Mean, Median and Mode do not coincide it pulls the Median and Mean away from Mode either left or right. In a skewed distribution, the frequency curve is not bell shaped and values do not lie on both sides of measure of central tendency equally. Here Mean Median and Mode fall at different points.

In symmetrical distribution curve, Mode coincides with Mean and Median. In positively skewed curve, the value of Mean and Median lie away from Mode values (right hand), the values are greater than Mode. In negatively skewed curve, the value of Mean and Median lie left hand to Mode value, the values are lesser than the Mode value.

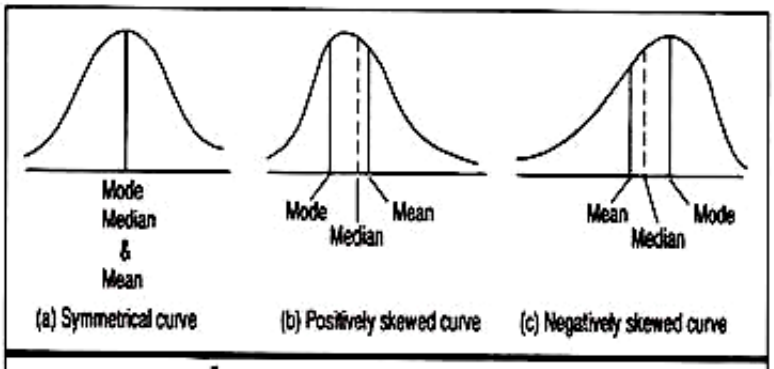


Fig. 10.1: Symmetrical and skewed curve

Measures of Central Tendency

Mean:

Arithmetic mean

It is most commonly used of all the averages. It is the value which we get by dividing the aggregate of various items of the same series by the total number of observations.

When observations are denoted by x values showing x1, x2, x3… xn; the total number of observations is calculated by summing up the observations and dividing the sum by the total number of observations (n)

Find out the average pod length of the plant.

$$\bar{x} = \frac{x_1 + x_2 + x_3 + \cdots + x_n}{n}$$

Example 1: The pod length of ten pods of a plant shows following data:

5.2 cm,	5.3 cm,	5.6 cm,	5.7 cm,	5.4 cm,
5.2 cm,	5.3 cm,	5.3 cm,	5.4 cm,	5.2 cm.

Find out the average pod length of the plant.

$$\bar{x} = \frac{\sum x}{n} = \frac{5.2 + 5.3 + 5.6 + 5.7 + 5.4 + 5.2 + 5.3 + 5.3 + 5.4 + 5.2}{10} \text{cm}$$

$$= \frac{53.6}{10} \text{cm} = 5.36 \text{cm}$$

Mean of Grouped Data:

$$\bar{x} = \frac{\sum fx}{n}$$

where: $\bar{x}$ = mean
f = frequency of each class
x = mid-interval value of each class
n = total frequency
$\sum fx$ = sum of the producst of mid − interval values and their corresponding frequency

Merits, Demerits and Uses of Arithmetic Mean:

Merits:

1. It has the simplest formula to calculate and it is easily understood.
2. It is rigidly defined mathematical formula the same result will come on repeated calculations.
3. The calculation is based on all the observations.
4. It is least affected by sampling fluctuation.
5. The arithmetic mean balances the value on either side.
6. It is the best measure to compare two or more series.
7. Arithmetic mean is totally dependent on values not on the position.

Demerits:

1. It cannot be calculated if all the values are not known.
2. The extreme values have greater effect on mean.
3. The qualitative data cannot be measured in this way.

Uses:

1. The arithmetic mean is mostly used in practical statistics.
2. Mean helps to calculate many other estimates in statistics.
3. The arithmetic mean is most popular method of any measurement used by common people to get the average of any data.

Mode:

Most frequent value in a series. Mode cannot be determined from a series of individual observations unless it is converted into either a discrete or continuous series. In a discrete series the value of the variable against which the frequency is the largest would be the modal value.

For example, 2, 4, 4, 4, 6, 9, 3, 2, 4, 6, 11, 13 mode is 4 as it is occurring maximum.

For example, 5, 3, 6, 3, 5, 10, 7, 2 mode is 3 and 5 such series is known as bi-modal series.

Similarly in a continuous frequency distribution the class interval having the maximum frequency would be the modal class.

Mode can be determined from grouped data using the following formula:

$$\text{Mode} = l_1 + \frac{\Delta_1}{\Delta_1 + \Delta_2} \times i,$$

where l_1 = lower limit of modal class, Δ_1 = difference of frequencies be-tween modal class and the preceding class, Δ_2 = difference of frequencies between modal class and post modal class and i = class interval.

Merits of Mode:

i. It is simple and easily understood.

ii. Mode is not affected by the values of extreme items provided they follow to the natural law relating to extremes.

iii. For determination of mode all values in the series are not considered.

Demerits of Mode:

i. As mode is not based on all observations of a series, therefore, it is not rigidly defined.

ii. Mode is not capable of further mathematical treatment.

iii. Mode may be unrepresentative in many cases and it may be impossible to set a definite value of mode as in a set of observations 2 or 3 or more modal values may occur.

Median:

Median is the value of the middle item of a series when arranged in order of magnitude (ascending or descending order).

M = size of (n+ 1)/2 th item, where M stands for median and n for the number of items.

For example,

from ungrouped data: 3, 15, 6, 9, 21, 7, 12, 10

arrange the series is magnitude: 3, 6, 7, 9, 10, 12, 15, 21

$n = 8$ *(the series is even)*

$$M = \text{size} = \frac{(8+1)}{2} = 4.5.$$

In such case 4th and 5th items would be added and divided by 2.

$$\therefore \text{Median} = \frac{9+10}{2} = 9.5.$$

For example,

series: 2, 12, 9, 6, 17, 5, 8, 14, 23, 27, 11

arrange in magnitude: 2, 5, 6, 8, 9, 11, 12, 14, 17, 23, 27

$n = 11$ *(odd series)*,

$$\text{Median} = \text{size of } \left(\frac{11+1}{2}\right) \text{th item} = \text{size of 6th item} = 11.$$

Median from grouped data can be calculated from the following formula:

$$\text{Median} = l_1 + \frac{N/2 - F}{fm} \times i,$$

Where, l_1 = lower limit of median class,

N = total frequency,

F = cumulative frequency,

f_m = frequency of the median class and

i = class interval of median class.

Merits of Median:

i. Easily calculated and better understood and not affected by the values of the extreme items.

ii. It can be located merely by inspection in many cases.

iii. It gives best result in a study of those phenomena which are incapable of direct quantitative measurement, for example, assessment of madness among mentally retarded patients. In such case it is possible to arrange a group of patients in ascending or descending order in relation to the degree of madness and thus to locate a person whose madness can be said to be average.

Demerits of Median:

i. Median may not be representative of a series in many cases.

ii. It is not suitable for further algebraic treatment.

iii. When median has to be calculated in continuous series it requires inter-polation.

iv. Median ignores the values of extremes in the series.

v. Median is likely to be affected by the fluctuations of sampling.

Example 1:

Find out mean, mode and median from the following data and draw the frequency distribution curve:

Solution:

No. of capsules/plant [Class interval]	*Class value* (x)	*No. of plants [frequency]*	*Cumulative frequency*	fx
1–5	3	3	3	9
6–10	8	7	10	56
11–15	13	12	22	156
16–20	18	20	42	360
21–15	23	27	69	621
26–30	28	32	101	896
31–35	33	21	122	693
36–40	38	16	138	608
41–45	43	10	148	430
46–50	48	6	154	288
51–55	53	2	156	106
		N = 156		Σfx = 4223

$$\text{Mean} = \bar{x} = \frac{\Sigma fx}{n} = \frac{4223}{156} = 27.07.$$

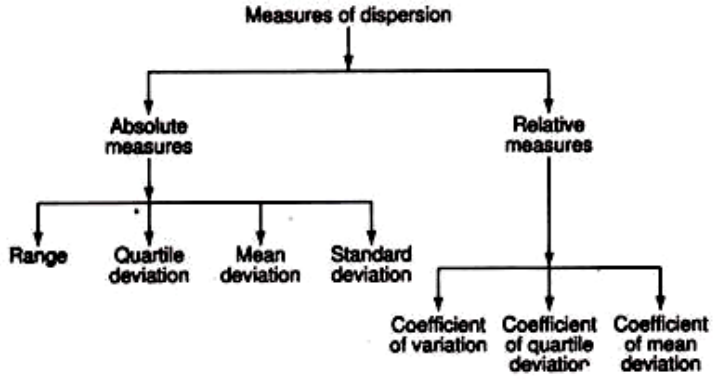
Mode = Modal class 26–30.

$$\text{Mode} = l_1 + \frac{\Delta_1}{\Delta_1 + \Delta_2} \times i = 26 + \frac{5}{5+11} \times 4 = 27.25.$$

$$\text{Median} = \text{the value of } \left(\frac{n+1}{2}\right)\text{th item} = \left(\frac{156+1}{2}\right) = 78.5, \text{ thus median class is 26–30.}$$

$$\text{Median} = l_1 + \frac{l_2 - l_1}{f_1}(m - c) = 26 + \frac{30-26}{21}(78.5 - 69) = 26 + \frac{4}{21}(9.5) = 27.81.$$

Measures of dispersion:



Range:

It is the difference between the largest and smallest observation.

Range = Maximum value — Minimum value.

Mean Deviation:

Mean deviation is the arithmetic mean of absolute deviation from mean or any other specified value.

Mean deviation = $\sum fd/n = \sum f(x - \bar{x})/n$

x = specified value,

$\bar{x}$ = mean value,

f = frequency,

n = total number of obser-vations.

Variance:

Variance is a measure of variation and is the sum of square of deviation (d) divided by the number of degree of freedom (n — 1).

Variance of the sample $S^2 = \sum fd^2/(n - 1)$

It is also denoted by σ^2.

Standard Deviation:

The best and most important measure of dispersion is standard deviation which was first worked out by Karl Pearson (1833). The standard deviation or mean error is an absolute measure of dispersion of an individual series or a frequency distribution, A statistical measuring the spread or variability of the sample around the mean or in other words it may be defined as the measure of dispersion of different variables around the central value..

It is square root of variance:

$$\sigma = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n-1}} = \sqrt{\frac{\Sigma d^2}{n-1}}, \text{ or, } \sqrt{\frac{\Sigma fd^2}{n-1}}.$$

Merits:

1. The calculation is based on all observations.
2. It is more rigidly defined.
3. Less affected by fluctuations of sampling compared to other measures of dispersion.
4. It summarizes the deviation of large number of observations from mean and is expressed as one unit of variation.

Demerits:

1. It requires a lengthy calculation, i.e., squaring of deviations and then again square root of summed up values.

2. Not very simple to understand.
3. The calculation gives more weightage to extreme values.

Uses of Standard Deviation:

1. It helps in correlating and comparing of different samples.
2. It helps in finding the suitable size of sample for valid conclusion.
3. It helps in finding the standard error which determines whether the difference between means of two similar samples by chance or real.
4. The value of mean and standard deviation help to comment on the population on the basis of observation of sample.

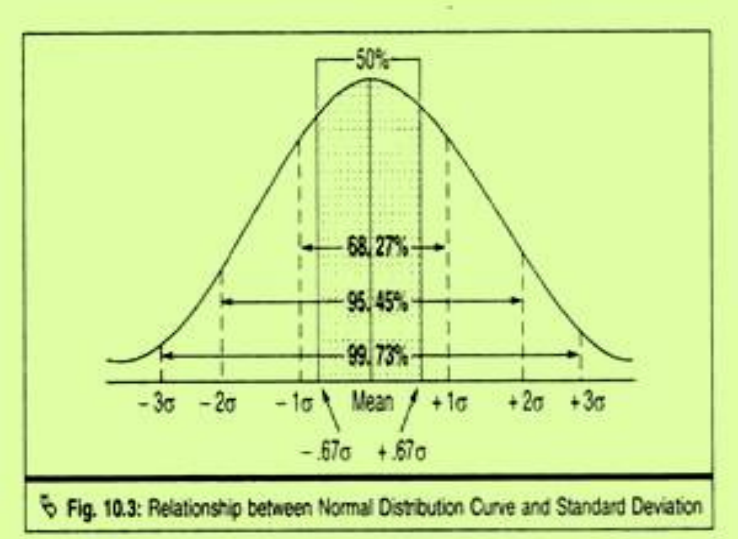


Fig. 10.3: Relationship between Normal Distribution Curve and Standard Deviation

(a) 50% of total observations lie in an area bounded by a distance of 0.6745 σ on both side of the mean.
(b) Mean ± 1σ covers the 68.27% area of the curve.
(c) Mean ± 2σ covers the 95.45% area of the curve.
(d) Mean ± 3a covers the 99.73% area of the curve.

Coefficient of Variation:

The standard deviation is an absolute measure of variation and is expressed in terms of the unit of the variable. For example, it would be in rupees for income, in cm for height and in kg or gm. for weight. For the purpose of comparative studies a relative measure of dispersion or variation is required.
Coefficient of variation serves this purpose as it does not have any unit. The ratio of standard deviation of a sample to its mean expressed in percentage is called coefficient of variation. Thus,

Coefficient of Variation = $$CV = \frac{\sigma}{\bar{x}} \times 100.$$

This measure was evolved by Karl Pearson. It is very useful for the study of variation in more than one sample or series. A sample in which coefficient of variation is higher would have greater variation than the one in which it is lower. In other words, when the coefficient of variation is high the sample is less consistent or more variable and when it is low the sample is more consistent or less variable.
In plant breeding, phenotypic, genotypic and environmental coefficients of variation are estimated from the corresponding variances, and are used for the assessment of variability. Simple measures of variability can be worked out from both un-replicated and replicated data.

Standard error:

Standard error is the approximate standard deviation of a statistical sample population. Standard error is a statistical term that measures the accuracy with which a sample represents a population. In statistics, a sample means deviates from the actual mean of a population; this deviation is the standard error.

The formula for the standard error of the mean is:

$$\sigma_M = \frac{\sigma}{\sqrt{N}}$$

Where, σ is the standard deviation of the original distribution and N is thesample size (the number of scores each mean is based upon).

Example 7:

In two different populations (Batch I and Batch II) the seed number/fruit is calculated:

Batch I 7, 9, 6, 8, 6, 5, 7, 8, 6, 8

Batch II 10, 8, 9, 10, 11, 10, 5, 6, 4, 7

Batch I

Seed number/fruit (x)	Frequency (f)	f × x	Deviation (|d|)	Deviation² (d²)	f × d²
5	1	5	2	4	4
6	3	18	1	1	3
7	2	14	0	0	0
8	3	24	1	1	3
9	1	9	2	4	4
	Σf = 10	Σfx = 70			Σfd² = 14

$$\bar{x} = \frac{\Sigma fx}{n} = \frac{70}{10} = 7$$

$$\sigma = \sqrt{\frac{\Sigma fd^2}{n-1}} = \sqrt{\frac{14}{9}} = 1.25$$

$$c.v. = \frac{\sigma}{\bar{x}} = \frac{1.25}{7} \times 100 = 17.8\%$$

Batch II

Seed number/fruit (x)	Frequency (f)	f × x	Deviation (|d|)	Deviation² (d²)	f × d²
4	1	4	4	16	16
5	1	5	3	9	9
6	1	6	2	4	4
7	1	7	1	1	1
8	1	8	0	0	0
9	1	9	1	1	1
10	3	30	2	4	12
11	1	11	3	9	9
	Σf = n = 10	Σfx = 80			Σfd² = 52

$$\bar{x} = \frac{\Sigma fx}{n} = \frac{80}{10} = 8$$

Solved Problem:

Find out mean, standard deviation, mean deviation, coefficient of variation and standard error from the given sample:

Class value (x)	*Frequency* (f)	fx	$(x - \bar{x}) = d$	fd	d^2	fd^2
48	8	384	−4.75	+38.00	22.56	180.50
50	32	1600	−2.75	+88.00	7.56	242.00
52	75	3900	−0.75	+56.25	0.56	42.18
54	52	2808	+1.25	+65.00	1.56	81.25
56	28	1568	+3.25	+91.00	10.56	295.75
58	5	290	+5.25	+26.25	27.56	137.81
	n = 200	Σfx = 10,550		Σfd = 364.50		979.49

$$\text{Mean } \bar{x} = \frac{\Sigma fx}{n} = \frac{10,550}{200} = \mathbf{52.75.}$$

$$\text{Mean deviation} = \frac{\Sigma fd}{n}$$

[Note: The positive signs exactly cancel negative sign of deviations and the summation of deviations gives a value of zero and, therefore, the spread of distribution cannot be shown. In calculation of mean deviation, the algebraic signs may be ignored.]

$$= \frac{364.50}{200} = 1.8225 = \mathbf{1.82.}$$

$$\text{Standard deviation } \sigma = \sqrt{\frac{\Sigma fd^2}{n-1}} = \sqrt{\frac{979.49}{200-1}}$$

$$= \sqrt{4.9221} = 2.2185 = \mathbf{2.22.}$$

$$\text{Coefficient of variation (CV)} = \frac{\sigma}{\bar{x}} \times 100 = \frac{2.22}{52.75} \times 100$$

$$= 4.2085 = \mathbf{4.21\%.}$$

$$\text{Standard error} = \frac{\sigma}{\sqrt{n}} = \frac{2.2}{\sqrt{200}} = 0.1556 = 0.16.$$

Therefore, mean is represented as 52.75 ± 0.16.

χ^2–Test:

It is used for testing the agreement of observed frequencies with those expected upon a given hypothesis or in other words it can be said that it is test of deviation between theoretical and observed frequencies and to see whether the deviation is significant or not. If the deviation is significant then the assumed hypothesis on which the test is performed is rejected.

$$\chi^2 = \sum \frac{(O-E)^2}{E}$$

O = Observed frequency;
E = Expected frequency;
Σ = Summation over all classes.

Salient Features of χ^2– Test:

1. Complete agreement of observed and expected frequencies will give χ^2-value as zero; but due to chance deviation (from sample fluctuations) positive val-ues will be scored.
2. The use of χ^2-test requires that the frequency in any class is not 5 or less.
3. The test is applicable only to comparisons of observed and expected values of absolute frequencies.

3. Applications of χ^2– Test:

(a) It can compare the values of two binomial samples when they are small.
(b) It can compare the frequencies of two multinomial samples.
(c) Chi-square measures the probability of association between two discrete attributes.
(d) The Chi-square test is applied as a test for goodness of fit which reveals the closeliness of observed frequency with those of the expected frequency. Thus it helps to answer whether physical or chemical factors did or did not have an effect.
(e) Occasionally it is desirable to compare one set of observations taken under particular conditions to those of a similar nature taken under different conditions. In this case there are no definite expected values, only the question is whether the results are dependent (contingent upon) or independent of conditions. Then the X^2-test is called as test for independence or contingency test.

Degrees of Freedom:

The number of degrees of freedom is calculated as the number of classes whose value is required to describe the outcome from all classes. The concept of degrees of freedom is important in experiments and genetic ratios because one must consider the total number of observed individuals in the experiment as a fixed or given quantity. This fixed quantity is composed of one or more classes some of which are variable.

In the experiment between tall and dwarf pea plants there are only two classes, tall and dwarf. As soon as the number of one class is set, the other can be determined. Thus when two classes are scored, there is one degree of freedom.

In an experiment where three classes are scored, there are two degrees of freedom, and so on. The rule states that for the kind of genetic experiments described, the degrees of freedom are equal to one less than the number of classes.

Level of Significance:

In the experiment described the actual ratio departs from that which is expected. We must now determine how significant is this discrepancy so that we can decide to accept or reject the results.

Small discrepancies are not significant; large discrepancies are significant and lead to rejection of a result or hypothesis. Therefore values are assigned to these two kinds of discrepancies—the large discrepancies are the largest 5% and small discrepancies are remaining 95%.

On this basis if the discrepancy lies in the large class it is significant and the result may be discarded. The 5% frequency value that enables us to reject the result is called the 5% level of significance. The level of significance can be changed.

If 5% is too high we can decide on a low level of significance say 1%. In this case it is not so easy to reject a result. Contrarily, if we decide on a high level of significance say 10%, it is easier to reject a result. Usually the accepted level of significance is between the two extremes, that is 5%.

After determining the degrees of freedom in an experiment and deciding on the level of significance, the actual size of the discrepancy between expected and observed is found by chi-square.

χ^2-Test For Goodness of Fit:

χ^2-test is applied to a wide range of studies relating to experimental biology and field studies. The aim of this test is to test the closeliness of observed frequencies with those of the expected, i.e., how well the observed frequency curve fits into theoretical curve.

If both the observed and expected frequency distribution are in complete agreement with each other then the χ2-value will be zero. But in experimental observations there is always some degree of deviation. The critical χ2-value will be exceeded due to sampling fluctuations.

For example, if a crossing experiment gives two different sizes of seeds in F_2 prog-eny then these two types seeds may segregate according to 3:1 (Mendelian Monohybrid), 1:1 (Monohybrid test cross), 9:7 (Complementary factor interaction), 13:3 (Inhibitory factor) or 15:1 (Duplicate gene interaction) ratio, etc.

Again if crossing experiment results in three types of seeds then these may be due to incomplete dominance (1:2:1), supplementary factor (9:3:4) or due to dominant epistasis (12:3:1) interaction, etc.

Likely, 4 types of seeds with 4 different combinations of two different characters may either follow the 9:3:3:1 (Mendelian dihybrid) or 1:1:1:1 (Dihybrid test cross) ratio for segregation.

Test for the goodness of fit is required in these above cases for studying the closeliness of observed data of the experiments with those of expected frequencies.

Steps to be followed to test the Goodness of Fit:

1. Deviation between the observed and the expected results should be calculated.
2. Comparing the minimum deviation the null hypothesis should be selected for X2-test.
3. χ^2-value should be determined.
4. Comparing the calculated χ^2-value with tabulated χ^2-value the conclusion has to be made.

Example:

Selfing of a hybrid plant produced a population with 120 pink flowers and 88 white flowers. Explain with χ^2-analysis, what does the result show?

Sample characters and sample size:

No. of classes	Sample character	Sample size
1	Pink flower	120
2	White flower	88

Total no. of samples = 208

Determination of deviation in different segregation ratio

	3:1		1:1		9:7		13:3		15:1	
Observed value	Exp	|d|	Exp	|d|	Exp	|d|	Exp	|d|	Exp	|d|
Pink 120	156	36	104	16	117	3	169	49	195	75
White 88	52	36	104	16	91	3	39	49	13	75
Total deviation		72		32		6		98		150

According to this table, we find that the deviation is minimum in case of 9:7 ratio. So, it is assumed that the observation should fit well with this ratio.

Determination of Chi-square value

No. of classes	Sample characters	Observed value (O)	Expected value (E)	Deviation (O–E)	$(O-E)^2$	$\frac{(O-E)^2}{E}$
1	Pink flower	120	117	3	9	0.076
2	White flower	88	91	3	9	0.098

Conclusion: $\chi^2 = \sum \frac{(O-E)^2}{E} = .076 + 0.098 = 0.175$

Since in the observation there are two classes, so the degree of freedom =2 – 1 = 1

The calculated χ^2 value is 0.175, which is much less than the table value 3.84 for 1 degree of freedom at 0.05 probability level.

The χ^2 value 0.175 lies between 50-70% probability range. Therefore this deviation from the expected value is insignificant and the observed ratio is in good fit with 9:7 ratio.

Comment:

It is concluded that the experimental result shows the characters with complementary factor interaction in F_2 generation.

As the observed samples are assumed to show complementary factor interaction, so the assumed genotypes are:

P_1 AAbb x aaBB

White flower White flower

gamete Ab aB

F_1 AaBb

Pink flower

F_2

♂ \ ♀	AB	Ab	aB	ab
AB	AABB Pink	AABb Pink	AaBB Pink	AaBb Pink
Ab	AABb Pink	AAbb White	AaBb Pink	Aabb White
aB	AaBB Pink	AaBb Pink	aaBB White	aaBb White
ab	AaBb Pink	Aabb White	aaBb White	aabb White

A-B- = Pink 9

A-bb = White 3

aaB- = White 3 } 7

aabb = White 1

Here, the analysis shows that 2 pairs of factors control the same character and two dominant genes A and B are complementary to each other. Each of which has no effect on expression of character, but when in combination shows their effect, i.e., pink colour.

Absence of any one of the them (A or B) leads to absence of pink colour and it can be concluded very easily that the colour character is controlled by two pairs of factors which are complementary to each other.

Note: Why it is (O – E)2/E?

1. To eliminate negative sign, the deviation is squared.
2. As the deviation from expected is tested, therefore, deviation is divided by expected.

Test of Linkage following the use of χ^2-Test:

Example:

A tall homozygous pea plant (TT) bearing yellow pods (YY) was crossed with a dwarf plant (tt) having green pods (yy).

The F_1 plants raised were all tall and yellow poded and on selfing of these plants F_2 plants were developed in the following frequencies:

Tall Yellow–120 Tall Green–10
Dwarf Yellow–10 Dwarf Green–20

Question:

Comment on the assortment of T and Y genes.

Solution:

χ2-test of goodness of fit will be applied to test the hypothesis 9 : 3 : 3 : 1 is the assumed hypothesis as it is a data from dihybrid cross.

	Phenotypic classes				
	Tall Yellow	*Tall Green*	*Dwarf Yellow*	*Dwarf Green*	*Total*
Observed	120	10	10	20	160
Expected	90	30	30	10	160
(O – E)	30	–20	–20	10	
$(O-E)^2$	900	400	400	100	
$\frac{(O-E)^2}{E}$	$\frac{900}{90}$	$\frac{400}{30}$	$\frac{400}{30}$	$\frac{100}{10}$	
$\chi^2 = \sum \frac{(O-E)^2}{E}$	10.0	13.33	13.33	10.0	
$\chi^2 = 46.66$					

Therefore, χ^2 = 46.66 at 3 DF (4 phenotypic classes; n — 1 =3).

As computed χ^2-value 46.66 at 3 DF is much higher than table value 7.8 at 3 DF, the deviations between observed and expected values are not good-fit and, therefore, the assumed hypothesis 9 : 3 : 3 : 1 is not accepted.

This significant deviation may be due to failure of independent assortment of T and Y genes as the consequence of linkage. High frequency of parental combinations than recombinant also indicated the possibility of linkage.

Linkage can be estimated from f2 data by the following formula:

$P^2 = E – M/N$,

where P = linkage value, E = sum of end classes (parental classes), M = sum of middle classes (recombinant classes) and N = number of progenies.

$p^2 = 140 – 20/160 = 0.75$; $P = \sqrt{0.75} = 0.8660$.

Therefore, percentage of linkage in the given data is 86.6. Recombination value is thus 13.4%. As 1% recombination is considered as 1 map unit, therefore, T and Y genes are 13.4 map units distance apart.

Determination of Linkage by Partitioning the Components of Dihybrid Ratio following the use of Orthogonal Function:

The method of partitioning χ^2 is also of great use in the detection of linkage. If the data under study is concerned with two traits segregating simultaneously, then it is important to know whether the two characters are inherited indepen-dently or tend to be associated.

For the purpose, it is necessary to test the segregation of two characters separately for their agreement with the respective expected ratios and if the segregation of individual trait is satisfied then the test of their independence can be proceed 1.

Thus, the objective can be achieved in a systematic manner by partitioning the total χ^2 for 3 degrees of freedom into components by the use of orthogonal function. Therefore, orthogonal function gives a precise knowledge of independent comparison in di-hybrid ratios.

Supposing the observed frequencies in the four classes are a_1, a_2, a_3, a_4, respectively, then following formulae are used:

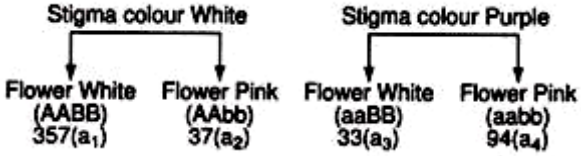
$$\chi^2_A = \frac{[(a_1 + a_2) - 3(a_3 + a_4)]^2}{3n}$$ for segregation of one trait (*let us consider* A *trait*) for 1 DF

$$\chi^2_B = \frac{[(a_1 + a_3) - 3(a_2 + a_4)]^2}{3n}$$ for segregation of another trait (*let* B *be the trait*) for 1 DF

$$\chi^2_{AB} = \frac{[(a_1 + 9a_4) - 3(a_2 + a_3)]^2}{9n}$$ for joint inheritance of A and B traits at 1 DF.

n = number of progenies.

Example:



So, here there are 2 traits:

(a) Stigma colour—controlled by A gene (A and a alleles.).

(b) Flower colour—controlled by B gene (B and b alleles,).

Solution: Segregation for A trait for 1 DF:

$$\chi^2_A = \frac{[(a_1 + a_2) - 3(a_3 + a_4)]^2}{3n}$$
$$= \frac{[(357 + 37) - 3(33 + 94)]^2}{3 \times 521}$$
$$= 0.108.$$

Segregation for B trait for 1 DF:

$$\chi^2_B = \frac{[(a_1 + a_3) - 3(a_2 + a_4)]^2}{3n}$$
$$= \frac{[(357 + 33) - 3(37 + 94)]^2}{3 \times 521}$$
$$= 0.006.$$

Joint inheritance of A and B genes for 1 DF

$$\chi^2_{AB} = \frac{[(a_1 + 9a_4 - 3(a_2 + a_3)]^2}{9n}$$
$$= \frac{[357 + 846 - 111 - 99]^2}{9 \times 521}$$
$$= 210.290.$$

Thus, we have the results:

Source	χ^2-*value*	DF
Stigma colour factor	0.108	1
Flower colour factor	0.006	1
Joint inheritance of both traits	210.290	1
Total	210.404	3

Same result would have come if χ^2-value was calculated directly.

Comment:

Results indicated that segregation of A (A and a alleles) and B (B and b alleles) factors independently were in agreement to theoretical expectations but not their joint inheritance. Thus, the discrepancy was due to failure of independent assortment between 'A' and 'B' factors.

Therefore, the merit of the method lies in locating precisely the source of discrepancy and in the present case it was due to linkage and not due to failure of segregation between alleles of A and B genes.

Example:

A tall homozygous pea plant (PP) having yellow pods (BB) was crossed with dwarf green poded plants having complementary genotypes (ppbb).

All F_1 plants were tall and yellow poded (PpBb) and were selfed to obtain F_2 progenies:

Question:

Tall Yellow—170 *Tall Green*—30

Dwarf Yellow—10 *Dwarf Green*—190

Use χ^2-test of goodness of fit to show the assortment (allelic interactions) of P and B genes.

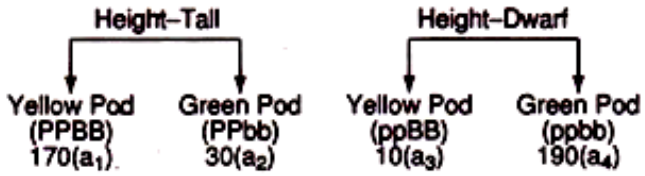
Solution:

Assumed hypothesis is 9 : 3 : 3 : 1.

Phenotypic classes	*Observed*	*Expected*	$\frac{(O-E)^2}{E}$
Tall Yellow	170	225	13.44
Tall Green	30	75	27.00
Dwarf Yellow	10	75	56.33
Dwarf Green	190	25	1089.00
		Total χ^2 = 1185.77 at 3 DF	

Thus, χ^2-value 1185.77 at 3 DF (table value 7.8 at 3DF, 5% level of signifi-cance) indicated that observed and expected values are not good-fit and conse-quently the hypothesis 9 : 3 : 3 : 1 has to be rejected. This rejection may be due to linkage between P and B genes, i.e., P and B factors have not assorted independently.

Let us consider this on the basis of orthogonal function.



Segregation for P trait for 1 DF:

$$\chi^2_P = \frac{[(a_1 + a_2) - 3(a_3 + a_4)]^2}{3n}$$
$$= \frac{[(170 + 30) - 3(10 + 190)]^2}{3 \times 400}$$
$$= 133.33.$$

Segregation for B trait for 1 DF:

$$\chi^2_B = \frac{[(a_1 + a_3) - 3(a_2 + a_4)]^2}{3n}$$
$$= \frac{[(170 + 10) - 3(30 + 190)]^2}{3 \times 400}$$
$$= 192.00.$$

Joint inheritance of P and B traits

$$\chi^2_{PB} = \frac{[(a_1 - 3a_2 - 3a_3 + 9a_4)]^2}{9n}$$
$$= \frac{[(a_1 + 9a_4) - 3(a_2 + a_3)]^2}{9n}$$
$$= \frac{[170 - 90 - 30 + 1710]^2}{9 \times 400}$$
$$= 860.44.$$

Source	χ^2-*value*	DF
Segregation for P factor	133.33	1
Segregation for B factor	192.00	1
Linkage (*Failure of independent assortment of* P *and* B *factors*)	860.44	1
	1185.77	3

Thus, by partitioning the components of dihybrid ratio it can be seen that high χ^2-value was not only due to linkage but also due to failure of segregation P (P and p alleles) and B (B and b alleles) factors.

Example: A plant with red flower (RR) with white stigma (WW) was crossed with a plant having white flower red stigma (rrww) and F_2 progenies were developed following selfing of F_1 plants:

F_2 progenies: Red flower red stigma-25; Red flower white stigma-100; White flower white stigma--25 and White flower and red stigma-100.

Use suitable statistical test to predict the mode of inheritance of the traits.

Solution: χ^2-test analysis is pertormed to predict the inheritance pattern of the concerned qualitative traits.

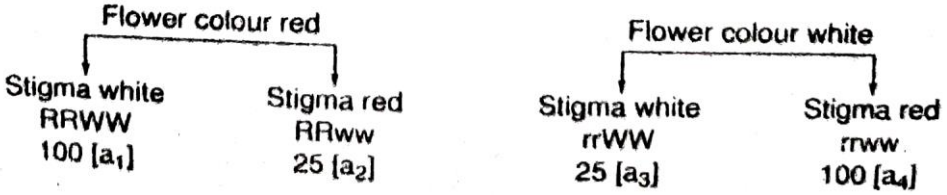
Null bypothesis (expecied): 9:3:3: 1 (F_2 progenies derived from $_{F1s}$).

Phenotypes	*Observed*	*Ratio*	*Expected*	$\frac{(O-E)^2}{E}$
1. Red flower, red stigma	25	3	46.8	10.15
2. Red flower, white stigma	100	9	140.6	11.72
3. White flower, white stigma	25	3	46.8	10.15
4. White flower, red stigma	100	1	15.6	456.62
				$\Sigma = 488.64$

$\chi^2 = 488.64$ at 3 DF.

The value of x is too high (488.64) at 3 DF than table value 7.8, thereby Indicating significant deviations between observed and expected values. In such case the hypothesis should be rejected but the problem given clearly suggested that it is a classical dihybrid cross and, therefore, the expected ratio should be 9 :3:3: 1. Thus, the deviation can be explained on the basis that R and W genes possibly failed to assort independently.

Use of Orthogonal Function (Partitioning the components of Dihybrid cross)



Let flower colour be 'A' trait and stigma be 'B' trait.

(a) $\chi^2_A = \frac{[(a_1 + a_2) - 3(a_3 + a_4)]}{3n}$ testing segregation of A factor alone at 1 DF

$= \frac{(125 - 375)^2}{3 \times 250} = \frac{(-250)^2}{750} = \frac{62500}{750} = \mathbf{83.3.}$

(b) $\chi^2_B = \frac{[(a_1 + a_3) - 3(a_2 + a_4)]^2}{3n}$ testing segregation of B factor alone at 1 DF

$= \frac{[125 - 3(125)]^2}{3n} = \frac{(-250)^2}{750} = \mathbf{83.3.}$

(c) $\chi^2_{AB} = \frac{[(a_1 - 3a_2 - 3a_3 + 9a_4)]^2}{9n}$ joint segregation of A and B factors—due to linkage at 1 DF

$= \frac{(100 - 75 - 75 + 900)^2}{9 \times 250} = \frac{(850)^2}{2250} = \mathbf{321.11.}$

Thus the following result is obtained:

Source	χ^2-*value*	DF
Flower colour factor (A-*factor*)	83.3	1
Stigma colour factor (B-*factor*)	83.3	1
Linkage	321.1	1
	487.7	3

Now it is confirmed that linkage was there between R and W genes-they have failed to assort independently. As χ^2 has been partitioned into components due to different causes it can be confirmed that discrepancy was also due to failure of segregation of single factor ratios and here lies the significance of the method.

Use of χ^2-Test in Binomial Distribution:

Binomial distribution is a discrete probability distribution and is defined by the p.m.f.

$f(x) = {}^nC_x p^x q^{n-x}$. (x = 0,1,2,…,n),

where, p and q are positive fractions (p + q = 1).

x	0	1	2	3	···	n	Total
f(x)	q^n	${}^nC_1 pq^{n-1}$	${}^nC_2 p^2 q^{n-2}$	${}^nC_3 p^3 qn^{n-3}$	···	p^n	1

The distribution is known as binomial because the probabilities are given by the binomial series

$(p + q)^n = q^n + {}^nC_1 pq^{n-1} + {}^nC_2 p^2 q^{n-2} + {}^nC_3 p^3 qn^{n-3} + \ldots + p^n$.

The two constant n and p appearing in the expression for f(x) are known as parameters of the binomial distribution. If the values of parameters are known, the distribution is completely known (q = 1 — p).

Binomial distribution takes into account the following:

(a) The result of any trial can be classified only under two categories (let us say, success and failure);

(b) The probability of success in each trials remains a constant;

(c) The trials are independent.

Example:

A total number of 160 families with 4 children have been surveyed and the following result has been obtained:

Girls	4	3	2	1	0
Boys	0	1	2	3	4
Families	7	50	55	32	16

Question:

Is the family distribution consistence with the hypothesis of equal num-ber of boys and girls?

Solution:

As boys and girls represent two set of terms, such problem can be solved by expanding the binomial (a + b)n.

Expansion of $(a + b)^n – a^n + a^{n-1}b + a^{n-2}b^2 + a^{n-3}b^3 + \ldots + b^n$.

Each term of the expansion has an appropriate coefficient and it can be calcu-lated from the general formula: n!/s!t!, where n = index of binomial, s = index of a in the term, t = index of b in the term and ! = factorial [e.g., 4! —4 x 3 x 2 x 1; 5! — 5 x 4 x 3 x 2 x 1; n! — 1 x 2 x 3 x 4 x … x n].

In the present problem $(a + b)^n$ can be written as $(a + b)^4$, where a = boy and b = girl.

Hypothesis to be assumed is boys and girls are equally (randomly/ consistent) distributed in the family. That is probability of girl is 1/2 and that of boy is also 1/2.

$(a + b)^4 = a^4 + a^3b + a^2b^2 + ab^3 + b^4$.

Using the formula n’/s!t!, the coefficient has been calculated and represented as:

$$a^4 + 4a^3b + 6a^2b^2 + 4ab^3 + b^4$$

$\begin{bmatrix}4\text{ girls}\\0\text{ boy}\end{bmatrix}$	$\begin{bmatrix}3\text{ girls}\\1\text{ boy}\end{bmatrix}$	$\begin{bmatrix}2\text{ boys}\\2\text{ girls}\end{bmatrix}$	$\begin{bmatrix}1\text{ girl}\\3\text{ boys}\end{bmatrix}$	$\begin{bmatrix}0\text{ girl}\\4\text{ boys}\end{bmatrix}$
$= a^4$	$= 4a^3b$	$= 6a^2b^2$	$= 4ab^3$	$= b^4$
$= (\frac{1}{2})^4$	$= 4 \times (\frac{1}{2})^3 (\frac{1}{2})$	$= 6 \times (\frac{1}{2})^2 (\frac{1}{2})^2$	$= 4 \times (\frac{1}{2}) (\frac{1}{2})^3$	$= (\frac{1}{2})^4$
$= \frac{1}{16}$	$= \frac{1}{4}$	$= \frac{6}{16}$	$= \frac{4}{16}$	$= \frac{1}{16}$
$= \frac{1}{16} \times 160$	$= \frac{1}{4} \times 160$	$= \frac{6}{16} \times 160$	$= \frac{4}{16} \times 160$	$= \frac{1}{16} \times 160$
$= 10$	$= 40$	$= 60$	$= 40$	$= 10$

	Families					*Total*
Observed	7	50	55	32	16	160
Expected	10	40	60	40	10	160

$$\frac{(O-E)^2}{E} = \frac{(7-10)^2}{10} \frac{(50-40)^2}{40} \frac{(55-60)^2}{60} \frac{(32-40)^2}{40} \frac{(16-10)^2}{10}.$$

$$\chi^2 = \sum \frac{(O-E)^2}{E} = \frac{9}{10} + \frac{5}{2} + \frac{5}{12} + \frac{8}{5} + \frac{18}{5}$$
$$= 0.9 + 2.5 + 0.42 + 1.6 + 3.6$$
$$= 9.02.$$

Degree of freedom n — 1 = 5 — 1 = 4. As the computed χ^2-values 9.02 at 4DF is not greater than the table value at 5% level (9.02 at 4DF), it can be concluded that expected and observed frequencies for families are good-fit. Thus the hypothesis assumed is accepted and can be inferred that the distribution of boys and girls within families is equal, i.e., random in nature.

Example: A Chloroxantha mutant has been spotted at M_2 of black cumin. Viable chloro phyll mutant (Chloroxantha-a chlorophyll-deficient mutant) can be used as genetic marker for efficient plant breeding in the species.

Reciprocal crosses were made between normal and Chloroxantha mutant plants and subsequently F_1 (all F_{1s} were of normal colour phenotypes) and F_2 plants were raised. The F_2 plants were used for estimating the segregation ratio tor seedling colours by using the χ^2-test analysis. The pattern or F_2 segregation (mutant as sigma parent in crossing with normal-25 normal, 15 mutant, $\chi^2 = 0.64$ for 9:7, p-value 0.40 to 0.50; mutant as pollen parent-48 normal, 28 mutant, $\chi^2 = 0.86$ tor 9:7, p-value 0.40 to 0.50) revealed that the inheritance of the Chloroxantha (seedling colour pale greenish yellow) mutant was recessive and was under the control of two gene loci.

Thus: Reciprocal crossing and use of a statistical test have thrown light on the nature of inheritance of a particular trait and thereby documented the genetics of the trait conçerned.

χ^2-test Independence:

Another common use of the χ^2-test is in testing independence of classifications in what are known as contingency table. The chi square test for independence of two variables is a test which uses a cross classification table to examine the nature of the relationship between these variables. These tables are sometimes referred to as contingency tables. These tables show the manner in which two variables are either related or are not related to each other. The test for independence examines whether the observed pattern between the variables in the table is strong enough to show that the two variables are dependent on each other or not. While the chi square statistic and distribution are used in this test, the test is quite distinct from the test of goodness of fit. The goodness of fit test examines only one variable, while the test of independence is concerned with the relationship between two variables.Data

are set out in a table with rows and columns, i.e., each observation is assigned to one of the cells in the table.

For example, if there are r rows and c columns, the table is generally called r x c contingency table, where r and c may represent any number and the simplest table of this kind is 2 x 2 contingency table.

			Total
	a	b	a + b
	c	d	c + d
Total	a + c	b + d	a + b + c + d

$$DF = (r - 1)(c - 1).$$

χ^2-value for 2 × 2 table is given by the following formula:

$$\chi^2 = \frac{(ad - bc)^2(a + b + c + d)}{(a + b)(b + d)(d + c)(c + a)}.$$

For a 2 x 2 table, there is only one degree of freedom, i.e., only one of the four cell frequencies can be arbitrarily given if the row and column total remain fixed. It is, therefore, necessary to make a correction of formula, so that its approximation to the continuous chi-square distribution can be improved. This is known as Yates correction for continuity.

$$\chi^2 = \frac{N\{(ad - bc) - N/2\}^2}{(a + b)(b + d)(d + c)(a + c)}.$$

Example:

In a survey of 200 boys of which 75 were intelligent, 40 had skilled fathers; while 85 of the unintelligent boys had unskilled fathers. Do these figures, support the hypothesis that skilled father have intelligent boys?

Solution:

The data are shown in the following 2 x 2 table:

Intelligence of sons	*Skill of fathers*		*Total*
	Skilled	*Unskilled*	
Intelligent	40	35	75
	(*a*)	(*b*)	
Unintelligent	40	85	125
	(*c*)	(*d*)	
Total	80	120	200

Null hypothesis—The two attributes skill of father and intelligence of son are inde-pendent (As it is a test of independence—the assumed hypothesis is considered to be independent between attributes).

$$\chi^2 = \frac{N\{(ab - bc) - N/2\}^2}{(a + b)(b + d)(d + c)(a + c)}$$

$$= \frac{200\left[(40 \times 85) - (35 \times 40) - \frac{200}{2}\right]^2}{80 \times 120 \times 75 \times 125}$$

$$= 8.02 \text{ at } 1 \text{ DF } [(r - 1)(c - 1) = (2 - 1)(2 - 1)] = 1.$$

As the observed value 8.02 at 1 DF is greater than the table value (3.84 at 1 DF) at 5% level of significance, there exist significant deviation between ob-served and expected cell frequencies and, therefore, the assumed hypothesis is rejected.

Inference:

By the use of χ^2-test of independence it can be concluded that skilled fathers have intelligent sons (the attributes are dependent).

Example:

Anaphase I chromosomal segregation in *Allium cepa* (2n = 16) showed seasonal variation and the data obtained has been represented in 2 x 2 contingency table. Test whether the attributes are independent or not.

	Anaphase I separations		
Seasons	*8 : 8*	*9 : 7*	*Total*
Summer	65(a)	35(a)	
Winter	80(c)	20(d)	100
Total	145	55	200

Solution:

Null hypothesis—Anaphase I separation of chromosomes is independent of seasons:

$$\chi^2 = \frac{N\{(ad - bc) - N/2\}^2}{(a+b)(b+d)(d+c)(a+c)}$$

$$= \frac{200\{(65 \times 20) - (35 \times 80) - 200/2\}^2}{100 \times 55 \times 100 \times 145}$$

$$= 6.42 \text{ at } 1 \text{ DF.}$$

The calculated χ^2-value 6.42 at 1 DF is greater than the table value 3.87 (1 DF at 5% level of significance) and, therefore, the hypothesis is rejected as there exist significant deviation between observed and expected cell frequencies.

Inference:

Anaphase I separation of *Allium cepa* chromosomes varied with seasons (the traits are dependent to one another).

Example:

500 PMCs of *Nigella sativa* (black cumin) were assessed in 2 diffe-ent seasons (summer and winter) for studying anaphase I segregation of chromosomes (2n = 12) and the data obtained have been tabulated in r x c contingency table. Test the independence of the characters (anaphase I chromosome segrega-tion and seasons,).

Seasons	*Anaphase I chromosome separation*			*Total*
	4 : 8	6 : 6	5 : 7	
Summer	42	137	61	240
	a	b	c	
Winter	58	113	89	260
	d	e	f	
Total	100	250	150	500

Solution:

Null hypothesis—Anaphase I chromosome segregation is independent to seasons.

The expected value for each class can be calculated from the following formula:

E = r x c / G, where r = row, c = column and G = grand total. G

Expected values:

$$a = \frac{100 \times 240}{500} = 48; \quad b = \frac{250 \times 240}{500} = 120;$$

$$c = \frac{150 \times 240}{500} = 72; \quad d = \frac{100 \times 260}{500} = 52;$$

$$e = \frac{250 \times 260}{500} = 130; \quad f = \frac{150 \times 260}{500} = 78.$$

$$\chi^2 = \frac{(42-48)^2}{48} + \frac{(137-120)^2}{120} + \frac{(60-72)^2}{72} + \frac{(58-52)^2}{52} + \frac{(113-130)^2}{130} + \frac{(89-78)^2}{78}$$

$$= 0.75 + 2.41 + 2.0 + 0.69 + 2.22 + 1.55$$

$$= 9.62 \text{ DF} = (r-1)(c-1) = (2-1)(3-1) = 2.$$

The calculated χ^2-value 9.62 at 2 DF is higher than table value (5.9 at 2 DF) at 5% level of significance, thereby indicating that the expected and observed frequencies are not good-fit rather showed significant deviations. Thus, the hy-pothesis is rejected.

Inference:

Anaphase I chromosome segregation is dependent to seasons.

Example:

Yield of wheat varieties in (quintal/hectare) relation to irrigation and non-irrigation has been presented in tabular form. Test whether yield of wheat varieties are related to irrigation.

Solution:

Wheat varieties	*Irrigation* (a)	*No irrigation* (a′)
Lerma rejo	15	10
Sarbati sonara	5	20
Kalyan sona	12	8
Sonalika	4	6
	$n_1 = 36$	$n_2 = 44$

Null hypothesis—Wheat varieties are independent to irrigation.

Following formula can be used to compute χ^2-value:

$$\chi^2 = \frac{1}{n_1 n_2} \sum \frac{(an_2 - a'n_1)^2}{a + a'}$$

n_1 and n_2 are column total, a and a′ are the observed frequencies

Lerma rejo $a + a' = 25 \quad \frac{(an_2 - a'n_1)^2}{a + a'} = 36.$

Sarbati sonara $a + a' = 25 \quad \frac{(an_2 - a'n_1)^2}{a + a'} = 100.$

Kalyan sona $a + a' = 20 \quad \frac{(an_2 - a'n_1)^2}{a + a'} = 2880.$

Sonalika $a + a' = 10 \quad \frac{(an_2 - a'n_1)^2}{a + a'} = 160.$

$$\chi^2 = \frac{1}{n_1 n_2} \sum \left\{ \frac{an_2 - a'n_1}{a + a'} \right\}^2 = \frac{1}{1584}\{36 + 100 + 2880 + 160\}$$

$$= \frac{3176}{1584} \text{ at 4 DF } [(r-1)(c-1) = (5-1)(2-1)]$$

$$= 2.01.$$

The calculated χ^2-value 2.01 at 4DF is less than the table (9.49 at 4DF) at 5% level of significance and, therefore, the hypothesis assumed have been ac-cepted (deviation between observed and expected frequencies non-significant).

Inference:

Yield of wheat varieties is independent to irrigation.

χ^2-test of Heterogeneity:

χ^2-test of heterogeneity is very similar to that of testing independence and widely used in genetic experiments, where the consistency (randomness) or otherwise (inconsistency = non-randomness = significant variation) of several groups of data can be tested. The advantage of this method is that it shows actual proportions observed in each class.

Metaphase I chromosome associations in tetraploid *Nigella damascena* (2n = 12) have been documented over 4 generations. Test whether chromosomal configurations (I, II and IV) per cell were consistent over the generations or not.

Generations	*No. of MI cells scored*	*Mean/cell*		
		I	*II*	*IV*
C_0	78	2.26	7.78	1.54
C_1	84	2.00	8.12	1.45
C_2	116	2.17	8.26	1.33
C_3	127	1.91	9.19	0.91
χ^2-value		3.69	13.97	20.06
p-value at 3 DF		0.30	> 0.01	> 0.001

Solution: Test of χ^2-test of heterogeneity has been performed.

For univalent/cell:

$$78 \times 2.26 = 176.28 — O_1 = C_0$$
$$84 \times 2.00 = 168.00 — O_2 = C_1$$
$$116 \times 2.17 = 251.72 — O_3 = C_2$$
$$127 \times 1.91 = 242.57 — O_4 = C_3$$
$$\text{Pooled} = 838.57$$

Total observation: 405.

Pooled expected: 2.07.

Expected for individual generation:

$$2.07 \times 78 = 161.46—E_1$$
$$2.07 \times 84 = 173.88—E_2$$
$$2.07 \times 116 = 240.12—E_3$$
$$2.07 \times 127 = 262.89—E_4$$

$$\chi^2 = \frac{(176.28 - 161.46)^2}{161.46} + \frac{(168.0 - 173.88)^2}{173.88} + \frac{(251.72 - 240.12)^2}{240.12}$$
$$= 1.36 + 0.20 + 0.56 + 1.57\frac{(242.57 - 262.89)^2}{262.89}$$
$$= \boxed{3.69}.$$

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		I	II	IV
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$$\chi^2 = \frac{(176.28 - 161.46)^2}{161.46} + \frac{(168.0 - 173.88)^2}{173.88} + \frac{(251.72 - 240.12)^2}{240.12}$$
$$= 1.36 + 0.20 + 0.56 + 1.57\frac{(242.57 - 262.89)^2}{262.89}$$
$$= \boxed{3.69}.$$

Thus, analysis of χ^2-test of heterogeneity of chromosome configurations over the generations at 3 DF revealed that frequency of quadrivalents ($p > 0.001$) and bivalents ($p > 0.01$) per cell at metaphase I among tetraploids were inconsis-tent (non-random/significant) over the generations; while, the univalents were random ($p = 0.30$) in distribution [uniformly distributed over the generations].

Example:

Univalent and bivalent frequency per cell have been presented in Table from 10 plants of *Ocimum basilicum* (basil 2n = 72).

Question:

Find out from the given data whether univalent and bivalent frequen-cies per cell were consistent among the plants.

Plant number	No. of MI cells scored	Frequency/cell	
		II	I
1	33	34.76	2.48
2	35	35.14	1.71
3	30	35.33	1.33
4	25	35.32	1.36
5	29	35.10	1.79
6	32	34.94	2.13
7	28	35.11	1.79
8	33	35.21	1.58
9	35	35.29	1.43
10	24	35.00	2.00

χ^2-test of heterogeneity has been performed to assess distribution of univalent and bivalent per cell over the plants. Result has been tabulated (process of calculation have been shown in the previous example).

Configuration	χ^2-value	DF	Probability level
II	0.26	9	> 0.95
I	21.27	9	< 0.05

Result indicated that bivalents/cell was random (consistent) among the plants; while, frequency of univalent/cell was non-random (significant variants occurred among plants).

Example:

Table: Meiotic analysis in tetraploid black cumin (*Nigella sativa* L.). Tetraploids No. of MI Mean number/cell at metaphase

Tetraploids	No. of MI cells studied	Mean number/cell at metaphase I				Mean chiasma number per cell	No. of AI cells scored	Frequency of cells with equal chromosomal (6/6)division(%)	Total pollen grains observed	Pollen fertility (%)
		I	II	III	IV					
C_0–1	116	2.37	8.09	0.11	1.27	16.4±0.7	63	55.6	163	52.8
C_1–1	24	4.16	5.75	0.00	2.08	14.4±0.5	26	76.9	192	68.8
C_1–2	32	1.17	8.06	0.00	1.63	19.1±0.6	23	82.6	215	74.9
C_1–3	42	2.85	7.23	0.00	1.67	16.6±0.4	35	80.0	307	71.0
C_1–4	44	1.82	8.68	0.00	1.23	18.4±0.7	38	78.9	97	73.2
C_2–1	32	1.87	8.12	0.00	1.38	16.3±0.4	60	85.0	97	73.3
C_2–2	32	0.50	9.00	0.00	1.37	18.5±0.6	30	90.0	112	81.3
C_2–3	44	3.00	8.14	0.00	1.18	12.6±0.4	25	72.0	66	63.6
C_2–4	28	2.00	7.28	0.00	1.86	15.8±0.4	36	72.2	127	63.0
C_2–5	30	1.73	9.53	0.00	0.80	16.2±0.4	51	86.3	82	78.1

Comment: Chi-square test of heterogeneity revealed that the frequency of bivalents and quadrivalents per cell among the tetraploids was random (that is there exist no significant variations) but the number of univalents per cell was non-random. The univalent frequency per cell among tetraploids demonstrated significant positive correlation with abnormal anaphase I cells (r = 0.81), the abnormal anaphase I cells showed significant negative correlation with pollen fertility (r =-0.99). Thus, cytological examination of chromosomes in the induced autotetraploids of black cumin leads to the conclusion that reduction in pollen fertility was the result of chromosomal disturbances arising from pairing irregularities.

Example: Secondary groupings in the form of 3 group class (3.13%), 6(28.83%), 9(12.50%), 12(43.75%), 15(8.33%), 18(9.38%) and > 18(2.08%) were observed in metaphase I cells (2n = 72) of *Ocimum basilicum* (sweet basil). Number of PMCS scored at Ml was 304 and 94.74% of the meiocytes were the groupings.
Bivalents and univalents lying in close proximity but always without any distinct material connections has been referred to as Secondary Associatton of Chromosomes and genetic inter-relationship has been ascribed to be the main causal factor for the phenomenon and has been studied in different plant species in tracing basic chromosome number and polyploid nature of the species.
Solution: χ^2-test analysis performed between the group classes (3 to > 18) revealed heterogeneity (χ^2= 251,46, DF 6, p-value < 0.001), thereby indicating that chromosomes have assorted themselves more preterentially into certain number of groups (predominantly 12 group class) than they do into others much against random distribution. In the species, observed cell frequencies for 12 and 6 group classes have only been found to be significantly higher than expected and it was verified by comparning the cell frequency of12 (observed 12 group class 126, rest 162, expected 12 group class 41.14, rest 246.86, total 288, χ^2 = 204.21 at 1 DF, p > 0.001) and 6 (observed 6 group class 60, rest 228, expected 6 group class 41.14, rest 246.86, total 288, χ^2 = 10.09 at 1 DF, p < 0.001) group classes against the pooled frequency of rest of the classes.

Comments: Secondary association of chromosomes in *O. basilicum* (sweet basil, Tulsi) has suggested secondary polyploid nature of the species and stastical analysis of cytological data has indicated that the basic chromosome number of the species is 12 which probably evolved from ancestral basic number x=6 through polyploidy.
Inference: *O. basilicum* is not a true diploid rather a derived polyploid (secondary polyploid) whose n = 36 but x= 12.

Probability:

Probability is a number expressed in a quantitative scale. When one event will not occur at all then the probability of that event is 0, and if there is any event which will happen positively without fail then the probability of that event is 1. But in biological science, mostly we find the probability of any event lies between impossibility to certainty i.e., the value ranges from 0 to 1.

Mathematically probability can be explained in the following way:

If an event can happen in ‘a’ number of ways, and fails to happen in ‘b’ number of ways, then the probability of its happening ‘p’ is written as.

$$p = \frac{a}{a+b} \quad \text{or} \quad p = \frac{\text{Number of events occurring}}{\text{Total number of events}}$$

Similarly **probability of failure** of any event is denoted as 'q', where,

$$q = \frac{b}{a+b} \quad \text{or} \quad q = \frac{\text{Number of events not happening}}{\text{Total number of events}}$$

Therefore, $p + q = \frac{a}{a+b} + \frac{b}{a+b} = 1$

So, if the probability of happening any event is 0.7, then the probability of not happening of that event is 0.3.

Events:

The results of any experiment in all possible forms are said to be events of that experiment. Such as, throwing of a dice has 6 possible outcomes, either 1 or 2 or 3 or 4 or 5 or 6. All these six outcomes are called events of that single experiment.

Null Event:

When there is no chance of getting an event is called null or impossible event. It is symbolically denoted by ϕ. Such as, survival of any human being forever is an impossible event or null event.

Sure Event:

If the likelihood of occurrence of any event is sure then the event is called sure event. Such as, the death of a human being is a sure event.

Equally likely events:

If the likelihood of the occurrence of every event in an experiment is same then those are called as equally likely events. Such as, when a dice is thrown, there is no biasness, there are the possibilities of coming any number 1 to 6 in equal probability, so the events are equally likely events.

Mutually Exclusive Events:

If in an experiment the occurrence of one event prevents or rules out the happening of all other events, then these are called as mutually exclusive events. Such as, when a coin is tossed either 'head' or 'tail' will come.

The occurrence of one event affects the occurrence of another event, both events cannot occur together, i.e., occurrence of 'head' rules out getting 'tail' in the same trial. Here the events are connected by the words 'either' or 'or'.

Simple Event and Compound Event:

Any event having only one sample point of a sample space is called simple event and if any event is decomposable into a number of simple events then it is called as compound event.

Such as, if a bag contains 4 white and 6 red balls, and if one ball is drawn then it is simple event, but if two balls are drawn together then the events will be — 'both the balls are white', 'both the balls are red', 'one ball is white and another ball is red' — these are compound events.

The compound events may be of two types:

Independent Event:

Two or more events are said to be independent events when the outcome of one event does not affect or is not affected by the other events. For example, if a coin is tossed twice, the result of second tossing would in no way be affected by the result of first tossing, so these are independent events.

Dependent Event:

The occurrence or non-occurrence of one event in one trail affects the probabilities of other events in other trails are called dependent events. For example, the probability of drawing a queen from a pack of 52 cards is 4/52, but if the card drawn for the first time (queen) is not replaced then the probability of second drawing of a queen is 3/51, as the pack now contains 3 queens and 51 cards.

Addition and Multiplication Rules:

Probability is estimated usually on the basis of following two rules of chances:

1. Addition rule
2. Multiplication rule

Addition Rule:

This rule is applied when two events are mutually exclusive, i.e., both events cannot occur simultaneously. The birth of a male child excludes the birth of a female child in the same trial. The probability that one of several mutually exclusive events will occur is the sum of the probabilities of the individual events.

Mathematically, when two events A and B are mutually exclusive, the chance of occurrence or probability of occurrence of either A or B can be calculated from the following formula:

p (A or B) = p(A) + p(B)

This rule is applicable to any number of mutually exclusive events as follows:

$p(E_1 \text{ or } E_2 \text{ or } E_3 \ldots E_n) = p(E_1) + p(E_2) + p(E_3) + \ldots + p(E_n)$

Example 1:

If a dice is rolled, what is the probability of getting either 3 or 5?

Probability of getting 3 is p(3) = 1/6

Probability of getting 5 is p(5) = 1/6

∴ Probability of getting either 3 or 5 is p(3) + p(5) = 1/6 + 1/6 = 1/3

Example 2:

What is the probability of getting a king or a joker from a pack of 54 cards?

Probability of getting a king is p(K) = 4/54 = 2/27

Probability of getting a joker is P(J) = 2/54 = 1/27

So, the probability of either a king or a joker is

p(K or J) = p(K) + p(J) = 2/27 +1/27 = 3/27 = 1/9

Addition rule changes when the events are not mutually exclusive, i.e., if two events A and B can occur simultaneously in few cases, then the rule becomes modified in the following way:

p(A or B) = p(A) + p(B) – p(A and B)

Example 3:

What is the probability of getting a king or club from a pack of 52 cards?

In this example, getting a king and a club are not mutually exclusive events as there will be one king which is king of club. So the chance or probability of getting that event should be subtracted.

p(King or Club) = p(King) + p(Club) – p(King and Club)

Probability of King = p(King) = 4/52 = 1/13

Probability of Club = p(Club) = 13/52 = 1/4

Probability of King and Club = p (King and Club) = 1/52

So, p(King or Club) = (1/13 + 1/4) – 1/52 = 4/13

Multiplication Rule:

(a) When the Events are Independent:

Probability of two or more independent events occurring together is the product of the probabilities of individual events.

Symbolically, if p(A) and p(B) are the probabilities of two respective events A and B, and the happening of these two events are independent then the probability of happening both the events together can be calculated with the following formula:

p(A and B) = p(A) x p(B)

Thus the rule may be extended to any number of independent events like E_1, E_2, E_3… E_n, and the formula will be as follows:

$p(E_1$ and E_2 and E_3 … and $E_n) = p(E_1)$ x $p(E_2)$ x $p(E_3)$ x … x $p(E_n)$

Example 4:

If two dice are thrown simultaneously what is the probability of getting 3 in both the dice?

The probability of getting 3 in first dice is p(A) = 1/6

The probability of getting 3 in 2nd dice is p(B) = 1/6

So, the probability of getting 3 in both the dice is

p(A and B) = p(A) × p (B)= 1/6 × 1/6 = 1/36

(b) When the Events are Dependent:

When the probability of happening one event is affected by the occurrence of another event then it is called conditional probability. Such as, conditional probability of happening A, when B has already happened, is denoted as p(A/B); conditional probability of B, and A has already happened, is denoted as p(B/A).

When the two events A and B are occurring simultaneously but any one event has conditional probability then the multiplication rule will be written as:

p(ab) = p(A)p(B/A) or p(B)-p(A/B)

where p(A/B) = Conditional probability of A given that B has happened

p(B/A) = Conditional probability of B given that A has happened

Example 5:

Four cards are drawn consecutively four times from a pack of 52 cards. Find the chances of drawing an ace, a king, a queen and a jack. The cards are not replaced after each withdrawal.

Probability of drawing an ace = p(A) = 4/52

Probability of drawing a king = p(K) = 4/51

Probability of drawing a queen = p(Q) = 4/50

Probability of drawing a jack = p(J) = 4/49

So, the combined probability

p(A and K and Q and J) = p(A) x p(K) x p(Q) x p(J)

= 4/52 x 4/51 x 4/40 x 4/49 = 0.317

Example 6:

Four cards are drawn in four consecutive drawals from a pack of 52 cards without replacing the cards after each drawal. What is the probability of drawing a king in each drawal?

The probability of getting a king in 1st drawal = 4/52

The probability of getting a king in 2nd drawal = 3/51

The probability of getting a king in 3rd drawal = 2/50

The probability of getting a king in 4th drawal = 1/49

So, the combined probability of getting a king in 4 consecutive drawals is

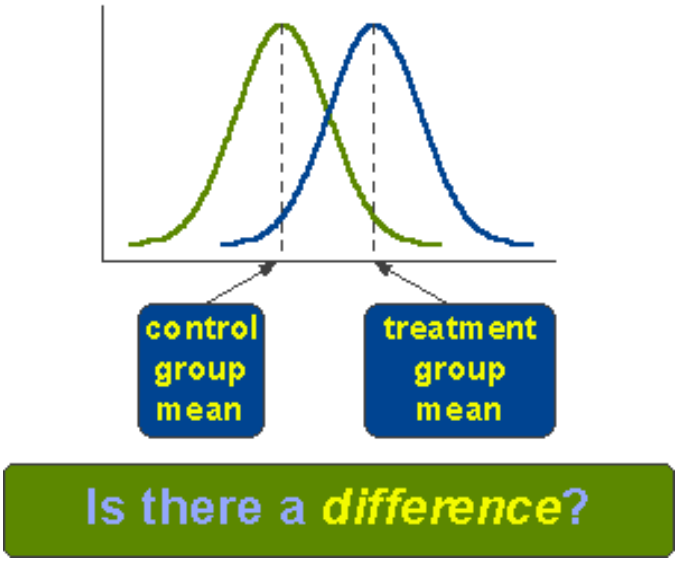
4/52 x 3/51 x 2/50 x 1/49 = 1/270725

Test of Significance:

In biological research when we compare any character of two samples, we calculate the significance of difference in the mean and variance to draw a meaningful conclusion.

Student's 't'-Test:

At the beginning of the 20th century, a statistician named William S. Gosset, an employee of Guinness Breweries in Ireland, was interested in making inferences about mean when σ was unknown. Because Guinness employees were not permitted to publish research work under their names, Gosset adopted the pseudonym "Student". The distribution that he developed has come to be known as Student's t- distribution (1921). t-test is used to test the significance of means of two



samples drawn from a population, as well as the significance of difference between the mean of small sample and hypothetical mean of population

In this test we make a choice between two alternatives:

1. To accept the null hypothesis (no difference between the two means);
2. To reject the null hypothesis (the difference between the means of two samples significant).

The test is applied to small sample (n<30), and samples must be drawn randomly from normal population. The 't' is defined as quantity representing the difference between the sample mean or true means or population mean expressed in terms of the standard error.

T = Difference between sample means/Standard error of the difference between means

Applications:

The t-distribution has a number of applications in Statistics and other disciplines, of which are:

I. t- test for significance of single mean, population variance being unknown. t-test for the significance of the difference between two sample means, the population variances being equal but unknown.

II. t- test for significance of an observed sample correlation coefficient.

III. t- test for significance of an observed regression coefficient

When two samples drawn from the normally distributed population, it is assumed that the means are normal distributed and are independent. The null hypothesis assumed states that the difference between population means μ_1 and μ_2 is zero ($\mu_1 = \mu_2$). Consequently, the sample means, X_1 and X_2 drawn from the respective populations are equal ($\bar{x}_1 = \bar{x}_2$) or it may be said that difference between two sample means is zero ($\bar{x}_1 - \bar{x}_2 = 0$).

To test whether deviation between 2 sample means is significant or not, student t-test is applied and expressed by the formula:

$$t = \frac{\text{difference of means of two-sample}}{\text{standard error of that difference}}.$$

The standard deviation of the difference of two means is commonly known as standard error of the difference. For estimation of standard error of difference we must calculate the variance of difference. The variance of the difference between the means can be calculated by adding the mean variance of the two samples.

$$\text{Mean variance} = \frac{\sigma^2}{n}.$$

$$\text{Variance of difference } (\sigma^2 d) = \frac{\sigma_A^2}{n_1} + \frac{\sigma_B^2}{n_2},$$

Where, $\sigma^2 d$ = variance of the difference of the two means,
σ^2_A = variance of population (sample) A;
σ^2_B = varance of population (sample) B;
n_1 = number of items in the sample A;
n_2=number of items in the sample B.
Standard error of difference

$$\sigma d = \sqrt{\frac{\sigma_A^2}{n_1} + \frac{\sigma_B^2}{n_2}}.$$

t-test from Unpaired Samples

Independent (or unpaired two sample) t-test is used to compare the means of two unrelated groups of samples.

As an example, we have a cohort of 100 individuals (50 women and 50 men). The question is to test whether the average weight of women is significantly different from that of men?

In this case, we have two independents groups of samples and **unpaired t-test** can be used to test whether the means are different.

Computation of 't' can be made for two large samples having means $\bar{x}_A$ and $\bar{x}_B$ and variance σ^2_A and σ^2_B with n_1 and n_2 observations (n > 30) in twe sanples using the formula:

$$t = \frac{\bar{x}_A - \bar{x}_B}{\sqrt{\frac{\sigma_A^2}{n_1} + \frac{\sigma_B^2}{n_2}}}, \text{ where } n > 30.$$

$$t = \frac{\bar{x}_A - \bar{x}_B}{\sigma d\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}, \qquad \sigma d = \sqrt{\frac{\Sigma d_A^2 + \Sigma d_B^2}{n_1 + n_2 - 2}}$$

this formula is used, where the sample size ($n < 30$) is less than 30.

Determination of Significance:

Probability of occurrence of any calculated value of 't' is determined by comparing it with the value given in the 't'-table corresponding to the calculated degree of freedom, derived from the number of observations in the samples under study. If the calculated value of 't' exceeds the value given at p = 0.05 (5% level) in the table, it is said to be significant. If the calculated value of 't' is less than the value given in 't'-table, it is not significant.

The 't'-test procedure is done to locate the observed value of 't' in the student's 't'- distribution curve. The 't' distribution curve is a symmetrical curve with mean zero, it extends to infinity on either sides. When degree of freedom is less in number, 't'- distribution resembles to normal distribution curve. If the calculated value of 't' is near the centre then the data is regarded as compatible with H_0, which

concludes that the observed deviation is due to chance factor or only due to sampling. If the 't' value is situated at tail region then $H_0$is not accepted.

Example: Seed yield (gm) of two-plant ypes (control-A and mutant-B) of *Nigella sativa* (black cumin) has been given:
Test whether mean seed yteld of two-plant types of *Nigella sativa* is significant or not

Sample–A	1.8	1.9	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.6
Sample–B	2.3	2.4	2.5	2.5	2.7	2.8	2.9	3.0	3.1	3.2

Solution: Assummed hypothests-Mean seed yield in two-plant types is same.

	Sample A (control)	*Sample B (mutant)*	d_A^2	d_B^2
1	1.8	2.3	0.1936	0.1936
2	1.9	2.4	0.1156	0.1156
3	2.0	2.5	0.0576	0.0576
4	2.1	2.5	0.0196	0.0576
5	2.2	2.7	0.0016	0.0016
6	2.3	2.8	0.0036	0.0036
7	2.4	2.9	0.0256	0.0256
8	2.5	3.0	0.0676	0.0676
9	2.6	3.1	0.1296	0.1296
10	2.6	3.2	0.1296	0.2116
	$\Sigma x_A = 22.4$, $\bar{x}_A = 2.24$	$\Sigma x_B = 27.4$, $\bar{x}_B = 2.74$	$\Sigma d_A^2 = 0.744$	$\Sigma d_B^2 = 0.864$

$$\sigma^d = \frac{\sqrt{0.744 + 0.864}}{18}$$

$$= 0.299$$

$$t = \frac{2.74 - 2.24}{0.299\sqrt{\frac{1}{10} + \frac{1}{10}}}$$

$$= \frac{0.50}{0.299 \times 0.447}$$

$$= 3.74 \text{ at } 18 \text{ DF } (10 + 10 - 2).$$

Tabulated value of t at 18 DF
2.10 at 0.05 probability level (5% level);
2.88 at 0.01 probability level (1% level);
3.92 at 0.001 probability level (0.1% level).
Comment: Calculated t-value is higher than the values at 5% and 1% levels (table values at 18 DF), therefore, mean seed yield of two-plant types of *N. sativa* differ significanty (null hypothesis rejected) at 1% level of significance.

Note 1. Level of significance is denoted by asterisk. *, ** and *** significant at 5%, 1% and 0.1% levels respectively.

Example: Variety A (1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.3, 2.4, 2.5 2.6) and B (2.3, 2.4, 2.5, 2.5, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2) of potato yielded tubers (gm). Does the mean yield of tubers of two potato varieties differ significantly?

Solution: Null hypothesis-Mean yield of tubers of 2 potato varieties is same.

Tuber No.	Variety A		Variety B	
	x_A	$(x-\bar{x})^2 = d^2$	x_B	$(x-\bar{x})^2 = d^2$
1	1.8 x_1	0.1681	2.3 x_1	0.1936
2	1.9 x_2	0.0961	2.4 x_2	0.1156
3	2.0 x_3	0.0441	2.5 x_3	0.0576
4	2.1 .	0.0121	2.5 .	0.0576
5	2.2 .	0.0001	2.7 .	0.0016
6	2.3 .	0.0081	2.8 .	0.0036
7	2.3 .	0.0081	2.9 .	0.0256
8	2.4 .	0.0361	3.0 .	0.0676
9	2.5 .	0.0841	3.1 .	0.1296
10	2.6 x_n	0.1521	3.2 x_n	0.2116
	$\Sigma x_A = 22.1$	$\Sigma d_A^2 = 0.6090$	$\Sigma x_B = 27.4$	$\Sigma d_B^2 = 0.8640$
	$\bar{x}_A = 2.21$		$\bar{x}_B = 2.74$	

$$t = \frac{\bar{x}_A - \bar{x}_B}{\sigma d\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

$$\sigma d = \sqrt{\frac{\Sigma d_A^2 + \Sigma d_B^2}{n_1 + n_2 - 2}} = \sqrt{\frac{0.6090 + 0.8640}{18}} = 0.286.$$

$$t = \frac{2.74 - 2.21}{0.286\sqrt{0.2}} = \frac{0.53}{0.286 \times 0.447} = \frac{0.53}{0.1279} = 4.14 \text{ at } 18 \text{ DF.}$$

Comment: The computed t-value 4.14 at 18 DF is greater than table value at 0.1 per cent level at 18 DF. Thus, the null hypothesis is rejected and it can be concluded that the mean yield of tubers of potato variety A and B differ significantly at 0.1% level.

t-test for Paired Samples

Paired 't'-Test:

Paired 't'-test is applied when each individual gives a pair of observations. Here the paired data of independent observation from one sample only to be compared.

For example, 20 mice received a treatment X for 3 months. The question is to test whether the treatment X has an impact on the weight of the mice at the end of the 3 months treatment. The weight of the 20 mice has been measured before and after the treatment. This gives us 20 sets of values before treatment and 20 sets of values after treatment from measuring twice the weight of the same mice.

This kind of observations is made available in biological sciences, such as:

(a) To study the effect of fertilizer, pesticide, drug on plants.

(b) To compare the effect of two different fertilizers or drugs.

(c) To compare the result of two techniques or the accuracy of two different instruments.

When we apply the null hypothesis we assume that the mean of the ditference between the pairs is zero, but in the observed experimental data we find a difference. Whether the difference is real or it occur by chance has to be tested statisticaly following the use of paired ttest.

$$t = \frac{\bar{D}}{SD/\sqrt{n}} = \frac{\bar{D}}{\text{SE of difference}} = \frac{\bar{D}}{\dfrac{\sqrt{\dfrac{\Sigma D^2 - \dfrac{(\Sigma D)^2}{n}}{n-1}}}{\sqrt{n}}}$$

D = difference between individual pair;

N = number of pairs

SD = standard deviation.

In paired t-test degree of freedom is n- 1.

Example: O_2 consumption (millilitre/hr) of 10 fishes (*Tilapia mossambica*) has been assessed in controt (before exposure) condition and after gamma irradiations (5KR doses applied). From the given data do you consider that mean O_2 consumption varied before and after irradiation?

Fish No.	1	2	3	4	5	6	7	8	9	10
Before irradiation	3.0	2.8	2.9	2.8	3.2	3.1	3.0	3.2	2.7	2.9
After irradiation	2.6	2.5	2.6	2.4	2.8	2.5	2.5	2.7	2.5	2.6

Solution: Paired t-test has to be performed as the same ishes where evaluated before and after treatments.

Null hypothesis -Mean O_2 consumption of fishes were same betore and after irradiations.

Fish No.	1	2	3	4	5	6	7	8	9	10
Before irradiation	3.0	2.8	2.9	2.8	3.2	3.1	3.0	3.2	2.7	2.9
After irradiation	2.6	2.5	2.6	2.4	2.8	2.5	2.5	2.7	2.5	2.6
D	+ 0.4	0.3	0.3	0.4	0.4	0.6	0.5	0.5	0.2	0.3
D^2	0.16	0.09	0.09	0.16	0.16	0.36	0.25	0.25	0.04	0.09

$\Sigma D = 3.9$

$\Sigma D^2 = 1.65$

$n = 10$

$\bar{D} = 0.39$

$$t = \frac{\bar{D}}{\dfrac{\sqrt{\dfrac{\Sigma D^2 - \dfrac{(\Sigma D)^2}{n}}{n-1}}}{\sqrt{n}}} = \frac{0.39}{\dfrac{\sqrt{\dfrac{1.65 - \dfrac{(3.9)^2}{10}}{9}}}{\sqrt{10}}}$$

$t = 10.26$ at 9 DF

Note. Mean should be always written as in t-test we are comparing two attributes in relation to their mean values

Table values at 9 DF:

t = 4.78: 0.1% level of significance,

t = 3.25: 1.0% level of significance

t= 2.31: 5.0% level of significance.

As the calculated t-value 10.26 is higher than the table value 2.31 at 5% 1evel, it can be said that mean O_2 consumption of the fishes varied significantly (in the present case at 0.1% level) before and after gamma irradiations.

Inference: Null hypothesis is rejected. Gamma irradiations have, thererore, affected O_2 consumption in *Tilapia* fishes.

Example: Blood sugar level (mg/ml) has been assessed from 10 patient before and after diet (before: 95, 100, 105, 90, 85, 90, 100, 85, 75 and I10; after: 110, 105, 115, 95, 90, 95, 120, 100, 85 and 140). Does the blood sugar level varied with diet among the patients?

Solution: Null hypothesis-Mean blood sugar level is same before and after diet

Patient	1	2	3	4	5	6	7	8	9	10
Blood sugar before diet	95	100	105	90	85	90	100	85	75	110
Blood sugar after diet	110	105	115	95	90	95	120	100	85	140
D	15	5	10	5	5	5	20	15	10	30
D^2	225	25	100	25	25	25	400	225	100	900

$$\Sigma D = 120$$
$$\Sigma D^2 = 2050$$
$$n = 10.$$
$$\bar{D} = \frac{\Sigma D}{n} = \frac{120}{10} = 12.$$

$$t = \frac{\bar{D}}{\frac{\sqrt{\Sigma D^2 - \frac{(\Sigma D)^2}{n}}}{n-1}}{\sqrt{n}}$$

$$t = \frac{12}{\frac{\sqrt{2050 - \frac{(120)^2}{n}}}{\frac{9}{\sqrt{n}}}} = \frac{12}{\frac{8.232726}{\sqrt{n}}} = \frac{12}{\frac{8.233}{3.162}} = \frac{12}{2.6037} = 4.61 \text{ at } 9 \text{ DF.}$$

$$t = 4.61 \text{ at } 9 \text{ DF}$$

Inference: The calculated t-value 4.61 at 9 DF is greater than values at 5% (2.31) and 1% (3.25) levels at 9DF has indicated that the deviation is significant at 0.01 probability level. Therefore, the assumed hypothesis is rejected and can be concluded that mean blood sugar level assessed among individuals, varied before and after diet.

t-test analysis is widely used in genetical experiments to evaluate significant difference between samples means.

Example: Ten black cumin (*Nigella sativa* L.) plants were assessed after 30 and 45 days for number of flowers produced by each plant and result obtained has been given below

Plant Nos.	1	2	3	4	5	6	7	8	9	10
30 Days	6	7	4	5	3	2	6	7	1	2
45 Days	10	12	8	12	4	2	10	8	4	6

Test whether mean number of flowers per plant in black cumin was same in two different dates.

Solution: Null hypothesis-Mean number ot flowers are same in two dates of recording.

Plant Nos.	1	2	3	4	5	6	7	8	9	10
30 Days	6	7	4	5	3	2	6	7	1	2
45 Days	10	12	8	12	4	2	10	8	4	6
D (Deviation)	+4	+5	+4	+7	+1	0	+4	+1	+3	+4
D^2	16	25	16	49	1	0	16	1	9	16

$\Sigma D^2 = 149; \quad \bar{D} = \frac{\Sigma D}{n} = \frac{33}{10} = 3.3.$

$$t = \frac{\bar{D}}{\sqrt{\frac{\Sigma D^2 - \frac{(\Sigma D)^2}{n}}{\frac{n-1}{\sqrt{n}}}}} = \frac{3.3}{\sqrt{\frac{149 - \frac{(33)^2}{10}}{\frac{9}{\sqrt{10}}}}} = \frac{3.3}{\frac{\sqrt{149 - 108.9}}{\frac{9}{\sqrt{10}}}} = \frac{3.3}{\frac{2.137}{3.162}}$$

$$= \frac{3.3}{0.676} = \mathbf{4.88.}$$

DF= n-1= 10-1 = 9.

Table value of t at 9 DF: 5% = 2.26, 1% = 3.25 and 0.1% = 4.78.

t-test analysis has been done-mean number of flowers were assessed in 2 dates. Further, paired t-test was computed as same plants were scored at 2 different dates.

Conclusion: The computed t-value is higher than table values at 5%, 1% and 0.1% levels, so the assumed hypothesis is rejected as there exist significant deviation (0.001 probability level) in mean flower number when assessed in two dates.

Model: Mean and standard error analyzed in normal and mutant plant types of black cumin.

Attributes	*Normal*		*Mutant*	
	Mean	*S.E.* (±)	*Mean*	*S.E.* (±)
Plant height (cm)	49.7	1.13	50.5	1.54
No. of primary branches/plant	6.8	0.15	7.0**	0.28
Total capsule numbers/plant	24.4	6.50	38.4**	4.03
Capsule length/fruit	1.2	0.02	1.2	0.03
Seed length (mm)	2.6	0.05	2.4*	0.04
Seed breadth (mm)	1.3	0.04	1.3	0.05
Seed number/plant	1535.76	153.47	2145.0	183.64
Seed weight/plant (gm)	2.7	0.16	2.7	0.25

* and ** = Significant at 5% and 1% levels respectively.

Comments: Mean data for different phenotypic variables have been compared statistically in two-plant types and results indicated that the mutant having similar height to control possessed significantly higher number of primary branches and capsules per plant and the capsules were with small sized seeds.

Fisher's Z-Test or Z-Test:

Z-test is based on the normal probability distribution and is used for testing the significance of several measures. The relevant test statistics is worked out and compared with its probable value (to be read; table showing the area under normal curve) at a given level of significance in order to judge the significance of measures concerned. Z-test is generally used to compare the mean of large sample; hypothetical mean for population.

Correlation:

Association between variables or attributes or characteristics at a given time is known as correlation.

Example:

(i) The amount of rainfall and yield of a certain crop;

(ii) Age of husband and wife;

(iii) Height and weight of students and

(iv) Different concentrations of mutagen and their effect on seed germination frequency.

In plant breeding the breeders targets improvement of yield. Relationship between yield and yield related traits (plant height, number of primary branches/ plant; total branches/plant, number of capsules/plant, capsule length, seeds/capsule, 100-seed weight, etc.) and between the yield related components can be worked out through correlation studies.

Significant correlation obtained will be helpful for selection and ascertaining the model plant type for the concerned species.

Precisely correlation may be defined as movement of one variable tends to be accompanied by corresponding movements in the other. Such simultaneous movement of two variables can be graphically plotted using value of one variable on x-axis and the other variable along y-axis.

Such representation of variables in-dicates the nature of association between the attributes and is called as scattered diagram or correlation chart.

Correlation may:

(a) Positive Correlation:

Increase in plant height is related to increase in number of branches per plant. On the scattered diagram the dots (each pair of obser-vation) representing the variables are in a linear path diagonally across the graph paper from bottom left-hand corner to the top right.

Positive correlation

Negative correlation

(b) Negative Correlation:

For example, increase in plant height of a species is re-lated to decrease in branch number per plant. The pattern of dots be such as to indicate a straight line path from the upper left-hand corner to the bottom right.

Zero correlation

Perfect correlation

(c) Zero Correlation:

The dots are scattered and do not indicate any straight line.

(d) Perfect Correlation:

When the dots lie exactly on a straight line.

In the present example height of plants represented independent variable and on the other hand the variable which changes with the change in the independent variable is called dependent variable (branches/plant).

It is customary to use the horizontal axis (x-axis) for the independent variable and the vertical axis (y-axis) for dependent variable.

The degree of relationship between 2 attributes can be determined by calcu-lating a coefficient called as correlation coefficient. The correlation coefficient is expressed by the letter 'r'. r varies from 0 to 1 and can be + (positive correlation) or — (negative correlation). Practically, r is never zero or 1 (complete/absolute).

Whenever correlation coefficient analysis is made, r-value ranges from 0 to 1 but it is necessary to compare the calculated r-value with table value at specific de-gree of freedom. If the value is significant, i.e., if the calculated r-value is greater than table value, then only we can say that the two attributes are statistically associated to one another. Degree of significance level has also to be assessed (5%, 1% and 0.1% levels).

$$r = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2\Sigma(y-\bar{y})^2}} = \frac{\Sigma d_x d_y}{\sqrt{\Sigma d_x^2 \Sigma d_y^2}},$$

Where, x and y are the variables.

where x and y are the variables.

$$\Sigma d_x d_y = \Sigma xy - \frac{\Sigma x \Sigma y}{n};$$

$$\Sigma d_x^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n};$$

$$\Sigma d_y^2 = \Sigma y^2 - \frac{(\Sigma y)}{n}.$$

In correlation degree of freedom is n — 1, where n represents pairs of obser-vations.

Degree of Correlation:

The degree of correlation is expressed by the value of coefficient of correlation which ranges between + 1 and -1.

The value of coefficient of correlation directly indicates the degree of correlation as detailed in the following table:

Value of Correlation Coefficient	*Degree of Correlation*
+ 1 value	Perfect positive correlation
- 1 value	Perfect negative correlation
Value between 1 and 0.75	High degree of correlation
Value between 0.75 and 0.5	Moderate degree of correlation
Value between 0.5 and 0	Low degree of correlation
0 (zero)	No correlation

Methods of Determining Correlation:

The following methods are generally used to determine simple correlation:

a. Graphic method.

b. Scatter diagram or Dotogram method.

c. Karl Pearson's method

d. Spearman's ranking method.

a. Graphic Method:

When the values of dependent series are plotted on O –X axis and independent series are plotted on O-Y axis of graph paper, a linear or non-linear graph will be obtained which will simply indicate the direction of correlation and not the numerical magnitude.

If the graph lines of two independent series move in upward direction from left to right, the correlation is positive, but if the graph line of one series moves upward from left to right and that of the other independent series moves downward from left to right, they show negative correlations.

If the values of two data series do not show either positive or negative trend then it should be inferred that there is no correlation.

b. Scatter Diagram or Dotogram Method:

This method is more or less similar to graphic method. In this method, the values of independent data series are plotted on O – X axis and those of dependent series on 0-Y axis and then the pairs of values are plotted on the graph paper.

In this ways, graphs of dots are obtained. These dots are scattered in different forms. Therefore, the graphs are called scatter diagrams or dotograms. The patterns of scatter diagrams indicate the Erection and magnitude of correlation.

The scatter diagram may indicate the following conditions:

(i) If the dots of the two series are advancing in a definite direction like a current, this condition indicates that the data series are definitely correlated.

Positive and Negative Correlation

(ii) When the arrays of dots advance from left to right in upward direction, the correlation is definitely positive

(iii) When the scatter diagram advances from left to right in downward direction, the correlation' is negative

(iv) When the dots are not in definite arrays and are scattered haphazardly, this condition indicates that there is no correlation between the data series [Fig. 34.2 (b)].

Perfect Negative Correlation, No Correlation and Perfect Positive Correlation

(v) When the dots appear to be situated on a line which advances upward at 45° angle from the O-X axis, this condition indicates perfect positive correlation among the data series.

(vi) If the dots appear to be situated on a line which moves from left to right in downward direction at 45° angle from 0-X axis, this condition is indicative of perfect negative correlation.

c. Karl Pearson's Coefficient of Correlation Method:

This is the best mathematical method of determining the correlation. Coefficient of correlation (r) is obtained by dividing the product of values of covariance of the two series by the product of their standard deviations

$$r = \frac{Cov.(X.Y)}{\sigma_X . \sigma_Y}$$

Where σX and σY are the standard deviation of variables of data series, X and Y. Covariance of tow series is obtained by dividing the sum of the products of deviations of two series and the arithmetic means by the number of observations

$$\text{Cov.}(X.Y) = \frac{\Sigma(X-\bar{X})(Y-\bar{Y})}{N}$$

$$\text{So,} \qquad r = \frac{\Sigma(X-\bar{X})(Y-\bar{Y})}{N.\sigma_X.\sigma_Y}$$

Where $\bar{X}$ = The arithmetic mean of series X

$\bar{Y}$ = Arithmetic mean of series Y

σ_X = Standard deviation of series X

σ_Y = Standard deviation of series Y

N = Number of observations

If $X-\bar{X}=x$ and $Y-\bar{Y}=y$

$$r = \frac{\Sigma xy}{n\sigma x\,\sigma y}$$

$$\text{Since } \sigma x = \frac{\sqrt{\Sigma x^2}}{N} \text{ and } \sigma y = \frac{\sqrt{\Sigma y^2}}{N}$$

$$\text{Therefore } r = \frac{\Sigma xy}{N\left(\frac{\sqrt{\Sigma x^2}}{N}.\frac{\sqrt{\Sigma y^2}}{N}\right)}$$

$$\text{or } r = \frac{\Sigma xy}{\sqrt{\Sigma x^2}\times\sqrt{\Sigma y^2}}$$

If the numbers of observations are small, Pearson's coeffcient of correlation is calculated by the following formula:

$$r = \frac{N\Sigma xy - \Sigma x.\Sigma y}{\sqrt{N\Sigma x^2-(\Sigma x)^2}\sqrt{N\Sigma y^2-(\Sigma y)^2}}$$

If the data in two series are classified, Pearson's coefficient of correlation is calculated by the following formula:

$$r = \frac{\Sigma dxdy - N(X - A_1)(Y - A_2)}{N\sigma x \sigma y}$$

Where, A_1 = assumed mean of series X

A_2 = assumed mean of series Y

dx = deviation from assumed mean of series – X or $(X - A)$

dy = deviation from assumed mean of series Y or $(Y - A_2)$

σx = standard deviation of series X

σy = standard deviation of series Y

The above formula can also be expressed as follows:

$$r = \frac{N\Sigma dxdy - \Sigma dx \Sigma dy}{\sqrt{N\Sigma dx^2 - (\Sigma dx)^2}\sqrt{N\Sigma dy^2 - (\Sigma dy)^2}}.$$

Example. Calculate Pearson's coefficient of correlation from the following data:

Series X—18, 20, 22, 27, 27, 26, 27, 29, 28, 26.

Series Y—23, 27, 28, 28, 29, 30, 31, 33, 35, 36.

Solution.

S. No.	Series X			Series Y			
	Observation (X)	Deviation $dx = X - \bar{X}$	dx^2	Observation (Y)	Deviation $dy = Y - \bar{Y}$	dy^2	$dx\,dy$
1	18	18 – 25 = – 7	49	23	23 – 30 = – 7	49	49
2	20	20 – 25 = – 5	25	27	27 – 30 = – 3	9	15
3	22	22 – 25 = – 3	9	28	28 – 30 = – 2	4	6
4	27	27 – 25 = 2	4	28	28 – 30 = – 2	4	– 4
5	27	27 – 25 = 2	4	29	29 – 30 = – 1	1	– 2
6	26	26 – 25 = 1	1	31	30 – 30 = 0	0	0
7	27	27 – 25 = 2	4	30	31 – 30 = 1	1	2
8	29	29 – 25 = 4	16	33	33 – 30 = 3	9	12
9	28	28 – 25 = 3	9	35	35 – 30 = 5	25	15
10	26	26 – 25 = 1	1	36	36 – 30 = 6	36	6
10	$\Sigma X = 250$		$\Sigma dx^2 = 122$	$\Sigma Y = 300$		Σdy^2	$\Sigma dx\,dy$
Mean	$\bar{X} = \frac{250}{10} = 25$			Mean $\bar{Y} = \frac{300}{10} = 30$		=138	=105–6 =99

$\bar{X} = 25$

$\bar{Y} = 30$

$$\sigma_X = \frac{\sqrt{\Sigma dx^2}}{n} = \frac{\sqrt{122}}{10} = 3.49$$

$$\sigma_Y = \frac{\sqrt{\Sigma dy^2}}{n} = \frac{\sqrt{138}}{10} = 3.71.$$

d. Spearman's Ranking Method:

Professor Charls Spearman worked out a method for determining correlation in which the values of all data of a series are assigned ranks in decreasing or increasing (ascending) order. In this ranking process, the highest value is given rank 1 and the next higher value is given rank 2 and so on. In some series the values of two or more data are similar.

In that case, the mean of the ranks will be equally shared by those data, as for example in one series there are two observations; one at S. No. 3 and the other at S. No. 10 of 67 each. In ranking process 67 at S. No. 3 and 67 at S. No. 10 instead of being ranked 6 and 7 respectively are ranked at 6.5 (mean of rank 6 and rank 7).

In the same way if there are three or more data in a series as have got same value, all those data will share the rank which will be the mean of their ranks. The number or frequency of the data with similar value is indicated by m.

In the next step, the difference between the ranks (D) of respective data of the two series arc obtained (D = R1-R2) which may be positive or negative figures. Then after, the values of D2 and sum of D2 (= ∑D2) are determined.

For two such series as are taking in data with similar values, the following formula is used to determine the coefficient of correlation by ranking method (Symbolized by Rho = p):

$$\rho = 1 - \frac{6\Sigma D^2}{N(N^2-1)}$$

e. Correlation Coefficient by Concurrent Deviation:

This method is used to indicate whether the correlation is in positive or negative direction especially in the data series characterized by short-term fluctuations of data.

Example 1:

Ten plants have been assessed in sesame (Til) for plant height (cm) and number of branches per plant. From the given data do you consider that there exists correlation (significant) between the variables?

No. of plants	*Plant height (cm) x variable*	*Branches per plant y variable*	x^2	y^2	xy
1	10	12	100	144	120
2	15	16	225	256	240
3	20	20	400	400	400
4	22	25	484	625	550
5	30	35	900	1225	1050
6	35	40	1225	1600	1400
7	40	45	1600	2025	1800
8	45	50	2025	2500	2250
9	50	52	2500	2704	2600
10	55	60	3025	3600	3300
$n=10$	$\Sigma x = 322$	$\Sigma y = 355$	$\Sigma x^2 = 12484$	$\Sigma y^2 = 15079$	$\Sigma xy = 13710$

$$r = \frac{\Sigma d_x \Sigma d_y}{\sqrt{\Sigma d_x^2 \Sigma d_y^2}}$$

$$\Sigma d_x d_y = \Sigma xy - \frac{\Sigma x \Sigma y}{n} = 13710 - \frac{322 \times 355}{10} = 2279.$$

$$\Sigma d_x^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n} = 12484 - \frac{(322)^2}{n} = 2115.6.$$

Inference:

The calculated value 0.996 for 9 DF is higher than the tabulated value at 5%, 1% and 0.1% levels and hence it can be suggested that the two vari-ables are positively and significantly correlated between them at 0.001 probabil-ity level.

The r-value can be represented as 0.996*** to show the level of significance.

Thus, selection of plants with higher height will facilitate selection of plants with enhanced number of branches.

1. Data from 10 basil (*Ocimum basilicum:* $2n = 72$) plants have been scored and given below:

No. Plants	*Univalent frequency/ cell at metaphase* I	*Balanced anaphase* I *cells (%)*	*Pollen fertility (%)*
1	0.56	68.6	52.8
2	0.23	82.0	66.7
3	0.12	93.0	80.2
4	0.92	52.4	47.6
5	1.14	27.8	26.3
6	0.02	98.2	90.4
7	0.16	95.5	92.6
8	0.72	60.2	49.3
9	1.00	30.0	34.0
10	2.00	12.0	10.0

From the given data can you draw (*using suitable statistical test*) a relation between cytological attributes and pollen fertility.

The cytological attributes are:

- Univalent frequency/cell at metaphase I and possible outcome of pairing defects.
- Balanced 36/36 segregation of chromosomes at anaphase I.

To find out whether the cytological attributes are related (*significantly*) or not—simple correlation analysis is done considering univalent frequency as independent variable (x) and anaphase I separation as dependent (y) variable.

$$\Sigma x = 6.87 \quad \Sigma x^2 = 8.07 \quad (\Sigma x)^2 = 47.20$$

$$\Sigma y = 619.70 \quad \Sigma y^2 = 47029.09 \quad (\Sigma y)^2 = 384028.09$$

$$\Sigma xy = 262.92$$

$$r = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\sqrt{\Sigma dx^2 \Sigma dy^2}}$$

$$\Sigma dx^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}$$

$$\Sigma dy^2 = \Sigma y^2 - \frac{(\Sigma y)^2}{n}$$

$$r = \frac{262.97 - \frac{6.87 \times 619.70}{10}}{\sqrt{3.35 \times 8626.28}}.$$

$$r = -\frac{162.81}{170.04} = -0.96$$

at 9 DF (*table value of* $r = 0.847$) at 0.001 probability level.

Therefore, the attributes are highly ($p > 0.001$) and negatively correlated between themselves, i.e., enhanced frequency of univalent per cell has reduced balanced anaphase I segregation of chromosomes.

Now, let us consider the attributes anaphase I segregation of chromosomes (x-*variable*) and pollen fertility (y-variable) and perform simple correlation between them to ascertain relationship.

$$\Sigma x = 619.70 \quad \Sigma x^2 = 47029.09 \quad (\Sigma x)^2 = 384028.09$$

$$\Sigma y = 549.90 \quad \Sigma y^2 = 37059.63 \quad (\Sigma y)^2 = 302390.01$$

$$\Sigma xy = 41603.90$$

$$r = \frac{7526.60}{7670.51} = 0.98$$

(significant at 0.001 probability level at 9 DF).

Thus, pollen fertility is the outcome of pairing defects (*resulting in univalent formation*) occurring in earlier stages of meiosis.

How to prepare Correlation Table from Experimental Data:

Following data has been given:

a. Plant height and number of primary branches/plant = 0.65
b. Plant height and total branches per plant = 0.57.
c. Height and number of capsules per plant = 0.81**.
d. Height and yield = 0.62.
e. Primary branches and total branches = 0.35.
f. Primary branches and capsules per plant = 0.80**.
g. Primary branches and yield = 0.87***.
h. Total branches and number of capsules = 0.52.
i. Total branches and yield = 0.43.
j. Capsules per plant and yield = 0.82**.

Attributes	*Plant height*	*Primary branches/ plant*	*Total branches per plant*	*Capsules per plant*	*Yield*
Plant height		0.65	0.57	0.81**	0.62
Primary branches per plant			0.35	0.80**	0.87***
Total branches per plant				0.52	0.43
Capsules/plant					0.82**

Inference:

Interrelationship between four yield related traits and their association with yield have been documented in tabular form. Result indicated positive and significant correlation between height and capsules/plant (1% level), primary branches/plant and capsules/plant (1% level), primary branches and yield (0.1% level) and capsules/plant and yield (1% level).

Thus, plants having higher number of primary branches with enhanced cap-sule number should be the selection indices for higher yield in the plant species.

11. Data representing phenotypic correlation analysis in sesame (*Sesamum indicum*). Comment on the given data:

Plant height (cm)	*No. of primary branches/ plant*	*Distance from base to first branch-ing (cm)*	*No. of capsules on the main axis*	*No. of capsules per plant*	*Capsule length (cm)*	*No. of seeds per capsule*	*Seed yield (gm)*
Plant height (cm)	–0.18	0.11	0.75**	0.72*	0.74**	0.68*	0.56
No. of primary branches/ plant		–0.23	–0.35	0.23	–0.33	0.44	0.22
Distance from base to first branching (cm)			0.23	–0.11	0.03	–0.27	0.05
No. of capsules on the main axis				0.48	0.79**	0.74**	0.29
No. of capsules per plant					0.49	0.52	0.72*
Capsule length (cm)						0.70*	0.25
No. of seeds/ capsule							0.62*

*, ** and *** Significant at 0.05, 0.01 and 0.001 probability level respectively.

Comment: Phenotypic correlation studies have been made between seven yield related traits and their association with yield with the objective to find a model plant type in sesame.

Result indicated that the most important selection criterion for sesame would be plants with enhanced number of capsules and those capsules should be with higher number of seeds. A model sesame plant must also be tall with higher number of capsules on the main axis.

Table: The genotypic and phenotypic correlation coefficients of different characters in sesame

Characters		Plant height (cm)	No. pr. branches/ plant	Total branches/ plant	Distance from base to first branching	Capsules on main axis	Capsules/ plant	Capsule length	Seeds/ capsule	Seed yield (gm)
Plant height (cm)	G		0.74***	0.68***	0.52***	0.79***	0.72***	0.67***	0.62***	0.57***
	P		0.54***	0.58***	0.43***	0.62***	0.68***	0.63***	0.59***	0.48***
No. pr. branches/	G			0.96***	0.39**	0.80***	0.91***	0.59***	0.62***	0.78***
plant	P			0.93***	0.35**	0.25*	0.74***	0.52***	0.54***	0.65***
Total branches/	G				0.33**	0.15	0.76***	0.41***	0.48***	0.67***
plant	P				0.30*	0.22	0.68***	0.41***	0.47***	0.55***
Distance from base to	G					0.09	0.12	0.12	0.26*	0.03
first branching	P					0.08	0.09	0.10	0.25*	0.01
Capsules on main axis	G						0.91***	0.92***	0.88***	0.89***
	P						0.68***	0.53***	0.58***	0.62***
Capsules/plant	G							0.71***	0.67***	0.86***
	P							0.62***	0.60***	0.69***
Capsule length	G								0.78***	0.90***
	P								0.68***	0.69***
Seeds/capsule	G									0.81***
	P									0.68***

Simple correlation described so far is of 3 types:

*, ** and *** significant at 5%, 1% and 0.1% level respectively.

Correlation studies showed that for all characters, genotypic and phenotypic associations were in the same direction and genotypic estimates were higher than the phenotypic ones indicating an inherited association between the traits. The yield showed a strong positive association (0.001 probability level) with all yield related traits excepting distance from base to first branching. Similarly, correlation among the yield components was mostly positive and significant (excepting distance from base to first branching with capsule or main axis, capsule/plant, and capsule length and between total branches/plant and capsule on the main axis).

Observable correlation between 2 variables and it includes both genotypic and environmental effects.

2. ***Genotypic:*** $r_g = \dfrac{G\,cov\,x \cdot y}{(GV_x \cdot GV_y)^{1/2}}$.

Such type of correlation takes into account the inherent association between two variables and it may be the outcome of pleiotropic action of genes or linkage or both.

3. ***Environmental:*** $r_e = \dfrac{E\,cov\,x \cdot y}{(EV_x \cdot EV_y)^{1/2}}$ —due to environmental effects.

P cov x. y, G cov x.y and E cov x.y are phenotypic, genotypic and environ-mental, respectively, covariance's between variables x and y; Vx and Vy are variances for x and y variables, respectively.

Partial Correlation:

X_1 and X_2 estimated by taking into account the effect of a 3rd variable X_3 and is denoted as $r_{12.3}$.

Partial correlation provides better relationship between the two variables X_1 and X_2 and is given by the formula:

$$r_{12.3} = \frac{(r_{12} - r_{13}r_{23})}{\sqrt{(1 - r_{13}^2)(1 - r_{23}^2)}}$$

r_{12}, r_{13} and r_{23} are the estimates of simple correlation coefficients between the variables X_1 and X_2, X_1 and X_3 and X_2 and X_3, respectively.

From the following data on yield in gms, quantity of manures (*in kg*) applied and rainfall in inches calculate the total and partial correlation coefficients and test their significance.

Sl. No.	*Yield in gms* (x)	*Quantity of manure applied* (y)	*Rainfall in inches* (z)
1	15	7	6
2	5	3	2
3	8	4	3
4	10	6	4
5	12	7	5
6	3	2	1
7	2	1	0
8	0	0	1
Total	55	30	22

x^2	y^2	z^2	xy	yz	xz
225	49	36	105	42	90
25	9	4	15	6	10
64	16	9	32	12	24
100	36	16	60	24	40
144	49	25	84	35	60
9	4	1	6	2	3
4	1	0	2	0	0
0	0	1	0	0	0
Total: 571	164	92	304	121	227

$$\Sigma(x-\bar{x})^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{N} = 571 - \frac{(55)^2}{8} = 571 - \frac{3025}{8}$$

$$= 571 - 378.13 = \mathbf{192.87.}$$

$$\Sigma(y-\bar{y})^2 = \Sigma y^2 - \frac{(\Sigma y)^2}{N} = 164 - \frac{(30)^2}{8} = 164 - \frac{900}{8}$$

$$= 164 - 112.5 = \mathbf{51.5.}$$

$$\Sigma(z-\bar{z})^2 = \Sigma z^2 - \frac{(\Sigma z)^2}{N} = 92 - \frac{(22)^2}{8} = 92 - \frac{484}{8}$$

$$= 92 - 60.50 = \mathbf{31.50.}$$

$$\Sigma(x-\bar{x})(y-\bar{y}) = \Sigma xy - \frac{\Sigma x \Sigma y}{N} = 304 - \frac{55 \times 30}{8}$$

$$= 304 - 206.25 = \mathbf{97.75.}$$

$$\Sigma(y-\bar{y})(z-\bar{z}) = \Sigma yz - \frac{\Sigma y \Sigma z}{N} = 121 - \frac{30 \times 22}{8}$$

$$= 121 - 82.5 = \mathbf{38.50.}$$

$$\Sigma(x-\bar{x})(z-\bar{z}) = \Sigma xz - \frac{\Sigma x \Sigma z}{N} = 227 - \frac{55 \times 22}{8}$$

$$= 227 - 151.25 = \mathbf{75.75.}$$

Total Correlation Coefficients

$$r_{xy} = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2 \times \Sigma(y-\bar{y})^2}} = \frac{97.75}{\sqrt{192.87 \times 51.50}}$$

$$= \frac{97.75}{99.66} = \mathbf{0.98.}$$

$$r_{xz} = \frac{\Sigma(x-\bar{x})(z-\bar{z})}{\sqrt{\Sigma(x-\bar{x})^2 \times \Sigma(z-\bar{z})^2}} = \frac{75.75}{\sqrt{192.87 \times 31.50}}$$

$$= \frac{75.75}{77.95} = \mathbf{0.97.}$$

$$r_{yz} = \frac{\Sigma(y-\bar{y})(z-\bar{z})}{\sqrt{\Sigma(y-\bar{y})^2 \times \Sigma(z-\bar{z})^2}} = \frac{38.50}{\sqrt{51.50 \times 31.50}}$$

$$= \frac{38.50}{40.03} = \mathbf{0.96.}$$

Test of Significance

$$t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}.$$

$$t \text{ for } r_{xy} = \frac{0.98\sqrt{8-2}}{\sqrt{1-(0.98)^2}} = \frac{0.98 \times 2.5}{\sqrt{1-0.96}} = \frac{2.45}{\sqrt{0.04}} = \frac{2.45}{0.2} = \mathbf{12.25.}$$

In the similar way the significance of correlation coefficients r_{xz} and r_{yz} can be tested.

Conclusion: Since the calculated value of t for r_{xy} is greater than the table t at DF 6, i.e., $8-2$ or $n-2$, the value of r_{xy} is significant which indicates that the yield and quality of manure are highly correlated.

Partial Correlation Coefficients

$$r_{xy\cdot z} = \frac{r_{xy} - r_{xz}\cdot r_{yz}}{\sqrt{(1-r^2xz)(1-r^2yz)}} = \frac{0.98 - 0.97 \times 0.96}{\sqrt{\{1-(0.97)^2\}\{1-(0.96)^2\}}}$$

$$= \frac{0.98 - 0.93}{\sqrt{(1-0.94)(1-0.92)}} = \frac{0.05}{\sqrt{0.06 \times 0.08}}$$

$$= \frac{0.05}{0.069} = \mathbf{0.72.}$$

$$r_{yz\cdot x} = \frac{r_{yz} - r_{xy}\cdot r_{xz}}{\sqrt{(1-r^2xy)(1-r^2xz)}} = \frac{0.96 - 0.98 \times 0.97}{\sqrt{\{1-(0.98)^2\}\{1-(0.97)^2\}}}$$

$$= \frac{0.96 - 0.95}{\sqrt{(1-0.96)(1-0.94)}} = \frac{0.01}{\sqrt{0.04 \times 0.06}}$$

$$= \frac{0.01}{\sqrt{0.0024}} = \mathbf{0.21.}$$

$$r_{xz\cdot y} = \frac{r_{xz} - r_{xy}\cdot r_{yz}}{\sqrt{(1-r^2xy)(1-r^2yz)}} = \frac{0.97 - 0.98 \times 0.96}{\sqrt{\{1-(0.98)^2\}\{1-(0.96)^2\}}}$$

$$= \frac{0.97 - 0.94}{\sqrt{(1-0.96)(1-0.92)}} = \frac{0.03}{\sqrt{0.04 \times 0.08}} = \frac{0.03}{\sqrt{0.0032}}$$

$$= \frac{0.03}{0.057} = \mathbf{0.53.}$$

Test of Significance

$$t = \frac{r\sqrt{n-2-1}}{\sqrt{1-r^2}}.$$

$$t \text{ for } r_{xy\cdot z} = \frac{0.72\sqrt{8-2-1}}{\sqrt{1-(0.72)^2}} = 2.35.$$

Conclusion: The value of the table t at DF $5(8-2-1)$ at 5% level is 2.571 which is greater than the calculated value of t. Therefore, $r_{xy\cdot z}$ is not significant which means that the partial correlation between yield and quantity of manure applied eliminating the effect of rainfall is not significant. In the similar way the partial correlation coefficients $r_{xy\cdot z}$ and $r_{yz\cdot x}$ may be tested for significance.

Multiple Correlation:

Estimate of joint influence of two or more variables on a dependent variable is called multiple correlation. Such an estimate helps in understanding the dependence of one variable, say X_1 on a set of independent variable say X_2, X_3

$$R^2_{1.23} = \frac{[(r^2_{12} + r^2_{13}) - 2(r_{12}\cdot r_{13}\cdot r_{23})]}{(1-r^2_{23})}.$$

The square root of $R_{21.23}$ is the estimate of multiple correlation coefficient. $R_{21.23}$ is coefficient of determination.

Linear Regression Analysis:

The statistical analysis employed to find out the exact position of the straight line is known as Linear regression analysis. From simple correlation analysis if there exist relationship between independent variable x and dependent variable y then the relationship can be expressed in a mathematical form known as Regression equation.

From regression equation we can work out the actual value of y variable (dependent) based on X variable (independent) and such values plot-ted graphically will give precise nature of the straight line (point of interception to y-axis can be noted).

Simple regression equation Yx = a + bx, where a and b are constant which minimize the residual error of Y. Y is the dependent variable.

The constants a and b can be obtained from the formula:

$$b = \frac{\Sigma dxdy}{\Sigma dx^2}, \quad \text{where } \Sigma dxdy = \Sigma xy - \frac{\Sigma x \Sigma y}{n};$$

$$\Sigma dx^2 = \Sigma x^2 - \frac{(\Sigma x)^2}{n}.$$

$$a = \bar{y} - b\bar{x}.$$

Regression coefficient is a statistical measure of the average functional relationship between two or more variables. In regression analysis, one variable is considered as dependent and other(s) as independent. Thus, it measures the degree of dependence of one variable on the other(s). Regression coefficient was first used for estimating the relationship between the heights of fathers and their sons.

Applications of Regression Coefficient in Genetics:

Regression analysis has wide applications in the field of genetics and breeding as given below:

1. It helps in finding out a cause and effect relationship between two or more plant characters.
2. It is useful in determining the important yield contributing characters.
3. It helps in the selection of elite genotypes by indirect selection for yield through independent characters.
4. It also helps in predicting the performance of selected plants in the next generation.

Example 2:

From the data find out the regression equation and draw a regression line on the graph paper.

Solution

.

No. of Branches (x)	*No. of Capsules* (y)	x^2	xy
2	4	4	8
5	10	25	50
8	15	64	120
10	20	100	200
15	25	225	375
20	30	400	600
25	40	625	1000
$\Sigma x = 85$	$\Sigma y = 144$	$\Sigma x^2 = 1443$	$\Sigma xy = 2353$

Solution:

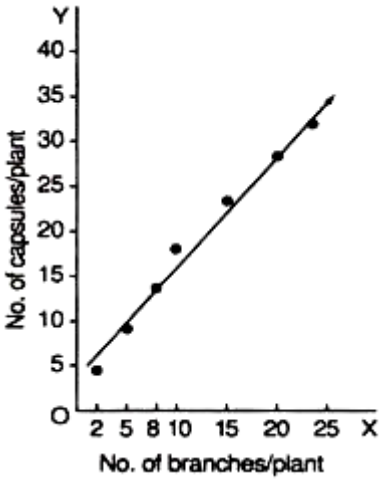
$$\bar{x} = \frac{85}{7} = 12.14; \quad \bar{y} = \frac{144}{7} = 20.57.$$

$$b = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}} = \frac{2353 - \frac{85 \times 144}{7}}{1443 - \frac{(85)^2}{7}} = 1.48.$$

$$a = \bar{y} - b\bar{x} = 20.57 - (1.48)(12.14) = 2.6.$$

Therefore, the regression equation $y_x = a + bx$, $y_x = 2.6 + 1.48x$.

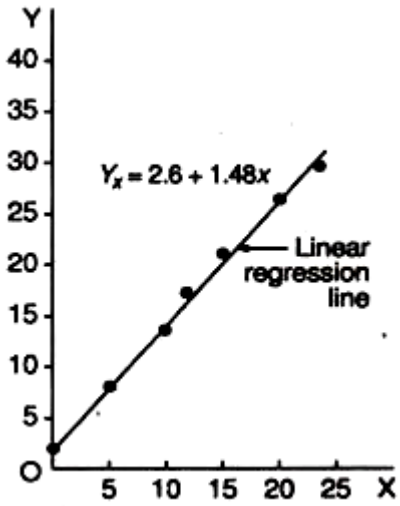
Using the regression equation yx = 2.6+1.48x the actual values of dependent variable can be worked out



If $x = 0$, $y = 2.60$
$x = 2$, $y = 5.56$
$x = 5$, $y = 10.00$
$x = 8$, $y = 14.44$
$x = 10$, $y = 17.40$
$x = 15$, $y = 24.80$
$x = 20$, $y = 32.20$
$x = 25$, $y = 39.60$

Using data of the given example the straight line is drawn but the point of interception to y-axis is lacking and, therefore, precise nature of the straight line is not understood. However, from the straight line it is evident that the variables were significantly and positively correlated between themselves.

These set of values plotted graphically will give a straight and the precise nature of the straight line can be obtained from x = 0, y = 2.6 (point of interception to y- axis can be found out).



Multiple Regression:

It involves more than two variables. In this, the number of independent variables may be two or more than two.

A linear regression problem is one in which the same change in dependent variable Y can be expected for a change in the independent variable X irrespective of the value of X. In this case, a mathematical function representing a straight line graph can be used to describe the relationship between the two variables.

In other words, the two variables show linear relationship when the change in independent variable X causes a change in dependent variable Y.

Example 3:

The following data giving mean yield (grain), mean ear number per plant and mean grain number per acre of 10 wheat varieties were obtained in low soil condition moisture plots in the experiment conducted at IARI during 2000-01 to study the influence of soil drought on the relation between yield and ear character.

Sl. No.	*Mean grain yield in gm (y)*	*Mean ear no. per plant (x_1)*	*Mean grain no. per ear (x_2)*
1	42.8	3.14	29.4
2.	40.8	3.04	24.2
3.	34.9	3.92	21.6
4.	34.3	3.36	27.5
5.	30.8	3.47	24.9
6.	29.6	3.40	22.5
7.	24.2	3.12	20.5
8.	18.0	4.28	16.1
9.	16.6	3.02	19.7
10.	12.4	3.16	16.3

Fit a multiple regression equation giving mean grain yield in terms of mean ear no. per plant and mean grain no. per ear.

Solution: Total of $y = 284.4$; $\bar{y} = 28.44$.
Total of $x_1 = 33.91$; $\bar{x}_1 = 3.391$.
Total of $x_2 = 222.7$; $\bar{x}_2 = 22.27$.

$$Sy^2 = \Sigma y^2 - \frac{(\Sigma y)^2}{n} = 9054.74 - \frac{(284.4)^2}{10} = 966.40$$

$$Sx_1^2 = \Sigma x_1^2 - \frac{(\Sigma x_1)^2}{n} = 116.52 - \frac{(33.91)^2}{10} = 1.53$$

$$Sx_2^2 = \Sigma x_2^2 - \frac{(\Sigma x_2)^2}{n} = 5132.31 - \frac{(222.7)^2}{10} = 172.78$$

$$Syx_1 = \Sigma yx_1 - \frac{\Sigma y \Sigma x_1}{n} = 959.86 - \frac{284.4 \times 33.91}{10} = -4.54$$

$$Syx_2 = \Sigma yx_2 - \frac{\Sigma y \Sigma x_2}{n} = 6690.73 - \frac{284.4 \times 222.7}{10} = 357.14$$

$$Sx_1x_2 = \Sigma x_1x_2 - \frac{\Sigma x_1 \Sigma x_2}{n} = 740.73 - \frac{33.91 \times 222.7}{10} = -5.45.$$

Multiple Regression Equation

$$Syx_1 = b_1Sx_1^2 + b_2Sx_1x_2 \quad (1)$$
$$Syx_2 = b_1Sx_1Sx_2 + b_2Sx_2^2 \quad (2)$$

or,
$$b_1Sx_1^2 + b_2Sx_1x_2 = Syx_1$$
$$b_1Sx_1x_2 + b_2Sx_2^2 = Syx_2$$

or,
$$\left.\begin{aligned} b_1 \times 1.53 + b_2 \times (-5.45) &= -4.54 \\ b_1 \times (-5.45) + b_2 \times (172.78) &= 357.14 \end{aligned}\right\} \begin{aligned} &\times 5.45 \\ &\times 1.53 \end{aligned}$$

$$\text{or, } 8.3385b_1 - 29.7025b_2 = -24.7430$$
$$-8.3385b_1 + 264.3534b_2 = 546.4242$$
$$\text{By adding } 234.6509b_2 = 521.6812$$
$$b_2 = \frac{521.6812}{234.6509} = 2.22$$

Again,

$$1.53b_1 - 5.45 \times 2.22 = -4.54$$
$$1.53b_1 = 12.10 - 4.54 = 7.56.$$
$$\therefore b_1 = \frac{7.56}{1.53} = 4.94.$$

$$\text{Now } b_0 = \bar{y} - b_1\bar{x}_1 - b_2\bar{x}_2 = 28.44 - 4.94 \times 3.391 - 2.22 \times 22.27$$
$$= 28.44 - 16.75 - 49.44 = -37.75.$$

Multiple Regression Equation $Y = b_0 + b_1x_1 + b_2x_2$.

$$\therefore Y = -37.75 + 4.94x_1 + 2.22x_2.$$

$$\therefore \text{SS Regression } (SR^2) = b_1S_yx_1 + b_2Syx_2$$
$$= 4.94 \times (-4.54) + 2.22 \times 357.14$$
$$= 22.43 + 792.85 = 770.42.$$

Anova Table

Source of Variation	df	SS	MS	F
Regression	2	$SR^2 = 770.42$	385.21	13.76
Deviation from regression	7	195.98	28.00	
Total	9	$Sy^2 = 966.40$		

$F_{0.05:2,7} = 4.75$
$F_{0.01:2,7} = 9.55$ } Table values.

Since the calculated value of F in respect of regression is greater than the table value both at 5% and 1% level of significance, the regression is highly significant. Thus, mean grain yield is significantly related to ear characters.

Analysis of Variance (ANOVA)

Analysis of Variance (ANOVA) is a hypothesis-testing technique used to test the equality of two or more population (or treatment) means by examining the variances of samples that are taken. ANOVA allows one to determine whether the differences between the samples are simply due to random error (sampling errors) or whether there are systematic treatment effects that cause the mean in one group to differ from the mean in another.

Most of the time ANOVA is used to compare the equality of three or more means, however when the means from two samples are compared using ANOVA it is equivalent to using a t-test to compare the means of independent samples.

ANOVA is based on comparing the variance (or variation) between the data samples to variation within each particular sample. If the between variation is much larger than the within variation, the means of different samples will not be equal. If the between and within variations are approximately the same size, then there will be no significant difference between sample means.

The analysis of variance is based on F-statistics which is a ratio of variances. The ratio of variances due to treatments and variances due to random differences within the treatment is calculated and the estimate, known as the 'F', is then used for comparison.

It is a collection of statistical models used to analyze the differences among group means and their associated procedures (such as "variation" among and between groups), developed by statistician and evolutionary biologist Ronald Fisher.

It is a measure of variation among treatments/genotypes/replications, etc., and is obtained by dividing the sum of squares (SS) by corresponding degrees of freedom (n — 1) to get mean sum of squares (MSS), i.e., variance. Variance parameter widely used in statistical analysis is the comparison among different sample means.

ANOVA statistical significance result is independent of constant bias and scaling errors as well as the units used in expressing observations

Assumptions of ANOVA:

(i) All populations involved follow a normal distribution.

(ii) All populations have the same variance (or standard deviation).

(iii) The samples are randomly selected and independent of one another.

Since ANOVA assumes the populations involved follow a normal distribution, ANOVA falls into a category of hypothesis tests known as parametric tests. If the populations involved did not follow a normal distribution, an ANOVA test could not be used to examine the equality of the sample means.

Instead, one would have to use a non-parametric test (or distribution-free test), which is a more general form of hypothesis testing that does not rely on distributional assumptions.

Grand Mean

Mean is a simple or arithmetic average of a range of values. There are two kinds of means that we use in ANOVA calculations, which are separate sample means $(\mu_1, \mu_2 \ \& \ \mu_3)$ and the grand mean (μ). . The grand mean is the mean of sample means or the mean of all observations combined, irrespective of the sample.

Hypothesis

Considering our above medication example, we can assume that there are 2 possible cases – either the medication will have an effect on the patients or it won't. These statements are called Hypothesis. A hypothesis is an educated guess about something in the world around us. It should be testable either by experiment or observation.

Just like any other kind of hypothesis that you might have studied in statistics, ANOVA also uses a Null hypothesis and an Alternate hypothesis. The Null hypothesis in ANOVA is valid when all the sample means are equal, or they don't have any significant difference. Thus, they can be considered as a part of a larger set of the population. On the other hand, the alternate hypothesis is valid when at least one of the sample means is different from the rest of the sample means. In mathematical form, they can be represented as:

$$H_o: \ \mu_1 = \mu_2 = \cdots = \mu_L \qquad \textit{Null hypothesis}$$

$$H_1: \ \mu_l \neq \mu_m \qquad \textit{Alternate hypothesis}$$

where μ_l and μ_m belong to any two sample means out of all the samples considered for the test. In other words, the null hypothesis states that all the sample means are equal or the factor did not have any significant effect on the results. Whereas, the alternate hypothesis states that at least one of the sample means is different from another. But we still can't tell which one specifically. For that, we will use other methods that we will discuss later in this article.

One Way ANOVA

A one way ANOVA is used to compare two means from two independent (unrelated) groups using the F-distribution. The null hypothesis for the test is that the two means are equal. Therefore, a significant result means that the two means are unequal.

Limitations of the One Way ANOVA

A one way ANOVA will tell you that at least two groups were different from each other. But it won't tell you which groups were different. If your test returns a significant f-statistic, you may need to run an ad hoc test (like the Least Significant Difference test) to tell you exactly which groups had a difference in means.

Two Way ANOVA

A Two Way ANOVA is an extension of the One Way ANOVA. With a One Way, you have one independent variable affecting a dependent variable. With a Two Way ANOVA, there are two independents. Use a two way ANOVA when you have one measurement variable (i.e. a quantitative variable) and two nominal variables. In other words, if your experiment has a quantitative outcome and you have two categorical explanatory variables, a two way ANOVA is appropriate.

For example, you might want to find out if there is an interaction between income and gender for anxiety level at job interviews. The anxiety level is the outcome, or the variable that can be measured.

Gender and Income are the two categorical variables. These categorical variables are also the independent variables, which are called factors in a Two Way ANOVA.
The factors can be split into levels. In the above example, income level could be split into three levels: low, middle and high income. Gender could be split into three levels: male, female, and transgender. Treatment groups are all possible combinations of the factors. In this example there would be 3 x 3 = 9 treatment groups.

What is MANOVA?

MANOVA is just an ANOVA with several dependent variables. It's similar to many other tests and experiments in that it's purpose is to find out if the response variable (i.e. your dependent variable) is changed by manipulating the independent variable. The test helps to answer many research questions, including:
Do changes to the independent variables have statistically significant effects on dependent variables?
What are the interactions among dependent variables?
What are the interactions among independent variables?

MANOVA Example

Suppose you wanted to find out if a difference in textbooks affected students' scores in math and science. Improvements in math and science mean that there are two dependent variables, so a MANOVA is appropriate.
An ANOVA will give you a single (univariate) f-value while a MANOVA will give you a multivariate F value. MANOVA tests the multiple dependent variables by creating new, artificial, dependent variables that maximize group differences. These new dependent variables are linear combinations of the measured dependent variables.

Interpreting the MANOVA results

If the multivariate F value indicates the test is statistically significant, this means that something is significant. In the above example, you would not know if math scores have improved, science scores have improved (or both). Once you have a significant result, you would then have to look at each individual component (the univariate F tests) to see which dependent variable(s) contributed to the statistically significant result.

Advantages and Disadvantages of MANOVA vs. ANOVA

Advantages

MANOVA enables you to test multiple dependent variables.
MANOVA can protect against Type I errors.

Disadvantages

MANOVA is many times more complicated than ANOVA, making it a challenge to see which independent variables are affecting dependent variables.
One degree of freedom is lost with the addition of each new variable.
The dependent variables should be uncorrelated as much as possible. If they are correlated, the loss in degrees of freedom means that there isn't much advantages in including more than one dependent variable on the test.

Analysis of Variance from RBD Design:

Randomization of any experiment is important for obtaining a precise result. Mean comparison between (genotypes/families/replications, etc.) treatments re-quires one or more conditions such as random allocation of the treatments to various plots (field experiments)/divisions.
Thus, for successful comparison of different treatments following aspects should be carefully dealt with:
1. Random allocation of treatments;

2. Replication of treatments—it average out as far as possible the effects of en-vironmental differences (including edaphic factors) so that the various treat-ments are given equal importance to demonstrate their performances.

For example, if we consider that the yield of 4 genotypes has to be compared in a given field condition those 4 genotypes have to be randomly allocated to plots in replications. The genotypes are so assigned that each one get equal scope for their performance. Randomization is non-deliberate allocation of different treatments and it will minimize (equalize) environmental effects.

In field experiments:

(a) Plot size should be equal;

(b) Distance between rows and plants in a plot should be uniform.

Layout of the experiment should be prepared before hand and finally implemented.

Example:

Four rice varieties were grown in 4 replications in randomized block design and their yield/plot was assessed. From yield data do you consider that the mean yield of varoieties differ among themselves?

Solution:

A_5	C_{10}	B_2	D_{10}
B_4	A_6	C_{12}	D_9
D_9	A_5	B_2	C_{11}
C_{12}	B_3	A_7	D_8

Significance:

No biasness over the experiment—Wide range of treatments with no restriction on number of replications.

Demerits:

Sectoral representation of some variety/treatments (that is, the treatments were not distributed uniformly) may occur.

Yield of the varieties—kg/plot.

Replication	*Varieties*				
	A	B	C	D	
1	5	2	10	10	27
2	6	4	12	9	31
3	5	2	11	9	27
4	7	3	12	8	30
	23	11	45	36	115 (Grand Total)

In the present case null hypothesis is assumed as:

(a) Varietal means are same, i.e., the varieties do not differ among themselves in yield.

(b) Varieties do not differ in replication.

First Step:
Add row and column totals to find out grand total = 115(T).
CF (Correction factor) = $T^2/n = (115)^2/16 = 826.56$.
Second Step:
Grand total sum of squares = $5^2 + 6^2 + 5^2 + 7^2 + \ldots + 9^2 + 9^2 + 9^2 + 8^2 = 1003$.
Third Step:
(Total SS = Grand total SS – CF = 1003 – 826.56 = 176.44.
Fourth Step:
SS due to variety (column) = $23^2 + 11^2 + 45^2 + 36^2/4$ – CF =166.19.
It is divided by 4 as each value is sum of four items.
Fifth Step:
SS due to replication (row) = $27^2 + 31^2 + 27^2 + 30^2/4$ – CF = 3.19.
Sixth Step:
SS due to error
= Total SS – (SS of variety + SS of replication)
= 176.44 — (166.19 + 3.19) = 7.06.

Anova Table

Sources	df	SS	MSS	F-value	*Table Value*		
					5%	1%	0.1%
Variety	$3(n_1)$	166.19	55.40	71.03***	3.9	7.0	13.9
Replication	$3(n_1)$	3.19	1.06	1.36			
Error	$9(n_2)$	7.06	0.78				
Total	15						

$$\text{MSS} = \text{mean sum of squares} = \frac{\text{SS}}{\text{df}}$$

$$\text{Calculated F-value} = \frac{\text{MSS of source}}{\text{MSS of error}}.$$

Results indicated that the varieties varied significantly among themselves at 0.1% level of significance; however, the varieties did not varied in replication.
Critical Difference (CD):
The square root of the error mean square measures the standard error per plot due to uncontrolled environmental effects. The varietal means were obtained from four plots (replication) and, therefore, standard error of varietal mean in the present case will be
$\sqrt{0.78/4} = 0.44$.
The standard error of difference of means of two varieties will be:
$0.44 \times \sqrt{2} = 0.44 \times 1.41 = 0.6204$.
From the value of standard error of difference we can calculate the value of the difference which will be just significant at a chosen level of significance. The difference is known as the critical difference for the particular level of significance (generally 5% level of significance is considered adequate).
Hence, CD is t-value x standard error of difference (5%) for 9 DF
= 2.26 (t-value at 5% for 9 DF in the present case) x 0.6204
= 1.4.

That is, if the difference between 2 varieties is 1.4, then it is significant.

	A	B	C	D
Total	23	11	45	36
Mean	5.75	2.75	11.25	9.0
CD at 5%	1.4			

From CD-value it is apparent that yield varied significantly between the varieties.

Estimation of Heritability from ANOVA:

$$\text{Genotypic variance} = \text{MSS due to genotypes (}\textit{variety}\text{)} - \frac{MS_e \text{ (error)}}{\text{No. of Replications}}$$

$$= 55.40 - \frac{0.78}{4} = 13.66.$$

$$\text{Phenotypic variance} = \text{Genotypic variance } (\sigma_g^2) + \sigma_e^2 \text{ (variance due to error} = MS_e)$$

$$= 13.66 + 0.78 = 14.44.$$

$$\text{Heritability (\%) (}\textit{broad sense}\text{)} = \frac{\sigma_g^2}{\sigma_p^2} \times 100 = \frac{13.66}{14.44} \times 100 = 94.6\%.$$

Genetic Advance = Genetic Gain:

The genetic gain is the difference between the mean of the progeny of selected individuals ($\bar{X}_p$) and the base population ($\bar{X}_0$)

R (genetic gain) = $\bar{X}_p - \bar{X}_0$.

Genetic gain can also be predicted using the following formula $R = ih.^2\sigma_p$; where, i is the standardized selection differential (5% level has been used mostly 2.06), h^2 is heritability, i.e., σ^2_g/σ^2_p, op is the phenotypic standard deviation.

Example: The numbersof spikelets/branch in rice under 3 different doses of fertilizers (RBD design)

Doses of Fertilizer

	Low	*Medium*	*High*
Irrigated lowland	10	15	8
Non-irrigated upland	8	12	6
Non-irrigated lowland	7	10	5

Do you consider that fertilizer doses and sites of irrigation have any effect on mean number of spikelet/branch?

In present case, 9 mean values are there and to find out the effect of fertilizers and site of irrigation on mean number of spikelet/branch one has to perform Analysis of Variance (ANOVA *test*-F-*test*). F-test denotes comparison among several mean values.

Grand total sum of squares = $10^2 + 8^2 + 7^2 \cdots 6^2 + 5^2 = 807.$

$$\text{Correction factor (CF)} = \frac{(\text{Grand total})^2}{n} = \frac{(81)^2}{9} = \mathbf{729.}$$

Total SS = Grand total SS − CF = 807 − 729 = **78.**

$$\text{SS due to fertilizer doses } (\textit{column}) = \frac{25^2 + 37^2 + 19^2}{3} - \text{CF} = \mathbf{56.}$$

$$\text{SS due to irrigation sites } (\textit{row}) = \frac{33^2 + 26^2 + 22^2}{3} - \text{CF} = \mathbf{20.67.}$$

SS due to error = Total SS − (SS due to doses and sites)
= 78 − (56 + 20.67) = **1.33.**

Anova Table

Sources	*Replication*	SS	MSS	F-*value calculated*	*Tabulated* F-*value*		
					0.05	0.01	0.001p
Doses	$2(n_1)$	56.0	28.0	84.9***	6.9	18.0	61.3
Sites	$2(n_1)$	20.67	10.34	31.3**	6.9	18.0	61.3
Error	$4(n_2)$	1.33	0.33				
Total	8						

From Anova Table it can be inferred that—(*a*) Mean number of spikelet/branch varied significantly ($p > 0.001$) among the fertilizer doses, (*b*) Spikelet number also showed significant variation at 0.01 probability level with si of irrigation.

$$\text{CD at 5\% level} = \sqrt{\frac{\text{Error MSS}}{\text{Replication}}} = \sqrt{\frac{0.3}{2}} = 0.4062 \times \sqrt{2} = \mathbf{0.5744}$$

t-value at 5% level at 4 DF (*Error* DF) = 2.78.

Therefore, CD = 0.5744 × 2.78 = **1.60.**

Fertilizer doses:

	Low	*Medium*	*High*
Mean:	25/3 = 8.3	37/3 = 12.3	19/3 = 6.3

CD at 5% is 1:6—Therefore, spikelet number/branch varied significantly be tween doses. Medium fertilizer dose is giving the best result.

Similarly, *sites of irrigation*.

	Irrigated lowland	*Non-irrigated upland*	*Non-irrigated lowland*
Mean:	33/3 = 11.0	26.0/3 = 8.7	22/3 = 7.3

CD = 1.6—From this result it may concluded that there exist no significant variation in mean spikelet number per branch when rice was grown in non-irrigated sites irrespective of upland and lowland.

Final conclusion derived from the data—Irrigated lowland and medium dose of fertilizer have been found to be effective for spikelet number/branch in rice.

3. In an experiment the yield of different rice varieties are studied in 3 different seasons. The result obtained have been given below:

	Rice varieties			
Seasons	*IR–8*	*Jaya*	*Padma*	*Basmati*
Summer	12	6	2	10
Rainy	7	5	1	6
Winter	7	3	3	5

State whether mean yield (*kg/acre*) of the rice varieties differ among themselves or not and the response of the seasons to varietal performance.

Anova (F-*test*) has to be performed as several means has been considered.

Row 1 total	30	Column 1 total	26
Row 2 total	19	Column 2 total	14
Row 3 total	18	Column 3 total	6
Grand total	67	Column 4 total	21
		Grand total	67

$$\text{CF (}\textit{Correction factor}\text{)} = \frac{\text{Grand total (T)}^2}{\text{Observations n}} = \frac{(67)^2}{12} = \mathbf{374.08.}$$

Grand total sum of squares $= 12^2 + 7^2 + 7^2 + 6^2 \cdots 6^2 + 5^2 = \mathbf{487.0.}$

Total SS = Grand total SS − CF = 487.0 − 374.1 = **112.9.**

$$\text{SS due to varieties (}\textit{column}\text{)} = \frac{26^2 + 14^2 + 6^2 + 21^2}{3} - \text{CF}$$

(It is divided by 3 as individual column total is sum of 3 values) = **75.59.**

SS due to seasons (*rows*) $= \dfrac{30^2 + 19^2 + 18^2}{4} - CF$

(It is divided by 4 as each row total is made up of 4 values) = **22.17.**

SS due to error = Total SS – [SS due to varieties + SS due to seasons]

= 112,9 – {75.59 + 22.17} = 112.9 – 97.76 = **15.14.**

Anova Table

Sources	DF	SS	MSS	F-*value*	0.05	0.01	0.00	P
Varieties	$3n_1$	75.59	25.2	10.0**	4.8	9.8	23.7	
Seasons	$2n_1$	22.17	11.09	4.4	5.1	—	—	
Error	$6n_2$	15.14	2.52					
Total	11							

From Anova Table it may be concluded that mean yield of the rice varieties differ significantly among themselves at 1% level; however, yield did not vary with seasons.

$$CD = \sqrt{\frac{2.52}{3}} = 0.917 \times \sqrt{2} = 1.30$$

t-value at 5% for 6 DF = 2.45.

Therefore, CD = 2.45 x 1.30 = 3.19.

	IR-8	*Jaya*	*Padma*	*Basmati*
Column total	26.0	14.0	6.0	21.0
Mean	8.6	4.6	2.0	7.0
CD	3.19			

Mean yield of IR-8 differs significantly with Jaya and Padma. Jaya diffe from Basmati. Padma differs from Basmati.

Table: Mean values for different quantitative characters in normal and three seed-coat mutants of black cumin.

Plant types with seed-coat colours	Plant height	No. of primary branches/ plant	No. of total branches/ plant	No. of capsules/ plant	No. of seta/ capsule	Capsule length/ fruit (cm)	Seed length (mm)	Seed breadth (mm)	Seed yield/ plant (gm)	Harvest index (%)
Black (normal)	50.1 ± 1.13	6.2 ± 0.15	23.8 ± 1.47	24.8 ± 1.47	5.4 ± 0.08	1.2 ± 0.02	2.6 ± 0.05	1.3 ± 0.04	2.7 ± 0.16	36.4
Dark reddish brown	52.9 ± 1.38	5.5 ± 0.19	22.3 ± 1.99	23.4 ± 1.99	5.7 ± 0.12	1.1 ± 0.02	2.4 ± 0.04	1.4 ± 0.03	2.0 ± 0.25	31.3
Yellowish brown	37.7 ± 1.9	6.0 ± 0.2	23.4 ± 2.18	24.4 ± 2.18	5.1 ± 0.10	1.1 ± 0.02	2.2 ± 0.04	1.3 ± 0.04	2.0 ± 0.19	31.0
Bicolour	39.7 ± 2.05	6.3 ± 0.67	22.7 ± 4.91	23.7 ± 4.91	5.1 ± 0.33	0.9 ± 0.0	2.2 ± 0.05	1.1 ± 0.04	0.9 ± 0.18	21.9
CD at 5% level	10.13	1.11	8.77	8.77	0.42	0.13	0.32	0.26	0.97	10.65

Experiment: Selfed progenies of seed-coat colour mutant plants and black seeded normal plants were grown in randomized block design with 3 replications. Observation in 5 randomly selected plants from each row and a total of 60 plants from 3 replications were recorded for different quantitative traits in control and in the mutant plant types and pooled mean with standard error have been presented in table. F-analysis among the plant types have been made for different traits and has been documented in the form of CD at 5% level.

Comment: Phenotypic data indicated that *dark reddish brown seed-coat* mutant was as productive as normal; while the *bicolour* and the *yellowish brown seed-coat* mutants were short sized and small seeded plants.

Experiment: Twenty-two genotypes of sesame (Til plant) were grown in RBD with 3 replications and data have been estimated for 8 yield related traits and yield from 5 randomly selected plants from each plot in each generation over 2 years (data pooled over generations) for studying genetic variability, character association and path analysis.

Objective: To understand the contribution of each yield related trait to yield and to select component maximizing yield.

Results obtained from Statistical Analysis

Table: Estimates of parameters of variability, heritability and genetic advance from different characters in sesame genotypes.

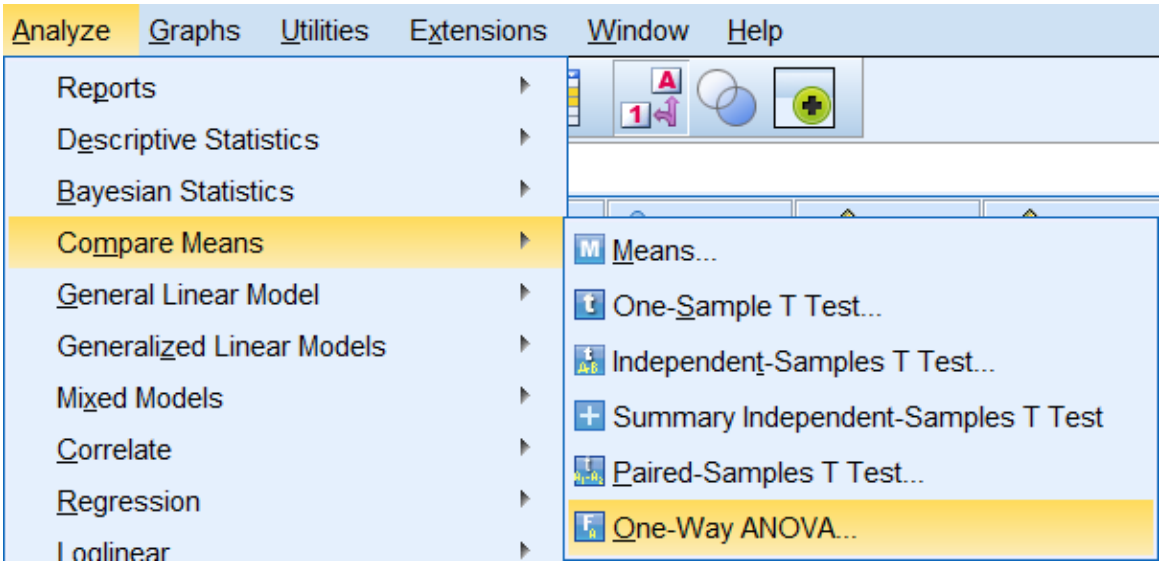
Attributes	*Population Mean ± S.E.*	*Coefficient of variability (%)*		*Heritability (%)*	*Genetic advance as % of mean*
		GCV	PCV		
Plant height (cm)	83.31 ± 2.65	24.60	25.79	91.00	48.34
Primary branches/plant	2.63 ± 0.15	27.94	46.72	35.76	34.42
Total branches/plant	2.88 ± 0.25	31.06	70.65	19.32	27.65
Distance from base to first branching	23.94 ± 1.35	36.55	45.69	63.98	60.23
Capsules on main axis	17.77 ± 0.97	29.35	44.32	43.86	40.04
Capsules/plant	37.84 ± 2.36	42.01	50.62	68.87	71.82
Capsule length (cm)	2.10 ± 0.04	8.25	15.50	25.00	8.50
No. of seeds/capsule	38.95 ± 0.92	16.36	19.20	72.66	28.74
Seed yield/plant	2.86 ± 0.22	41.37	62.35	44.03	56.55

The estimates of genetic parameters indicated that the magnitude of GCV (genotypic coefficient of variation) and PCV (phenotypic coefficient of variation) was of high order for all the traits and PCV was higher than the corresponding GCV values. The heritability estimated ranged from 19.32% to 91.0% among the traits. High degree of heritability was obtained for plant height, seeds/capsule, number of capsules/plant and distance from base to first branching; while, it was moderate to low for other traits. High heritability coupled with high genetic advance is being considered as effective criteria for selection for improvement in yield and has been noted for number of capsules/plant and distance from base to first branching.

One-way ANOVA in SPSS software:

The following steps reflect SPSS's dedicated **One-Way ANOVA** procedure. However, since the One-Way ANOVA is also part of the General Linear Model (GLM) family of statistical tests, it can also be conducted via the Univariate GLM procedure ("univariate" refers to one dependent variable). This latter method may be beneficial if your analysis goes beyond the simple One-Way ANOVA and involves multiple independent variables, fixed and random factors, and/or weighting variables and covariates (e.g., One-Way ANCOVA). We proceed by explaining how to run a One-Way ANOVA using SPSS's dedicated procedure.

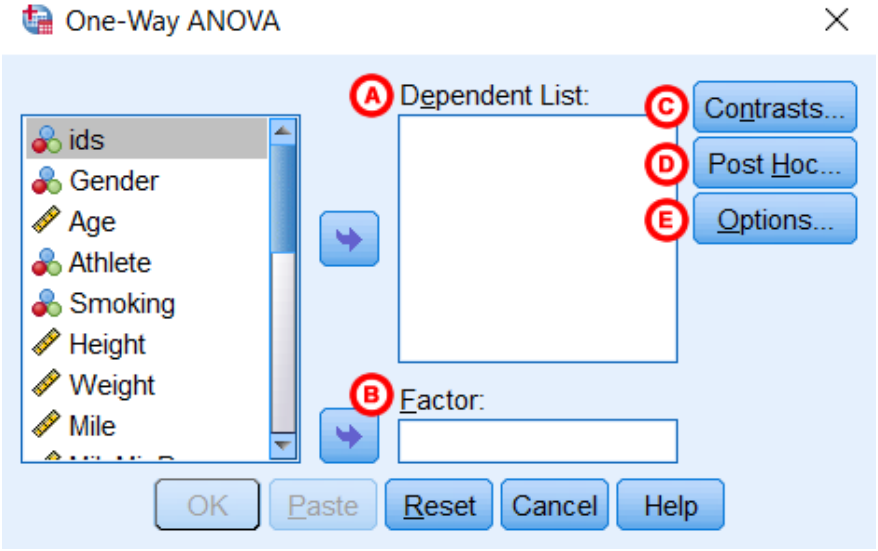
To run a One-Way ANOVA in SPSS, click **Analyze > Compare Means > One-Way ANOVA**.



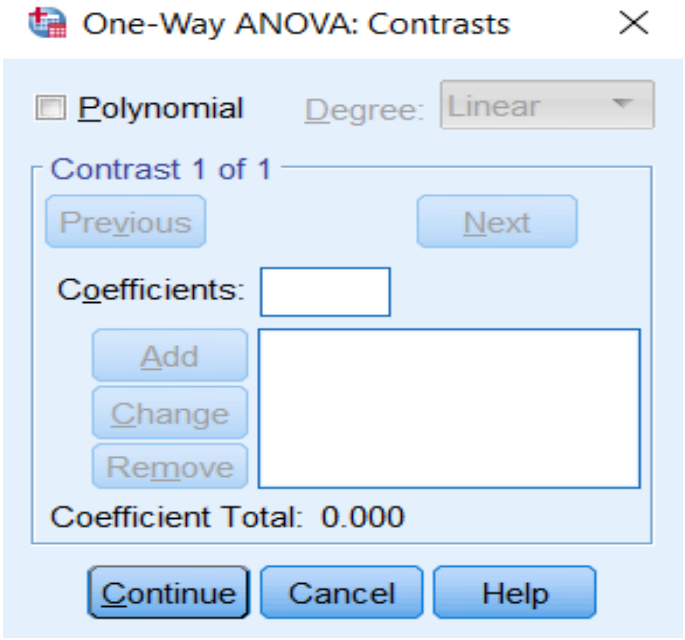
The One-Way ANOVA window opens, where you will specify the variables to be used in the analysis. All of the variables in your dataset appear in the list on the left side. Move variables to the right by selecting them in the list and clicking the blue arrow buttons. You can move a variable(s) to either of two areas: **Dependent List** or **Factor**.

A Dependent List: The dependent variable(s). This is the variable whose means will be compared between the samples (groups). You may run multiple means comparisons simultaneously by selecting more than one dependent variable.

B Factor: The independent variable. The categories (or groups) of the independent variable will define which samples will be compared. The independent variable must have at least two categories (groups), but usually has three or more groups when used in a One-Way ANOVA.

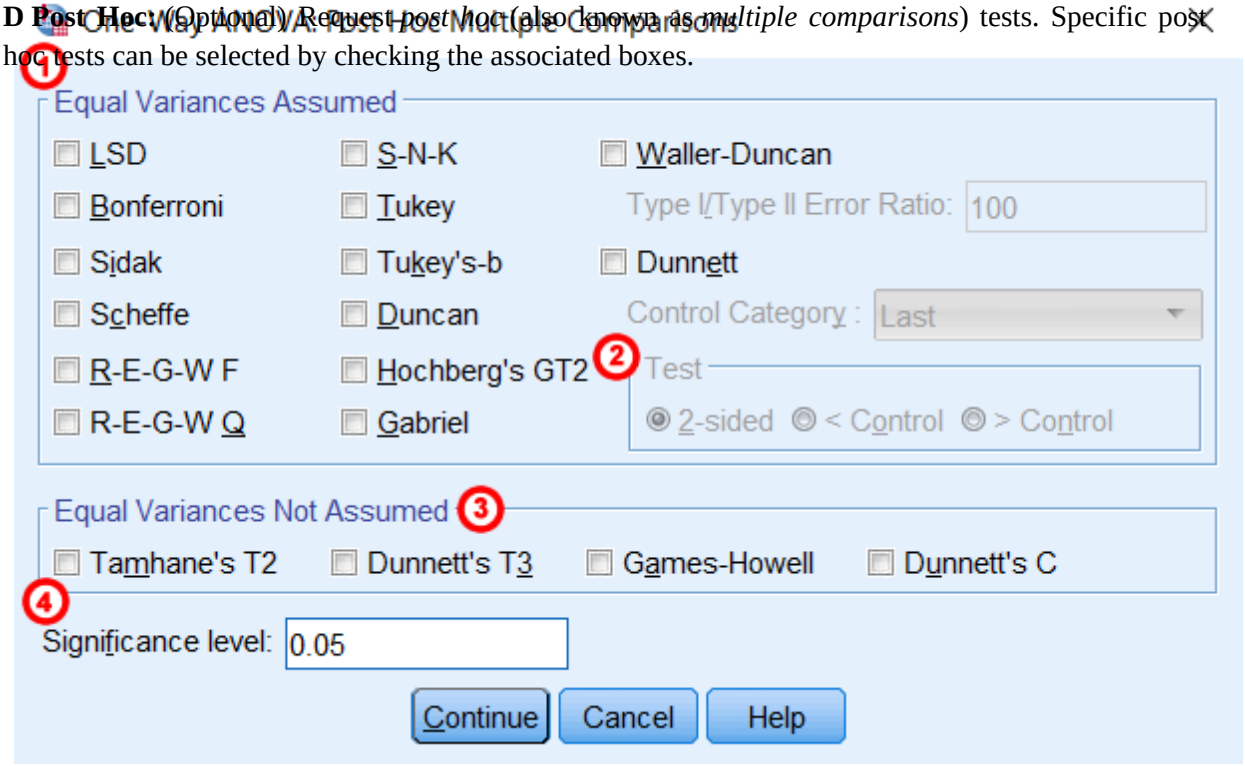


C Contrasts: (Optional) Specify contrasts, or planned comparisons, to be conducted after the overall ANOVA test.



When the initial *F* test indicates that significant differences exist between group means, contrasts are useful for determining which specific means are significantly different *when you have specific hypotheses that you wish to test*. Contrasts are decided **before** analyzing the data (i.e., *a priori*). Contrasts break down the variance into component parts. They may involve using weights, non-orthogonal comparisons, standard contrasts, and polynomial contrasts (trend analysis).
Many online and print resources detail the distinctions among these options and will help users select appropriate contrasts. For more information about contrasts, you can open the IBM SPSS help manual from within SPSS by clicking the "Help" button at the bottom of the One-Way ANOVA dialog window.

D Post Hoc: (Optional) Request *post hoc* (also known as *multiple comparisons*) tests. Specific post hoc tests can be selected by checking the associated boxes.



1 Equal Variances Assumed: Multiple comparisons options that assume *homogeneity of variance* (each group has equal variance). For detailed information about the specific comparison methods, click the **Help** button in this window.

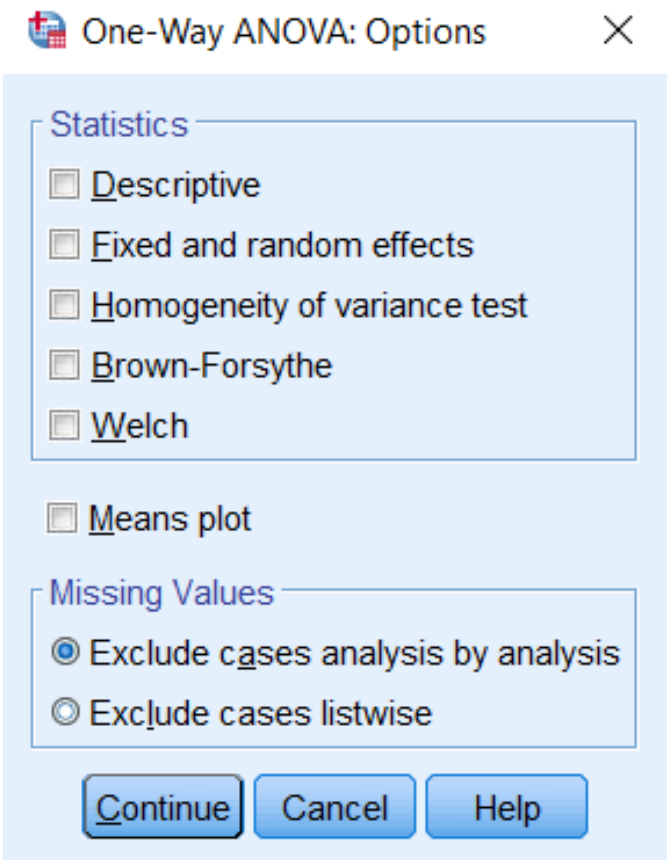
2 Test: By default, a 2-sided hypothesis test is selected. Alternatively, a directional, one-sided hypothesis test can be specified if you choose to use a Dunnett post hoc test. Click the box next to **Dunnett** and then specify whether the **Control Category** is the Last or First group, numerically, of your grouping variable. In the **Test** area, click either **< Control** or **> Control**. The one-tailed options require that you specify whether you predict that the mean for the specified control group will be less than (**> Control**) or greater than (**< Control**) another group.

3 Equal Variances Not Assumed: Multiple comparisons options that do not assume equal variances. For detailed information about the specific comparison methods, click the **Help** button in this window.

4 Significance level: The desired cutoff for statistical significance. By default, significance is set to 0.05.

When the initial *F* test indicates that significant differences exist between group means, post hoc tests are useful for determining which specific means are significantly different *when you do not have specific hypotheses that you wish to test*. Post hoc tests compare each pair of means (like t-tests), but unlike t-tests, they correct the significance estimate to account for the multiple comparisons.

E Options: Clicking **Options** will produce a window where you can specify which **Statistics** to include in the output (Descriptive, Fixed and random effects, Homogeneity of variance test, Brown-Forsythe, Welch), whether to include a **Means plot**, and how the analysis will address **Missing Values** (i.e., **Exclude cases analysis by analysis** or **Exclude cases listwise**). Click **Continue** when you are finished making specifications.



Click **OK** to run the One-Way ANOVA.

Example

To introduce one-way ANOVA, let's use an example with a relatively obvious conclusion. The goal here is to show the thought process behind a one-way ANOVA.

PROBLEM STATEMENT

In the sample dataset, the variable *Sprint* is the respondent's time (in seconds) to sprint a given distance, and *Smoking* is an indicator about whether or not the respondent smokes (0 = Nonsmoker, 1 = Past smoker, 2 = Current smoker). Let's use ANOVA to test if there is a statistically significant difference in sprint time with respect to smoking status. Sprint time will serve as the dependent variable, and smoking status will act as the independent variable.

BEFORE THE TEST

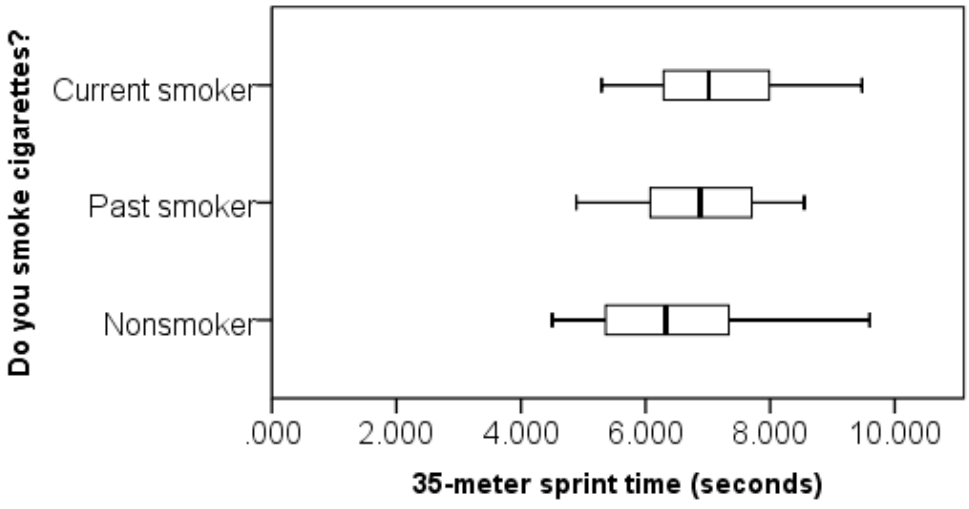
Just like we did with the paired *t* test and the independent samples *t* test, we'll want to look at descriptive statistics and graphs to get picture of the data before we run any inferential statistics.

The sprint times are a continuous measure of time to sprint a given distance in seconds. From the Descriptives procedure (**Analyze > Descriptive Statistics > Descriptives**), we see that the times exhibit a range of 4.5 to 9.6 seconds, with a mean of 6.6 seconds (based on n=374 valid cases). From the Compare Means procedure (**Analyze > Compare Means > Means**), we see these statistics with respect to the groups of interest:

	N	Mean	Std. Deviation
Nonsmoker	261	6.411	1.252
Past smoker	33	6.835	1.024
Current smoker	59	7.121	1.084
Total	353	6.569	1.234

Notice that, according to the Compare Means procedure, the valid sample size is actually n=353. This is because Compare Means (and additionally, the one-way ANOVA procedure itself) requires there to be nonmissing values for both the sprint time and the smoking indicator.

Lastly, we'll also want to look at a comparative boxplot to get an idea of the distribution of the data with respect to the groups:



From the boxplots, we see that there are no outliers; that the distributions are roughly symmetric; and that the center of the distributions don't appear to be hugely different. The median sprint time for the nonsmokers is slightly faster than the median sprint time of the past and current smokers.

RUNNING THE PROCEDURE

1. Click **Analyze > Compare Means > One-Way ANOVA**.
2. Add the variable *Sprint* to the **Dependent List** box, and add the variable *Smoking* to the **Factor** box.
3. Click **Options**. Check the box for **Means plot**, then click **Continue**.
4. Click **OK** when finished.

Output for the analysis will display in the ***Output Viewer*** window.

SYNTAX

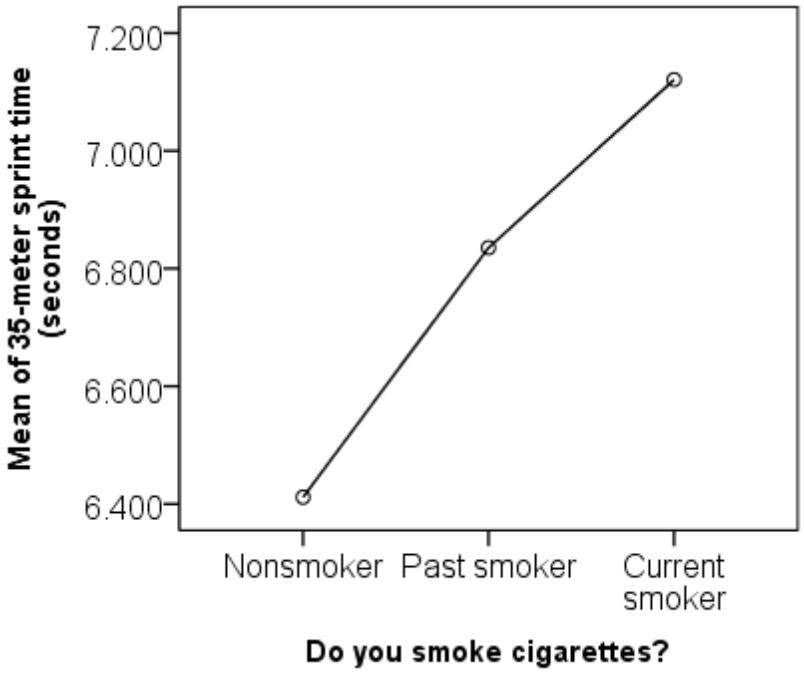
```
ONEWAY Sprint BY Smoking
  /PLOT MEANS
  /MISSING ANALYSIS.
```

OUTPUT

The output displays a table entitled **ANOVA**.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26.788	2	13.394	9.209	.000
Within Groups	509.082	350	1.455		
Total	535.870	352			

After any table output, the Means plot is displayed.



The Means plot is a visual representation of what we saw in the Compare Means output. The points on the chart are the average of each group. It's much easier to see from this graph that the current smokers had the slowest mean sprint time, while the nonsmokers had the fastest mean sprint time.

DISCUSSION AND CONCLUSIONS

We conclude that the mean sprint time is significantly different for at least one of the smoking groups ($F_{2,\ 350} = 9.209$, $p < 0.001$). Note that the ANOVA alone does not tell us specifically which means

were different from one another. To determine that, we would need to follow up with *multiple comparisons* (or *post-hoc*) tests.

RBD (Anova test) from Unequal Replications:

Example:

Performance of mean height of 4 lines of rice are to be tested. They are sown in the field as single row. Plant number in them were unequal. Plant heights are in cm.

Line 1 12, 12, 13, 16, 14, 10, 10, 14, 15, 13, 17, 11, 11, 10, 7, 14.

Line 2 7, 6, 7, 11, 10, 11, 8, 11, 4, 9, 10.

Line 3 5, 14, 9, 9, 8, 10, 10, 9.

Solution:

Total of Line 1 = 199		No. of observations in Line 1 = 16	
" " " 2 = 94		" " " " " 2 = 11	
" " " 3 = 74		" " " " " 3 = 8	
" " " 4 = 151		" " " " " 4 = 11	
Grand Total = 518		Total No. of observations = 46	

$$CF = \frac{(518)^2}{46} = \frac{268324}{46} = \mathbf{5833.13.}$$

$$\text{Total SS} = \left[12^2 + 12^2 + 13^2 + \ldots + 12^2 + 11^2 + 10^2\right] - CF$$
$$= 6506 - 5833.13 = \mathbf{672.87.}$$

$$\text{Between line SS} = \left[\frac{(199)^2}{16} + \frac{(94)^2}{11} + \frac{(74)^2}{8} + \frac{(151)^2}{11}\right] - CF$$
$$= [2475.06 + 803.27 + 684.50 + 2072.82] - CF$$
$$= 6035.65 - 5833.13 = \mathbf{202.52.}$$

Anova Table

Sources	df	SS	MSS	F
Between lines	3	202.52	67.507	5.631**
Error (*within lines = between plants*)	42	470.35		
Total	45	672.87		

** Significant at 1% level.

Conclusion:

Since the observed value of F(5.631) is greater than the value of F at 1% at df $n_1 = 3$, $n_2 = 42$, the test is highly significant indicating thereby that there are significant difference in plant height between different lines.

Treatment means, i.e., line means:

Line 1 = 199/16 = 12.44 cm;

Line 2 = 94/11 = 8.55 cm;
Line 3 = 74/8 = 9.25 cm;
Line 4 = 151/11 = 13.73 cm;
From the above mean heights of the four lines it is found that the mean value of line 2 is lowest and that of line 4 is highest.

Significance of Difference of Means of Line 2 and Line 4:

Standard deviation (σ) of Line 2 and 4

$$= \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}} = \sqrt{\frac{[\text{Line } 2^2 + \text{Line } 4^2] - \frac{[\Sigma \text{ Line } 2 + \Sigma \text{ Line } 4]^2}{n}}{n-1}}$$

$$= \sqrt{\frac{(858 + 2345) - \frac{(94 + 151)^2}{22}}{22-1}} = \sqrt{\frac{3203 - \frac{60025}{22}}{21}}$$

$$= \sqrt{\frac{474.6}{21}} = \sqrt{22.6} = 4.75.$$

Now, t between Line 2 and Line 4

$$= \frac{\text{Difference of Line 2 and 4}}{\sqrt{\frac{1}{\text{No. of obs. of Line 2}} + \frac{1}{\text{No. of obs. of Line 4}}}}$$

S = pooled SD of Line 2 and 4

$$= \frac{13.75 - 8.55}{4.75\sqrt{\frac{1}{11} + \frac{1}{11}}} = \frac{5.18}{4.75\sqrt{\frac{1}{22}}} = \frac{5.18}{1.012} = 5.128.$$

Conclusion:

The observe value of t, i.e., 5.128 is greater than the table value of t at 20($n_1 + n_2 - 2 = 11 + 11 - 2 = 20$) at 1% level. Therefore, the value of observed t is highly significant which indicates that the difference in plant height between the Line 2 and 4 is highly significant.

Analysis of Variance using Latin Square Design:

Here the treatments are so designed that each treatment is represented once in each row and once in each column. Number of treatments must be same as number of replications.

Four genotypes of wheat (A, B, C and D) in four replications 4 x 4 assignment.

A	D	B	C
B	C	D	A
C	B	A	D
D	A	C	B

As randomization is non-biased representation, it may so happens that varieties/families/treatments, etc., in field may have sectorial representation which maybe a hindrance to minimization of error (soil fertility). However, Latin square design will be only accommodative in square or rectangular field.

Example:

Seeds of 4 wheat genotypes (A,B,C and D) were given mutagenic treatments of ethyl methane sulphonate at 0.25%, 3h, 0.50%, 3h and 1.0%, 3h (treatments: 0-control, 0.25%, 0.5% and 1.0%) and grown in 4 replications in field plots. On harvest yield was assessed per plots (kg/plot).

Solution:

Test:

1. Yield of the varieties differed or not.
2. Variety yield in relation to replication.
3. Variety yield in relation to treatments.

Given data in tabular form:

Replication	*Treatments (%)*			
	0	0.25	0.50	1.0
1	4(A)	5(D)	12(B)	10(C)

Rep	*Genotypes*				*Total*
	A	B	C	D	
1	4	12	10	5	31
2	10	6	8	16	40
3	14	6	2	10	32
4	10	6	8	6	30
Total	38	30	28	37	133

$$\text{Correction factor} = \frac{(133)^2}{16} = \mathbf{1105.56.}$$

$$\text{Grand total SS} = 4^2 + 10^2 \cdots 10^2 + 6^2 = \mathbf{1313.}$$

$$\text{SS due to genotypes} = \frac{38^2 + 30^2 + 28^2 + 37^2}{4} - \text{CF} = \mathbf{18.69.}$$

$$\text{SS due to replication} = \frac{31^2 + 40^2 + 32^2 + 30^2}{4} - \text{CF} = \mathbf{15.69.}$$

Solution:

Null hypothesis—Mean yield of the genotypes is same:

(a) among themselves,
(b) among replication and
(c) among treatments.

(a) Varieties (Genotypes) in relation to replication:

(b) Genotypes in relation to treatments:

Genotypes	Treatments			
	0	0.25	0.50	1.0
A	4	10	14	10
B	6	6	12	6
C	2	8	8	10
D	6	5	16	10
Total	18	29	50	36

$$\text{SS due to treatments} = \frac{18^2 + 29^2 + 50^2 + 36^2}{4} - \text{CF} = \mathbf{134.69.}$$

$$\text{Total SS} = \text{Grand total SS} - \text{CF} = 1313 - 1105.56 = \mathbf{207.44.}$$

$$\begin{aligned}\text{SS due to error} &= \text{Total SS} - (\text{SS due to genotypes} + \text{replication} + \text{treatments}) \\ &= 207.44 - (18.69 + 15.69 + 134.69) \\ &= \mathbf{38.37.}\end{aligned}$$

Anova Table

Sources	df	SS	MSS	F-*value*	*Table-values* (F-*value*)		
					0.05	0.01	0.001
Genotypes	$3(n_1)$	18.69	6.23	0.97	4.8	9.8	23.7
Replications	$3(n_1)$	15.69	5.23	0.82			
Treatments	$3(n_1)$	34.69	44.90	7.02*			
Error	$6(n_2)$	38.37	6.40				
Total	15						

Inference:

1. Yield (mean) of the genotypes do not differ among themselves significantly.
2. Mean yield of the genotypes did not vary in replication.
3. Mean yield of the genotypes varied significantly in treatments.

Analysis of Variance from Split Plot Design:

In split plot design the field is divided into identical blocks and such blocks are considered similar to replications. Each block is divided into main plots, where the first factor (treatment) is assigned at random and subsequently each main plot is subdivided into sub-plots to be allotted randomly to the levels of the second factor. It is breeder's choice to consider the treatments as first or second factor. Significance of this layout is that it gives effective control over treatments and minimizes error. Further, interaction between two factors can also be analyzed.

Thus, in the design the field is:

1. Divided into blocks = replications.
2. Each block is divided into main plots, where the main treatment will be assigned.
3. Each main plot is sub-divided in sub-plots = sub-treatment.

4. Size of the main plots and sub-plots should be identical.
5. Random distribution of the treatments into plots.

Layout of the Experiment with a Specific Example:

Treatments:

1. Date of sowing:

(a) S_1—1st November
(b) S_2—1st December
(c) S_3—1st January
(d) S_4—1st February

} Main treatment ↓ Assigned to main plots

2. Methods of sowing:

(a) m_1—Broadcasting
(b) m_2—Drilling
(c) m_3—Line

Blocks = Replications = 3.

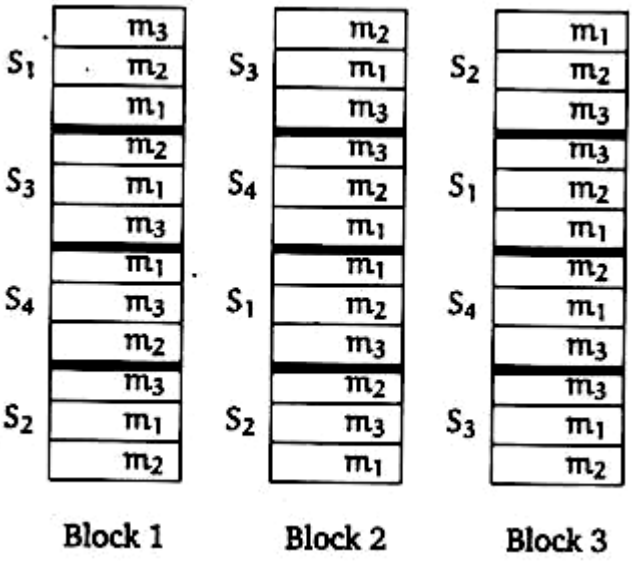


Diagram showing how field is divided in split plot design.

Example:

Split plot design experiment has been conducted to evaluate the differences in yield in relation to sowing dates (A—15th November; B—1st December; C—15th December; D—1st January and E—15th January) in 8 genotypes of Nigella sativa (control and 7 mutant lines) to evaluate the yield differences (seed yield in gm). The experiment was conducted in 3 blocks.

Solution:

Main treatment = Sowing dates = Main plots;
Sub-treatment = Genotypes = Sub-plots.
Results have been tabulated.

Table 7.1: Two-way table of blocks and dates of sowing (*replication and main treatment*)

Blocks	*Dates of sowing*					*Total*
	A	B	C	D	E	
1	12.35	7.46	4.45	1.79	1.78	27.77
2	11.00	11.49	7.78	1.18	1.15	32.60
3	10.91	14.59	4.15	1.44	1.35	32.44
Total	34.26	33.48	16.38	4.41	4.28	92.81

$$CF = \frac{(92.81)^2}{120} \quad [8 \text{ genotypes} \times 5 \text{ sowing dates} \times 3 \text{ blocks} = 120]$$

$$= \mathbf{71.78.}$$

$$\text{Grand total SS} = \frac{(12.35)^2 + (11.0)^2 + \cdots (1.15)^2 + (1.35)^2}{8} - CF$$

[as each x_i-value is sum total of 8 values obtained from genotypes]

$$= \mathbf{41.06.}$$

$$\text{SS due to replication (\textit{blocks})} = \frac{(27.77)^2 + (32.60)^2 + (32.44)^2}{40} - CF \quad (8 \times 5 = 40)$$

$$= \mathbf{0.37.}$$

SS due to planting dates (*main treatment*)

$$= \frac{(34.26)^2 + (33.48)^2 + (16.38)^2 + (4.41)^2 + (4.28)^2}{24} - CF \quad (8 \times 3 = 24)$$

$$= \mathbf{36.58.}$$

Error a = Total SS – [SS due to planting + SS due to replication]

= 41.06 – [0.37 + 36.58]

= 41.06 – 36.95

= **4.11.**

Table 7.2: Two-way table of treatment totals for split plot experiment (*main treatments and sub-treatments*)

Genotypes	*Planting dates*					*Total*
	A	B	C	D	E	
1	3.88	6.33	3.44	0.60	0.50	14.75
2	6.25	3.48	1.25	0.71	0.66	12.35
3	3.78	6.85	4.15	0.77	0.60	16.15
4	3.60	2.54	2.58	0.46	0.44	9.62
5	5.94	3.39	1.05	0.63	0.75	11.76
6	6.69	8.17	2.82	0.48	0.60	18.76
7	2.30	1.26	0.75	0.47	0.38	5.16
8	1 82	1.46	0.34	0.29	0.35	4.26
Total	34.26	33.48	16.38	4.41	4.28	92.81

SS due to planting × genotypes

$$= \frac{(3.88)^2 + (6.25)^2 + \cdots + (0.38)^2 + (0.35)^2}{3} - CF$$

[value obtained from 3 replications/blocks]

$= 64.71.$

SS due to genotypes

$$= \frac{(14.75)^2 + (12.35)^2 + \cdots + (5.16)^2 + (4.26)^2}{15} - CF$$

(3 replications × 5 dates = 15)

$= 12.12.$

SS due to main treatment (*planting dates*) × sub-treatment (*genotypes*)

$= 64.71 - (12.12 + 36.58)$

$= 16.01.$

Total SS = (Sum of squares of individual values Σx_i's)

$= 154.42 - 71.78(CF)$

$= 82.63.$

Error b = 82.63 − [SS due to replication + SS due to dates + SS due to genotypes + SS due to interaction + SS due to error a]

$= 82.63 - [0.37 + 36.58 + 12.12 + 16.01 + 4.11]$

$= 13.44.$

Table 7.3: Complete analysis of variance

Sources of variation	df	SS	MSS	F-*value*
Replication	2	0.37	0.185	0.35
Planting dates	4	36.58	9.145	17.59***
Error a	8	4.11	0.52	
Genotypes	7	12.12	1.73	9.01***
Planting dates × genotypes	28	16.01	0.57	2.96***
Error b	70	13.44	0.192	
Total	119			

*** Significant at 0.001 probability level (0.1%).

Inference:

It has been observed that both treatments and their interactions have been significant at 0.1% level. It has also been noted that the differences between genotypes were of a much smaller order than those between dates of planting.

The estimates of the two error variance were 0.52 [Error a] and 0.192 [Error b], thus confirming the expectation that the main plot error is likely to be higher than sub-plot error. Further, it may also be concluded that sowing period A (1st November) gives maximum yield irrespective of genotypes.

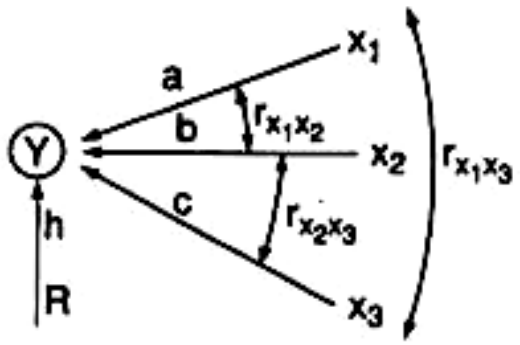
Path Analysis:

Path analysis was proposed by Wright in 1921 and was used first for plant selection by Dewey and Lu in 1959. Path analysis is simply standardized partial regression coefficient partitioning the correlation coefficients into the measures of direct and indirect effects of set of independent variables on the dependent variable. It is also known as cause and effect relationship.

For example, in sesame, yield (x_4) is mostly contributed by plant height (x_1), total capsules/plant (x_2) and seeds/capsule (x_3).

Path analysis will indicate whether the association of the yield related traits with yield is due to their direct effect on yield (true association and selection can be made for improvement), or is a consequence of their indirect effect via some other traits (s) and in such cases geneticist has to select the trait through which the indirect effect has been exerted.

Path analysis is carried out using the estimates of correlation coefficients.



Path diagram showing cause and effect relationship.

Y is yield (effect) of the causal factors x_1, x_2 and x_3 (yield-related components'); r designate association between variables; a, b, c and h are path coefficients due to respective variables and R is residual effect.

Accounts of Path Co-Efficient analysis:

Path coefficient analysis takes into account the followings:

1. Direct Effect:

Using the values of correlation coefficients and with the help of path diagram following simultaneous equation can be set up:

$$r_{14} = P_{14} + r_{12}P_{24} + r_{13}P_{34}$$
$$r_{24} = r_{12}P_{14} + P_{24} + r_{23}P_{34}$$
$$r_{34} = r_{13}P_{14} + r_{23}P_{24} + P_{34}.$$

In the path diagram P_{14} = a, P_{24} = b and P_{34} = c and are direct effects of variables x_1, x_2 and x_3 respectively on the dependent variable Y (yield). Putting the values of the correlation coefficients in the above equations, the values of P_{14}, P_{24} and P_{34} are estimated by the process of elimination.

Example 1:

In sesame plant height (x_1), capsules/plant (x_2) and seeds/ capsule (x_3) are yield components and their associations and relationship with yield (4) are as follows.

$r_{12} = 0.72$ $r_{23} = 0.52$

$r_{13} = 0.68$ $r_{24} = 0.72$

$r_{14} = 0.56$ $r_{34} = 0.62.$

2. Indirect Effects and Direct Effects:

Values of correlation coefficient and those of direct effects are put to compute indirect effects.

(a) Plant Height (x_1) and Yield (x_4):

Direct effect	$P_{14} = -0.1732$
Indirect effect via total capsules/plant	$P_{24}r_{12} = 0.4550$
Indirect effect via seeds/capsule	$P_{34}r_{13} = 0.2781$
Total (direct + indirect) effect	0.5599

(b) Total Capsules/Plant (x_2) and Yield (x_4):

Direct effect	$P_{24} = 0.6320$
Indirect effect via plant height	$P_{14}r_{12} = -0.1247$
Indirect effect via seeds/capsule	$P_{34}r_{23} = 0.2127$
Total	0.72

(c) Seeds/Capsule (x_3) and Yield (x_4):

Direct effect	$P_{34} = 0.4090$
Indirect effect via plant height	$P_{14}r_{13} = 0.1178$
Indirect effect via capsules/plant	$P_{24}r_{23} = 0.3286$
Total	0.6198

3. Residual Effect can be calculated from:

$1 = P^2R_4 + P^2_{14} + P^2_{24} + P^2_{34} + 2P_{14}r_{12}P_{24} + 2P_{14}r_{13}P_{34} + 2P_{24}r_{23}P_{34}$.

P^2R_4 is the square of residual effect = 0.6224.

Path coefficient analysis revealed that the direct contribution of total number of capsules/plant was high and positive (P_{24} = 0.6320) which was followed by seeds/capsule (P_{34} = 0.4090). Poor negative direct contribution was recorded for plant height (P_{14} = —0.1732).

The indirect effects via plant height were poor and negative in all cases; while indirect effects via total capsule/plant and seeds/capsule were positive and substantial. Total (direct and indirect) effect of plant height, capsules/plant and seeds/capsule has been recorded as 0.5599, 0.72 and 0.6198, respectively. High residual effect (0.6224) was observed.

Table: Direct and indirect effects of contributing characters on seed yield of sesame

Characters	Plant height (cm)	No. pr. branches/ plant	Total branches/ plant	Distance from base to first branching	Capsules on main axis	Capsules/ plant	Capsule length	Seeds/ capsule	Correlation with seed yield
Plant height (cm)	-0.406	-0.719	0.538	0.027	-0.752	0.895	0.706	0.281	0.57***
No. pr. branches/plant	-0.300	**-0.971**	0.759	0.020	-0.761	1.131	0.622	0.281	0.78***
Total branches/plant	-0.276	-0.933	**0.391**	0.017	-0.523	0.945	0.432	0.281	0.67***
Distance from base to first branching	-0.211	-0.379	0.261	**0.051**	-0.086	0.149	0.126	0.118	0.03
Capsules on main axis	-0.321	-0.777	0.435	0.005	**-0.952**	1.131	0.969	0.399	0.89***
Capsules/plant	-0.292	-0.884	0.601	0.006	-0.866	**1.243**	0.748	0.304	0.86***
Capsule length	-0.272	-0.573	0.324	0.006	-0.876	0.883	**1.054**	0.354	0.90***
Seeds/capsule	-0.252	-0.602	0.379	0.013	-0.837	0.833	0.822	**0.454**	0.81***

Residual effect 0.0802; Figures in bold are the direct effects; *** Significant at 0.1% level.

In addition to the degree of associations path coefficient analysis takes into account the cause and effect relationship and has been performed to partition the genotypic correlation into direct and indirect effects for understanding the relative importance of the component characters on yield. The perusal of path analysis revealed that capsules per plant (1.243) had maximum positive direct effect followed by capsule (1.243) had maximum positive direct effect followed by capsule length (1.054), seeds/capsule (0.454) and total branches/plant (0.691). Further, indirect contribution via these traits on yield was also very high and positively significant. Thus, path analysis led to the inference that number of total branches and capsules per plant, capsule length and seeds per capsule are the important selection criteria for yield improvement.

Stepwise regression analysis performed with yield components as indepen dent variables (X_1) and seed yield as dependent variable (Y) revealed that $Y = 4.67 + 0.03X_1 + 0.07X_2 + 1.71X_3$; as the best fitted equation where X_1 seeds per capsule, X_2 = capsule length and X_3 = seeds per capsule; R^2 0.62, standard error of estimate 1.06) and these three traits (X_1, X_2 and X_3) are explaining more than 62.0 per cent variables of the total variations. This statistical analysis laid emphasis on simultaneous selection of capsules per plant, capsule length and seeds per capsule.

Inference: Different statistical analysis has been performed to select rais for improvenent in sesame genotypes.

(a) Extension of statistical analysis gave precise account of the traits to be selected-those are maximizing yield.

(b) These traits will be helpful for efficient breeding in sesame through the development of recombinant genotypes.

13. Let's sum up

- Male sterility is defined as an absence or non-function of pollen grain in plant or incapability of plants to produce or release functional pollen grains as a result of failure of formation or development of functional stamens, microspores or gametes.

- The male sterility may be due to mutation, chromosomal aberrations, cytoplasmic factors or interaction of cytoplasmic and genetic factors.

- Cytoplasmic male sterility is governed by the cytoplasm (mitochondrial or chloroplast genes). Result of mutation in mitochondrial genome (mtDNA)-Mitochondrial dysfunction. Genetic male sterility is governed by nuclear genes. Male sterility geness are generally recessive (*ms ms*) but dominant gene governing male sterility are also occur in safflower.

- Cytoplasmic genetic male sterility is determined by the cytoplasm, and the plasmagenes producing male sterility are ordinarily located in mtDNA just as is the case of CMS. But a nuclear gene, called restorer gene, restores male fertility and, thereby, eliminates the effects of male sterile cytoplasm

- Self-incompatibility or intraspecific incompatibility is a well-designed genetic mechanism by which certain plants recognize and reject their own pollen thus forcing outbreeding. It is defined as inability of the plant producing functional gametes to set seed upon self-pollination.
- Its genetic system is based on a single locus, the sterility (*S*) locus, with multiple alleles. Pollen germination or pollen tube growth is blocked when the pollen grain and the stigma upon which it lands have the same allele at the same locus. Besides the genetic factors, intraspecific incompatibility is also associated with different lengths of stamens and style in flowers on same plant.
- The organisms having more than two sets of chromosomes by the addition of another chromosome is called polyploidy. It may arise as a result of abnormal mitosis where chromosomes divide by cytoplasm fails to divide during cytokinesis. The basic set of chromosomes undergoes multiplications.
- Polyphyletic origin of polyploid species: Provides source for intra and intergenomic admixture following hybridisation event resulting in (1) higher level of heterozygosity, (2) less inbreeding depression, (3) higher genetic diversity, (4) genome rearrangement and (5) occurrence of large region of homologous and homeologous sequences in DNA.

- Hybridization between individuals from different species belonging the same genus (interspecific hybridization) or two different genera of same family (intergeneric hybridization) is termed as distant hybridization and such crosses are known as distant crosses or wide crosses..

- When two parents are incompatible, a third parent that is compatible with both the parents can be used for bridge crosses and thus it becomes possible to perform cross between the original parents.

- In backcross method two plants are selected and crossed and hybrid successively backcross to one of their parents. As a result the grain hybrid backcross progeny becomes increasingly similar to that of the parents to which it identical with the parent used for backcrossing. In this method the desirable variety which are lacking in some characteristics known as a recurrent

or recipient parent, while the undesirable variety on wild variety processing only one or two desirable characteristics known as donor parent or non recurrent parent.

- Multiline variety is a mixture of several pure lines of similar phenotype(height, seed color flowering time, maturity time and various other agronomic characteristics) but have different genes for the character under consideration the disease resistance means these are isogenic lines.

- Marker assisted breeding is defined as the application of molecular markers in combination with linkage maps and genomics to alter and improve plant traits on the basis of genotypic assays. This term is used to describe several modern breeding strategies including marker-assisted selection. MAS is based on the concept that it is possible to infer the presence of a gene from the presence of a marker which is tightly linked to the gene of interest.

- Identifying a gene or QTL with in a plant genome is like finding the needle in a hay stack. QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. The markers are used to partition the mapping population in to different genotypic classes based on genotypes at the marker locus, and apply the correlative statistics to determine whether the individual of one genotype differ significantly with the individuals of other genotype with respect to the trait under study.

- A trait that defines some aspect of produce quality is called a quality trait. Each crop has a specific set of quality traits, which may be classified into the following groups: (i) morphological, (ii) organoleptic, (iii) nutritional, (iv) biological, and (v) others. For example, the important quality traits of wheat are grain size, grain colour, grain texture, protein content, gluten content, Polshenke value, sedimentation value, alveogram and mixogram

- The breeding approaches for quality traits are as follows: (i) screening of germplasm, (ii) mutagenesis, (ii) hybridization, (iv) interspecific hybridization (e.g. fruit colour, fruit size, provitamin A and vitamin C contents in tomato), (v) somaclonal variation eg. Scarlet variety of sweet potato), and (vi) genetic engineering.

- Genetic engineering has enabled the development of insect pest and/or disease resistant varieties in many crop species; many of varieties are already in cultivation. It has also become feasible to develop such varieties that are suited to specific consumer, industrial, etc. needs. Such varieties are popularly termed as 'designer crops' since they possess such specific features as were specified before their development, i.e., they are specifically designed to meet certain specified requirements. The designer crops may have a specified protein, fat or starch quality; nutriuonal feature like vitamin content, elimination of an antinutritional factor, colour, flavour, taste, etc. of fruit/grain; keeping quality: etc., or may produce a specific novel biochemical of pharmaceutical/industrial value, or may have some other features that make them more useful to humankind.
- The emergence of the novel “omics” technologies, such as genomics, proteomics, and metabolomics, is now allowing researchers to identify the genetic behind plant stress responses. These omics technologies enable a direct and unbiased monitoring of the factors affecting plant growth and development and provide the data that can be directly used to investigate the complex interplay between the plant, its metabolism, and also the stress caused by the environment or the biological threats (insects, fungi, or other pathogens). Plant responses to stress are mediated via profound changes in gene expression which result in changes in composition of plant transcriptome, proteome, and metabolome

- Disease is a series of invisible and visible responses of plant cells and tissues to a pathogenic microorganism or an environmental factor that result in adverse changes in form, function or integrity of plant and may lead to partial impairment or death of the plant or it parts.

- Plant disease resistance protects plants from pathogens in two ways: by preformed structures and chemicals, and by infection-induced responses of the immune system.

- Marker assisted breeding is defined as the application of molecular markers in combination with linkage maps and genomics to alter and improve plant traits on the basis of genotypic assays. This term is used to describe several modern breeding strategies including marker-assisted selection. Heritability is a statistic used in the fields of breeding and genetics that estimates the degree of variation in a phenotypic trait in a population that is due to genetic variation between individuals in that population. Heritability is estimated by comparing individual phenotypic variation among related individuals in a population. Heritability is an important concept in quantitative genetics, particularly in selective breeding and behavior genetics.

- Heritability, amount of phenotypic variation in a population that is attributable to individual genetic differences. Heritability, in a general sense, is the ratio of variation due to differences between genotypes to the total phenotypic variation for a character or trait in a population. The concept typically is applied in behaviour genetics and quantitative genetics, where heritability estimates are calculated by using either correlation and regression methods or analysis of variance methods.

- Mean is the value which we get by dividing the aggregate of various items of the same series by the total number of observations. Mode is the most frequent value in a series and median is the value of the middle item of a series when arranged in order of magnitude.

- In biological research when we compare any character of two samples, we calculate the significance of difference in the mean and variance to draw a meaningful conclusion.

- $\chi 2$ test is used for testing the agreement of observed frequencies with those expected upon a given hypothesis.

- The analysis of variance is based on F-statistics which is a ratio of variances. The ratio of variances due to treatments and variances due to random differences within the treatment is calculated and the estimate, known as the 'F', is then used for comparison.

- Association between variables or attributes or characteristics at a given time is known as correlation. The statistical analysis employed to find out the exact position of the straight line is known as Linear regression analysis.

14. Suggested Reading

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15. Assignment

1. How will you identify male sterility?
2. What is genotype of Pin and Thrum?
3. What are ways and means to overcome self incompatibility?
4. How will you classify SI on the basis of site of expression?
5. What will be the result when both parents have different alleles (S_1S_2 x S_3S_4) in the gametophytic self incompatibility?
6. What are male gametocides?
7. Briefly describe the molecular basis of self incompatibity.
8. What is gene pyramiding?
9. Explain the significance of self incompatibility.
10. Compare self incompatibility and male sterility.
11. How can you maintain a CGMS line?
12. What is R line?
13. In gametophytic self incompatibility what will be the outcome of cross S_1S_2 x S_1S_2?
14. Give a brief description of the various types male sterility and their utilization in plant breeding.
15. Write down the main features of genetic male sterility.
16. What is cytoplasmic genetic male sterility?
17. A wheat variety A is excellent in yield and adaptability, but susceptible to leaf blight. Another wheat variety B hass a recessive gene conferring resistance to leaf blight. Briefly described the breeding procedure to be follow for transfer of leaf blight resistance gene to susceptible variety A.
18. What is recurrent parent?
19. What do you mean by compatible solutes? Give an example.
20. Name to ion that induces salinity stress.
21. What are halophytes?
22. Comment on the role of proline, polyols and stress induced proteins in the abiotic stressed conditions
23. What is segmental allopolyploids?
24. Enumerate the genetic insight of polyploidy in angiosperms.
25. Define multiline varieties.
26. What are the prerequisites of MAS selection?
27. What is NIL?
28. Mention the barriers of distant hybridization.
29. What is bridge species?
30. Defined ideotypes? How are ideotypes developed?
31. Describe the breeding approaches for oil quality
32. What is antisense RNA technology? Mention its role in nutritional quality improvement.
33. Explain two of the most successful strategies for producing transgenic male sterility.
34. What is normal curve?

35. Briefly describe the Steps of QTL mapping.
36. What is SIM?
37. How do you calculate $\chi 2$ test of heterogeneity?
38. Short note on a) CV, b) Standard error, c) ANOVA, d) Gene for gene hypothesis.
39. Give the differences between vertical and horizontal disease resistance?
40. Describe the merits and dedmirits of mean, median and mode.
41. What is RAPD, ISSR? States its significance in plant breeding program?
42. What are the possible methods of breeding for disease resistance?
43. Enumerate the broad sense and narrow sense heritability with classical examples.
44. Why CD is important?
45. How does split plot design differ from randomized block and Latin square designs?
46. How can you differentiate Student's t test from F-test?
47. What does 't-test' signify?
48. Mention the equation for simple regression analysis.
49. What is a basic chromosome number?
50. Mention the chromosome number of cultivated cotton.
51. Cite two examples to explain disease resistance in plant species
52. What is vertifolia effect?
53. How can you calculate CD from ANOVA table?
54. What are the measures of dispersion?
55. How can you calculate mode from grouped data with regular class interval?
56. What is mean shift?
57. What is the genetical significance of $\chi 2$-test analysis?
58. Mention the names of test of significance.
59. What is 'r' and how it varies?
60. State two applications of student 't' test.
61. What is degree of freedom?
62. What is amphidiploids?
63. Name one released autopolyploid variety.
64. Ten plants have been assessed in sesame for plant height (cm) and number of branches per plant. From the given data do you consider that there exists correlation (significant) between variables? Test whether the two varieties differ in respect of this character.

A	25	23	26	25	22	26	23	21	26	25
B	22	24	29	24	23	18	19	23	24	19

65. Four rice varieties have been tested for its grain yield (kg/hectare) in four replication and following result has been obtained. Test whether the varieties differ in respect of this character.

	A	B	C	D
Rep I	12	17	13	10
Rep II	9	8	15	10
Rep III	11	12	15	22
Rep IV	23	10	7	12

All the materials are self writing and collected from ebook, journals and websites.

BOTANY

POST GRADUATE DEGREE PROGRAMME
(CBCS CURRICULUM)

SEMESTER: IV

PAPER: BOET 4.3

Microbiology Course - I

Directorate of Open and Distance Learning
UNIVERSITY OF KALYANI
Kalyani, Nadia
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Website : www.klyuniv.ac.in

POST GRADUATE DEGREE PROGRAMME
(CBCS)
IN
BOTANY

SEMESTER - IV

Course: BOET4.3

(Microbiology Course – I)

Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI
KALYANI - 741 235,
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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMS). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMS for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMS, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMS, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks are also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMS. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

SYLLABUS
COURSE - BOET4.3
Microbiology (Course – I)
(Full Marks – 80)

Course	Group	Details Contents Structure		Study hour
BOET4.3	Microbiology (Course – I)	**Unit1. Environmental Microbiology**	Microbes in environment – their evolution and diversity, RNA world, evolution of bacterial species- 16s rRNA based Phylogeny	1
		Unit2.Microbial interaction	Microbial interaction, Biofilm, Biosensor	1
		Unit3. Aeromicrobiology	Aeromicrobiology- Assessment of aeromicroflora, microbes as atmospheric pollutant	1
		Unit4. Water microbiology	Water microbiology- Assessment of water quality through physiochemical and microbiological parameters	1
		Unit5. Waste water treatment microbiology	Waste water treatment microbiology- Treatment of waste water by microorganisms	1
		Unit6. Soil microbiology	Soil microbiology- Biotic and abotic factors regulating the population dynamics of soil microflora	1
		Unit7. Microbial biogeochemical cycling	Microbial biogeochemical cycling- Nitrogen cycle, phosphorus cycle, sulfur cycle	1
		Umit8. Role of microbes in environmental management	Role of microbes in environmental management- microbial bioremediation, role of microorganisms in mineral recovery, microbial bioleaching of metals, bioterrorism	1
		Unit9. Microbial desulfurization	Microbial desulfurization of coal, degradation of xenobiotics by microorganisms, biodegradation of halocarbons, synthetic polymers, oil, biopolymer	1

Course	Group	Details Contents Structure		Study hour
BOET4.3	Microbiology (Course – I)	**Unit10. Agriculture microbiology**	Microbial exploitation for improvement and crop protection, biopesticides, biofertilizers, bio-composting	1
		Unit11. Clinical microbiology	Air, water, soil and food borne diseases, Venereal diseases	1
		Unit12. Food microbiology	Micro-organism associated with food; food spoilage; food poisoning and intoxication; food preservation, concept of probiotics	1

Content

COURSE - BOET4.3
Microbiology (Course – I)

Elective Paper **Credit : 4**

Content Structure

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1. Introduction
2. Course Objective

a. Microbes in environment- their evolution and diversity
b. Microbial interaction
c. Aeromicrobiology
d. Water microbiology
e. Waste water treatment microbiology
f. Soil microbiology : Soil microbiology is the study of microorganisms in soil, their functions, and how they affect soil properties.
g. Microbial biogeochemical cycling
h. Role of microbes in environmental management : Microbes and their biosynthetic capabilities have been invaluable in finding solutions for several intractable problems mankind has encountered in maintaining the quality of the environment.
i. Microbial desulfurization

3. Agriculture microbiology :MICROBIAL EXPLOITATION FOR IMPROVEMENT AND CROP PROTECTION
4. CLINICAL MICROBIOLOGY : Perhaps more than a million microbial species exist in nature, but only a few hundred species cause disease
5. Food microbiology
6. Suggest reading
7. Assignment

1. Introduction

A microbe, or microorganism, is a microscopic organism that comprises either a single cell (unicellular); cell clusters; or multicellular, relatively complex organisms. The study of microorganisms is called microbiology, a subject that began with Anton van Leeuwenhoek's discovery of microorganisms in 1675, using a microscope of his own design.

Microorganisms are very diverse; they include bacteria, fungi, algae, and protozoa; microscopic plants (green algae); and animals such as rotifers and planarians. Some microbiologists also include viruses, but others consider these as nonliving. Most microorganisms are unicellular, but this is not universal, since some multicellular organisms are microscopic. Some unicellular protists and bacteria, like *Thiomargarita namibiensis*, are macroscopic and visible to the naked eye.

Microorganisms live in all parts of the biosphere where there is liquid water, including soil, hot springs, on the ocean floor, high in the atmosphere, and deep inside rocks within the Earth's crust. Most importantly, these organisms are vital to humans and the environment, as they participate in the Earth's element cycles, such as the carbon cycle and the nitrogen cycle.

Microorganisms also fulfill other vital roles in virtually all ecosystems, such as recycling other organisms' dead remains and waste products through decomposition. Microbes have an important place in most higher-order multicellular organisms as symbionts, and they are also exploited by people in biotechnology, both in traditional food and beverage preparation, and in modern technologies based on genetic engineering. Pathogenic microbes are harmful, however, since they invade and grow within other organisms, causing diseases that kill humans, animals, and plants.

2. Course Objective

Recognize and describe the characteristics of important pathogens and spoilage microorganisms in foods. Upon successful completion of this course the student will be able to: This course provides learning opportunities in the basic principles of medical microbiology and infectious disease

The course is designed to develop your ability to apply the techniques used in the different phases of industrial microbiology: discovery, production(including fermentation and scale-up),bioprocessing and cell banking. It includes the principles and practices in the main applications of micro-organisms to the industrial production of foods, pure chemicals, proteins and other useful products, including the use of genetically modified organisms. This course aims to enable graduates to enter industry with an appropriate level of understanding of the need for both the science and business aspects to be achievable to make a viable product.

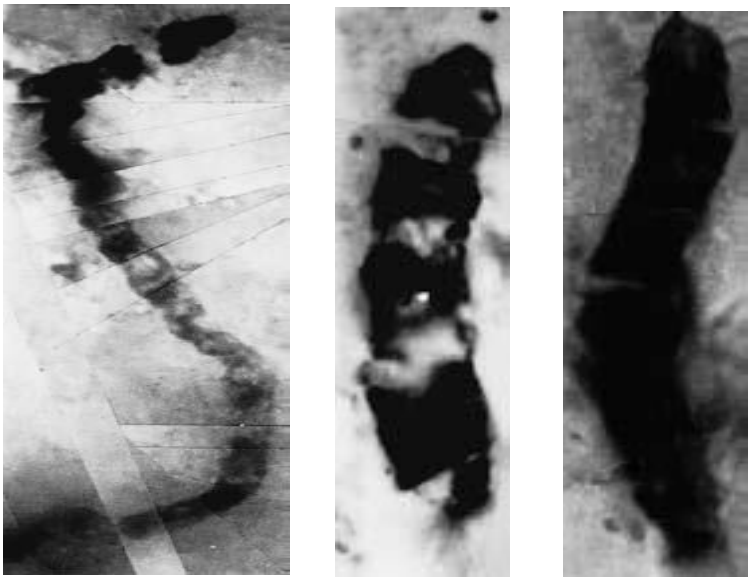
a. Microbes in environment- their evolution and diversity, RNA World, evolution of bacterial species- 16s rRNA based phylogeny.

Microbial Evolution:

Biological diversity is usually thought of in terms of plants and animals; yet, the assortment of microbial life forms is huge and largely uncharted. Consider the metabolic diversity of microorganisms— this alone suggests that the number of habitats occupied by microbes vastly exceeds that of all larger organisms. How has microbial life been able to radiate to such a bewildering level of diversity? To answer this question, one must consider microbial evolution. The field of microbial evolution, like any other scientific endeavor, is based on the formulation of hypotheses, the gathering of data, the analysis of the data, and the reformation of hypotheses based on newly acquired evidence. That is to say, the study of microbial evolution is based on the scientific method. To be sure, it is sometimes more difficult to amass evidence when considering events that occurred millions, and often billions, of years ago, but the advent of molecular biology has offered scientists a living record of life's ancient history. This chapter describes the outcome of this scientific research.

The Origin of Life

Dating meteorites through the use of radioisotopes places our planet at an estimated 4.5 to 4.6 billion years old. However, conditions on Earth for the first hundred million years or so were far too harsh to sustain any type of life. The first direct evidence of cellular life was discovered in 1977 in a geologic formation in South Africa known as the Swartkoppie chert, a granular type of silica. These microbial fossils as well as those from the Archaean Apex chert of Australia have been dated at about 3.5 billion years old (figure 1.1). Despite these findings, the microbial fossil record is understandably sparse. Thus to piece together the very early events that led to the origin of life, biologists must rely primarily on indirect evidence. Each piece of evidence must fit together like a jigsaw puzzle for a coherent picture to emerge.

(a) 

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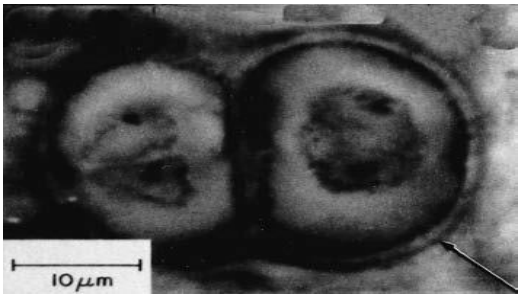

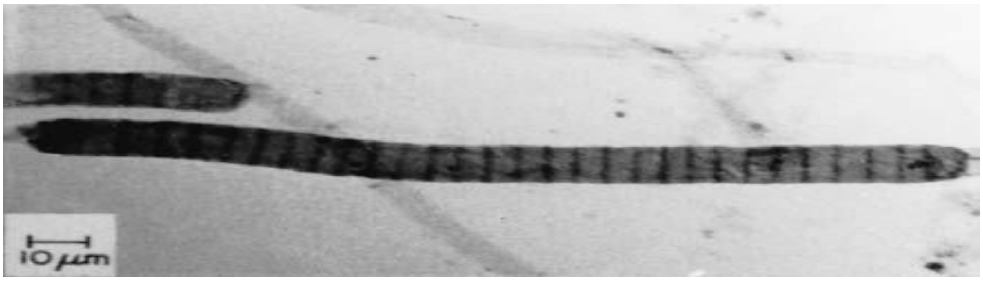

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Figure 1.1. Fossilized Bacteria. Severalmicrofossils resembling bacteria are shown, some with interpretive drawings. (a) Thin sections of Archean Apex chert from Western Australia; the fossilized remains of procaryotes are about 3.5 billion years old. (b) *Gloeodiniopsis,* about 1.5 billion years old, from carbonaceous chert in the Satka Formation of the southern Ural Mountains.The arrow points to the enclosing sheath. (c) *Palaeolyngbya,* about 950 million years old, from carbonaceous shale of the Lakhanda Formation of the Khabarovsk region in eastern Siberia.

Microbial Diversity:

In this section, we examine the wide diversity of microbial life. We shall also consider some specific examples, particularly with respect to their effect on humans. By way of introduction, however, we need to say something on the subject of the classification of microorganisms. In any discussion on biological classification, it is impossible to avoid mentioning Linnaeus, the Swedish botanist who attempted to bring order to the naming of living things by giving each type a Latin name. He even gave himself one – his real name was Carl von Linn´e! It was Linnaeus who was responsible for introducing the binomial system of nomenclature, by which each organism was assigned a genus and a species. To give a few familiar examples, you and I are Homo sapiens, the fruit fly that has contributed so much to our understanding of genetics is Drosophila melanogaster, and, in the microbial world, the bacterium responsible for causing anthrax is Bacillus anthracis. Note the following conventions, which apply to the naming of all living things (the naming of viruses is something of a special case:

- The generic (genus) name is always given a capital letter
- The specific (species) name is given a small letter
- The generic and specific name are italicised, or, if this isn't possible, underlined

The science of *taxonomy* involves not just naming organisms,but grouping them with other organisms thatshare common properties. In the early days, classification appeared relatively straightforward, with all livingthings apparently fitting into one of two *kingdoms*. Tooversimplify the matter, if it ran around, it was an animal,if it was green and didn't, it was a plant! As

ourawareness of the microbial world developed, however, itwas clear that such a scheme was not satisfactory to accommodate all life forms, and inthe mid-19th century, Ernst Haeckel proposed a third kingdom, the *Protista*, to includethe bacteria, fungi, protozoans and algae.

In the 20th century, an increased focus on the cellular and molecular similaritiesand dissimilarities between organisms led to proposals for further refinements to thethree-kingdom system. One of the most widely accepted of these has been the *five kingdomsystem* proposed by Robert Whittaker in 1969 (Figure A1).

Like some of itspredecessors, this took into account the fundamental difference in cell structure betweenprokaryotes and eukaryotes, and so placed prokaryotes (bacteria) in theirown kingdom, the *Monera*, separate from single-celled eukaryotes. Another feature ofWhittaker's scheme was to assign the *Fungi* to their own kingdom, largely on account oftheir distinctive mode of nutrition. Table A1 shows some of the characteristic featuresof each kingdom.

Molecular studies in the 1970s revealed that the *Archaea* differed from all other bacteriain their 16S rRNA sequences, as well as in their cell wall structure, membrane lipidsand aspects of protein synthesis. These differences were seen as sufficiently important for the recognition of a third basic cell type to add to the prokaryotes and eukaryotes.

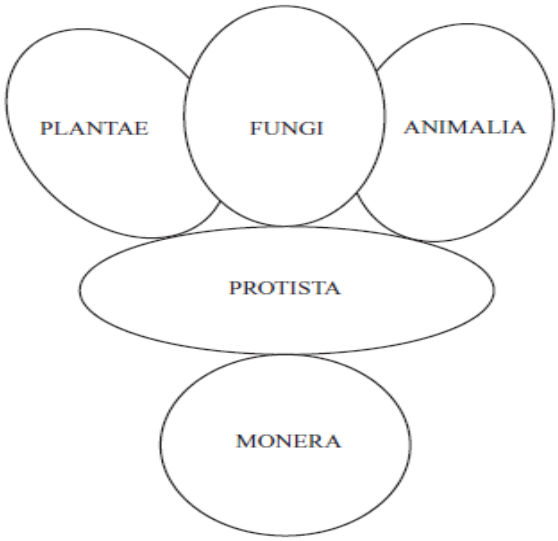


Figure A.1 Whittaker's five-kingdom system of classification

Table A.1 Characteristics of Whittaker's five kingdoms

	Monera (procaryotae)	Protista	Fungi	Plantae	Animalia
Cell type	Procaryotic	Eucaryotic	Eucaryotic	Eucaryotic	Eucaryotic
Cell organization	Unicellular; occasionally grouped	Unicellular; occasionally multicellular	Unicellular or multicellular	Multicellular	Multicellular
Cell wall	Present in most	Present in some, absent in others	Present	Present	Absent
Nutrition	Absorption, some photosynthetic, some chemosynthetic	Ingestion or absorption, some photosynthetic	Absorption	Absorptive, photosynthetic	Ingestion; occasionally in some parasites by absorption
Reproduction	Asexual, usually by binary fission	Mostly asexual, occasionally both sexual and asexual	Both sexual and asexual, often involving a complex life cycle	Both sexual and asexual	Primarily sexual

This led to the proposal of a three-domain scheme of classification, in which prokaryotes are divided into the Archaea and the Bacteria. The third domain, the Eucarya represents all eukaryotic organisms. The domains thus represent a level of classification that goes even higher than the kingdoms. Although the Archaea (the word means 'ancient') represent a more primitive bacterial form than the Bacteria, they are in certain respects more closely related to the eukaryotes, causing biologists to revise their ideas about the evolution of the eukaryotic state.

In hierarchical systems of classification, related species are grouped together in the same genus, genera sharing common features are placed in the same family, and so on. Table A2 shows a modern classification scheme for the gut bacterium *Escherichia coli.* The boundaries between long-standing divisions such as algae and protozoa have become considerably blurred in recent years, and alternative classifications based on molecular data have been proposed. This is very much a developing field, and no definitive alternative classification has yet gained universal acceptance.

of the word, that is, unicellular eukaryotic forms. It retains the traditional distinction between protozoans, algae and other protists (water moulds and slime moulds), but also offers an alternative, 'molecular' scheme, showing the putative phylogenetic relationship between the various groups of organisms. Microbiology has traditionally embraced anomalies such as the giant seaweeds, as it has encompassed all organisms that fall outside of the plant and animal kingdoms. This book offers only a brief consideration of such macroscopic forms, and for the most part confines itself to the truly microbial world.

The viruses, it ought to be clear by now, are special cases. Because an understanding of viruses requires an appreciation of the basics of DNA replication and protein synthesis.

Table A.2 A modern hierarchical classification for *E. coli*; note that in this classification, there are no kingdoms

Domain	Bacteria
Phylum	Proteobacteria
Class	Zymobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*coli*

Ever since bacteria were first identified, microbiologists have attempted to bring order to the way they are named and classified. The range of morphological features useful in the differentiation of bacteria is fairly limited (compared, say, to animals and plants), so other characteristics have also been employed. These include metabolic properties, pathogenicity, nutritional requirements, staining reactions and antigenic properties. The first edition of Bergey's Manual of Systematic Bacteriology (henceforth referred to as 'Bergey'), published in the mid-1980s, and mainly used phenotypic characteristics such as these to classify bacteria. The result placed bacteria into taxonomic groups that may or may not reflect their evolutionary relationship to one another. In the years since the first edition of Bergey, the remarkable advances made in molecular genetics have led to a radical reappraisal of the classification of bacteria. Comparison of nucleic acid sequences, notably those of 16S ribosomal RNA genes, has led to a new, phylogenetically based scheme of classification, that is, one based on how closely different groups of bacteria are thought to be related, rather than what morphological or physiological features they may share. Ribosomal RNA occurs in all organisms, and serves a similar function, thus to a large extent these sequences are conserved (remain similar) in all organisms. The nature and extent of any differences that have crept in during evolution will, therefore, be an indication of the relatedness of different organisms.

The second edition of Bergey aims to reflect this change of approach and reassign many bacteria according to their phylogenetic relationship, as deduced from molecular evidence. Due to be issued in five volumes over a number of years, the first volume was published in 2001. As an example, the genus Pseudomonas previously contained some 70 species on the basis of phenotypicsimilarities, but in the second edition of Bergey, taking into account 16S rRNA information, many of these are assigned to newly created genera.

It must be stressed that Bergey (second edition) does not represent the definitive final word on the subject, and that the classification of bacteria is very much a developing science, in a constant process of evolution. Indeed, microbiologists are by no means unanimous in their acceptance of the 'molecular' interpretation of bacterial taxonomy. Some point to perceived inadequacies in the collection of data for thescheme, as well as errors in the data arising from the sequencing and amplification techniques utilized. Other critics question the validity of a scheme based on 16S rRNA data when it seems increasingly likely that lateral gene transfer played an important role in bacterial evolution.

In the following pages, the major taxonomic groupings are discussed according to their arrangement in the second edition of Bergey. Figure 7.1 shows a phylogenetic tree, reflecting current ideas on the relationship be- tween the major bacterial groups, as determined by 16S rRNA sequencing.

Domain: Archaea

Studies on 16S ribosomal RNA sequences by Carl Woese and colleagues allowed the construction of phylogenetic trees for the prokaryotes, showing their evolutionary relatedness. Figure 7.1 shows how the major procaryotic groups are thought to be related, based on 16S rRNA data. The work of Woese also revealed that one group of prokaryotes differed from all the others. Archaea are now regarded as being quite distinct from the Bacteria (sometimes called Eubacteria). Together with the Eucarya, these form the three domains of life. As can be seen in Table 7.1, archaeashare some

features in common with other bacteria and some with eukaryotes. Extending nucleic acid analysis to other genes has shown that members of the Archaea possess many genes not found in any other type of bacteria.

Table 7.1 The three domains of life: Archaea share some features with true bacteria and others with eucaryotes

	Archaea	Bacteria	Eucarya
Main genetic material	Single closed circle of dsDNA	Single closed circle of dsDNA	True nucleus with multiple linear chromosomes
Histones	Present	Absent	Present
Introns	Common	Common	Present Rare
Gene structure	Present	Present	Absent
Plasmids			
Polycistronic mRNA	70S	70S	80S
Ribosomes Protein synthesis	Not sensitive to streptomycin, chloramphenicol	Sensitive to streptomycin, chloramphenicol	Not sensitive to streptomycin, chloramphenicol
Initiator tRNA	Methionine	N-formyl methionine	Methionine
Membrane fatty acids	Ether-linked, branched	Ester-linked, straight chain	Ester-linked, straight chain
Internal organelles	Absent	Absent	Present Mitochondria
Site of energy generation Cell wall	Cytoplasmic membrane Muramic acid absent	Cytoplasmic membrane Muramic acid present	Muramic acid absent

General features of the Archaea

Members of the Archaea show considerable diversity of both morphology and physiology. In view of the fact that the Archaea remained unidentified as a separate group for so many years, it should come as no surprise that they do not display any obvious morphological differences from true bacteria, and all the main cell shapes are rep- resented. More unusual shapes are also encountered in archaea; members of the genus *Haloarcula* have flattened square or triangular cells! Both Gram-positive and Gram- negative forms of archaea are found, but neither possesses true peptidoglycan. Some types have a so-called pseudomurein, composed of different substituted polysaccharides and L-amino acids (Figure 7.2). Most archaea, however, have cell walls composed of a layer of proteinaceous subunits known as an S-layer, directly associated with the cell membrane. This difference in cell wall chemistry means that members of the Archaea are not susceptible to antibacterial agents such as lysozyme and penicillin, whose action is directed specifically towards peptidoglycan. Differences are also found in the make-upof Archaean membranes, where the lipid component of membranes contains branched isoprenes instead of fatty acids, and these are joined to glycerol by ether-linkages, rather than the ester-linkages found in true bacteria (Figure 7.3). The diversity of archaea ex- tends into their adopted means of nutrition and metabolism: aerobic/anaerobic and autotrophic/heterotrophic forms are known. Many members of the Archaea are found in extreme environments such as deep-sea thermal vents and salt ponds. Some extreme thermophiles are able to grow at temperatures well over 100 ◦C, while psychrophilic

forms constitute a substantial proportion of the microbial population of Antarctica. Similarly, examples are to be found of archaea that are active at extremes of acidity, alkalinity or salinity. Initially it was felt that archaea were limited to such environments because there they faced little competition from true bacteria or eucaryotes. Recent stud- ies have shown however that archaea are more widespread in their distribution, making up a significant proportion of the bacterial biomass found in the world's oceans, and also being found in terrestrial and semiterrestrial niches. The reason that this lay unde- tected for so long is that these organisms cannot as yet be cultured in the laboratory, and their presence can only be inferred by the use of modern DNA-based analysis.

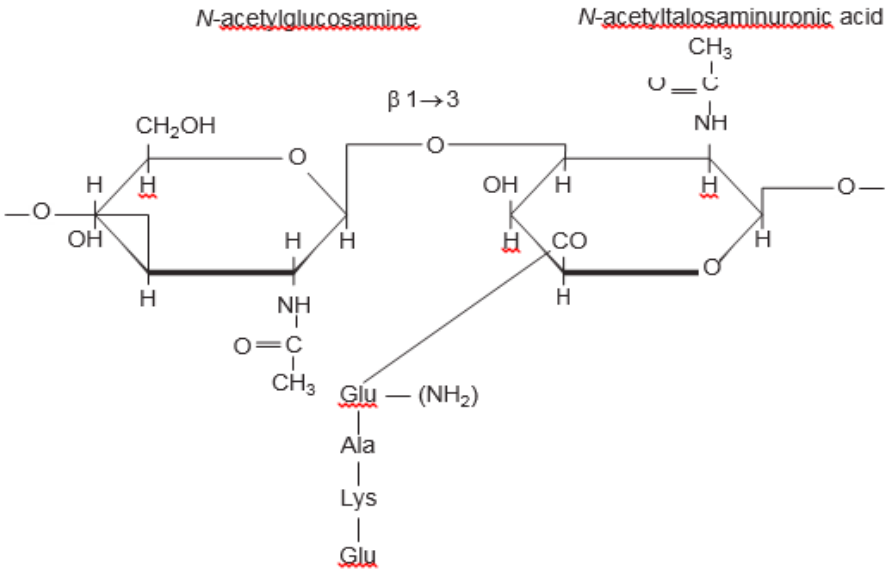


Figure 7.2 Pseudomurein, found in the cell walls of certain members of the Archaea, comprises subunits of *N*-acetylglucosamine and *N*-acetyltalosaminuronic acid. The latter replaces the *N*-acetylmuramic acid in true peptidoglycan. As with peptidoglycan, the amino acids in the peptide chain may vary, however in pseudomurein they are always of the L-form. The components in parentheses are not always present.

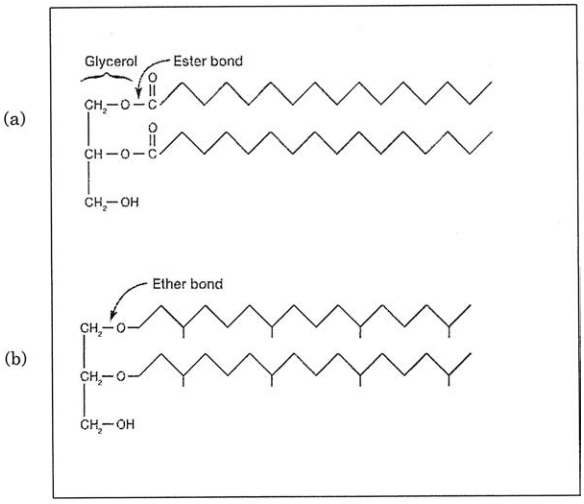


FIGURE7.3MembranelipidsinArchaeaandBacteria.Compositional differences inmembrane lipidsb etween (a)Bacteriaand (b)Archaea.Notetheetherlinkagesand branched fattyacidsin(b)

Classification of the Archaea

According to the second edition of Bergey, the Archaea are divided into two phyla, the Euryarchaeota and Crenarchaeota. A third phylum, the Korarchaeota has beenproposed, whose members are known only from molecular studies, while the recent discovery of Nanoarchaeum equitans has led to the proposal of yet another archaean phylum. Countless more species of archaea are thought to exist, which like the Korarchaeota, have not yet been successfully cultured in the laboratory.

The phylum Eurychaeota is a bigger group than the Crenarchaeota, and includes halophilic and methanogenic forms. The former are aerobic heterotrophs, requiring a chloride concentration of at least 1.5 m (generally 2.0–4.0 m) for growth. One species, *Halobacterium salinarum*, is able to carry out a unique form of photosynthesis using the bacterial pigment bacteriorhodopsin, and uses the ATP so generated for the active transport into the cell of the chloride ions it requires.

Members of the Euryarchaeota such as *Methanococcus* and *Methanobacterium* are unique among all life forms in their ability to generate methane from simple carbon com- pounds. They are strict anaerobes found in environments such as hot springs, marshes and the gut of ruminant mammals. The methane is derived from the metabolism of various simple carbon compounds such as carbon dioxide or methanol in reactions linked to the production of ATP. e.g.

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$$
$$CH_3OH + H_2 \longrightarrow CH_4 + H_2O$$

In addition, a few species can cleave acetate to produce methane:

$$CH_3COO^- + H_2O \longrightarrow CH_4 + HCO_3^-$$

This acetotrophic reaction is responsible for the much of the methane production in sewage sludges. Although sharing the unique facility to generate methane, some of the methanogenic genera are quite distantly related to one another.

Other representatives of the Eurychaeota include the Thermoplasmata and the Thermococci. Thermoplasmata are highly acidophilic and moderately ther- mophilic; they completely lack a cell wall, and are pleomorphic. A unique membrane lipid composition allows them to withstand temperatures of well over 50 ◦C. Thermococci are anaerobic extreme thermophiles found in anoxic thermal waters at temperatures as high as 95 ◦C. Enzymes isolated from thermococci have found a variety of applications. A thermostable DNA polymerase from *Pyrococcus furiosus* is used as an alternative to *Taq* polymerase (see Phylum 'Deinococcus-Thermus' later in this chapter) in the polymerase chain reaction (PCR).

Representative genera: *Methanobacterium, Halobacterium*

Members of the **Crenarchaeota** are nearly all extreme thermophiles, many of them capable of growth at temperatures in excess of 100 ◦C, including *Pyrolobus fumarii,* which has an optimum growth temperature of 106 ◦C, and can survive autoclaving at 121 ◦C.

Many utilise inorganic sulphur compounds as either a source or acceptor of electrons (respectively, oxidation to H_2SO_4 or reduction to H_2S).

Crenarchaeotes are mostly anaerobic, and are thought by many to resemble the common ancestors of all bacteria.

Representative genera- *Thermoproteus, Sulfolobus*

Domain: Bacteria

All the remaining bacterial groups belong to the domain Bacteria. This is divided into 23 phyla (Table 7.2), the more important of which are discussed in the following pages, according to their description in the second edition of *Bergey*. As with the Archaea, many other forms are known only through molecular analysis and it is estimated that these represent at least another 20 phyla.

Phylum: Proteobacteria : We start our survey of the Bacteria with the Proteobacteria. This is by far the biggest single phylum, and occupies the whole of volume 2 in the second edition of *Bergey.* The size of the group is matched by its diversity, both morphological and physiological; most forms of metabolism are represented, and the wide range of morphological forms gives rise to the group's name. (Proteus was a mythological Greek god who was able to assume many different forms.)

Table 7.2 Phyla of domain Bacteria

Phylum	**Aquificae**
Phylum	**Thermotogae**
Phylum	Thermodesulfobacteria
Phylum	**'Deinococcus–Thermus'** *
Phylum	Chrysiogenetes
Phylum	**Chloroflexi**
Phylum	Thermomicrobia
Phylum	**Nitrospira**
Phylum	Deferribacteres
Phylum	**Cyanobacteria**
Phylum	**Chlorobi**
Phylum	**Proteobacteria**
Phylum	**Firmicutes**
Phylum	**Actinobacteria**
Phylum	**Planctomycetes**
Phylum	**Chlamydiae**
Phylum	**Spirochaetes**
Phylum	Fibrobacteres
Phylum	Acidobacteria
Phylum	**Bacteroidetes**
Phylum	Fusobacteria
Phylum	**Verrucomicrobia**
Phylum	Dictyoglomi

The reason such a diverse range of organismshave been assigned to a single taxonomic grouping is that their 16S rRNA indicates a common ancestor (thought to be photosynthetic, though few members now retain this ability). At the time of writing more than 460 genera and 1600 species had been iden- tified, all of them Gram-negative and representing almost half of all accepted bacterial genera. These include many of the best known Gram-negative bacteria of medical, in- dustrial and agricultural importance. For taxonomic purposes, the Proteobacteria

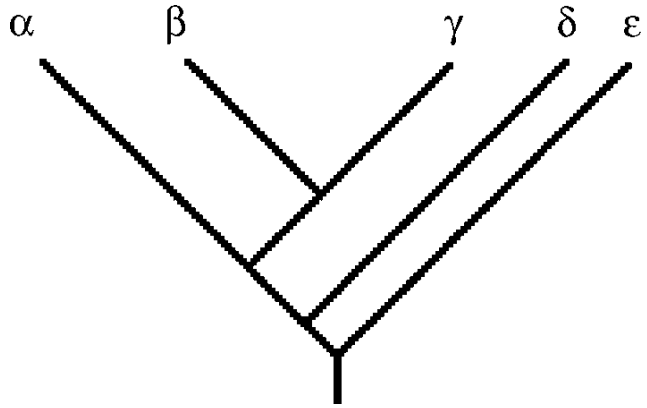
have been divided into five classes reflecting their presumed lines of descent and termed the Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria andEpsilonproteobacteria(Figure7.4).Itshouldbestressedthatbecauseclassification is based on molecular relatedness rather than shared phenotypic traits, few if any mor- phological or physiological properties can be said to be characteristic of all members of each class. Equally, organisms united by a particular feature may be found in more than one of the proteobacterial classes, for example nitrifying bacteria are to be found in the ***α***, ***β*** and ***γ*** Protobacteria. For this reason, in the following paragraphs we describe the Proteobacteria interms of their *phenotypi c*characteristics ratherthan attempt to group them phylogenetically.

Photosynthetic Proteobacteria

The purple sulphur and purple non-sulphur bacteria are the only members of the Pro- teobacteria to have retained the photosynthetic ability of their presumed ancestor. The type of photosynthesis they carry out, however, is quite distinct from that carried out by plants, algae and cyanobacteria (see later in this chapter), differing in two important respects:

- it is anoxygenic – no oxygen is produced by the process
- it utilizes bacteriochlorophyll *a* and/or *b*, which have different absorbance properties to chlorophylls *a* and *b*.

Like organisms that carry out green photosynthesis, however, they incorporate CO_2 into carbohydrate by means of the Calvin cycle (see Chapter 6). All are at least facultativelyanaerobic, and are typically found in sediments of stagnant lakes and salt marsh pools, where they may form extensive coloured blooms. Because the absorption spectrum of bacteriochlorophylls lies mostly in the infrared part of the spectrum, they are able to utilise



light energy that penetrates beyond the surface layers of water.

FIGURE7.4The phylogenetic relationships of the Proteobacteria, based on 16S rRNA sequences

The coloration, ranging from orange/brown to purple, is due to the presence of carotenoid pigments such as lycopene and spirillixanthin, which mask the blue/green colour of the bacteriochlorophylls. The photosynthetic pigments are located on highly folded extensions of the plasmamembrane.Photosynthetic proteobacteria include rods, cocci and spiralforms.

Under anaerobic conditions, the purple sulphur bacteria typically utilise hydrogen sulphide or elemental sulphur as an electron donor for the reduction of CO_2.

$$H_2S + CO_2 \longrightarrow (CH_2O)_n + S^0$$
$$S^0 + CO_2 + H_2O \longrightarrow (CH_2O)_n + H_2SO_4$$

Many store sulphur in the form of intracellular granules. The purple sulphur bacteria allbelongtotheγ-Protobacteria.They are typically found in surface muds,and sulphur springs,habitats that provide the right combination of light and anaerobic conditions.

Representative genera: *Thiospirillum, Chromatium*

The purple non-sulphur bacteria were distinguished from the above group because of their apparent inability to use H_2S as an electron donor. It is now known, however,that the majority *can* do this, but are able to tolerate much lower concentrations in comparison with the purple sulphur bacteria. The purple non-sulphur bacteria are fac- ultative anaerobes able to grow as photoheterotrophs, that is, with light as an energy source and a range of organic molecules such as carbohydrates and organic acids as sources of both carbon and electrons. In addition, many are able to grow aerobically as chemo heterotrophs in the absence of light.Under present classification systems,purple nonsulphur bacteria are divided betweentheα-andβ-Proteobacteria.

Representative genera: *Rhodospirillum, Rhodopseudomonas*

Nitrifying Proteobacteria : This group comprises aerobic Gram-negative chemolithoautotrophs that derive their energy from the oxidation of inorganic nitrogen compounds (either ammonia or nitrite), and their carbon from CO_2. The nitrifying bacteria are represented in both the α- and β-Proteobacteria.

Representative genera: *Nitrosomonas* ($NH_4^+ \longrightarrow NO_2^-$)
Nitrobacter ($NO_2^- \longrightarrow NO_3^-$)

Iron- and sulphur-oxidising Proteobacteria

Two further groups of environmentally significant chemolithoautotrophs derive their energy through the oxidation of reduced iron and sulphur respectively.

Among the sulphur oxidisers, perhaps the best studied are members of the genus *Acidithiobacillus**, which includes extreme acidophiles such as *A. thiooxidans* that are capable of growth at a pH as low as 1.0! These may utilise sulphur in its elemental form, as H_2S, metal sulphides, or other forms of reduced sulphur such asthiosulphate:

$2S^0 + 3O_2 + 2H_2O \longrightarrow 2H_2SO_4\ S^{2-} +$
$4O_2 \longrightarrow 2SO_4^{2-}$

$S_2O_3^{2-} + 2O_2 + H_2O \dashrightarrow 2SO_4^{2-} + 2H^+$

Hydrogen-oxidising Proteobacteria

This diverse group of bacteria are united by their ability to derive energy by using hydrogen gas as a donor of electrons, and oxygen as an acceptor:

$$2H_2 + O_2 \dashrightarrow 2H_2O$$

Nearly all the members of this group are facultative chemolithotrophs, i.e. they can also grow as heterotrophs, utilising organic compounds instead of CO_2 as their carbon source,andindeedmostgrowmoreefficientlyinthisway.

Representative genera: *Alcaligenes*, *Ralstonia*

Nitrogen-fixing Proteobacteria

The α-Proteobacteria includes certain genera of **nitrogen-fixing bacteria**. These are able to fix (reduce) atmospheric N_2 as NH_4^+ for subsequent incorporation into cellular materials, a process that requires a consid- erable input of energy in the form of ATP:

$N_2 + 8e^- + 8H^+ + 16ATP \dashrightarrow, 2NH_4^+ + 16ADP + 16Pi$

Nitrogen-fixing bacteria may be free-living in the soil (e.g. *Azotobacter*), or form a symbiotic relationship with cells on the root hairs of leguminous plants such as peas, beansandclover(e.g.*Rhizobium*).The nitrogen as eresponsible for therea ction(actually a complex of two enzymes) is highly sensitive to oxygen; many nitrogen fixers are anaerobes, while others have devised ways of keeping the cell interior oxygen-free. NitrogenfixationisdiscussedfurtherinChapters15and16.

Closely related to Rhizobium,but unable to fix nitrogen,are members of the genus Agrobacterium. LikeRhizobium,these enterthetissues of plants,but instead of forming a mutually beneficial association, cause cell proliferation and tumour formation. A. tumefaciens has proved to be a valuable tool in the genetic engineering of plants.

Representative genera: Rhizobium, Azotobacter

Methanotrophic Proteobacteria

Methanotrophs are strict aerobes, requiring oxygen for the oxidation of methane. The methane generating bacteria, however, as we've seen are anaerobes; methanotrophs are consequently to be found at aerobic/anaerobic interfaces such as topsoil, where theycan find both the oxygen and the methane they require. The methane is firstly oxidised to methanol, then to formaldehyde, by means of separate enzyme systems. Some of the carbon in formaldehyde is assimilated into organic cellular material, while some is further oxidised to carbon dioxide.

Bacteria able to utilise other single-carbon com- pounds such as methanol (CH_3OH) or methylamine (CH_3NH_2) are termed *methylotrophs*. Depending on whether they possess the enzyme methane monooxygenase (MMO), they may also be methanotrophs.

Representative genera: *Methylomonas, Methylococcus*

Sulphate- and sulphur-reducing Proteobacteria

Some 20 genera of anaerobic δ-Proteobacteria reduce either elemental sulphur or oxi- dised forms of sulphur such as sulphate to hydrogen sulphide. Organic compounds such as pyruvate, lactate or certain fatty acids act as electron donors.

Sulphate and Sulphur reducers are found in anaerobic muds and play an important role in the global sulphur cycle.

Representative genera: *Desulfovibrio* (sulphate), *Desulfuromonas* (sulphur)

Enteric Proteobacteria

This is a large group of rod-shaped bacteria, mostly motile by means of peritrichous flagella, which all belong to the γ -Proteobacteria. They are facultative aerobes, charac- terised by their ability in anaerobic conditions to carry out fermentation of glucose and other sugars to give a variety of products. The nature of these products allows division into two principal groups, the mixed acid fermenters and the butanediol fermenters (Figure 6.23). All the enteric bacteria test negative for cytochrome *c* oxidase (see *Vibrio* and related genera below). In view of their similar appearance, members of the group are distinguished from one another largely by means of their biochemical characteris- tics. An unknown isolate is subjected to a series of tests including its ability to utilise substrates such as lactose and citrate, convert tryptophan to indole, and hydrolyse urea. On the basis of its response to each test, a characteristic profile can be built up for the isolate, and matched against those of known species (see Table 7.3).

The most thoroughly studied of all bacteria, *Escherichia coli* (*E. coli*) is a member of this group, as are a number of important pathogens of humans such as *Salmonella*, *Shigella* and *Yersinia* (the causative agent of plague).

Representative genera: *Escherichia, Enterobacter*

Table 7.3 Identification of enteric bacteria on the basis of their biochemical and other properties

Some of the tests used to identify isolates of enteric bacteria are listed below. The table on the next page indicates typical results obtained for common – genera; note, however, that for many cases, the result of a test may vary for different species within a genus. The symbols and indicate that most or all species within a genus give a positive or negative result, whilst / denotes that results are more variable within a genus.

Test	Description
Indole	Tests for ability to produce indole from the amino acid tryptophan.
Methyl Red	Acid production causes methyl red indicator to turn red.
Voges-Proskauer	Tests for ability to ferment glucose to acetoin.
Citrate utilisation	Demonstrates ability to utilise citrate as sole carbon source.
Urease	Demonstrates presence of the enzyme urease by detecting rise in pH due to urea being converted to ammonia and CO_2.
Gas from sugars	Production of gas from sugars such as glucose is demonstrated by collection in a Durham tube (a small inverted tube placed in a liquid medium).
H_2S production	Production of H_2S from sulphate reduction or from sulphur-containing amino acids is demonstrated by the formation of black iron sulphide in an iron-rich medium.
Ornithine decarboxylase	Growth on medium enriched in ornithine leads to pH change when enzyme is present.
Motility	Diffusion through soft agar demonstrates cellular movement.
Gelatin liquefaction	Demonstrates presence of proteolytic enzymes capable of liquefying a medium containing gelatin.
% age GC	Nucleotide composition determined by melting point measurements.

Vibrio and related genera

A few other genera, including *Vibrio* and *Aeromonas*, are also facultative anaerobes able to carry out the fermentative reactions described above, but are differentiated from the enteric bacteria by being oxidase-positive (Table 7.4). *Vibrio* and *Photobacterium* both include examples of marine *bioluminescent*species; these are widely found both in seawater and associated with fish and other marine life. The luminescence, which requires the presence of oxygen, is due to an oxidation reaction carried out by the enzyme *luciferase*.

Vibrio cholerae is the causative agent of cholera, a debilitating and often fatal form of acute diarrhoea transmitted in faecally contaminated water. It remains a major killer in many third world countries. Several species of *Vibrio*, including *V. cholerae*, have been shown to possess two circular chromosomes instead of the usual one.

Representative genera: *Vibrio, Aeromonas*

Acetic acid bacteria

Acetobacter and *Gluconobacter* are two genera of the α-Proteobacteria that convert ethanol into acetic acid, a highly significant reaction in the food and drink industries (see Chapter 17). Both genera are strict aerobes, but unlike *Acetobacter*, which can oxidise the acetic acid right through to carbon dioxide and water, *Gluconobacter* lacks all the enzymes of the TCA cycle, and cannot oxidise it further.

Acetobacter species also have the ability, rare in bacteria, to synthesise cellulose; the cells become surrounded by a mass of extracellular fibrils, forming a pellicle at the surface of an unshaken liquid culture.

Representative genera: *Acetobacter, Gluconobacter*

Stalked and budding Proteobacteria : The members of this group of aquatic Proteobacteria differ noticeably in their appear- ance from typical bacteria by their possession of extracellular extensions known as *prosthecae*; these take a variety of forms but are always narrower than the cell itself. They are true extensions of the cell, containing cytoplasm, rather than completely ex- tracellular appendages.

In the stalked bacteria such as *Caulobacter* (Figure 7.5), the prostheca serves both as a means of attaching the cell to its substratum, and to enhance nutrient absorption byincreasing the surface area-to-volume ratio of the cell. The latter enables such bacteria to live in waters containing extremely low levels of nutrient. *Caulobacter* lives part of its life cycle as a free-swimming swarmer cell with no prostheca but instead a flagellum for mobility.

The iron oxidiser *Gallionella* (see Nitrifying Proteobacteria above) may be regarded as a stalked bacterium, however it is not truly prosthecate, as its stalk does not contain cytoplasm.

In the budding bacteria, the prostheca is involved in a distinctive form of reproduc- tion, in which two cells of unequal size are produced (c.f. typical binary fission, which results in two identical daughter cells). The daughter cell buds off from the mother cell, either directly, or as *Hyphomicrobium* spp. at the end of a hypha (stalk) (Figure 7.6). Once detached, the daughter cell grows to full size and eventually produces its own buds. *Hyphomicrobium* is a methanotroph and a methylotroph, so it also belongs to the methanotrophs described earlier.

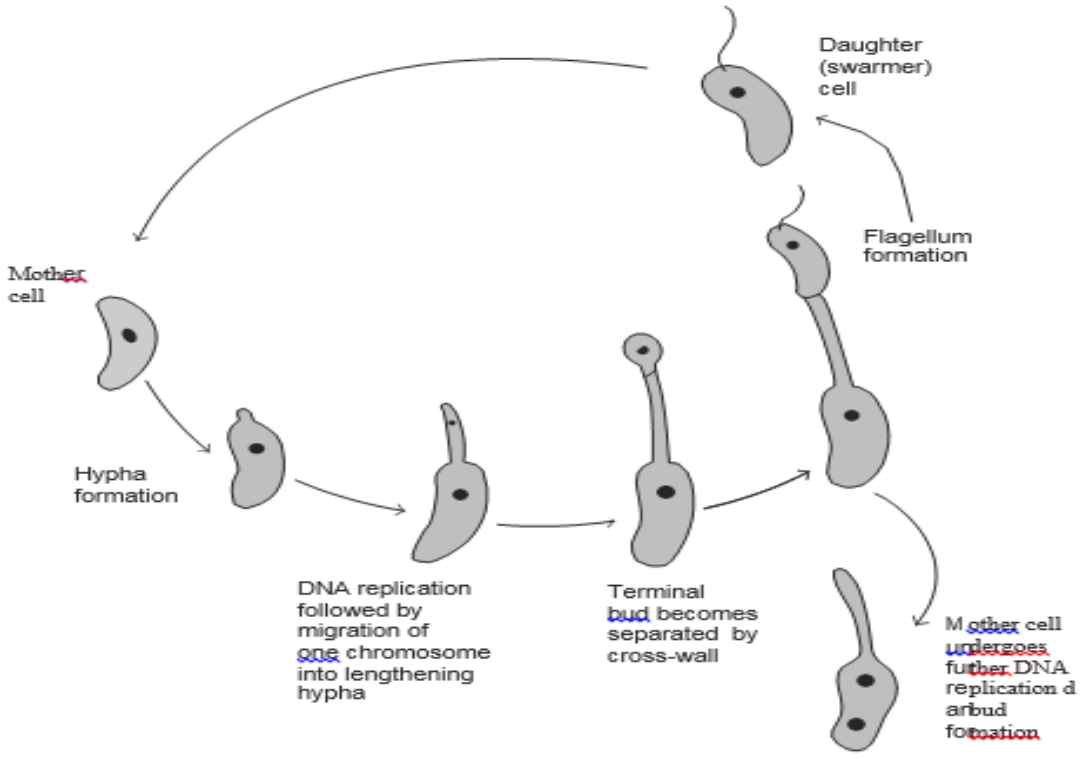


FIGURE 7.6 The budding bacteria: reproduction in *Hyphomicrobium*. Before reproduction takes place, the vegetative cell develops a stalk or hypha, at the end of which a bud develops. This produces a flagellum, and separates to form a motile swarmer cell

In some bacteria, more than one prostheca is found per cell; these *polyprosthecate* forms include the genus *Stella*, whose name ('a star') derives from its six symmetrically arranged buds.

Representative genera: *Caulobacter, Hyphomicrobium*

Sheathed Proteobacteria

Some genera of β Proteobacteria exist as chains of cells surrounded by a tube-like sheath, made up of a carbohydrate/protein/lipid complex. In some cases, the sheath contains deposits of manganese oxide or ferric hydroxide, which may be the product of chemical or biological oxidation. Empty sheaths encrusted with oxides may remain long after the bacterial cells have died off or been released. As with the stalked bacteria (see above) the sheath helps in the absorption of nutrients, and may also offer protection against predators.

The sheathed bacteria have a relatively complex life cycle. They live in flowing water, and attach with one end of the chain to, for example, a plant or rock. Free-swimming single flagellated cells are released from the distal end and settle at another location, where a new chain and sheath are formed (Figure 7.7).

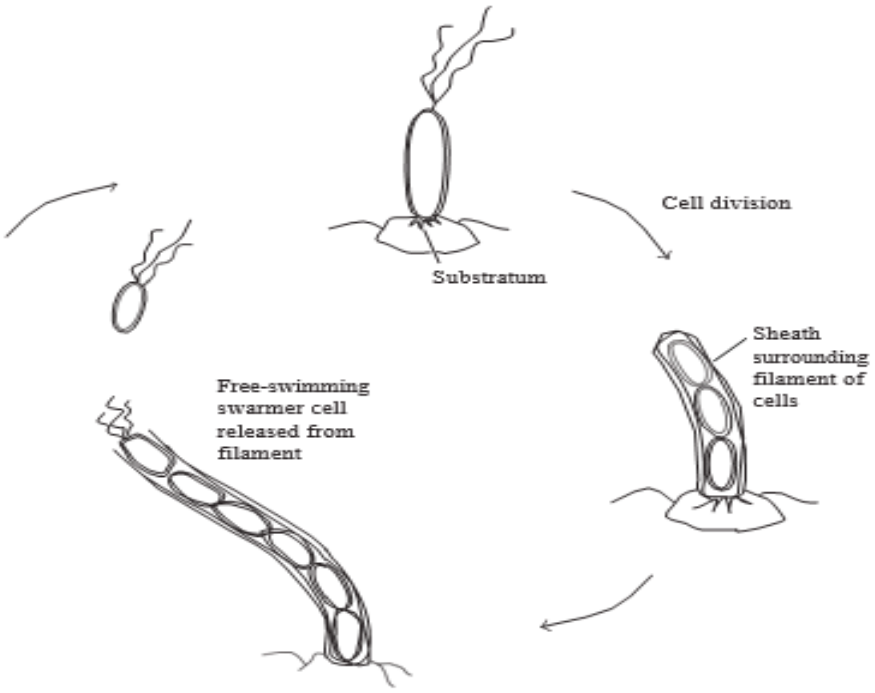


FIGURE 7.7 The sheathed bacteria. The life cycle of *Sphaerotilus*. Free-swimming swarmer cells settle on an appropriate substratum and give rise to long filaments contained within a sheath. New locations become colonised when flagellated cells are released into the water to complete the cycle

Sphaerotilus forms thick 'streamers' in polluted water, and is a familiar sight around sewage outlets.

Representative genera: *Sphaerotilus, Leptothrix*

Predatory Proteobacteria

Bdellovibrio is a unique genus belonging to the *δ*- Proteobacteria. It is a very small comma-shaped bacterium, which actually attacks and lives inside other Gram-negative bacteria (Figure 7.8). Powered by its flagellum, it collides with its prey at high speed and penetrates even thick cell walls by a combination of enzyme secretion and mechanical boring. It takes up residence in the periplasmic space, between the plasma membrane and cell wall. The host's nucleic acid and protein synthesis cease, and its macromolecules are degraded, pro- viding nutrients for the invader, which grows into a longhelical cell. This eventually divides into several motile progeny cells, which are then released.

Representative genus: *Bdellovibrio*

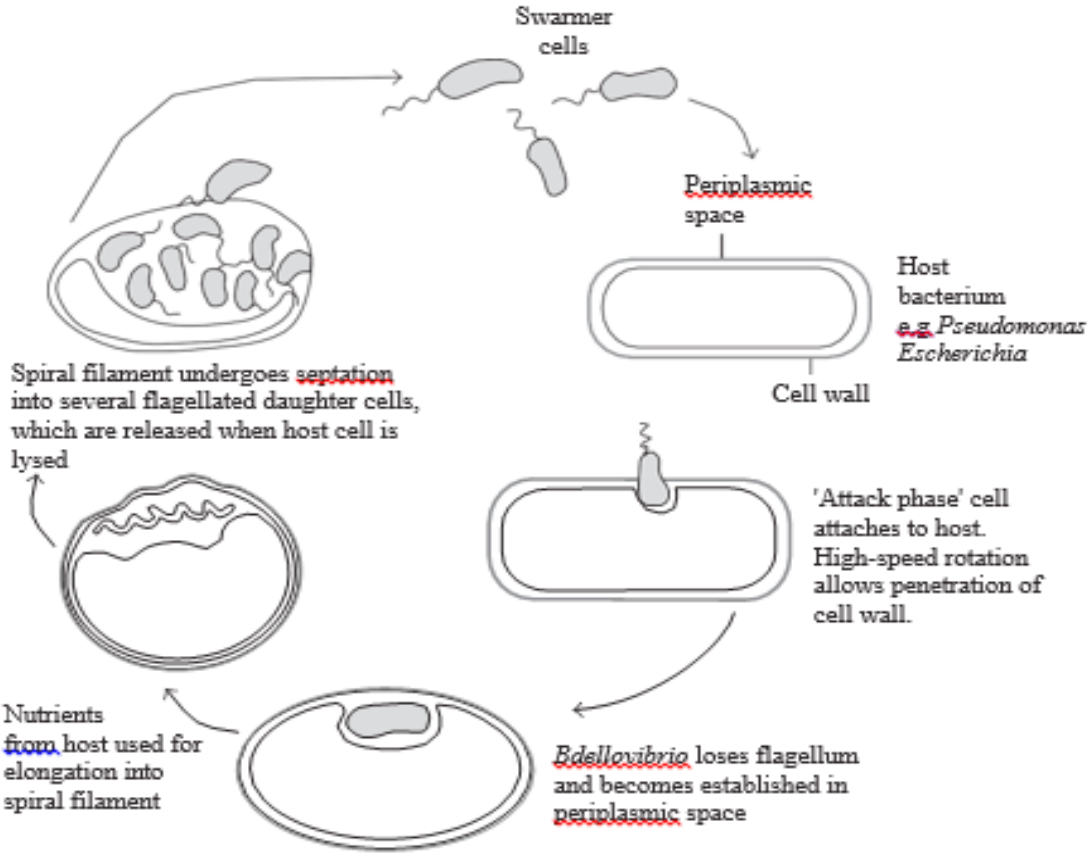


FIGURE 7.8 The life cycle of *Bdellovibrio*, a bacterial predator. Once *Bdellovibrio* has taken up residence in the periplasmic space of its host, it loses its flagellum and becomes non-motile. In nutrient-rich environments, *Bdellovibrio* is also capable of independent growth

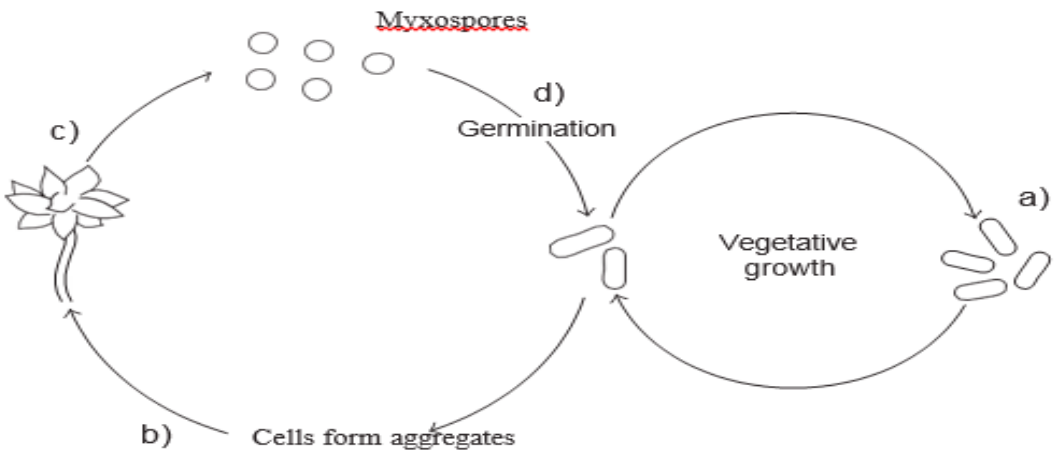


FIGURe 7.9 The Myxobacteria: a complex bacterial life cycle. When nutrients are in plen- tiful supply, myxobacteria divide by binary fission (a). On depletion of nutrients, they form aggregates of cells, which leads to the formation of a fruiting body (b). Within the fruit- ing body, some cells form myxospores, enclosed within a sporangium (c). Myxospores re- main dormant until environmental conditions are favourable, then germinate into vegetative cells (d)

Another group of bacteria that may be regarded as predatory are the Myxobacteria (Figure 7.9). These are rod-shaped bacteria lacking flagella, which yet are motile by gliding along a solid surface,aided by the excretion of extracellular polysaccharides. For this reason they are some times referred to as the *glidingbacteria*.Theyareheterotrophs, typically requiring complex organic nutrients, which they obtain by the lysis of other types of bacteria. Thus, unlike *Bdellovibrio*, they digest their prey before they ingest it. Whenarichsupplyofnutrientsisnotavailable,many thousands of ellsmayaggregate to form *fruiting bodies*, inside which *myxospores* develop. These are able to resist drought and lack of nutrients for many years. Myxobacteria exhibit the most complex lifecyclesofanyprocaryotesofarstudied.

Representative genera: *Myxococcus, Chondromyces*

Spirilla

Spirilla such as *Aquaspirillum* and *Magnetospirillum* contain *magnetosomes*, intra- cellular particles of iron oxide (magnetite, Fe_3O_4). Such magnetotactic bacteria have theremarkableabilitytoorientatethemselveswithrespecttotheearth's magneticfield (*magnetotaxis*).

Two important pathogens of humans are included in the spirilla; *Campylobacter jejuni* is responsible for foodborne gastroenteritis,while *Helicobacterpylori*hasinrecent timesbeenidentifiedasthecauseofmanycasesofpepticulcers.

Representative genera: *Magnetospirillum, Campylobacter*

Rickettsia : This group comprises arthropod-borne intracellular parasites of vertebrates, and in- cludes the causative agents of human diseases such as *typhus* and *Rocky Mountain spotted fever*. The bacteria are taken up by host phagocytic cells, where they multiply and eventually cause lysis.

The Rickettsia are aerobic organotrophs, but some possess an unusual mode of en- ergy metabolism, only being able to oxidise intermediate metabolites such as glutamate and succinate, which they obtain from their host. *Rickettsia* and *Coxiella*, the two main genera, are not closely related phylogenetically and are placed in the α- and γ - Proteobacteria, respectively.
Representative genera: *Rickettsia, Coxiella*

Neisseria and related Proteobacteria
All members of this loose collection of bacteria are aerobic non-motile cocci, typically seen as pairs, with flattened sides where they join. Some however only assume this morphologyduringstationarygrowthphase.Manyarefoundinwarm-bloodedanimals, and some species are pathogenic. The genus *Neisseria* includes species responsible for gonorrhoea and meningitis inhumans.

Other Gram-negative phyla The following section considers those Gram-negative bacteria not included in the Pro- teobacteria. These phyla are not closely related in the phylogenetic sense, either to each other or to the Proteobacteria.

PHYLUMCyanobacteria:the BLUE-GReenbacteria

The Cyanobacteria are placed in volume 1 of the second edition of *Bergey*, along with the Archaea (see above), the deeply branching bacteria, the 'Deinococcus–Thermus' group, and the green sulphur and green non-sulphur bacteria.

Members of the Cyanobacteria were once known as blue-green algae because they carry out the same kind of *oxygenic photosynthesis* as algae and green plants

They are the only group of procaryotes capable of carrying out this form of photosynthesis;all the other groups of photosynthetic bacteria to be discusse dinthis chapter carry out an anoxygenic form. When it became possible to examine cell struc- ture in more detail with th eelectron microscope,it became clear at the cyanobacteria were in fact procaryotic, and hence quite distinct from the true algae. Old habits die hard, however, andthe term 'blue-green algae' is still encountered, particularly in the popular press. Being procaryotic, cyanobacteria do not possess chloroplasts; however they contain lamellar membranes called *thylakoids*, which serve as the site of photo- synthetic pigments and as the location for both light-gathering and electron transfer processes. Early members of the Cyanobacteria evolved when the oxygen content of the earth's atmosphere was much lower than it is now, and these organisms are thought to have been responsible for its gradual increase, since photosynthetic eucaryotes did not arise until many millions of years later.

Cyanobacteria are Gram-negative bacteria which may be unicellular or filamentous; in spite of the name by which they were formerly known, they may also appear variously as red, black or purple, according to the pigments they possess. A characteristic ofmanycyanobacteriaistheabilitytofixatmosphericnitrogen,thatis,toreduceitto ammonium ions (NH_4^+) for incorporation into cellular constituents.

In filamentous forms, this activity is associated with specialised, enlarged cells called *heterocysts*(Figure 7.10).

The tiny unicellular cyanobacterium *Prochlorococcus* is found in oceans throughout the tropical and temperate regions and is thought to be the most abundant photosynthetic organism on our planet. It has several strains adapted to different light conditions. Some cyanobacteria are responsible for the production of unsightly (and smelly!) 'Algal' blooms in waters rich in nutrients such as phosphate. When they die, their decomposition by other bacteria leads to oxygen depletion and the death of other aquatic life forms. Bloom-forming species contain gas vacuoles to aid their buoyancy

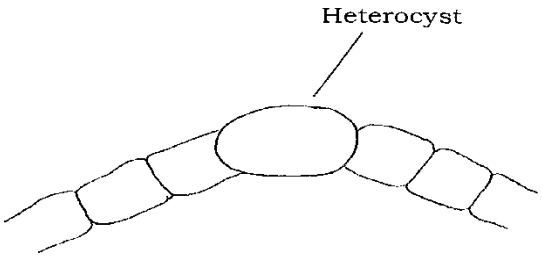


FIGURE7.10 Cyanobacteria.Nitrogenfixation takes place in specialized cells called hetero-cysts,which develop from ordinary cells when supplies of available nitrogen(e.g.ammonia) aredepleted.The heterocyst losesits ability to photosynthesiseand,therefore,to produc eoxygen. This is essential because oxygen is highly inhibitory to the nitrogenase enzyme complex

Phylum Chlorobi (green sulphur bacteria) and phylum Chloroflexi (green non-sulphur bacteria)

We have already come across three distinct groups of photosynthetic bacteria in this chapter: the purple sulphur and purple nonsulphur bacteria and the Cyanobacteria: here we consider the remaining two groups, the green sulphur and green non-sulphur bacteria.

The green sulphur bacteria (phylum Chlorobi), like their purple counterparts (see above), are anaerobic photolithotrophs that utilise reduced sulphur compounds instead of water as an electron donor, and generate elemental sulphur. They differ, however, in a number of respects. The sulphur is deposited *outside* the cell, and CO_2 is assimilated not by the Calvin cycle, but by a reversal of the steps of the TCA cycle (see Chapter 6). The photosynthetic pigments in the green sulphur bacteria are contained in sac-like structures called *chlorosomes* that are associated with the inside of the plasma membrane.

Most members of the green non-sulphur bacteria (phylum Chloroflexi) are filamen- tous thermophiles, living in non-acid hot springs, where they form thick bacterial mats. Like the purple non-sulphur bacteria, they are photoheterotrophs, but can also grow in the dark as chemoheterotrophs.

Representative genera: *Chlorobium* (green sulphur), *Chloroflexus* (green non-sulphur).

Phylum Aquificae and phylum Thermotogae: the deeply branching bacteria

These two phyla are regarded as the two deepest branches in the evolution of the Bacteria and both comprise highly thermophilic Gram-negative rods. They are the only members of the Bacteria that can compare with the Archaea in their ability to live at high temperatures (optimal growth >80 °C). The two phyla differ in their mode of nutrition: the Aquificae are autotrophs capable of oxidising hydrogen or sul-phur,while the Therm otogaeareanaerobic heterotrophs,fermenting carbohydrates.

Members of the Thermotogae are characteristically surrounded, sometimes in a chain, by a proteinaceous sheath (or 'toga').

Representative genera: *Aquifex* (phylum Aquificae), *Thermotoga* (phylum Thermotogae)

PHYLUM'DEINOCOCCUS–THERMUS

Only thre genera are included in this phylum,but two of these are of particularinter- est because of remarkable physiological properties. *Thermus* species are thermophiles whose best known member is *T.aquaticus*;this is the source of the thermostableen- zyme *Taq* polymerase, used in the polymerase chain reaction (Chapter 12).*Deinococcus* species show an extraordinary degree of resistance to radiation,due,itseems,toanun- usually powerful DNA repairsystem.This also enables *Deinococcus* to resist chemical mutagens(seeChapter12).Both*Deinococcus* and

*Thermu s*species have anunusualre- finement to the structure of the irpeptidoglycan,with ornithine replacing diaminopimelic acid (Figure7.11).

Representative genera: *Deinococcus*, *Thermus*

PHYLUMPlanctomycetes

This very ancient group of bacteria has a number of unusual properties, including cell division by budding, the lack of any peptidoglycan in their cell walls and the presenceof a degree of internal compartmentalisation. (Recall from Chapter 3 that membrane- bound compartments are regarded as a quintessentially *eucaryotic* feature.)

The recently discovered reaction known as *anammox* (anaerobic ammonium oxida- tion), whereby ammonium and nitrite are converted to nitrogen gas:

$$NH_4^+ + NO_2^- = N_2 + 2H_2O$$

has been attributed to certain members of the Planctomycetes. It is thought that this reaction may be responsible for much of the nitrogen cycling in the world's oceans. The bacteria concerned have only been identified by means of their rRNA gene sequences, and have as yet been assigned only provisional generic and specific names. They are anaerobic chemo

lithoautotrophs, however this is not typical of the Planctomycetes, most of which are aerobic chemoorganoheterotrophs.

Recent studies propose that the Planctomycetes should be placed much closer to the root of any proposed phylogenetic tree than had previously been proposed.

Representative genera: *Planctomyces, Pirellula*

Phylum Chlamydiae

Formerly grouped with the Rickettsia (see above), these non-motile obligate parasites of birds and mammals are now assigned a separate phylum comprising only five genera, of which *Chlamydia* is the most important. Like the Rickettsia, members of the Chlamydiae have extremely small cells, and very limited metabolic capacities, and depend on the host cell for energy generation. Unlike that group, however, they are not dependent on an arthropod vector for transmission from host to host.

Chlamydia trachomatis is the causative agent of trachoma, a major cause of blindness in humans. Different strains of this same species are responsible for one of the most common forms of sexually transmitted disease. *C. psittaci* causes the avian disease psittacosis, and *C. pneumoniae* causes chlamydial pneumonia in humans as well as being linked to some cases of coronary artery disease.

Representative genus: *Chlamydia*

PHYLUMSpirochaetes

The Spirochaetes are distinguished from all other bacteria by their slender helical mor- phology and corkscrew-like movement. This is made possible by *endoflagella* (axial filaments),so-called because they are enclose dinthespace between the cell and a flexi- ble sheath that surroundsit.

Spirochaetes comprise both aerobic and anaerobic bacteria that inhabit a wide range of habitats, including water and soil as well as the gut and oral cavities of both vertebrate and invertebrate animals. Some species are important pathogens of humans, including *Treponema pallidum* (syphilis) and *Leptospira interrogans* (leptospirosis).

Phylum Bacteroidetes

No unifying phenotypic feature characterises this diverse group, but their phylogenetic closeness causes them to be placed together. In light of this, we can only consider ex- amples, without claiming them to be in any way representative.

The genus *Flavobacterium* takes its name from the yellow carotenoid pigments se- creted by its members. These are aerobic, free-living, aquatic forms, although they are also associated with food spoilage.

In contrast, *Bacteroides* species are obligate anaerobes found in the human gut, where they ferment undigested food to acetate or lactate. Here they outnumber all other microbial forms, and are responsible for a significant percentage of the weight of human faeces. Some species can also

be pathogenic, and may cause peritonitis in cases where the large intestine or appendix has become perforated.

Representative genera: *Bacteroides, Flavobacterium*

Phylum: Verrucomicrobia

Members of the Verrucomicrobia form several prosthecae per cell; these are similar to those described for certain Proteobacteria (see above). Although widespread in terres- trial, freshwater and marine environments, only a handful of representatives have been isolated in pure culture.

Representative genera: *Verrucomicrobium, Prosthecobacter*

The Gram-positive bacteria: phylum Firmicutes and phylum Actinobacteria

In the second edition of *Bergey*, the Gram-positive bacteria are divided into two large phyla, the Firmicutes and the Actinobacteria. Some 2500 species are known, but a substantial proportion of these belong to just a handful of genera. Gram-positive bacteria mostly have a chemoheterotrophic mode of nutrition and include among their number several important human pathogens, as well as industrially significant forms.

The base composition of an organism's DNA can be expressed as the percentage of cytosine and guanine residues (per cent GC content); the technique is used widely in microbial taxonomy, and the Gram-positive bacteria are divided into those whose GC content is significantly over or under 50 per cent. It is convenient to consider groupings within the high GC and low GC forms as follows

Phylum Firmicutes(lowGC):	spore-forming
	non-spore forming mycoplasma
Phylum Actinobacteria (highGC):	actinomycetes
	coryneform bacteria

PHYLUMFIRMICUTES:ThelowGCGram-positivebacteria

The low GC Gram-positive bacteria form volume 3 of the second edition of *Bergey*.The *spore-forming* Gram-positive bacteria include two large genera, *Clostridium* and *Bacillus*. Although not particularly close in phylogenetic terms, they are both capable of propagation by *endospores*.

Clostridium species are obligate anaerobes, and common inhabitants of soil. Sugars are fermented to various end-products such as butyric acid, acetone or butanol. Lacking an electron transport system, they obtain all their ATP from substrate-level phosphorylation.

Several species of *Clostridium* are serious human pathogens including *C. botulinum* (botulism) and *C. tetani* (tetanus). *C. perfringens* causes gas gangrene, and if ingested, can also result in gastroenteritis. All these conditions are due to the production of bacterial exo- toxins. The

resistance of spores to heating is thus highly relevant both in medicine and in the food industry. Related to *Clostridium* are the heliobacteria, two genera of anaerobic photoheterotrophic rods, some of which produce endospores. They are the only known photo- synthetic Gram-positive bacteria.

Bacillus species are aerobes or facultative anaerobes. They are chemoheterotrophs and usually motile by means of peritrichous flagella. Only a few species of *Bacillus* are pathogenic in humans, notably *B. anthracis*, the causative agent of anthrax. This is seen by many as a potential agent of bioterrorism, and here again the relative indestructibility of its spores is a crucial factor. Other species, conversely, are positively beneficial to hu- mans; antibiotics such as bacitracin and polymixin areproduced by *Bacillus* species, whilst the toxin from *B. thuringiensis* has been used as a natural insecticide.

Representative genera: *Bacillus*, *Clostridium*

The non-spore-forming low GC Gram-positive bacteria include a number of medically and industrially significant genera, a few of which are discussed below.

The lactic acid bacteria are a taxonomically diverse group containing both rods (*Lac- tobacillus*) and cocci (*Streptococcus*, *Lactococcus*), all characterised by their fermentative metabolism with lactic acid as end-product. Although they are able to tolerate oxygen, these bacteria do not use it in respiration. They are said to be *aero-tolerant*. Like the clostridia, they lack cytochromes, and are therefore unable to carry out electron transport phosphorylation. The lactic acid bacteria have limited synthetic capabilities, so they are dependent on a supply of nutrients such as amino acids, purines/pyrimidines and vitamins. There has been growing interest in recent years in the use of certain lactic acid bacteria as *probiotics*.

The genus *Streptococcus* remains a large one, although some members have been assigned to new genera in recent years, e.g. *Enterococcus*, *Lactococcus*. Streptococci are classified in anumber of ways on the basis of phenotypic characteristics, but these do not correspond to phylogenetic relationships. Many species produce *haemolysis* when grown on blood agar, due to the production of toxins called*haemolysins*. In α-haemolysis,haemoglobin is reduced to methaemoglobin, resulting in a partial clearance of the medium and a characteristic green colour. β-Haemolysis causes a complete lysis of theredbloodcells,leavinganareaofclearingintheagar.Afewspeciesarenon-haemolytic.
Streptococci are also classified on the basis of carbohydrate antigens found in the cell wall; this system, which assigns each organism to a lettered group, is named after its devisor, RebeccaLancefield.

Pathogenic species of *Streptococcus* include *S. pyogenes* ('strep' sore throat, as well as the more serious rheumatic fever), *S. pneumoniae* (pneumococcal pneumonia) and*S. mutans* (tooth decay). Cells of *Streptococcus* exist mostly in chains, but in *S. pneu- moniae* they are characteristically paired.

Lactobacillus is used very widely in the food and drink industry in the production of such diverse foodstuffs as yoghurt, cheeses, pickled foods (e.g. sauerkraut) and certain beers.The cells of staphylococci occur in irregular bunches rather than ordered chains. They also produce lactic acid

but can additionally carry out aerobic respiration involving cytochromes, and lack the complex nutritional requirements of the lactic acid bacteria. They are resistant to drying and able to tolerate relatively high concentrations of salt. These properties allow *Staphylococcus aureus* to be a normal inhabitant of the human skin, where it can sometimes give rise to dermatological conditions such as acne, boils and impetigo. It is also found in the respiratory tract of many healthy individuals, to whom it poses no threat, but in people whose immune system has been in some way compromised, it can cause serious respiratory infections. *S. aureus* can also cause a type of food poisoning and is the causative agent of toxic shock syndrome. Widespread antibiotic use has been largely responsible for the development of resistant forms of*S. aureus*, which have become ubiquitous inhabitants of hospitals (methicillin-resistant *Staphylococcus aureus*: MRSA). The problem of antibiotic resistance.

Representative genera: *Streptococcus, Staphylococcus*

The Mycoplasma (Class Mollicutes) lack a cell wall and hence have a fluid shape (*pleomorphic*). Since the Gram test is based on the peptidoglycan content of a cell wall, why are these organisms grouped with the Gram-positive bacteria? The answer is that althoughtheydonotgiveapositiveGramtest,they are clearly related atthegenetic level to other members of the low GC Gram-positive group. The membranes of mycoplasma contain sterols; these help in resisting osmotic lysis, and are often essential as agrowth requirement.Saprophytic,commensaland parasitic forms are known,and some species are associated with respiratory diseases in animals. Mycoplasma frequently occur as contaminants in the culture of animal cells, because their small size allows them to pass through filters, and they are resistant to antibiotics directed at cell wallsynthesis.Members of the Mycoplasma are among the smallest of all known cells and have some of the smallest genomes (just over half a million base pairs).

Representative genera: *Mycoplasma, Ureoplasma*

Phylum Actinobacteria: The high GC Gram-positive bacteria

The high GC gram-positive bacteria make up volume 4 of the second edition of *Bergey.* The actinomycetes are aerobic, filamentous bacteria that form branching *mycelia* superficially similar to those of the Fungi. Remember, however, that the actinomycetes are procaryotes and the fungi are eucaryotes, so the mycelia formed by the former are appreciably smaller. In some cases, the mycelium extends clear of thesubstratum and bears asexual *conidiospores* at the hy- phal tips. These are produced by the formation of cross-walls and pinching off of spores, which are often coloured. The best known actinomycete genus is *Strepto- myces*, which contains some 500 species, all with a char- acteristically high GC content (69–73 per cent). *Strepto- myces* are very prevalent in soil, where they saprobically degrade a wide range of complex organic substrates by means of extracellular enzymes. Indeed, the characteris- tic musty smell of many soils is due to the production of a volatile organic compound called *geosmin*. A highproportion of therapeutically useful antibiotics derive from *Streptomyces* species, in- cluding well-known examples such as streptomycin, erythromycin and tetracycline.

Most actinomycetes, including *Streptomyces*, are aerobic; however, members of the genus *Actinomyces* are facultative anaerobes.

Representative genus: *Streptomyces*

The coryneform bacteria are morphologically half way between single celled bacilli and the branching filamentous actinomycetes. They are rods that show rudimentary branching, giving rise to characteristic 'V' and 'Y' shapes. Among the genera in this group are *Corynebacterium*, *Mycobacterium, Propionibacterium* and *Nocardia.*

Propionibacterium species ferment lactic acid to propionic acid. Some species are important in the production of Swiss cheeses, whilst *P. acnes* is the main cause of acne in humans.

Representative genera: *Corynebacterium, Propionibacterium*

Bacteria and human disease

Although a detailed discussion of bacterial diseases falls outside the remit of this in- troductory text, their effect on the human race is too huge to fail to mention them completely. Some important examples have been mentioned briefly in the preceding text and Table 7.5 summarises the principal bacterial diseases of humans. This chap- ter about bacteria concludes with a brief discussion of four bacterial diseases, each providing an example of a different mode of transmission.

Table 7.5 Some bacterial diseases of humans

	Genus	Disease
Gram-positive	*Staphylococcus*	Impetigo, food poisoning, endocarditis, bronchitis, toxic shock syndrome
	Streptococcus	Pneumonia, pharyngitis, meningitis, scarlet fever, dental caries
	Enterococcus	Enteritis
	Listeria	Listeriosis Anthrax
	Bacillus	Tetanus, botulism, gangrene
	Clostridium	Diphtheria
	Corynebacterium	Leprosy, tuberculosis Acne
	Mycobacterium	Pneumonia, vaginosis
	Propionibacterium	
	Mycoplasma	
Gram-negative	*Salmonella*	Salmonellosis
	Escherichia	Gastroenteritis
	Shigella	Dysentery
	Neisseria	Gonorrhoea, meningitis
	Bordetella	Whooping cough
	Legionella	Legionnaires' disease
	Pseudomonas	Infections of burns Cholera
	Vibrio	Gastroenteritis
	Campylobacter	Peptic ulcers
	Helicobacter	Bronchitis, pneumonia
	Haemophilus	Syphilis
	Treponema	Pneumonia, urethritis, trachoma
	Chlamydia	

Phylogenetic Trees : Phylogenetic relationships are illustrated in the form of branched diagrams or trees. A **phylogenetic tree** is a graph made of branches that connect nodes (**figure 19.13**). The nodes represent taxonomic units such as species or genes; the external nodes at the end of the branches represent living (extant) organisms. As in the universal phylogenetic tree (figure 19.3), the length of the branches represents the number of molecular changes that have taken place between the two nodes. Finally, a tree may be unrooted or rooted. An unrooted tree (figure 19.13*a*) simply represents phylogenetic relationships but does not provide an evolutionary path. Figure 19.13*a* shows that Ais more closely related to C than it is to either B or D, but does not specify the common ancestor for the four species or the direction of change. In contrast, the rooted tree (figure 19.13*b*) gives a node that serves as the common ancestor and shows the development of the four species from this root. It is much more difficult to develop a rooted tree. For example, there are 15 possible rooted trees that connect four species, but only three possible unrooted trees. Phylogenetic trees are developed by comparing nucleotide or amino acid sequences. To compare two molecules, their sequences must first be aligned so that similar parts match up. The object is to align and compare homologous sequences, ones that are similar because they had a common origin in the past. This is not an easy task, and computers and fairly complex mathematics must be employed to minimize the number of gaps and mismatches in the sequences being compared. Once the molecules have been aligned, the number of positions that vary in the sequences are determined. These data are used to calculate a measure of the difference between the sequences. Often the difference is expressed as the **evolutionarydistance.** This is simply a quantitative indication of the number of positions that differ between two aligned macromolecules.

Statistical adjustments are made for back mutations and multiple substitutions that may have occurred. Organisms are then clustered together based on similarity in the sequences. The most similar organisms are clustered together, then compared with the remaining organisms to form a larger cluster associated together at a lower level of similarity or evolutionary distance. The process continues until all organisms are included in the tree. Phylogenetic relationships also can be estimated by techniques such as **parsimony analysis.** In this approach, relationships are determined by estimating the minimum number of sequence changes required to give the final sequences being compared.

It is presumed that evolutionary change occurs along the shortest pathway with the fewest changes or steps from an ancestor to the organism in question. The tree or pattern of relationships is favored that is simplest and requires the fewest assumptions.

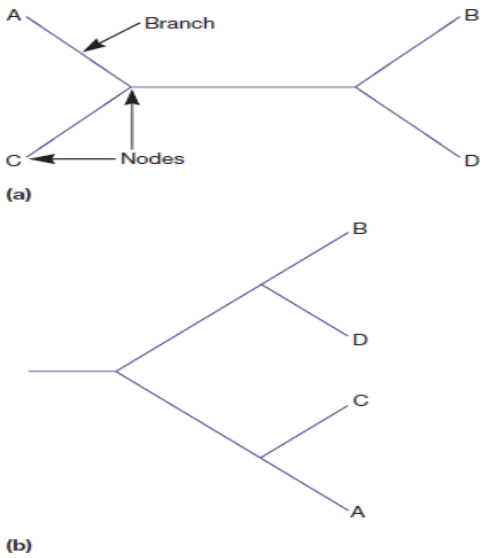


Figure 19.13 Examples of Phylogenetic Trees. (a) Unrooted tree joining four taxonomic units. (b) Rooted tree. See text for details.

Molecular versus Organismal Trees

In this text, phylogenetic trees derived from 16S rRNA sequences are presented because these data are most extensive and are thought to be most accurate by the majority of microbiologists and evolutionary biologists. Phylogenetic trees based on the analysis of molecules like proteins or nucleic acids, are considered molecular phylogenetic trees. It should be remembered that these trees are based on individual genes, not whole organisms. Prior to the advent of molecular phylogeny, trees were constructed that classified eukaryotic organisms without significant consideration of the vast diversity of microbes or their evolutionary history. Such organismal trees generally reflect the organizationrequirements of zoologists and botanists at the expense ofphylogenetics.

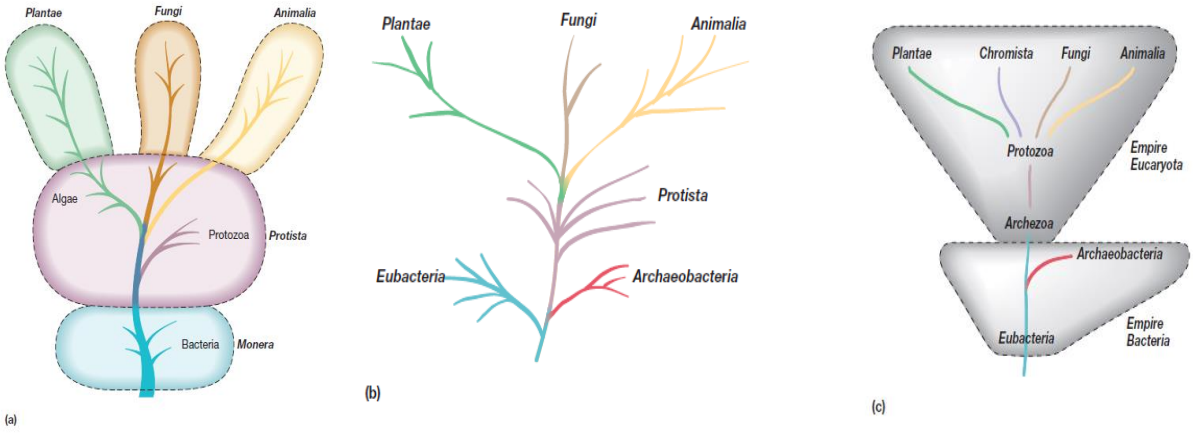


Figure 19.16 Systems of Eucaryotic and Procaryotic Phylogeny. Simplified schematic diagrams of the (a) five-kingdom system (Whittaker), (b) six-kingdom system, and (c) eight-kingdom system (Cavalier-Smith).

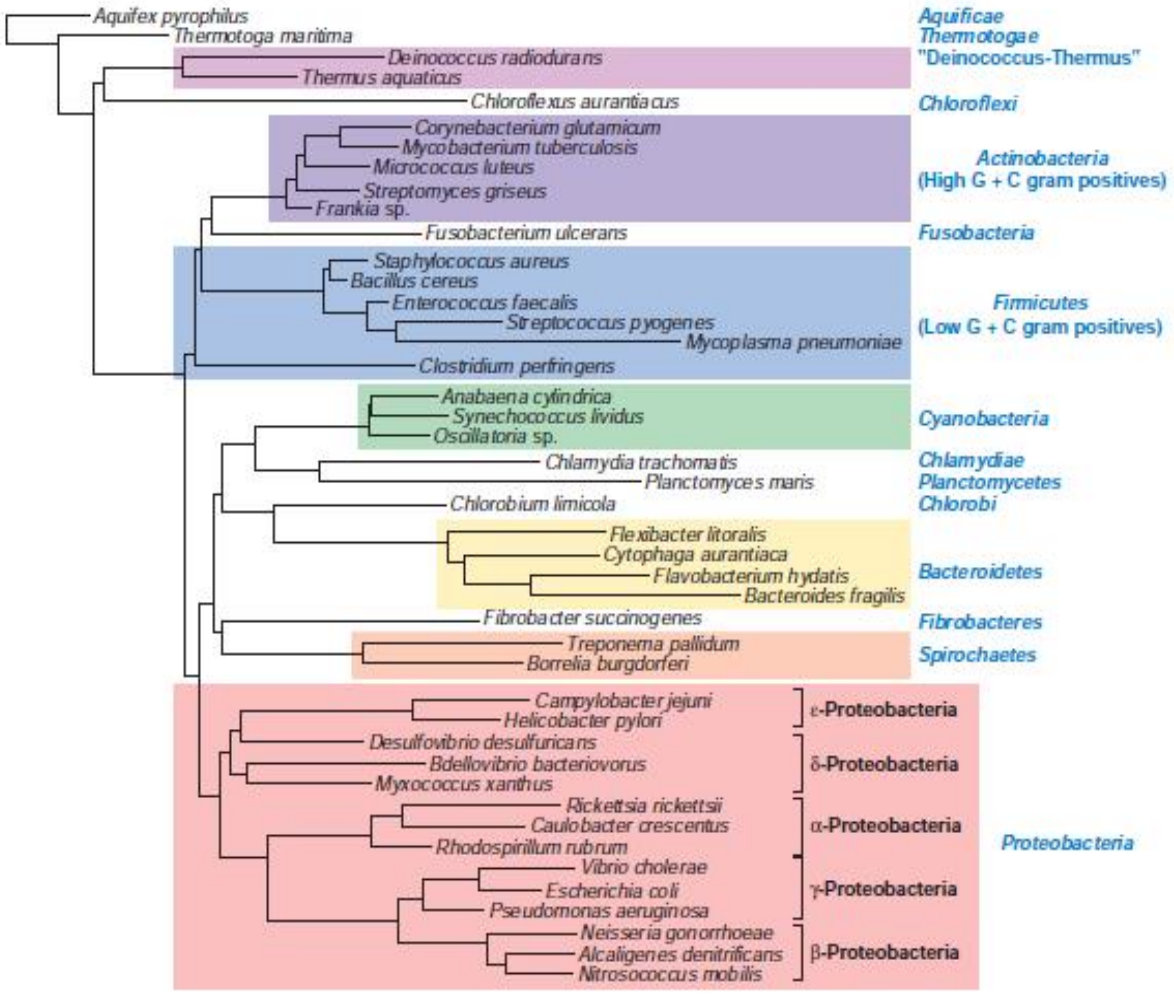


Figure 19.18 Phylogeny of the Bacteria. The tree is based on 16S rRNA comparisons. See text for discussion. *Source: The Ribosomal Database Project.*

The First Self-Replicating Entity:The RNA World

The origin of life rests on a single question: How did early cells arise? No one can say for certain; however, it seems likely that the first self-replicating entity was much simpler than even the most primitive modern, living cells. Before there was life, Earth was a cauldron of chemicals that reacted with one another, randomly "testing" the stability of the resulting molecules. This means that the first cells evolved when Earth was a very different place: hot and anoxic, with an atmosphere rich in gases like hydrogen, methane, carbon dioxide, nitrogen, and ammonia. To account for the evolution of life, one must consider the three essential cellular molecules: DNA, RNA, and proteins—one of these molecules presumably developed first and holds the key to understanding all that followed. Proteins are capable of performing cellular work but cannot replicate, while just the opposite is true of DNA. For life to evolve, a molecule was needed that could both replicate and perform cellular work. A possible solution to this problem was suggested in 1981 when Thomas Cech discovered self-splicing RNA in the eucaryotic microbe *Tetrahymena*. Three years later, Sidney Altman found that RNaseP in *Escherichia coli* is an RNA molecule that cleaves phosphodiester bonds. RNAmolecules that possess catalytic activity are called ribozymes and to

some, the ability of RNA to catalyze biochemical reactions suggests a precellularRNAworld, a term coined byWalter Gilbert in 1986. Thishypothesis suggests that the first self-replicating molecule was RNA, which is capable of storing, copying, and expressing geneticinformation, and possesses enzymatic activity as well. In thisversion of early life, various forms of molecules were assembledand destroyed over roughly half a billion years, until ultimately anentity something like modern RNAenclosed in a lipid vesicle was generated.

Apart from its ability to replicate and perform enzymatic activities, the function of RNA suggests its ancient origin. Consider that much of the cellular pool of RNA in modern cells exists in the ribosome, a structure that consists largely of rRNAand it uses mRNA and tRNA to construct proteins. In fact, rRNA itself catalyzes peptide bond formation during protein synthesis. Thus RNA seems to be well poised for its importance in the development of proteins. Because RNA and DNA are structurally similar, RNA could have given rise to double-stranded DNA. It is posited that once DNA evolved it became the storage facility for cellular functions because it provided a more chemically stable structure. Two other pieces of evidence support the RNA world hypothesis: the fact that the energy currency of the cell, ATP, is a ribonucleotide, and the more recent discovery that RNA can regulate gene expression. So it would seem that proteins, genes, and cellular energy all can be traced back to RNA.

Others are skeptical of the RNAworld hypothesis. They claim conditions on Earth 4 billion years ago would have prevented the stable formation of ribose, phosphate, purines, and pyrimidines— all needed to construct RNA. In fact, while purine bases have been generated abiotically in a heated mixture of hydrogen cyanide and ammonia, scientists have so far been unable to make pyrimidines in a similar fashion. Another problem with the RNA world hypothesis is the instability of RNAonce it is assembled. In 1996, James Ferris and colleagues were able to overcome the problem of RNA degradation by adding the clay mineral montmorillonite to a solution of chemically charged nucleotides. They showed that the rate of RNA synthesis was faster than its degradation.

Later they and others showed in similar experiments that amino acids in solution with the minerals hydroxyapatite and illite could also polymerize into polypeptides of about 50 amino acid residues. To some, these experiments provide experimental evidence for the biosynthesis of early organic polymers. However, one additional problem with the RNA world hypothesis concerns the ability of early RNA to self-replicate. Recall that in modern cells, RNA is synthesized by the enzyme RNA polymerase, a protein. Replication of early RNA without a protein was presumably accomplished by an ancient ribozyme. So far, no such ribozyme has been found, nor has it been generated experimentally. Thus although it is clear that microbial life ultimately emerged from a random mixture of chemicals, the actual mechanism by which the first cell-like entity arose is a controversy that may never be resolved.

Early cellular life, although primitive compared to modern life, was still relatively complex. Cells had to derive energy from a harsh, anoxic environment. When scientists attempt to reconstruct the nature of very ancient life, they look to extant (living) microbes for clues. For instance, it is thought

that the FeS-based metabolism seen in some hyperthermophilic archaea may be a remnant of the firstform of chemiosmosis. Here it is suggested that the energy-yieldingreaction
$FeS + H_2S \rightarrow FeS_2 + H_2$ provided the reducing power (H_2) to produce a proton motive force. Photosynthesis also appears to have evolved early in Earth's history. There is fossil evidence to place the evolution of cyanobacteria and oxygenic photosynthesis atabout 3 billion years ago. Stromatolites are layered rocks, often domed, that are formed by the incorporation of mineral sediments into microbial mats dominated by cyanobacteria **(figure 1.2).** Recent evidence has shown that some fossilized stromatolites formed in a similar fashion. The presence of oxygen was critical because it enabled the evolution of a wider variety of energy-capturing strategies, including aerobic respiration. Ironically, the study of the most ancient organisms is one of the youngest disciplines in the biological sciences. The ability to culture and examine microorganisms was developed only about150 years ago. Almost immediately, early microbiologists attempted to classify microbes and organize them according to possible relationships to one another. Two important elements not understood until the late twentieth century made this especially difficult. First, only about 1% of all microbes have been cultured in the laboratory. Second, the most accurate assessment of evolutionary relationships between organisms is obtained by comparing nucleotide and amino acid sequences. Prior to the advent of sequence-based techniques, it was impossible to discern evolutionary relationships among microorganisms.

Figure 1.2 Stromatolites. These are stromatolites at Shark Bay,Western Australia.Modern stromatolites are layered or stratified rocks formed by the incorporation of calcium sulfates, calcium carbonates, and other minerals into microbial mats.The mats are formed by cyanobacteria and other microorganisms.

b. Microbial interaction, Biofim, Biosensor

Microbial interaction

Microorganisms can associate physically with other organismsin a variety of ways. One organism can be located on the surfaceof another, as an **ectosymbiont.** In this case, the ectosymbiontusually is a smaller organism located on the surface of a largerorganism. In contrast, one organism can be located within anotherorganism as an **endosymbiont.** While the simplest microbialinteractions involve two members, a symbiont and its host,a number of interesting organisms host more than one symbiont.The term **consortium** can be used to describe this physical relationship.

For example, *Thiothrix* species, a sulfur-using bacterium,is attached to the surface of a mayfly larva and itself contains a parasitic bacterium. Fungi associated with plant root (mycorrhizal fungi) often contain endosymbiotic bacteria, aswell as having bacteria living on their surfaces. These physical associations can be intermittent and cyclic orpermanent. Examples of intermittent and cyclic associations ofmicroorganisms with plants and marine animals are shown in**table 30.1.** Important human diseases, including listeriosis,malaria, leptospirosis, legionellosis, and vaginosis also involvesuch intermittent and cyclic symbioses. Interesting permanent relationshipsalso occur between bacteria and animals, as shown in **table 30.2.**

Hosts include squid, leeches, aphids, nematodes, and mollusks.In each of these cases, an important characteristic of the host animalis conferred by the permanent bacterial symbiont.

Table 30.1 Intermittent and Cyclical Symbioses of Microorganisms with Plants and Marine Animals

Symbiosis	Host	Cyclical Symbiont
Plant-bacterial	*Gunnera* (tropical angiosperm)	*Nostoc* (cyanobacterium)
	Azolla (rice paddy fern)	*Anabaena* (cyanobacterium)
	Phaseolus (bean)	*Rhizobium* (N_2 fixer)
	Ardisia (angiosperm)	*Protobacterium*
Marine animals	Coral coelenterates	*Symbiodinium* (dinoflagellate)
	Luminous fish	*Vibrio, Photobacterium*

Adapted from L. Margulis and M. J. Chapman, 1998. Endosymbioses: Cyclical and permanent in evolution. *Trends in Microbiology* 6(9):342–46, tables 1, 2, and 3.

Table 30.2 Examples of Permanent Bacterial-Animal Symbioses and the Characteristics Contributed by the Bacterium to the Symbiosis

Animal Host	Symbiont	Symbiont Contribution
Sepiolid squid (*Euprymna scolopes*)	Luminous bacterium (*Vibrio fischeri*)	Luminescence
Medicinal leech (*Hirudo medicinalis*)	Enteric bacterium (*Aeromonas veronii*)	Blood digestion
Aphid (*Schizaphis graminum*)	Bacterium (*Buchnera aphidicola*)	Amino acid synthesis
Nematode worm (*Heterorhabditis* spp.)	Luminous bacterium (*Photorhabdus luminescens*)	Predation and antibiotic synthesis
Shipworm mollusk (*Lyrodus pedicellatus*)	Gill cell bacterium	Cellulose digestion and nitrogen fixation

Source: From E. G. Ruby, 1999. Ecology of a benign "infection": Colonization of the squid luminous organ by *Vibrio fischeri*. In *Microbial ecology and infectious disease*, E. Rosenberg, editor, American Society for Microbiology, Washington, D.C., 217-31, table 1.

Although it is possible to observe microorganisms in these varied physical associations with other organisms, the fact that there is some type of physical contact provides no information on the nature of the interactions that might be occurring. These interactions include mutualism, cooperation, commensalism, predation, parasitism, amensalism, and competition (**figure 30.1**).

These interactions are now discussed-

Mutualism-

Mutualism [Latin *mutuus*, borrowed or reciprocal] defines the relationship in which some reciprocal benefit accrues to bothpartners. This is an obligatory relationship in which the **mutualist**and the host are dependent on each other. When separated, inmany cases, the individual organisms will not survive. Severalexamples of mutualism are presented next.

Microorganism-Insect Mutualisms:

Mutualistic associations are common in the insects. This is relatedto the foods used by insects, which often include plant sapor animal fluids lacking in essential vitamins and amino acids.The required vitamins and amino acids are provided by bacterialsymbionts in exchange for a secure habitat and ample nutrients. The aphid is an excellentexample of this mutualistic relationship. This insect harbors the -proteobacterium *Buchnera aphidicola* in its cytoplasm,and a mature insect contains literally millions of these bacteria inits body. The *Buchnera* provides its host with 10 essential aminoacids, and if the insect is treated with antibiotics, it dies. Likewise,*B. aphidicola* is an obligate mutualistic symbiont. The inabilityof either partner to grow without the other indicates thatthe two organisms demonstrate coevolution, or have evolved together.It is estimated that the *B. aphidicola*-aphid endosymbiosiswas established about 150 million years ago. The genomes oftwo different *B. aphidicola* strains have been sequenced and annotatedto reveal extreme genomic stability. These strains diverged50 to 70 million years ago, and since that time there have been no gene duplications, translocations, inversions, or genesacquired by horizontal transfer. The genomes are small, only0.64 Mb each with 93% of their genes common to both strains.

Furthermore, only two genes have no orthologs in their close relative*E. coli*. This tremendous degree of stability implies that althoughthe initial acquisition of the endosymbiont by ancestralaphids enabled their use of an otherwise deficient food source(sap), the bacteria have not continued to expand the ecologicalniche of their insect host through the acquisition of new traits that might be advantageous.

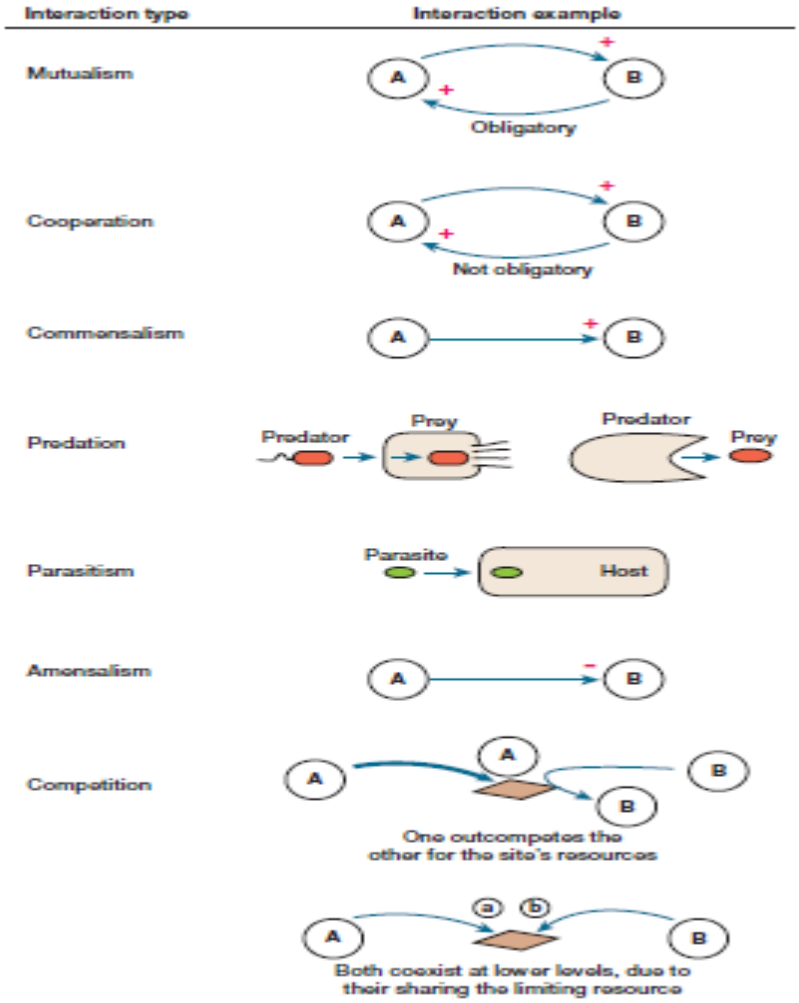


Figure 30.1 Microbial Interactions. Basic characteristics of symbiotic interactions that can occur between different organisms.

The protozoan-termite relationship is another classic exampleof mutualism in which the flagellated protozoa live in the gut oftermites and wood roaches (**figure 30.2*a***). These flagellates existon a diet of carbohydrates, acquired as cellulose ingested by theirhost **(figure 30.2*b*).** The protozoa engulf wood particles, digest thecellulose, and metabolize it to acetate and other products. Termitesoxidize the acetate released by their flagellates. Because the hostis almost always incapable of synthesizing cellulases (enzymesthat catalyse the hydrolysis of cellulose), it is dependent on the

mutualisticprotozoa for its existence. This mutualistic relationshipcan be readily tested in the laboratory if wood roaches are placedin a bell jar containing wood chips and a high concentration of O_2.Because O_2 is toxic to the flagellates, they die. The wood roachesare unaffected by the high O_2 concentration and continue to ingestwood, but they soon die of starvation due to a lack of cellulases.

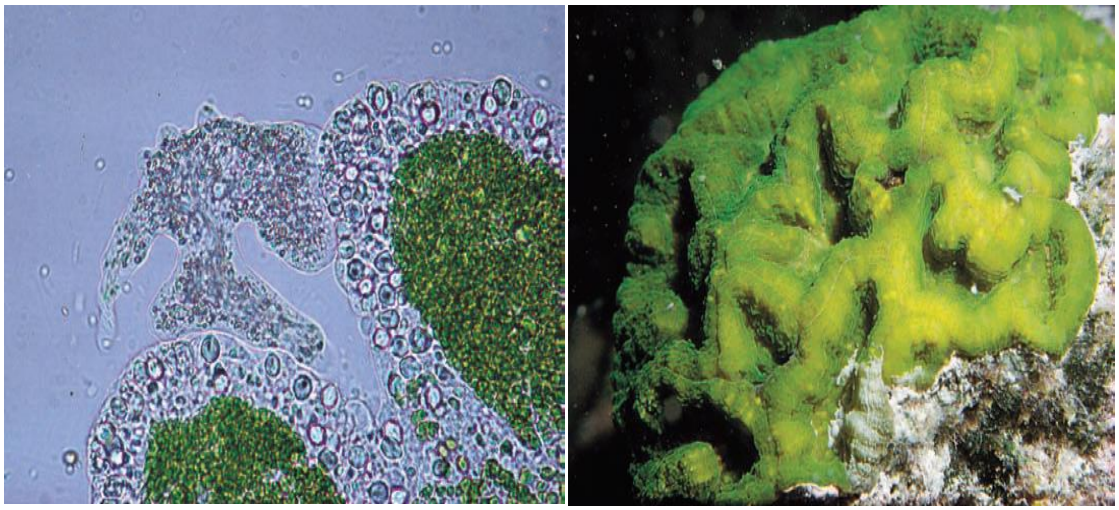
(a) ***(b)***

Figure 30.2 Mutualism. Light micrographs of **(a)** a worker termite of the genus *Reticulitermes* eating wood(×10), and **(b)** *Trichonympha*, a multiflagellated protozoan from the termite's gut (×135). Notice the many flagella that occur over most of its length. The ability of *Trichonympha* to break down cellulose allows termites to use wood as a food source.

Zooxanthellae:

Many marine invertebrates (sponges, jellyfish, sea anemones,corals, ciliates) harbor endosymbiotic dinoflagellates called**zooxanthellae** within their tissue (**figure 30.3*a***). Because the degreeof host dependency on the mutualistic protist is somewhatvariable, only one well-known example is presented. The hermatypic (reef-building) corals **(figure 30.3*b*)** satisfymost of their energy requirements using their zooxanthellae,which are found at densities between 5×10^5 and 5×10^6cellsper square centimeter of coral animal. In exchange for up to 95%of their photosynthate (fixed carbon), zooxanthellae receive nitrogenouscompounds, phosphates, CO2, and protection from UVlight from their hosts. This efficient form of nutrient cycling andtight coupling of trophic levels accounts for the stunning successof reef-building corals in developing vibrant ecosystems. However,during the past several decades, the number of coral bleachingevents has increased dramatically. **Coral bleaching** is definedas a loss of either the photosynthetic pigments from the

corals orexpulsion of the zooxanthallae. It has been determined that damageto photosystem II of the zooxanthellae generates reactive oxygenspecies (ROS); it is these ROS that appear to be the directcause of damage (recall that photosystem II uses water as the electronsource resulting in the evolution of oxygen). Coral bleachingappears to be caused by a variety of stressors, but it has been experimentallydetermined, as well as observed in field sites, that temperature increases as small as 2°C above the average summermaxima can cause coral bleaching. Sadly, evidence suggests thatmany corals will be unable to evolve quickly enough to keep pacewith the observed and predicted increases in ocean temperaturesif global warming continues unchecked.



(a) ***(b)***

Figure 30.3 Zooxanthellae. (a) Zooxanthellae (green) within the tip of a hydra tentacle (×150). **(b)** The green color of this rose coral (*Manilina*) is due to the abundant zooxanthellae within its tissues.

Sulfide-Based Mutualisms:

Tube worm-bacterial relationships exist several thousand metersbelow the surface of the ocean, where the Earth's crustal platesare spreading apart (**figure 30.4**). Vent fluids are anoxic, contain high concentrations of hydrogen sulfide, and can reach a temperatureof 350°C. However, because of increased atmospheric pressure,the water does not boil. The seawater surrounding these ventshas sulfide concentrations around 250 _M and temperatures 10 to20°C above the ambient seawater temperature of about 2°C.Giant (_1 m in length), red, gutless tube worms (*Riftia* spp.;**figure 30.5*a***) near these hydrothermal vents provide an exampleof a unique form of mutualism and animal nutrition in whichchemolithotrophic bacterial endosymbionts are maintainedwithin specialized cells of the tube worm host (**figure 30.5 *b,c,d***).

The *Riftia* tube worms live at the interface between the hot,anoxic fluids of the vents and the cold, oxygen-containing seawater.Here, reduced sulfides from the vents react rapidly andspontaneously with oxygen in the seawater. In order to provideboth reduced sulfur and oxygen to their bacterial endosymbionts,*Riftia's* blood contains a unique kind of hemoglobin, which accountsfor the bright-

red plume extending out of their tubes. Hydrogensulfide (H_2S) and O_2 are removed from the seawater bythe worm's hemoglobin, and delivered to a special organ calledthe trophosome. The trophosome is packed with chemolithotrophicbacterial endosymbionts that fix CO_2 using theCalvin cycle (*see* ***figure 30.4*****)** with electrons provided by H_2S.

The CO_2 is carried to the endosymbionts in three ways: (1) freelyin the bloodstream, (2) bound to hemoglobin, and (3) as organicacids such as malate and succinate. When these acids are decarboxylated,they release CO_2. This process is similar to carbon fixationby plants and cyanobacteria, but it occurs in the deepest,darkest reaches of the ocean. This mutualism is enormously successful:not only do the worms grow to an astounding size indensely packed communities, but the bacteria (which have notyet been cultured in the laboratory) reach densities of up to 1011cells per gram of worm tissue.

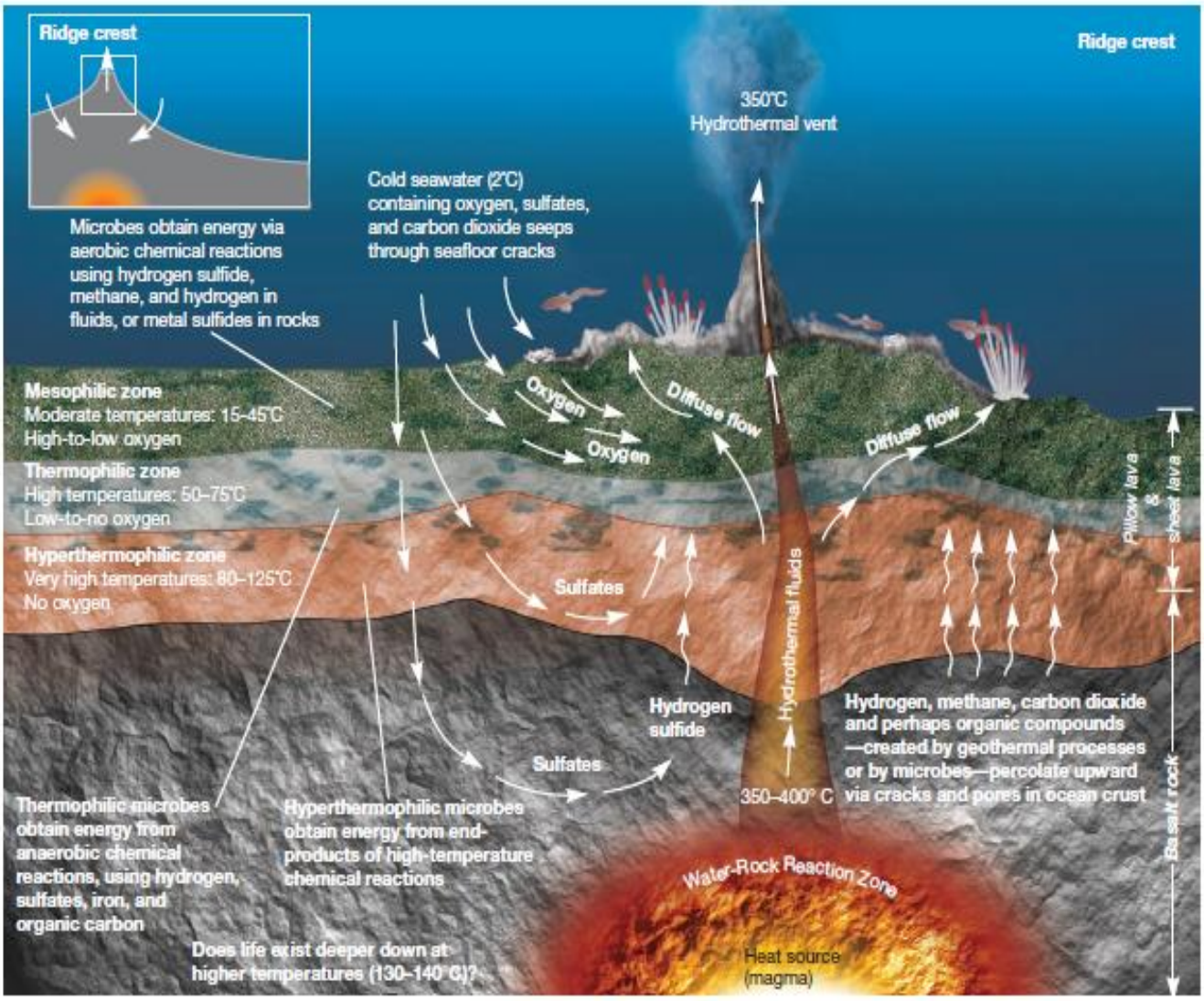


Figure 30.4 Hydrothermal Vents and Related Geological Activity. The chemical reactions between seawater and rocks that occur over a range of temperatures on the seafloor supplies the carbon and energy that support a diverse collection of microbial communities in specific niches within the vent system.

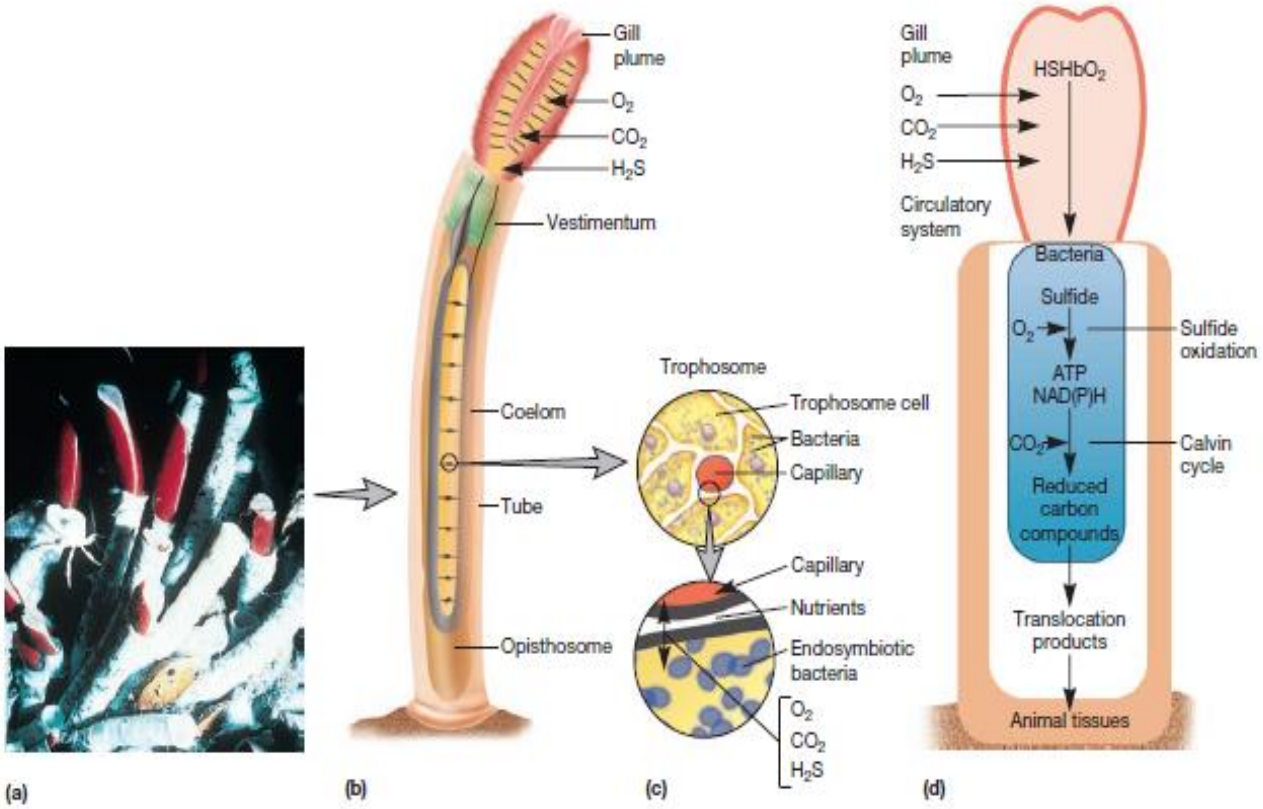


Figure 30.5 The Tube Worm–Bacterial Relationship. **(a)** A community of tube worms (*Riftia pachyptila*) at the Galápagos Rift hydrothermal vent site (depth 2,550 m). Each worm is more than a meter in length and has a 20 cm gill plume. **(b, c)** Schematic illustration of the anatomical and physiological organization of the tube worm. The animal is anchored inside its protective tube by the vestimentum. At its anterior end is a respiratory gill plume. Inside the trunk of the worm is a trophosome consisting primarily of endosymbiotic bacteria, associated cells, and blood vessels. At the posterior end of the animal is the opisthosome, which anchors the worm in its tube. **(d)** Oxygen, carbon dioxide, and hydrogen sulfide are absorbed through the gill plume and transported to the blood cells of the trophosome. Hydrogen sulfide is bound to the worm's hemoglobin ($HSHbO_2$) and carried to the endosymbiont bacteria. The bacteria oxidize the hydrogen sulfide and use some of the released energy to fix CO_2 in the Calvin cycle. Some fraction of the reduced carbon compounds synthesized by the endosymbiont is translocated to the animal's tissues.

Methane-Based Mutualisms

Other unique food chains involve methane-fixing microorganisms. By converting methane to carbohydrate, these bacteria perform the first step in providing organic matter for consumers. Methanotrophs are bacteria capable of using methane as a sole carbon source. They occur as intracellular symbionts of methanevent mussels. In these mussels the thick, fleshy gills are filledwith bacteria. In the Barbados Trench, methanotrophic carnivorous sponges have been discovered in a mud volcano at a depth of 4,943 m. Abundant methanotrophic symbionts were confirmed by the presence of enzymes related to methane oxidation in sponge tissues. These sponges are not satisfied with just bacteria;they also trap swimming prey to give variety to their diet.

Methanotrophic microorganisms are important in ecosystems outside of methane vents. For example, a methanotrophic endosymbiont was recently discovered to reduce the flux of methane from peat bogs; wetlands are the largest natural source of this greenhouse gas. *Sphagnum* moss, the principal plant in peat bogs (and a favorite among florists), can grow when submerged in water. Methanotrophic -proteobacteria living within the outer cortex cells of *Sphagnum* stems oxidize methane as it diffuses through the water column:

$$CH_4 + 2O_2 \rightarrow CO_2 + H_2O$$

The resulting CO_2 is then readily fixed by the plant, which uses the Calvin cycle:

$$2CO_2 + 2H_2O \rightarrow 2CH_2O + 2O_2$$

This enables extremely efficient carbon recycling within this ecosystem:

$$CH_4 + 2CO_2 \rightarrow 2CH_2O$$

The Rumen Ecosystem

Ruminants are the most successful and diverse group of mammals on Earth today. Examples include cattle, deer, elk, bison, water buffalo, camels, sheep, goats, giraffes, and caribou. These animals spend vast amounts of time chewing their cud—a smallball of partially digested grasses that the animal has consumed but not yet completely digested. It is thought that the ruminants evolved an "eat now, digest later" strategy because their grazing can often be interrupted by predator attacks.

These herbivorous animals have stomachs that are divided into four chambers (**figure 30.6**). The upper part of the ruminant stomach is expanded to form a large pouch called the **rumen** anda smaller, honeycomb-like region, the reticulum. The lower portion is divided into an antechamber, the omasum, followed by the "true" stomach, the abomasum. The rumen is a highly muscular, anaerobic fermentation chamber where huge amounts of grasses eaten by the animal are digested by a diverse microbial community that includes bacteria, archaea, fungi, and protists. This microbial community is large—about 1012 organisms per milliliter of digestive fluid. When the animal eats plant material, it ismixed with saliva and swallowed without chewing to enter the rumen. Here the material is churned and thoroughly mixed.

Eventually, microbial attack and mixing coats the grass with microbes, reducing it to a pulpy, partially digested, mass. At this point the mass moves into the reticulum where it is regurgitated as cud, chewed, and re-swallowed by the animal. As this process proceeds, the grass becomes progressively more liquefied and flows out of the rumen into the omasum and then the abomasum.

Here the nutrient-enriched grass material meets the animal's digestive enzymes and soluble organic and fatty acids are absorbed into the animal's bloodstream. The microbial community in the rumen is extremely dynamic. The rumen is slightly warmer than the rest of the animal and with a redox potential of about -30 mV, all resident microorganisms must carry out anaerobic metabolism. Not only does the animal have a mutualistic relationship with the microbial community, but within the microbial community there are very specific interactions.

One population of bacteria produce extracellular cellulases that cleave the ß(1 → 4) linkages between the successive D-glucose molecules that form plant cellulose. The D-glucose isthen fermented to organic acids such as acetate, butyrate, and propionate. These organic acids, as well as fatty acids are the true energy source for the animal. In some ruminants, the processing of organic matter stops at this stage. In others, such as cows, acetate, CO_2, and H_2 are used by methanogenic archaea to generate methane (CH_4), a greenhouse gas. In fact, a single cow can produce as much as 200 to 400 liters of CH_4 per day. The animal releases this CH_4 by a process called eructation (Latin *eructare,* to belch). Although the methanogens consume acetate that could be used by their animal hosts, they provide most of the vitamins needed by the ruminant.

In fact, rumen microbes are so effective in fortifying the grass consumed by the animal, unlike humans, most ruminants have no required dietary amino acids.

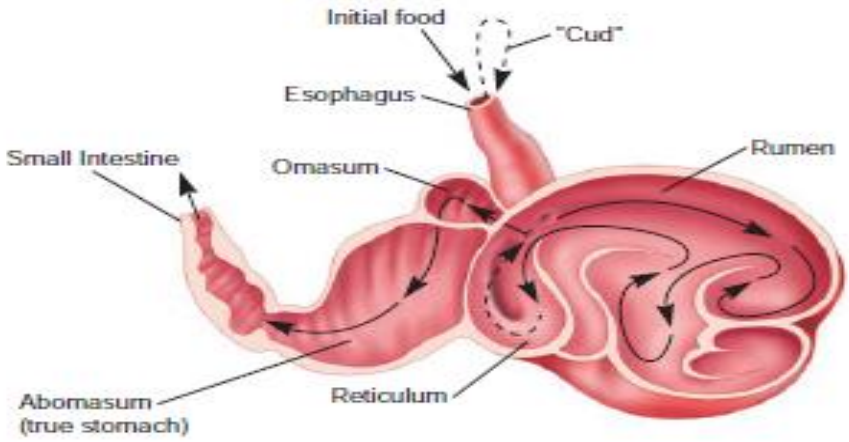


Figure 30.6 Ruminant Stomach. The stomach compartments of a cow. The microorganisms are active mainly in the rumen. Arrows indicate direction of food movement.

Cooperation:

Cooperation and commensalism are two positive but not obligatory types of symbioses found widely in the microbial world. These involve syntrophic relationships. **Syntrophism** [Greek*syn,* together, and *trophe,* nourishment] is an association in which the growth of one organism either depends on or is improved by growth factors, nutrients, or substrates provided by another organismgrowing nearby. Sometimes both organisms benefit.

Cooperation benefits both organisms (figure 30.1). Acooperative relationship is not obligatory and, for most microbial ecologists, this nonobligatory aspect differentiates cooperation from mutualism. Unfortunately, it is often difficult to distinguish obligatory from nonobligatory because

that which is obligatory in one habitat may not be in another (e.g., the laboratory). Nonetheless, the most useful distinction between cooperation and mutualism is the observation that cooperating organisms can be separated from one another and remain viable, although they may not function as well.

Two examples of a cooperative relationship include the association between *Desulfovibrio* and *Chromatium* (**figure 30.7*a***), in which the carbon and sulfur cycles are linked, and the interactionof a nitrogen-fixing microorganism with a cellulolytic organism such as *Cellulomonas* (figure 30.7*b*). In the second example, the cellulose-degrading microorganism liberates glucose from the cellulose, which can be used by nitrogen-fixing microbes.

An excellent example of a cooperative biodegradative association is shown in **figure 30.8.** In this case degradation of the toxin 3-chlorobenzoate depends on the functioning of microorganisms with complementary capabilities. If any one of the three microorganisms is not present and active, the degradation of the substrate will not occur. This example points out how the sum of the microbes in a community can be considered greater than the contribution made by any single microorganism.

In other cooperative relations, sulfide-dependent autotrophic filamentous microorganisms fix carbon dioxide and synthesize organic matter that serves as a carbon and energy source for a heterotrophic organism. Some of the most interesting include thepolychaete worms *Alvinella pompejana* (**figure 30.9**), the Pompeii worm, and also *Paralvinella palmiformis*, the Palm worm.

Both have filamentous bacteria on their dorsal surfaces. These filamentous bacteria can tolerate high levels of metals such as arsenic, cadmium, and copper. When growing on the surface of the animal, they may provide protection from these toxic metals, as well as thermal protection; in addition, they appear to be used as a food source. A deep-sea crustacean has been discovered that usessulfur-oxidizing autotrophic bacteria as its food source. Thisshrimp, *Rimicaris exoculata* (**figure 30.10**) has filamentoussulfur-oxidizing bacteria growing on its surface (figure 30.10*b*).

When these are dislodged the shrimp ingests them. This nominally "blind" shrimp use a reflective organ to respond to the glow emitted by geothermally active black smoker chimneys. The organ is sensitive to a light wavelength that is not detectable by humans.

Another interesting example of bacterial epigrowth is shown by nematodes, including *Eubostrichus parasitiferus*, that live at the interface between oxic and anoxic sulfide-containing marine sediments (**figure 30.11*a,b***). These animals are covered by sulfide-oxidizing bacteria that are present in intricate patterns (figure 30.11*b*). The bacteria not only decrease levels of toxic sulfide, which often surround the nematodes, but they also serve as a food supply.

In 1990, hydrothermal vents were discovered in a freshwater environment at the bottom of Lake Baikal, the oldest (25 million years old) and deepest lake in the world. This lake is located in the far east of Russia (**figure 30.12*a,b***) and has the largest volume of any freshwater lake (not the largest area—which is Lake Superior). Microbial mats featuring long, white strands are in the center of the vent field where the highest temperatures are found. At the edge of the vent field, where the water temperature is lower, the microbial mats end, and sponges, gastropods, and other

organisms, which use the sulfur-oxidizing bacteria as a food source, are present (figure 30.12*b*). Similar although less developed areas have been found in Yellowstone Lake, Wyoming.

A form of cooperation also occurs when a population of similar microorganisms monitors its own density—the process of quorum sensing. The microorganisms produce specific autoinducer compounds, and as the population increases and the concentration of these compounds reaches critical levels, specific genes are expressed.These responses are important for microorganisms that form associations with each other, plants, and animals. Intercellular communication is critical for the establishment of biofilms and for colonization of hosts by pathogens.

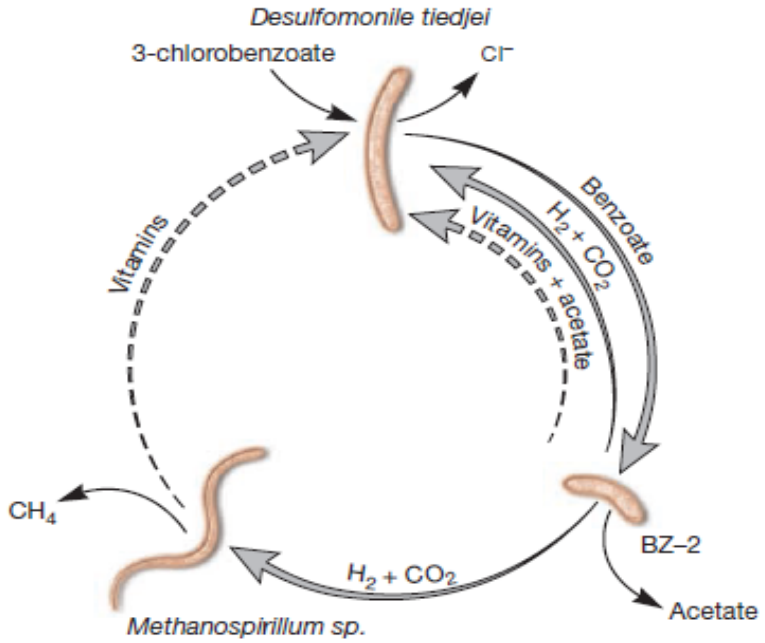
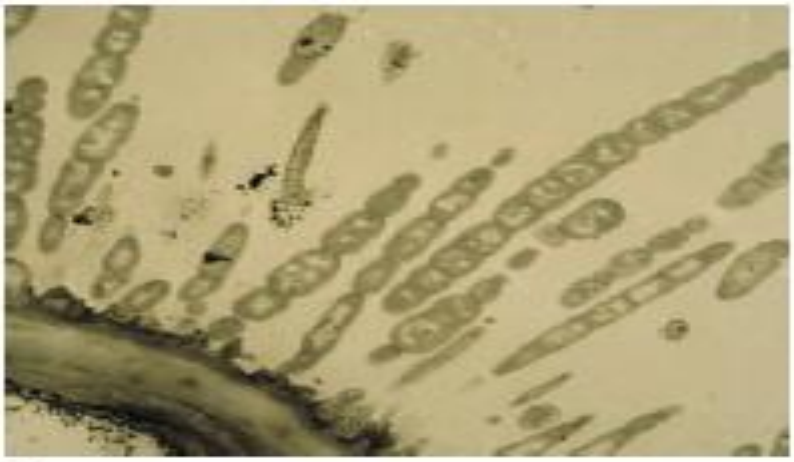


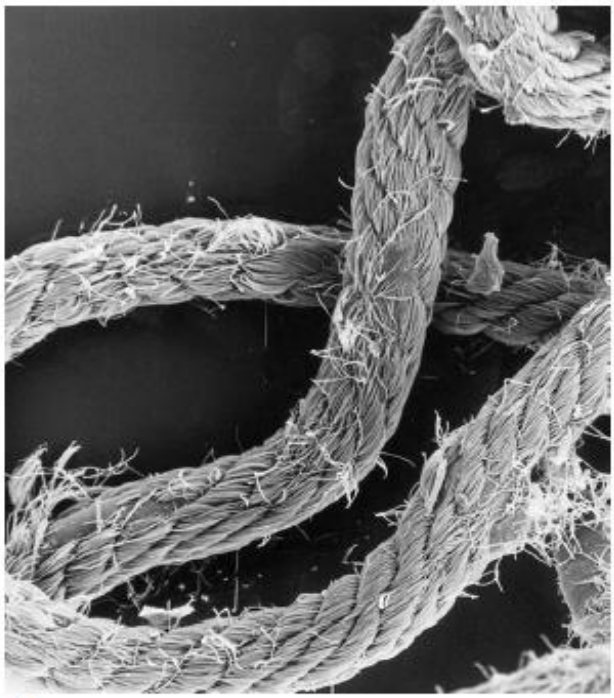
Figure 30.8 Associations in a Defined Three-Membered Cooperative and Commensalistic Community That Can Degrade 3-Chlorobenzoate. If any member is missing, degradation will not take place. The solid arrows demonstrate nutrient flows, and the dashed lines represent hypothesized flows.

(a)

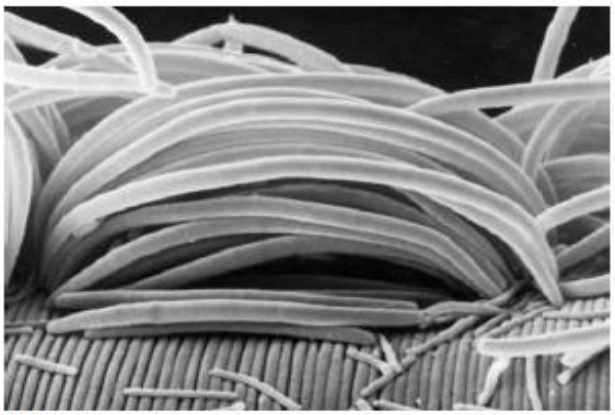


(b)

Figure 30.10 A Marine Crustacean-Bacterial Cooperative Relationship. **(a)** A picture of the marine shrimp *Rimicaris exoculata* clustered around a hydrothermal vent area, showing the massive development of these crustaceans in the area where chemolithotrophic bacteria grow using sulfide as an electron and energy source. The bacteria, which grow on the vent openings and also on the surface of the crustaceans, fix carbon, and serve as the nutrient for the shrimp. **(b)** An electron micrograph of a thin section across the leg of the marine crustacean *Rimicaris exoculata*, showing the chemolithotrophic bacteria that cover the surface of the shrimp. The filamentous nature of these bacteria, upon which this commensalistic relationship is based, is evident in this thin section.

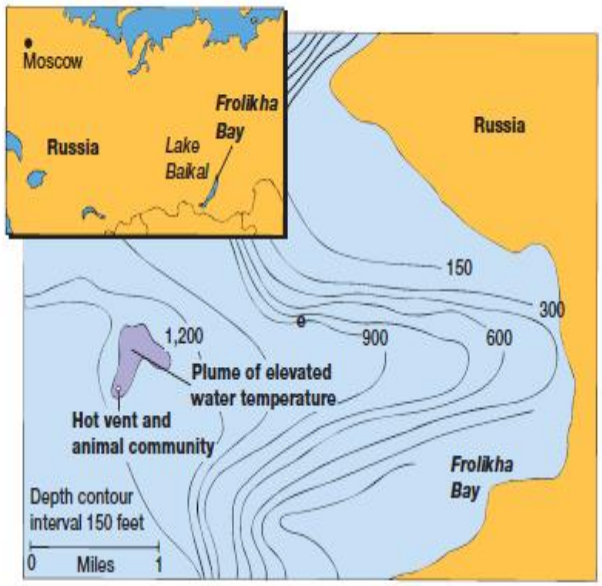


(a)



(b)

Figure 30.11 A Marine Nematode-Bacterial Cooperative Relationship. Marine free-living nematodes, which grow at the oxidized-reduced interface where sulfide and oxygen are present, are covered by sulfide-oxidizing bacteria. The bacteria protect the nematode by decreasing sulfide concentrations near the worm, and the worm uses the bacteria as a food source. **(a)** The marine nematode *Eubostrichus parasitiferus* with bacteria arranged in a characteristic helix pattern. **(b)** The chemolithotrophic bacteria attached to the cuticle of the marine nematode *Eubostrichus parasitiferus*. Cells are fixed to the nematode surface at both ends.



(a)

(b)

Figure 30.12 Hydrothermal Vent Ecosystems in Freshwater Environments. Lake Baikal has been found to have low temperature hydrothermal vents. **(a)** Location of Lake Baikal, site of the hydrothermal vent field. **(b)** Bacterial filaments and sponges at the edge of the vent field. *(a) Source: Data from the National Geographic Society.*

Commensalism

Commensalism [Latin *com*, together, and *mensa*, table] is a relationship in which one symbiont, the **commensal,** benefits while the other (sometimes called the host) is neither harmed nor helped, as shown in figure 30.1. This is a unidirectional process. Often both the host and the commensal "eat at the same table." The spatial proximity of the two partners permits the commensal to feed on substances captured or ingested by the host, and the commensal often obtains shelter by living either on or in the host.

The commensal is not directly dependent on the host metabolically and causes it no particular harm. When the commensal is separated from its host experimentally, it can survive without the addition of factors of host origin.

Commensalistic relationships between microorganisms include situations in which the waste product of one microorganism is a substrate for another species. One good example is nitrification, the oxidation of ammonium ion to nitrite by microorganisms such as *Nitrosomonas,* and the subsequent oxidation of the nitrite to nitrate by *Nitrobacter* and similar bacteria. *Nitrobacter* benefits from its association with *Nitrosomonas* because it uses nitrite to obtain energy for growth. A second example of this type of relationship is found in anoxic methanogenic ecosystems such as sludge digesters, anoxic freshwater aquatic sediments, and flooded soils. In these environments, fatty acids can be degraded to produce H_2 and methane by the interaction of two different bacterial groups. Methane production by methanogens depends on **interspecies hydrogen transfer.** A fermentative bacterium generates hydrogen gas, and the methanogen uses it quickly as a substrate for methane gas production.

Various fermentative bacteria produce low molecular weight fatty acids that can be degraded by anaerobic bacteria such as *Syntrophobacter* to produce H_2 as follows:

Propionic acid → acetate + CO_2+ H2

Syntrophobacter uses protons ($H^+ + H^+ \rightarrow H_2$) as terminal electron acceptors in ATP synthesis. The bacterium gains sufficient energy for growth only when the H_2 it generates is consumed. The products H_2 and CO_2 are used by methanogenic archaea such as *Methanospirillum* as follows:

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$

By synthesizing methane, *Methanospirillum* maintains a low H_2concentration in the immediate environment of both microbes. Continuous removal of H_2 promotes further fatty acid fermentation and H_2 production. Because increased H_2 production and consumption stimulate the growth rates of *Syntrophobacter* and*Methanospirillum,* both participants in the relationship benefit.

Commensalistic associations also occur when one microbial group modifies the environment to make it more suited for another organism. For example, common, nonpathogenic strains of *Escherichiacoli* live in the human colon, but also grow quite well outside the host, and thus are typical commensals. When oxygenis used up by facultatively anaerobic *E. coli,* obligate anaerobes

such as *Bacteroides* are able to grow in the colon. The anaerobes benefit from their association with the host and *E. coli,* but *E. coli*derives no obvious benefit from the anaerobes. In this case the commensal *E. coli* contributes to the welfare of other symbionts.

Commensalism can involve other environmental modifications. The synthesis of acidic waste products during fermentation stimulate the proliferation of more acid-tolerant microorganisms, which are only a minor part of the microbial community at neutral pH. A good example is the succession of microorganisms during milk spoilage. Biofilm formation provides another example. The colonization of a newly exposed surface by one type of microorganism (an initial colonizer) makes it possible for other microorganisms to attach to the microbially modified surface.

Commensalism also is important in the colonization of the human body and the surfaces of other animals and plants. The microorganisms associated with an animal's skin and body orifices can use volatile, soluble, and particulate organic compounds from the host as nutrients. Under most conditions these microbes do not cause harm, other than possibly contributing to body odor. Sometimes when the host organism is stressed or the skin is punctured, these normally commensal microorganisms may become pathogenic by entering a different environment.

Predation:

As is the case with larger organisms, **predation** among microbes involves a predator species that attacks and usually kills its prey. Over the last several decades, microbiologists have discovered a number of fascinating bacteria that survive by their ability to prey upon other microbes. Several of the best examples are *Bdellovibrio,Vampirococcus,* and *Daptobacter* (**figure 30.13**).

Bdellovibrio is an active hunter that is vigorously motile, swimming about looking for susceptible gram-negative bacterial prey. Upon sensing such a cell, *Bdellovibrio* swims faster until it collides with the prey cell. It then bores a hole through the outer membrane of its prey and enters the periplasmic space. As it grows, it forms a long filament that eventually septates to produce progeny bacteria. Lysis of the prey cell releases new *Bdellovibrio* cells. *Bdellovibrio* will not attack mammalian cells, and gram-negative prey bacteria have never been observed to acquire resistance to *Bdellovibrio* attack. This has raised interest in the use of *Bdellovibrio* as a "probiotic" to treat infected wounds. Although this is has not yet been tried, one can imaginethat with the rise in antibiotic-resistant pathogens, such forms of treatments may become viable alternatives.Although *Vampirococcus* and *Daptobacter* also kill their prey, they gain entry in a less-dramatic fashion. *Vampirococcus*attaches itself as an epibiont to the outer membrane of its prey. It then secretes degradative enzymes that result in the release of the prey's cytoplasmic contents. In contrast, *Daptobacter* penetrates the prey cell and consumes the cytoplasmic contents directly. A surprising finding is that predation has many beneficial effects, especially when one considers interactive populations of predators and prey, as summarized in **table 30.3.**

Simple ingestion and assimilation of a prey bacterium can lead to increased rates ofnutrient cycling, critical for the functioning of the microbial loop. In this process, organic matter produced through photosynthetic and chemotrophic activity is mineralized before it reaches higher consumers, allowing the minerals to be made available to the primary producers. Ingestion and

short-term retention of bacteria also are critical for ciliate functioning in the rumen, where methanogenic bacteria contribute to the health of the ciliates by decreasing toxic hydrogen levels by using H_2 to produce methane, which then is passed from the rumen.

Predation also can provide a protective, high-nutrient environment for particular prey. Ciliates ingest the gram-positive bacterium *Legionella* and protect this important pathogen from chlorine, which often is used in an attempt to control *Legionella*in cooling towers and air-conditioning units. The ciliate serves as a reservoir host. *Legionella pneumophila* also has been found to have a greater potential to invade macrophages and epithelial cells after predation, indicating that ingestion not only provides protection but also may enhance pathogenicity. A similar phenomenon of survival in protozoa has been observed for *Mycobacteriumavium*, a pathogen of worldwide concern. These protective aspects of predation have major implications for survival and control of disease-causing microorganisms in the biofilms present in water supplies and air-conditioning systems.

Fungi often show interesting predatory skills. Some fungi can trap protozoa by the use of sticky hyphae or knobs, sticky networks of hyphae, or constricting or nonconstricting rings. Aclassic example is *Arthrobotrys*, which traps nematodes by use of constricting rings. After the nematode is trapped, hyphae grow into the immobilized prey and the cytoplasm is used as a nutrient. Other fungi have conidia that, after ingestion by an unsuspectingpredator, grow and attack the susceptible host from inside the intestinaltract. In this situation the fungus penetrates the host cellsin a complex interactive process.

Clearly predation in the microbial world is not straightforward. It often has a fatal and final outcome for an individual prey organism but it can have a wide range of beneficial effects on prey populations. Predation is clearly critical in the functioning of natural environments.

Table 30.3 The Many Faces of Predation

Predation Result	Example
Digestion	The microbial loop. Soluble organic matter from primary producers is normally used by bacteria, which become a particulate food source for higher consumers. Flagellates and ciliates prey on these bacteria and digest them, making the nutrients they contain available again in mineral form for use in primary production. In this way a large portion of the carbon fixed by the photosynthetic microbes is mineralized and recycled and does not reach the higher trophic levels of the ecosystem (*see figure 27.13*).
	Predation also can reduce the density-dependent stress factors in prey populations, allowing more rapid growth and turnover of the prey than would occur if the predator were not active.
Retention	Bacteria retained within the predator serve a useful purpose, as in the transformation of toxic hydrogen produced by ciliates in the rumen to methane. Also, trapping of chloroplasts (kleptochloroplasty) by protozoa provides the predator with photosynthate.
Protection and increased fitness	The intracellular survival of *Legionella* ingested by ciliates protects it from stresses such as heating and chlorination. Ingestion also results in increased pathogenicity when the prey is again released to the external environment, and this may be required for infection of humans. The predator serves as a reservoir host.
	Nanoplankton may be ingested by zooplankton and grow in the zooplankton digestive system. They are then released to the environment in a more fit state. Dissemination to new locations also occurs.

Parasitism: **Parasitism** is one of the most complex microbial interactions; the line between parasitism and predation is difficult to define (figure 30.1). This is a relationship between two organisms in which one benefits from the other, and the host is usually harmed. This can involve

nutrient acquisition and/or physical maintenance in or on the host. In parasitism there is always some co-existence between host and parasite. Successful parasites have evolved to co-exist in equilibrium with their hosts. This is because a host that dies immediately after parasite invasion may prevent the microbe from reproducing to sufficient numbers to ensure colonization of a new host. But what happens if the host-parasite equilibrium is upset? If the balance favors the host (perhaps by a strong immune defense orantimicrobial therapy), the parasite loses its habitat and may be unable to survive. On the other hand, if the equilibrium is shifted to favor the parasite, the host becomes ill, and depending on the specific host-parasite relationship, may die.

One good example is the disease typhus. This disease is caused by the rickettsia *Rickettsiatyphi,* which is harbored in fleas that live on rats. It is transmitted to humans who are bitten by such fleas, so in order to contract typhus, one must be in close proximity to rats. Humans often live in association with rats, and in such communities there is always a small number of people with typhus—that is to say, typhus is endemic.

However, during times of war or when people are forced to become refugees, lack of sanitation and overcrowding result in an increased number of rat-human interactions. Typhus can then reach epidemic proportions. During the Crimean War (1853–1856), about 213,000 men were killed or wounded in combat while over 850,000 were sickened or killed by typhus. On the other hand, a controlled parasite-host relationship can be maintained for long periods of time. For example, **lichens** (**figure30.14**) are the association between specific ascomycetes (a fungus) and certain genera of either green algae or cyanobacteria.

In a lichen, the fungal partner is termed the **mycobiont** and the algal or cyanobacterial partner, the **phycobiont.** In the past the lichen symbiosis was considered to be a mutualistic interaction. It recently has been found that a lichen forms only when the two potential partners are nutritionally deprived. In nutrient-limited environments, the relationship between the fungus and its photosynthetic partner has coevolved to the point where lichen morphology and metabolic relationships are extremely stable. In fact, lichens are assigned generic and species names. The characteristic morphology of a given lichen is a property of the association and is not exhibited by either symbiont individually.Because the phycobiont is a photoautotroph—dependent only on light, carbon dioxide, and mineral nutrients—the fungus can get its organic carbon directly from the alga or cyanobacterium. The fungus often obtains nutrients from its partner by projections of fungal hyphae called haustoria, which penetrate the phycobiont cell wall. It also uses the O_2 produced during phycobiont photophosphorylation in carrying out respiration. In turn the fungus protects the phycobiont from high light intensities, provideswater and minerals to it, and creates a firm substratum within which the phycobiont can grow protected from environmental stress. The invasive nature of the fungal partner is why lichens are considered parasitic relationships.

An important aspect of many symbiotic relationships, including parasitism, is that over time, the symbiont, once it has established a relationship with the host, will tend to discard excess, unused genomic information, a process called **genomic reduction.**This is clearly the case with the aphid endosymbiont *Buchneraaphidicola* (p. 718) and it has also occurred with the parasite *Mycobacterium leprae,* and with the microsporidium *Encephalitozooncuniculi*. The latter

organism, which parasitizes a wide range of animals, including humans, now can only survive inside the host cell.

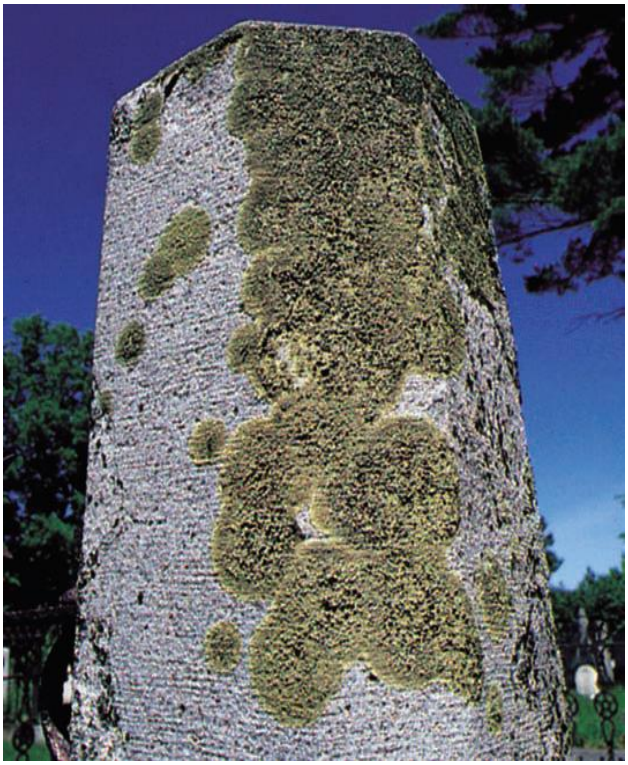


Figure 30.14 Lichens. Crustose (encrusting) lichens growing on a granite post.

Amensalism:

It describes he adverse effect that one organism has on another organism (figure 30.1). This is a unidirectional process based on the release of a specific compound by one organism which has a negative effect on another organism. A classic example of amensalism is the production of antibiotics that can inhibit or kill a susceptible microorganism (**figure 30.15*a***). Community complexity is demonstrated by the capacity of attine ants (ants belonging to a New World tribe) to take advantage of an amensalistic relationship between an actinomycete and the parasitic fungi *Escovopsis*. This amensalistic relationship enables the ant to maintain a mutualism with another fungal species, *Leucocoprini*. Amazingly, these ants cultivate a garden of *Leucocoprini* for their own nourishment (figure 30.15b). To prevent the parasitic fungus *Escovopsis* from decimating their fungal garden, the ants also promote the growth of an actinomycete of the genus *Pseudonocardia,* which produces an antimicrobial compound that inhibits the growth of *Escovopsis*.

This unique amensalistic process appears to have evolved 50 to 65million years ago in South America. Thus this relationship has been subject to millions of years of coevolution, such that particular groups of ants cultivate specific strains of fungi that are then subject to different groups of *Escovopsis* parasites. In addition, the ants have developed intricate crypts within their exoskeletons for the growth of the antibiotic-producing *Pseudonocardia*.As shown in figure

30.15*c*, these crypts have been modified throughout the ants' evolutionary history. The most primitive "paleo-attine" ants carry the bacterium on their forelegs, "lower" and "higher" attines have evolved special plates on their ventral surfaces, while the entire surface of the most recent attines, leaf-cutter ants of the genus *Acromyrmex*, are covered with the bacterium. Related ants that do not cultivate fungal gardens (e.g., *Atta* sp.) do not host *Pseudonocardia*.

This unique multipartner relationship has enabled scientists to explore the behavioral, physiological, and structural aspects of the organisms involved. Other important amensalistic relationships involve microbial production of specific organic compounds that disrupt cell wall orplasma membrane integrity of target microorganisms. These include the bacteriocins. The bacteriocin nisin has been used as an additive for controlling the growth of undesired pathogens in dairy products for over 40 years. Antibacterial peptides also can be released by the host in the intestine and other sites. These molecules, called cecropins in insects and defensins in mammals, are effector molecules that play significant roles in innate immunity.

In vertebrates these molecules are released by phagocytes and intestinal cells, and have powerful antimicrobial activity. Human sweat is also antimicrobial. Sweat glands produce an antimicrobial peptide called dermicidin. The skin also produces similar compounds including an antimicrobial peptide called cathelicidin.Finally, metabolic products, such as organic acids formed in fermentation, can produce amensalistic effects. These compounds inhibit growth by changing the environmental pH, for example, during natural milk spoilage.

Competition:

Competition arises when different organisms within a population or community try to acquire the same resource, whether this is a physical location or a particular limiting nutrient (figure 30.1). If one of the two competing organisms can dominate the environment, whether by occupying the physical habitat or by consuming a limiting nutrient, it will outgrow the other organism. This phenomenon was studied by E. F. Gause, who in 1934 described it as the **competitive exclusion principle.** He found that if two competingciliates overlapped too much in terms of their resource use, oneof the two protozoan populations was excluded. In chemostats,competition for a limiting nutrient may occur among microorganisms with transport systems of differing affinity. This can lead to the exclusion of the slower-growing population under a particular set of conditions. If the dilution rate is changed, the previously slowergrowing population may become dominant. Often two microbial populations that appear to be similar nevertheless coexist. In this case, they share the limiting resource (space, a limiting nutrient) and coexist while surviving at lower population levels.

◆ HUMAN-MICROBE INTERACTIONS

As we have seen, many microorganisms live much of their lives in a special ecological relationship: an important part of their environment is a member of another species. Here we discuss microorganisms normally associated with the human body, the **normal microbial flora** or **microbiota.** If we consider the human body as a diverse environment in and on which specific niches are formed, the normal flora may be discussed as the microbial ecology of a human.

The application of ecological principles can assist in our understanding of host-microberelationships. Interactions between host and microbe are dynamic, permitting niche fulfillment that maximizes benefit to the microbe and, in some cases, the host. Tolerating a normal flora likewise suggests that the host derives benefit. Acquisition of a normal microbial flora represents a selective process, where a niche may be defined by cellular receptors, surface properties, or secreted products. It should be noted that microbial niche variations are also related to age, gender, diet, nutrition, and developmental stage of the host. In general, the adult human microbial flora is relatively constant and can thus be mapped.

The survival of a host, such as a human, depends upon an elaborate network of defenses that keeps harmful microorganismsand other foreign material from entering the body. Should they gain access, additional host defenses are summoned to preventthem from establishing another type of relationship, one of parasitism or **pathogenicity.** Pathogenicity is the ability to produce pathologic changes or disease. A **pathogen** [Greek *patho,*and *gennan,* to produce] is any disease-producing microorganism. Here we introduce the normal human microbiota, which function not as pathogens but as symbionts that are part of the host's first line of defense against harmful infectious agents.

◆ GNOTOBIOTIC ANIMALS

To determine the role of the normal microorganisms associated with a host and evaluate the consequences of colonization, it is possibleto deliver an animal by cesarean section and raise that animal in the absence of microorganisms—that is, germfree. These microorganism- free animals provide suitable experimental models for investigating the interactions of animals and their microorganisms.

Comparing animals possessing normal microorganisms (conventional animals) with germfree animals permits the elucidation of many complex relationships between microorganisms, hosts, and specific environments. Germfree experiments also extend and challenge the microbiologist's "pure culture concept" to in vivo research.

The term **gnotobiotic** [Greek *gnotos,* known, and *biota,* the flora and fauna of a region] has been defined in two ways. Some think of a gnotobiotic environment or animal as one in which allthe microbiota are known; they distinguish it from one that is truly germfree. We shall use the term in a more inclusive sense. Gnotobiotic refers to a microbiologically monitored environment or animal that is germfree (**axenic** [*a,* neg, and Greek *Xenos,* a stranger]) or in which the identities of all

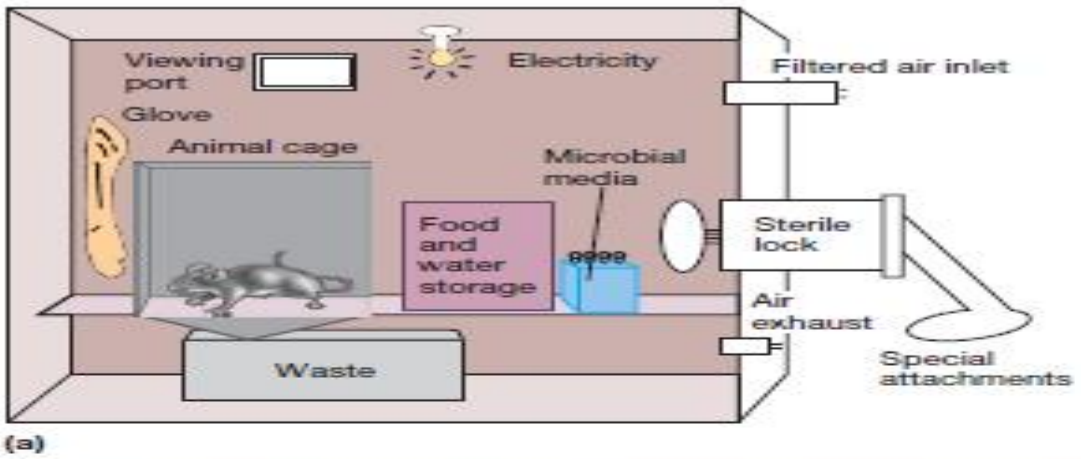
microbiota are known.Development of a lifelong symbiotic relationship with microbes begins during birth. The infant's exposure to the vaginal mucosa, skin, hair, food, and other nonsterile objects quickly results in the acquisition of a predominantly commensal normal flora. The human fetus in utero (as is the case in most mammals) is usually free from microorganisms. As an infant begins to acquire a normal microbiota, the microbial population stabilizes during the first week or two of life.

Colonization of the newborn varies with respect to its environment. The newborn likely acquires external flora from those who provide its care. Likewise, internal flora are acquired through its diet. Bifidobacteria represent more than 90% of the total intestinal bacteria in breast-fed infants, with *Enterobacteriaceae*and enterococci in smaller proportions. This suggests that human milk may act as a selective medium for nonpathogenic bacteria, as bottle-fed babies appear to have a much smaller proportion of intestinal bifidobacteria. Switching tocow's milk or solid food (mostly polysaccharide) appears to resultin the loss of bifidobacteria predominance, as *Enterobacteriaceae*,enterococci, bacteroides, lactobaccili, and clostridia increase in number. Bacterial chemotaxis and trophism may explain the high frequency of bacterial-tissue associations. Additionally, the host may partly direct microbe-tissue associations as seen in the selective destruction of gram-positive bacteria by the antimicrobial peptide angiogenin-4 secreted by special intestinal cells called **Paneth cells.**

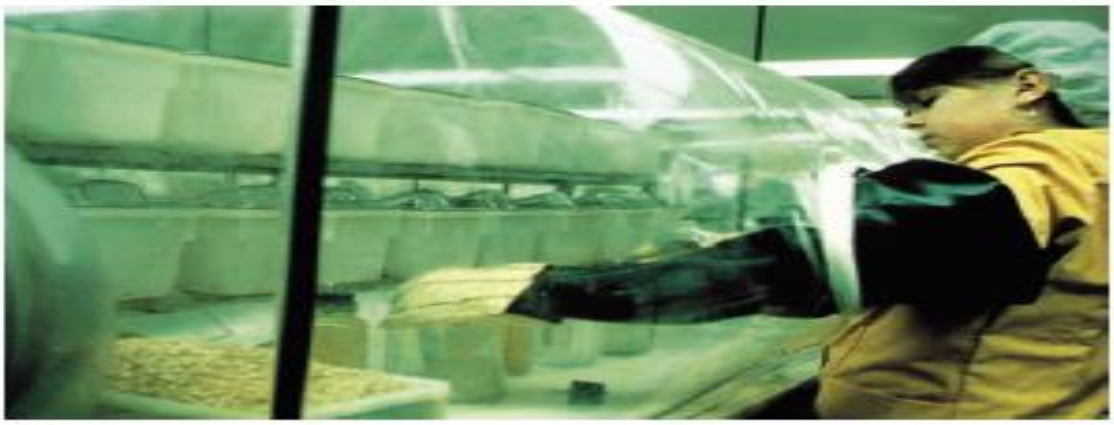
Louis Pasteur first suggested that animals could not live in the absence of microorganisms. Attempts between 1899 and 1908 togrow germfree chickens had limited success because the birds died within a month. Thus it was believed that intestinal bacteria were essential for the adequate nutrition and health of the chickens. It was not until 1912 that germfree chickens were shown to be as healthy as normal birds when they were fed an adequate diet. Since then, gnotobiotic animals and systems have become commonplace in research laboratories (**figure 30.16**).

What have we learned from germfree animals? Germfree animals are usually more susceptible to pathogens. With the normal commensal microbiota absent, foreign and pathogenic microorganismsestablish themselves very easily. The number of microorganisms necessary to infect a germfree animal and produce a diseased state is much smaller. Conversely, germfree animals are almost completely resistant to the intestinal protozoan *Entamoebahistolytica* that causes amebic dysentery.

This resistance results from the absence of the bacteria that *E. histolytica* uses as a food source. Germfree animals also do not show any dental caries or plaque formation. However, if they are inoculated with cariogenic (caries or cavity-causing) streptococci of the *Streptococcusmutans–Streptococcus gordonii* group and fed a highsucrose diet, they develop caries.



(a)



(b)

Figure 30.16 Raising Gnotobiotic Animals. **(a)** Schematic of a gnotobiotic isolator. The microbiological culture media monitor the sterile environment. If growth occurs on any of the cultures, gnotobiotic conditions do not exist. **(b)** Gnotobiotic isolators for rearing colonies of small mammals.

◆ NORMALMICROBIOTA OF THE HUMAN BODY

In a healthy human the internal tissues (e.g., brain, blood, cerebrospinal fluid, muscles) are normally free of microorganisms. Conversely, the surface tissues (e.g., skin and mucous membranes) areconstantly in contact with environmental microorganisms and become readily colonized by various microbial species. The mixture of microorganisms regularly found at any anatomical site is referred to as the normal microbiota, the indigenous microbial population, the microflora, or the normal flora. For consistency, the term normal microbiota is used in this chapter. An overview of the microbiota native to different regions of the body is presented next.

Because bacteria make up most of the normal microbiota, they are emphasized over the fungi (mainly yeasts) and protists.There are many reasons to acquire knowledge of the normalhuman microbiota. Three specific examples include:

1. An understanding of the different microorganisms at particular locations provides greater insight into the possible infections that might result from injury to these body sites.

2. A knowledge of the normal microbiota helps the physician investigator understand the causes and consequences of colonization and growth by microorganisms normally absent at a specific body site.

3. An increased awareness of the role that these normal microbiota play in stimulating the host immune response can be gained. This awareness is important because the immune system provides protection against potential pathogens.

As noted previously, three of the most important types of symbiotic relationships are commensalism, mutualism, and parasitism. Within each category the association may be either ectosymbiotic or endosymbiotic. In the following subsections,examples are presented of both ecto- and endosymbiotic relationships.Both commensalistic and mutualistic relationships are also considered.

Skin-

The adult human is covered with approximately 2 square meters of skin. It has been estimated that this surface area supports about1012 bacteria. Recall that commensalism is a symbiotic relationship in which one species benefits and the other is unharmed. Commensal microorganisms living on or in the skin can be either resident (normal) or transient microbiota. Resident organisms normally grow on or in the skin. Their presence becomes fixed in well-defined distribution patterns. Those that are temporarily present are transient microorganisms. Transients usually do not become firmly entrenched and are unable to multiply.

It should be emphasized that the skin is a mechanically strong barrier to microbial invasion. Few microorganisms canpenetrate the skin because its outer layer consists of thick, closely packed cells called keratinocytes. In addition to direct resistance to penetration, continuous shedding of the outer epithelial cells removes many of those microorganisms adhering to the skin surface.

The anatomy and physiology of the skin vary from one part of the body to another, and the normal resident microbiota reflect these variations. The skin surface or epidermis is not a favorable environment for microbial colonization. In addition to a slightly acidic pH, a high concentration of sodium chloride, and a lack ofmoisture in many areas, certain inhibitory substances (bactericidaland/or bacteriostatic) on the skin help control microbial colonization.

For example, the sweat glands release lysozyme (muramidase), an enzyme that lyses *Staphylococcus epidermidis* and other gram-positive bacteria by hydrolyzing the ß(1 → 4) glycosidic bond connecting *N*-acetylmuramic acid and *N*-acetylglucosaminein the bacterial cell wall peptidoglycan. Sweatglands also produce antimicrobial peptides called **cathelicidins**(Latin *catharticus,* to purge, and *cida,* to kill) that help protect against infectious agents by forming pores in bacterial plasma membranes.The oil glands secrete complex lipids that may be partially degraded by the enzymes from certain gram-positive bacteria (e.g., *Propionibacterium acnes*). These bacteria can change the secreted lipids to unsaturated fatty acids such as oleic acid that have

strong antimicrobial activity against gram-negative bacteria and some fungi. Some of these fatty acids are volatile and may be associated with a strong odor. Therefore many deodorants contain antibacterial substances that act selectively against gram-positive bacteria to reduce the production of volatile unsaturated fatty acids and body odor.

Most skin bacteria are found on superficial cells, colonizing dead cells, or closely associated with the oil and sweat glands. Secretionsfrom these glands provide the water, amino acids, urea, electrolytes, and specific fatty acids that serve as nutrients primarily for *S. epidermidis* and aerobic corynebacteria. Gramnegative bacteria generally are found in the moister regions. The yeasts *Pityrosporum ovale* and *P. orbiculare* normally occur on the scalp.

The most prevalent bacterium in the oil glands is the grampositive, anaerobic, lipophilic rod *Propionibacterium acnes*. Thisbacterium usually is harmless; however, it is associated with the skin disease acne vulgaris. Acne commonly occurs during adolescence when the endocrine system is very active. Hormonal activity stimulates an overproduction of **sebum,** a fluid secreted by the oil glands. A large volume of sebum accumulates within the glands and provides an ideal microenvironment for *P. acnes*. In some individuals this accumulation triggers an inflammatory response that causes redness and swelling of the gland's duct and produces a **comedo** [pl., comedones], a plug of sebum and keratin in the duct. Inflammatory lesions (papules, pustules, nodules) commonly called "blackheads" or "pimples" can result when pores or ducts clog with cebum or bacteria. *P. acnes* produces lipases that hydrolyse the sebum triglycerides into free fatty acids. Free fatty acids are especially irritating because they can enter the dermis and promote inflammation. Because *P. acnes*is extremely sensitive to tetracycline, this antibiotic may aidacne sufferers. Retin A and accutane, synthetic forms of vitamin A, are also used.

Nose and Nasopharynx-

The normal microbiota of the nose is found just inside the nostrils. *Staphylococcus aureus* and *S. epidermidis* are the predominant bacteria present and are found in approximately the same numbers as on the skin of the face. The nasopharynx, that part of the pharynx lying above the level of the soft palate, may contain small numbers of potentially mpathogenic bacteria such as *Streptococcus pneumoniae, Neisseriameningitidis,* and *Haemophilus influenzae*. Diphtheroids, a large group of nonpathogenic gram-positive bacteria that resemble *Corynebacterium,* are commonly found in both the nose and nasopharynx.

Oropharynx-

The oropharynx is that division of the pharynx lying between the soft palate and the upper edge of the epiglottis. The most important bacteria found in the oropharynx are the various alpha-hemolytic streptococci (*S. oralis, S. milleri, S. gordonii, S. salivarius*); large numbers of diphtheroids; *Branhamella catarrhalis;* and small gram-negative cocci related to *N. meningitidis*. The palatine and pharyngeal tonsils harbor a similar microbiota, except within the tonsillar crypts, where there is an increase in *Micrococcus* and the anaerobes *Porphyromonas, Prevotella,* and *Fusobacterium*.

Respiratory Tract- The upper and lower respiratory tracts (trachea, bronchi, bronchioles, alveoli) do not have a normal microbiota. This is because microorganisms are removed in at least three ways. First, a continuous stream of mucus is generated by the goblet cells. This entraps microorganisms, and the ciliated epithelial cells continually move the entrapped microorganisms out of the respiratory tract. Second, alveolar macrophages phagocytize and destroy microorganisms. Finally, a bactericidal effect is exerted by the enzyme lysozyme, which is present in the nasal mucus.

Eye At birth and throughout human life, a small number of bacterial commensals are found on the conjunctiva of the eye. The predominant bacterium is *S. epidermidis* followed by *S. aureus,Haemophilus* spp., and *S. pneumoniae*.

External Ear- The normal microbiota of the external ear resemble those of the skin, with coagulase-negative staphylococci and *Corynebacterium*predominating. Mycological studies show the following fungi to be normal microbiota: *Aspergillus, Alternaria, Penicillium,Candida,* and *Saccharomyces.*

Mouth- The normal microbiota of the mouth or oral cavity contains organisms that resist mechanical removal by adhering to surfaces like the gums and teeth. Those that cannot attach are removed by the mechanical flushing of the oral cavity contents to the stomach where they are destroyed by hydrochloric acid. The continuous desquamation (shedding) of epithelial cells also removes microorganisms.

Those microorganisms able to colonize the mouth finda very comfortable environment due to the availability of water and nutrients, the suitability of pH and temperature, and the presence of many other growth factors. The oral cavity is colonized by microorganisms from the surrounding environment within hours after a human is born.

Initially the microbiota consists mostly of the genera *Streptococcus, Neisseria, Actinomyces, Veillonella,* and *Lactobacillus*.Some yeasts also are present. Most microorganisms that invadethe oral cavity initially are aerobes and obligate anaerobes. Whenthe first teeth erupt, anaerobes (*Porphyromonas, Prevotella,* and*Fusobacterium*) become dominant due to the anoxic nature of thespace between the teeth and gums. As the teeth grow, *Streptococcus parasanguis* and *S. mutans* attach to their enamel surfaces;*S. salivarius* attaches to the buccal and gingival epithelialsurfaces and colonizes the saliva. These streptococci produce aglycocalyx and various other adherence factors that enable themto attach to oral surfaces. The presence of these bacteria contributesto the eventual formation of dental plaque, caries, gingivitis,and periodontal disease.

Stomach-

As noted earlier, many microorganisms are washed from the mouth into the stomach. Owing to the very acidic pH values (2 to3) of the gastric contents, most microorganisms are killed. As a result the stomach usually contains less than 10 viable bacteria per milliliter of gastric fluid. These are mainly *Streptococcus, Staphylococcus, Lactobacillus, Peptostreptococcus,* and yeasts such as *Candida* spp. Microorganisms may survive if they pass rapidly through the stomach or if the organisms ingested with food areparticularly resistant to gastric pH (e.g., mycobacteria).

Small Intestine- The small intestine is divided into three anatomical areas: the duodenum, jejunum, and ileum. The duodenum (the first 25 cm of the small intestine) contains few microorganisms because of the combined influence of the stomach's acidic juices and the

inhibitory action of bile and pancreatic secretions that are added here. Of the bacteria present, gram-positive cocci and rods comprise most of the microbiota. *Enterococcus faecalis, lactobacilli,* diphtheroids, and the yeast *Candida albicans* are occasionally found in the jejunum. In the distal portion of the small intestine (ileum), the microbiota begin to take on the characteristics of the colon microbiota. It is within the ileum that the pH becomes more alkaline. As a result anaerobic gram-negative bacteria and members of the family *Enterobacteriaceae* become established.

Large Intestine (Colon)- The large intestine or colon has the largest microbial community in the body. Microscopic counts of feces approach 1012 organismsper gram wet weight. Over 400 different species have been isolated from human feces. The microbiota consist primarily of anaerobic, gram-negative bacteria and gram-positive, spore-forming, and nonsporing rods. Not only are the vast majority of microorganisms anaerobic, but many different species are present in large numbers. Several studies have shown that the ratio of anaerobic to facultative anaerobic bacteria is approximately 300 to 1. Besides the many bacteria in the large intestine, the yeast *Candida albicans* and certain protozoa may occur as harmless commensals. *Trichomonas hominis, Entamoebahartmanni, Endolimax nana,* and *Iodamoeba butschlii*are common inhabitants.

The importance of the microbes living within the human colon, which can be likened to an anaerobic bioreactor, has prompted a number of investigations using culture-independent molecular approaches. Recent 16S rRNA analysis of microbes shed in feces, as well as microbes collected from gut epithelium, reveals that the majority of procaryotes are currently uncultivated.

However, one bacterium, *Bacteroides thetaiontaomicron,* has been the focus of recent interest. This microbe is well suitedfor survival in the gut, where it is able to degrade complex dietarypolysaccharides. Genome analysis reveals that *B. thetaiontaomicron* has a large collection of genes that encode proteins neededfor the acquisition and metabolism of carbohydrates. It resides ina specific microenvironment: rather than adhering to the intestinalepithelium, it produces substrate-specific binding proteinsthat allow it to colonize exfoliated host cells, food particles, andeven sloughed mucus (**figure 30.18**). It is thought that such attachmenthelps retain the microbes in the gut and, once bound,the induced expression of extracellular hydrolases enables efficientdigestion. Of course, the diversity and density of microbeswithin the colon suggests that such "nutrient rafts" are colonizedby a community of bacteria. For example, methanogenic bacteriaare thought to remove the products of fermentation by convertingH_2 and CO_2 to methane, just as they do in the rumenmicrobial community.

Various physiological processes move the microbiota through the colon so an adult eliminates about 3×10^{13} microorganisms daily. These processes include peristalsis and desquamation of the surface epithelial cells to which microorganisms are attached, and continuous flow of mucus that carries adhering microorganisms with it. To maintain homeostasis of the microbiota, the body must continually replace lost microorganisms.

The bacterial population in the human colon usually doubles once or twice a day. Under normal conditions the resident microbial community is self-regulating. Competition and mutualism between different microorganisms and between the microorganisms and their host serve to

maintain a status quo. However, if the intestinal environment is disturbed, the normal microbiota may change greatly. Disruptive factors include stress, altitude changes, starvation, parasitic organisms, diarrhea, and use of antibiotics or probiotics. Finally, it should be emphasized that the actual proportions of the individual bacterial populations within the indigenous microbiota depend largely on a person's diet.

Genitourinary Tract- The upper genitourinary tract (kidneys, ureters, and urinary bladder) is usually free of microorganisms. In both the male and female, a few bacteria (*S. epidermidis, E. faecalis*, and *Corynebacterium*spp.) usually are present in the distal portion of the urethra. In contrast, the adult female genital tract, because of its large surface area and mucous secretions, has a complex microbiota that constantly changes with the female's menstrual cycle. The major microorganisms are the acid-tolerant lactobacilli, primarily *Lactobacillus acidophilus*, often called Döderlein's bacillus. They ferment the glycogen produced by the vaginal epithelium, forming lactic acid. As a result the pH of the vagina and cervix is maintained between 4.4 and 4.6, inhibiting other microorganisms.

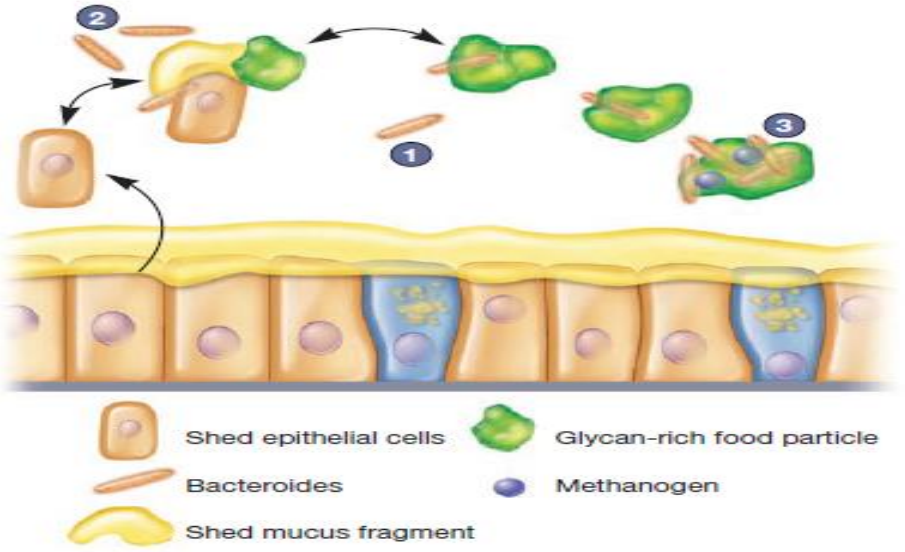


Figure 30.18 ***Bacteriodes thetaiontaomicron*** **as a Model for Colon Microbial Physiology and Community Dynamics.**
(1) *B. thetaiontaomicron* is rapidly eliminated if it remains planktonic; however, (2) it efficiently adheres to substrates within the lumen of the gut rather than the gut itself (3) where, together with other microbes including methanogenic archaea, it degrades complex carbohydrates.

◆ THE RELATIONSHIP BETWEEN NORMAL MICROBIOTA AND THE HOST

The interaction between a host and a microorganism is a dynamic process in which each partner acts to maximize its survival. In some instances, after a microorganism enters or contacts a host, a

positive mutually beneficial relationship occurs that becomes integral to the health of the host. These microorganisms become the normal microbiota. In other instances, the microorganism produces or induces deleterious effects on the host; the end result may be disease or even death of the host.

Our environment is teeming with microorganisms and we come in contact with many of them every day. Some of these microorganisms are pathogenic—that is, they cause disease. Yet these pathogens are at times prevented from causing disease by competition provided by the normal microbiota. In general, the normal microbiota use space, resources, and nutrients needed by pathogens. In addition, they may produce chemicals that repel invading pathogens. These normal microbiota prevent colonization by pathogens and possible disease through "bacterial interference."

For instance, the lactobacilli in the female genital tract maintain a low pH and inhibit colonization by pathogenic bacteriaand yeast, and the corynebacteria on the skin produce fattyacids that inhibit colonization by pathogenic bacteria. This is anexcellent example of amensalism.Products made by colonic bacteria (such as vitamins B andK) also benefit the host. Interestingly, studies using germfree animals suggest a strong correlation between the establishment of a stable microbial flora and the induction of immune compentency. For example, the introduction of normal fecal flora to germfree rodents stimulates the production and secretion of angiogenin-4, an antimicrobial peptide of intestinal Paneth cells.

Furthermore, the reconstitution of germfree rodents with flora from conventionally raised siblings causes the abnormal gutassociated lymphoid (GALT) tissue and intestinal lamina propria to resemble that of the conventional animals (i.e., their lymphoid tissues and immunity are normalized). Even cell wall fragmentsfrom gram-positive bacteria can induce these changes. This normalization also includes an increase in the local lymphocyte populations and increased mucosal antibody production.Although normal microbiota offer some protection from invading pathogens, they may themselves become pathogenic and produce disease under certain circumstances, and then are termed **opportunistic microorganisms** or **pathogens.** These opportunistic microorganisms are adapted to the noninvasive mode of life defined by the limitations of the environment in which they are living. If removed from these environmental restrictions and introduced into the bloodstream or tissues, disease can result. For example, streptococci of the viridans group are the most common resident bacteria of the mouth and oropharynx. If they are introduced into the bloodstream in large numbers (e.g., following tooth extraction or a tonsillectomy), they may settle on deformed or prosthetic heart valves and cause endocarditis.

Opportunistic microorganisms often cause disease in compromised hosts. A **compromised host** is seriously debilitated and has a lowered resistance to infection. There are many causes of this condition including malnutrition, alcoholism, cancer, diabetes,leukemia, another infectious disease, trauma from surgery or an injury, an altered normal microbiota from the prolonged use of antibiotics, and immunosuppression by various factors (e.g., drugs, viruses [HIV], hormones, and genetic deficiencies). For example, *Bacteroides* species are one of the most common residents in the large intestine (figure 30.18) and are quite harmless in that location. If introduced into the

peritoneal cavity or into the pelvic tissues as a result of trauma, they cause suppuration (the formation of pus) and bacteremia (the presence of bacteria in the blood).

The important point here is that the normal microbiota are harmless and are often beneficial in their normal location in the host and in the absence of coincident abnormalities. However, they can produce disease if introduced into foreign locations or compromised hosts.

BIOFILMS:

Although scientists observed as early as the 1940s that more microbes in aquatic environments were found attached to surfaces (sessile) rather than were free-floating (planktonic), only relativelyrecently has this fact gained the attention of microbiologists. These attached microbes are members of complex, slime-encased communities called **biofilms.** Biofilms are ubiquitous in nature. There they are most often seen as layers of slime on rocks or other objects in water (**figure 6.27*a***). When they form on the hulls of **(a) (b)**boats and ships, they cause corrosion, which limits the life of the ships and results in economic losses. Of major concern is the formation of biofilms on medical devices such as hip and knee implants (figure 6.27*b*). These biofilms often cause serious illness and failure of the medical device. Biofilm formation is apparently an ancient ability among the procaryotes, as evidence for biofilms can be found in the fossil record from about 3.4 billion years ago.

Biofilms can form on virtually any surface, once it has been conditioned by proteins and other molecules present in the environment (**figure 6.28**). Microbes reversibly attach to the conditioned surface and eventually begin releasing polysaccharides,proteins, and DNA. These polymers allow the microbes to stick more stably to the surface. As the biofilm thickens and matures, the microbes reproduce and secrete additional polymers. The end result is a complex, dynamic community of microorganisms. The microbes interact in a variety of ways. For instance, the waste products of one microbe may be the energy source for another microbe. The cells also communicate with each other as described next. Finally, the presence of DNA in the extracellular slime can be taken up by members of the biofilm community. Thus genes can be transferred from one cell (or species) to another. While in the biofilm, microbes are protected from numerous harmful agents such as UV light, antibiotics, and other antimicrobial agents. This is due in part to the extracellular matrix in which they are embedded, but it also is due to physiological changes. Indeed, numerous proteins synthesized or activated in biofilm cells are not observed in planktonic cells and vice versa.

The resistance of biofilm cells to antimicrobial agents has serious consequences. When biofilms form on a medical device such as a hip implant (figure 6.27*b*), they are difficult to kill and can cause serious illness. Often the only way to treat patients in this situation is by removing the implant. Another problem with biofilms is that cells are regularly sloughed off (figure 6.28). This can have many consequences. For instance, biofilms in a city’s water distribution pipes can serve as a source of contaminationafter the water leaves a water treatment facility.

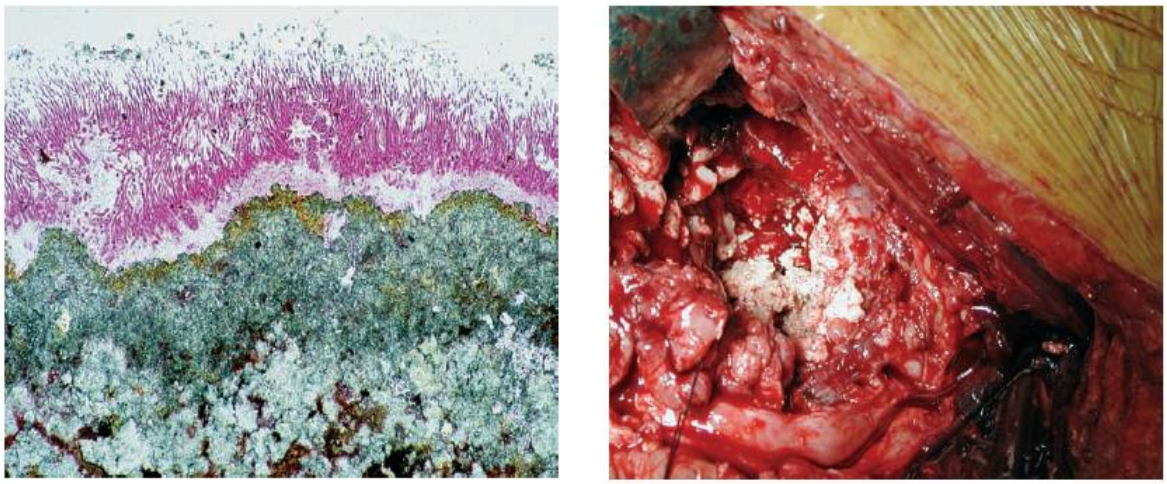


Figure 6.27 Examples of Biofilms. Biofilms form on almost any surface exposed to microorganisms. (**a**) Biofilm on the surface of a stromatolite in Walker Lake (Nevada, USA), an alkaline lake. The biofilm consists primarily of the cyanobacterium *Calothrix*. (**b**) Photograph taken during surgery to remove a biofilm-coated artificial joint. The white material is composed of pus, bacterial and fungal cells, and the patient's white blood cells.

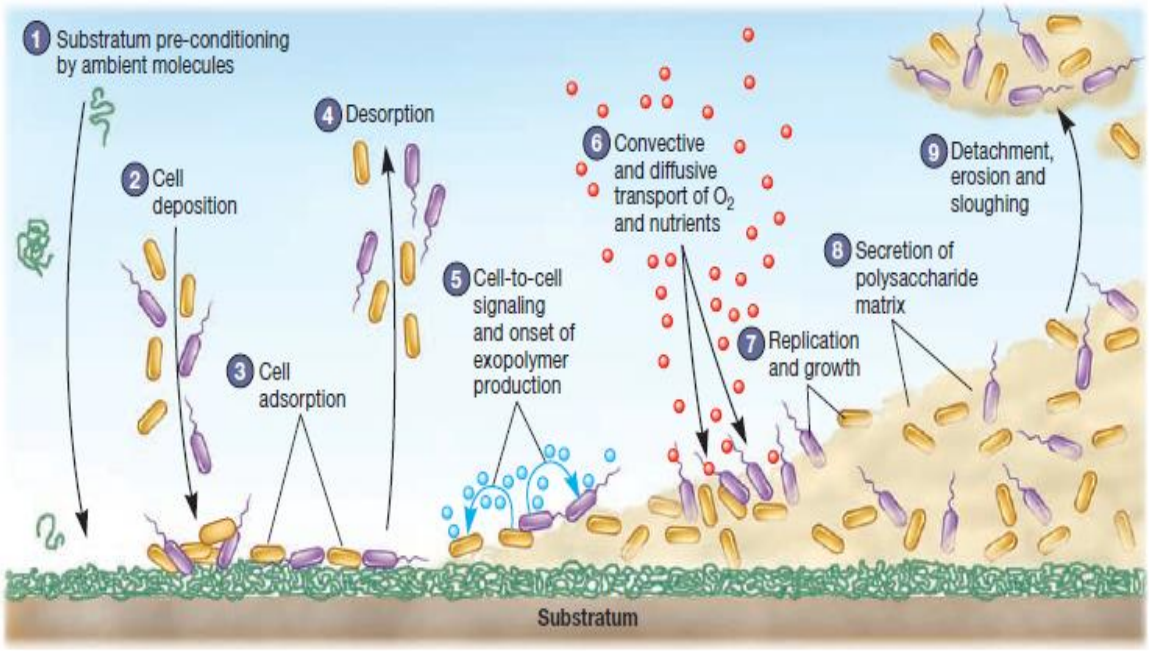


Figure 6.28 Biofilm Formation.

Cell-Cell Communication within Microbial Populations : For decades, microbiologists tended to think of bacterial populations as collections of individual cells growing and behaving independently. But about 30 years ago, it was discovered that the marine luminescent bacterium *Vibrio fischeri* controls its ability to glow by producing a small, diffusible substance called

autoinducer. The autoinducer molecule was later identified as an **acylhomoserinelactone (AHL).** It is now known that many gram-negative bacteriamake AHL molecular signals that vary in length and substitution at the third position of the acyl side chain (**figure 6.29**).

In many of these species, the AHL is freely diffusible across the plasma membrane. Thus at a low cell density it diffuses out of the cell. However, when the cell population increases and AHL accumulates outside the cell, the diffusion gradient is reversed so that the AHL enters the cell. Because the influx of AHL is cell density dependent, it enables individual cells to assess population density. This is referred to as **quorum sensing;** a quorum usually refers to the minimum number of members in an organization, such as a legislative body, needed to conduct business. When AHL reaches a threshold level inside the cell, it serves to induce the expression oftarget genes that regulate a number of functions, depending on the microbe. These functions are most effective only if a large number of microbes are present. For instance, the light produced by one cellis not visible, but cell densities within the light organ of marine fish and squid reach 1010 cells per milliliter. This provides the animal with a flashlight effect while the microbes have a safe and nutrient enriched habitat (**figure 6.30**). In fact, many of the processes regulated by quorum sensing involve host-microbe interactions such as symbioses and pathogenicity.

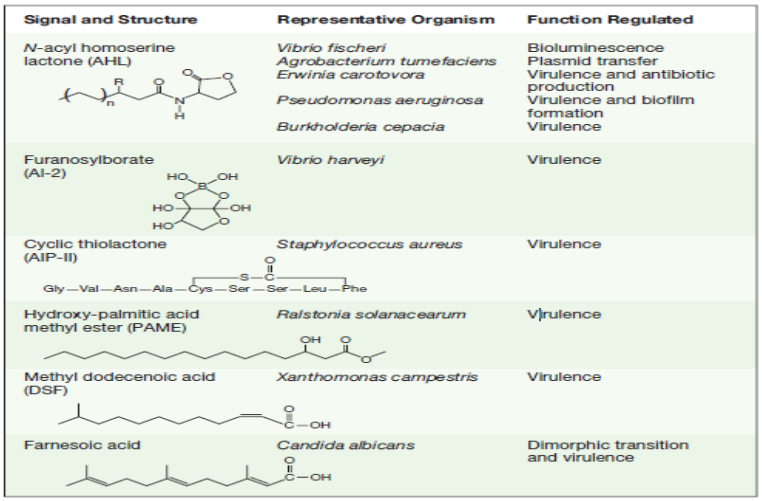


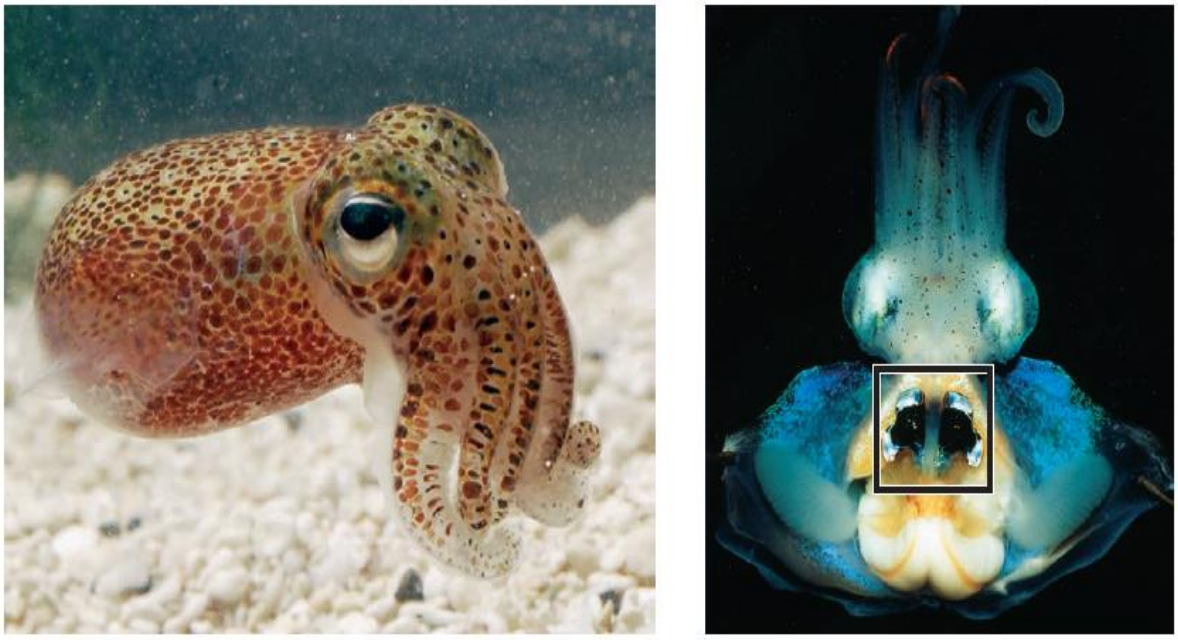
Figure 6.29 Representative Cell-Cell Communication Molecules.

Many different bacteria use AHL signals. In addition to *V. fischeri* bioluminescence, the opportunistic pathogens *Burkholderia cepacia* and *Pseudomonas aeruginosa* use AHLs to regulatethe expression of virulence factors (figure 6.29). These gramnegativebacteria cause debilitating pneumonia in people who areimmunocompromised, and are important pathogens in cystic fibrosispatients. The plant pathogens *Agrobacterium tumefaciens* will not infect a host plant and *Erwinia carotorvora* will not produceantibiotics without AHL signaling. Finally, *B. cepacia*, *P. aeruginosa*, as well as *Vibrio cholerae* use AHL intercellular communication to control biofilm formation, an important strategyto evade the host's immune system.

The discovery of additional molecular signals made by a variety of microbes underscores the importance of cell-cell communication in regulating procaryotic processes. For instance, while only gram-negative bacteria are known to makeAHLs, both gramnegative and gram-positive bacteria make autoinducer-2 (AI-2). Gram-positive bacteria usually exchange short peptides called

oligopeptides instead of autoinducer-like molecules. Examples include *Enterococcus faecalis*, whose oligopeptide signal is used todetermine the best time to conjugate (transfer genes). Oligopeptidecommunication by *Staphylococcus aureus* and *Bacillus subtilis* is used to trigger the uptake of DNAfrom the environment. The soil microbe *Streptomyces griseus* produces a gamma-butyrolactone known as A-factor. This small molecule regulates both morphological differentiation and the production of the antibiotic streptomycin. Eucaryotic microbes also rely on cell-cell communication to coordinate key activities within a population. For example, the pathogenic fungus *Candida albicans* secretes farnesoic acid to govern morphology and virulence.

These examples of cell-cell communication demonstrate what might be called multicellular behavior in that many individual cells communicate and coordinate their activities to act as a unit. Other examples of such complex behavior is pattern formation in colonies and fruiting body formation in the myxobacteria.



(a) *E. scolopes*, the bobtail squid (b) Light organ

Figure 6.30 ***Euprymna scolopes.*** (a) *E. scolopes* is a warm-water squid that remains buried in sand during the day and feeds at night. (b) When feeding it uses its light organ (boxed, located on its ventral surface) to provide camouflage by projecting light downward. Thus the outline of the squid appears as bright as the water's surface to potential predators looking up through the water column. The light organ is colonized by a large number of *Vibrio fischeri* so autoinducer accumulates to a threshold concentration, triggering light production.

BIOSENSORS:

Arapidly developing area of biotechnology, is that of **biosensor** production. In this field of bioelectronics, living microorganisms (or their enzymes or organelles) are linked with electrodes,and biological reactions are converted into electrical currents(**figure 41.29**). Biosensors have been developed to measure specificcomponents in beer, to monitor pollutants, to detect

flavorcompounds in food, and to study environmental processessuch as changes in biofilm concentration gradients. It is possibleto measure the concentration of substances from many differentenvironments (**table 41.17**). Applications include thedetection of glucose, acetic acid, glutamic acid, ethanol, andbiochemical oxygen demand. Biosensors have been developedusing immunochemical-based detection systems. These newbiosensors will detect pathogens, herbicides, toxins, proteins,and DNA. Since the bioterrorism attacks of 2001, the U.S. governmenthas stepped up funding for research and developmentof biosensors capable of detecting minute levels of potential airbornepathogens. Many of these biosensors are based on the useof a streptavidin-biotin recognition system.

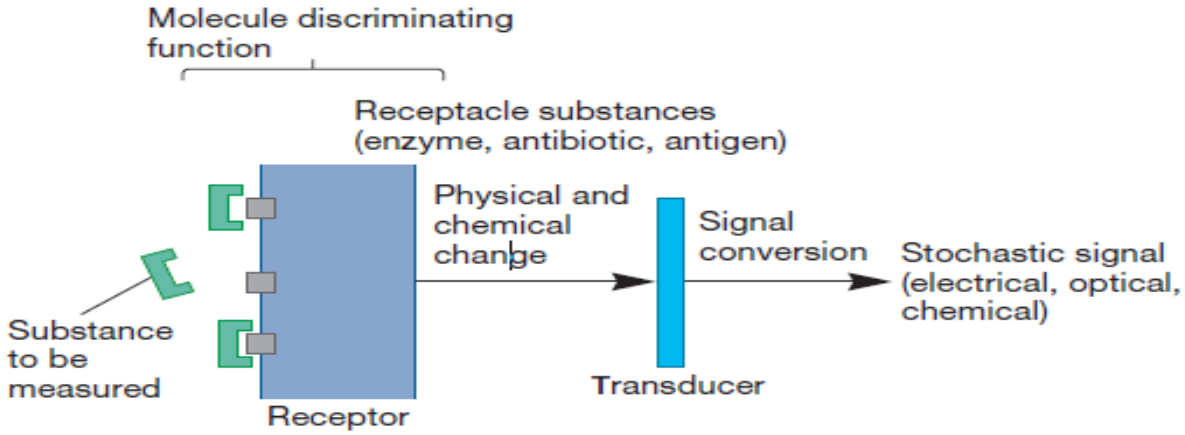


Figure 41.29 Biosensor Design. Biosensors are finding increasing applications in medicine, industrial microbiology, and environmental monitoring. In a biosensor a biomolecule or whole microorganism carries out a biological reaction, and the reaction products are used to produce an electrical signal.

c. Aeromicrobiology :

Aeromicrobiology is the study of living microbes which are suspended in the air. These microbes are referred to as bioaerosols (Brandl et. al, 2008). Though there are significantly less atmospheric microorganisms than there are in oceans and in soil, there is still a large enough number that they can affect the atmosphere (Amato, 2012). Once suspended in the air column, these microbes have the opportunity to travel long distances with the help of wind and precipitation, increasing the occurrence of widespread disease by these microorganisms. These aerosols are ecologically significant because they can be associated with disease in humans, animals and plants. Typically microbes will be suspended in clouds, where they are able to perform processes that alter the chemical composition of the cloud, and may even induce precipitation.

Physical Environment:

There are many factors within the physical environment that affect the launching, transport and deposition of bioaerosols. Particles which become suspended in the air column arise mainly from

terrestrial and aquatic environments and are typically launched by air turbulence (Pepper 2011). Winds are the primary means of transport for bioaerosols. Bioaerosols can be deposited by a number of mechanisms, including gravity pulling them down, making contact with surfaces, or combining with rain which pulls the particles back down to earth's surface.

Atmosphere

Along with water droplets, dust particles and other matter, air contains microbes (Al-Dagal 1990). Microbes follow a particular pathway in which they are suspended into the atmosphere. First they are launched into the air. The source of the launching of airborne microbes stems from humans, animals and vegetation. (Al-Dagal 1990). then they are transported (by various methods including winds, machinery and people) and finally are deposited somewhere new. The atmosphere can have a variety of physical characteristics, and can be very extreme in terms of the relative humidity, temperature and radiation. These factors play a huge role in what kinds of microbes will survive in the atmosphere and how long they will stay alive (Pepper 2011).

Clouds

One area that bioaerosols can be found in is within clouds. Cloud water is a mixture of organic and inorganic compounds suspended within moisture (contribution of microbial activity yo clouds). The conditions in clouds are not conducive to much life, as microbes present there must withstand freezing temperatures, the threat of desiccation, and extreme UV rays. Clouds are also an acidic

environment, with a pH ranging from 3 to 7. Nevertheless, there are extremophile microbes which can withstand all of these environmental pressures. Clouds serve as a transport for these microbes, dispersing them over long distances (Amato 2012).

Microbes exist within the atmosphere, and can be transported within clouds. Photo by Hamed Saber/flickr/Creative Commons.

Physical Environment Stresses

The atmosphere is a difficult place for a microbe to survive. Dessication is the primary stress that aeromicrobes face, and it limits the amount of time that they can survive while suspended in the air (Pepper 2011). Humidity within the air is a second factor which can affect the survival of organisms. Certain bacteria, including Gram + bacteria, are more tolerant of high humidity in the air, while others are more tolerant of dessication and dry conditions, such as Gram + cells (Pepper 2011). Temperature must be in an intermediate range, as too hot of temperatures can denature proteins, and too cold of temperatures can cause ice crystal formation (Pepper 2011). Finally, radiation poses a potential hazard for aeromicrobes, as it can damage DNA within the cells.

Microbial Communities:

Many different microorganisms can be in aerosol form in the atmosphere, including viruses, bacteria, fungi, yeasts and protozoans. In order to survive in the atmosphere, it is important that these microbes adapt to some of the harsh climatic characteristics of the exterior world, including temperature, gasses and humidity. Many of the microbes that are capable of surviving harsh conditions can readily form endospores, which can withstand extreme conditions (Al-Dagal 336).

Many of these microorganisms can be associated with specific and commonly known diseases. Below are two tables. Table 1 below shows examples of Airborne Plant pathogens, and Table 2 shows examples of airborne human pathogens.

Bacterial

One such bacterial microorganism that can resist environmental stresses is *Bacillus anthracis*. It is a gram positive rod shaped bacteria that utilizes spore formation to resist environmental stresses. The spore is a dehydrated cell with extremely thick cell walls which can remain inactive for many years. This spore makes *Bacillus anthracis* a highly resilient bacteria, allowing it can survive extreme temperatures, chemical contamination, and low nutrient environments (Gatchalian 2010). This bacteria is associated with Anthrax, which is a severe respiratory disease that infects humans.

Fungal

Another such microorganism that can resist environmental stresses is *Aspergillus fumigatus*, which is a major airborne fungal pathogen (McCormick 2010). This pathogen is capable of causing many human diseases when conidia are inhaled into the lungs. While *A. fumigatus* lacks virulence traits, it is very adaptable to changing environmental conditions and therefore is still capable of mass infection. (McCormick 2010).

Viral

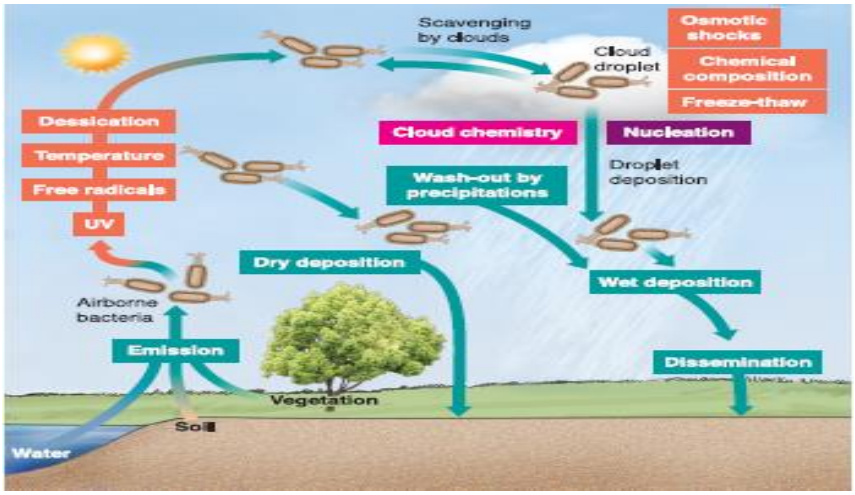
An example of a viral airborne pathogen is the Avian Influenza Virus, which is a single stranded RNA visur that can infect a broad range of animal species as well as humans and cause the Avian Influenza

Microbial Processes: The figure on the bottom right depicts the processes that a microbe undergoes during its life cycle. The microbes undergo the emission process, in which they are emitted from surfaces such as water, soil or vegetation and become airborne and transported into the airstream. The red boxes indicate some of the harsh environmental conditions that the microbes must withstand while airborne. The microbes that are able to withstand and survive these environmental pressures are the more resistant varieties. The microbes make it into clouds, where they can begin the breakdown of organic compounds. Finally, the microbes are "rained" out of the clouds through wet deposition, and they begin colonization of their new location (Amato 2012).

Human Disease	Pathogen
Bacterial diseases	
Pulmonary tuberculosis	Mycobacterium tuberculosis
Pneumonia	Klebsiella pneumoniae
Pulmonary anthrax	Bacillus anthracis
Legionellosis	Legionella spp.
Whopping cough	Bordetella pertussis
Diptheria	Cornebacterium diptheriae
Fungal diseases	
Aspergillosis	Aspergillus fumigatus
Coccidioidomycosis	Coccidioides immitis
Viral disease	
Influenza	Influenza virus
Hantavirus pulmonary synd.	Hantavirus
Hepatitis	Hepatitis virus
Chicken pox	Herpesvirus
Common cold	Picornavirus
Dengue fever	Flavivirus

Droplet Formation:

The emission process mentioned above, in which microbes are lifted in the air often involves microbes being suspended in droplets, which are large enough to keep the microbes hydrated and large enough to maintain a virulent amount of pathogen, but are still small enough to stay suspended in the air (Robinson 2012).



Studies about bioaerosols are difficult to conduct because identifying specific microbes and fungi in the air is a daunting task. One study conducted by Mark Hayes and associated looked into the feasibility of identifying specific particles in the air using fingerprinting techniques. It discussed sampling and analysis techniques that can help to identify cellular material. This is important because the particles in the air are information rich and can tell us a lot about where they originated or certain biochemical information about the organisms (Hayes 2012).

A great deal of the research conducted on bioaerosols studies their effects on humans, since human disease is one of the effects of bioaerosols. One such study conducted by Brandl and associates studied the distribution and dynamics of particles within the air in a lecture hall. Knowing what is in the air is important as human mortality rates increase with the rise of airborne particulate populations (Brandl 2008). Additionally, people are continually spending more time indoors, and knowing what exists within the air they breathe is important. This study found that bioaerosol populations were highest when there were a great deal of students in the hallways and the lecture area, proving that humans are a vector for the transport of bioaerosols.

Another study conducted this year by Vinni Hansen looked into components in both greenhouses and open fields to see which affected the levels of fungal bioaerosols. This study was conducted primarily to determine which is more detrimental to farmworkers. The study concluded that the bioaerosol levels were related to the environment, the work tasks, and the vegetable crops.

d. Water Microbiology

Water microbiology refers to the study of the microorganisms that live in water, or which can be transported from one habitat to another by water.

Water can support the growth of many types of microorganisms. This can be advantageous. For example, the chemical activities of certain strains of yeasts provide us with beer and bread. As well, the growth of some bacteria in contaminated water can help digest the poisons from the water.

However, the presence of other disease causing microbes in water is unhealthy and even life threatening. For example, bacteria that live in the intestinal tracts of humans and other warm blooded animals, such as *Escherichia coli, Salmonella, Shigella,* and *Vibrio,* can contaminate water

if feces enters the water. Contamination of drinking water with a type of *Escherichia coli* known as O157:H7 can be fatal. The contamination of the municipal water supply of Walkerton, Ontario, Canada in the summer of 2000 by strain O157:H7 sickened 2,000 people and killed seven people.

The intestinal tract of warm-blooded animals also contains viruses that can contaminate water and cause disease. Examples include rotavirus, enteroviruses, and coxsackievirus.

Another group of microbes of concern in water microbiology are protozoa. The two protozoa of the most concern are *Giardia* and *Cryptosporidium*. They live normally in the intestinal tract of animals such as beaver and deer. *Giardia* and *Cryptosporidium* form dormant and hardy forms called cysts during their life cycles. The cyst forms are resistant to chlorine, which is the most popular form of drinking water disinfection, and can pass through the filters used in many water treatment plants. If ingested in drinking water they can cause debilitating and prolonged diarrhea in humans, and can be life threatening to those people with impaired immune systems. *Cryptosporidium* contamination of the drinking water of Milwaukee, Wisconsin with in 1993 sickened more than 400,000 people and killed 47 people. Illness caused by these protozoans are becoming more prevalent in the United States, as urban areas continue to expand into what was previously relatively undisturbed wilderness.

Many microorganisms are found naturally in fresh and saltwater. These include bacteria, cyanobacteria, protozoa, algae, and tiny animals such as rotifers. These can be important in the food chain that forms the basis of life in the water. For example, the microbes called cyanobacteria can convert the energy of the sun into the energy it needs to live. The plentiful numbers of these organisms in turn are used as food for other life. The algae that thrive in water is also an important food source for other forms of life.

A variety of microorganisms live in fresh water. The region of a water body near the shoreline that is termed the littoral zone is well lighted, shallow, and warmer than other regions of the water. Photosynthetic algae and bacteria that use light as energy thrive in this zone. Further away from the shore is the limnitic zone, which can be colder and sunlight only in the upper 100 feet or so. Photosynthetic microbes also live here. As the water deepens, temperatures become colder and the oxygen concentration and light in the water decrease. Now, microbes that require oxygen do not thrive. Instead, purple and green sulfur bacteria, which can grow without oxygen, dominate. Finally, at the bottom of fresh waters (the benthic zone), few microbes survive. Bacteria that can survive in the absence of oxygen and sunlight, such as methane producing bacteria, thrive.

Salt water presents a different environment to microorganisms. The higher salt concentration, higher pH, and lower nutrients, relative to freshwater, are lethal to many microorganisms. But, salt loving (halophilic) bacteria abound near the surface, and some bacteria that also live in freshwater are plentiful (i.e., *Pseudomonas* and *Vibrio*). Also, in 2001, researchers demonstrated that the ancient form of microbial life known as archaebacteria is one of the dominant forms of life in the ocean. The role of archaebacteria in the ocean food chain is not yet known, but must be of vital importance.

Microorganisms exist at all depths in the ocean, even at the ocean floor, thousands of feet below the surface. Indeed, at locations such as hydrothermal vents, bacteria are the basis of the community of organisms that flourishes around the vents.

Another microorganism found in saltwater are a type of algae known as dinoflagellelates. The rapid growth and multiplication of dinoflagellates can turn the water red. This "red tide" depletes the water of nutrients and oxygen, which can cause many fish to die. As well, humans can become ill by eating contaminated fish.

Water can also be an ideal means of transporting microorganisms from one place to another. For example, the water that is carried in the hulls of ships to stabilize the vessels during their ocean voyages is now known to be a means of transporting microorganisms around the globe. One of these organisms, a bacterium called *Vibrio cholerae,* causes life threatening diarrhea in humans.

Drinking water is usually treated to minimize the risk of microbial contamination. The importance of drinking water treatment has been known for centuries. For example, in pre-Christian times the storage of drinking water in jugs made of metal was practiced. Now, the anti-bacterial effect of some metals is known. Similarly, the boiling of drinking water, as a means of protection of water has long been known.

Chemicals such as chlorine or chlorine derivatives have been a popular means of killing bacteria such as *Escherichia coli* in water since the early decades of the twentieth century. Other bacteria-killing treatments that are increasingly becoming popular include the use of a gas called ozone and the disabling of the microbe's genetic material by the use of ultraviolet light. Microbes can also be physically excluded from the water by passing the water through a filter. Modern filters have holes in them that are so tiny that even particles as miniscule as viruses can be trapped.

An important aspect of water microbiology, particularly for drinking water, is the testing of the water to ensure that it is safe to drink. Water quality testing can be done in several ways. One popular test measures the turbidity of the water. Turbidity gives an indication of the amount of suspended material in the water. Typically, if material such as soil is present in the water then microorganisms will also be present. The presence of particles even as small as bacteria and viruses can decrease the clarity of the water. Turbidity is a quick way of indicating if water quality is deteriorating, and so if action should be taken to correct the water problem.

In many countries, water microbiology is also the subject of legislation. Regulations specify how often water sources are sampled, how the sampling is done, how the analysis will be performed, what microbes are detected, and the acceptable limits for the target microorganisms in the water sample. Testing for microbes that cause disease (i.e., *Salmonella typhymurium* and *Vibrio cholerae*) can be expensive and, if the bacteria are present in low numbers, they may escape detection. Instead, other more numerous bacteria provide an indication of fecal pollution of the water. *Escherichia coli* has been used as an indicator of fecal pollution for decades. The bacterium is present in the intestinal tract in huge numbers, and is more numerous than the disease-causing bacteria and viruses. The chances of detecting *Escherichia coli* is better than detecting the actual disease causing microorganisms. *Escherichia coli* also has the advantage of not being capable of growing and reproducing in the water (except in the warm and food-laden waters of tropical

countries). Thus, the presence of the bacterium in water is indicative of recent fecal pollution. Finally, *Escherichia coli* can be detected easily and inexpensively.

Bacteria, viruses, and fungi are widely distributed throughout aquatic environments. They can be found in fresh water rivers, lakes, and streams, in the surface waters and sediments of the world's oceans, and even in hot springs. They have even been found supporting diverse communities at **hydrothermal vents** in the depths of the oceans.

Microorganisms living in these diverse environments must deal with a wide range of physical conditions, and each has specific adaptations to live in the particular place it calls home. For example, some have adapted to live in fresh waters with very low **salinity** , while others live in the saltiest parts of the ocean. Some must deal with the harsh cold of arctic waters, while those in hot springs are subjected to intense heat. In addition, aquatic microorganisms can be found living in environments where there are extremes in other physical parameters such as pressure, sunlight, organic substances, dissolved gases, and water clarity.

Aquatic microorganisms obtain nutrition in a variety of ways. For example, some bacteria living near the surface of either fresh or marine waters, where there is often abundant sunlight, are able to produce their own food through the process of **photosynthesis** . Bacteria living at hydrothermal vents on the ocean floor where there is no sunlight can produce their own food through a process known as **chemosynthesis** , which depends on preformed organic **carbon** as an energy source. Many other microorganisms are not able to produce their own food. Rather, they obtain necessary nutrition from the breakdown of organic matter such as dead organisms.

Aquatic microorganisms play a vital role in the cycling of nutrients within their environment, and thus are a crucial part of the **food chain/web**. Many microorganisms obtain their nutrition by breaking down organic matter in dead plants and animals. As a result of this process of decay, nutrients are released in a form usable by plants. These aquatic microorganisms are especially important in the cycling of the nutrients **nitrogen**, **phosphorus**, and carbon. Without this **recycling**, plants would have few, if any, organic nutrients to use for growth.

In addition to breaking down organic matter and recycling it into a form of nutrients that plants can use, many of the microorganisms become food themselves. There are many types of animals that graze on bacteria and fungi. For example, some deposit-feeding marine worms ingest sediments and digest numerous bacteria and fungi found there, later expelling the indigestible sediments. Therefore, these microorganisms are intimate members of the food web in at least two ways.

Humans have taken advantage of the role these microorganisms play in **nutrient** cycles. At **sewage treatment** plants, microscopic bacteria are cultured and then used to break down human wastes. However, in addition to the beneficial uses of some aquatic microorganisms, others may cause problems for people because they are pathogens, which can cause serious diseases. For example, viruses such as *Salmonella typhi*, *S. paratyphi*, and the Norwalk **virus** are found in water contaminated by sewage can cause illness. Fecal coliform (*E. coli*) bacteria and Enterococcus bacteria are two types of microorganisms that are used to indicate the presence of disease causing microorganisms in aquatic environments.

Bacteriological water quality analysis: **Bacteriological water analysis** is a method of analysing water to estimate the numbers of bacteria present and, if needed, to find out what sort of bacteria they are. It represents one aspect of water quality. It is a microbiological analytical procedure which uses samples of water and from these samples determines the concentration of bacteria. It is then possible to draw inferences about the suitability of the water for use from these concentrations. This process is used, for example, to routinely confirm that water is safe for human consumption or that bathing and recreational waters are safe to use.

E. coli culture on a Petri dish

The interpretation and the action trigger levels for different waters vary depending on the use made of the water. Whilst very stringent levels apply to drinking water, more relaxed levels apply to marine bathing waters, where much lower volumes of water are expected to be ingested by users.

The common feature of all these routine screening procedures is that the primary analysis is for indicator organisms rather than the pathogens that might cause concern. Indicator organisms are bacteria such as non-specific coliforms, *Escherichia coli* and *Pseudomonas aeruginosa* that are very commonly found in the human or animal gut and which, if detected, may suggest the presence of sewage. Indicator organisms are used because even when a person is infected with a more pathogenic bacteria, they will still be excreting many millions times more indicator organisms than pathogens. It is therefore reasonable to surmise that if indicator organism levels are low, then pathogen levels will be very much lower or absent. Judgements as to suitability of water for use are based on very extensive precedents and relate to the probability of any sample population of bacteria being able to be infective at a reasonable statistical level of confidence.

Analysis is usually performed using culture, biochemical and sometimes optical methods. When indicator organisms levels exceed pre-set triggers, specific analysis for pathogens may then be undertaken and these can be quickly detected (where suspected) using specific culture methods or molecular biology.

Methodologies- The most reliable methods are direct plate count method and membrane filtration method. mEndo Agar is used in the membrane filtration while VRBA Agar is used in the direct plate count method. VRBA stands for violet red bile agar. A media that contains bile salts which promotes the growth of gram negative and has inhibitory characteristic to gram positive although not complete inhibitory. These media contain lactose which is usually fermented by lactose fermenting bacteria producing colonies that can be identified and characterised. Lactose fermenting produce colored colonies while non lactose fermenting produce colorless ones. Because the

analysis is always based on a very small sample taken from a very large volume of water, all methods rely on statistical principles.

Multiple tube method- One of the oldest methods is called the multiple tube method. In this method a measured sub-sample (perhaps 10 ml) is diluted with 100 ml of sterile growth medium and an aliquot of 10 ml is then decanted into each of ten tubes. The remaining 10 ml is then diluted again and the process repeated. At the end of 5 dilutions this produces 50 tubes covering the dilution range of 1:10 through to 1:10000.

The tubes are then incubated at a pre-set temperature for a specified time and at the end of the process the number of tubes with growth in is counted for each dilution. Statistical tables are then used to derive the concentration of organisms in the original sample. This method can be enhanced by using indicator medium which changes colour when acid forming species are present and by including a tiny inverted tube called a Durham tube in each sample tube. The Durham inverted tube catches any gas produced. The production of gas at 37 degrees Celsius is a strong indication of the presence of *Escherichia coli*.

ATP Testing- An ATP test is the process of rapidly measuring active microorganisms in water through detection adenosine triphosphate (ATP). ATP is a molecule found only in and around living cells, and as such it gives a direct measure of biological concentration and health. ATP is quantified by measuring the light produced through its reaction with the naturally occurring enzyme firefly luciferase using a luminometer. The amount of light produced is directly proportional to the amount of biological energy present in the sample.

Second generation ATP tests are specifically designed for water, wastewater and industrial applications where, for the most part, samples contain a variety of components that can interfere with the ATP assay.

Plate count-

The plate count method relies on bacteria growing a colony on a nutrient medium so that the colony becomes visible to the naked eye and the number of colonies on a plate can be counted. To be effective, the dilution of the original sample must be arranged so that on average between 30 and 300 colonies of the target bacterium are grown. Fewer than 30 colonies makes the interpretation statistically unsound whilst greater than 300 colonies often results in overlapping colonies and imprecision in the count. To ensure that an appropriate number of colonies will be generated several dilutions are normally cultured. This approach is widely utilized for the evaluation of the effectiveness of water treatment by the inactivation of representative microbial contaminants such as *E. coli* following ASTM D5465.

The laboratory procedure involves making serial dilutions of the sample (1:10, 1:100, 1:1000, etc.) in sterile water and cultivating these on nutrient agar in a dish that is sealed and incubated. Typical media include plate count agar for a general count or MacConkey agar to count Gram-negative bacteria such as *E. coli*. Typically one set of plates is incubated at 22 °C and for 24 hours and a second set at 37 °C for 24 hours. The composition of the nutrient usually includes reagents that resist the growth of non-target organisms and make the target organism easily identified, often by a colour change in the medium. Some recent methods include a fluorescent agent so that counting of

the colonies can be automated. At the end of the incubation period the colonies are counted by eye, a procedure that takes a few moments and does not require a microscope as the colonies are typically a few millimetres across.

Membrane filtration- Most modern laboratories use a refinement of total plate count in which serial dilutions of the sample are vacuum filtered through purpose made membrane filters and these filters are themselves laid on nutrient medium within sealed plates. The methodology is otherwise similar to conventional total plate counts. Membranes have a printed millimetre grid printed on and can be reliably used to count the number of colonies under a binocular microscope.

Pour plate method-

When the analysis is looking for bacterial species that grow poorly in air, the initial analysis is done by mixing serial dilutions of the sample in liquid nutrient agar which is then poured into bottles which are then sealed and laid on their sides to produce a sloping agar surface. Colonies that develop in the body of the medium can be counted by eye after incubation.

The total number of colonies is referred to as the total viable count (TVC). The unit of measurement is cfu/ml (or colony forming units per millilitre) and relates to the original sample. Calculation of this is a multiple of the counted number of colonies multiplied by the dilution used.

Pathogen analysis: When samples show elevated levels of indicator bacteria, further analysis is often undertaken to look for specific pathogenic bacteria. Species commonly investigated in the temperate zone include *Salmonella typhi* and *Salmonella Typhimurium*. Depending on the likely source of contamination investigation may also extend to organisms such as *Cryptosporidium spp*. In tropical areas analysis of *Vibrio cholerae* is also routinely undertaken.

Types of nutrient media used in analysis-

MacConkey agar is culture medium designed to grow Gram-negative bacteria and stain them for lactose fermentation. It contains bile salts (to inhibit most Gram-positive bacteria), crystal violet dye (which also inhibits certain Gram-positive bacteria), neutral red dye (which stains microbes fermenting lactose), lactose and peptone. Alfred Theodore MacConkey developed it while working as a bacteriologist for the Royal Commission on Sewage Disposal in the United Kingdom.

Endo agar contains peptone, lactose, dipotassium phosphate, agar, sodium sulfite, basic fuchsin and was originally developed for the isolation of *Salmonella typhi*, but is now commonly used in water analysis. As in MacConkey agar, coliform organisms ferment the lactose, and the colonies become red. Non-lactose-fermenting organisms produce clear, colourless colonies against the faint pink background of the medium.

mFC medium is used in membrane filtration and contains selective and differential agents. These include rosolic acid to inhibit bacterial growth in general, except for faecal coliforms, bile salts inhibit non-enteric bacteria and aniline blue indicates the ability of faecal coliforms to ferment lactose to acid that causes a pH change in the medium.

TYEA medium contains tryptone, yeast extract, common salt and L-arabinose per liter of glass distilled water and is a non selective medium usually cultivated at two temperatures (22 and 36 °C) to determine a general level of contamination (a.k.a. colony count).

Water Quality Analysis: A rapid method for testing the total bacterial levels in water is the *standard plate count.* In this technique, a small sample of water is spread over the surface of a solid medium. The numbers of colonies that develop provide an estimate of the total viable population without differentiating coliforms from other species. This information is particularly helpful in evaluating the effectiveness of various water purifi cation stages. Another general indicator of water quality is the level of dissolved oxygen it contains called the *biological oxygen demand* (*BOD*). It is established that water containing high levels of organic matter and bacteria will have a lower oxygen content because of consumption by aerobic respiration.

Coliform Assays-

Water quality departments employ some standard assays for routine detection and quantification of coliforms. The techniques available are

- simple tests, such as presence-absence broth, that detect coliform activity but do not quantify it;
- rapid tests that isolate coliform colonies and provide quantities of coliforms present; and
- rapid tests that identify specifi c types of coliforms and determine their numbers.

In many circumstances (drinking water, for example), it is important to *differentiate* between facultative **coliforms** (*Enterobacter*) that are often found in other habitats (soil, water) *and* true **fecal coliforms** that live mainly in human and animal intestines. At one time, it was too diffi cult to differentiate *E. coli* from the closely related species of *Citrobacter, Klebsiella,* and *Enterobacter,* so laboratories instead simply reported whether a sample contained any of these isolates. All of these bacteria ferment lactose and are phenotypically similar. The terminology adopted was "coli*f orm-",* meaning *E. coli*- like. In other words, such bacteria present in the sample were not necessarily *E. coli.* Recently, microbiologists have noted serious problems with the use of coliform counts to indicate fecal contamination. The main issue is that the three other bacterial genera already mentioned, among others, are commonly found growing in nonfecal environments such as freshwater and plants that eventually become food. In other words, if the tests are not specifi cally for *E. coli,* you can't be sure that feces are present.

Microbiologists are now advocating that *E. coli* alone—not just any coliforms—be used as an indicator of fecal contamination. More specific identification techniques make this as simple as the standard coliform tests covered here.

Standard Coliform Testing: The *membrane filter* method is a widely used rapid method that can be used in the field or lab to process and test larger quantities of water. This method is more suitable for dilute fluids, such as drinking water, that are relatively free of particulate matter, and it is less suitable for water containing heavy microbial growth or debris. This technique is related to the method for sterilizing fluids by filtering out microbial contaminants, except that in this system, the filter containing the trapped microbes is the desired end product. The steps in membrane filtration are diagrammed in **figure 26.18 *a, b*.** After filtration, the membrane fi lter is placed in a Petri dish containing selective and differential media. After incubation, both nonfecal and fecal coliform colonies can be counted and often presumptively identified by their distinctive characteristics on these media **(figure 26.18 *c, d)*.**

Another more time-consuming but useful technique is the *most probable number* (*MPN*) procedure, which detects coliforms by aseries of *presumptive*, *confi rmatory*, and *completed* tests. The presumptivetest involves three subsets of fermentation tubes, eachcontaining different amounts of lactose or lauryl tryptose broth.The three subsets are inoculated with various-size water samples.After 24 hours of incubation, the tubes are evaluated for gas production.

A positive test for gas formation is presumptive evidence of coliforms; negative for gas means no coliforms. The number of positive tubes in each subset is tallied, and this set of numbers is applied to a statistical table to estimate the most likely or probable concentration of coliforms (see appendix C and table C.1).

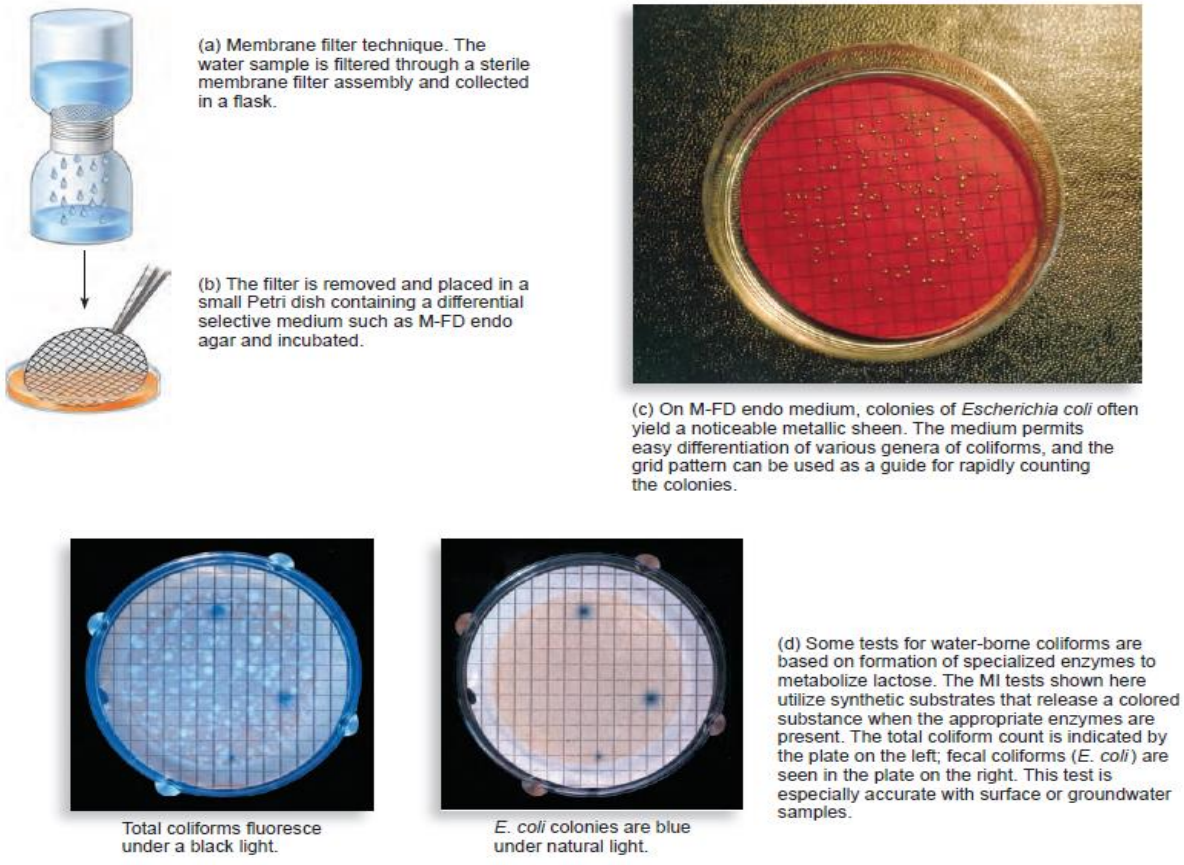


Figure 26.18 Rapid methods of water analysis for coliform contamination.

e. Wastewater treatment microbiology

Wastewater (or **waste water**) is any water that has been contaminated by human use. Wastewater is "used water from any combination of domestic, industrial, commercial or agricultural activities, surface runoff or stormwater, and any sewer inflow or sewer infiltration".[1] Therefore, wastewater is a byproduct of domestic, industrial, commercial or agricultural activities. The characteristics of wastewater vary depending on the source. Types of wastewater include: domestic wastewater from households, municipal wastewater from communities (also called sewage) and industrial wastewater. Wastewater can contain physical, chemical and biological pollutants.

Households may produce wastewater from flush toilets, sinks, dishwashers, washing machines, bath tubs, and showers. Households that use dry toilets produce less wastewater than those that use flush toilets.

Wastewater may be conveyed in a sanitary sewer that conveys only sewage. Alternatively, wastewater can be transported in a combined sewer that conveys both stormwater runoff and sewage, and possibly also industrial wastewater. After treatment at a wastewater treatment plant, treated wastewater (also called effluent) is discharged to a receiving water body. The terms "wastewater reuse" and "water reclamation" apply if the treated waste is used for another purpose. Wastewater that is discharged to the environment without suitable treatment can cause water pollution.

In developing countries and in rural areas with low population densities, wastewater is often treated by various on-site sanitation systems and not conveyed in sewers. These systems include septic tanks connected to drain fields, on-site sewage systems (OSS), vermifilter systems and many more.

Wastewater treatment is a process used to remove contaminants from wastewater or sewage and convert it into an effluent that can be returned to the water cycle with minimum impact on the environment, or directly reused. The latter is called water reclamation because treated wastewater can be used for other purposes. The treatment process takes place in a wastewater treatment plant (WWTP), often referred to as a Water Resource Recovery Facility (WRRF) or a Sewage Treatment Plant (STP). Pollutants in municipal wastewater (households and small industries) are removed or broken down.

The treatment of wastewater is part of the overarching field of sanitation. Sanitation also includes the management of human waste and solid waste as well as stormwater (drainage) management.[1] By-products from wastewater treatment plants, such as screenings, grit and sewage sludge may also be treated in a wastewater treatment plant.

What are they used for?

Biological wastewater treatment is the most common sanitation method in the world. This technology uses different types of bacteria and other microorganisms for the treatment and purification of polluted water.

Wastewater treatment is as essential to human health as it is to the protection of the environment. The use of these bacteria accelerates the process of treating pollution on a small surface: the wastewater treatment plant. It’s better than letting the river handle it, because even though it’s the same purification process that occurs in nature, the quantities of pollution discharged today are too high to keep the natural cycle intact. Thus, sewage treatment plants can prevent eutrophication of rivers, for example, but also prevent the diffusion of diseases.

Municipal and industrial effluent is the main source of wastewater. And thanks to the use of micro-organisms, we are able to degrade the content of these organic wastes as they are used as a source of food and energy to grow and multiply.

You got it, bacteria are the heart of the process. And finally, a wastewater treatment plant is like a farm where micro-organisms are grown on a large scale.

Where are bacteria present? Everywhere, from the water arriving at the treatment plant to its outlet. The operating parameters defined in the treatment tanks influence the development of various microbial structures and species. This complex combination of micro-organisms, rich in species, achieves a high level of biodegradation over a wide range of substrates, unlike the use of single species. This is the main factor influencing the quality of treated wastewater.

Usually, these organisms swarm and aggregate into a flake-like structure within the free culture called the Floc. These flocs, visible to the naked eye, contain living and dead cells of bacteria, fungi, protozoa and metabolic products. They agglomerate around the suspended organic matter on which they feed. This is the case, for example, with activated sludge. In addition, in fixed cultures, similar biofilms develop on contact surfaces. For example, biofilters and biological disks are fixed cultures.

Some plants are equipped with UV reactors or chlorine injection to eliminate the remaining bacteria in the outlet water before discharge into the river. One example is Australia and New Zealand.

Who are these microorganisms?

First, before we know who they are, we need to understand the parameters that influence their growth. Firstly, geographical location. Secondly, the type of pond in which bacteria will be grown. Thirdly, the characteristics of the wastewater entering the plant. Finally, the operating parameters of the system, such as aeration, agitation, chemical injection. All of these factors create quantitative changes between autotrophic and heterotrophic bacteria. In municipal wastewater treatment plants, for example, gram-negative bacteria of the proteobacteria type are predominant (21-65%) of which Betaproteobacteria is the most abundant class, largely responsible for the elimination of organic elements and nutrients. The other branches are Bacteroidetes, Acidobacteria and Chloroflexi (Nielsen et al., 2010; Nguyen et al., 2011; Wan et al., 2011; Hu et al., 2012; Wang et al., 2012). The most numerous types of bacteria are Tetrasphaera, Trichococcus, Candidatus Microthrix, Rhodoferax, Rhodobacter, Hyphomicrobium (McIllory et al., 2015).

Among fungi, Ascomycetes are the most common, accounting for 6.3 to 7.4% of micro-organisms. Then come the archaeobacteria, with Euryarcheota (1.5% of micro-organisms, Wang et al., 2014b). In addition, in presence of ammonia and oxygen, Nitrosomonas is very present. Finally, a high sludge age allows protozoa and rotifers to colonize the environment.

Temperature affects the presence of certain species. Thus, the effect of geographic location affects species composition. On the other hand, in industry, for example, the presence of predominantly well-defined micro-organisms can be explained by their ability to biodegrade specific components of industrial wastewater.

Bacteria are further categorized by how they get oxygen. In wastewater treatment, there are three types of bacteria used to treat wastewater entering the treatment plant: aerobic, anaerobic and facultative.

Their impacts and the treatment solutions:

The presence of bad bacteria (or the absence of good ones) can cause in particular:

- Low biogas efficiency of the anaerobic digester

- Poor flocculation and sedimentation
- An excess of filamentous bacteria
- Excess of phosphorus
- Low nitrogen removal efficiency (NH4, NO3)
- The production of unpleasant odours
- Excess consumption of chemical products
- In an anaerobic digester, foam production

There are generally three ways to restore an effective treatment. First, by changing the operating settings, and waiting for the right species to colonize the environment again. Second, by completely removing the microorganisms in place when the first solution did not work. Be careful, this method is not recommended because the biomass will take several days to develop, so the water will not be properly treated during this period. The third solution consists in injecting specially selected, cultured and multiplied bacteria in order to recover the advantage over the undesirable bacteria present in the environment.

Frequent applications:

Microbial biotechnology offers innovative scientific applications of high ecological and economic interest. It maximizes the natural degradation processes and thus eliminates pollution at significantly lower costs than conventional physicochemical or mechanical treatment processes.

The use of bacteria differs from common process techniques in that it involves simple and natural methods, the end-result of which makes it possible to eliminate pollution without generating new pollution. Most of the time, their implementation requires the use of a dedicated bioreactor, as well as the nutrients needed to multiply them in large numbers. The dosing is easy and requires very little operating time.

Accelerate plant startup / Get a quick start on bacterial seeding for a mobile plant:

The colonization of an environment by the needed bacteria and microorganisms necessary for the purification generally lasts between 4 and 8 weeks. Once again, it is the temperature that has the most influence on this growth time.

There are solutions to reduce this time to about a week, through seeding with selected and multiplied bacteria. There are two main advantages here:

- Reduce the start-up time of a wastewater treatment plant
- Accelerate the start-up of a mobile processing unit (e. g. in case of accident at the main plant)

The technique consists in recirculating a well-adapted combination of substrate and selected bacteria so that they settle very quickly. Under these favorable conditions, bacteria develop flocs or biofilms very quickly. Under these favorable conditions, bacteria develop flocs or biofilms very quickly. Finally, after a few days, the habitat is ready and wastewater can be discharged.

We have selected a range of bacteria to start your installation in one week under normal conditions, with water temperatures between 12 and 30°C.

Solving the presence of undesirable bacteria:

On activated sludge plants, the presence of filamentous bacteria is a real problem. First, the solution consists of extracting as much sludge as possible and increasing aeration. The good bacteria can take several days to recover the environment. If this does not work, then it is possible to destroy these bacteria with chlorine. The problem is that it kills all bacteria. Then it will take a few weeks for normal conditions to be reached again.

While the majority of operators continue to inject chlorine, we recommend the injection of dedicated bacteria. As for the accelerated start-up of a plant, the massive addition of these good populations makes it possible to quickly restore the balance in the tanks.

For example, here is an illustration of the removal of floats in a clarifier.

Too much scum on activated sludge	Clarifier BEFORE bacterial treatment	Clarifier AFTER bacterial treatment
		

How to improve treatment efficiency: By eliminating the fats and oils responsible for the habitat degradation

Lipophilic bacteria are specialized in the decomposition of animal and vegetable fats and oils in urban WWTPs and industrial treatment plants. These bacteria are easily adaptable to all current treatment systems.

On the market, there are products such as completely natural bacteria and enzymes, designed and selected for their ability to dissolve and digest fats and sludge. Some bacteria are so specialized in the degradation of fats that they are capable of degrading high loads, up to 300,000 mg/L COD.

By increasing the presence of good bacteria:

- Activated sludge (fine bubble ventilation)
- Natural and artificial lagoons and ponds
- Biofilters
- Thrickling filter
- Rotating biological contactors

By adding bacteria for the treatment of cold or hot water:

The majority of micro-organisms generally develop more rapidly at high temperatures, up to 38°c max. However, their development becomes very slow below 12°c, or almost nil below 5°C. These low temperatures are often reached when sewage treatment plants are located in geographic areas such as Canada or northern Europe. During the snow melting, these bacteria must treat the pollution while living in cold water. The main parade consists in significantly increase the size of the plant to compensate the lack of microbial activity. However, this solution, although still widely practiced, is very expensive.

By contrast, some industrial processes generate water above 38°C. The most common bacteria cannot survive under these conditions.

This is why there are effective bacterial mixtures for the treatment of different types of water. Thus, before a cold event, for example, it is possible to pre-seed the biological reactor with specially selected bacteria for these conditions. They will then take over the existing populations, and ensure effective treatment under these difficult conditions.

We have a selection of bacteria for these difficult conditions:

- cold water (between 1°C and 12°C),
- hot water (between 30°C and 50°C or more)

Organisms involved in water purification:

Most **organisms involved in water purification** originate from the waste, wastewater or water stream itself or arrive as resting spore of some form from the atmosphere. In a very few cases, mostly associated with constructed wetlands, specific organisms are planted to maximise the efficiency of the process.

Role of biota-

Biota are an essential component of most sewage treatment processes and many water purification systems. Most of the organisms involved are derived from the waste, wastewater or water stream itself or from the atmosphere or soil water. However some processes, especially those involved in removing very low concentrations of contaminants, may use engineered eco-systems created by the introduction of specific plants and sometimes animals. Some full scale sewage treatment plants also use constructed wetlands to provide treatment

Pollutants in wastewater-

Pathogens: Parasites, bacteria and viruses may be injurious to the health of people or livestock ingesting the polluted water. These pathogens may have originated from sewage or from domestic or wild bird or mammal feces. Pathogens may be killed by ingestion by larger organisms, oxidation, infection by phages or irradiation by ultraviolet sunlight unless that sunlight is blocked by plants or suspended solids.

Suspended solids: Particles of soil or organic matter may be suspended in the water. Such materials may give the water a cloudy or turbid appearance. The anoxic decomposition of some organic materials may give rise to obnoxious or unpleasant smells as sulphur containing compounds are released.

Nutrients:

Compounds containing nitrogen, potassium or phosphorus may encourage growth of aquatic plants and thus increase the available energy in the local food-web. This can lead to increased concentrations of suspended organic material. In some cases specific micro-nutrients may be required to allow the available nutrients to be fully utilised by living organisms. In other cases, the presence of specific chemical species may produce toxic effects limiting growth and abundance of living matter.

Metals:

Many dissolved or suspended metal salts exert harmful effects in the environment sometimes at very low concentrations. Some aquatic plants are able to remove very low metal concentrations, with the metals ending up bound to clay or other mineral particles.

Organisms-

Saprophytic bacteria and fungi can convert organic matter into living cell mass, carbon dioxide, water and a range of metabolic by-products. These saprophytic organisms may then be predated upon by protozoa, rotifers and, in cleaner waters, Bryozoa which consume suspended organic particles including viruses and pathogenic bacteria. Clarity of the water may begin to improve as the protozoa are subsequently consumed by rotifers and cladocera.Purifying bacteria, protozoa, and rotifers must either be mixed throughout the water or have the water circulated past them to be effective. Sewage treatment plants mix these organisms as activated sludge or circulate water past organisms living on trickling filters or rotating biological contactors.

Aquatic vegetation may provide similar surface habitat for purifying bacteria, protozoa, and rotifers in a pond or marsh setting; although water circulation is often less effective. Plants and algae have the additional advantage of removing nutrients from the water; but some of those nutrients will be returned to the water when the plants die unless the plants are removed from the water. Because of the complex chemistry of Phosphorus much of this element is in an unavailable form unless decomposition creates anoxic conditions which render the phosphorus available for re-uptake. Plants also provide shade, a refuge for fish, and oxygen for aerobic bacteria. In addition, fish can limit pests such as mosquitoes. Fish and waterfowl feces return waste to the water, and their feeding habits may increase turbidity. Cyanobacteria have the disadvantageous ability to add nutrients from the air to the water being purified and to generate toxins in some cases.

The choice of organism depends on the local climate different species and other factors. Indigenous species usually tend to be better adapted to the local environment.

Macrophytes:

A water-purifying plant (*Iris pseudacorus*) in growth after winter (leaves die at that time of year)

The choice of plants in engineered wet-lands or managed lagoons is dependent on the purification requirements of the system and this may involve plantings of varying plant species at a range of depths to achieve the required goal.

Plants purify water by consuming excess nutrients and by providing surfaces upon which a wide range of other purifying organisms can live. They also are effective oxygenators in sunlight. They also have the ability to translocate chemicals between their submerged foliage and their root systems and this is of significance in engineered wet-lands designed to de-toxify waste waters. Plants that have been used in temperate climates include *Nymphea alba*, *Phragmites australis*, *Sparganium erectum*, *Iris pseudacorus*, *Schoenoplectus lacustris* and *Carex acutiformis*.

Where oxygenation is a critical requirement *Stratiotes aloides*, *Hydrocharis morsus-ranae*, *Acorus calamus*, *Myriophyllum* species and *Elodea* have been used. *Hydrocharis morsus-ranae* and *Nuphar lutea* have been used where shade and cover are required

Rotifers: Rotifers are microscopic complex organisms and are filter feeders removing fine particulate matter from water. They occur naturally in aerobic lagoons, activated sludge processes, in trickling filters and in final settlement tanks and are a significant factor in removing suspended bacterial cells and algae from the water column.

The aim of wastewater treatment is the removal of undesirable substances and hazardousmicroorganisms in order that the water may safely enter a watercourse such asa river or stream. Further purification procedures are required before it can be use as drinking water. Wastewater treatment is fundamental to any developed society, and greatly reduces the incidence of waterborne diseases such as cholera. Wastewater maycome from domestic or commercial sources; highly toxic industrial effluents may requirepre-treatment before entering a water treatment system. Sewage is the term usedto describe liquid wastes that contain faecal matter (human or animal).

The effectiveness of the treatment process is judged chiefly by the reduction of thewastewaters *biochemical oxygen demand* (BOD). This is a measure of the amount ofoxygen needed by microorganisms to oxidise its organic content. A high BOD leads tothe removal of oxygen from water, a certain indicator of pollution.Wastewater treatment usually occurs in stages, the first of which (primary treatment)is purely physical, and involves the removal of floating objects followed by sedimentation,a process that removes up to a third of the BOD value. Secondary treatment involves microbial oxidation, leading to a substantial further reduction in BOD. Thismay take one of two forms, both of which are aerobic, the traditional *trickling filter* andthe more recent *activated sludge* process (Figure 16.6). In the former, the wastewater ispassed slowly over beds of stones or pieces of moulded plastic. These develop a biofilm comprising bacteria, protozoans, fungi and algae, and the resulting treated water has itsBOD reduced by some 80–85 per cent. Activated sludge plants achieve an even higher degree of BOD reduction. Here the wastewater is aerated in tanks that have been seededwith a mixed microbial sludge. The main component of this is the bacterium *Zoogloea*,which secretes slime, forming aggregates called *flocs*, around which other microorganismssuch as protozoans attach. Some of the water's organic content is not immediatelyoxidised, but becomes incorporated into the flocs. After a few hours' residence in thetank, the sludge is allowed to settle out, and the treated water passes out of the system.

Before being discharged to a watercourse, it is treated with chlorine to remove anypathogenic microorganisms that may remain.The principal operating problem encountered with activated sludge is that of *bulking*.This is caused by filamentous bacteria such as *Sphaerotilus natans*; it results in the sludgenot settling properly and consequently passing out with the treated water.Both secondary treatment processes result in some surplus sludge, which undergoes*anaerobic digestion*, resulting in the production of methane and CO2. The methane canbe used as a fuel to power the plant, and any remaining sludge is dewatered and usedas a soil conditioner. Care must be taken in this context, however, that the sludge doesnot have a high heavy metal content.

Wastewater Treatment Processes An aerial photograph of a modern sewage treatment plant isshown in **figure 35.4a.** Wastewater treatment involves a numberof steps that are spatially segregated (figure 35.4 *b*). The first threesteps are called primary, secondary, and tertiary treatment (**table35.3)**. At the end of the process, the water is usually chlorinated(itself an emerging environmental and human health problem)before it is released.

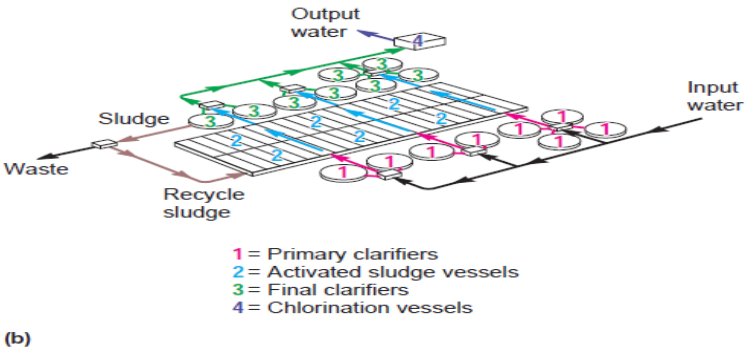
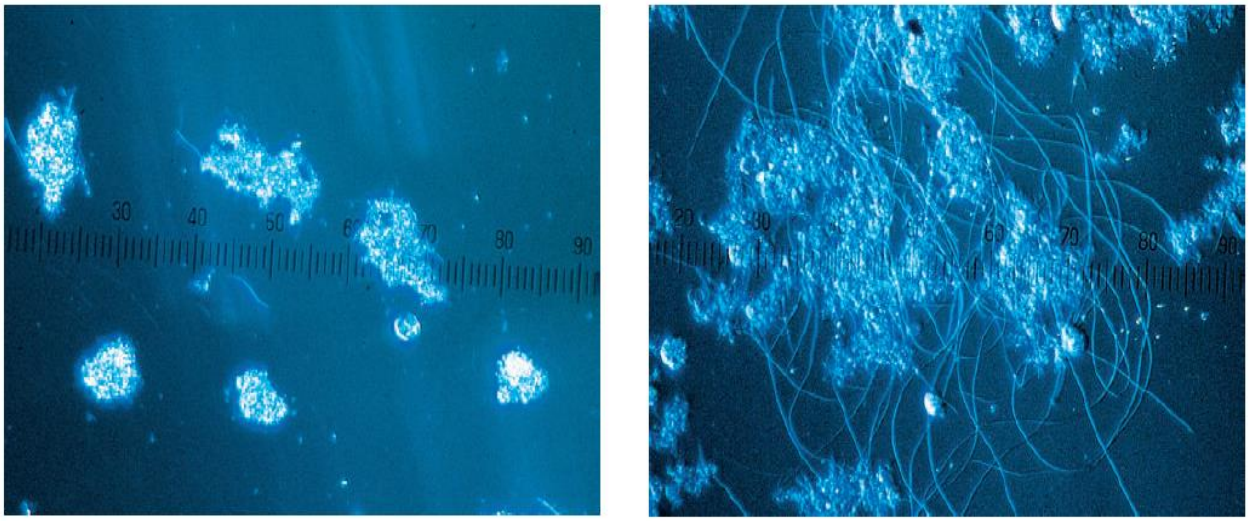


Figure 35.4 An Aerial View of a Conventional Sewage Treatment Plant. Sewage treatment plants allow natural processes of self-purification that occur in rivers and lakes to be carried out under more intense, managed conditions in large concrete vessels. (a) A plant in New Jersey. (b) A diagram of flows in the plant.

Primary treatment physically removes 20 to 30% of the BOD present in particulate form. In this treatment, particulatematerial is removed by screening, precipitation of small particulates,and settling in basins or tanks. The resulting solid materialis usually called **sludge.**

Secondary treatment promotes the biological transformationof dissolved organic matter into microbial biomass andcarbon dioxide. About 90 to 95% of the BOD and many bacterialpathogens are removed by this process. Several approaches canbe used in secondary treatment to remove dissolved organic matter.All of these techniques involve similar microbial activities.Under oxic conditions, dissolved organic matter will be transformedinto additional microbial biomass plus carbon dioxide.When microbial growth is completed, under ideal conditions themicroorganisms will aggregate and form stable flocs that settle.Minerals in the water also may be tied up in microbial biomass.As shown in **figure 35.5** ***a***, a healthy settleable floc is compact. In contrast, poorly formed flocs have a network of filamentousmicrobes that retard settling (figure 35.5 *b*).When these processes occur with lower O 2 levels or with amicrobial community that is too young or too old, unsatisfactory floc formation and settling can occur. The result is a **bulking sludge,**caused by the massive development of filamentous bacteria such as*Sphaerotilus* and *Thiothrix* ,together with many poorly characterized filamentous organisms. These important filamentous bacteria form flocs that do not settle well and thus produce effluent quality problems. <<*Class* Betaproteobacteria: *Order* Burkholderiales *(section20.2); Class* Gammaproteobacteria: *Order* Thiotrichales.



(a) (b)

Figure 35.5 Proper Floc Formation in Activated Sludge. Microorganisms play a critical role in the functioning of activated sludge systems. (a) The operation is dependent on the formation of settleable flocs. (b) If the plant does not run properly, poorly settling flocs can form due to such causes as low aeration, sulfide, and acidic organic substrates. These flocs do not settle properly because of their open or porous structure. As a consequence, the organic material is released with the treated water and lowers the quality of the final effluent.

An aerobic **activated sludge** system (**figure 35.6 *a***) involvesthe horizontal fl ow of materials with recycling of sludge—theactive biomass that is formed when organic matter is oxidized anddegraded by microorganisms. Activated sludge systems can bedesigned with variations in mixing. In addition, the ratio of organicmatter added to the active microbial biomass can be varied. A lowratesystem (low nutrient input per unit of microbial biomass), withslower growing microorganisms, will produce an effluent with lowresidual levels of dissolved organic matter.

A high-rate system(high nutrient input per unit of microbial biomass), with fastergrowing microorganisms, will remove more dissolved organic carbonper unit time but produce a poorer quality effluent.Aerobic secondary treatment also can be carried out with a**trickling filter** (figure 35.6 *b*). The waste effluent is passed overrocks or other solid materials upon which microbial biofilms havedeveloped, and the microbial community degrades the organic waste.

A sewage treatment plant can be operated to produce less sludgeby employing the **extended aeration** process (figure 35.6 *c*).Microorganisms grow on the dissolved organic matter, and the newlyformed microbial biomass is eventually consumed to meet maintenanceenergy requirements. This requires extremely large aerationbasins and long aeration times. In addition, with the biologicalself-utilization of the biomass, minerals originally present in themicroorganisms are again released to the water.

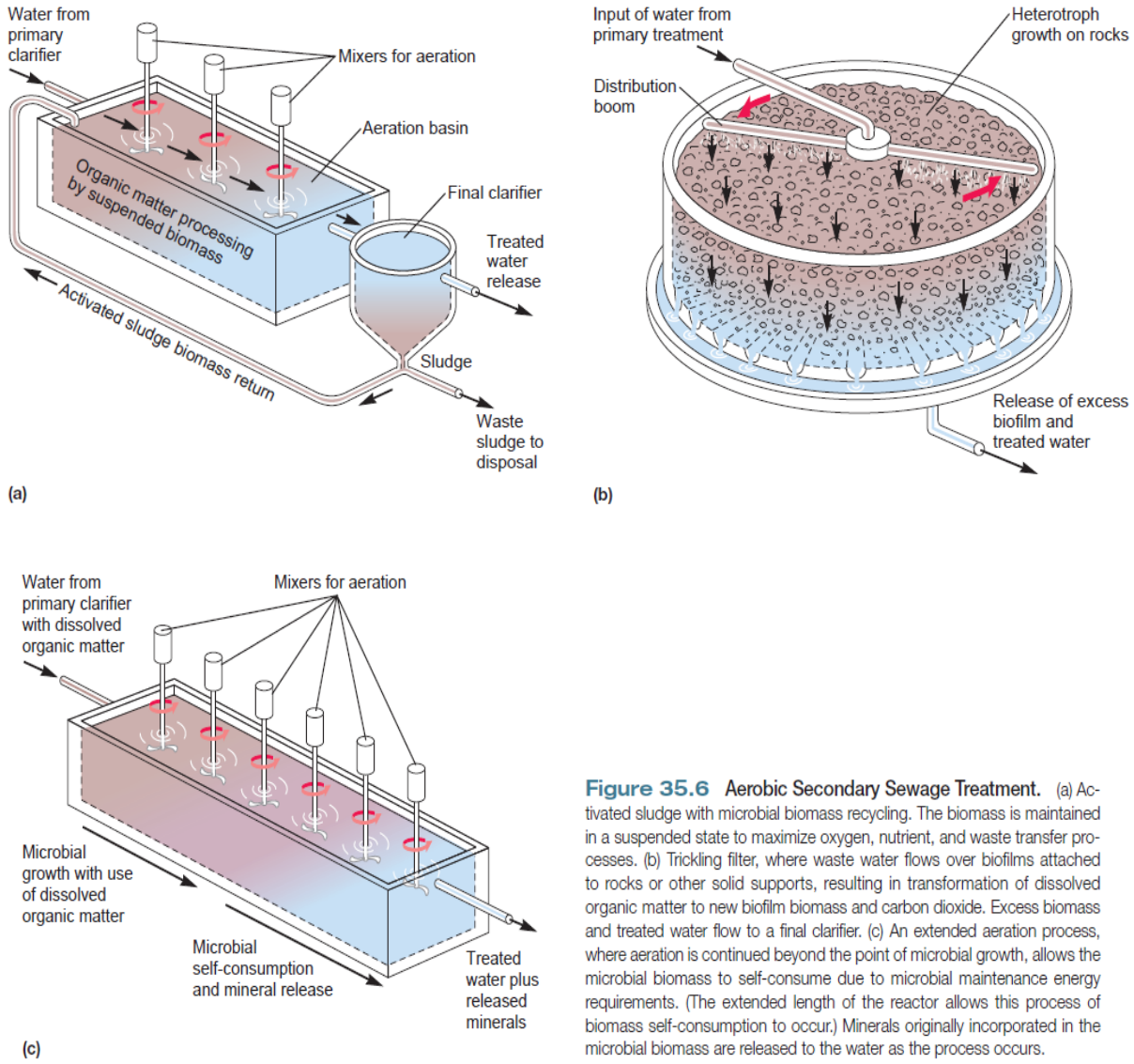


Figure 35.6 Aerobic Secondary Sewage Treatment. (a) Activated sludge with microbial biomass recycling. The biomass is maintained in a suspended state to maximize oxygen, nutrient, and waste transfer processes. (b) Trickling filter, where waste water flows over biofilms attached to rocks or other solid supports, resulting in transformation of dissolved organic matter to new biofilm biomass and carbon dioxide. Excess biomass and treated water flow to a final clarifier. (c) An extended aeration process, where aeration is continued beyond the point of microbial growth, allows the microbial biomass to self-consume due to microbial maintenance energy requirements. (The extended length of the reactor allows this process of biomass self-consumption to occur.) Minerals originally incorporated in the microbial biomass are released to the water as the process occurs.

All aerobic processes produce excess microbial biomass, orsewage sludge, which contains many recalcitrant organics. Oftenthe sludges from aerobic sewage treatment, together with the materialssettled out in primary treatment, are further treated by anaerobicdigestion. **Anaerobic digesters** are large tanks designed to operatewith continuous input of untreated sludge and removal of the final, stabilized sludge product. Methane is vented and often burned forheat and electricity production. This digestion process involvesthree steps: (1) the fermentation of the sludge components to formorganic acids, including acetate; (2) production of the methanogenic substrates: acetate, CO_2, and hydrogen; and finally,(3) methanogenesis by the methane producers. These methanogenicprocesses, summarized in **table 35.4**, involve critical balancesbetween electron acceptors and donors. To function most efficiently,the hydrogen concentration must be maintained

at a low level.

Table 35.4 Sequential Reactions in the Anaerobic Digester

Process Step	Substrates	Products	Major Microorganisms	
Fermentation	Organic polymers	Butyrate, propionate, lactate, succinate, ethanol, acetate,[a] H_2,[a] CO_2[a]	*Clostridium* *Bacteroides* *Peptostreptococcus*	*Peptococcus* *Eubacterium* *Lactobacillus*
Acetogenic reactions	Butyrate, propionate, lactate, succinate, ethanol	Acetate, H_2, CO_2	*Syntrophomonas* *Syntrophobacter*	
Methanogenic reactions	Acetate H_2 and HCO_3^-	$CH_4 + CO_2$ CH_4	*Methanosarcina* *Methanothrix* *Methanobrevibacter* *Methanomicrobium*	*Methanogenium* *Methanobacterium* *Methanococcus* *Methanospirillum*

[a]Methanogenic substrates produced in the initial fermentation step.

If hydrogen and organic acids accumulate, methane production can beinhibited, resulting in a stuck digester. Anaerobic digestion has many advantages. Most of the microbialbiomass produced in aerobic growth is used for methaneproduction in the anaerobic digester. Also, because the process ofmethanogenesis is energetically very inefficient, the microbes mustconsume about twice the nutrients to produce an equivalent biomassas that of aerobic systems. Consequently, less sludge isproduced and it can be easily dried. Dried sludge removed fromwell-operated anaerobic systems can even be sold as organic gardenfertilizer. However, sludge can be dangerous if the system is not properly managed because heavy metals and other environmentalcontaminants may be concentrated in it.

Tertiary treatment further purifies wastewaters. It is particularlyimportant to remove nitrogen and phosphorus compoundsthat can promote eutrophication. Organic pollutants can be removed with activated carbon filters. Phosphate usually is precipitated ascalcium or iron phosphate (e.g., by the addition of lime). To removephosphorus, oxic and anoxic conditions are used alternately in aseries of treatments, and phosphorus accumulates in microbial biomassas polyphosphate. Excess nitrogen may be removed by"stripping," volatilization of NH_3 at high pHs. Ammonia itself canbe chlorinated to form dichloramine, which is then converted tomolecular nitrogen. In some cases, microbial processes can be used to remove nitrogen and phosphorus. A widely used processfor nitrogen removal is denitrification. Here, nitrate, produced bymicrobes under aerobic conditions, is used as an electron acceptorunder conditions of low oxygen with organic matter added as anenergy source. Nitrate reduction yields nitrogen gas (N_2) andnitrous oxide (N_2O) as the major products. In addition to denitrification, the anammox process is also important. In this reaction,ammonium ion (used as the electron donor) is reacted with nitrite(the electron acceptor) produced by partial nitrification (i.e., theoxidation of ammonium to nitrite). The anammox process can convertup to 80% of the beginning ammonium ion to N 2 gas. Tertiarytreatment is expensive and is usually not employed except wherenecessary to prevent obvious ecological disruption.

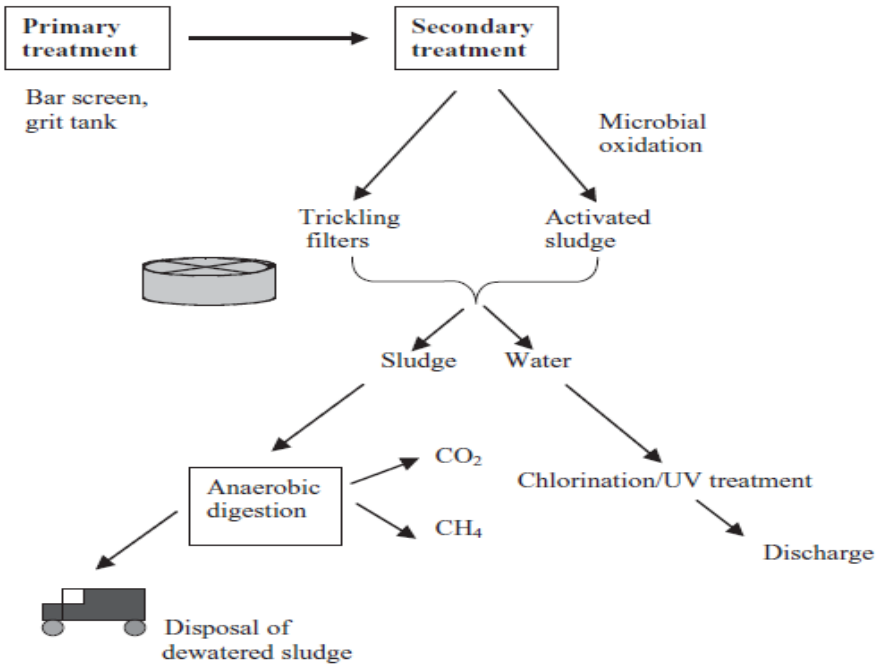


Figure 16.6 Wastewater treatment. Wastewater treatment achieves a reduction of the biochemical oxygen demand of the water by primary (physical) and secondary (biological) treatment

Wetlands are a vital natural resource and a critical part of ourenvironment, and increasingly efforts are being made to protectthese fragile aquatic communities from pollution. A major meansof wastewater treatment is the use of constructed wetlands,where the basic components of natural wetlands (soils, aquaticplants, waters) are used as a functional waste treatment system.Constructed wetlands now are increasingly employed in thetreatment of liquid wastes and for bioremediation. This system uses floating, emergent, orsubmerged plants, as shown in **figure 35.7**. The aquatic plantsprovide nutrients in the root zone, which support microbialgrowth. Especially with emergent plants, the root zone can bemaintained in an anoxic state in which sulfide, produced by*Desulfovibrio* using root zone organic matter as an energysource, can trap metals. Constructed wetlands also are beingused to treat acid mine drainage and industrial wastes in many parts of the world.

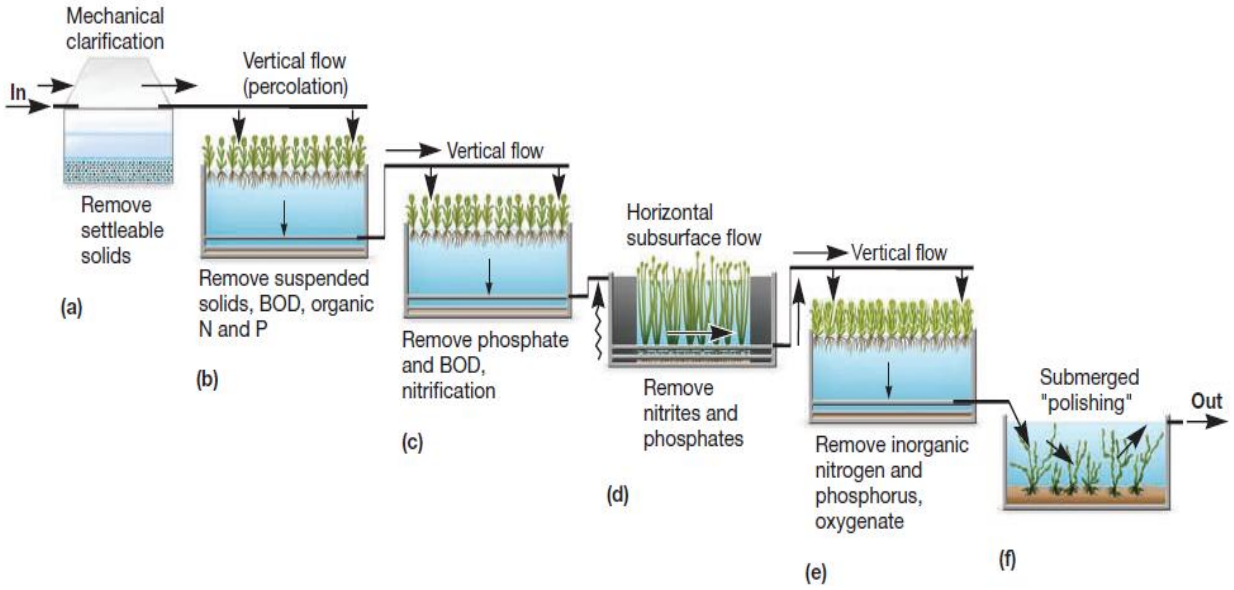


Figure 35.7 Constructed Wetland for Wastewater Treatment. Multistage constructed wetland systems can be used for organic matter and phosphate removal. (a) Free-floating macrophytes (b,c,e), such as duckweed and water hyacinth, can be used for a variety of purposes. Emergent macrophytes (d), such as bulrush, allow surface flow as well as vertical and horizontal subsurface flow. Submerged vegetation (f), such as waterweed, allows final "polishing" of the water. These wetlands also can be designed for nitrification and metal removal from waters.

f. The microbiology of soil

Soil microbiology is the study of microorganisms in soil, their functions, and how they affect soil properties. It is believed that between two and four billion years ago, the first ancient bacteria and microorganisms came about on Earth's oceans. These bacteria could fix nitrogen, in time multiplied, and as a result released oxygen into the atmosphere. This led to more advanced microorganisms, which are important because they affect soil structure and fertility. Soil microorganisms can be classified as bacteria, actinomycetes, fungi, algae and protozoa. Each of these groups has characteristics that define them and their functions in soil. Up to 10 billion bacterial cells inhabit each gram of soil in and around plant roots, a region known as the rhizosphere. In 2011, a team detected more than 33,000 bacterial and archaeal species on sugar beet roots.

The composition of the rhizobiome can change rapidly in response to changes in the surrounding environment.

The microbial population of asoil will vary according to the amount of available waterand organic matter, and different organisms colonisedifferent strata in the soil.The organic content of a soil derives from the remainsof dead plants and animals. These are brokendown in the soil by a combination of invertebrates andmicroorganisms (mainly bacteria and fungi) known asthe *decomposers*. Their action results in the release ofsubstances that can be used by plants and by other microorganisms. Much organic material is easily degraded,while the more resistant fraction is referred to as *humus,*and comprises lignin together with various othermacromolecules. The humus content of a soil, then, isa reflection of how favorable (or otherwise) conditionsare for its decomposition; the value usually falls between2 and 10 per cent by weight. The inorganic fraction

ofa soil derives from the weathering of minerals. Microorganisms may be present in soilsin huge numbers, mostly attached to soil particles. Their numbers vary according tothe availability of suitable nutrients. Bacteria (notably actinomycetes) form the largestfraction of the microbial population, together with much smaller numbers of fungi,algae and protozoans. Published values of bacterial numbers range from overestimates(those that do not distinguish between living and dead cells) and underestimates (thosethat depend on colony counts and therefore exclude those organisms we are not yetable to grow in the laboratory – 99 per cent according to some experts!). Suffice tosay that many millions (possibly billions) of bacteria may be present in a single gramof topsoil. In spite of being present in such enormous numbers, microorganisms onlyrepresent a minute percentage of the volume of most soils. Fungi, although present inmuch smaller numbers than bacteria, form a higher proportion of the soil biomass,due to their greater size. The majority of soil microorganisms are aerobic heterotrophs,involved in the decomposition of organic substrates; thus, microbial numbers diminishgreatly the further down into the soil we go, away from organic matter and oxygen.The proportion of anaerobes increases with depth, but unless the soil is waterlogged,they are unlikely to predominate.

Other factors affecting microbial distribution include pH, temperature, and moisture.Broadly speaking, neutral conditions favour bacteria, while fungi flourish in mildlyacidic conditions (down to about pH4), although extremophiles survive well outsidethese limits. Actinomycetes favour slightly alkaline conditions. Bacterial forms occurringcommonly in soils include *Pseudomonas*, *Bacillus*, *Clostridium*, *Nitrobacter* and the nitrogen-fixing *Rhizobium* and *Azotobacter*, as well as cyanobacteria such as *Nostoc*and *Anabaena*. Commonly found actinomycetes include *Streptomyces* and *Nocardia*. As we have noted elsewhere, actinomycetes are notable for their secretion of antimicrobial compounds into their surroundings. This provides an example of how the presence of one type of microorganism in a soil population can influence the growth of others, forming a dynamic, interactive ecosystem. In addition, bacteria may serve as prey for predatory protozoans, and secondary colonisers may depend on a supply of nutrients from, for example, cellulose degraders. Important fungal genera common in soil include the familiar *Penicillium* and *Aspergillus*; these not only recycle nutrients by breaking down organic material, but also contribute to the fabric of the soil, by binding together microscopic soil particles. Soil protozoans are mostly predators that ingest bacteria or protists such as yeasts or unicellular algae. All the major forms of protozoans may be present (flagellates, ciliates and amoebas), moving around the water-lined spaces between soil particles. Algae are of course phototrophic, and are therefore to be found mostly near the soil surface, although it will be recalled from that some forms are capable of heterotrophic growth, and may thus survive further down.

The surface of soil particles is a good natural habitat for the development of *biofilms*,complex structures comprising microbial cells held together in a polysaccharide matrix.The microorganisms themselves produce the polysaccharide, which also allows the passageof nutrients from the environment. Biofilms can form on almost any surface, and areoften to be found in rapidly flowing waters. Biofilms may be beneficial or harmful (e.g. infections resulting from growth in catheters)to humans.

Although we have emphasized the importance of organic matter in soil ecosystems,microorganisms may also be found growing on or even within rocks. The growth of suchorganisms, together with the action of wind and rainfall, contribute to the weatheringof rocks.

Terrestrial Microbiology: The Composition

Descriptions such as “soiled” or “dirty” may suggest to some that soil is an undesirable, possibly harmful substance; or its appearance mightsuggest a somewhat homogeneous, inert substance. At the microscopiclevel, however, soil is a dynamic ecosystem that supports complexinteractions between numerous geologic, chemical, and biologicalfactors.

This rich region, part of the lithosphere, teems with microbes,serves a dynamic role in biogeochemical cycles, and is an importantrepository for organic detritus and dead terrestrial organisms.

The abiotic portion of soil is a composite of mineral particles,water, and atmospheric gas. The development of soil begins whengeologic sediments are mechanically disturbed and exposed toweather and microbial action.

Rock decomposition releases various-size particles rangingfrom rocks, pebbles, and sand grains to microscopic morsels that liein a loose aggregate **(figure 26.10).** The porous structure of soil creates various-size pockets or spaces that provide numerous microhabitats.Some spaces trap moisture and form a liquid phase in whichmineral ions and other nutrients are dissolved. Other spaces trap airthat will provide gases to soil microbes, plants, and animals. Becauseboth water and air compete for these pockets, the water content ofsoil is directly related to its oxygen content. Water-saturated soilscontain less oxygen, and dry soils have more. Gas tensions in soil canalso vary vertically. In general, the concentration of O 2 decreases andthat of CO 2 increases with the depth of soil. Aerobic and facultativeorganisms tend to occupy looser, drier soils, whereas anaerobeswould adapt to waterlogged, poorly aerated soils.

Within the superstructure of the soil are varying amounts of**humus, *** the slowly decaying organic litter from plant and animaltissues. This soft, crumbly mixture holds water like a sponge. It isalso an important habitat for microbes that decompose the complexlitter and recycle nutrients.

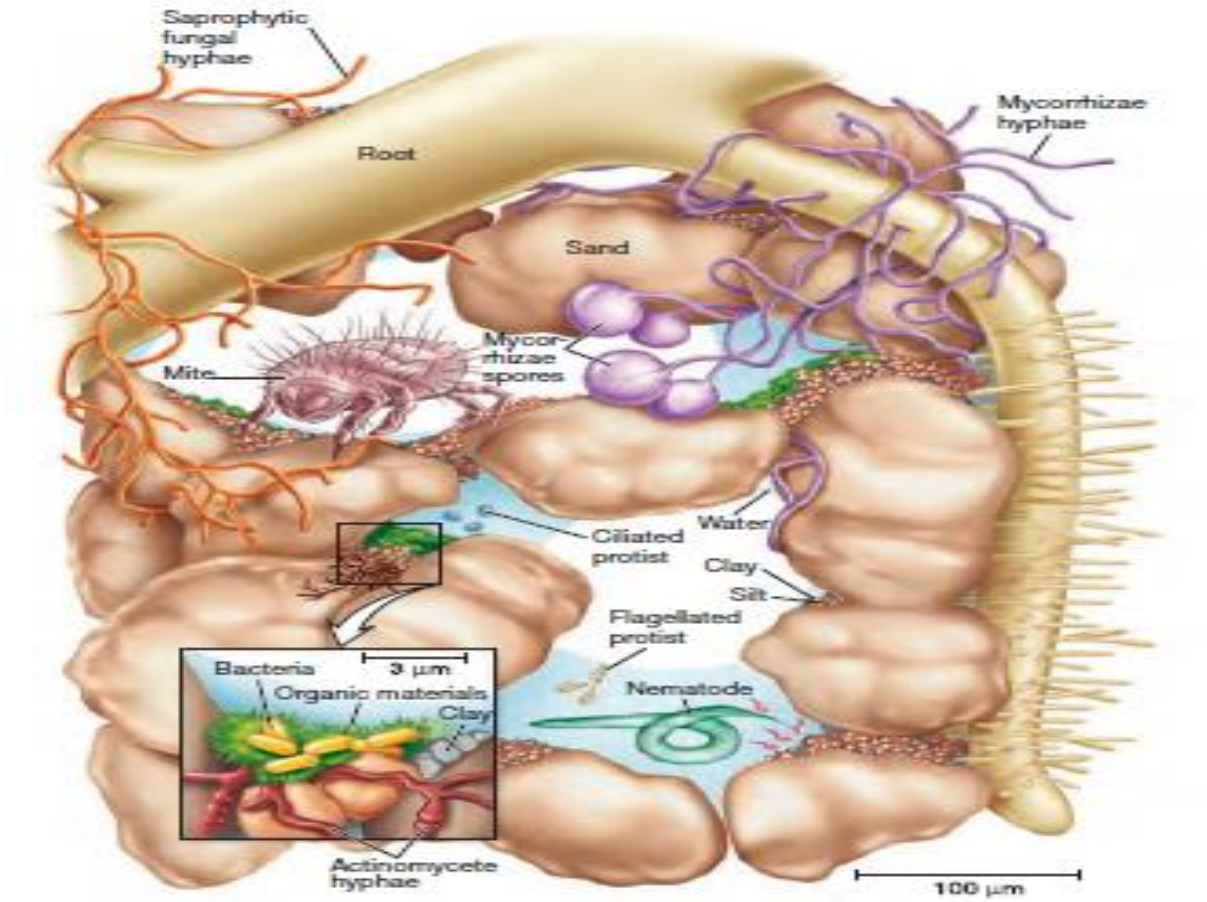


Figure 26.10 The soil habitat. A typical soil habitat contains a mixture of clay, silt, and sand along with soil organic matter. Roots and animals (e.g., nematodes and mites), as well as protozoa and bacteria, consume oxygen, which rapidly diffuses into the soil pores where the microbes live. Note that two types of fungi are present: mycorrhizal fungi, which derive their organic carbon from plant roots; and saprophytic fungi, which help degrade organic material.

The humus content varies with climate, temperature, moistureand mineral content, and microbial action. Warm, tropical soilshave a high rate of humus production and microbial decomposition. Because nutrients in these soils are swiftly released and used up,they do not accumulate. Fertilized agricultural soils in temperateclimates build up humus at a high rate and are rich in nutrients. Thevery low content of humus and moisture in desert soils greatly reducesits microbial biota, rate of decomposition, and nutrient content.Bogs are likewise nutrient-poor due to a slow rate ofdecomposition of the humus caused by high acid content and lackof oxygen. Humans can artifi -cially increase the amount of humusby mixing plant refuse and animal wastes with soil and allowingnatural decomposition to occur, a process called *composting.* Compostingis a very active metabolic process that generates a great dealof heat. The temperature inside a well-maintained compost canreach 80°C to 100°C.

Living Activities in Soil The rich culture medium of the soil supports a fantastic array ofmicroorganisms (bacteria, fungi, algae, protozoa, and viruses). Agram of moist loam soil with high humus content can have a microbecount as high as 10 billion, each competing for its own nicheand microhabitat. Some of the most distinctive biological interactionsoccur in the **rhizosphere,** the zone of soil surrounding theroots of plants, which contains associated bacteria, fungi, and protozoa

(**See figure 26.10**). Plants interact with soil microbes in a trulysynergistic fashion. Studies have shown that a rich microbial community grows in a biofilm around the root hairs and other exposed Surfaces. Their presence stimulates the plant to exude growth factorssuch as carbon dioxide, sugars, amino acids, and vitamins.

These nutrients are released into fluid spaces, where they can bereadily captured by microbes. Bacteria and fungi likewise contributeto plant survival by releasing growth factors andprotective substances. They also convert minerals into forms usableby plants. We saw numerous examples in the nitrogen, sulfur, andphosphorus cycles.

We previously observed that plants can form close symbioticassociations with microbes to fi x nitrogen. Other mutualistic partnershipsbetween plant roots and microbes are **mycorrhizae.** Theseassociations occur when various species of basidiomycetes, ascomycetes,or zygomycetes attach themselves to the roots of vascularplants **(figure 26.11).** The plant feeds the fungus through photosynthesis,and the fungus sustains the relationship in several ways. Byextending its mycelium into the rhizosphere, it helps anchor theplant and increases the surface area for capturing water from drysoils and minerals from poor soils. Plants with mycorrhizae can inhabitsevere habitats more successfully than plants without them.

The topsoil, which extends a few inches to a few feet from thesurface, supports a host of burrowing animals such as nematodes,termites, and earthworms. Many of these animals are decomposerreducerorganisms that break down organic nutrients through digestionand also mechanically reduce or fragment the size of particlesso that they are more readily mineralized by microbes. Aerobicbacteria initiate the digestion of organic matter into carbon dioxideand water and generate minerals such as sulfate, phosphate, andnitrate, which can be further degraded by anaerobic bacteria. Fungal enzymes increase the efficiency of soil decomposition by hydrolyzingcomplex natural substances such as cellulose, keratin, lignin, chitin, and paraffin.

The soil is also a repository for agricultural, industrial, anddomestic wastes such as insecticides, herbicides, fungicides, manufacturingwastes, and household chemicals. Applied microbiologists,

Using expertise from engineering, biotechnology, andecology, work to explore the feasibility of harnessing indigenoussoil microbes to break down undesirable hydrocarbons and pesticides.

Figure 26.11 Mycorrhizae, symbiotic associations between fungi and plant roots, favor the absorption of water and minerals from the soil.

g. Microbial Biogeochemical cycle

Environmental ecosystems are exposed to the sun, which constantly infuses them with a renewable source of energy. In contrast, thebioelements and nutrients that are essential components of cells andmulticellular organisms are supplied exclusively by sources some-where in the biosphere and are not being continually replenished from outside the earth. In fact, the lack of a required nutrient in the immediate habitat is one of the chief factors limiting organismic and population growth. It is for these reasons that there must be continuous and sustained recycling of elements and nutrients in the biosphere. Essential elements such as carbon, nitrogen, sulfur, phosphorus, oxygen, and iron are recharged through biological, geologic, and chemical mechanisms called biogeochemical cycles. Although these cycles vary in certain specific characteristics, they share several general qualities, as summarized in the following list:

All elements ultimately originate from a nonliving, long-term reservoir in the atmosphere, the lithosphere, or the hydrosphere. They cycle in pure form (N_2) or as compounds (PO_4).

Elements make the rounds between the abiotic environment and the biotic environment.

Recycling maintains a necessary balance of nutrients in the biosphere so that they do not build up or become unavailable.

Cycles are complex systems that rely upon the interplay of producers, consumers, and decomposers. Often the waste products of one organism become a source of energy or building material for another.

All organisms participate directly in recycling, but only certain categories of microorganisms have the metabolic pathways for converting inorganic compounds from one nutritional form to another.

The English biologist James Lovelock has postulated a conceptcalled the Gaia * theory, after the mythical Greek goddess of earth.Its primary idea proposes that the biosphere contains a diversity ofhabitats and niches favorable to life because living things have made it that way. Not only does the earth shape the character of living things,but living things shape the character of the earth. After all, we knowthat the compositions of the aquatic environment, the atmosphere, andeven the soil would not exist as they do without the actions of livingthings. For billions of years, microbes have played prominent roles inthe formation and maintenance of the earth's crust, the developmentof rocks and minerals, and the formation of fossil fuels. This revolutionin understanding the biological involvement in geologic processes has given rise to a new field called *geomicrobiology*.

In the next several sections, we examine how, jointly and overa period of time, the varied microbial activities affect and are themselvesaffected by the abiotic environment.

Atmospheric Cycles

- ***The Carbon Cycle*** Because carbon is the fundamental atom in all biomolecules and accountsfor at least one-half of the dry weight of biomass, the **carboncycle** is more intimately associated with the energy transfers andtrophic patterns in the biosphere than are other elements. Carbonexists predominantly in the mineral state and as an organic reservoirin the bodies of organisms. A much smaller amount of carbon alsoexists in the gaseous state as carbon dioxide (CO_2), carbon monoxide(CO), and methane (CH_4). In general, carbon is recycled through ecosystems via carbon fixation, respiration, or fermentation of organicmolecules, limestone decomposition, and methane production.

A convenient starting point from which to trace the movementof carbon is with carbon dioxide, which occupies a central positionin the cycle and represents a large common pool that diffuses into allparts of the ecosystem **(figure 26.6).** As a general rule, the cycles ofoxygen and hydrogen are closely allied to the carbon cycle.The principal users of the atmospheric carbon dioxide poolare photosynthetic autotrophs (photoautotrophs) such as plants,algae, and bacteria. An estimated 165 billion tons of organic material per year are produced by terrestrial and aquatic photosynthesis.

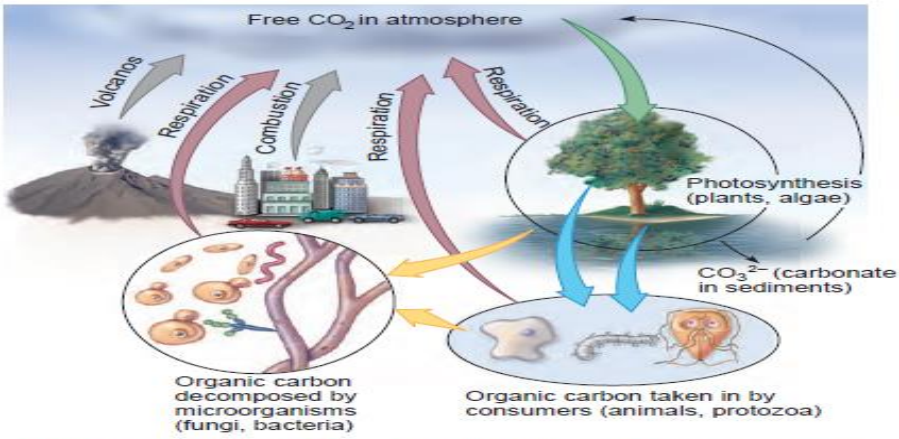
Although we don't yet know exactly how many autotrophsexist in the earth's crust, a small amount of CO_2 is used by thesebacteria (chemolithoautotrophs) that derive their energy frombonds in inorganic chemicals. Phototrophs use energy from the sun to fix CO_2 into organic compoundssuch as glucose that can be used in synthesis. Photosynthesisis also the primary means by which the atmospheric supply of O_2 is regenerated.

Just as photosynthesis removes CO_2 from the atmosphere,other modes of generating energy, such as respiration and fermentation,can be used to remove and return it. As you may recall fromthe discussion of aerobic respiration in chapter 8, in the presence ofO_2 , organic compounds such as glucose are degraded completely to CO_2 , with the release of energy and the formation of H_2O. Carbondioxide is also released by anaerobic respiration and by certaintypes of fermentation reactions.

A small but important phase of the carbon cycle involves certainlimestone deposits composed primarily of calcium carbonate ($CaCO_3$). Limestone is produced when marine organisms such asmollusks, corals, protozoans, and algae form hardened shells bycombining carbon dioxide and calcium ions from the surroundingwater. When these organisms die, the durable skeletal componentsaccumulate in marine deposits. As these immense deposits aregradually exposed by geologic upheavals or receding ocean levels, various decomposing agents liberate CO_2 and return it to the CO_2pool of the water and atmosphere.

The complementary actions of photosynthesis and respiration,along with other natural CO_2 -releasing processes such as limestoneerosion and volcanic activity, have maintained a relatively stableatmospheric pool of carbon dioxide. Thisbalance is being disturbed as humans burn *fossil fuels* and otherorganic carbon sources. Fossil fuels, including coal, oil, and naturalgas, were formed through millions of years of natural biologicaland geologic activities. Humans are so dependent upon this energysource that, within the past 30 years, the proportion of CO_2 in theatmosphere has steadily increased from 32 ppm to 39 ppm. Althoughthis increase may seem slight and insignificant, most scientistsnow feel it has begun to disrupt the delicate temperature balance of the biosphere.

Compared with carbon dioxide, methane gas (CH_4) plays asecondary part in the carbon cycle, though it can be a significantproduct in anaerobic ecosystems dominated by **methanogens**
(methane producers). In general, when methanogens reduce CO_2 bymeans of various oxidizable substrates, they give off CH_4.



Process Figure 26.6 **The carbon cycle.** This cycle traces carbon from the CO_2 pool in the atmosphere to the primary producers (green) where it is fixed into protoplasm. Organic carbon compounds are taken in by consumers (blue) and decomposers (yellow) that produce CO_2 through respiration and return it to the atmosphere (pink). Combustion of fossil fuels and volcanic eruptions also add to the CO_2 pool. Some of the CO_2 is carried into inorganic sediments by organisms that synthesize carbonate (CO_3) skeletons. In time, natural processes acting on exposed carbonate skeletons can liberate CO_2.

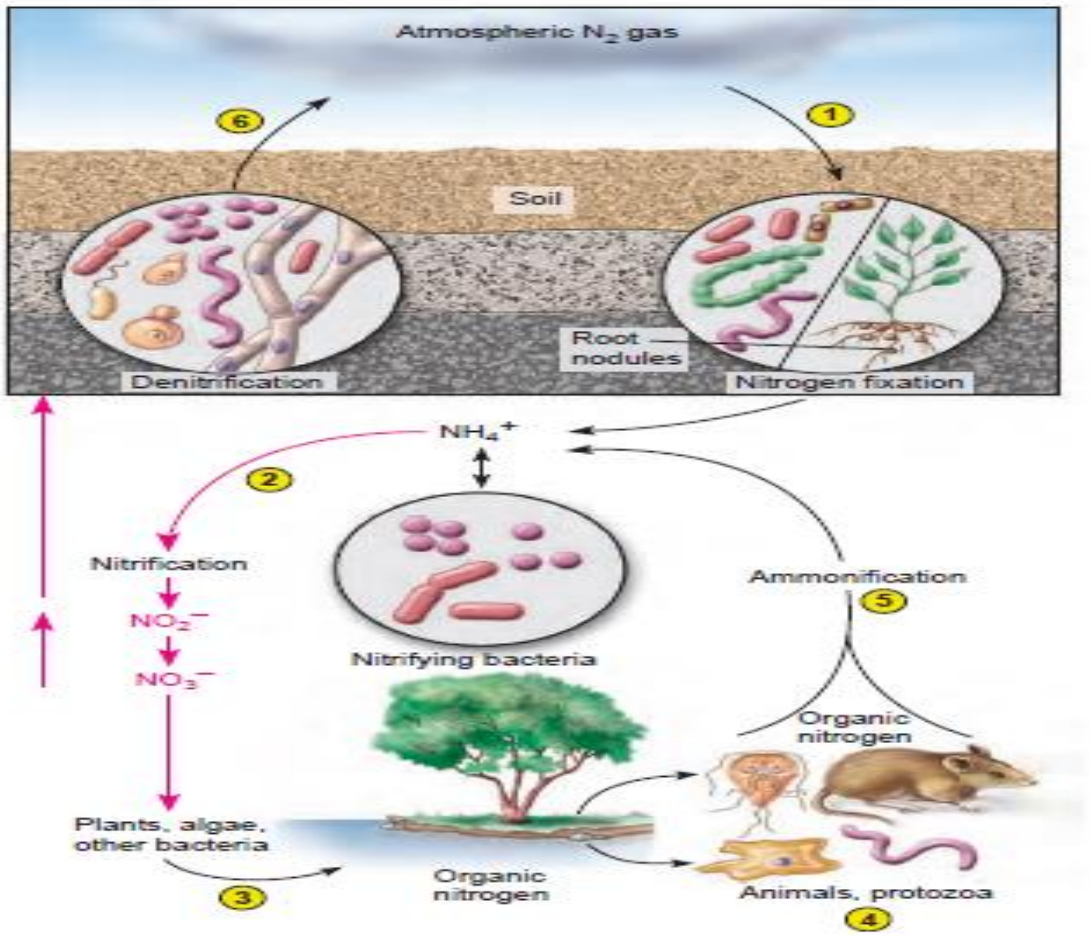
- ***The Nitrogen Cycle***

Nitrogen (N_2) gas is the most abundant component of the atmosphere,accounting for nearly 79% of air volume. As we will see, this extensivereservoir in the air is largely unavailable to most organisms. Onlyabout 0.03% of the earth's nitrogen is combined (or fixed) in some other form such as nitrates (NO_3), nitrites (NO_2), ammonium ion (NH_4^+), and organic nitrogen compounds (proteins, nucleic acids).

The **nitrogen cycle** is relatively more intricate than other cyclesbecause it involves such a diversity of specialized microbes to maintain the flow of the cycle. In many ways, it is actually more ofa nitrogen "web" because of the array of adaptations that occur. Higher plants can utilize $NO_3^-$and NH_4^+; animals must receivenitrogen in organic form from plants or other animals; however,microorganisms can use all forms of nitrogen: NO_2^-, NO_3^-, NH_4^+,N_2, and organic nitrogen. The cycle includes four basic types of reactions: nitrogen fixation, ammonification, nitrification, and denitrification **(figure 26.7).**

Nitrogen Fixation The biosphere is most dependent upon the only process that can remove N_2 from the air and convert it to a formusable by living beings. This process, called **nitrogen fixation,** is thebeginning step in the synthesis of virtually all nitrogenous compounds. Nitrogen fixation is brought about primarily by nitrogen-fixingbacteria in soil and water, though a small amount is formed through nonliving processes involving lightning. Nitrogen-fixing microbes have developed a unique enzyme system capable of breaking the triple bonds of the N_2 molecule and reducing the N atoms, an anaerobicprocess that requires the expenditure of considerable ATP. The primary product of nitrogen fixation is the ammonium ion, NH_4^+. Nitrogenfixing bacteria live free or in a symbiotic relationship with plants.

Among the common free-living nitrogen fixers are the aerobic *Azotobacter*and *Azospirillum*, certain members of the anaerobic genus*Clostridium*, and the cyanobacteria *Anabaena* and *Nostoc.*



Process Figure 26.7 Simplified events in the nitrogen cycle. (1) In nitrogen fixation, gaseous nitrogen (N_2) is acted on by nitrogen-fixing bacteria, which give off ammonia (NH_4^+). (2) Ammonia is converted to nitrite (NO_2^-) and nitrate (NO_3^-) by nitrifying bacteria in nitrification. (3) Plants, algae, and bacteria use nitrates to synthesize nitrogenous organic compounds (proteins, amino acids, nucleic acids). (4) Organic nitrogen compounds are used by animals and other consumers. (5) In ammonification, nitrogenous macromolecules from wastes and dead organisms are converted to NH_4^+ by ammonifying bacteria. NH_4^+ can be either directly recycled into nitrates or (6) returned to the atmospheric N_2 form by denitrifying bacteria (denitrification).

Root Nodules: Natural Fertilizer Factories

A significant symbiotic association occurs between **rhizobia *** (bacteria in the generan such as *Rhizobium, Bradyrhizobium*, and *Azorhizobium*) and **legumes ***(plants such as soybeans, peas, alfalfa, and clover that characteristicallyproduce seeds in pods). The infection of legume roots by these gramnegative,motile, rod-shaped bacteria causes the formation of special nitrogen-fixing organs called **root nodules (figure 26.8).** Nodulation begins when rhizobia colonize specific sites on root hairs. From there, the bacteria invade deeper root cells and induce the cells to form tumor like masses. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the

plant, and the plant furnishes nutrients and energy for the activities of the bacterium. The legume uses the $NH_4^+$to aminate (add an amino group to) various carbohydrate intermediates and thereby synthesize amino acids and other nitrogenous compounds that are used in plant and animal synthesis.

Plant-bacteria associations have great practical importance in agriculture, because an available source of nitrogen is often a limiting factor in the growth of crops. The self-fertilizing nature of legumes makes them valuable food plants in areas with poor soils and in countries with limited resources. It has been shown that crop health and yields can be improved by inoculating legume seeds with pure cultures of rhizobia, because the soil is often deficient in the proper strain of bacteria for forming nodules.

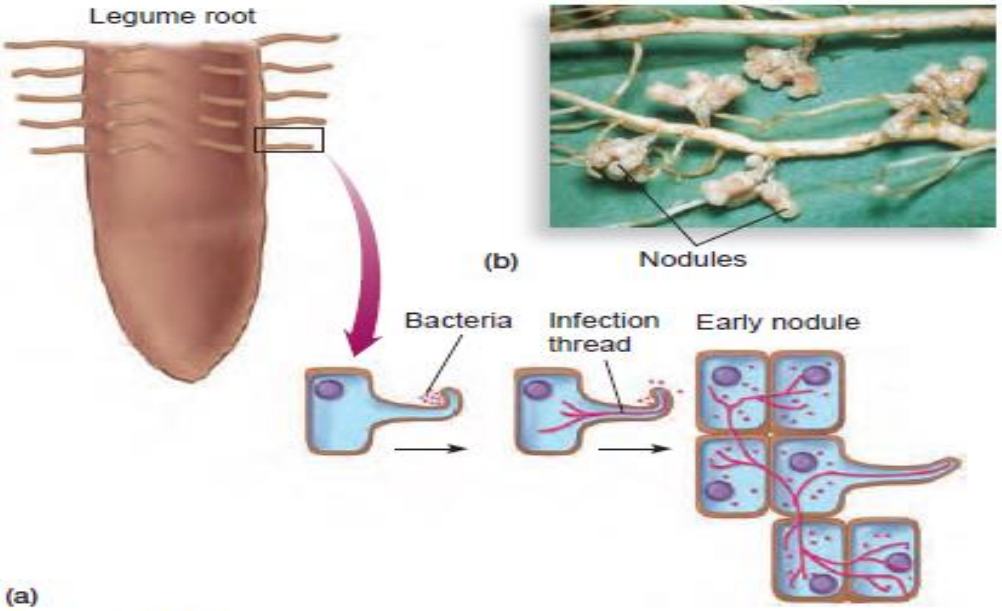


Figure 26.8 Nitrogen fixation through symbiosis.
(a) Events leading to formation of root nodules. Cells of the bacterium *Rhizobium* attach to a legume root hair and cause it to curl. Invasion of the legume root proper by *Rhizobium* initiates the formation of an infection thread that spreads into numerous adjacent cells. The presence of bacteria in cells causes nodule formation. **(b)** Mature nodules that have developed in a sweet clover plant.

Ammonification, Nitrification, and Denitrification:

Inanother part of the nitrogen cycle, nitrogen-containing organicmatter is decomposed by various bacteria (*Clostridium, Proteus,* forexample) that live in the soil and water. Organic detritus consistsof large amounts of protein and nucleic acids from dead organismsand nitrogenous animal wastes such as urea and uric acid. Thedecomposition of these substances splits off amino groups andproduces NH_4^+. This process is thus known as **ammonification.**

The ammonium released can be reused by certain plants or converted to other nitrogen compounds, as discussed next.The oxidation of $NH_4^+$to NO_2^- and $NO_3^-$is called **nitrification.**It is an essential conversion process for generating the mostoxidized form of nitrogen (NO_3). This reaction occurs in two phasesand involves two different kinds of lithotrophic bacteria in soil and water. In the first phase, certain gram-negative genera such as *Nitrosomonas, Nitrosospira,* and *Nitrosococcus*

oxidize NH_3 to NO_2^-as ameans of generating energy. Nitrite is rapidly acted upon by a second group of nitrifiers, including *Nitrobacter, Nitrosospira,* and *Nitrococcus,*which perform the final oxidation of NO_2^- to NO_3^-. Nitrates canbe assimilated through several routes by a variety of organisms(plants, fungi, and bacteria). Nitrate and nitrite are also important inanaerobic respiration where they serve as terminal electron acceptors;some bacteria use them as a source of oxygen as well.

The nitrogen cycle is completed through a process of **denitrification.**This occurs when nitrogen compounds undergo a series of reactionsthat convert NO_3^-through intermediate steps to atmosphericnitrogen. The first step, which involves the reduction of nitrate tonitrite, is so common that hundreds of different bacterial species cando it. Several genera such as *Bacillus, Pseudomonas, Spirillum,* and*Thiobacillus* can carry out denitrification to completion as follows:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2\,(\text{gas})$$

The fi nal product of this series returns nitrogen gas to its primary reservoir. Along the way, other gases—notably, nitrous oxide (N_2O)—are given off during incomplete denitrification. This is the main source of N_2O in the atmosphere and an important contributor to the greenhouse effect.

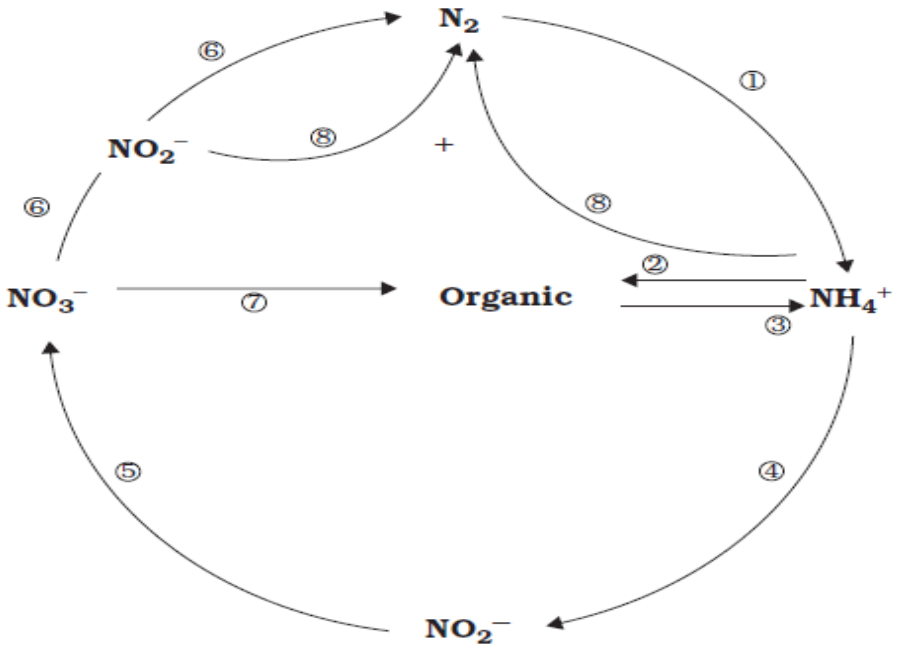


Figure 16.3 The nitrogen cycle. See the text for further details of reactions. Numbered arrows refer to reactions described in the text

The Sulfur Cycle

Sulphur is found in living organisms in the form of compounds such as amino acids, coenzymes and vitamins. It can be utilised by different types of organisms in several forms; Figure 16.4 shows the principal components of the sulphur cycle.

The **sulfur cycle** resembles the phosphorus cycle in that both elementsexist mostly in solid form and originate from natural sedimentarydeposits in rocks, oceans, and lakes and not from the atmosphere.Sulfur exists in the elemental form (S) and as hydrogen sulfide gas(H 2 S), sulfate (SO 4), and thiosulfate (S 2 O 3). Most of the oxidationsand reductions that convert one form of inorganic sulfur to another areaccomplished by bacteria. Plants and many microorganisms can assimilateonly SO 4 , and animals must have an organic source. Organicsulfur occurs in the amino acids cystine, cysteine, and methionine,which contain sulfhydryl (—SH) groups and form disulfi de (S—S)bonds that contribute to the stability and configuration of proteins.

One of the most remarkable contributors to the cycling of sulfurin the biosphere are the Thiobacilli .These gram-negative, motile rodsflourish in mud, sewage, bogs, mining drainage, and brackish springsthat can be inhospitable to organisms that require complex organicnutrients. But the metabolism of these specialized lithotrophic bacteria is adapted to extracting energy by oxidizing elemental sulfur, sulfides, and thiosulfate. One species, *Thiobacillus thiooxidans,* is so efficient at this process that it secretes large amounts of sulfuric acidinto its environment, as shown by the following equation:

$$Na_2S_2O_3 + H_2O + O_2 \rightarrow Na_2SO_4 + H_2SO_4 \text{ (sulfuric acid)} + 4S$$

The marvel of this bacterium is its ability to create and survive inthe most acidic habitats on the earth. It also plays an essential partin the phosphorus cycle, and its relative, *T. ferrooxidans,* participatesin the cycling of iron. Other bacteria that can oxidize sulfur to sulfates are the photosynthetic sulfur bacteria.

The sulfates formed from oxidation of sulfurous compoundsare assimilated into biomass by a wide variety of organisms. Thesulfur cycle reaches completion when inorganic and organic sulfurcompounds are reduced. Bacteria in the genera *Desulfovibrio* and*Desulfuromonas* anaerobically reduce sulfates to hydrogen sulfide (H_2S) or metal sulfide as the final step in electron transport. Sites inocean sediments and mud where these bacteria live usually emanate a strong, rotten-egg stench from H_2S and may be blackened by theiron they contain.

In its elemental form, sulphur is unavailable to most organisms; however, certainbacteria such as *Acidithiobacillus* are able to oxidise it to sulphate (**1**), a form that canbe utilised by a much broader range of organisms:

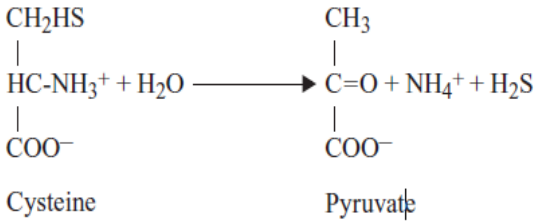
$$2S + 3O_2 + 2H_2O \longrightarrow H_2SO_4$$

Powdered sulphur is often added to alkaline soils in order to encourage this reactionand thereby reduce the pH.Sulphate-reducing bacteria convert the sulphate to hydrogen sulphide gas (**2**) usingeither an organic compound or hydrogen gas as electron donor:

$$8H^+ + SO_4^{2-} \longrightarrow H_2S + 2H_2O + 2OH^-$$

These bacteria are obligate anaerobes, and the process is termed *dissimilatory* sulphatereduction.

Plants are also able to utilise sulphate, incorporating it into cellular constituents suchas the amino acids methionine and cysteine (**3**) (*assimilatory* sulphate reduction).When the plants die, these compounds are broken down, again with the release ofhydrogen sulphide (**4**):



Green and purple photosynthetic bacteria and some chemoautotrophs use hydrogensulphide as an electron donor in the reduction of carbon dioxide, producing elemental sulphur and thus completing the cycle (**5**):

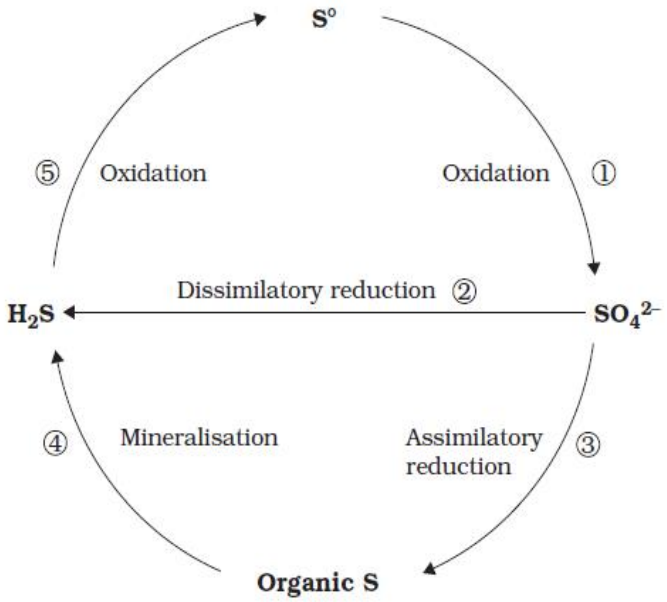


Figure 16.4 The sulphur cycle. See the text for further details of reactions. Numbered arrows refer to reactions described in the text

◆ Phosphorus Cycle

Phosphorus exists almost exclusively in nature as phosphate; however, this is cycled between soluble and insoluble forms. This conversion is pH-dependent, and if phosphate is only present in an insoluble form, it will act as a limiting nutrient. This explains the sudden surge in the growth of plants, algae and cyanobacteria when a source of soluble phosphate (typically fertilizer or detergent) enters a watercourse. Unlike the elements, phosphorus hardly exists in a gaseous form, so its main ‘reservoir’ is in the sea rather than the atmosphere.

Phosphorus is an integral component of DNA, RNA, and ATP, and all life depends upon a constant supply of it. It cycles between the abiotic and biotic environments almost exclusively as inorganic **phosphate** (PO_4) rather than its elemental form **(figure 26.9).**

The chief inorganic reservoir is phosphate rock, which contains the insoluble compound fluorapatite, $Ca_5(PO_4)_3F$. Before it can enter biological systems, this mineral must be *phosphatized*—converted into more soluble PO_4^{3-} by the action of acid. Phosphate is released naturally when the sulfuric acid produced by *Thiobacillus* dissolves phosphate rock. Soluble phosphate in the soil and water is the principal source for autotrophs, which fi x it onto organic molecules and pass it on to heterotrophs in this form. Organic phosphate is returned to the pool of soluble phosphate by decomposers, and it is finally cycled back to the mineral reservoir by slow geologic processes such as sedimentation. Because the low phosphate content of many soils can limit productivity, phosphate is added to soil to increase agricultural yields. The excess runoff of fertilizer into the hydrosphere is often responsible for overgrowth of aquatic pests.

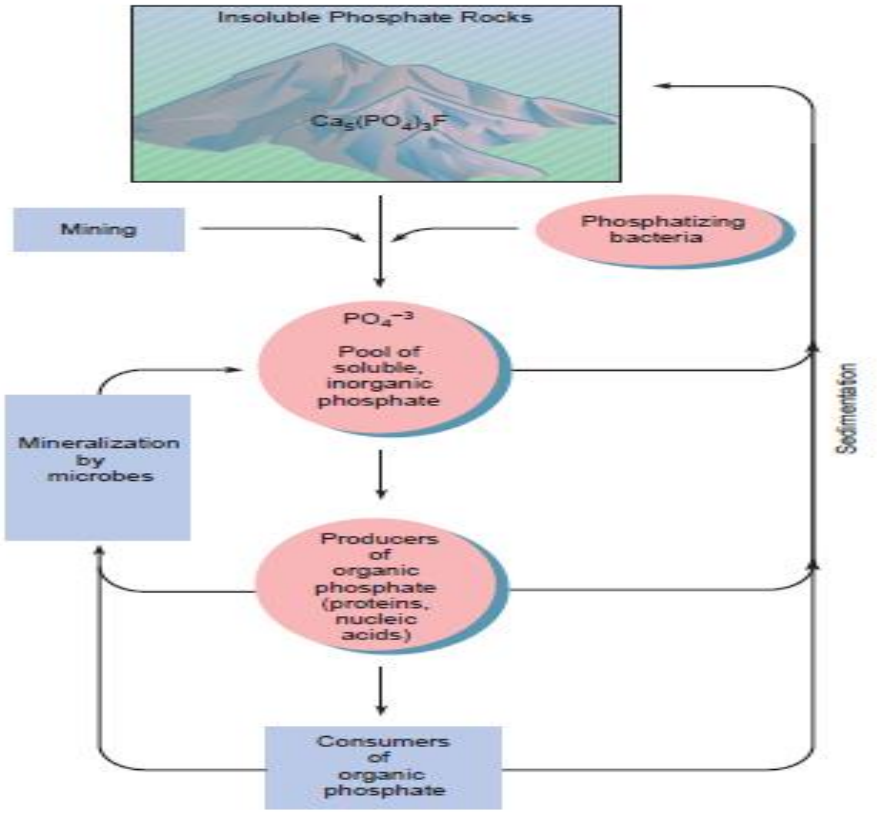


Figure 26.9 The phosphorus cycle. The pool of phosphate existing in sedimentary rocks is released into the ecosystem either naturally by erosion and microbial action or artificially by mining and the use of phosphate fertilizers. Soluble phosphate (PO_4^{3-}) is cycled through producers, consumers, and decomposers back into the soluble pool of phosphate, or it is returned to sediment in the aquatic biosphere.

h. Role of Microbes in environmental management

Microbes and their biosynthetic capabilities have been invaluable in finding solutions for several intractable problems mankind has encountered in maintaining the quality of the environment. They have, for example, been used to positive effect in human and animal health, genetic engineering, environmental protection, and municipal and industrial waste treatment. Microorganisms have enabled feasible and cost-effective responses which would have been impossible via straightforward chemical or physical engineering methods. Microbial technologies have of late been applied to a range of environmental problems, with considerable success. This survey of recent scientific progress in usefully applying microbes to both environmental management and biotechnology is informed by acknowledgement of the polluting effects on the world around us of soil erosion, the unwanted migration of sediments, chemical fertilizers and pesticides, and the improper treatment of human and animal wastes. These harmful phenomena have resulted in serious environmental and social problems around the world, problems which require us to look for solutions elsewhere than in established physical and chemical technologies. Often the answer lies in hybrid applications in which microbial methods are combined with physical and chemical ones. When we remember that these highly effective microorganisms, cultured for a variety of applications, are but a tiny fraction of those to be found in the world around us, we realize the vastness of the untapped and beneficial potential of microorganisms. At present, comprehending the diversity of hitherto uncultured microbes involves the application of metagenomics, with several novel microbial species having been discovered using culture-independent approaches. Edited by recognized leaders in the field, this penetrating assessment of our progress to date in deploying microorganisms to the advantage of environmental management and biotechnology will be widely welcomed.

The many and varied metabolic activities of microbes assure that they participate in chemical reactions in almost every environment on earth. Microbes require an energy producing system (including an electron acceptor) to sustain life and nutrients, including liquid water, in order to grow and reproduce. Since microbes have been present on earth longer than other organisms, they have evolved the ability to thrive in almost any environment that meets these minimal criteria. Energy comes from one of two sources, light (photosynthesis) or the oxidation of reduced molecules. Oxidizable molecules may be organic (e.g. sugar, protein or any of the other foods we humans relish) or a variety of inorganic molecules such as sulfur, iron, hydrogen, carbon monoxide, or ammonia or even a combination of organic/inorganic molecules. Microbes exist that prosper inside of eukaryotic cells, at temperatures of >100^{o}C, in the presence of toxic metals like copper or mercury, at pH's ~2.0 and ~11.0, down to 3.5 km below the earth's surface and in saturated salt solutions at 0^{o}C. Microbes have broadened the environments they can live in by evolving enzymes that allow them to utilize sunlight for energy as well as a diversity of electron donor/acceptors pairs so they can perform energy-yielding oxidative reactions on available energy sources. That this evolution is ongoing is shown by the isolation of microbes that can metabolize numerous man-made chemicals (ones not found in nature). The range of electron acceptors includes gaseous oxygen (like us), sulfate, nitrate, nitrite, carbon dioxide, carbon monoxide, iron and magnesium. Indeed, evolutionary principles predict that microbes should have evolved to utilize any niche meeting the minimal physical and chemical requirements. Recently, bacteria that

live ~3.5 km below the earth's surface in rocks at high temperatures have been discovered. Since these conditions cover the entire earth, even that portion under the oceans, these bacterial forms may make up the largest single mass of life on (or in) earth.
Below is an abbreviated list of the roles microbes play in our lives:

- They maintain soil fertility and soil tilth.
- They clean up all the dead organic material; without them we would be up to our ears in dead things, like our ancestors.
- They fix gaseous nitrogen into forms that can be used by plants to maintain the fertility of soils
- They can be used to extract minerals from ores.
- They are the prime food for all the marine and freshwater life; even whales depend on them directly or indirectly for their nutrition.

The Role of Microbes on the Earth:

Outside of major planetary occurrences such as earthquakes, volcanoes and continental drift, there are very few events on earth that are not affected in some way by microorganisms. For example, marine algae that routinely cover huge patches of ocean absorb, and convert into heat, sunlight, which would otherwise be reflected back into space. This absorbed heat raises the oceans' temperature. Since oceans are the "the earth's weather engine", microbes thus affect the weather. Most (some might say all) multicellular forms of life live in intimate association with a host of microbes and in some cases the multicellular forms could not exist without their microbe associates. More to the point, there is probably no human endeavor in which microbes fail to play a role in at some level. Because of hubris humans tend to consider microbial activities only in terms of their primary effect on themselves (e.g. diseases) and their commercial enterprises (e.g. wine production). However, it is quite clear that without microbes life on earth could not exist. Not only were bacteria the first life on earth, and the progenitors of all subsequent forms of life, but in many ways they remain the predominate life form on the planet, both in terms of the degree to which they affect earthly things, and perhaps even in terms of total protoplasmic mass; given their small size this means there are humongous numbers of them out there. In this chapter, you will learn just a bit of the extent of the role of microbes in your life.

When considering the impact of bacteria and other microbes on the earth it is important to grasp the concept of "**MICROENVIRONMENTS**" or "**MICROHABITATS**". Microenvironments are small spaces or locations within the Earth's ecosystem where some environmental factors differs from another that is nearby. This cartoon illustrates a microenvironment situation in the soil. For example, within a square cm of normal soil 100s or even 1000s of different environments (microhabitats) may exist separated by only a mm or less. One part of the soil may contain a tiny fragment of organic matter (a cellulose chunk) that is only utilized (eaten) by a few species of microbes (those that produce cellulase). Thus mostly cellulase-producing microbes would be found there and they would, in turn, produce waste products that would change the pH in a sphere ~0.1

mm around the cellulose chunk. Whereas 1 mm away is a different nutrient source with its unique microbes affecting the immediate environment around it (e.g. by producing an antibiotic that inhibits likely competitors). Essentially the entire world is composed of microenvironments. In fact microenvironments are so prevalent that they are assumed to be far more common than macro environments (like beer fermentation tanks). Even the water in a lake or the ocean is composed of distinct microenvironments depending on environmental factors like light, nutrients, temperature etc. Our mouths contain a host of microhabitats that determine it health and halitosis.

- **Microbial Bioremediation:**

The removal of toxic industrial products in soils and aquatic environments has become a daunting and necessary task. Compounds such as perchloroethylene (PCE), trichloroethylene (TCE), and polychlorinated biphenyls (PCBs) are common contaminants. These compounds adsorb onto organic matter in the environment, making decontamination using traditional approaches difficult or ineffective. The use of microbes to transform these contaminants to nontoxic degradation products is called **bioremediation.**

To understand how bioremediation takes place at the level of an ecosystem, we first must consider the biochemistry of biodegradation. Degradation of complex compounds requires several discrete stages, usually performed by different microbes. Initially contaminants are converted to less-toxic compounds that are more readily degraded.

The first step for many contaminants, including organochloride pesticides, alkyl solvents, and aryl halides, is **reductivedehalogenation.** This is the removal of a halogen substituent (e.g., chlorine, bromine, fluorine) while at the same time adding electrons to the molecule. This can occur in two ways. In hydrogenolysis, the halogen substituent is replaced by a hydrogen atom. Alternatively, dihaloelimination removes two halogen substituents from adjacent carbons while inserting an additional bond between the carbons. Both processes require an electron donor. The dehalogenation of PCBs uses electrons derived from water; alternatively hydrogen can be the electron donor for the dehalogenation of different chlorinated compounds.

Major genera that carry out this process include *Desulfi tobacterium, Dehalospirillum,*and *Desulfomonile.*Reductive dehalogenation usually occurs under anoxic conditions. In fact, humic acids (polymeric residues of lignin decomposition that accumulate in soils and waters) have been found to play a role in anaerobic biodegradation processes. They can serve as terminal electron acceptors under what are called "humic acid-reducing conditions." The use of humic acids as electron acceptors has been observed with the anaerobic dechlorination of vinyl chloride and dichloroethylene.Once the anaerobic dehalogenation stepsare completed, degradation of the main structure of many pesticides and other xenobiotics often proceeds more rapidly in the presence of O_2. Thus the degradation of halogenated toxic compounds generally requires the action of several microbial genera, sometimes referred to as a consortium.

Structure and stereochemistry are critical in predicting the fate of a specific chemical in nature. When a constituent is in the *meta*as opposed to the *ortho* position, the compound will be degraded at a much slower rate.

So, Bioremediation is a process used to treat contaminated media, including water, soil and subsurface material, by altering environmental conditions to stimulate growth of microorganisms and degrade the target pollutants. In many cases, bioremediation is less expensive and more

sustainable than other remediation alternatives. Biological treatment is a similar approach used to treat wastes including wastewater, industrial waste and solid waste.

Most bioremediation processes involve oxidation-reduction reactions where either an electron acceptor (commonly oxygen) is added to stimulate oxidation of a reduced pollutant (e.g. hydrocarbons) or an electron donor (commonly an organic substrate) is added to reduce oxidized pollutants (nitrate, perchlorate, oxidized metals, chlorinated solvents, explosives and propellants). In both these approaches, additional nutrients, vitamins, minerals, and pH buffers may be added to optimize conditions for the microorganisms. In some cases, specialized microbial cultures are added (bioaugmentation) to further enhance biodegradation. Some examples of bioremediation related technologies are phytoremediation, mycoremediation, bioventing, bioleaching, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation.

Chemistry:

Most bioremediation processes involve oxidation-reduction (Redox) reactions where a chemical species donates an electron (electron donor) to a different species that accepts the electron (electron acceptor). During this process, the electron donor is said to be oxidized while the electron acceptor is reduced. Common electron acceptors in bioremediation processes include oxygen, nitrate, manganese (III and IV), iron (III), sulfate, carbon dioxide and some pollutants (chlorinated solvents, explosives, oxidized metals, and radionuclides). Electron donors include sugars, fats, alcohols, natural organic material, fuel hydrocarbons and a variety of reduced organic pollutants. The redox potential for common biotransformation reactions is shown in the table.

Process	Reaction	Redox potential (E_h in mV)
aerobic	$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$	600 ~ 400
anaerobic		
denitrification	$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$	500 ~ 200
manganese IV reduction	$MnO_2 + 2e^- + 4H^+ \rightarrow Mn^{2+} + 2H_2O$	400 ~ 200
iron III reduction	$Fe(OH)_3 + e^- + 3H^+ \rightarrow Fe^{2+} + 3H_2O$	300 ~ 100
sulfate reduction	$SO_4^{2-} + 8e^- + 10\ H^+ \rightarrow H_2S + 4H_2O$	0 ~ −150
fermentation	$2CH_2O \rightarrow CO_2 + CH_4$	−150 ~ −220

Aerobic: Aerobic bioremediation is the most common form of oxidative bioremediation process where oxygen is provided as the electron acceptor for oxidation of petroleum, polyaromatic hydrocarbons (PAHs), phenols, and other reduced pollutants. Oxygen is generally the preferred electron acceptor because of the higher energy yield and because oxygen is required for some enzyme systems to initiate the degradation process. Numerous laboratory and field studies have

shown that microorganisms can degrade a wide variety of hydrocarbons, including components of gasoline, kerosene, diesel, and jet fuel. Under ideal conditions, the biodegradation rates of the low- to moderate-weight aliphatic, alicyclic, and aromatic compounds can be very high. As the molecular weight of the compound increases, so does the resistance to biodegradation.

Common approaches for providing oxygen above the water table include landfarming, composting and bioventing. During landfarming, contaminated soils, sediments, or sludges are incorporated into the soil surface and periodically turned over (tilled) using conventional agricultural equipment to aerate the mixture. Composting accelerates pollutant biodegradation by mixing the waste to be treated with a bulking agent, forming into piles, and periodically mixed to increase oxygen transfer. Bioventing is a process that increases the oxygen or air flow into the unsaturated zone of the soil which increases the rate of natural in situ degradation of the targeted hydrocarbon contaminant.

Approaches for oxygen addition below the water table include recirculating aerated water through the treatment zone, addition of pure oxygen or peroxides, and air sparging. Recirculation systems typically consist of a combination of injection wells or galleries and one or more recovery wells where the extracted groundwater is treated, oxygenated, amended with nutrients and reinjected. However, the amount of oxygen that can be provided by this method is limited by the low solubility of oxygen in water (8 to 10 mg/L for water in equilibrium with air at typical temperatures). Greater amounts of oxygen can be provided by contacting the water with pure oxygen or addition of hydrogen peroxide (H_2O_2) to the water. In some cases, slurries of solid calcium or magnesium peroxide are injected under pressure through soil borings. These solid peroxides react with water releasing H_2O_2 which then decomposes releasing oxygen. Air sparging involves the injection of air under pressure below the water table. The air injection pressure must be great enough to overcome the hydrostatic pressure of the water and resistance to air flow through the soil.

Anaerobic: Anaerobic bioremediation can be employed to treat a broad range of oxidized contaminants including chlorinated ethenes (PCE, TCE, DCE, VC), chlorinated ethanes (TCA, DCA), chloromethanes (CT, CF), chlorinated cyclic hydrocarbons, various energetics (e.g., perchlorate, RDX, TNT), and nitrate. This process involves the addition of an electron donor to: 1) deplete background electron acceptors including oxygen, nitrate, oxidized iron and manganese and sulfate; and 2) stimulate the biological and/or chemical reduction of the oxidized pollutants. Hexavalent chromium (Cr[VI]) and uranium (U[VI]) can be reduced to less mobile and/or less toxic forms (e.g., Cr[III], U[IV]). Similarly, reduction of sulfate to sulfide (sulfidogenesis) can be used to precipitate certain metals (e.g., zinc, cadmium). The choice of substrate and the method of injection depend on the contaminant type and distribution in the aquifer, hydrogeology, and remediation objectives. Substrate can be added using conventional well installations, by direct-push technology, or by excavation and backfill such as permeable reactive barriers (PRB) or biowalls. Slow-release products composed of edible oils or solid substrates tend to stay in place for an extended treatment period. Soluble substrates or soluble fermentation products of slow-release substrates can potentially migrate via advection and diffusion, providing broader but shorter-lived treatment zones. The added organic substrates are first fermented to

hydrogen (H_2) and volatile fatty acids (VFAs). The VFAs, including acetate, lactate, propionate and butyrate, provide carbon and energy for bacterial metabolism.

Heavy Metals:

Heavy metals including cadmium, chromium, lead and uranium are elements so they cannot be biodegraded. However, bioremediation processes can potentially be used to reduce the mobility of these material in the subsurface, reducing the potential for human and environmental exposure. The mobility of certain metals including chromium (Cr) and uranium (U) varies depending on the oxidation state of the material. Microorganisms can be used to reduce the toxicity and mobility of chromium by reducing hexavalent chromium, Cr(VI) to trivalent Cr (III).Uranium can be reduced from the more mobile U(VI) oxidation state to the less mobile U(IV) oxidation state. Microorganisms are used in this process because the reduction rate of these metals is often slow unless catalyzed by microbial interactionsResearch is also underway to develop methods to remove metals from water by enhancing the sorption of the metal to cell walls. This approach has been evaluated for treatment of cadmium,chromium, and lead. Phytoextraction processes concentrate contaminants in the biomass for subsequent removal.

Additives:

In the event of biostimulation, adding nutrients that are limited to make the environment more suitable for bioremediation, nutrients such as nitrogen, phosphorus, oxygen, and carbon may be added to the system to improve effectiveness of the treatment.

Many biological processes are sensitive to pH and function most efficiently in near neutral conditions. Low pH can interfere with pH homeostasis or increase the solubility of toxic metals. Microorganisms can expend cellular energy to maintain homeostasis or cytoplasmic conditions may change in response to external changes in pH. Some anaerobes have adapted to low pH conditions through alterations in carbon and electron flow, cellular morphology, membrane structure, and protein synthesis.

Limitations of bioremediation:

Bioremediation can be used to completely mineralize organic pollutants, to partially transform the pollutants, or alter their mobility. Heavy metals and radionuclides are elements that cannot be biodegraded, but can be bio-transformed to less mobile forms.[18][19][20] In some cases, microbes do not fully mineralize the pollutant, potentially producing a more toxic compound. For example, under anaerobic conditions, the reductive dehalogenation of TCE may produce dichloroethylene (DCE) and vinyl chloride (VC), which are suspected or known carcinogens.[18] However, the microorganism *Dehalococcoides* can further reduce DCE and VC to the non-toxic product ethene. Additional research is required to develop methods to ensure that the products from biodegradation are less persistent and less toxic than the original contaminant.Thus, the metabolic and chemical pathways of the microorganisms of interest must be known. In addition, knowing these pathways will help develop new technologies that can deal with sites that have uneven distributions of a mixture of contaminants.

Also, for biodegradation to occur, there must be a microbial population with the metabolic capacity to degrade the pollutant, an environment with the right growing conditions for the microbes, and the right amount of nutrients and contaminants. The biological processes used by these microbes are highly specific, therefore, many environmental factors must be taken into account and regulated as well.Thus, bioremediation processes must be specifically made in accordance to the conditions at the contaminated site.Also, because many factors are interdependent, small-scale tests are usually performed before carrying out the procedure at the contaminated site. However, it can be difficult to extrapolate the results from the small-scale test studies into big field operations.In many cases, bioremediation takes more time than other alternatives such as land filling and incineration.

Genetic engineering:

The use of genetic engineering to create organisms specifically designed for bioremediation is under preliminary research.Two category of genes can be inserted in the organism: degradative genes which encode proteins required for the degradation of pollutants, and reporter genes that are able to monitor pollution levels. Numerous members of *Pseudomonas* have also been modified with the lux gene, but for the detection of the polyaromatic hydrocarbon naphthalene. A field test for the release of the modified organism has been successful on a moderately large scale.

There are concerns surrounding release and containment of genetically modified organisms into the environment due to the potential of horizontal gene transfer.Genetically modified organisms are classified and controlled under the Toxic Substances Control Act of 1976 under United States Environmental Protection Agency. Measures have been created to address these concerns. Organisms can be modified such that they can only survive and grow under specific sets of environmental conditions.[26] In addition, the tracking of modified organisms can be made easier with the insertion of bioluminescence genes for visual identification.

Genetically modified organisms have been created to treat oil spills and break down certain plastics (PET).

- **Role of microorganisms in mineral recovery:**

Microorganisms are used in large-scale heap or tank aeration processes for the commercial extraction of a variety of metals from their ores or concentrates. These include copper, cobalt, gold and, in the past, uranium. The metal solubilization processes are considered to be largely chemical with the microorganisms providing the chemicals and the space (exopolysaccharide layer) where the mineral dissolution reactions occur. Temperatures at which these processes are carried out can vary from ambient to 80°C and the types of organisms present depends to a large extent on the process temperature used. Irrespective of the operation temperature, biomining microbes have several characteristics in common. One shared characteristic is their ability to produce the ferric iron and sulfuric acid required to degrade the mineral and facilitate metal recovery. Other characteristics are their ability to grow autotrophically, their acid-tolerance and their inherent metal resistance or ability to acquire metal resistance. Although the microorganisms that drive the process have the above properties in common, biomining microbes usually occur in consortia in which cross-feeding may occur such that a combination of microbes including some with heterotrophic tendencies may contribute to the efficiency of the process. The remarkable adaptability of these organisms is assisted by several of the processes being continuous-flow systems that enable the continual selection of microorganisms that are more efficient at mineral

degradation. Adaptability is also assisted by the processes being open and non-sterile thereby permitting new organisms to enter. This openness allows for the possibility of new genes that improve cell fitness to be selected from the horizontal gene pool. Characteristics that biomining microorganismshaveincommonandexamplesoftheirremarkableadaptabilityaredescribed,

Introduction

The solubilization of metals due to the action of microbes and the subsequent recovery of the metals from solution has deep historical roots that have been extensively reviewed. Similarly, an indication of the number and sizes of the operations that employ microbes for the recovery of mainly copper, gold, cobalt and uranium has also been reviewed. These processes use the action of microbes for one of two purposes. Either to convert insoluble metal sulfides (or oxides) to water soluble metal sulfates or as a pretreatment process to open up the structure of the mineral thereby permitting other chemicals to better penetrate the mineral and solubilize the desiredmetal. An example of the first type of process is the con- version of insoluble copper present in minerals such as covellite (CuS) or chalcocite (Cu2S) to soluble copper sulfate.

An example of the second, is the removal of iron,arsenic and sulfur from gold-bearing arsenopyrite so that the gold that remains in the mineral is more easily extracted by subsequent treatment with cyanide. Both are oxidation processes, but where the metal to be recovered is extracted into solution the process is known as bioleaching, whereas when the metal remains in the min- eral, bioleaching is an inappropriate term and the process should strictly be referred to as biooxidation. Nevertheless, the term bioleaching is frequently used for both.

Not all types of mineral are amenable to biologically- assisted leaching. In general, the mineral should contain iron or a reduced form of sulfur. Alternately, a mineral lacking in these compounds may be leached if it occurs together with another mineral that contains iron and reduced sulfur, provided that the mineral is subject to attack by ferric iron and/or sulfuric acid.

Metalsincertainnon-sulfidemineralsmaybesolubilized byaprocessofcomplexationwithoxalic,citricorother organicacids.Theseorganicacidsaretypicallyproduced by certain types of fungi. Varioustypes of mineral biooxidation organisms have in common. However,before discussing these general characteristicsit is necessary to describebriefly the mechanism of leaching and the technology of them talrecovery processes.

Mechanisms

Metal leaching is now recognized as being mainly a chemical process in which ferric iron and protons are responsible for carry out the leaching reactions. The role of the microorganisms is to generate the leaching chemicals and to create the space in which the leaching reactions take place. Microorganisms typically form an exopolysaccharide (EPS) layer when they adhere to the surface of a mineral but not when growing as planktonic cells. It is within this EPS layer rather than in the bulk solution that the biooxidation reactions take place most rapidly and efficiently and therefore the EPS serves as the reaction space.

The mineral dissolution reaction is not identical for all metal sulfides and the oxidation of different metal sulfides proceeds via different intermediates. Briefly, a **thiosulfate mechanism** has been proposed for the oxidation of acidinsoluble metal sulfides such as pyrite (FeS_2) and molybdenite

(MoS_2), and a **polysulfide mechanism** for acid soluble metal sulfides such as sphalerite (ZnS), chalcopyrite (CuFeS2) or galena (PbS).

In the thiosulfate mechanism, solubilization is through ferric iron attack on the acid-insoluble metal sulfides with thiosufate being the main intermediate and sulfate the main end-product. Using pyrite as an example of a min- eral, the reactions may be represented as:

$$FeS_2 + 6\ Fe^{3+} + 3\ H_2O \rightarrow S_2O_3^{2-} + 7\ Fe^{2+} + 6\ H^+ \quad (1)$$

$$S_2O_3^{2-} + 8\ Fe^{3+} + 5\ H_2O \rightarrow 2\ SO_4^{2-} + 8\ Fe^{2+} + 10\ H^+ \quad (2)$$

Microbes such as *Acidithiobacillusthiooxidans*or *Acidithio- bacillus caldus* (reaction 5below).

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5\ H_2S_n + Fe^{2+} (n \geq 2) \quad (3)$$

$$0.5\ H_2S_n + Fe^{3+} \rightarrow 0.125\ S_8 + Fe^{2+} + H^+ \quad (4)$$

$$0.125\ S_8 + 1.5\ O_2 + H_2O \xrightarrow{\text{microbes}} SO_4^{2-} + 2\ H^+ \quad (5)$$

The ferrous iron produced in reaction 1 to 4 may be reoxidized to ferric iron by iron oxidizing microorganisms like *Acidithiobacillusferrooxidansor* bacteria of the genera*Leptospirillum* or*Sulfobacillus*.

$$2\ Fe^{2+} + 0.5\ O_2 + 2\ H^+ \xrightarrow{\text{microbes}} 2\ Fe^{3+} + H_2O \quad (6)$$

The role of the microorganisms in the solubilization of metal sulfides is, therefore, to provide sulfuric acid (reaction 5) for a proton attack and to keep the iron in the oxidized ferric state (reaction 6) for an oxidative attack on the mineral.

Commercial metal extraction operations

1. **Heap leaching processes-**

Commercial bioleaching can take place using what may be considered to be a low technology process, the irrigation of waste ore dumps. The metal recovery process may be made more efficient by the construction and irrigation of especially-designed heaps rather than by the irrigation of an existing dump that has not been designed to optimize the leaching process. When building a heap, agglomerated ore is piled onto an impermeable base and supplied with an efficient leach liquor distribution and collection system. Acidic leaching solution is percolated through the crushed ore and microbes growing on the surface of the mineral in the heap produce the ferric iron and acid that result in mineral dissolution and metal solubilization. Aeration in such processes can be passive, with air being draw into the reactor as a result of the flow of liquid, or active with air blown into the heap through piping installed near the bottom. Metal-containing leach solutions that drain from the heap are collected and sent for metal recovery. Heap reactors are cheaper to construct and operate and are therefore more suited to the treatment of lower grade ores. However, compared with tank reactors, heap reactors are more difficult to aerate efficiently and the undesirable formation of gradients of pH and nutrient levels as well as liquor channeling are difficult to manage. Furthermore, although one can rely on the natural movement of microbes to eventually inoculate the heap, initial rates of bioleaching can be improved by effective heap inoculation, but this is difficult to achieve.

Copper is the metal recovered in the largest quantity by means of heap reactors. Although comparisons are difficult as data are presented in different ways, examples of large copper leaching operations are those by Sociedad Contractual Minera El Abra and the Codelco Division Radimiro Tomic both in Chile and pro- ducing 225 000 and 180 000 tonnes Cu per annum respectively. Gold ore is also pretreated by bioleaching in heaps by Newmont Mining, in the Carlin Trend region, Nevada, USA.

2. Tank leaching processes-

In stirred tank processes highly aerated, continuous-flow reactors placed in series are used to treat the mineral. Finely milled mineral concentrate or ore is added to the first tank together with inorganic nutrients in the form ofammonia- and phosphate-containing fertilizers. The mineral suspension flows through series of highly-aerated tanks that are pH and temperature-controlled. Mineral solubilization takes place in days in stirred-tank reactors compared with weeks or months in heap reactors. Stirred tank reactors that operate at 40°C and 50°C have proven to be highly robust and very little process adaptation is required for the treatment of different mineral types. A major constraint on the operation of stirred tank reactors is the quantity of solids (pulp density) that can be maintained in suspension. This is limited to about 20% as at pulp densities >20%, physical mixing and microbial problems occur. The liquid becomes too thick for efficient gas transfer and the shear force induced by the impellers causes' physical damage to the microbial cells. This limitation in solids concentration plus considerably higher capital and running costs in tank compared with heap reactors has meant that the use of stirred reactors has been restricted to high value minerals or mineral concentrates.

Stirred tanks are used as a pretreatment process for gold- containing arsenopyrite concentrates with the first of these having been built at the Fairview mine, Barberton, South Africa in 1986. The largest is at Sansu in the Ashanti goldfields of Ghana, West Africa. These two oper- ations currently treat 55 and 960 tonnes of gold concen- trate per day respectively. Another example is the use of stirred tanks to treat 240 tonnes of cobalt-containing pyrite in 1300 m3 tanks at Kasese, Uganda.

Types of Microorganisms In general, the types of microorganisms found in heapleaching processes are similar to those found in stirred tank processes, however, the proportions of the microbes may vary depending on the mineral and the conditions under which the heaps or tanks are operated. In processes that operate from ambient temperatures to about 40°C, the most important microorganisms are considered to be a consortium of Gram-negative bacteria. These are the iron- and sulfur-oxidizing *Acidithiobacillus ferrooxidans*(previously *Thiobacillus ferrooxidans*), the sulfur-oxidizing*Acidithiobacillus thiooxidans* (previously *Thiobacillus thiooxidans*) and *Acidithiobacillus caldus* (previously *Thiobacilluscaldus*), and the iron-oxidizing leptospirilli, *Leptospirillumferrooxidans* and *Leptospirillum ferriphilum*. If ferrous iron is added to the leaching solutions (lixiviants) that are circulated through a heap or dump, then *At. Ferrooxidans* may dominate the iron-oxidizers.

In continuous flow, stirred tank processes, the steady state ferric iron concentration is usually high and under such conditions *At. ferrooxidans*is less important than a combination of *Leptospirillum* and *At. thiooxidans*or *At. caldus*. Gram-positive iron and sulfur-oxidizing bacteriarelatedto*Sulfobacillusthermosulfidooxidans*have alsobeenidentified.The consortium of

bioleaching microbes frequently includes acidophilic heterotrophic organisms such as bacteria belonging to the genus *Acidiphilium* or *Ferroplasma*-like archaea.A fluidized-bedreactoroperatingat37°CandpH1.4was dominated by *L. ferriphilum* with a small proportion of *Ferroplasma*-likearchaea.'Heterotrophicallyinclined' microbesarebelievedtoassistthegrowthofiron-oxidiz- ing bacteria like *At. ferrooxidans*and the leptospirilli.This is thoughttobeduetotheirabilitytopro-videessentialnutrientsortoremovetoxicorganiccom- pounds or other inhibitory substances. How muchthis abilitycontributestotheoverallmineralbiooxidation efficiencyofamicrobialconsortiuminpracticeisstill unclear

There are fewer commercial processes that operate in the 45–50°C range and therefore studies on microorganisms that dominate these bioleaching consortia have been less well reported. Rawlings et al., identified *At. caldus*and a species of *Leptospirillum* as being the dominant microbes in a continuous-flow biooxidation tanks processing several mineral ores operating in this tempera- ture range. *At. caldus, Sulfobacillus thermosulfidooxidans* and bacteria of the informally recognized species '*Sulfobacillus montserratensis*' together with an uncultured thermal soil bacterium were found to dominate the consortium of organisms oxidizing chalcopyrite concentrate at 45°C. The same bacteria dominated the culture irrespective of whether chalcopyrite, pyrite or an arsenic pyrite concen- trate was being oxidized. In a pilot scale, stirred-tank operation in which three tanks in series were used to treat a polymetallic sulfide ore at 45°C, *At. caldus*-like, *L. ferriphilum*-like and *Sulfobacillus*like bacteria were found to dominate the first tank. The proportions of these bac- teria decreased in the second tank with the numbers of *At. caldus*and *Ferroplasma*-like archaea being equally domi- nant. The *Ferroplasma*-like archaea completely dominated the third tank with the number of leptospirilli being reduced to undetectable levels. When combinations of pure cultures were tested, a mixed culture containing both autotrophic (*Leptospirillum* MT6 and *At. caldus*) and heter- otrophic moderate thermophiles (*Ferroplasma* MT17) was the most efficient. The presence of *Ferroplasma*-like organisms is being increasing recognized in bioleaching processes that operate at very low pH (1.4 or less). These archaea appear to be able to oxidize minerals like pyrite in pure culture although not without a small quantity of yeast extract. Species of the gram-positive genus, *Acidimi- crobium* may occur together with sulfobacilli in cul- tures that grow at 45°C.

General characteristics of mineral degrading bacteria

As would be gathered from the above, the most important microbes involved in the biooxidation of minerals are those that are responsible for producing the ferric iron and sulfuric acid required for the bioleaching reactions. These are the iron- and sulfur-oxidizing chemolithrophic bacteria and archaea. Irrespective of the type of process or temperature at which they are employed, these microbes have a number of features in common that make them especially suitable for their role in mineral solubilization. Four of the most important characteristics are;

a) they grow autotrophically by fixing CO_2 from the atmosphere; b) they obtain their energy by using either ferrous iron or reduced inorganic sulfur compounds(some use both) as an electron donor, and generally use oxygen as the electron acceptor; c) they are acidophiles and grow in low pH environments (pH.1.4 to 1.6 is typical) and d) they are remarkably tolerant to a wide range of metal ions, though there is considerable variation within and between species.

The modest nutritional requirements of these organisms are provided by the aeration of an iron- and/or sulfur containing mineral suspension in water or the irrigation of a heap. Small quantities of inorganic fertilizer can be added to ensure that nitrogen, phosphate, potassium and trace element limitation does not occur.

A further advantageous characteristic of mineral biooxidation operations is that they are usually not subject to contamination by unwanted microorganisms. In the case of continuous-flow tank leaching processes, the continual wash-out of mineral together with their attached microbes as well as the organisms in suspension provides strong selection for improved microorganisms.

- **Microbial Leaching**

Bioleaching is the use of microorganisms, which produce acids from reduced sulfur compounds, to create acidic environments that solubilize desired metals for recovery. This approach is used to recover metals from ores and mining tailings with metal levels too low for smelting. Bioleaching carried out by natural populations of *Leptospirillum*-like species, *Thiobacillus thiooxidans,* and related thiobacilli, for example, allows recovery of up to 70% of the copper in low-grade ores. As shown in **figure 41.27,** this involves the biological oxidation of copper present in these ores to produce soluble copper sulfate.

It is apparent that nature will assist in bioremediation if given a chance. The role of microorganisms in biodegradation is now better appreciated. An excellent example is the xenobiotic metabolism of the versatile fungus *Phanerochaete chrysosporium.*

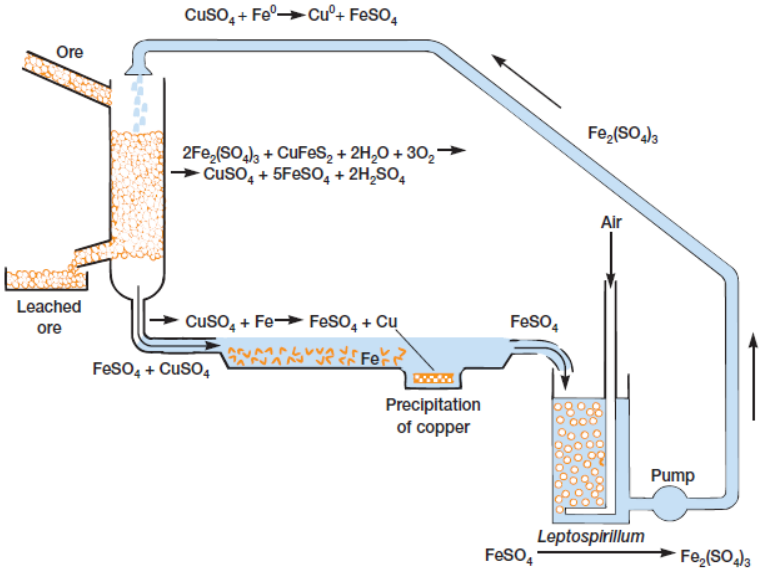


Figure 41.27 Copper Leaching from Low-Grade Ores. The chemistry and microbiology of copper ore leaching involve interesting complementary reactions. The microbial contribution is the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}). *Leptospirillum ferrooxidans* and related microorganisms are very active in this oxidation. The ferric ion then reacts chemically to solubilize the copper. The soluble copper is recovered by a chemical reaction with elemental iron, which results in an elemental copper precipitate.

The acid production and dissolution of pyrite (FeS_2) by acidophilicbacteria can be put to use inthe mining of metal ores. Sulfide (HS-) forms insoluble mineralswith many metals, and many ores mined as sources of these metalsare sulfide ores. If the concentration of metal in the ore is low,it may be economically feasible to mine the ore only if the metalsof interest are first concentrated by **microbial leaching**. Leachingis especially useful for copper ores because copper sulfate($CuSO_4$), formed during the oxidation of copper sulfide ores, isvery water-soluble. Indeed, approximately a quarter of all coppermined worldwide is obtained by microbial leaching.

The Leaching Process

We have seen how Acidithiobacillus ferrooxidans and othermetal-oxidizing chemolithotrophic bacteria can catalyze the oxidationof sulfide minerals, thus aiding in solubilization of the metal. The susceptibility to oxidation varies among minerals, and those minerals that are most readily oxidizedare most amenable to microbial leaching. Thus, iron andcopper sulfide ores such as pyrrhotite (FeS) and covellite (CuS)are readily leached, whereas lead and molybdenum ores aremuch less so. In microbial leaching, low-grade ore is dumped in alarge pile called the leach dump and a dilute sulfuric acid solutionat pH 2 is percolated down through the pile (**Figure 24.15**). Theliquid emerging from the bottom of the pile (Figure 24.15b) isrich in dissolved metals and is transported to a precipitationplant (Figure 24.15c) where the desired metal is precipitated andpurified (Figure 24.15d). The liquid is then pumped back to thetop of the pile and the cycle repeated. As needed, acid is added tomaintain an acidic pH.We illustrate microbial leaching of copper with the commoncopper ore CuS, in which copper exists as Cu^{2+}. A. ferrooxidansoxidizes the sulfide in CuS to SO_4^{2-}, releasing Cu^{2+}as shown in**Figure 24.16**. However, this reaction can also occur spontaneously.

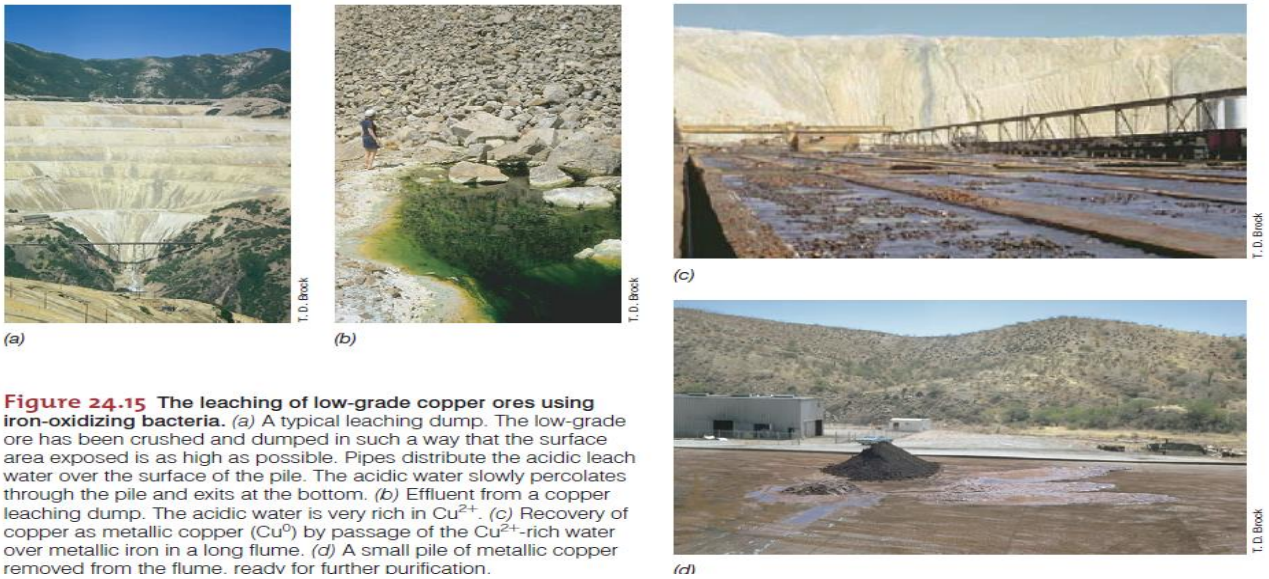


Figure 24.15 The leaching of low-grade copper ores using iron-oxidizing bacteria. *(a)* A typical leaching dump. The low-grade ore has been crushed and dumped in such a way that the surface area exposed is as high as possible. Pipes distribute the acidic leach water over the surface of the pile. The acidic water slowly percolates through the pile and exits at the bottom. *(b)* Effluent from a copper leaching dump. The acidic water is very rich in Cu^{2+}. *(c)* Recovery of copper as metallic copper (Cu^0) by passage of the Cu^{2+}-rich water over metallic iron in a long flume. *(d)* A small pile of metallic copper removed from the flume, ready for further purification.

Indeed, the key reaction in copper leaching is actuallynot the bacterial oxidation of sulfide in CuS but the spontaneousoxidation of sulfide by ferric iron (Fe^{3+}) generated from the bacterialoxidation of ferrous iron (Fe^{2+}) (Figure 24.16). In any copperore, FeS_2 is also present, and its oxidation by bacteria leads to theformation of Fe^{3+} (Figures 24.11c and 24.16). The spontaneousreaction of CuS with Fe^{3+} proceeds in the absence of O_2 andforms Cu^{2+} plus Fe^{2+}; importantly for efficiency of the leachingprocess, this reaction can take place deep in the leach dumpwhere conditions are anoxic.

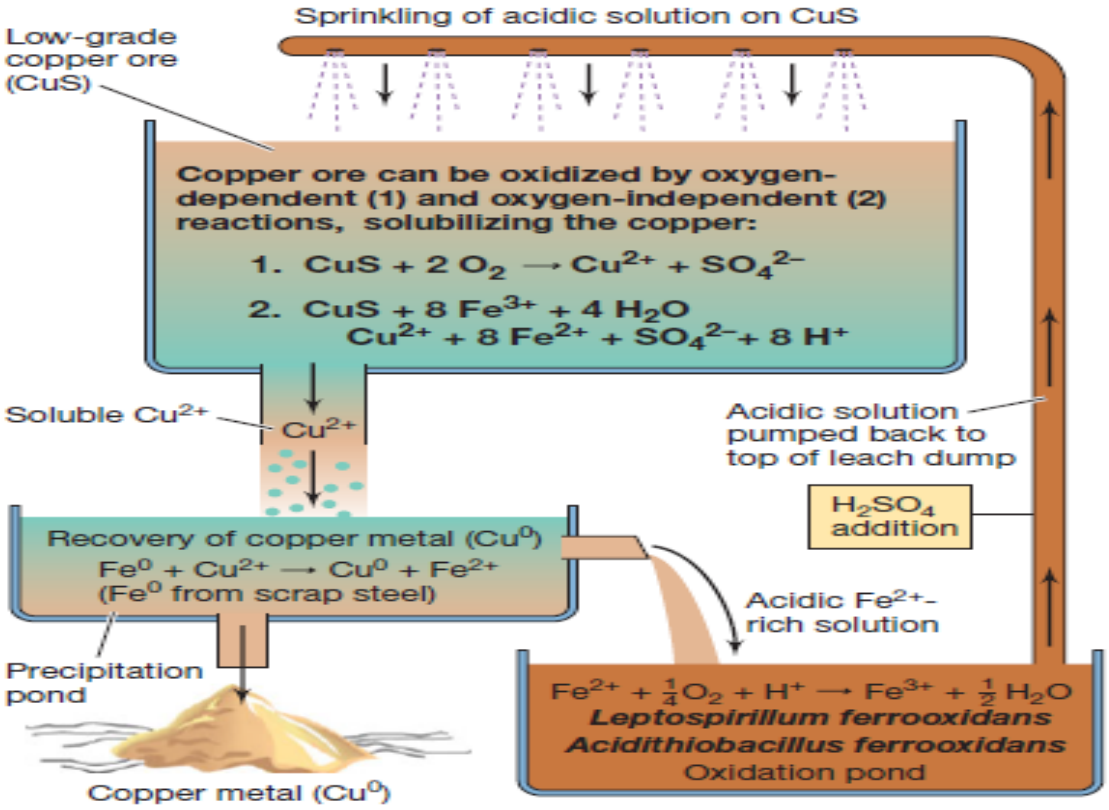


Figure 24.16 **Arrangement of a leaching pile and reactions in the microbial leaching of copper sulfide minerals to yield metallic copper.** Reaction 1 occurs both biologically and chemically. Reaction 2 is strictly chemical and is the most important reaction in copper-leaching processes. For reaction 2 to proceed, it is essential that the Fe^{2+} produced from the oxidation of sulfide in CuS to sulfate be oxidized back to Fe^{3+} by iron chemolithotrophs (see chemistry in the oxidation pond).

Metal Recovery

The precipitation plant is where the Cu2+ from the leachingsolution is recovered (Figure 24.15c, d). Shredded scrap iron (asource of Fe0) is added to the precipitation pond to recover copperfrom the leach liquid by the chemical reaction shown in thelower part of Figure 24.16. This results in a Fe^{2+}-rich liquid thatis pumped to a shallow oxidation pond where iron-oxidizingchemolithotrophs oxidize the Fe^{2+} to Fe^{3+}. This now ferriciron–rich acidic liquid is pumped to the top of the pile and theFe^{3+} is used to oxidize more CuS (Figure 24.16). The entire CuSleaching operation is thus driven by the oxidation of Fe^{2+} to $Fe^{3+}$by iron-oxidizing bacteria.

Temperatures rise in a leaching dump and this leads to shifts inthe iron-oxidizing microbial populations. A. ferrooxidans is amesophile, and when heat generated by microbial activities raisestemperatures above about 308C inside a leach dump, this bacteriumis outcompeted by mildly thermophilic iron-oxidizingchemolithotrophs such as Leptospirillum ferrooxidans andSulfobacillus. At even higher temperatures (60–80^{0}C), hyperthermophilicArchaea such as Sulfolobus predominatein the leach dump.

Other Microbial Leaching Processes:

Uranium and Gold

Bacteria are also used in the leaching of uranium (U) and gold(Au) ores. In uranium leaching, A. ferrooxidans oxidizes U^{4+} toU^{6+} with O_2 as an electron acceptor. However, U leachingdepends more on the abiotic oxidation of U^{4+} by Fe^{3+} with A. ferrooxidanscontributing to the process mainly through the reoxidationof Fe^{2+} to Fe^{3+}, as in copper leaching (Figure 24.16). Thereaction observed is as follows:

$$\underset{(U^{4+})}{UO_2} + \underset{(Fe^{3+})}{Fe_2(SO_4)_3} \longrightarrow \underset{(U^{6+})}{UO_2SO_4} + \underset{(Fe^{2+})}{2FeSO_4}$$

Unlike UO_2, the uranyl sulfate (UO_2SO_4) formed is highly solubleand is concentrated by other processes.Gold is typically present in nature in deposits associated withminerals containing arsenic (As) and FeS_2. *A. ferrooxidans* andrelated bacteria can leach the arsenopyrite minerals, releasing thetrapped Au:

$$2\,FeAsS[Au] + 7\,O_2 + 2\,H_2O + H_2SO_4 \longrightarrow Fe_2(SO_4)_3 + 2\,H_3AsO_4 + [Au]$$

The Au is then complexed with cyanide (CN^-) by traditional goldminingmethods. Unlike copper leaching, which is done in a hugedump (Figure 24.15a), gold leaching is done in small bioreactortanks (**Figure 24.17**), where more than 95% of the trapped Au canbe released. Moreover, the potentially toxic As and CN^-residuesfrom the mining process are removed in the gold-leaching bioreactor.Arsenic is removed as a ferric precipitate, and $CN^-$is removedby its bacterial oxidation to CO_2 plus urea in later stages of the Aurecovery process. Small-scale microbial-bioreactor leaching hasthus become popular as an alternative to the environmentally devastatinggold-mining techniques that leave a toxic trail of As andCN^-at the extraction site. Pilot processes are also being developedfor bioreactor leaching of zinc, lead, and nickel ores.

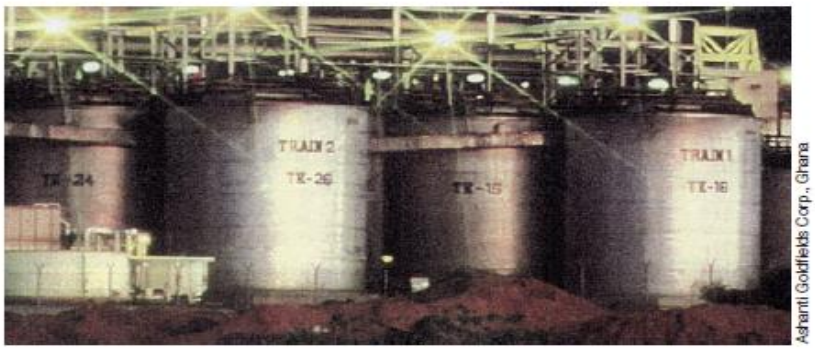


Figure 24.17 Gold bioleaching. Gold leaching tanks in Ghana (Africa). Within the tanks, a mixture of *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans*, and *Leptospirillum ferrooxidans* solubilizes the pyrite/arsenic mineral containing trapped gold, which releases the gold.

Bioterrorism : Bioterrorism, the deliberate, private use of biological agents to harm and frighten the people of a state or society, is related to the military use of biological, chemical, and nuclear weapons. Attacks with biological agents are among the most insidious and breed the greatest fear. Attacks could go undetected for a long time, potentially exposing a vast number of people, who are unaware of the threat.

Bioterrorism covers a very broad spectrum of concerns, from catastrophic terrorism with mass casualties, to micro events using low technology but producing civil unrest, disruption, disease, disabilities, and death. The threat of bioterrorism, long ignored and denied, has heightened over the past few years. We are ill prepared to deal with a terrorist attack that employs biological weapons. As was done in response to the nuclear threat, the medical community should educate the public and policy makers about the threat.

Bioterrorist Attacks The use of biological weapons, living organisms that cause harm to humans, animals, or plants, has been noted throughout recorded history. Biological weapons are cheap, generally easy to make, and can often go undetected. This makes them the theoretical ideal weapon for terrorists. Though large attacks require a certain amount of sophistication, smaller attacks do not.

The evolution of bioterrorism response has undergone dramatic changes in recent years. Although historically bioterrorism and response to such attacks have been perceived to be within the bailiwick of governmental agencies such attacks have recently become of concern to industry as-well-as government. Bioterrorism response is no longer seen as being the responsibility of any single agency, organization, or level of government. It has become a combined effort of various levels and branches of government, as well as organizations that historically have no responsibility for emergency response. Never-the-less, the efforts of individual non-governmental organizations to establish an effective Incident Response Plan to these threats has proceeded slowly, mainly due to the fact that bioterrorist threats are threats against populations not organizations in the strict sense. While organizations can implement certain preventative measures such as air filtration and ventilation systems, the use of latex gloves to open mail, respirators and eye goggles, efforts to respond to an attack in progress that involves a biological or chemical agent is an attack that exceeds the responsive abilities of the organization as such attacks require at that point the intervention of medical and health organizations along with governmental agencies tasked with responsibility to respond to these attacks.

Recent events have focused the attention of the International Community on bioterrorist attacks that employ Anthrax (attacks utilizing the postal service within the United States in 2001-2002), Sarin (attacks by a radical organization within the subway in Japan and military use of sarin most recently by Saddam Hussein in highly lethal attacks against several Kurdish communities during the 19880s), and the threat of an epidemic brought on by smallpox or another similar highly contagious disease. Attention has also focused on the possibility of a terrorist attack involving a "Dirty Bomb" employing a non-nuclear device to spread radio-active debris throughout a large metropolitan region. It also goes without saying that security organizations continue to search for ways to mitigate what has been the terrorist attack of choicethe bomb. We are daily reminded of the difficulty in managing risks associated with thethreats and vulnerabilities that societies face daily from the threat of attacks from bombs borne by various means.

A key point that facilitates success of bioterrorist attacks is that the attacker relies on the inability of defenders to effectively secure the means by which the threat is propagated usually this involves some natural environment for example atmospheric factors such as wind and the inability to "fence off" the atmosphere in order to deny access to the attacker. Air- borne, water-borne, or human carrier borne components in these attacks present unique and additionally difficult natural occurring vulnerabilities that further exploit the vulnerability of the human defender to pro-actively mediate and control the threat and/or the attack.

This study will focus on scenarios involving anthrax, sarin, and smallpox will be examined along with some of the major issues in incident response and control of these threats. The potential for the development of an effective Incident Response Plan (IRP), Disaster Response (DR), and Business Continuity (BC) Plan will be considered in light of these scenarios.

Anthrax Scenario

Anthrax Types:

Anthrax is a zoonotic disease caused by *Bacillus anthracis.* Anthrax is considered an effective bioterrorism agent because the bacterial spore (dormant form) is highly stable and storable, and because of the disease's relatively high lethality. Various strains of anthrax exhibit different levels of lethality.

There are three types of this disease: cutaneous anthrax, inhalation anthrax, and gastrointestinal anthrax.

Cutaneous anthrax develops when a bacterial organism from infected animal tissues becomes deposited under the skin. When a patient contracts cutaneous anthrax, there develops a small elevated lesion on the skin which becomes a skin ulcer, frequently surrounded by swelling or edema. The lymph gland near the lesion may also swell from the infection. If the lesion occurs on the neck or on or about the eye, it may cause complications. The incubation period for cutaneous anthrax is from one to seven days. When a patient does not receive an effective antibiotic, the mortality rate for cutaneous anthrax is 10-20%. With treatment, the mortality rate falls to less than 1%.

Inhalation anthrax develops when the bacterial organism is inhaled into the lungs.

A progressive infection follows. Since inhalation anthrax is usually not diagnosed in time for treatment, the mortality rate in the United States is 90-100%.(FAS, 2006c)

Gastrointestinal anthrax occurs when someone eats anthrax-contaminated meat. The disease usually develops within one week, and can affect the mouth, esophagus, intestines, and colon. The infection can spread to the bloodstream, and may result in death (Hurtado, 2005).

Attack Scenario:

Production: While cultivating anthrax from naturally occurring sources is relatively simple, producing an effective a form that can be used as a weapon is technically difficult. The anthrax spores must be specially processed to prevent clumping and allow for greater inhalation leading to a fatal infection.

Delivery: As previously indicated humans can become infected with anthrax in three ways- ingestion, inhalation, and cutaneous (skin) exposure. A deliberate anthrax attack, however, would likely rely on inhalation since it is the most deadly. Finely milled powder and aerosolized spray

anthrax are easily inhaled. A biological attack with anthrax spores delivered by aerosol would cause inhalation anthrax, an extraordinarily rare form of the naturally occurring disease.

Mechanism: Once in the body, anthrax becomes active, multiplies, and releases a three-part protein toxin of which one part is deadly to humans: the lethal factor. The lethal factor interferes with the normal functioning of the body's immune system cells.

Effects of an Attack:

The disease begins after an incubation period varying from 1-6 days, presumably dependent upon the dose of inhaled organisms. Onset is gradual and nonspecific, with fever, malaise, and fatigue, sometimes in association with a nonproductive cough and mild chest discomfort. In some cases, there may be a short period of improvement. The initial symptoms are followed in 2-3 days by the abrupt development of severe respiratory distress with dyspnea, diaphoresis, strider, and cyanosis. Physical findings may include evidence of pleural effusions, edema of the chest wall, and meningitis. Chest x-ray reveals a dramatically widened mediastinum, often with pleural effusions, but typically without infiltrates. Shock and death usually follow within 24-36 hours of respiratory distress onset.

An epidemic of inhalation anthrax in its early stage with nonspecific symptoms could be confused with a wide variety of viral, bacterial, and fungal infections. Progression over 2-3 days with the sudden development of severe respiratory distress followed by shock and death in 24-36 hours in essentially all untreated cases eliminates diagnoses other than inhalation anthrax. The presence of a widened mediastinum on chest x-ray, in particular, should alert one to the diagnosis. Other suggestive findings include chest-wall edema, hemorrhagic pleural effusions, and hemorrhagic meningitis.

Remedies:

There are two primary modes of prevention of anthrax. For individuals who have been truly exposed to anthrax (but have no signs and symptoms of the disease), preventive antibiotics may be offered, such as ciprofloxacin, penicillin, or doxycycline, depending on the particular strain of anthrax. An anthrax vaccine is available to selected military personnel, but not to the general public. It is given in a 6-dose series. There is no known transmission of cutaneous anthrax from person to person. Household contacts of individuals with cutaneous anthrax do not need antibiotics unless they have also been exposed to the same source ofanthrax. (Hurtado,2005)

Historically, penicillin has been regarded as the treatment of choice, with 2 million units given intravenously every 2 hours. Tetracycline and erythromycin have been recommended in penicillin-sensitive patients. The vast majority of anthrax strains are sensitive*in vitro* to penicillin. However, penicillin-resistant strains exist naturally, and one has been recovered from a fatal human case. Moreover, it is not difficult to induce resistance to penicillin, tetracycline, erythromycin, and many other antibiotics through laboratory manipulation of organisms. All naturally occurring strains tested to date have been sensitive to erythromycin, chloramphenicol, gentamicin, and ciprofloxacin.

Vaccines are available against some forms of anthrax, but their efficacy against abnormally high concentrations of the bacteria is uncertain. A licensed, alum-precipitated preparation of purified *B.anthracis* protective antigen (PA) has been shown to be effective in preventing or significantly

reducing the incidence of inhalation anthrax. Limited human data suggest that after completion of the first three doses of the recommended six-dose primary series (0, 2, 4 weeks, then 6, 12, 18 months), protection against both cutaneous and inhalation anthrax is afforded. As with all vaccines, the degree of protection depends upon the magnitude of the challenge dose; vaccine-induced protection is undoubtedly overwhelmed by extremely high spore challenge.

If there is information indicating that a biological weapon attack is imminent, prophylaxis with ciprofloxacin (500 mg orally twice a day), or doxycycline (100 mg orally twice a day) is recommended. If unvaccinated, a single 0.5 mL dose of vaccine should also be given subcutaneously. Should the attack be confirmed as anthrax, antibiotics should be continued for at least 4 weeks in all exposed.

Sarin Scenario

General Information: Sarin is a colorless, odorless, tasteless, human-made chemical warfare agent. It was originally developed in Germany in the 1930's as a pesticide. Sarin is a nerve agent that disrupts the functioning of the nervous system. Nerve agents are the most toxic and rapidly acting of all known chemical warfare agents. Sarin is highly toxic in both its liquid and vapor states.

Attack Scenario:

Delivery: Following the release of sarin into the air, people can be exposed to it through contact with skin or eyes. Sarin can also be inhaled as a gas. Sarin mixes easily with water, and since it is odorless, people would not be aware of sarin in drinking water.Furthermore, sarin in water can be absorbed through the skin.

Production: Sarin is made by mixing several commercially available chemicals in the right amounts and in the right sequence. It is debatable how easy it is for the layperson to synthesize sarin. It is somewhat complicated and dangerous to produce.

Mechanism: Sarin disrupts the ability of the body to regulate nerve impulses. When this happens, both the voluntary and involuntary glands and muscles of the body are continually stimulated, leading to system fatigue. The victim will lose control over his bodily functions. Ultimately, the victim will fall into a coma and suffocate.

Effects of an Attack:

Effects: The first signs of sarin exposure are a runny nose, tightness in the chest, pinpoint pupils, eye pain, and blurred vision. The victim will then experience drooling, excessive, sweating, coughing, chest pain, diarrhea, increased urination, confusion, drowsiness, weakness, headache, nausea, and vomiting. Exposure to large doses of sarin will result in loss of consciousness, involuntary twitching and jerking, paralysis, coma, and eventually, death.

Remedies:

Treatment: There are antidotes to sarin, but they must be provided very soon after exposure to be effective. Clothing can retain sarin, so it must be removed. The victim should move quickly to

fresh air. As quickly as possible after exposure, the victim should wash thoroughly with soap and water.

Smallpox Scenario

Attack Scenario:

Smallpox is caused by the double-stranded DNA orthopoxviruses *Variola major* and *Variola minor*. The virus no longer occurs naturally. Under natural conditions, the virus is transmitted by direct (face-to face) contact with an infected case, by fomites, and occasionally by aerosols. Smallpox virus is highly stable and retains infectivity for long periods outside of the host. A smallpox attack would likely rely on victims inhaling *Variola* via an aerosol or through an infectious individual deliberately infected with the virus. Clothing, blankets, and other such material can harbor the virus for up to a week. Infection with *Variola* could be accomplished with as little as 10-100 viral particles.

Production: Two noted production methods include incubation inside the embryos of chicken eggs and culturing the virus with cells susceptible to infection.

Effects of an Attack:

Effects: Flu-like symptoms, including headache, fever, and fatigue, usually first occur 12 days after exposure. The infected person is also contagious at this stage. Within the next 4 days, the initial lesions containing *Variola* appear and spread to the arms, torso, and legs. Over the next two weeks, the virus continues to damage the body, particularly the immune and circulatory systems. When the last rash has scabbed over and fallen off, the person is no longer contagious. Permanent scars, blindness, and arthritis can result from the infection. Smallpox isfatal in 30% of infections. In 2% to 6% of smallpox infections, lesions are classified as hemorrhagic, characterized by bleeding sores, or flat; where the lesions are soft and flat. The mortality rates for those types of infections are over 95%.

Remedies:

Treatment: The vaccine used to eradicate *Variola*, routinely used in the U.S. until 1972, prevents infections for an undetermined amount of time. It can also prevent or lessen smallpox if administered within four days of exposure. Mild to life-threatening risks are associated with the vaccine. No antivirals are available for unvaccinated individuals who contract the virus. While there is no specific treatment available although some evidence suggests that vaccinia-immune globulin is of some value in treatment if given early in the course of the illness.

After the symptoms develop, medications, and intravenous fluid can be administered to make the patient more comfortable. Antibiotics can reduce potential secondary bacterial infections*Vaccinia* virus is a live poxvirus vaccine that induces strong crossprotection against smallpox for at least 5 years and partial protection for 10 years or more.

The vaccine is administered by dermal scarification or intradermal jet injection; appearance of a vesicle or pustule within several days is indication of a "take." Vaccinia-immune human globulin at a dose of 0.3 mg/kg body weight provides $\geq$70% protection against naturally occurring smallpox if given during the early incubation period. Administration immediately after or within the first 24 hours of exposure would provide the highest level of protection, especially in unvaccinated

persons. The antiviral drug, Marboran afforded protection in some early trials, but not others, possibly because of noncompliance due to unpleasant gastrointestinal side effects.

Patients with smallpox should be treated by vaccinated personnel using universal precautions. Objects in contact with the patient, including bed linens, clothing, ambulance, etc. require disinfection by fire, steam, or sodium hypochlorite solution.

Managing the Bioterrorism Response

Bioterrorism incident response management is unique in that much of the responsibility for an effective response relies heavily on the coordination with, and preparation of, the healthcare community in particular the Department of Health and Human Services Centers for Disease Control and Prevention (DHHS-CDC). While the predominant role of healthcare services is required for operational success in a bioterrorism event, all responders are expected to use and operate under the mandates of the National Incident Management System (NIMS) and the National Response Plan (NRP) (Costa).

Biological weapons and infectious diseases share several fundamental characteristics that can be leveraged to counter both of these threats more effectively. Both a bio- weapons attack and a natural pandemic, such as avian flu, can be detected in similar ways, and the effectiveness of any response to an outbreak of infectious disease, whether natural or caused deliberately by terrorists, hinges on the strength of the U.S. public health and medical systems — the network of federal, state, local, and private-sector entities responsible for the health of the nation's population. Natural pandemic outbreaks and bioterrorist attacks would place different stresses on these systems at the outset, yet the basic response and containment mechanisms would be essentially the same (Grotto, 2006).

Difficulty in establishing an effective Incident Response Plan (IRP) to manage a bioterrorist attack stems from several problematic issues including but not limited to:

- Magnitude of diverse incident responders – an incident will trigger responses from several different organizations at potentially many levels of government, business, or both. Coordination of efforts by these diverse organizations and authorities is a major logistical problem.
- Nature of an attack – the three scenarios demonstrate that if a threat agent is able to trigger an attack, then containment presents a series problem for any response team. The release of anthrax, sarin, or smallpox into an eco-system assures the attacker the likelihood of a high degree of success in the manipulation of the natural occurring vulnerabilities of the eco-system.
- A highly mobile population poses additional risk when coupled with the incubation period of many bio-threat agents. An infected individual could travel on several air- flights to a number of different cities across several countries exposing thousands to the biological threat all before the nature of the threat would be identified. An example is the recent history of the spread of severe acute respiratory syndrome (SARS).
- High costs associated with prevention or mitigation of an attack. The bio-chemical nature of these threats requires that an effective IRP must include a vast array of equipment:
    - to assure the containment of an air-borne attack using filters and ventilation

equipment
    - to assure decontamination of air or water-borne attack agents specialized environmental suits and breathing equipment, specialized chemicals, solvents, and cleaning agents to neutralize the bioterrorist threat agent such as sarin.
    - relocation of populations in the event of a dirty-bomb, radio-active contamination
    -
- Education and communal knowledge of the initial signs that an attack has occurred are critical.
    - It is imperative for quick containment of any bioterrorist attack that organizations have in place explicit measures that will allow for directly affected individuals to identify the threat and put in motion steps to contain it.
    - The fact that these threats require a high degree of specialized knowledge to correctly determine the threat poses a great impediment to early containment.
    - The ability to react to a physical substance such as the white powder that an anthrax attack might employ is more manageable than the orderless properties of many gaseous bio-chemical terrorist threats, such as sarin.
    - The incubation period of many diseases is prohibitive of any manageable early detection, containment, and control, such as SARS.
- Commitment of adequate financial resources -- as with any security policy and plan the assurance that adequate financing is available resides at the heart of implementation of the risk management.
- Exponential growth in sources of potential threats and threat agents – more governments globally have acquired the ability and capacity to develop biological and chemical sources of materials that lend themselves to the bioterrorist.

The effort to establish a viable Incident Response Plan to meet the challenges presented by the bioterrorist is different and definitely more complex then the challenges that the development of a successful IRP for a technologically orientated threat present. As has been demonstrated by the anthrax attacks, detection of the threat agents has proven to be an extremely vexing task, clean-up took considerable resources in terms of the time, neutralizing agents, and man-hours required to effective neutralize the threat and restore the compromised facilities to their pre-attack status. And, unfortunately there was a high human cost in that several individuals either died as a result of the attacks or have remained asymptomatic suffering continued symptoms after their proclaimed recovery.

A business or community that has endured an incident involving the implementation of a bioterrorist attack must consider that an essential aspect of any disaster recovery (DR) or business continuity (BC) planning must take into consideration the longevity that containment and decontamination has been found to take. The containment and decontamination of the federal buildings during the anthrax attacks took from a few weeks in the case of the Congressional buildings, to well over a year in the case of the Brentwood Postal facility in Washington, DC. As the postal facility demonstrated an organization will need to have the equivalent of a fully operational *Hot Site* available for immediate and long term use in order to maintain business continuity. Similar DR and BC will prove necessary for any response to a successful attack

involving biological, chemical, or nuclear threat agents. While recovery from a minor incident although costly would in all likelihood be within an organizations budgetary means, recovery from a major disaster incident could easily prove to be devastating in terms of the costs associated with business continuity as the organization would have to literally recreate itself in terms of the physical plant and the hardware components of its information system. This is assuming that the organization had operationally secure backups of all its data, operating systems and necessary software ready to implement at its Hot Site.

Solution Approaches:

As the President's Council of Advisors on Science and Technology (Council) has determined in their report on combating terrorism, the recent studies of the experiences of first responders in terrorist events suggest a number of approaches to providing more appropriate equipment. First responders have indicated that the highest priorities for equipment development should be assigned to respirators that offer both practicality and comfort for extended use, escape hoods with an air supply for emergency medical service personnel, and thinner, yet effective, thermal protection gloves for firefighters. In particular, research and development for future personal protective equipment should strive for higher levels of protection while placing much greater emphasis on making it possible for responders to perform their emergency response duties with a minimum of equipment-related interference. In addition, techniques for faster and more accurate hazard monitoring should be developed to enable first responders to evaluate environments for themselves or to receive early hazard assessment information. Personal protective equipment selection decisions will require such information as long as equipment that is specific to a single hazard type continues to be used. Broader-spectrum personal protective equipment useful for a range of hazards needs to be developed, particularly for respiratory protection, which is obviously one of the most essential elements of protection. This might best be achieved in stages, with the initial stage being to develop and field equipment with an intermediate level of protection against a wider range of hazards than is now available while still meeting weight, flexibility and decontamination requirements at an affordable cost. Emergency- response "caches" managed at regional and national levels are needed and can be used to promote standardization. Federal agencies should be required to purchase the same equipment, or equipment that, at a minimum, is compatible with other equipment—unless there are sound and specific reasons for doing otherwise. Cost and logistical considerations dictate that this activity be coordinated with the Federal Response Plan and the Strategic National Stockpile program.

Pre-disaster training should be conducted under more realistic, high-pressure conditions and should include the participation of engineering, construction and transportation personnel.

Procedures are needed to permit the communication of accurate hazard information to responders as quickly as the nature of a hazard is determined. Finally, flexible and dynamic procedures need to be developed to insure an effective incident management authority that can quickly establish control at a disaster site, account for individuals working in dangerous environments and assure that the proper personal protective equipment is selected for use (Augustine, 2003).

Physical Approaches to Defense against Bioterrorism:

The Council further determined that while the discussion thus far has dealt with medical or

biological defenses against bioterrorism, there is also an important role for other approaches involving the physical sciences. To be harmful, a biological agent must reach its intended victim; however, in today's world, many of the most attractive targets for bioterrorism are cities with their large concentrated populations spending much of the time indoors. Many buildings already provide their occupants with filtered air since filters are a part of every heating, ventilating and air conditioning system. These filters can be upgraded to greatly reduce the level of air-borne biological contaminants. Upgrading to the highest commercial grade provides a concomitantly high degree of protection. Similar measures are possible for individual homes, and more advanced filtering capabilities can be developed. Filtering and other physical methods—such as safe rooms—have an important strategic advantage over purely biological defenses. Filtering methods can be devised which will stop almost all particles larger than a specified size. If the particle is, for example, an anthrax spore or similar particle, the same filter will stop all such particles; however, each may require a different medical approach, and as the characteristics of the agent may not be known in advance, that medical approach may not be available. In addition, if filters are always in use, they provide automatic protection in advance of detection. Recent research has investigated the modification of certain plants to be responsive as sensors to various bioterrorist agents, with the plant turning a different color in the presence of the threat agent.

Barbara Rosenberg in an analysis of the anthrax attacks notes that although biodefense has gotten a shot in the arm, it is important to understand that the goal of defending against bioweapons is not primarily public protection--which is largely impossible, as recent attacks demonstrated. It is rather "to allow the military forces of the United States to survive and successfully complete their operational missions ... in battle space environments contaminated with chemical or biological warfare agents," according to the annual report of the Department of Defense's Chemical and Biological Defense Program. Biological weapons are preeminently anti-population weapons. But it would be impossible to provide the entire country with protective suits, masks, detectors, shelters, training and vaccinations against the large and growing array of potential agents. In any event, vaccinations can have serious side effects and can be overcome if the dose of the pathogenic agent is large or if the agent has been engineered to evade the vaccine. Instead of protection, the civil defense response is entirely concerned with limiting the damage should an attack occur. There are also paradoxes here. Because of the rush to "do something," large amounts of government money are being thrown, without sufficient forethought, at research involving potential biological weapons agents.

- As noted the bioterrorist attacker is primarily focused on population centers not the individual organization, in particular not a small business or non-governmental organization. That said an attack on a population center could be initiated with an attack on a small organization, such as the release of a biological or chemical agent at a sports arenacontaminating a select group of individuals with the intention of using them as carriers of the contaminate to the larger population. The responses that such an organization could provide are greatly limited as it would be likely that without a prior warning or the use of special senor equipment that would alert to the threat the event would pass without anyone being aware that an attack had

taken place, in particular if the threat agent were a biological that had an incubation period longer then the event itself. But, as noted such specialized equipment as special sensors that alert to biological or chemical threat agents are still very expensive and have a high maintenancecost.

- Organizations are indeed undertaking steps to develop IRPs, DRPs, and BCPs, to the extent that such plans are able to address the vulnerabilities that are within the effective control of the organization. Such plans would include the use of filters and ventilation systems, ability to contain a contaminated area if the threat is recognized sufficiently early in the attack, measures to evacuate employees from a contaminated area again if the threat is recognized at an early point in the attack, designation of all pertinent responders within and outside the organization, along with explicit instructions as to the procedures that are to be followed at each step of response to an attack. However, as pointed out these are highly improbable "ifs" that do not give any consideration to the more serious aspects of bioterrorist threats the medicalissues. These aspects are beyond the scope and ability of an organization to address. They fall within the responsibility of the various governmental and increasingly private aspects of the national and international health community where efforts to develop appropriate plans are still in their infancy.

i. Microbial desulfurization of coal, Degradation of xenobiotics by microorganisms, Biodegradation of halocarbon, synthetic carbon, oil, biopolyme

MICROBIAL DESULFURIZATION OF COAL : Energy is essential to modern civilization. Its consumption is roughly proportional to the human population on the earth. With a growing population, energy demand will increase in a long-term trend. It is, therefore, one of the most important tasks of mankind to find and develop new energy sources for future supply. In the immediate future, increase in the use of nuclear power as an alternative to fossil fuels is not very likely because of the adverse public opinion. World energy consumption in terms of coal equivalent is about ten billion tons per year, with industrialized countries consuming about 80% of the total. Most of the demand is projected to be satisfied by fossil fuels (Haggin, 1989). Fossil energy sources include gas, oil and coal. Their reserves likely will meet energy demand well into the next millennium, with oil and gas gradually giving way to coal in the long term. Since the oil crises in 1973 and 1919 it has been realized that coal must play a more important role in the world's fuel supply and chemical feedstock. As recently as the 19409, coal was the primary basis of the organic "chemical industry (Spitz, 1988). In the 19503, petrochemistry underwent a tremendous surge in its industrial development. Today, oil is the primary organic chemical feedstock for all the leading industrial nations. However, even the most optimistic estimates of oil reserves show that, at currently projected production and consumption rates, oil will only last a few more decades (Agreda, 1990). In contrast, the coal reserves are far more abundant than those of other fossil fuels (Schilling and Wiegand, 1987). Known coal reserves

should provide fuel and chemical feedstocks for several centuries (Falbe, 1981). Therefore the development of long-term use of coal as energy and chemical sources is a most important task for the future. A major disadvantage to the use of coal as fuel is that pollutants are released into the environment during combustion. The sulfut ooddes and nitrogen oxides released are of increasing coneern with respect to air quality and acid rain control. Regulations governing sulfur dioxide emissions have created new criteria for determining the depee to which coal must be cleaned before it *can* be used as a fuel. These regulations are likely to become more stringent as the individual states and the Congress become increasingly concerned about the environmental impact of burning "dirty" coal. For this reason, coal research has moved especially in the direction of developmenl and improvement of clean-coal technologies. There are different possibilities for reducing the emission of sulfur oxides. Sulfur compounds can be removed before combustion by using the traditional sink-float techniques if the inorganic sulfdic minerals are present as coarse particles. For the removal of finely distributed sulfidic minerals, physical separation techniques have beensuggested, such as froth flotation, high-gradient magnetic separation and electrostatic separation. For the removal of both inorganic and organic sulfur, the possibilities of chemical methods have been studied. Morrison concluded that these chemical processes are able to remove inorganic sulfur from coal. He stated that the extent of organic sulfur removal is much less certain because of the inaccuracies involved in the determination of organic sulfur content in coal. On the other hand, sulfur dioxide can be removed during combustion by fluidized-bed combustion of aal-chalk mixtures by simply injecting lime into the flame. The removal of sulfur dioxide can also be achieved after combustion by flue-gas desulfurization (Prior, 1977). None of the techniques mentioned meets the problem of nitrogen-oxides emission, only a small part of which has its origin in the nitrogen

content of coal. Nitrogen oxides are mainly produced in the flame from atmospheric nitrogen and oxygen. The generation of nitrogen oxides during coal combustion can be reduced by lowering the flame temperature and by changes in the geometry of the burnen. In parallel to the development of chemical and physical techniques for clean-coal technologies in the last decades, hiotechnological alternatives are also being explored. The U.S. Department of Energy (DOE) has had a wide-ranging research and development program of coal bioprocessing in place since the mid-1980's. During the fiscal years 1984-88, DOE fossil energy programs committed approximately $16 million to coal biopromsing, with about $4 million for microbial desulfurization of coal.The Electric Power Research Institute (EPRI) is also actively committed to coal bioprocessing research. Although bioprocessing of coal is stiU in its infancy, this technology may provide a potential means to give greater Uexibility in the use of coal. Currently, coal bioprocessing is developing along three broad fronts. Microorganisms or, in some cases, microbially produced enzymes are being evaluated as agents for the following purposes: (1) to reduce the sulfur content of coal, (2) to solubilize coal and convert the soluble product to liquid or gaseous fuels, and (3) to convert coal-derived synthesis gas to liquid or gaseous fuels. To the extent that they can produce liquids, options 2 and 3 are termed *direct* and *indirect liquefaction,* respectively

Microbial removal of inorganic sulphur compound in coal:

Much of the sulfur in coal, especially in the eastern United States, is present as the iron disulfide (FeSz) minerals, pyrite and marcasite (both with the same molecular formula but with different crystalline structures). Consequently, most research on microbial desulfurization of coal has been directed to the removal of inorganic sulfur compounds from coal. It is the best studied and established technology among bioproeessing technologies of coal.

Principle:

Some microorganisms have been found to oxidize the inorganic **sulfur** compounds in coal and convert them into water-soluble products which canbe leached from the coal. The microorganisms responsible belong to thegroup of acidophilic sulfur- and/or iron-oxidizing bacteria of these themesophilic, autotrophic organism Thiobacillus feroozidons is the beststudied. Microbial pyrite removal is a result of the combined effects of direct bacterial attack and indirect chemical (i.e. ferric iron) solubilization, although the relative importance of these two mechanisms is still in dispute.

$$4FeS_2 + 15O_2 + 2H_2O \longrightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4$$

The direct microbial oxidation of pyrite requires the intimate contact between bacteria and pyrite surfaces. Several studies have clearly shown the cell attachment and apparent pit formation on surfaces of pyrite in coal samples treated with microorganisms. An analysis has been made recently to explain the selective adsorption of Thiobacilli to dislocation sites on pyrite surfaces. The faster diffusion of sulfur atoms along the dislocations than that through the pyrite crystals was proposed to provide a great advantage to bacteria adsorbed at dislocation sites on the pyrite surface because sulfur oxidation has a much higher yield of bee energy than iron oxidation.

Microbial desulfurization of coal

In the indirect chemical soluhilization of pyrite, ferric iron serves as the strong oxidizing agent and reacts with metal sulfides, including the pyrite. The ferrous iron thus produced is readily reconverted to ferric iron by *T.*ferroozidam at low pH as a source of metabolic energy:

$$FeS_2 + Fe_2(SO_4)_3 \longrightarrow 3FeSO_4 + 2S^0 \qquad (2)$$

$$4FeSO_4 + O_2 + 2H_2SO_4 \longrightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad (3)$$

The role of bacteria in the indirect oxidation of pyrite is thus to regenerate the ferric iron which in turn oxidizes metal sulfides. An important experimental evidence supporting the significance of this mechanism was provided by Wakao and co-workers (1984). In their study surfaceactive agents such as Tween 20 were found to accelerate pyrite oxidation. Since increases in the number of unattached cells were also observed in the surfactant-containing suspensions, they suggested that the indirect leaching mechanism predominates and that unattached cells oxidize Fe+2 more rapidly than attached wUs, resulting in faster regeneration of Fe+3 to react with pyrite.

Another significant role of bacteria in the process of depyritization is to oxidize the elemental sulfur produced in Reaction 2 to sulfuric acid. This reaction is important because it is believed to prevent a layer of elemental sulfur from accumulating on pyrite surfaces, which would otherwise inhibit further oxidation reactions. Since *T. ferroozidans* is considered to be a poor oxidizer of elemental sulfur, mixed cultures containing another sullur-oxidizing organism such as *Thiobacillus thioozidans* to remove the sulfur formed on the pyrite surface have been used by many researchers. Inaddition, a positive effect of acidophilic, oligotrophic heterotrophs, and facultative acidophilic autotrophs, such as *Acidiphilium crypturn* and *Thiobacillus* acidophilw, respectively, on the performance of *T. ferroozidans* cultures has been described. Heterotrophic bacteria may be able to metabolize excretion products of *T. ferroon'dans* which could be inhibitory. Another explanation may be the local production of additional carbon dioxide by heterotrophs growing on (trace) organic substrates. These explanations are only tentative and need thorough checking for their validation. Nevertheless, mixed populations generally performed better than pure cultures in the process.

Besides the mesophiles such as *T. ferroozidatls and T. thioozidans* mentioned above, moderate thermophiles (*Thiobacillw*-iike bacteria) with optimal temperatures around 50'C (Murr and Mehta, 1982; Gokcay and Yurteri, 1983) and extreme tbermophiles such as *Sulfolobus* species with optimal temperatures around 70'C have also been studied or suggested for microbial removal of inorganic sulfur compounds from coal. In contrast to *T. ferroozidans*, which is an obligate autotroph, the moderate and the extreme thermophiles are facultative autotropbs. Advantages attainable by using thermophilic bacteria include accelerated reaction rates and lack of cooling system requirements. However, the corrosion of container and equipment materials will likely increase at elevated temperatures.

Microbial removal of organic sulfur compound from coal

The removal of inorganic sulfur from coal is considered to be only a partial solution to the reduction of sulfur oxides emission from wal-fired installations. This provides a strong incentive to study the possibility of biologically removing organic sulfur from coal. Recently, there have been two experimental trends in microbiological removal of organic sulfur wntents of fossil fuels. One has emphasized oxidation of S-heterocycles to polar thiophene derivatives, which are subsequently removed by water washing. This approach has the advantage that many different microbes readily effect the oxidations, but the disadvantage of removing the valuable hydroeartmn moiety along with the sulfur. The other has stressed specific removal of the thiophenic sulfur atom from dibenzothiophene, yielding sulfate and leaving the carbon skeleton intact. However, to date only a few organisms with this capability have been described, thus limiting the flexibility of study and process development. The former approach has been primarily explored in biodesuifurization of petroleum and the latter has focused on removal of organic sulfur from wal. There is also interest in the former approach for waste remediation, due to its potential use to destroy some ring-structured sulfur compounds that are organic pollutants in soils around power plants and various other places (Dugan *el al,* 1990). An understanding of

microbial metabolism of model organosulfur compounds is necessary to the development of a rational approach to microbial desulfurization.

Removal of Oreanic Sulfur from Coal

Although metabolism of pure organic sulfur compounds has been demonstrated, degradation of the wrresponding wnstituents chemically Downloaded by bound in coal is influenced by biological factors, such as toxicity and enzyme induction, and by chemical-physical properties of the coal, such as partied size, porosity, surface properties, etc. Several studies have been reported on organic sulfur removal from coal by microorganisms. A mixed heterotrophic culture, isolated from soil and enriched on DBT, to remove organic sulfur from coal. Nearly 20% reduction in organic sulfur wntent of Indian bituminous coal was reported.

Thiobacillus type organism (the strain TH1, originally isolated from an Icelandic thermal spring and initially characterized and for the removal of both pyritic and organic sulfur from Turkish lignite. The lignite contained 1.5% pyritic sulfur and 2.2% organic sulfur. After 25 days of incubation, 90 - 95% of pyritic sulfur was removed from the coal (53 - 177 µm particles). In flasks containing yeast extract or nutrient salts, 50 - 57% of organic sulfur was also removed. DBT-adapted organism S. *acidocaldan'us* has also been shown to remove organic sulfur from wal. S. *acidocaldan'us* is an acidophilic (pH 1 to 4), thermophilic (60° - 90°C), facultative autotrophic, sulfur-oxidizing organism originally isolated from acid hot springs of Yellowstone National Park. Pittsburgh coal samples, which contained 2.1% pyritic sulfur and 1.9% organic sulfur, were first treated either with hot 2N HNO3 or by incubation with *Sulfolobus* for two weeks in order to remove pyritic sulfur. The DBT-adapted culture was then investigated for capabilities of removing organic sulfur from inorganicsulfur-free samples of pretreated coal and petroleum pitch. Nearly 44% of the initial organic sulfur content was removed from slurries of 10% acid-treated coal at 70°C in about four weeks.A removal of about 19% of organic sulfur was observed in the microbially pretreated coal. The higher extent of organic sulfur removal from acid pretreated coal was attributed to partial oxidation of some organic sulfur compounds by nitric acid pretreatment. The petroleum pitch sample contained only organic sulfur (w 3.1%) and was, therefore, not pretreated. In four weeks, the organic sulfur removal from the petroleum pitch was found to be nearly 25%.

A mixed bacterial culture was evaluated for its desulfurization ability in coal treatment. The culture was shown to be capable of sulfur opecific metabolism, i.e., removing the sulfur but leaving the carbon skeleton intact. In coal treatment with the mixed culture, theremoval of organic sulfur (>25%) was observed.

The removal of organic sulfur by a genetically modified bacterkm CB1, which had been shown capable of specifically removing the thiophenic sulfur from DBT as sulfate, was investigated using a variety of coal samples, including run-of-mine (ROM) coals (coals as mined; no washing or cleaning to remove contaminants) and several low-sulfur coals in which more than 90% of the sulfur content was present as organic sulfur. These low-sulfur coals were purposely selected for study in order to reduce to be analytical problems associated with quantifying organic sulfur content. As mentioned earlier, organic sulfur content in coal is generally calculated as the

difference between the amount of total sulfur and the combined amount of pyritic and sulfatic sulfur. It is difficult to analyze accurately the inorganic sulfur content in coal containing a high percentage of impurities; inaccuracies in the determination of inorganic sulfur content lead to inaccurate organic sulfur values. Depending on the coals investigated, organic sulfur reductions in these coals ranged from 18 to 36%. The coal was pulverized to -200 mesh and treated with 200 mM NaOH at ambient temperature (23 - 25°C). The soluble coal was then filtered, precipitated by adjusting pH to 1.5 with 6N HCI, collected by centrifugation, washed with 1N HCI and, finally, freeze-dried. The use of water-soluble coal products for investigation was to minimize the accessibility problems inherent with a solid coal substrate and to virtually eliminate the presence of inorganic sulfur compounds, *e.g.,* sulfate and pyrite. For instance, the soluble product of Illinois no. 6 coal contained 2.05% organic sulfur, with little pyritic (0.04%) and sulfatic (0.22%) sulfur, while the raw Illinois no. 6 coal contained 2.02% organic sulfur, 0.3% pyritic sulfur, and 1.77% sulfate sulfur. While the analysis by Fourier transform infrared spectroscopy (FTIR) showed several modifications of the coal structure by the microorganisms, such as increases in carbonyl functionalities and aliphatic methyl and methylene groups, the elemental analysis indicated that the content ratio of sulfur to carbon in the coal product did not change as the result of microbial treatment.

- **DEGRADATION OF XENOBIOTICS BY MICROORGANISMS**

More than ten million organic compounds are generated by biosynthetic pathways in animals, plants, and microorganisms, by other natural processes, and by industrial synthesis. Whilst the organic structures found in nature are created by many organisms and processes, microorganisms (bacteria and fungi) perform most of the biodegradation of both natural products and industrial chemicals. Collectively, microorganisms play a key role in the biogeochemical cycles of the Earth.

The substances transformed or degraded by microorganisms are used as a source of energy, carbon, nitrogen, or other nutrient, or as final electron acceptor of a respiratory process [see also - Cell thermodynamics and energy metabolism]. 'Biodegradation' involves the breakdown of organic compounds, usually by microorganisms, into biomass and less complex compounds, and ultimately to water, carbon dioxide, and the oxides or mineral salts of other elements present. The complete breakdown of an organic compound into inorganic components is termed 'mineralization', but (ultimate/complete) biodegradation' and '(complete) mineralization' are often used interchangeably, although 'biodegradation' involves the formation of biomass as well as inorganic compounds. Of course, biomass finally will also undergo mineralization. Degradation of an organic compound to a less complex organic compound is referred to as 'incomplete (partial) biodegradation'.

'Biotransformation' is the metabolic modification of the molecular structure of a compound, resulting in the loss or alteration of some characteristic properties of the original compound, with no (or only minor) loss of molecular complexity. Biotransformation may effect the solubility, mobility in the environment, or toxicity of the organic compound.

A microbial population growing on one compound may fortuitously transform a contaminating chemical that cannot be used as carbon and energy source, a process referred to as 'co-metabolism'. The phenomenon has also been called 'co-oxidation' and 'gratuitous' or 'fortuitous' metabolism. Usually, the primary substrate induces production of (an) enzyme(s) that fortuitously alter(s) the molecular structure of another compound. The organisms do not benefit from the co-metabolic process. Co-metabolic transformation may result in a minor modification of the molecule, or it may lead to incomplete or even complete degradation.

The products of partial biodegradation, or biotransformation, or co-metabolic conversion of a xenobiotic may be less harmful as the original compound, or they may be as hazardous or even more hazardous as the original compound. For example, tetrachloroethene and trichloroethene can be microbially reduced to vinyl chloride, a known carcinogen, in anoxic habitats. In natural environments, the products of bioconversion processes may be further transformed or degraded by other microorganisms, maybe eventually leading to complete degradation by the microbial consortium. Co-metabolic processes, and biodegradation by microbial consortia are thought to be of enormous ecological importance. However, persistent xenobiotics and metabolic dead-end products will accumulate in the environment, become part of the soil humus, or enter the food chain leading to biomagnification.

What are Xenobiotics? Xenobiotics (greek*xenos* = strange, foreign, foreigner) are chemically synthesized compounds that do not occur in nature and thus are 'foreign to the biosphere'. They have 'unnatural' structural features to which microorganisms have not been exposed to during evolution. Xenobiotics may resist biodegradation, or they undergo incomplete biodegradation or just biotransformation.

The definition of xenobiotics as compounds 'foreign to life' exhibiting 'unnatural' structural features does not necessarily imply that xenobiotics are toxic compounds, but many xenobiotics indeed are harmful to living organisms. Whereas xenobiotics may persist in the environment for months and years, most biogenic compounds are biodegraded rapidly. Exceptions are lignin, the structural\ polymer of woody plants, and, above all, the melanin polymers which are constituents of the cell wall of the spores of a number of fungi. Recalcitrance (i.e., the structureimmanent stability) of a xenobiotic molecule is mainly due to 'unphysiological' chemical bonds and/or substituents, which block the attack by microbial catabolic enzymes (see Table 1 and Figure 2). Type, number and position of bonds and substituents affect the xenobiotic character. However, it is not always easy to determine which structural moieties indeed are xenobiotic in the sense of 'foreign to life'. Some natural compounds show principally the same unusual structural features as xenobiotics, such as halogen substituents or nitro groups found in some antibiotics, or they contain stable chemical bonds like the ether and carbon-carbon bonds stabilizing lignin. Moreover, microorganisms throughout geological time have also been exposed to a variety of chemicals produced by abiotic natural processes:

•	High molecular mass
•	Low solubility in water
•	Condensed benzene and pyridine rings, especially: polycyclic structures
•	Three-fold substituted N atoms
•	Quarternary C atoms
•	Unphysiological bonds and substituents R-X (especially, polysubstitution): -X = -O-R -N=N- -F, -Cl, -Br $-NO_2$ $-CF_3$ $-SO_3H$

Table 1. Typical features of recalcitrant organic compounds. Type, number, and position of 'unphysiological' substituents influence recalcitrance.

It should be noted that organic chemicals of anthropogenic origin are not necessarily recalcitrant. There are a number of industrial products that are degraded by microorganisms. These compounds obviously are readily recognized by microbial catabolic enzymes. Besides, research in biodegradation has demonstrated that a number of xenobiotic compounds such as polychlorinated biphenyls (PCBs) and nitroaromatics which once were thought to be recalcitrant are subject to microbial attack.

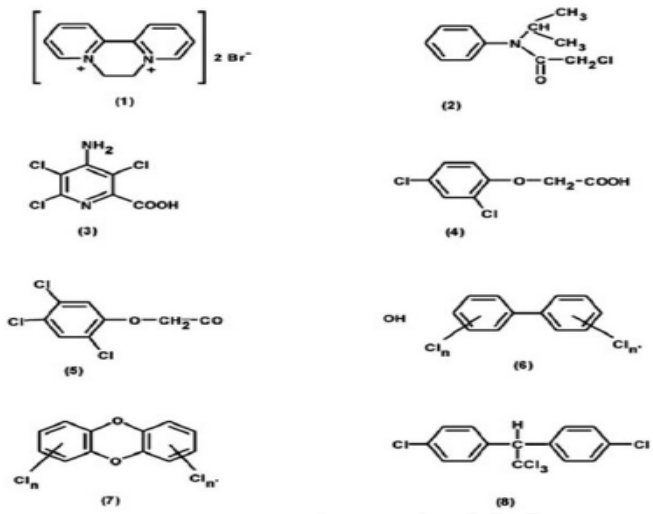


Figure 2. Examples of relatively persistent xenobiotics showing 'typical' features of recalcitrant compounds.
(1) The herbicide diquat-dibromide, DT90 (degradation time for 90% removal) is up to 12 months; (2) the herbicide propachlor, DT90 is 2 to 12 months; (3) the herbicide picloram, DT90 is 2 to 18 months; (4) the herbicide 2,4-D, DT90 is 0.5 to 1.5 months; (5) the herbicide 2,4,5-T, DT90 is 6 to 12 months; (6) PCBs, used e.g. as insulating and cooling fluids, hydraulic fluids, and additives for lubricants; DT90 depends on the position and extent of substitution, but is generally much higher than 12 months; (7) PCDDs, by-products in the synthesis of herbicides such as 2,4- D and 2,4,5-T, and by-products of combustion processes; DT90 is much higher than 12 months; (8) the insecticide DDT, DT90 is many years.

PARAMETER INFLUENCING BIOAVAILAVABILITY AND RATE OF BIODEGRADATION

The extent of biodegradation and the rate at which it occurs depend on the chemical structure and concentration of the compound being degraded, the type and number of microorganisms present, and the physicochemical properties of the environment. In laboratory as well as environmental systems, only the fraction of the xenobiotic pollutant that is dissolved in the aqueous phase is generally assumed to be available to the microorganisms for degradation. Bioavailability is controlled by parameters such as the physical state of the pollutant compound (solid, liquid, gaseous), its solubility in water, and its tendency to adsorb or bind to soil or sediment particles. In soil aggregates or other solids, microbes may be excluded from entering the smaller micropores. Xenobiotics present in the micropores are thus unavailable to the microorganisms and must diffuse through pore water to the grain surface in order to be degraded. However, diffusion in soil systems may well be sorption-limited. Actually, sorption, immobilization and micropore entrapment are major causes for the persistence of many xenobiotics. 'Aging', i.e. the length of time a soil or sediment has been exposed to contamination, also affects bioavailability: Pollutants may undergo reactions that lead to strong binding to soil and sediment material, becoming increasingly unavailable to microorganisms with the progress of time. Many xenobiotics, for example the polycyclic aromatic hydrocarbons and the polychlorinated biphenyls, are poorly soluble in water, and tend to adsorb to and be immobilized by the soil matrix and sediment material.

As mentioned above, the structure of xenobiotic molecules is characterized by 'unphysiological' substituents and stable chemical bonds, which impede or even prevent biodegradation. Unfavorable concentrations of the xenobiotic compound also affect biodegradation. In high concentrations, many xenobiotics are toxic to organisms, including the degradative bacteria. On the other hand, there may be a minimum concentration below which a compound is not degraded any more. Synthesis of catabolic enzymes may not occur when the concentration of a chemical is below a level that is effective for induction of the corresponding catabolic genes. Besides, the minimal threshold concentration depends mainly on the kinetic parameters of growth and metabolism, but also on the thermodynamics of the overall transformation reaction. Actually, the substrate affinity constant is the most important parameter with respect to the biodegradation of contaminants to very low concentrations. Typical minimal substrate concentrations for aerobic systems may be in the range of 0.1 to 1.0 mg L^{-1}, but the desired end concentrations in environmental systems often are 1 μg L^{-1} or less. Other factors that influence biodegradation involve environmental conditions such as temperature, pH, water content and salinity, presence of inhibitory chemicals, availability of electron donors and nutrients, and availability of oxygen or other electron acceptors. In soil, for example, oxygen availability is very often the limiting factor of aerobic biodegradation processes. Moreover, the presence of competing microorganisms, or of predators grazing on the microbial consortium, also affect biodegradation. When determining biodegradation rates, it is important to keep in mind that an observed 'disappearance' of a xenobiotic from an ecosystem does not necessarily mean that it was

biodegraded, since loss can also occur by partial degradation, biotransformation, or by volatilization, leaching, or chemical conversion (polymerization, modification, breakdown). In monitoring the environmental fate of a chemical, one must also monitor the products formed, not simply the disappearance of the parent compound. The rates of xenobiotic biodegradation in the environment may range from days and weeks to years and decades. The organophosphate insecticide malathion disappears from soil within approximately one week, and the herbicide 2,4-D (2,4- dichlorophenoxyacetic acid) is degraded within four to six weeks in soil. Modern herbicides are designed to undergo biodegradation within one cropping season. On the other hand, there are recalcitrant xenobiotics that persist in the environment for many years. Simple structural changes of a molecule, such as the addition of a chlorine substituent, can convert a readily biodegradable compound such as 2,4-D into a more persistent substance such as 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) which is degraded in soil within approximately six to twelve months (see Figure 2). A prominent example of a very persistant xenobiotic is the insecticide DDT, which was used extensively from the 1930s until its ban in 1979. DDT was found to persist with an average half-life of 4.5 years in field soils, and a half-life in anoxic soils of about 700 days. Stable metabolites of DDT have been detected in soil, groundwater, and in the tissue of organisms.

- **BIODEGRADATION OF HALOCARBONS**

Research on the biodegradation of environmental pollutants, mostly halogenated aliphatic and some aromatic compounds, has been a major research topic in the group for almost 20 years. The work was inspired by many cases of environmental pollution discovered in the early 80s, as well as the lack of scientific knowledge at that time on biodegradation pathways, evolution of new activities and enzyme mechanisms. In fact, the transformation of synthetic organic compounds by microorganisms and microbial enzymes is a scientifically intriguing process of considerable biotechnological importance. Many synthetic compounds have a xenobiotic molecular structure that did not occur on earth in biologically significant concentrations before the development of industrial organic synthesis some 70-100 years ago. Microbial degradation of such compounds therefore is expected to require the evolution of new catabolic pathways, either by acquisition of novel genes orby mutation of existing genes. This makes microorganisms that degrade xenobiotics very attractive for studying the evolution of enzyme specificity and the assembly of new metabolic routes. Furthermore, microorganisms that transform synthetic organic compounds often possess enzymes with interesting regionselectivities and stereoselectivities. Exploiting and engineering the catalytic potential of microbial enzymes can lead to the development of new biological processes for the production of valuable fine chemicals.

Bacterial growth on halogenated chemicals

Many chlorinated and brominated chemicals that were originally expected to be recalcitrant to biodegradation now appear to be able to serve as a carbon source for some specialized microorganisms. Such organisms have developed a catabolic pathway that harvests energy and

carbon from the halocarbon compound when degrading it. Examples of compounds for which we found organisms are listed in Table 1.

Other organohalogens can only be degraded under aerobic conditions by cometabolism or by anaerobic, reductive reactions. The former reactions are particularly well carried out by methanotrophic bacteria that produce a soluble type of methane monooxygenase. This enzyme has a very broad substrate range and can cooxidize various recalcitrant organohalogens, including trichloroethylene and dichloroethylenes. A drawback is that these cometabolic conversion often are accompanied by toxic effects, which are cause by the fact that various reactive transformation products can be formed.

In view of these effects, the isolation of organisms that can degrade and grow on organohalogens still is an important bottleneck towards the degradation of an efficient treatment process. How an organism that can degrade an organohalogen can be applied in bioremediation was nicely illustrated by Stucki and Thuer who developed a full scale process for groundwater treatment employing 1,2dichloroethane degrading bacteria. Experiences of a large-scale application of 1,2-dichloroethane degrading microorganisms for groundwater treatment.

Isolated organisms that grow on organohalogens as sole carbon source.

Compound	Organism	Reference
dichloromethane	*Hyphomicrobium* GJ21	unpublished
1,2-dichloroethane	*Xanthobacter autotrophicus* GJ10, *Ancylobacter aquaticus* AD20	Janssen et al., 1985, van den Wijngaard et al., 1992
2-chloroethyl-vinylether	*Ancylobacter aquaticus* AD27	van den Wijngaard et al., 1992
2-chloroethanol	*Pseudomonas* GJ1	van der Ploeg et al., 1996
1,6-dichlorohexane	*Rhodococcus* GJ70	Janssen et al., 1987
epichlorohydrin	*Agrobacterium radiobacter* AD1	van den Wijngaard et al., 1989
1-chloro-2,3-propanediol	*Arthrobacter* AD2	van den Wijngaard et al., 1989
1,3-dichloropropene	*Pseudomonas pavonaceae* 170	Poelarends et al., 2000a
1,2-dibromoethane	*Mycobacterium* GP1	Poelarends et al., 2000b
chlorobenzene	*Pseudomonas* GJ31	Mars et al., 1997
1,2-dichlorobenzene	*Pseudomonas* GJ60	Oldenhuis et al., 1989

Dehalogenases cleave carbon-halogen bonds

Halogenated aliphatic compounds are widely used as solvents and intermediates in the production of agrochemicals and polymers. Studies on the biochemistry of catabolic pathways in organisms that use such compounds as a carbon source for growth have led to the discovery of several new dehalogenating enzymes.

Four different classes of aliphatic dehalogenases are now well defined with X-ray structures solved and reliable mechanistic insight available.

These are: 1) haloalkane dehalogenases (e.g. DhlA from Xanthobacter autotrophicus and DhaA from Rhodococcus erythropolis), a group of proteins belonging to the α/β-hydrolase fold family; 2) halocarboxylic acid dehalogenases (e.g. DhlB of X. autotrophicus) of the HAD superfamily of proteins, which includes phosphatases; 3) halohydrin dehalogenases (e.g. HheC from Agrobacterium radiobacter), a class of proteins that shares similarity to the short chain dehydrogenase-reductase family of proteins; 4) chloroacrylic acid dehalogenases (e.g. CaaD from a 1,3-dichloropropene degrading Pseudomonas cichorii), enzymes that share similarity to the 4-oxalocrotonate tautomerase superfamily.

From the study of these dehalogenases, a number of important conclusions have emerged. First, it appears that enzymes that act on halogenated compounds really have evolved to carry out dehalogenation reactions. Thus, the capacity to cleave carbon-halogen bonds in xenobiotic structural elements is not just a side reaction of an enzyme that has a function in the conversion of some non-halogenated natural metabolite. Dehalogenases possess a specific halide-binding site, formed by groups that can donate hydrogen bonds to the halide ion, and that can bind both the halogenated substrate and the displaced halide.

Second, dehalogenases appear to be evolutionarily related to proteins that carry out conversions with natural compounds. For example, the halohydrin dehalogenases are related to the SDR (short-chain dehydrogenase-reductase) family of proteins, which is a diverse group of enzymes that oxidize alcohols and various other compounds.

Third, various open reading frames that show sequence similarity to known dehalogenases are present in many organisms, as shown by genome sequencing projects. The activity and function of the encoded proteins is not always clear, but they could be involved in the biodegradation of natural organohalogens, which may be formed by haloperoxidases or specific halogenating enzymes in a variety of environments. The abundance of these putative dehalogenase sequences varies a lot, some being rather common, such as genes that encode chlorocarboxylic acid dehalogenases, whereas others are very rare, e.g. the putative halohydrin dehalogenase gene sequences.

Haloalkane dehalogenase

The best studied dehalogenases are undoubtedly the haloalkane dehalogenases, especially the enzyme from *Xanthobacter autotrophicus* GJ10, a hydrolytic α/β-hydrolase fold enzyme, composed of a main domain with the general fold and a cap domain, which seems to be involved

in determining the substrate specificity (Pries et al., 1994; Pikkemaat and Janssen, 2002). Its mechanism and kinetics have been investigated in detail.

A dehalogenase similar to the *Xanthobacter* enzyme has been discovered in a gram positive organism growing on 1-chlorobutane. This enzyme (DhaA) shares only about 28 % sequence identity with the haloalkane dehalogenase from *Xanthobacter* (DhlA). When we studied the genes involved in the initial dehalogenation reactions of 1,3-dichloropropylene in *Pseudomonas pavonaceae* 170 and Mycobacterium sp. GP1, it appeared that these organisms possessed haloalkane dehalogenase genes that were (almost) identical to the one found in *Rhodococcus* (Poelarends et al., 2000a, b). To understand the distribution of these genes, we have cloned and compared the sequences of the gene regions encoding the dehalogenases in these three organisms. The results indeed suggest that the dehalogenase gene clusters in bacteria growing on 1,2-dibromoethane and 1,3-dichloropropene originate from the more widespread 1-chlorobutane gene cluster of *Rhodococcus erythropolis*.

Classification of some important dehalogenases (see Janssen et al., 2001, 2005).

Type (example)	Family	Mechanism
Haloalkane dehalogenase (DhlA, DhaA)	α,β-hydrolase fold, similar to lipases	catalytic triad, Asp as nucleophile, distinct halide-binding site, covalent intermediate
halocarboxylic acid dehalogenase (DhlB)	haloacid dehalogenase-phosphatase fold	catalytic Asp close to N-terminus as nucleophile, covalent intermediate
halohydrin dehalogenase (HheA, HheB, HheC)	short chain dehydrogenase-reductase family (SDR proteins)	catalytic triad for H^+ abstraction, halide binding site, non-covalent catalysis
chloroacrylic acid dehalogenase (CaaD)	4-OT family of hexameric enzymes	N-terminal nucleophilic Pro, no covalent intermediate, hydratase-like mechanism

Other dehalogenases

After the initial studies on haloalkane dehalogenases, several otherdehalogenases were mechanistically and structurally investigated. The Table gives a few examples. Halocarboxylic acid dehalogenases of the HAD family are enantioselective with 2-chloropropionic acid and related to a broad family of proteins that share a conserved topology and positioning of the catalytic residues.

Halohydrin dehalogenases belong to the SDR family of proteins (van Hylckama Vlieg et al., 2001). They do not catalyze a hydrolytic reaction, but instead convert vicinal halohydrins to epoxides. Chloroacrylic acid dehalogenases convert 3-chloroacrylic acid to malonic semialdehyde. These proteins belong to 4-OT family of tautomerases-isomerases.

- **SYNTHETIC POLYMER**

Synthetic polymers are those which are human-made polymers. Polymers are those which consists of repeated structural units known as monomers. Polyethylene is considered to be as one of the simplest polymer, it has ethene or ethylene as the monomer unit whereas the linear polymer is known as the high density polyethylene-HDPE. Many of the polymeric materials have chain-like structures which resemble polyethylene.

Synthetic polymers are sometimes referred as "plastics", of which the well-known ones are nylon and polyethylene. The polymers which are formed by linking monomer units, without the any change of material, are known to as addition polymers or also called as chain-growth polymers. All these are said to be synthetic polymers.

Some of the synthetic polymers which we use in our everyday life include nylons used in fabrics and textiles, Teflon used in non-stick pans, polyvinyl chloride used in pipes. The PET bottles we use are commonly made up of synthetic polymer called as polyethylene terephthalate. The covers and plastic kits comprises of synthetic polymers such as polythene, and the tyres of vehicles are manufactured from the Buna rubbers. But on the other side, there also arises environmental issues by the use of these synthetic polymers such as the bioplastics and those made from petroleum as they are said to be non-biodegradable.

Types of Synthetic Polymers with Examples-

There are various synthetic polymers developed so far. Let us study in brief about few of the synthetic polymers used in everyday life-

Nylon-

Nylon belongs to the synthetic polymers family and is also known as polyamides. It was produced on February 28 in the year 1935 by person naming Wallace Carothers at the DuPont's research facility. Nylon is widely used polymers. The backbone of it called as amide causes it to become hydrophilic than other polymers. Nylon gets engaged in hydrogen bonding with water, not like the pure hydrocarbon polymers which make most of the plastics.

Polyvinyl Chloride –

- Polyvinyl Chloride or PVC is third-most majorly produced plastics coming after polypropylene and polyethylene. This PVC is used for construction purposes as it is known to be stronger and cheaper than other alternatives like copper or iron. PVC is also used in the clothing, electrical cable insulation including many other applications replacing rubber.

Low-Density Polyethylene–

- The Low-Density Polyethylene polymers are the most common kind of synthetic polymers, which are widely used in households. LDPE is a kind of thermoplastic which is prepared from the monomer called ethylene.

Polypropylene–

- Polypropylene also called as polypropene is a kind of thermoplastic synthetic polymer which is used in variety of applications such as packaging, labeling, stationery, textiles, plastics and in reusable containers, laboratory equipments and etc.

Some of the other examples includes Thermoplastic Polyurethane, Teflon, Polystyrene, High Density Polyethylene, Neoprene, etc.

Uses of Synthetic Polymers

Some of the uses are given below-

1. The polymer called Polyethylene is used in plastic bags and film wraps.
2. Polyethylene is utilized in the bottles, electrical insulation, toys, etc
3. Polyvinyl Chloride(PVC) is used in siding, pipes, flooring purposes.
4. The synthetic polymer Polystyrene is used in cabinets and in packaging.
5. Polyvinyl acetate is used in adhesives and latex paints.

- **OIL**

An **oil** is any nonpolar chemical substance that is a viscous liquid at ambient temperatures and is both hydrophobic (does not mix with water, literally "water fearing") and lipophilic (mixes with other oils, literally "fat loving"). Oils have a high carbon and hydrogen content and are usually flammable and surface active.
The general definition of oil includes classes of chemical compounds that may be otherwise unrelated in structure, properties, and uses. Oils may be animal, vegetable, or petrochemical in origin, and may be volatile or non-volatile. They are used for food (e.g., olive oil), fuel (e.g., heating oil), medical purposes (e.g., mineral oil), lubrication (e.g. motor oil), and the

manufacture of many types of paints, plastics, and other materials. Specially prepared oils are used in some religious ceremonies and rituals as purifying agents.

Organic oils

Organic oils are produced in remarkable diversity by plants, animals, and other organisms through natural metabolic processes. *Lipid* is the scientific term for the fatty acids, steroids and similar chemicals often found in the oils produced by living things, while oil refers to an overall mixture of chemicals. Organic oils may also contain chemicals other than lipids, including proteins, waxes (class of compounds with oil-like properties that are solid at common temperatures) and alkaloids.

Lipids can be classified by the way that they are made by an organism, their chemical structure and their limited solubility in water compared to oils. They have a high carbon and hydrogen content and are considerably lacking in oxygen compared to other organic compounds and minerals; they tend to be relatively nonpolar molecules, but may include both polar and nonpolar regions as in the case of phospholipids and steroids

Mineral oils

Crude oil, or petroleum, and its refined components, collectively termed *petrochemicals*, are crucial resources in the modern economy. Crude oil originates from ancient fossilized organic materials, such as zooplankton and algae, which geochemical processes convert into oil. The name "mineral oil" is a misnomer, in that minerals are not the source of the oil—ancient plants and animals are. Mineral oil is organic. However, it is classified as "mineral oil" instead of as "organic oil" because its organic origin is remote (and was unknown at the time of its discovery), and because it is obtained in the vicinity of rocks, underground traps, and sands. *Mineral oil* also refers to several specific distillates of crude oil.

Cooking

Several edible vegetable and animal oils, and also fats, are used for various purposes in cooking and food preparation. In particular, many foods are fried in oil much hotter than boiling water. Oils are also used for flavoring and for modifying the texture of foods (e.g. Stir Fry). Cooking oils are derived either from animal fat, as butter, lard and other types, or plant oils from the olive, maize, sunflower and many other species.

Cosmetics Oils are applied to hair to give it a lustrous look, to prevent tangles and roughness and to stabilize the hair to promote growth. See hair conditioner.

Religion Oil has been used throughout history as a religious medium. It is often considered a spiritually purifying agent and is used for anointing purposes. As a particular example, holy anointing oil has been an important ritual liquid for Judaism and Christianity.

Painting Color pigments are easily suspended in oil, making it suitable as a supporting medium for paints. The oldest known extant oil paintings date from 650 AD.

Heat transfer Oils are used as coolants in oil cooling, for instance in electric transformers. Heat transfer oils are used both as coolants (see oil cooling), for heating (e.g. in oil heaters) and in other applications of heat transfer.

Lubrication Synthetic motor oil Given that they are non-polar, oils do not easily adhere to other substances. This makes them useful as lubricants for various engineering purposes. Mineral oils are more commonly used as machine lubricants than biological oils are. Whale oil is preferred for lubricating clocks, because it does not evaporate, leaving dust, although its use was banned in the USA in 1980.

It is a long-running myth that spermaceti from whales has still been used in NASA projects such as the Hubble Telescope and the Voyager probe because of its extremely low freezing temperature. Spermaceti is not actually an oil, but a mixture mostly of wax esters, and there is no evidence that NASA has used whale oil.

Fuel

Some oils burn in liquid or aerosol form, generating light, and heat which can be used directly or converted into other forms of energy such as electricity or mechanical work. In order to obtain many fuel oils, crude oil is pumped from the ground and is shipped via oil tanker or a pipeline to an oil refinery. There, it is converted from crude oil to diesel fuel (petrodiesel), ethane (and other short-chain alkanes), fuel oils (heaviest of commercial fuels, used in ships/furnaces), gasoline (petrol), jet fuel, kerosene, benzene (historically), and liquefied petroleum gas. A 42-US-gallon (35 imp gal; 160 L) barrel of crude oil produces approximately 10 US gallons (8.3 imp gal; 38 L) of diesel, 4 US gallons (3.3 imp gal; 15 L) of jet fuel, 19 US gallons (16 imp gal; 72 L) of gasoline, 7 US gallons (5.8 imp gal; 26 L) of other products, 3 US gallons (2.5 imp gal; 11 L) split between heavy fuel oil and liquified petroleum gases,[12] and 2 US gallons (1.7 imp gal; 7.6 L) of heating oil. The total production of a barrel of crude into various products results in an increase to 45 US gallons (37 imp gal; 170 L). Not all oils used as fuels are mineral oils, see biodiesel, vegetable oil fuel, and olive oil.

In the 18th and 19th centuries, whale oil was commonly used for lamps, which was replaced with natural gas and then electricity.

- **BIOPOLYMER** There has been heightened interest in using biopolymers in recent years due to the increasing concern with the sustainability of using petroleum-based polymers. Nonetheless, there is no clear

definition for the word biopolymer as many different concepts exist as to what is a biopolymer. Terms such as biopolymers, bioplastics, and biodegradable plastics are used synonymously in certain contexts; however, each has a unique meaning. A biodegradable plastic is one that degrades due to the action of living organisms such as microbes and fungi. A bioplastic can be defined as a polymer that is manufactured into a commercial product from a natural source or renewable resource. A bioplastic can be biodegradable, but a biodegradable plastic does not mean the material was derived fully or in part from a biological source. For example, polymers such as polycaprolactone (PCL) and poly(butylene succinate) (PBS) are biodegradable but

petroleum based. It is important to distinguish between biopolymers and biodegradability. The biodegradability of a polymer signifies whether its chemical structure can be metabolized by microorganisms and fungi and turned into shorter polymer segments. Biopolymers are one type of polymer composed primarily of a few types of repeating units containing carbon which are used in or originate from living organisms. Based on this board definition, biopolymers include natural polymers, bio-based polymers, also known as bioplastics, which are extracted from biomass (i.e., natural polymers) or polymerized from bio-based monomers and those polymers produced in microorganisms and extracted. Polymers used in the field of health sciences are classified as biopolymers (or biomedical polymers) because of their use in biological systems. These materials are used in biomedical applications such as pharmaceutical, medical device coatings, and resorbable implants that require biocompatibility and nontoxicity. Obviously, a biomedical polymer can be petroleum-based or bio-based. Due to a vast range of applications and material variations, biomedical polymers will not be discussed. Reference gives more detailed coverage of biomedical polymers.

According to the definition of biopolymers given above, complex carbon-based macromolecular arrangements such as DNA, RNA, and proteins do not fall under this definition. Although many refer to these molecules as natural polymers or biopolymers since they are produced by living organisms, this is not entirely accurate due to the fact that they contain many different types of repeating units connected in somewhat complex sequences. Precisely speaking, they are bio macromolecules. In bio macromolecules, covalent bonds exist between different types of amino acids, parallel to the covalent bonds that connect monomer groups within a polymer. DNA, RNA, and proteins have unique structures. One example is DNA's double helix which is caused by secondary bonds and interactions such as hydrogen bonds, ionic bonds, van der Waals forces, and hydrophobic interactions. Due to the strong influence of secondary bonds and interactions on the structure of these complex macromolecules, the environment plays a significant role in their conformation, which in turn determines functionality. A slight change in environmental conditions such as pH or temperature may cause a protein to change conformation and lose functionality, known as protein denaturing. Some diseases, like Alzheimer's, can be traced back to changes in protein conformation which resulted in a loss of function. For further reading on protein conformation diseases or the fundamentals of biochemistry, consult

There are two major groups of protein structures, fibrous and globular, present in the body. Fibrous proteins have more regularity in their monomer sequences than the other type of protein. Some examples of fibrous proteins include keratin (hair, nails, feathers, and horns), collagen (connective tissue), fibroin (silk), and myosin (muscle). The combination of these fibrous proteins into plastics is a topic of current interest. Other proteins such as soy protein and wheat gluten are used in bioplastics and biomedical applications.

Natural Polymers : Natural polymers are polymers produced in living organisms and as such are essential to all life. Some of these natural polymers can be extracted and modified into plastics for commercial use, known as bio-based polymers or bioplastics.

Natural Rubbers

Natural rubber is probably the most widely used natural polymer in modern life. It is a polyterpene synthesized by enzymatic polymerization of isopentenyl pyrophosphate and the repeat unit structure is isoprene (1-17). Natural rubber consists of 97% cis-1,4-polyisoprene (1-18) and exists in the natural latex form in the rubber tree Hevea braziliensis. The other 3% contains various proteins that sometimes trigger allergies. Guayule, a plant shrub, also grows rubber that does not trigger allergic reactions. Natural rubber is obtained by tapping the bark of a tree that grows wild in South America and is cultivated in other parts of the world. Since natural rubber has a glass transition temperature of 270 °C and very high abrasion resistance, it has been extensively used to make tires. However, to turn natural rubber (a soft material at room temperature) into a tire, cross-linking of the cis-1,4-polyisoprene molecules, a process called vulcanization, is needed. It is worth noting that natural rubber is very reactive due to the presence of many double bonds in its structure. And such a chemical moiety is vulnerable to free radical and oxidation reactions. It is because natural rubber is used in its cross-linked form that it is not biodegradable and not easily recycled.

Polysaccharides

Otherwise known as carbohydrates, are polymeric structures in biological systems. Polysaccharides are made of many monosaccharides, which are composed primarily of carbon, oxygen, and hydrogen. The most common types of monosaccharide units contain either five or six carbon atoms. Due to the presence of several hydroxyl groups within the monosaccharide structure, hydrogen bonds are formed between polymer chains. Glucose is the most abundant sixcarbon sugar and is the monomeric unit of both cellulose and starch. Two types of linkages are possible from the polymerization of glucose: alpha or beta acetal linkages. These different linkages are possible since glucose primarily exists in alpha or beta anomers. An anomer is one of two stereoisomers of a cyclic sugar that differ only in configuration at the anomeric carbon of the hemiacetal. In a glucose ring, when the OH group of the hemiacetal is in the down position it is called α-glucose, compared to when the OH group is in the up position it is called β-glucose. The β-glucose anomer is the most stable because all of the substituents larger than hydrogen are in the more spacious equatorial position, including the OH group. The same naming applies to polymerized forms of the glucose molecules. When the bond between two glucose molecules at the anomeric carbon is up, it is a β linkage, the bond angles influence the structure of the polymer. For example, cellulose is an almost linear chain, whereas amylose forms a spiral structure.

Starch

It is an abundant polymer stored in plants for energy consumption in the form of granules. Although glucose is the monomer in both starch and cellulose, starch differs from cellulose in bonding by two ways: (1) the glucose rings are connected through carbons 1 and 4 by α linkages rather than β linkages and (2) frequent branching occurs at carbon 6. Starch consists of two polysaccharides, amylose and amylopectin, illustrated in Fig. 13.2, which can be separated due to differences in solubility. The ratio of amylopectin to amylose in natural starch varies based on plant species; however, amylopectin is always more abundant. Amylose is mainly linear in

structure which can have a degree of polymerization as high as 600. Amylose resembles cellulose apart from the α-1, 4 linkage between glucose rings. Amylopectin is a branched polymer with α-1, 6 branch points. Amylopectin contains 4-6% of branch points which is a significant difference from the structure of amylose, a linear chain. The branches in amylopectin have been classified into A-, B-, and C-chains, where A-chains do not carry any other chains, B-chains carry one or more chains, and the C-chain is the original chain carrying the sole reducing end Starch is used in a wide range of commercial products from filler in toothpaste to food products as well as in plastics. The processing and manufacturing of starch plastics will be discussed along with blending of starch with other polymers.

Cellulose is the most abundant biopolymer and is the largest organic carbon source on earth. Cellulose is synthesized by plants and makes up a large portion of a plant's chemical structure. The content of cellulose in plants varies from 90% in cotton to 4050% in wood. Cellulose is a hydrophilic linear polymer consisting of D-anhydroglucose ($C_6H_{11}O_5$) repeat units containing three hydroxyl groups with the repeat units joined by β-1, 4 ether linkages at C1 and C4 positions. Cellulose provides strength and rigidity to plants due to crystalline packing of the linear polymer chains. The two other biopolymers found in plants are hemicelluloses and lignin; see Fig. 13.3. The amount of each constituent in a plant is dependent on both species and growing conditions. Hemicellulose is very hydrophilic and is composed of multiple polysaccharides of 5- and 6-carbon ring sugars with branched pendant groups. Lignin is an amorphous and highly complex cross-linked molecule with aliphatic and aromatic constituents. Lignin provides plant tissue and individual fibers with compressive strength and stiffens the cell wall of the fibers to protect polysaccharides, cellulose, and hemicelluloses from chemical and physical damage. Due to the complex nature of lignin, it is very hard for the majority of microorganisms to break it down. However, certain species of fungi are capable of degrading lignin. Lignin has been deemed the limiting step in the degradation of wood and plant fibers.

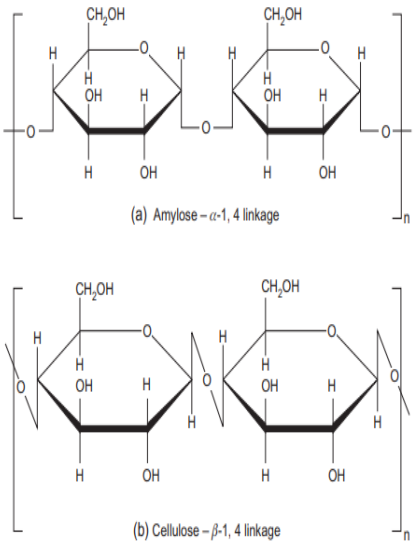


FIGURE 13.1

Two types of glucose linkages (a) α-1,4 (amylose) and (b) β-1,4 (cellulose). Each star denotes the anomeric carbon used to determine the position of the ether bond (down position, α, or up position β).

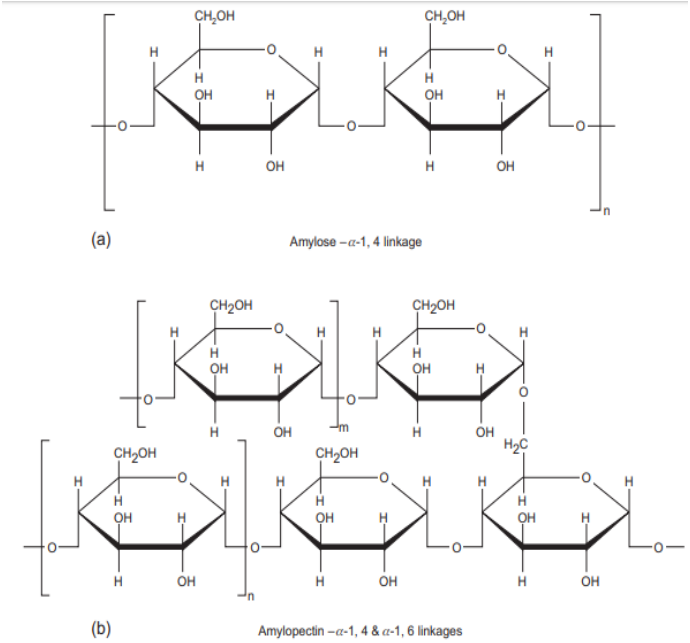


FIGURE 13.2

Chemical structure for the two main components of starch (a) amylose and (b) amylopectin.

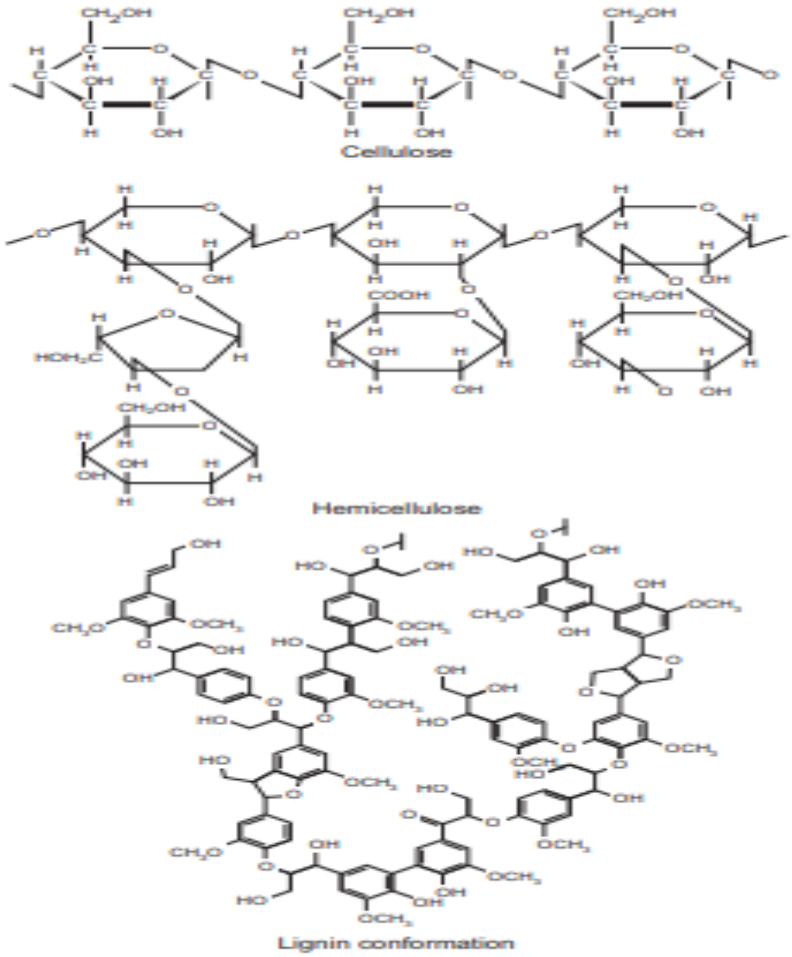


FIGURE 13.3

Chemical structures of the three most common biopolymers in plants. Cellulose (top), hemicelluloses (middle), and one configuration of lignin (bottom).

Chitin

It is a skeletal polysaccharide, poly(2-acetamido-2-deoxy-D-glucopyranose), which makes up the basic constituent of shells such as lobsters, shrimps, and insects. Chitosan, poly(2-amino-2-deoxy-D-glucopyranose) is obtained by the deacetylation of chitin. The extraction and modification of this natural polymer demonstrate unique properties such as biocompatibility, antimicrobial activity, biodegradability, film and fiber forming ability as well as heavy metal absorption. Chitinous fibers are manufactured into artificial skin, absorbable sutures, carriers for controlled delivery of drugs, and bone tissues. Natural polymers without modification and processing are not acceptable to be used in many functional engineering applications, like conventional plastics. It is the modification of these natural polymers that allows them to be made into useful materials. This concludes the brief overview of naturally occurring polymers, one of three classifications of biopolymers. The next section focuses on bio-based polymers, the second sub-classification of biopolymers.

Bio-Based Polymers (Bioplastics)

A bio-based polymer is derived fully or in part from biomass or microorganisms as an alternative to petroleum plastics. Many of these polymers are commonly referred to as bioplastics. A bioplastic is similar to a conventional plastic, like polypropylene, in the fact that it is used to make commercial products. There are three generic pathways to producing plastics from natural materials such as (1) the extraction and modification of natural polymers from biomass; (2) the polymerization of bio-based monomers; and (3) the extraction of polymers produced in microorganisms. An explanation of each pathway along with specific examples of bioplastics, processing, properties, and applications will be detailed. A general overview of synthetic pathways to a bioplastic is illustrated in Fig. 13.4, along with examples of products.

Extraction and Modification of Natural Polymers in Plants

Many different types of natural polymers exist in the environment. The extraction and/or modification of these polymers enable them to be used as commercial materials, a bioplastic. Examples of these products include the vulcanization of natural rubber, thermoplastic starch, and regenerated cellulose.

Natural Rubber As mentioned, natural rubber is collected by tapping rubber trees, Hevea braziliensis, by making an incision into the bark of the tree. Natural rubber is unique to "bioplastics" as the tree producing the rubber is not destroyed to harvest the material. When polyisoprene is vulcanized with heat and sulfur, the resulting product is very useful in commercial products such as tires.

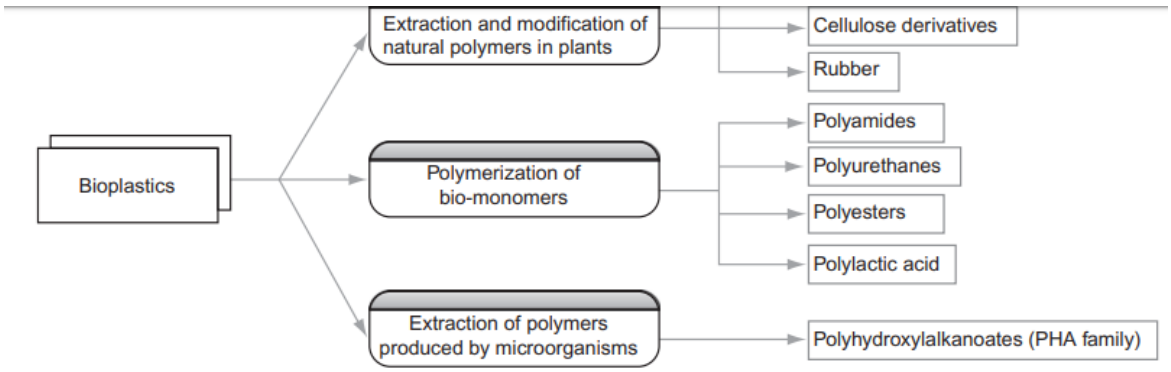


FIGURE 13.4

Flow chart of different pathways used to synthesize bioplastics.

Starch : Starch, in its native form, is a granular structure. This form of starch is incorporated into plastics as a type of filler. Starch is heavily used in food processing, such as cereal, snacks, canned goods, frozen food, dairy products, and much more. Starch is also used in a variety of industries such as adhesives, mining, metal, paper, cosmetics and pharmaceutical, construction, and textile in many products. In order to use starch as the polymer for a bioplastic application, the polymer structures amylose and amylopectin are extracted. Extraction occurs by disrupting the granular structure by solubilization of the starch granules in water at elevated temperatures, called gelatinization. In the presence of water the granules swell and eventually burst, leading to the release of polymer into the surrounding water. Products used for a range of applications can

be obtained depending on the level of destruction of the granule caused by the water content, time, and temperature of the extraction. For starch to be able to be classified as a plastic, usable interchangeably with traditional plastics, it must undergo further processing before it is classified as thermoplastic starch. Thermoplastic starch (TPS) is defined as destructurized or gelatinized starch that is combined with one or more additives (plasticizer) to produce a material that can be treated like other thermoplastic polymers. Common plasticizers used are glycerol, sorbitol, polyethylene glycol, or other polyols that are added to the extracted polymer. The plasticizers are able to separate the melting temperature from the thermal degradation temperature to allow the extracted polymers to be processed using conventional machines, such as extrusion and injection molding. The resulting material is expelled from the extrusion die as a homogeneous molten phase and then pelletized after cooling. Thermoplastic starch blended with other biopolymers increases biodegradability. Focus in research has been placed on engineering TPS for packaging applications.

Cellulose : Cellulose can be harvested from many plant sources as it is the major part of the cell wall. Also cellulose can be produced by fermentation of certain bacteria. Fermentation occurs in a bioreactor and is the process of microorganisms metabolically breaking down organic substances in the absence of oxygen, anaerobic metabolism. Important parameters controlled during the process are temperature, pH, and sugar content from the feedstock material. Fermentation can also be carried out with genetically engineered microorganisms (GEMs). GEMs can be designed to break down specific substrate materials during fermentation, as well as surviving the harsh conditions. Cellulose produced by bacteria can be obtained as a continuous film by cultivating the bacteria in a glucose solution. Cellulose is also secreted by marine chordates such as the sea squire. Cellulose from plant sources can be extracted by chemical solubilisation type processes such as pulping, which results in a refined structure. The cellulose structure is disrupted and the cellulose remaining in solution is then precipitated. This method is used to prepare regenerated cellulose. Regenerated cellulose is different from native cellulose because extensive degradation takes place during the process of dissolution and the final product is usually less crystalline. Other products derived from cellulose include cellulose nitrate, cellulose ester, and cellulose acetate. Work has been conducted on grafting cellulose as copolymer, as well as the potential to cross-link cellulose. There is interest in cellulose chemistry and polymerization due to the abundance of cellulose as a renewable resource.

Biopolymer Blends and Biocomposites

Many other uses of biopolymers exist than are discussed in the previous sections. A biopolymer can be blended with (1) another biopolymer, (2) a biodegradable synthetic polymer, or (3) a non-degradable synthetic polymer. Also biopolymers can be combined with different reinforcing materials such as mineral particles or natural fibers to create a bio-polymer matrix composite. In fact, natural rubbers are usually compounded with various inorganic fillers, antioxidants, pigments, to name a few, to make it more useful.

Biopolymer Blends

Bending is a useful strategy to modify the materials properties for specific applications. Biopolymers are no exception. Biopolymers are often blended with one another to improve total degradation time as well as mechanical properties. For instance, TPS is more susceptible to microbial action and is more ductile compared to PLA, and a blend leads to intermediate properties. Bioplastics are often blended with synthetic polymers in order to reduce the cost of the material. Along with the reduction of cost, if the bioplastic chosen is biodegradable, the overall material may be considered biodegradable depending on the particle size of the remaining synthetic plastic once biodegradation has been completed.

Biocomposites

A biocomposite is a material composed of two or more distinct constituent materials (one being naturally derived) which are combined to yield a new material with improved performance over individual constituent materials. The constituent materials are the matrix and reinforcing component. The reinforcing component is the primary load-carrying element, which can be in the form of fibers, whiskers, particles and flakes. The matrix serves to bind the reinforcing components together and provide mechanical support. A frequently studied biocomposite is natural-fiber-reinforced biopolymer composite. The reinforcing component is natural fiber or cellulose extracts combined with a bioplastic matrix. The natural fiber adds further strength to the weaker biopolymer matrix, allowing for the material to be used in more applications. Significant research is being conducted in embedding nanoparticles or particulates such as layered silicates, carbon nanotubes, hydroxyapaptite, cellulose, and talc into bioplastics. The most commonly used in PLA bioplastics is layered silicate clay, as it has been attributed to a dramatic increase in material properties such as improved tensile and flexural properties, elevated heat distortion temperature, enhanced barrier properties, and accelerated biodegradation. Processing issues associated with these nano-biocomposites are distribution and dispersion of the reinforcement within the biopolymer.

2. AGRICULTURAL MICROBIOLOGY

MICROBIAL EXPLOITATION FOR IMPROVEMENT AND CROP PROTECTION

Human beings and animals are largely dependent on the plants for meeting their energy requirements. Also, continuous change in the environmental conditions due to climate change and global warming is having adverse effects on agricultural crops in commercial sectors. Conditions for plants become very drastic which are not suitable for their survival. So, at this stage, i.e., in the changing environment, it becomes essential to improve our agriculture system in such a way that we should not just aim for higher production but should also be looking for betterment in plant protection and adaptability, simultaneously.

In the recent decade, we have seen huge increase in production in the agriculture sector which is largely attributed to the extensive use of synthetic chemicals, but this is not a long-term strategy for getting sustainable production. Due to high consumer pressure and new policies made by regulatory authorities, the withdrawal of these chemicals had been seen on a larger scale in order to reduce toxic residue in soil. In addition to this, the production, development, and registration cost for these synthetic chemicals has been inclining very rapidly, which is further limiting available control strategies for the growers. The pursuit for substitute solutions for agriculture has encouraged researchers to have a second look at the variety of microorganisms, recognized earlier to impart assistances to agricultural production, and is providing options for biocontrol agents and plant growth-promoting microbes In spite of the purpose for these microorganisms applied to crops, they must be produced at commercial or large scale and used in a way that maintains their activity and functionality in the target conditions also. So far, these formulations are available as liquids (sprays, drenches, root dips) mainly for experimentation or as dehydrated powder forms, which are supplied at the time of plantation. But most of these strategies do not work on mass production scale, due to a huge amount of microbial inoculum which is required. The beneficial microbes can be applied to the seeds for placing the microbial flora into the soil, where they will start colonizing and will provide protection by interacting against different insects and pests which are feeding on plant roots and reducing the yield. In addition, they will also synthesize essential nutrients which are required for proper growth and development of plants. This is not a new technique and is already established and demonstrated at small scale by different researchers in different conditions. However, despite the fact that they have been well demonstrated and available in various formulations in association with legume crops, they are still not used on commercial scale by the farmers due to lack of knowledge and interests.

Plant-microbiome interactions represent a very promising solution for providing protection and improving agricultural yield sustainably. In this chapter, we tried to merge the fundamental basics and applied aspects of beneficial plant-microbial interactions effectively. This is our humble approach and sincere effort for advancing the agriculture by providing details about available microbial solutions.

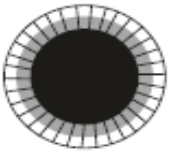
	Bioprimed	*Film coated*	*Slurry coated*	*Pelleted*
	 *Inoculant within seed*	 *Inoculant in thin layer on seed surface*	 *Inoculant in (peat) carrier stuck to outside of seed*	 *Inoculant applied to seed along with conventional seed additives*
Method	Seed soaked in saline / inoculant suspension	Inoculant suspended (e.g. sugar. methyl cellulose) and dried	Inoculant grown in solid carrier medium applied to seed using sticker Often dusted with lime to ensure flowability	Typical commercial process
Utility	Experimental limited commercial use	Mainly for experimental use only	Widely used for rhizobial inoculants prior to sowing	Not yet but desired by seed companies and growers
Inoculant survival	Good long term survival	Short term survival	Variable	Poor survival unless resistant (spore-former) inoculants used

Fig. 26.1 Methods and preparations available for microbial seed inoculation

Microorganisms for Sustainable Plant Growth

Plants are unsurprisingly accompanying multifaceted microbiomes, which are known to boost plant growth and stress tolerance, backing plant nutrition and antagonizing plant pathogens. The main properties of microorganisms for subsidiary plant growth and development

Harmonizing Soil Ecology

Microorganisms are a fundamental part of almost every soil ecosystem. Soil is a hub of various kinds of biological and biochemical activities, and most of them are carried out by microorganisms. Metabolic activities of PGPR, mycorrhiza, cyanobacteria, and certain soil fauna have been reported to improve soil health and increase crop productivity. Most of the beneficial microorganisms need carbon as a sole source of energy, so that's why it has been observed that the soils which are poor in organic matter have less microbial activities. Moreover, the extensive use of synthetic fertilizers, insecticides, and pesticides in the last decades further lowered down the level of organic matter and even worsens the situation by having adverse effects on microbialactivities. This led to decrease, or in many cases, a number of beneficial soil microorganisms such as PGPR, fungi, earthworms, actinomycetes, etc. have been extinct from those areas. Similar kind of situation prevails in almost all parts across the country.

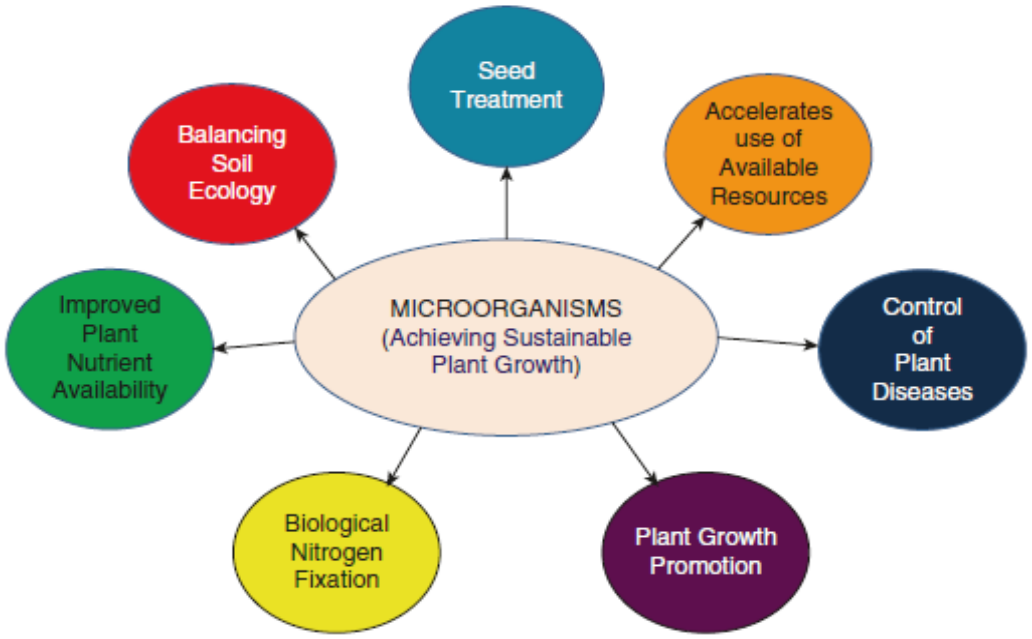


Fig. 26.2 Role of microorganism in plant growth

Biological Fixation of Nitrogen

Nitrogen is the most abundant gas on earth, but it is impossible to use nitrogen in its gaseous form by the plants. Also, nitrogen is a primary essential macro nutrient which is required by the plants and is a part of most of the biomolecules which has a role in physiological function and metabolism. There are many microorganisms which have the potential to convert gaseous nitrogen to its usable form that is nitrates through a process called as biological nitrogen fixation (BNF). BNF is the main process and source of nitrogen for legumes and other important crops. BNF provides the largest input of nitrogen to agricultural soils worldwide. *Rhizobium* inoculation as biofertilizer in the crops like groundnut, pigeon pea, soybean, etc. reported to provide 19–22 kg of nitrogen per hectare with 17–33% of total increase in crop yield. Likewise, the use of *Azotobacter*, which is a nonsymbiotic bacterium, and *Azospirillum* in wheat, sorghum, tomato, cotton, and sugarcane contributed nitrogen supply to crops to an extent of 20–30 kg per hectare providing 10–30% increased crop yield. Wherever water, sunlight, and carbon dioxide are available, phototrophic microorganisms like blue-green algae or cyanobacteria can grow. Therefore, rice ecosystem provides an ideal environment for the growth and development of these self-supporting organisms such as *Anabaena*, *Nostoc*, *Aulosira*, *Calothrix*, *Tolypothrix*, etc. They colonize the rice field soils, compete well with the native strains, thus grow profusely near the rhizosphere, and release fixed nitrogen through exudation or through microbial decomposition after the algae dies. So, in rice fields, the degradation of algal biomass most frequently results in maintenance of soil fertility. The residual effects influence the succeeding crops also. Apart from fixing nitrogen and adding organic matter to soil, BGA are also known to produce and excrete plant promoting substances like indoleacetic acid. Also, the continuous use

of the BGA biofertilizers for 2–3 years adequately builds up the population of these organisms in the soil.

The relative contribution of BGA as a percentage of total nitrogen fixed in paddy fields varies widely and is estimated to be 15–35 kg nitrogen per hectare in India. In areas where chemical nitrogen is not used for various reasons, algal inoculation enhances minimum of 4% to a maximum of 32.8% crop yield with an overall average of 16.1%. Even at the levels of chemical nitrogen fertilizers being used in different states, the application of BGA biofertilizers resulted in an increased crop yield of 8.85%. Plant growth-promoting rhizobacteria (PGPR) are low-cost input from nature; besides nitrogen fixers, many bacteria colonize plant roots. Some of them promote plant growth significantly. They help in mobilization of the soil nutrients and production of phytohormones or growth-regulating substances. These phytohormone producing microbes have been classified as PGPR. Of the many such bacteria identified, the role of fluorescent *Pseudomonas* and *Bacillus* species has attracted much attention. The substances produced by them have natural biocontrol and plant growth-promoting capabilities. Increased amount of nutrient uptake by plants inoculated by *Pseudomonas putida* has been attributed to the production of growth regulators by the bacterium at root surface which stimulates root development. Pseudomonad (group of *Pseudomonas* sp.) inoculants produce indoleacetic acid-like substances (plant hormone) in the rhizosphere of wheat grown in field conditions. Many PGPR, for example, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, also produce substances such as siderophores and saponins, which are responsible for the removal of heavy metal toxicity. These organisms are also responsible for enhancement of rhizospheric competitive ability by antagonistic effects on other harmful bacteria; control of plant diseases that affect root density; and production of chemicals that interfere with the organisms infecting plant roots, enhancing the availability of nutrients that improve the efficacy of plants. PGPR are therefore being widely evaluated for their role in sustainable resource management as biocontrol agent and Biofertilizer.

Table 26.1 Growth-promoting substances released by some important plant growth-promoting bacteria

Beneficial bacteria	Growth-promoting properties/compounds
Pseudomonas putida	Siderophores, IAA, ammonia, phosphate solubilization, HCN, exo-polysaccharides
Pseudomonas aeruginosa	Siderophores, IAA, ammonia, phosphate solubilization, HCN, exo-polysaccharides
Klebsiella sp.	Siderophores, IAA, ammonia, phosphate solubilization, HCN, exo-polysaccharides
Enterobacter asburiae	Siderophores, IAA, ammonia, phosphate solubilization, HCN, exo-polysaccharides
Mesorhizobium sp.	HCN, ammonia, IAA, exo-polysaccharides, siderophores
Acinetobacter sp.	Phosphate solubilization, IAA, siderophores
Rhizobium sp.(pea)	HCN, ammonia, IAA, exo-polysaccharides, siderophores
Rhizobium sp.(lentil)	HCN, ammonia, IAA, exo-polysaccharides, siderophores
Pseudomonas sp. A3R3	IAA, siderophores
Psychrobacter sp. SRS8	Heavy metal mobilization
Bradyrhizobium sp.	HCN, ammonia, IAA, exo-polysaccharides, siderophores
Pseudomonas aeruginosa 4EA	Siderophores
Bradyrhizobium sp.750	Heavy metal mobilization
Bacillus species PSB10	Ammonia, IAA, siderophores, HCN
Paenibacillus polymyxa	Siderophores, IAA
Rhizobium phaseoli	IAA
Stenotrophomonas maltophilia	Nitrogenase activity, phosphate solubilization, IAA, ACC deaminase
Rahnella aquatilis	ACC deaminase, phosphate solubilization, IAA
Proteus vulgaris	Siderophores
Pseudomonas sp.	Siderophore, phosphate solubilization, IAA
Azospirillum amazonense	Biocontrol potentials, nitrogenase activity, HCN, IAA
Mesorhizobium sp.	IAA, siderophores, HCN, ammonia
Pseudomonas sp.	ACC deaminase, IAA, siderophore
Serratia marcescens	IAA, siderophore, HCN
Pseudomonas fluorescens	ACC deaminase, phosphate solubilization
Enterobacter sp.	Phosphate solubilization, siderophore, N2 fixation, phosphate solubilization, ACC deaminase, IAA

IAA indole-3-acetic acid, *HCN* hydrogen cyanide, *ACC* 1-aminocyclopropane-1-carboxylate

Increasing the Potential Gain of Available Resources

Mycorrhizae play a dominant role in making unavailable soil nutrients available to plant roots and increasing the potential gain of available resources. These organisms ensure easy availability of organic carbon and complex organic nitrogen and phosphorus sources and increase phosphorus solubilization and availability in clay soils. These fungi work upon large volumes of soil. Their hyphae extend outwardly from the roots ranging from a few centimeters to several meters in the soil. This results in increasing the effective absorbing surface of the host root by as

much as ten times, resulting in enhanced absorption of immobile nutrients such as phosphorus, zinc, copper, etc. in the soil by 60 times.

Mycorrhizal fungi also transport many other nutrients including calcium, magnesium, sodium, sulfur, iron, chlorine, etc., all essential for plant growth and development. It has been reported that plants with mycorrhizal association are more tolerant to heavy metal toxicity. These plants survive well in drought and arid conditions as improved water movement is facilitated by mycorrhiza. Theoretically, the most efficient level of nutrients is the concentration of mineral elements in the plant tissue just above the "critical level" necessary for optimum growth. Further addition of chemical fertilizers may be taken up by plants, as "luxury concentration." This adds very little to plant growth. Now, these microorganisms help in constituting the "optimum level" of minerals in the plant tissue even at low level of fertilizer inputs. They fix nitrogen, solubilize phosphorus, and facilitate uptake of minerals by roots. Thus, these microorganisms in the form of biofertilizers are essential for maintaining good soil fertility, better soil conditions, and sustainable agricultural productivity.

Seed Treatments

There is a growing curiosity in the use of soil microorganisms which are beneficial for plant development as potential substitutes to synthetic fertilizers and pesticides in agricultural production. Seed inoculation techniques developed for research purposes are often not possible to be implemented at a commercial scale because of significant obstacles or challenges like technical aspects for maintaining viable microbial inocula throughout complete seed treatment process and seed storage. Further research advances in these technologies are required for imparting benefits of a wide range of environmentally sensitive potential seed inoculants in the field of agriculture. Presently, there are no solutions available for commercialization of seed inoculation treatments at commercial scale. So, there is an urgent need for association of scientific fields like soil science, microbiology, biotechnology, agriculture, and adjuvant chemistry to develop a sustainable protocol for making these technologies commercially viable and available to farmers.

Control of Plant Diseases and Plant Growth Promotion

Seeds are exposed to fungicides and bactericides in order to prevent crop failure because of seed- or soil-borne pathogens. Normally these treatments are chemical techniques which are cytotoxic; it means they can also have adverse effects on the viability of seeds and their germination potential. Microbial inocula, which are antagonistic to soil-borne pathogens, is an ideal delivery system as it directly introduces inoculum to the rhizosphere of the plant where plant pathogens like *Pythium* and *Rhizoctonia* are active, causing diseases like seed rots in the spermosphere and damping-off disease in seedling. Various bacterial and fungal antagonists have been identified and developed experimentally and commercially for this purpose, but their use as seed treatments is still very limited

BIOPESTICIDES

The term biopesticides defines compounds that are used to manage agricultural pests by means of specific biological effects rather than as broader chemical pesticides. It refers to products containing biocontrol agents – i.e., natural organisms or substances derived from natural materials (such as animals, plants, bacteria, or certain minerals), including their genes or metabolites, for controlling pests. According to the FAO definition, biopesticides include those biocontrol agents that are passive agents, in contrast to biocontrol agents that actively seek out the pest, such as parasitoids, predators, and many species of entomopathogenic nematodes. Thus biopesticides cover a wide spectrum of potential products that can be classified as follows: l Microbial pesticides and other entomopathogens: pesticides that contain microorganisms, like bacteria, fungi, or virus, which attack specific pest species, or entomopathogenic nematodes as active ingredients. Although most of these agents attack insect species (called entomopathogens; products referred to as bioinsecticides), there are also microorganisms (i.e., fungi) that control weeds (bioherbicides). l Plant-Incorporated Protectants (PIPs): these include pesticidal substances that are produced in genetically modified plants/organisms (GMO) (i.e., through the genetic material that has been incorporated into the plant). l Biochemical pesticides: pesticides based on naturally occurring substances that control pests by non-toxic mechanisms, in contrast to chemical pesticides that contain synthetic molecules that directly kill the pest. Biochemical pesticides fall into different biologically functional classes, including pheromones and other semiochemicals, plant extracts, and natural insect growth regulators. Biopesticides generally have several advantages compared to conventional pesticides (Kaya and Lacey 2007, Kaya and Vega 2012). While chemical pesticides are responsible for extensive pollution of the environment, a serious health hazard due to the presence of their residues in food, development of resistance in targeted insect pest populations, a decrease in biodiversity, and outbreaks of secondary pests that are normally controlled by natural enemies, biopesticides, in contrast, are inherently less toxic to humans and the environment, do not leave harmful residues, and are usually more specific to target pests. Often they affect only the target pest and closely related organisms, substantially reducing the impact on non-target species. A further advantage of most microbial pesticides is that they replicate in their target hosts and persist in the environment due to horizontal and vertical transmission, which may cause long-term suppression of pest populations even without repeating the application. Since the use of the biopesticides is markedly safer for the environment and users, and more sustainable than the application of chemicals, their use as alternatives to chemical pesticides, especially as components in Integrated Pest Management (IPM) strategies, is of growing interest. Several biopesticides of the different classes have proved to be very effective in controlling potato pests; however, there are certain disadvantages associated with their use that have prevented them from being used on a wider basis in potato production today. The very high specificity of the products might be a disadvantage when a complex of pest species needs to be controlled. Since biopesticides often contain living material, the products have reduced shelf lives. Also, their efficacy is often variable due to the influence of various biotic and abiotic factors. For using biopesticides

effectively, users need to have good knowledge about managing the particular pests or pest complexes. Due to limited commercial use (niche products) biopesticides often are developed by research institutions rather than by the traditional pesticide industry. While effective active ingredients have been discovered, products might lack appropriate formulation for efficient field use. A broader set of perspectives in the design and launch of a biopesticide would be helpful. Farmers consider biopesticides often as an alternative to a chemical pesticide, in which the active ingredient is thought to be synthetic, having a similar mode of action to the chemical pesticide. But the truth is that biopesticides differ in their modes of action from conventional chemical pesticide considerably; their modes of action are almost always specific. Therefore, using biopesticides efficiently requires specific user knowledge on the agent and the target pest for optimizing application time, field rates, and application intervals. Biopesticides should not be considered as a one-for-one replacement of chemical pesticides. As pesticides in general, biopesticides need to be approved and registered as such in most countries before they can be used, sold, or supplied. Since biopesticides pose fewer risks than conventional pesticides, authorities generally require fewer data for their registration. For example, the Environmental Protection Agency (EPA) in the USA often registers new biopesticides in less than a year, compared with an average of more than 3 years for conventional pesticides. However, in some cases it is difficult to determine whether a product meets the criteria for classification as a biopesticide, and the decision by local agencies might vary depending on the regulations in each country. There might be specific requirements pertinent to the different categories of biopesticides. In this chapter, the major biopesticides of potato pests and their potential for integrated pest management are reviewed according to their categories. Because knowledge about the mode of action of each type of active ingredient is crucial, each subsection includes a brief description of the biocontrol agent.

Factors	Benefits of biopesticides
Cost effectiveness	Costlier but reduced number of applications
Persistence and residual effect	Low, mostly biodegradable and self perpetuating
Knockdown effect	Delayed
Handling and Bulkiness	Bulky: Carrier based Easy:Liquid formulation
Pest resurgence	Less
Resistence	Less prone
Effect on beneficial flora	Less harmful on beneficial pests
Target specificity	Mostly host specific
Waiting time	Almost nil
Nature of control	Preventive
Shelf life	Less

Biopesticides fall into three major categories:

(1) **Microbial pesticides** contain a microorganism (bacterium, fungus, virus, protozoan or alga) as the active ingredient. Microbial pesticides can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest[s]. For example, there are

fungi that control certain weeds, and other fungi that kill specific insects. The most widely known microbial pesticides are varieties of the bacterium *Bacillus thuringiensis*, or Bt, which can control certain insects in cabbage, potatoes, and other crops. Bt produces a protein that is harmful to specific insect pests. Certain other microbial pesticides act by out-competing pest organisms. Microbial pesticides need to be continuously monitored to ensure they do not become capable of harming non-target organisms, including humans.

(2) **Plant-pesticides** are pesticidal substances that plants produce from genetic material that has been added to the plant. For example, scientists can take the gene for the Bt pesticidal protein, and introduce the gene into the plants own genetic material. Then the plant, instead of the Bt bacterium manufactures the substance that destroys the pest. Both the protein and its genetic material are regulated by EPA; the plant itself is not regulated.

(3) **Biochemical pesticides** are naturally occurring substances that control pests by non-toxic mechanisms. Conventional pesticides, by contrast, are synthetic materials that usually kill or inactivate the pest. Biochemical pesticides include substances that interfere with growth or mating, such as plant growth regulators, or substances that repel or attract pests, such as pheromones. Because it is sometimes difficult to determine whether a natural pesticide controls the pest by a non-toxic mode of action, EPA has established a committee to determine whether a pesticide meets the criteria for a biochemical pesticide. The growth of total world production of biopesticides is rising and therefore demand and use is also increasing. In India, biopesticide consumption has shown its increased use over the time (Figure 1). In 2005-06, consumption of biopesticides in India stands at 1920 MT.

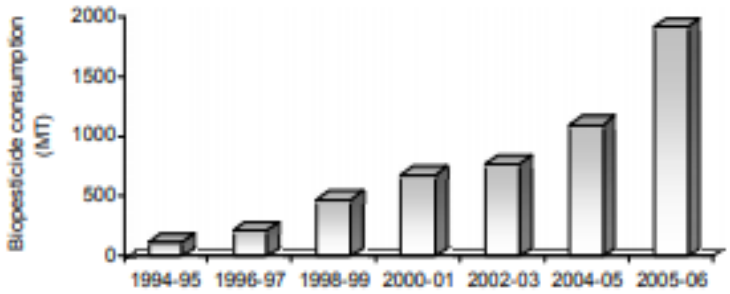


Figure 1. Consumption of biopesticides (MT)

India has a vast potential for biopesticides. However, its adoption by farmers in India needs education for maximizing gains. Biopesticides represent only 2.89% (as on 2005) of the overall pesticide market in India and is expected to exhibit an annual growth rate of about 2.3% in the coming years (Thakore, 2006). In India, so far only 12 types of biopesti cides have been

registered under the Insecticide Act, 1968.

S. No.	Name of the Biopesticide
1.	*Bacillus thuringiensis var. israelensis*
2.	*Bacillus thuringiensis var. kurstaki*
3.	*Bacillus thuringiensis var. galleriae*
4.	*Bacillus sphaericus*
5.	*Trichoderma viride*
6.	*Trichoderma harzianum*
7.	*Pseudomonas fluoresens*
8.	*Beauveria bassiana*
9.	NPV of *Helicoverpa armigera*
10.	NPV of *Spodoptera litura*
11.	*Neem based pesticides*
12.	Cymbopogan

Neem

Derived from the neem tree (*Azadirachta indica*), this contains several chemicals, including 'azadirachtin', which affects the reproductive and digestive process of a number of important pests. Recent research carried out in India and abroad has led to the development of effective formulations of neem, which are being commercially produced. As neem is non-toxic to birds and mammals and is non-carcinogenic, its demand is likely to increase. However, the present demand is very small. Although more than 100 firms are registered to produce neem-based pesticides in India, only a handful are actually producing it. Furthermore, very little of the production is sold locally, most being for export markets.

Bacillus thuringiensis (Bt)

Bacillus thuringiensis is the most commonly used biopesticide globally. It is primarily a pathogen of lepidopterous pests like American bollworm in cotton and stem borers in rice. When ingested by pest larvae, Bt releases toxins which damage the mid gut of the pest, eventually killing it. Main sources for the production of BT preparations are the strains of the subspecies kurstaki, galeriae and dendrolimus.

Baculoviruses

These are target specific viruses which can infect and destroy a number of important plant pests. They are particularly effective against the lepidopterous pests of cotton, rice and vegetables. Their large-scale production poses certain difficulties, so their use has been limited to small areas. They are not available commercially in India, but are being produced on a small scale by various IPM centres and state agricultural departments.

Trichoderma

Trichoderma is a fungicide effective against soil born diseases such as root rot. It is particularly relevant for dryland crops such as groundnut, black gram, green gram and chickpea, which are susceptible to these diseases. Preparation of Trichoderma biopesticide is cheap and requires only basic knowledge of microbiology.

Trichogramma *Trichogramma* are minute wasps which are exclusively eggparasites. They lay eggs in the eggs of various lepidopteran pests. After hatching, the *Trichogramma* larvae feed on and destroy the host egg. *Trichogramma* is particularly effective against lepidopteran pests like the sugarcane internode borer, pink bollworm and sooted bollworms in cotton and stem borers in rice. They are also used against vegetable and fruit pests. *Trichogramma* is the most popular biocontrol agent in India, mainly because it kills the pest in the egg stage, ensuring that the parasite is destroyed before any damage is done to the crop. *Trichogramma* eggs have to be used within a short period (before the eggs hatch). This limits their production and marketing on a large scale, and is also the reason why *Trichogramma* is not sold through dealers and shopkeepers. Some success stories about successful utilization of biopesticides and bio-control agents in Indian agriculture include: 1. Control of diamondback moths by *Bacillus thuringiensis*, 2. Control of mango hoppers and mealy bugs and coffee pod borer by *Beauveria*, 3. Control of Helicoverpa on cotton, pigeon-pea, and tomato by *Bacillus thuringiensis*, 4. Control of white fly on cotton by neem products, 5. Control of Helicoverpa on gram by N.P.V., 6. Control of sugarcane borers by Trichogramma and 7. Control of rots and wilts in various crops by *Trichoderma*-based products.

Opportunities

The area under organic cultivation (crops) in India is estimated to be around 1,00,000 hectare. Besides, there are lakhs of hectare of forest area being certified as organic. Further, some states like Uttaranchal and Sikkim have declared their states as organic. Moreover, the area under organic crop cultivation may rise because of the growing demand of organic food, a result of increasing health consciousness among the people. This indicates that there is huge scope for growth of the biopesticide sector in India. At the same time increasing population can be fed by organic farming dependence is a big question and unless organic farming yield can be brought equal to that of conventional farming involving the use of agrochemicals etc, the organic farming may not be feasible at the moment. Analysts believe that there would be a greater development in the biopesticides sector (Desai, 1997). Due to its rich biodiversity India offers plenty of scope in terms of sources for natural biological control organisms as well as natural plant based pesticides. The rich traditional knowledge base available with the highly diverse indigenous communities in India may provide valuable clues for developing newer and effective biopesticide. The stress on organic farming and on residue free commodities would certainly warrant increased adoption of biopesticides by the farmers. Increased adoption further depends on1.Concrete evidences of efficacy of biopesticides in controlling crop damage and the resultant increase in crop yield, 2. Availability of high quality products at affordable prices, 3. Strengthning of supply chain management in order to increase the usage of biopesticides. In this regard, an efficient delivery system from the place of production (factory) to place of utilization (farm) of biopesticides is quite essential. The National Farmer Policy 2007 has strongly recommended the promotion of biopesticides for increasing agricultural production, sustaining the health of farmers and environment. It also includes the clause that biopesticides would be treated at par with chemical pesticides in terms of support and promotion. Further research and

development of biological pest control methods must be given priority and people in general and agriculturists in particular must be educated about the handling and use of such control measures. All this will lead to a general understanding about the benefits of biopesticides as green alternative. However, the need in the present day context is on IPM, INM, ICM and GAP and by practicing these the quality of life and health will be assured.

BIOFERTILIZER

A **biofertilizer** (also **bio-fertilizer**) is a substance which contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonize the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. Biofertilizers add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth-promoting substances. Biofertilizers can be expected to reduce the use of synthetic fertilizers and pesticides. The microorganisms in biofertilizers restore the soil's natural nutrient cycle and build soil organic matter. Through the use of biofertilizers, healthy plants can be grown, while enhancing the sustainability and the health of the soil. Since they play several roles, a preferred scientific term for such beneficial bacteria is "plant-growth promoting rhizobacteria" (PGPR). Therefore, they are extremely advantageous in enriching soil fertility and fulfilling plant nutrient requirements by supplying the organic nutrients through microorganism and their byproducts. Hence, biofertilizers do not contain any chemicals which are harmful to the living soil.

Biofertilizers provide "eco-friendly" organic agro-input. Biofertilizers such as *Rhizobium*, *Azotobacter*, *Azospirilium* and blue green algae (BGA) have been in use a long time. *Rhizobium* inoculant is used for leguminous crops. *Azotobacter* can be used with crops like wheat, maize, mustard, cotton, potato and other vegetable crops. *Azospirillum* inoculations are recommended mainly for sorghum, millets, maize, sugarcane and wheat. Blue green algae belonging to a general cyanobacteria genus, *Nostoc* or *Anabaena* or *Tolypothrix* or *Aulosira*, fix atmospheric nitrogen and are used as inoculations for paddy crop grown both under upland and low-land conditions. *Anabaena* in association with water fern *Azolla* contributes nitrogen up to 60 kg/ha/season and also enriches soils with organic matter.

Other types of bacteria, so-called phosphate-solubilizing bacteria, such as *Pantoea agglomerans* strain P5 or *Pseudomonas putida* strain P13, are able to solubilize the insoluble phosphate from organic and inorganic phosphate sources. In fact, due to immobilization of phosphate by mineral ions such as Fe, Al and Ca or organic acids, the rate of available phosphate (P_i) in soil is well below plant needs. In addition, chemical P_i fertilizers are also immobilized in the soil, immediately, so that less than 20 percent of added fertilizer is absorbed by plants. Therefore, reduction in P_i resources, on one hand, and environmental pollutions resulting from both production and applications of chemical P_i fertilizer, on the other hand, have already demanded the use of phosphate-solubilizing bacteria or phosphate biofertilizers.

Types:

Nitrogen Biofertilizers:-

This type of biofertilizers helps the agriculturists to determine the nitrogen level in the soil. Nitrogen is a necessary component which is used for the growth of the plant. Plants need a limited amount of nitrogen for their growth. The type of the crops also determines the level of nitrogen. Some crops need more nitrogen for their growth while some crops need fewer amounts. The type of the soil also determines that which type of biofertilizers is needed for this crop. Fr example, *Azotobacteria* is used for the non legume crops; Rhizobium is needed for the legume crops. Similarly blue green algae are needed to grow rice while *Acetobacter* is used to grow sugarcane.

Phosphorus biofertilizers:- Phosphorus biofertilizers are used to determine the phosphorus level in the soil. The need of phosphorus for the plant growth is also limited. Phosphorus biofertilizers make the soil get the required amount of phosphorus. It is not necessary that a particular phosphorus biofertilizers is used for a particular type of crop. They can be used for any types of the crops for example; *Acetobacter, Rhizobium* and other biofertilizers can use phosphotika for any crop type.

Compost Biofertilizers:-

Compost biofertilizers are those which make use of the animal dung to enrich the soil with useful microorganisms and nutrients. To convert the animals waste into a biofertilizers, the microorganisms like abcteria undergo biological processes and help in breaking down the waste. Cellulytic fungal culture and *Azetobacter* cultures can be used for the compost biofertilizers.

Advantages of biofertilizers:-

1) They help to get high yield of crops by making the soil rich with nutrients and useful microorganisms necessary for the growth of the plants.

2) Biofertilizers have replaced the chemical fertilizers as chemical fertilizers are not beneficial for the plants. They decrease the growth of the plants and make the environment polluted by releasing harmful chemicals.

3) Plant growth can be increased if biofertilizers are used, because they contain natural components which do not harm the plants but do the vice versa.

4) If the soil will be free of chemicals, it will retain its fertility which will be beneficial for the plants as well as the environment, because plants will be protected from getting any diseases and environment will be free of pollutants.

5) Biofertilizers destroy those harmful components from the soil which cause diseases in the plants. Plants can also be protected against drought and other strict conditions by using biofertilizers.

6) Biofertilizers are not costly and even poor farmers can make use of them.

7) They are environment friendly and protect the environment against pollutants.

Applications of biofertilizers to crop:-

Seedling root dip:-

This method is applied to the rice crop. A bed of water is spread on the land where the crop has

to grow. The seedlings of rice are planted in the water and are kept there for eight to ten hours.

Seed treatment:-

In this method, the nitrogen and phosphorus fertilizers are mixed together in the water. Then seeds are dipped in this mixture. After the applications of this paste to the seeds, seeds are dried. After they dry out, they have to be sown as soon as possible before they get damaged by harmful microorganisms.

Soil treatment:-

All the biofertilizers along with the compost fertilizers are mixed together. They are kept for one night. Then the next day this mixture is spread on the soil where seeds have to be sown.

BIOCOMPOSTING

Compost is organic matter that has been decomposed in a process called composting. This process recycles various organic materials otherwise regarded as waste products and produces a soil conditioner (the *compost*).

Compost is rich in nutrients. It is used, for example, in gardens, landscaping, horticulture, urban agriculture and organic farming. The compost itself is beneficial for the land in many ways, including as a soil conditioner, a fertilizer, addition of vital humus or humic acids, and as a natural pesticide for soil. In ecosystems, compost is useful for erosion control, land and stream reclamation, wetland construction, and as landfill cover.

At the simplest level, the process of composting requires making a heap of wet organic matter (also called green waste), such as leaves, grass, and food scraps, and waiting for the materials to break down into humus after a period of months. However, composting can also take place as a multi-step, closely monitored process with measured inputs of water, air, and carbon- and nitrogen-rich materials. The decomposition process is aided by shredding the plant matter, adding water and ensuring proper aeration by regularly turning the mixture when open piles or "windrows" are used. Fungi, earthworms and other detritivores further break up the material. Bacteria requiring oxygen to function (aerobic bacteria) and fungi manage the chemical process by converting the inputs into heat, carbon dioxide, and ammonium.

Fundamentals

Composting is an *aerobic method* (meaning that it requires the presence of air) of decomposing organic solid wastes. It can therefore be used to recycle organic material. The process involves decomposition of organic material into a humus-like material, known as compost, which is a good fertilizer for plants. Composting requires the following three components: human management, aerobic conditions, and development of internal biological heat.

Composting organisms require four equally important ingredients to work effectively:

- **Carbon —** for energy; the microbial oxidation of carbon produces the heat, if included at suggested levels. High carbon materials tend to be brown and dry.

- **Nitrogen —** to grow and reproduce more organisms to oxidize the carbon. High nitrogen materials tend to be green (or colorful, such as fruits and vegetables) and wet.
- **Oxygen —** for oxidizing the carbon, the decomposition process.
- **Water —** in the right amounts to maintain activity without causing anaerobic conditions.

Certain ratios of these materials will provide microorganisms to work at a rate that will heat up the pile. Active management of the pile (e.g. turning) is needed to maintain sufficient supply of oxygen and the right moisture level. The air/water balance is critical to maintaining high temperatures 130–160 °F (54–71 °C) until the materials are broken down.

The most efficient composting occurs with an optimal carbon:nitrogen ratio of about 25:1. Hot container composting focuses on retaining the heat to increase decomposition rate and produce compost more quickly. Rapid composting is favored by having a C/N ratio of ~30 or less. Above 30 the substrate is nitrogen starved, below 15 it is likely to outgas a portion of nitrogen as ammonia.

Nearly all plant and animal materials have both carbon and nitrogen, but amounts vary widely, with characteristics noted above (dry/wet, brown/green). Fresh grass clippings have an average ratio of about 15:1 and dry autumn leaves about 50:1 depending on species. Mixing equal parts by volume approximates the ideal C:N range. Few individual situations will provide the ideal mix of materials at any point. Observation of amounts, and consideration of different materials as a pile is built over time, can quickly achieve a workable technique for the individual situation.

Microorganisms

With the proper mixture of water, oxygen, carbon, and nitrogen, micro-organisms are able to break down organic matter to produce compost. The composting process is dependent on micro-organisms to break down organic matter into compost. There are many types of microorganisms found in active compost of which the most common are:

- **Bacteria**- The most numerous of all the microorganisms found in compost. Depending on the phase of composting, mesophilic or thermophilic bacteria may predominate.
- **Actinobacteria**- Necessary for breaking down paper products such as newspaper, bark, etc.
- **Fungi**- molds and yeast help break down materials that bacteria cannot, especially lignin in woody material.
- **Protozoa**- Help consume bacteria, fungi and micro organic particulates.
- **Rotifers**- Rotifers help control populations of bacteria and small protozoans.

In addition, earthworms not only ingest partly composted material, but also continually re-create aeration and drainage tunnels as they move through the compost.

Phases of composting

Under ideal conditions, composting proceeds through three major phases:

- **Mesophilic phase:** An initial, mesophilic phase, in which the decomposition is carried out under moderate temperatures by mesophilic microorganisms.

- **Thermophilic phase:** As the temperature rises, a second, thermophilic phase starts, in which the decomposition is carried out by various thermophilic bacteria under high temperatures.
- **Maturation phase:** As the supply of high-energy compounds dwindles, the temperature starts to decrease, and the mesophiles once again predominate in the maturation phase.

Slow and rapid composting

There are many proponents of rapid composting that attempt to correct some of the perceived problems associated with traditional, slow composting. Many advocate that compost can be made in 2 to 3 weeks.Many such short processes involve a few changes to traditional methods, including smaller, more homogenized pieces in the compost, controlling carbon-to-nitrogen ratio (C:N) at 30 to 1 or less, and monitoring the moisture level more carefully. However, none of these parameters differ significantly from the early writings of compost researchers, suggesting that, in fact, modern composting has not made significant advances over the traditional methods that take a few months to work. For this reason and others, many scientists who deal with carbon transformations are skeptical that there is a "super-charged" way to get nature to make compost rapidly.

Both sides may be right to some extent. The bacterial activity in rapid high heat methods breaks down the material to the extent that pathogens and seeds are destroyed, and the original feedstock is unrecognizable. At this stage, the compost can be used to prepare fields or other planting areas. However, most professionals recommend that the compost be given time to cure before using in a nursery for starting seeds or growing young plants. The curing time allows fungi to continue the decomposition process and eliminating phytotoxic substances.

An alternative approach is anaerobic fermentation, known as bokashi. It retains carbon bonds, is faster than decomposition, and for application to soil requires only rapid but thorough aeration rather than curing. It depends on sufficient carbohydrates in the treated material.

Materials that can be composted

Composting is a process used for resource recovery. It can recycle an unwanted by-product from another process (a waste) into a useful new product.

Organic solid waste (green waste)

A large compost pile that is steaming with the heat generated by thermophilic microorganisms.

Composting is a process for converting decomposable organic materials into useful stable products. Therefore, valuable landfill space can be used for other wastes by composting these materials rather than dumping them on landfills. It may however be difficult to control inert and plastics contamination from municipal solid waste.

Co-composting is a technique that processes organic solid waste together with other input materials such as dewatered fecal sludge or sewage sludge.

Industrial composting systems are being installed to treat organic solid waste and recycle it rather than landfilling it. It is one example of an advanced waste processing system. Mechanical sorting of mixed waste streams combined with anaerobic digestion or in-vessel composting is called mechanical biological treatment. It is increasingly being used in developed countries due

to regulations controlling the amount of organic matter allowed in landfills. Treating biodegradable waste before it enters a landfill reduces global warming from fugitive methane; untreated waste breaks down anaerobically in a landfill, producing landfill gas that contains methane, a potent greenhouse gas.

Animal manure and bedding

On many farms, the basic composting ingredients are animal manure generated on the farm and bedding. Straw and sawdust are common bedding materials. Non-traditional bedding materials are also used, including newspaper and chopped cardboard. The amount of manure composted on a livestock farm is often determined by cleaning schedules, land availability, and weather conditions. Each type of manure has its own physical, chemical, and biological characteristics. Cattle and horse manures, when mixed with bedding, possess good qualities for composting. Swine manure, which is very wet and usually not mixed with bedding material, must be mixed with straw or similar raw materials. Poultry manure also must be blended with carbonaceous materials - those low in nitrogen preferred, such as sawdust or straw.

Human waste and sewage sludge

Human waste can be added as an input to the composting process since human excreta is a nitrogen-rich organic material. It can be either composted directly, as in composting toilets, or indirectly (as sewage sludge), after it has undergone treatment in a sewage treatment plant. Feces contain a wide range of microorganisms including bacteria, viruses and parasitic worms and its use in home composting can pose significant health risks.

Urine can be put on compost piles or directly used as fertilizer. Adding urine to compost can increase temperatures and therefore increase its ability to destroy pathogens and unwanted seeds. Unlike feces, urine does not attract disease-spreading flies (such as houseflies or blowflies), and it does not contain the most hardy of pathogens, such as parasitic worm eggs.

Uses : Compost can be used as an additive to soil, or other matrices such as coir and peat, as a tilth improver, supplying humus and nutrients. It provides a rich *growing medium* as absorbent material (porous). This material contains moisture and soluble minerals, which provides support and nutrients. Although it is rarely used alone, plants can flourish from mixed soil, sand, grit, bark chips, vermiculite, perlite, or clay granules to produce loam. Compost can be tilled directly into the soil or growing medium to boost the level of organic matter and the overall fertility of the soil. Compost that is ready to be used as an additive is dark brown or even black with an earthy smell.

Generally, direct seeding into a compost is not recommended due to the speed with which it may dry and the possible presence of phytotoxins in immature compost that may inhibit germination, and the possible tie up of nitrogen by incompletely decomposed lignin. It is very common to see blends of 20–30% compost used for transplanting seedlings at cotyledon stage or later.

Compost can be used to increase plant immunity to diseases and pests.

Composting technologies Various approaches have been developed to handle different ingredients, locations, throughput and applications for the composted product.

Industrial-scale Industrial-scale composting can be carried out in the form of in-vessel composting, aerated static pile composting, vermicomposting, or windrow composting.

Vermicomposting Vermicompost is the product or process of organic material degradation using various species of worms, usually red wigglers, white worms, and earthworms, to create a heterogeneous mixture of decomposing vegetable or food waste (excluding meat, dairy, fats, or oils), bedding materials, and vermicast. Vermicast, also known as worm castings, worm humus or worm manure, is the end-product of the breakdown of organic matter by species of earthworm.

Vermicomposting can also be applied for treatment of sewage sludge.

Composting toilets A composting toilet collects human excreta. These are added to a compost heap that can be located in a chamber below the toilet seat. Sawdust and straw or other carbon rich materials are usually added as well. Some composting toilets do not require water or electricity; others may. If they do not use water for flushing they fall into the category of dry toilets. Some composting toilet designs use urine diversion, others do not. When properly managed, they do not smell. The composting process in these toilets destroys pathogens to some extent. The amount of pathogen destruction depends on the temperature (mesophilic or thermophilic conditions) and composting time.

Composting toilets with a large composting container (of the type Clivus Multrum and derivations of it) are popular in United States, Canada, Australia, New Zealand and Sweden. They are available as commercial products, as designs for self-builders or as "design derivatives" which are marketed under various names.

Black soldier fly larvae Black soldier fly (*Hermetia illucens*) larvae are able to rapidly consume large amounts of organic material when kept at around 30 °C. Black soldier fly larvae can reduce the dry matter of the organic waste by 73% and convert 16–22% of the dry matter in the waste to biomass.[25][26] The resulting compost still contains nutrients and can be used for biogas production, or further traditional composting or vermicomposting.The larvae are rich in fat and protein, and can be used as, for example, animal feed or biodiesel production. Enthusiasts have experimented with a large number of different waste products.

Bokashi Bokashi is not composting as defined earlier, rather an alternative technology. It ferments (rather than decomposes) the input organic matter and feeds the result to the soil food web (rather than producing a soil conditioner). The process involves adding *Lactobacilli* to the input in an airtight container kept at normal room temperature. These bacteria ferment carbohydrates to lactic acid, which preserves the input. After this is complete the preserve is mixed into soil, converting the lactic acid to pyruvate, which enables soil life to consume the result.

Bokashi is typically applied to food waste from households, workplaces and catering establishments, because such waste normally holds a good proportion of carbohydrates; it is also applied to other organic waste by supplementing carbohydrates. Household containers ("bokashi bins") typically give a batch size of 5–10 kilograms (11–22 lb), accumulated over a few weeks. In horticultural settings batches can be orders of magnitude greater.

Inside a recently started bokashi bin. Food scraps are raised on a perforated plate (to drain runoff) and are partly covered by a layer of bran inoculated with *Lactobacilli*

Bokashi offers several advantages:

- Fermentation retains all the original carbon and energy. (In comparison, composting loses at least 50% of these and 75% or more in amateur use; composting also loses nitrogen, a macronutrient of plants, by emitting ammonia and the potent greenhouse gas nitrous oxide.)[30]
- Virtually the full range of food waste is accepted, without the exclusions of composting. The exception is large bones.
- Being airtight, the container inherently traps smells, and when opened the smell of fermentation is far less offensive than decomposition. Hence bokashi bins usually operate indoors, in or near kitchens.
- Similarly the container neither attracts insect pests nor allows them ingress.
- The process is inherently hygienic because lactic acid is a natural bactericide and anti-pathogen; even its own fermentation is self-limiting.
- Both preservation and consumption complete within a few weeks rather than months.
- The preserve can be stored until needed, for example if ground is frozen or waterlogged.
- The increased activity of the soil food web improves the soil texture, especially by worm action - in effect this is in-soil vermicomposting.

The importance of the first advantage should not be underestimated: the mass of any ecosystem depends on the energy it captures. Plants depend upon the soil ecosystem making nutrients available within soil water. Therefore, the richer the ecosystem, the richer the plants. (Plants can also take up nutrients from added chemicals, but these are at odds with the purpose of composting).

Other systems at household level

The practice of making raised garden beds or mounds filled with rotting wood is also called *hügelkultur* in German. It is in effect creating a nurse log that is covered with soil.

Benefits of *hügelkultur* garden beds include water retention and warming of soil. Buried wood acts like a sponge as it decomposes, able to capture water and store it for later use by crops planted on top of the *hügelkultur* bed.

Compost tea

Compost teas are defined as water extracts leached from composted materials. Compost teas are generally produced from adding one volume of compost to 4–10 volumes of water, but there has also been debate about the benefits of aerating the mixture. Field studies have shown the benefits of adding compost teas to crops due to the adding of organic matter, increased nutrient availability and increased microbial activity. They have also been shown to have an effect on plant pathogens.

Related technologies :

Organic ingredients intended for composting can also be used to generate biogas through anaerobic digestion. This process stabilizes organic material. The residual material, sometimes in combination with sewage sludge can be treated by a composting process before selling or giving away the compost.

Regulations

There are process and product guidelines in Europe that date to the early 1980s (Germany, the Netherlands, Switzerland) and only more recently in the UK and the US. In both these countries, private trade associations within the industry have established loose standards, some say as a stop-gap measure to discourage independent government agencies from establishing tougher consumer-friendly standards.

The USA is the only Western country that does not distinguish sludge-source compost from green-composts, and by default in the USA 50% of states expect composts to comply in some manner with the federal EPA 503 rule promulgated in 1984 for sludge products.[39]

Compost is regulated in Canada and Australia as well.

Many countries such as Wales and some individual cities such as Seattle and San Francisco require food and yard waste to be sorted for composting.

3. CLINICAL MICROBIOLOGY

Perhaps more than a million microbial species exist in nature, but only a few hundred species cause disease. Many microorganisms are closely associated with plants or animals, including humans, in beneficial relationships. However, pathogenic species have profoundly negative effects on host organisms. Here, we consider diseases transmitted from person to person. For example, influenza virus and streptococci cause diseases with overlapping symptoms, although the causal agents, one viral and one bacterial, are very different. Here these pathogens are discussed together because they are spread from person to person via a respiratory route. Using this approach, we will establish the connections between biologically diverse, but ecologically and pathogenically related, disease agents.

Airborne Transmission of Disease:

Aerosols, such as those generated by a human sneeze, are important vehicles for person-to-person transmission of many infectious diseases. Respiratory diseases are spread in this fashion. For example, Mycobacterium tuberculosis, the bacterium that causes the disease tuberculosis, has spread in this way to infect at least one-third of the world's population. In addition, respiratory spread of influenza and cold viruses is so efficient that virtually everyone has been infected, sometimes several times a year, as in the case of colds.

Fig 33.1High-speed photograph sneezing unstifled sneeze.

Airborne Pathogens

Microorganisms found in air are derived from soil, water, plants, animals, people, and other sources. In outdoor air, soil organisms predominate. Indoors, the concentration of microorganisms is considerably higher than outdoors, especially for organisms that originate in the human respiratory tract. Most microorganisms survive poorly in air. As a result, pathogens are effectively transmitted among humans only over short distances. Certain pathogens, however, survive under dry conditions and can remain alive in dust for long periods of time. Because of their thick, rigid cell walls, gram-positive bacteria (Staphylococcus, Streptococcus) are generally more resistant to drying than gram-negative bacteria. Likewise, the waxy layer of Mycobacterium cell walls resists drying and promotes survival. The endospores of endospore-forming bacteria are extremely resistant to drying but are not generally passed from human to human in the endospore form.

Large numbers of moisture droplets are expelled during sneezing (Figure 33.1), and a sizable number are expelled during coughing or simply talking. Each infectious droplet is about 10 μm in diameter and may contain one or two microbial cells or virions. The initial speed of the droplet movement is about 100 m/sec (more than 325 km/h) in a sneeze and ranges from 16 to 48 m/sec during coughing or shouting. The number of bacteria in a single sneeze varies from 10,000 to 100,000. Because of their small size, the moisture droplets evaporate quickly in the air, leaving behind a nucleus of organic matter and mucus to which bacterial cells are attached.

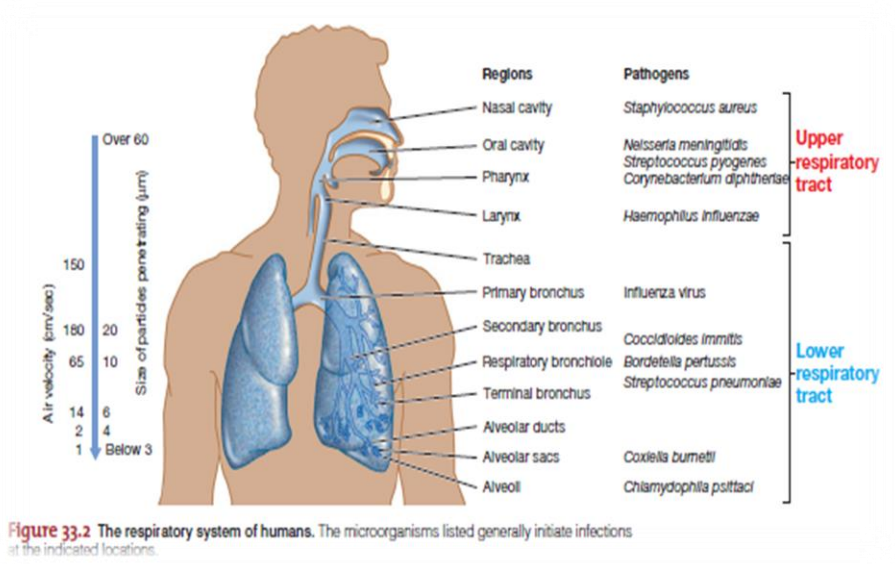


Figure 33.2 **The respiratory system of humans.** The microorganisms listed generally initiate infections at the indicated locations.

Respiratory Infections Humans breathe about 500 million liters of air in a lifetime, much of it containing microorganism-laden dust. The speed at which air moves through the respiratory tract varies, and in the lower respiratory tract the rate is quite slow. As air slows down, particles in it stop moving and settle. Large particles settle first and the smaller ones later; only particles smaller than 3 _m travel as far as the bronchioles in the lower respiratory tract (**Figure 33.2**). Of course, most pathogens are much smaller than this, and different organisms characteristically colonize the respiratory tract at different levels. The upper and lower respiratory tracts offer decidedly different environments, favoring different microorganisms.

Bacterial and Viral Pathogens

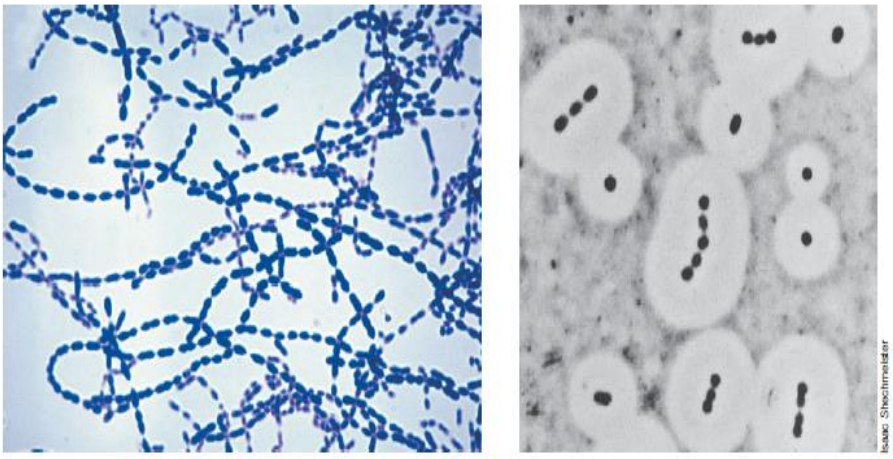
Most human respiratory pathogens are transmitted from person to person because humans are the only reservoir for the pathogens; pathogen survival thus depends on person-to-person transmission. Here we discuss some of the pathogens that are transmitted primarily via the respiratory route. However, many of these such as Streptococcus spp., cold viruses, and influenza can also be transmitted via direct contact or on fomites. A fewrespiratory pathogens such as Legionella pneumophila (legionellosis,or Legionnaires' disease) are transmitted primarily fromwater or soil and thus do not require person-to-person propagation. Bacterial and viral respiratory infections, serious in themselves, often initiate secondaryproblems that can be life-threatening. Thus, accurate and rapiddiagnosis and treatment of respiratory infections can limit hostdamage. Many bacterial and viral pathogens can be controlled byimmunization. Most respiratory bacterial pathogens respondreadily to antibiotic therapy, but antiviral drug treatment optionsare generally limited.

Streptococcal Diseases The bacteria Streptococcus pyogenes and Streptococcus pneumonia (**Figure 33.3**) are important human respiratory pathogens;both organisms are transmitted by the

respiratory route.S. pneumoniae is found in the respiratory flora of up to 40% ofhealthy individuals. Although endogenous strains do not causedisease in most normal individuals, they can cause severerespiratory disease in compromised individuals.Streptococci are nonsporulating, homofermentative, aerotolerant, anaerobic gram-positive cocci. Cells ofS. pyogenes (Figure 33.3a) typically grow in elongated chains, as domany other members of the genus. Pathogenicstrains of S. pneumoniae typically grow in pairs or short chains,and virulent strains produce an extensive polysaccharide capsule(Figure 33.3b).

***Streptococcus pyogenes*: Epidemiologyand Pathogenesis**

Streptococcus pyogenes, also called group A Streptococcus(GAS)(Figure 33.3a), is frequently isolated from the upper respiratorytract of healthy adults. Although numbers of endogenousS. pyogenes are usually low, if host defenses are weakened or anew, highly virulent strain is introduced, acute suppurative(pus-forming) infections are possible. S. pyogenes is the cause of streptococcal pharyngitis, or "strep throat." Most isolates fromclinical cases of streptococcal pharyngitis produce a toxin thatlyses red blood cells in culture media, a condition called beta-hemolysis. Streptococcal pharyngitis is characterizedby a severe sore throat, enlarged tonsils with exudate,tender cervical lymph nodes, a mild fever, and general malaise.S. pyogenes can also cause related infections of the middle ear(otitis media), the mammary glands (mastitis), infections of thesuperficial layers of the skin (pyoderma or impetigo) (impetigocan also be caused by Staphylococcus aureus) (**Figure 33.4**),erysipelas, an acute streptococcal skin infection (**Figure 33.5**),necrotizing fasciitis, an infection of subcutaneous tissue, andseveral conditions linked to the after effects of streptococcalinfections.



(a) (b)

Figure 33.3 ***Streptococcus* pathogens.** *(a) Streptococcus pyogenes* grows in chains. The cells range in size from 1 to 2 μm in diameter. *(b)* India ink negative stain of *Streptococcus pneumoniae*. An extensive capsule surrounds the cells, which are about 0.5–1.2 μm in diameter.

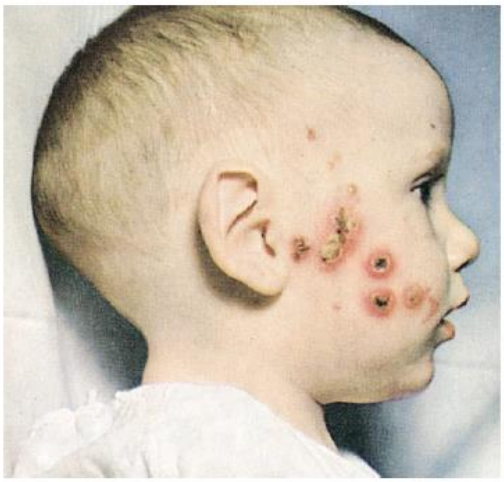


Figure 33.4 **Typical lesions of impetigo.** Impetigo is commonly caused by *Streptococcus pyogenes* or *Staphylococcus aureus*.

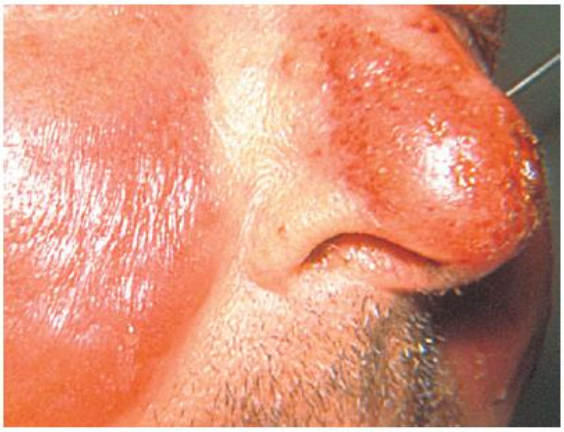


Figure 33.5 **Erysipelas.** Erysipelas is a *Streptococcus pyogenes* infection of the skin, shown here on the nose and cheeks, characterized by redness and distinct margins of infection. Other commonly-infected body sites include the ears and the legs.

About half of the clinical cases of severe sore throat are due to Streptococcus pyogenes, with most others due to viral infections. An accurate, rapid determination of the cause of the sore throat is important. If the sore throat is due to S. pyogenes, rapid, complete treatment of streptococcal sore throat is important because untreated streptococcal infections can lead to serious diseases such as scarlet fever, rheumatic fever, acute glomerulonephritis, and streptococcal toxic shock syndrome. On the other hand, if the sore throat is due to a virus, treatment with antibacterial drugs (antibiotics) will be useless, and may promote antimicrobial drug resistance. Certain GAS strains carry a lysogenic bacteriophage that encodes streptococcal pyrogenic exotoxin A (SpeA), SpeB, SpeC, and SpeF. These exotoxins are responsible for most of the symptoms of streptococcal toxic shock syndrome (STSS) and **scarletfever.** Streptococcal pyrogenic exotoxins are superantigens that recruit large numbers of T cells to the infected tissues. Toxic shock results when the activated T cells secrete cytokines, which in turn activate large numbers ofmacrophages and neutrophils, causing local and systemic inflammationand tissue destruction.Occasionally GAS causes fulminant (sudden and severe) invasivesystemic infection such as cellulitis, a skin infection in subcutaneouslayers, and necrotizing fasciitis, a rapid and progressivedisease resulting in extensive destruction of subcutaneous tissue,muscle, and fat. Necrotizing fasciitis is responsible for the dramaticreports of “flesh-eating bacteria.” In these cases, SpeA, SpeB,SpeC, and SpeF, as well as the bacterial cell surface M protein,function as superantigens. These diseases cause inflammationresulting in extensive tissue destruction.Invasive streptococcal disease including cellulitis, necrotizingfasciitis, scarlet fever, and STSS occur in an estimated 11,000patients per year. Death occurs in up to 15% of these patients(about 50% in STSS). In all of these cases, timely and adequatetreatment of the GAS infection stops production of the superantigenand its effects.

Other Streptococcal Diseases

Untreated or insufficiently treated S. pyogenes infections may leadto other serious diseases, even in the absence of active infection.These severe nonsuppurative (non-pus-forming) poststreptococcaldiseases usually occur about 1 to 4 weeks after the onset of astreptococcal infection. The immune response to the invadingpathogen produces antibodies that cross-react with host tissueantigens on the heart, joints, and kidneys, resulting in damage tothese tissues. The most serious of these diseases is **rheumaticfever** caused by rheumatogenic strains of S. pyogenes. Thesestrains contain cell surface antigens that are similar to heart valveand joint antigens. Rheumatic fever is an autoimmune disease;antibodies directed against streptococcal antigens also react withheart valve and joint antigens, causing inflammation and tissue Destruction. Damage to host tissues may bepermanent, and is often exacerbated by later streptococcal infectionsthat lead to recurring bouts of rheumatic fever.Another nonsuppurative disease is acute poststreptococcalglomerulonephritis, a painful kidney disease. This immune complexdisease develops following infection with S. pyogenes due tothe formation of streptococcal antigen–antibody complexes inthe blood. The immune complexes lodge in the glomeruli (filtrationmembranes of the kidney), causing inflammation of the kidney(nephritis) accompanied by severe pain. Within several days,the complexes are usually dissolved and the patient returns tonormal. Unfortunately, even timely antibacterial treatment maynot prevent glomerulonephritis. Only a few strains of S. pyogenes,so-called nephritogenic strains, produce this painful disease, butup to 15% of infections with nephritogenic strains cause glomerulonephritis. Because infection induces strain-specific immunity, reinfectionby a particular S. pyogenes strain is rare. However, there maybe up to 150 different strains defined by distinct cell surface Mproteins. Thus, an individual can be infected multiple times bydifferent S. pyogenes strains. There are no available vaccines toprevent S. pyogenes infections.

Diagnosis of *Streptococcus pyogenes*

Several rapid antigen detection (RAD) systems have been developedfor identification of S. pyogenes. Surface antigens are firstextracted by enzymatic or chemical means directly from a swabof the patient’s throat. The antigens are then detected using antibodiesspecific for surface proteins of S. pyogenes with immunologicalmethods such as latex bead agglutination, fluorescentantibody staining, and enzyme immunoassay (EIA). Using these methods, clinical specimenscan be quickly processed, sometimes in just a few minutes.Rapid diagnostic tests allow the physician to initiate appropriateantibiotic therapy to treat GAS infections and prevent more seriousdisease.

A more accurate confirmation of GAS infection is a positiveculture from the throat or lesion grown on sheep blood agar. Although the RAD tests are nearly as specific asthroat cultures, they can be up to 40% less sensitive, leading tofalse-negative reports. Throat cultures take up to two days toprocess, hence the popularity of the RAD tests. Serology tests arethe most sensitive tests available for identifying recent streptococcalinfections. Patients are examined for the presence

orincrease of antibodies (rise in titer) to streptococcal antigens.The detection of new antibodies or an increase in the quantity ofexisting antibodies confirms a recent streptococcal infection.

Streptococcus pneumoniae

The other major pathogenic streptococcal species, Streptococcus pneumoniae (Figure 33.3b), causes invasive lung infections that often develop as secondary infections to other respiratory disorders. Strains of S. pneumoniae that are encapsulated are particularly pathogenic because they are potentially very invasive. Cells invade alveolar tissues (lower respiratory tract) in the lung, where the capsule enables the cells to resist phagocytosis and elicit a strong host inflammatory response. Reduced lung function, called pneumonia, can result from accumulation of recruited phagocytic cells and fluid. The S. pneumoniae cells can then spread from the focus of infection as a bacteremia, sometimes resulting in bone infections, middle ear infections, and endocarditis. Untreated invasive pneumococcal disease has a mortality rate of about 30%. Even with aggressive antimicrobial treatment, individuals hospitalized with pneumococcal pneumonia have up to 10% mortality. Laboratory diagnosis of S. pneumoniae is based on the culture of gram-positive diplococci from either patient sputum or blood. There are over 90 different serotypes (antigenic capsule variants), and, as for S. pyogenes, infection induces immunity to only the infecting serotype of S. pneumoniae.

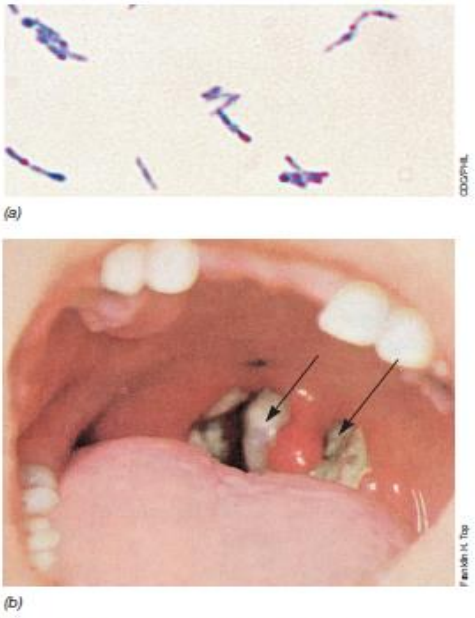


Figure 33.7 ***Corynebacterium*** **and diphtheria.** *(a)* Cells of *Corynebacterium diphtheriae* showing typical club-shaped appearance. The gram-positive cells are 0.5–1.0 μm in diameter and may be several micrometers in length. *(b)* Pseudomembrane (arrows) in an active case of diphtheria caused by the bacterium *C. diphtheriae*.

Prevention and treatment

Effective vaccines are available for prevention of infection by the most common strains of S. pneumoniae. A vaccine for adultsconsists of a mixture of 23 capsular polysaccharides from the most prevalent pathogenic strains. The vaccine is recommendedfor the elderly, healthcare providers, individuals with compromisedimmunity, and others at high risk for respiratory infections.A vaccine containing seven capsular polysaccharidesconjugated to diphtheria protein is recommended for children,age 2–23 months, to prevent ear infections. Novaccine is available for GAS.

Both GAS and S. pneumoniae can be treated with antibiotics. Penicillin G and its many derivatives are the agents of choice for treating GAS infections. Erythromycin and other antibacterial drugs are used in individuals who have penicillin allergies. S. pneumoniae infections respond quickly to penicillin G therapy, but up to 30% of pathogenic isolates now exhibit resistance to penicillin. Erythromycin and cefotaxime resistance is also found in some strains, and a few strains exhibit multiple drug resistance. Thus, each pathogenic isolate must be tested for antibiotic sensitivity. All strains are sensitive to vancomycin. Invasive disease such as pneumonia caused by drug-resistant S. pneumonia is now a reportable disease in the United States; more than 3000 cases are reported annually.

Diphtheria and Pertussis

Corynebacterium diphtheriae causes diphtheria, a severe respiratorydisease that typically infects children. Diphtheria is preventableand treatable. C. diphtheriae is a gram-positive, nonmotile, aerobic bacterium that forms irregular rods that may appear asclub-shaped cells during growth (**Figure 33.7*a***).**Pertussis** or **whooping cough** is a serious respiratory diseasecaused by infection with Bordetella pertussis, a small, gramnegative,aerobic coccobacillus that is a member of the Betaproteobacteria(**Figure 33.8**). Pertussis affects mostly children butcan cause serious respiratory disease for anyone. The disease ispreventable and curable.

Diphtheria Epidemiology, Pathology,Prevention, and Treatment

Diphtheria was once a major childhood disease, but it is nowrarely encountered because an effective vaccine is available. Inthe United States and other developed countries, the disease is virtually unknown. Worldwide, over 5000 fatal cases of diphtheriaoccur per year, largely because of a lack of effective immunizationprograms in less developed countries.Corynebacterium diphtheriae enters the body, infecting thetissues of the throat and tonsils. The organism spreads fromhealthy carriers or infected individuals to susceptible individualsby airborne droplets. Previous infection or immunization provides resistance to the effects of the potent diphtheria exotoxin. Throattissues respond to C. diphtheriae infection by forming a characteristiclesion called a pseudomembrane (Figure 33.7b), which consists of damaged host cells and cells of C. diphtheriae. Pathogenic strains of C. diphtheriae lysogenized by bacteriophage β produce a powerful exotoxin called diphtheria toxin that inhibitseukaryotic protein synthesis, leading to cell death. Death from diphtheria is usually due to a combination of the effects of partial suffocation and tissue destruction by exotoxin. In untreated infections, the toxin can cause systemic damage to the heart (about 25% of diphtheria patients develop myocarditis), kidneys, liver, and adrenal glands. C. diphtheria isolated from the throat is diagnostic for diphtheria. Nasal or throat swabs are used to inoculate blood agar, tellurite medium, or the selective Loeffler's medium that inhibits the growth of most other respiratory pathogens. Prevention of diphtheria is accomplished with a highly effective toxoid vaccine, part of the DTaP (diphtheria toxoid, tetanus toxoid, and acellular pertussis) vaccine. Penicillin,

erythromycin, and gentamicin are generally effective for stopping C. diphtheriae growth and further toxin production, but do not alter the effects of preformed toxin. Diphtheria antitoxin (an antiserum produced in horses) contains neutralizing antibodies, but is available only for serious acute cases of diphtheria. Early administration of both antibiotics and antitoxin is necessary for effective treatment of the acute disease.

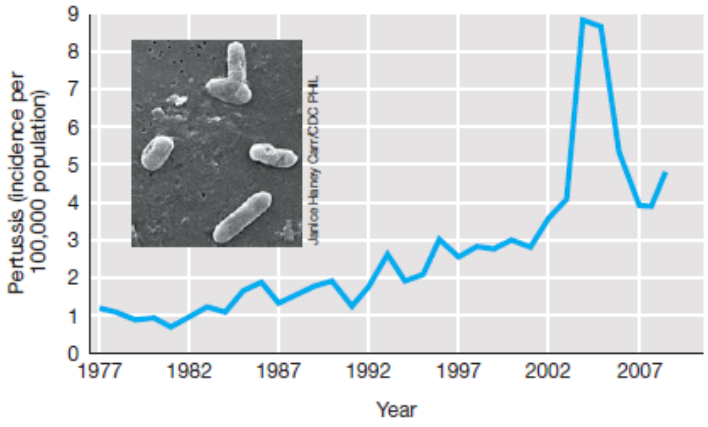


Figure 33.8 ***Bordetella* and pertussis.** The scanning electron micrograph (inset) shows the coccobacillus *Bordetella* sp. The variably shaped organisms range from 0.2 to 0.5 μm in diameter and are up to 1.0 μm in length. The graph shows the incidence of pertussis per 100,000 population caused by respiratory infection with *Bordetella pertussis*. There were 25,616 cases of pertussis in 2005, mostly in infants and school-age children, triple the number of 2001. After 2005, the incidence declined significantly, but was rising again by 2009. Data are from the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

Pertussis Pertussis, also known as whooping cough, is an acute, highly infectious respiratory disease now observed frequently in children under 19 years of age. Infants less than 6 months of age, who are

too young to be effectively vaccinated, have the highest incidence of disease and also have the most severe disease. B. pertussis attaches to cells of the upper respiratory tract by producing a specific adherence factor called filamentous hemagglutinin antigen,which recognizes a complementary molecule on the surfaceof host cells. Once attached, B. pertussis grows and producespertussis exotoxin. This potent toxin induces synthesis of cyclicadenosine monophosphate (cyclic AMP), which is at least partiallyresponsible for the events that lead to host tissue damage.B. pertussis also produces an endotoxin, which may inducesome of the symptoms of whooping cough. Clinically, whoopingcough is characterized by a recurrent, violent cough that can lastup to 6 weeks. The spasmodic coughing gives the disease itsname; a whooping sound results from the patient inhaling deepbreaths to obtain sufficient air. Worldwide, there are up to 50million cases and over 250,000 pertussis deaths each year, mostin developing countries. B. pertussis is endemic worldwide andpertussis remains a problem, even in developed countries, usuallydue to inadequate immunization.

Pertussis Epidemiology In the United States there has been an upward trend of B. pertussisinfections and disease since the 1980s, reversing a trend thatstarted with the introduction of an effective pertussis vaccine.

In 1976, the year of lowest prevalence and incidence, therewere only 1010 reported cases of pertussis. By contrast, in 2005,there were 25,616 cases. Although the numbers of infections have declined in recent years compared to the peak incidence in2004–2005, the incidence is still significantly higher than in the1990s (Figure 33.8). In the United States pertussis causes about 14 deaths per year. About 60% of recent cases were in adolescentsand adults of all ages who lacked appropriate immunity. About13% of cases were in children less than 6 months of age who hadnot yet received all of the recommended doses of pertussis vaccine.Up to 32% of coughs lasting 1 to 2 weeks or longer may becaused by B. pertussis. Pertussis is an endemic disease; incidencerises cyclically as populations become susceptible and areexposed to the pathogen. Lack of appropriate immunization at allages may be adding to the overall higher incidence of pertussis ascompared to recent decades.

Pertussis Diagnosis, Prevention, and Treatment Diagnosis of whooping cough can be made by fluorescent antibodystaining of a nasopharyngeal swab specimen or by actual culture of the organism. For best recovery of B. pertussis, a nasopharyngealaspirate is inoculated directly onto a blood–glycerol–potatoextract agar plate (although not selective, this rich medium supportsgood recovery of B. pertussis). The β-hemolytic coloniescontaining small gram-negative coccobacilli are tested forB. pertussis by a latex bead agglutination test or are stained witha fluorescent antibody specific for B. pertussis for positive identification.A polymerase chain reaction (PCR) test is consideredthe most sensitive and preferred diagnostic test. Improveddiagnostic and reporting techniques may be one reason for therecent observed increase in pertussis cases in the UnitedStates, but the disease may still be underreported, especially inadolescents and adults.A vaccine consisting of proteins derived from B. pertussis ispart of the routinely administered DTaP vaccine. This vaccine is normally given to children at appropriate intervals beginning soon after birth. The acellular pertussis vaccine has fewer side effects than the older pertussis vaccines and has caused no deaths. It is also recommended for adolescents and certain populations of adults (healthcare and childcare workers)as well as young children. Worldwide, immunization programs should be targeted tochildren, but immunization of adolescents and adults should alsobe a priority because vaccinated individuals lose effective immunitywithin 10 years and can transmit B. pertussis to youngchildren. Vaccination of a large percentage of the population isnecessary to build herd immunity.Cultures of B. pertussis are killed by ampicillin, tetracycline, anderythromycin, although antibiotics alone do not seem to be sufficientto kill the pathogen in vivo: A patient with whooping coughremains infectious for up to 2 weeks following commencement ofantibiotic therapy, indicating that the immune response may bemore important than antibiotics for eliminating B. pertussis fromthe body.

***Mycobacterium*, Tuberculosis, and Hansen's disease :** Tuberculosis (TB) is caused by the gram-positive, acid-fast bacillus Mycobacterium tuberculosis. The Germanmicrobiologist Robert

Koch isolated and described the causative agent in 1882. A related Mycobacterium species,Mycobacterium leprae, causes Hansen's disease (leprosy). All
mycobacteria share acid-fast properties due to the waxy mycolicacid constituent of their cell wall. Mycolic acid allows theseorganisms to retain carbol-fuchsin, a red dye, after washing in 3% hydrochloric acid in alcohol (**Figure 33.9**).

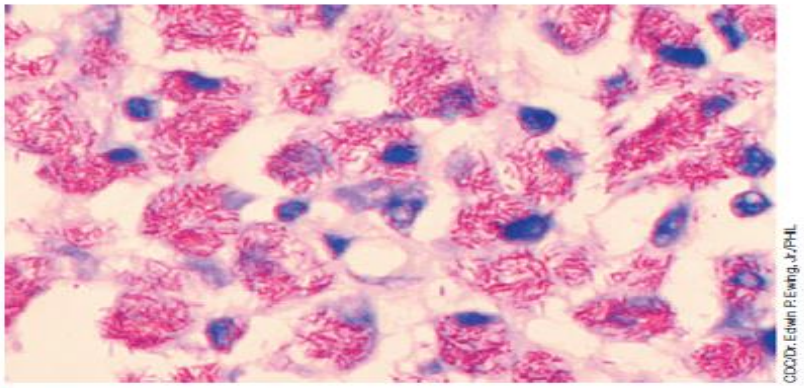


Figure 33.9 Mycobacteria. Here an acid-fast stained lymph node biopsy from a patient with HIV/AIDS displays *Mycobacteroum avium*. Multiple bacilli, stained red with carbol-fuchsin, are evident inside each cell. The individual rods are about 0.4 μm in diameter and up to 4 μm in length.

Tuberculosis Epidemiology

Mycobacterium tuberculosis is easily transmitted by the respiratory route; even normal conversation can spread the organism from person to person. At one time, TB was the most important infectious disease of humans and accounted for one-seventh of all deaths worldwide. Presently, over 13,000 new cases of TB and over 600 deaths occur each year in the United States. Worldwide, TB still accounts for over 1.4 million deaths per year, almost 11% of all deaths due to infectious disease. About one-third of the world's population has been infected with M. tuberculosis. Many new TB cases in the United States occur in acquired immunodeficiency syndrome (AIDS) patients.

Tuberculosis Pathology

The interaction of the human host and the bacterium M. tuberculosisis determined both by the virulence of the strain and the resistanceof the host. Cell-mediated immunity plays a critical role inthe prevention of active disease after infection. TB can be a primaryinfection (initial infection) or postprimary infection (reinfection).Primary infection typically results from inhalation of dropletscontaining viable M. tuberculosis bacteria from an individualwith an active pulmonary infection. The inhaled bacteria settle inthe lungs and grow. The host mounts an immune response toM. tuberculosis, resulting in a delayed-type hypersensitivity reaction
and the formation of aggregates of activatedmacrophages, called tubercles. Mycobacteriaoften survive and grow within the macrophages, even with anongoing immune response. In individuals with low resistance,the bacteria are not controlled and the pulmonary infectionbecomes acute,

leading to extensive destruction of lung tissue,the spread of the bacteria to other parts of the body, and death.In these cases, M. tuberculosis survives both the low pH andthe effects of the oxidative antibacterial products found in thelysosomes of phagocytes such as macrophages.In most cases of TB, however, an obvious acute infection doesnot occur. The infection remains localized, is usually inapparent,and appears to end. But this initial infection hypersensitizes the individual to the bacteria or their products and consequentlyalters the response of the individual to subsequent or post primary infections by M. tuberculosis. A diagnostic skin test, calledthe **tuberculin test**, can be used to measure this hypersensitivity.

In a hypersensitive individual, tuberculin, a protein extract from M. tuberculosis, elicits a local immune inflammatory reactionwithin 1–3 days at the site of an intradermal injection. The reactionis characterized by induration (hardening) and edema (swelling). An individual exhibiting this reactionis said to be tuberculin-positive, and many healthy adults showpositive reactions as a result of previous inapparent infections.A positive tuberculin test does not indicate active disease, butonly that the individual has been exposed to the organism in thepast and has generated a cell-mediated immune response againstM. tuberculosis.For most individuals, this cell-mediated immunity is protective and lifelong. However, some tuberculin-positive patients developpostprimary tuberculosis through reinfection from outsidesources or as a result of reactivation of bacteria that haveremained dormant in lung macrophages, often for years. For example, advanced age, malnutrition, overcrowding, stress, andhormonal changes may reduce effective immunity in untreatedindividuals and allow reactivation of dormant infections. Becauselatent M. tuberculosis can become activated many years after theinitial exposure and immune response, individuals who have apositive tuberculin test are treated with antimicrobial agents forlong periods of time. Postprimary mycobacterial infections oftenprogress to chronic infections that result in destruction of lungtissue, followed by partial healing and calcification at the infectionsite. Chronic postprimary TB often results in a gradualspread of tubercular lesions in the lungs. Bacteria are found inthe sputum in individuals with active disease, and areas of destroyed tissue can be seen in X-rays (**Figure 33.10**).

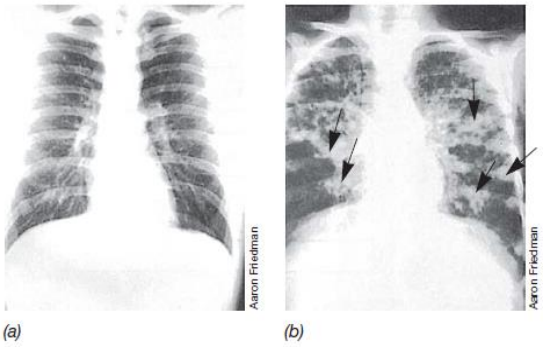


Figure 33.10 **Tuberculosis X-ray.** *(a)* Normal chest X-ray. The faint white lines are arteries and other blood vessels. *(b)* Chest X-ray of an advanced case of pulmonary tuberculosis; white patches (arrows) indicate areas of disease. These patches, or tubercles as they are called, may contain viable cells of *Mycobacterium tuberculosis*. Lung tissue and function is permanently destroyed by these lesions.

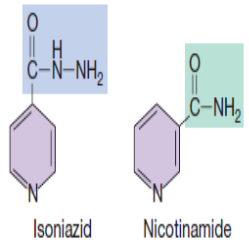


Figure 33.11 **Structure of isoniazid (isonicotinic acid hydrazide).** Isoniazid is an effective chemotherapeutic agent for tuberculosis. Note the structural similarity to nicotinamide.

Tuberculosis Prevention and Treatment

Individuals who have active cases of TB may spread the diseasesimply by coughing or speaking. Because TB is highly contagious,the U.S. Occupational Safety and Health Administration has stringent requirements for the protection of healthcare workerswho are responsible for TB patient care. For example, patientswith infectious tuberculosis must be hospitalized in negativepressurerooms. In addition, healthcare workers who havepatient contact must be provided with personally fitted facemasks having high-efficiency particulate air (HEPA) filters toprevent the passage of M. tuberculosis cells in sputum or on dustparticles.

Antimicrobial therapy of TB has been a major factor in controlof the disease. Streptomycin was the first effective antibiotic, butthe real revolution in treatment came with the discovery of isonicotinicacid hydrazide, called isoniazid (INH) (**Figure 33.11**). Thisdrug, specific for mycobacteria, is effective, inexpensive, relativelynontoxic, and readily absorbed when given orally. Although themode of action of isoniazid is not completely understood, itaffects the synthesis of mycolic acid by Mycobacterium. Mycolicacid is a lipid that complexes with peptidoglycan in the mycobacterialcell wall.Isoniazid probably functions as a growth factor analog of thestructurally related molecule, nicotinamide. As such, isoniazidwould be incorporated in place of nicotinamide and inactivateenzymes required for mycolic acid synthesis. Treatment ofmycobacteria with very small amounts of isoniazid (as little as 5picomoles [pmol] per 10^9 cells) results in complete inhibition ofmycolic acid synthesis, and continued incubation results in lossof outer areas of the cell wall, a loss of cellular integrity, and death. Following treatment with isoniazid, mycobacteria losetheir acid-fast properties, in keeping with the role of mycolic acidin this staining property.Treatment is typically achieved with daily doses of isoniazidand rifampin for 2 months, followed by biweekly doses for a total of 9 months. This treatment eradicates the pathogen and preventsemergence of antibiotic-resistant organisms. Failure tocomplete the entire prescribed treatment may allow the infection to be reactivated, and reactivated organisms are often resistant tothe original treatment drugs. Incomplete treatment encouragesantibiotic resistance because a high rate of spontaneous mutationsin surviving M. tuberculosis promotes rapid acquisition ofresistance to single antibiotics. To ensure treatment and thus discouragedevelopment of antibiotic-resistant organisms, directobservation of treatment may be necessary for noncompliantindividuals. In populations such as hospitals and nursing homes,where resistant mycobacterial strains are most likely to be present,patients are routinely treated with up to four drugs for2 months, followed by rifampin–isoniazid treatment for a totalof 6 months. Multiple drug therapy reduces the possibility thatstrains having resistance to more than one drug will emerge.Resistance of M. tuberculosis to isoniazid and other drugs,however, is increasing, especially in AIDS patients. A number ofstrains that are resistant to both isoniazid and rifampin havealready emerged. Treatment of these strains, called multidrug-resistant tuberculosis strains (MDR TB), requires the use ofsecond-line tuberculosis drugs that are generally more toxic, lesseffective, and more costly than rifampin and isoniazid. A WorldHealth Organization (WHO) survey indicated that up to 20% ofMDR TB strains are extensively drug-

resistant (XDR TB) strains.XDR TB strains have resistance to virtually all TB drugs, includingthe second-line drugs. Preventing emergence of these strains requires better diagnostic and drug susceptibility tests in addition to new anti-TB treatment drugs and regimens.In many countries, immunization with an attenuated strain of
Mycobacterium bovis, the Bacillus Calmette-Guerin (BCG) strain,is routine for prevention of TB. However, in the United Statesand other countries where the prevalence of *Mycobacterium tuberculosis* infection and disease is relatively low, immunizationwith BCG is discouraged. The live BCG vaccine induces adelayed-type hypersensitivity response, and all individuals who receive it develop a positive tuberculin test. *This compromisesthe tuberculin test as a diagnostic and epidemiologic indicatorfor the spread of M. tuberculosis infection.*

***Mycobacterium leprae* andHansen's Disease (Leprosy)**

Mycobacterium leprae, discovered by the Norwegian scientistG.A. Hansen in 1873, causes Hansen's disease, also known asleprosy. M. leprae is the only Mycobacterium species that has notbeen grown on artificial media. The armadillo is the only experimentalanimal that has been successfully used to grow M. lepraeand achieve symptoms similar to those in the human disease.The most serious form of Hansen's disease is lepromatousleprosy, characterized by folded, bulblike lesions on the body,especially on the face and extremities (**Figure 33.12**). The lesionsare due to the growth of M. leprae cells in the skin and maycontain up to 109 bacterial cells per gram of tissue. Like othermycobacteria, M. leprae from the lesions stain deep red with carbol-fuchsin in the acid-fast staining procedure, providing arapid, definitive demonstration of active infection. Lepromatousleprosy has a very poor prognosis. In severe cases the disfiguring

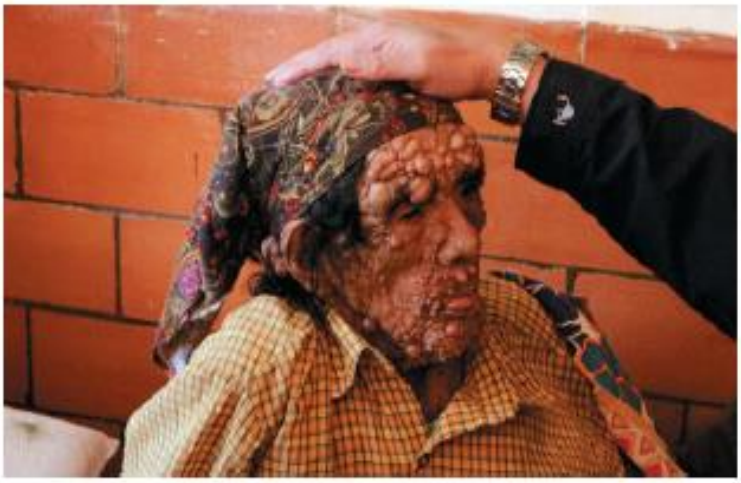


Figure 33.12 Lepromatous leprosy lesions on the skin. Lepromatous leprosy is caused by infection with *Mycobacterium leprae*. The lesions can contain up to 10^9 bacterial cells per gram of tissue, indicating an active uncontrolled infection with a poor prognosis.

lesions lead to destruction of peripheral nerves and loss of motor function. Many Hansen's disease patients exhibit less-pronounced lesions from which no bacterial cells can be recovered.

These individuals have the tuberculoid form of the disease. Tuberculoid leprosy is characterized by a vigorous delayed-type hypersensitivity response and a good prognosis for spontaneous recovery. Hansen's disease of either form, and the continuum ofintermediate forms, is treated using a multiple drug therapy(MDT) protocol, which includes some combination of dapsone(4,4'-sulfonylbisbenzeneamine), rifampin, and clofazimine. As inTB, drug-resistant strains have appeared, especially after inadequatetreatment or treatment with single drugs. Extended drugtherapy of up to 1 year with a MDT protocol is required foreradication of the organism.The pathogenicity of M. leprae is due to a combination ofdelayed hypersensitivity and the invasiveness of the organism.Transmission is by direct contact as well as respiratory routes, but Hansen's disease is not as highly contagious as TB. The timefrom exposure to onset of disease varies from several weeks toyears, or even decades. During this time, M. leprae cells growwithin macrophages, causing an intracellular infection that canresult in large numbers of bacteria within the skin, leading to thecharacteristic lesions.In many areas of the world, the incidence of Hansen's disease isvery low. Worldwide, however, over 750,000 new cases of the diseaseare reported each year. About 100 cases are reported annuallyin the United States, mostly in southern states, or amongimmigrants from the Caribbean islands or Central America.

Ninety percent of worldwide cases are in Madagascar, Mozambique,Tanzania, and Nepal. Up to 2 million people are permanentlydisabled as a result of Hansen's disease, but because of the chronic nature and long latent period of the disease, it may beunrecognized and unreported in as many as 12 million people.

Other Pathogenic *Mycobacterium* Species

A common pathogen of dairy cattle, Mycobacterium bovis ispathogenic for humans as well as other animals. M. bovis entershumans via the intestinal tract, typically from the ingestion of unpasteurized milk. After a localized intestinal infection, theorganism eventually spreads to the respiratory tract and initiatesthe classic symptoms of TB. M. bovis is a different organism from M. tuberculosis, although the genomes of the two organisms arevery similar. There is no observed difference in their infectivityand pathogenesis in humans, although the genome of M. bovishas several gene deletions compared with that of M. tuberculosis.Pasteurization of milk and elimination of diseased cattle haveeradicated bovine-to-human transmission of TB in developedcountries.

A number of other Mycobacterium species are also occasionalhuman pathogens. For example, M. kansasii, M. scrofulaceum,M. chelonae, and a few other mycobacterial species can cause disease.Respiratory disease due to the Mycobacterium avium complexof organisms (including M. avium and M. intracellulare)is particularly dangerous in AIDS patients or other immunecompromisedindividuals; these opportunistic pathogens rarelyinfect healthy individuals.

Neisseria meningitidis, Meningitis,and Meningococcemia

Meningitis is an inflammation of the meninges, the membranesthat line the central nervous system, especially the spinal cordand brain. Meningitis can be caused by viral, bacterial, fungal, orprotist infections. Here we will deal with infectious bacterialmeningitis caused by Neisseria meningitidis and a related infection,**meningococcemia**.Neisseria meningitidis, often called *meningococcus*, is a gramnegative,nonsporulating, obligately aerobic, oxidase-positive,encapsulated diplococcus, about0.6–1.0 _m in diameter. At least 13 pathogenic strains of *N. meningitidis* are recognized. Antigenic differences in capsularpolysaccharides distinguish each strain.

Epidemiology and Pathology

Meningococcal meningitis often occurs in epidemics, usually inclosed populations such as military installations and college campuses.It typically strikes older school-age children and youngadults. Up to 30% of individuals carry N. meningitidis in thenasopharynx with no apparent harmful effects. In epidemic situations,the prevalence of carriers may rise to 80%. The trigger forconversion from the asymptomatic carrier state to pathogenicacute infection is unknown.In an acute meningococcus infection, the bacterium is transmittedto the host, usually via the airborne route, and attaches to thecells of the nasopharynx. Once there, the organism gains access tothe bloodstream, causing bacteremia and upper respiratory tractsymptoms. The bacteremia sometimes leads to fulminantmeningococcemia, characterized by septicemia, intra vascular coagulation, shock, and death in over 10% of cases. *Meningitis* is another possible serious outcome of infection. Meningitis is characterizedby sudden onset of headache, vomiting, and stiff neck,and can progress to coma and death in a matter of hours. Up to 3%of acute meningococcal meningitis victims die.In the United States, there were 1057 cases of serious meningococcaldisease in 2008, the lowest number since 1977. The longterm decreased incidence indicates the success of widespreadvaccination in susceptible populations. However, the mortalityrate in recent years was over 10%.

Diagnosis, Prevention, and Treatment

Specimens isolated from nasopharyngeal swabs, blood, or cerebrospinalfluid are inoculated onto modified Thayer–Martin medium, a selective medium that suppressesthe growth of most normal flora, but allows the growth of thepathogenic members of the genus, *N. meningitidis* and Neisseriagonorrhoeae. Colonies showing gram-negative diplococcus morphologyand a positive oxidase test are presumptively identifiedas Neisseria. Due to the rapid onset of life threatening symptoms, preliminary diagnosis is often based onclinical symptoms and treatment is started before culture testsconfirm infection with *N. meningitidis*.Penicillin G is the drug of choice for the treatment of*N. meningitidis* infections. However, resistant strains have beenreported. Chloramphenicol is the accepted alternative agent fortreatment of infections in penicillin-sensitive individuals. Anumber of broad-spectrum cephalosporins are also effective.Naturally occurring strain-specific antibodies acquired by subclinicalinfections are effective for preventing infections in mostadults. Vaccines consisting of purified polysaccharides or polysaccharidesfrom the most prevalent pathogenic strains conjugatedto diphtheria toxin are available and are used to

immunizesusceptible individuals. The vaccines are used to prevent infectionin certain susceptible populations such as military recruitsand students living in dormitories. In addition, rifampin is oftenused as a chemoprophylactic antimicrobial drug to eradicate thecarrier state and prevent disease in close contacts of infectedindividuals.

Other Causes of Meningitis

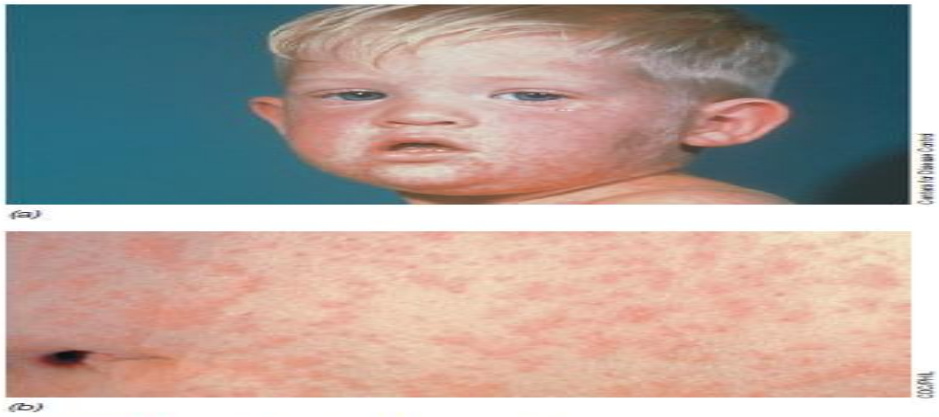
A number of other organisms can also cause meningitis. Acutemeningitis is usually caused by one of the pyogenic bacteria suchas Staphylococcus, Streptococcus, or *Haemophilus influenzae.H. influenzae* primarily infects young children. An effective vaccinefor preventing *H. influenzae* meningitis is available and isrequired in the United States for school-age children. Several viruses also cause meningitis. Among these are herpessimplex virus, lymphocytic choriomeningitis virus, mumps virus,and a variety of enteroviruses. In general, viral meningitis is lesssevere than bacterial meningitis.

Viruses and Respiratory Infections

The most prevalent human infectious diseases are caused byviruses. Most viral diseases are acute, self-limiting infections, butsome can be problematic in healthy adults. We begin here by describing measles, mumps, rubella, and chicken pox, all common,endemic viral diseases transmitted in infectious droplets byan airborne route.

Measles

Measles (rubeola or 7-day measles) affects susceptible children asan acute, highly infectious, often epidemic disease. The measlesvirus is a paramyxovirus, a negative-strand RNA virus that enters the nose and throat by airborne transmission,quickly leading to systemic viremia. Symptoms startwith nasal discharge and redness of the eyes. As the disease progresses,fever and cough appear and rapidly intensify, followed bya characteristic rash (**Figure 33.14**); symptoms generally persistfor 7–10 days. Circulating antibodies to measles virus are measurableabout 5 days after initiation of infection; the serum antibodiesand T-cytotoxic lymphocytes combine to eliminate thevirus from the system. Possible postinfection complicationsinclude middle ear infection, pneumonia, and, in rare cases,measles encephalomyelitis. Encephalomyelitis has a mortalityrate of nearly 20% and can cause neurological disorders includinga form of epilepsy. Of the 131 measles cases that occurred in 2008, 15 of the infected individuals were hospitalized.



(a)

(b)

Figure 33.14 Measles in children. (a) The light pink rash starts on t
head and neck, and (b) spreads to the chest, trunk, and limbs. Discret
papules coalesce into blotches as the rash progresses for several days

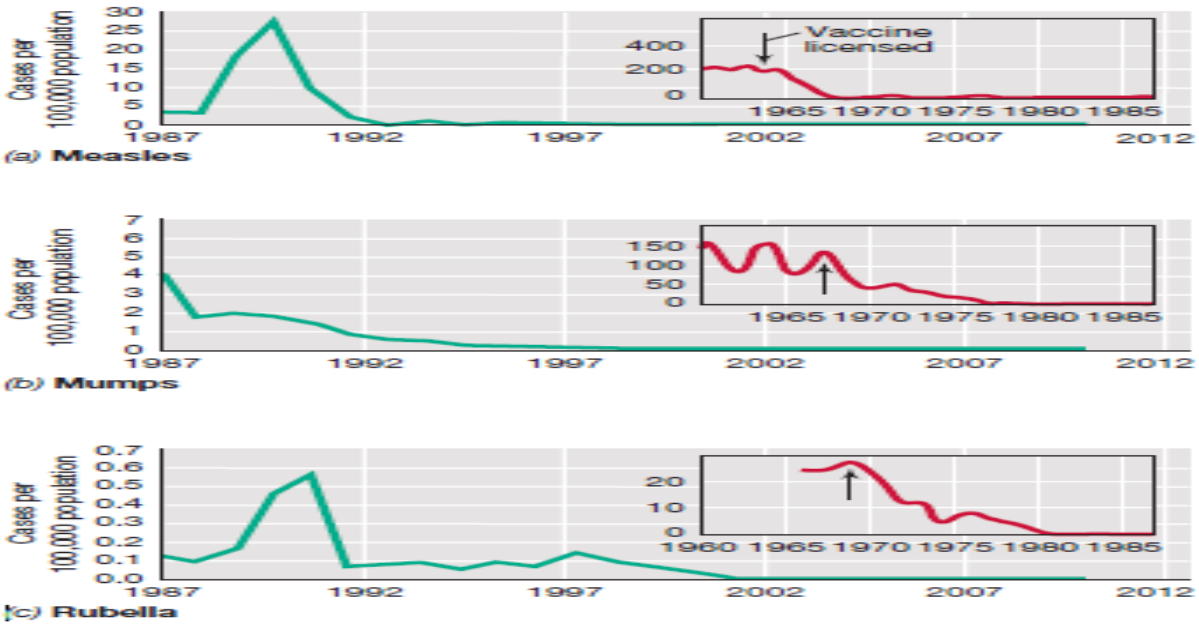


Figure 33.15 Viral diseases and vaccines. Major childhood viral diseases are now controlled by the MMR (measles, mumps, rubella) vaccine in the United States. Data are from the Centers for Disease Control and Prevention, Atlanta, Georgia.

Although once a common childhood illness, measles is generallylimited now to rather isolatedoutbreaks in the United Statesbecause of widespread immunization programs begun in the mid-1960s (**Figure 33.15*a***). Outbreaks generally occur only inpopulations that were not immunized or were inadequatelyimmunized. Over 90% of the cases were either acquired outsidethe United States or were associated with contact with travelersto foreign countries. Worldwide, measles remains endemic andstill causes over 400,000 annual deaths, mostly in children.Because the disease is highly infectious, all public school systemsin the United States require proof of immunization before a childcan enroll. Active immunization is done with an attenuated viruspreparation as part of the MMR (measles, mumps, rubella) vaccine. A childhood case of measles generallyconfers lifelong immunity to reinfection.

Mumps

Mumps, like measles, is caused by a paramyxovirus and is alsohighly infectious. Mumps is spread by airborne droplets, and thedisease is characterized by inflammation of the salivary glands,leading to swelling of the jaws and neck (**Figure 33.16**). The virusspreads through the bloodstream and may infect other organs, including the brain, testes, and pancreas. Severe complicationsmay include encephalitis and, very rarely, sterility. The hostimmune response produces antibodies to mumps virus surfaceproteins, and this generally leads to a quick recovery and lastingimmunity to reinfection. An attenuated vaccine is highly effectivefor preventing mumps. Hence, the prevalence of mumps in developedcountries is usually very low, with disease generally restrictedto individuals who did not receive the MMR vaccine (Figure33.15b). In 2006, however, an outbreak centered in the MidwesternUnited States involved more than 5000 cases, significantly up froma normal number of less than 300 cases per year since 2001. Theoutbreak

affected mainly young adults (18–34). As a result, recommendationsfor immunizations were revised to target school-agechildren, healthcare workers, and adults at high risk.

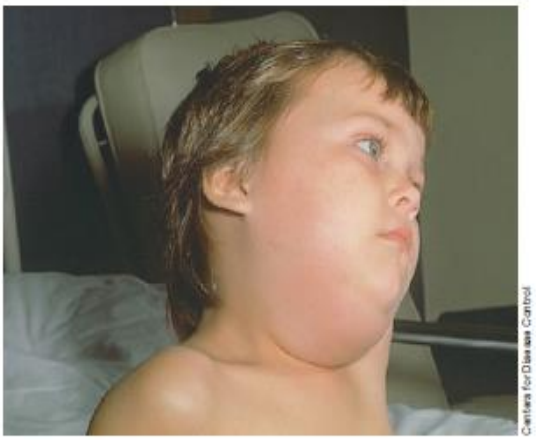


Figure 33.16 **Mumps.** Glandular swelling characterizes infection with the mumps virus.

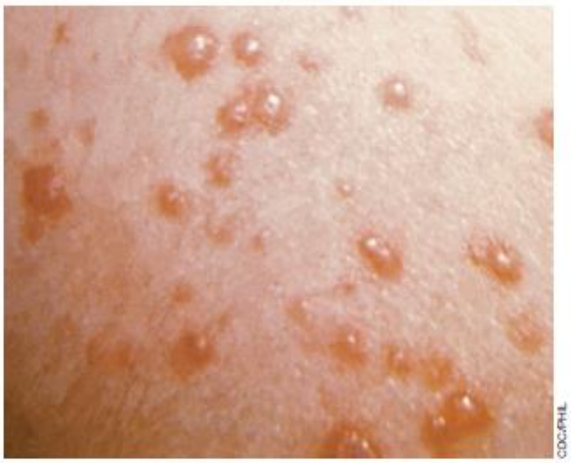


Figure 33.17 **Chicken pox.** Mild papular rash associated with the infection by varicella-zoster virus (VZV), the herpesvirus that causes chicken pox.

Rubella

Rubella (German measles or 3-day measles) is caused by a singlestranded,positive-sense RNA virus of the togavirus group. Disease symptoms resemble measles but are generallymilder. Rubella is less contagious than measles, and thus asignificant proportion of the population has never been infected.During the first three months of pregnancy, however, rubellavirus can infect the fetus by placental transmission and causeserious fetal abnormalities including stillbirth, deafness, heartand eye defects, and brain damage. Thus, women should not beimmunized with the rubella vaccine or contract rubella duringpregnancy. For this reason, routine childhood immunizationagainst rubella should be practiced. An attenuated virus isadministered as part of the MMR vaccine. Thelow incidence of cases since 2001, coupled with the high degreeof protection from the vaccine and the relatively low infectivity ofthe virus, suggest that rubella is no longer endemic in the UnitedStates (Figure 33.15c).

Chicken Pox and Shingles

Chicken pox (varicella) is a common childhood disease caused bythe varicella-zoster virus (VZV), a DNA herpesvirus. VZV is highly contagious and is transmitted by infectiousdroplets, especially when susceptible individuals are in close contact.In schoolchildren, for example, close confinement duringthe winter months leads to the spread of VZV through airbornedroplets from infected classmates and through contact with contaminatedfomites. The virus enters the respiratory tract, multiplies,and is quickly disseminated via the bloodstream, resultingin a systemic papular rash that quickly heals, rarely leaving disfiguringmarks (**Figure 33.17**). An attenuated virus vaccine is nowused in the United States. The reported annual incidence of chicken pox, now about 40,000 cases per year, is about onefourthof the number of cases reported prior to 1995, the year thevaccine was licensed for use. Since 2003, VZV infections havebeen

nationally notifiable, resulting in an increased number ofreported cases.VZV establishes a lifelong latent infection in nerve cells. Thevirus occasionally migrates from this reservoir to the skin surface,causing a painful skin eruption referred to as shingles(zoster). Shingles most commonly strikes immune suppressedindividuals or the elderly. The prophylactic use of human hyperimmuneglobulin prepared against the virus is useful for preventingthe onset of symptoms of shingles. Such therapy is advisedonly for patients for whom secondary infections such as pneumoniaor encephalitis, occasionally associated with shingles, maybe life-threatening. To prevent shingles, a vaccine is recommendedfor individuals over 60 years of age. The vaccine stimulatesantibody and T-cytotoxic cell immunity to VZV, keepingVZV from migrating out of nerve ganglia to skin cells.

Colds Colds are the most common of infectious diseases.People acquire about ten colds for every other
infectious disease, except influenza. Colds are viral infectionsthat are transmitted via droplets spread from person to person incoughs, sneezes, and respiratory secretions. Colds are usually of short duration, lasting 1 week or less, and the symptoms aremilder than other respiratory diseases such as influenza. **Table33.1** compares the symptoms of colds and influenza.Each person averages more than three colds per year throughouthis or her lifetime (Figure 33.18). Cold symptoms includerhinitis (inflammation of the nasal region, especially the mucousmembranes), nasal obstruction, watery nasal discharges, and ageneral feeling of malaise, usually without fever. Rhinoviruses,positive-sense, single-stranded RNA viruses of the picornavirus group (**Figure 33.19*a*** and Section 21.8), are the most commoncauses of colds. At least 115 different rhinoviruses havebeen identified. About 25% of colds are due to infections with other viruses. Coronaviruses (Figure 33.19b) cause 15% of allcolds in adults. Adenoviruses, coxsackie viruses, respiratory syncytialviruses (RSV), and orthomyxoviruses are collectively responsible for about 10% of colds. Each of these viruses may alsocause more serious disease. For example, one adenovirus strainproduces a severe and sometimes lethal respiratory infection.

Colds generally induce a specific, local, neutralizing IgA antibodyresponse. However, the number of potential infectious agents makes immunity due to previous exposure very unlikely.The sheer numbers of viruses that might cause a cold also precludethe development of useful vaccines.Aerosol transmission of the virus is probably the major meansof spreading colds, although experiments with volunteers suggestthat direct contact and fomite contact are also methods of transmission.Most antiviral drugs are ineffective against the commoncold, but a pyrazidine derivative (**Figure 33.20*a***) has shownpromise for preventing colds after virus exposure. In addition,new experimental antiviral drugs are being designed based oninformation derived from three-dimensional structures. For example, the antirhinovirus drug WIN 52084 (Figure 33.20b)binds to the virus, changing its three-dimensional surface configurationand disrupting rhinovirus binding to the host cell receptorICAM-1 (intercellular adhesion molecule-1), thus preventinginfection. Alpha interferon, a cytokine, is also effective in preventingthe onset

of colds. Thus, there are several experimentalpossibilities for cold prevention and treatment, although noneare widely accepted as effective and safe. Because colds are generally
brief and self-limiting, treatment is aimed at controllingsymptoms, especially nasal discharges, with antihistamine anddecongestant drugs.

Table 33.1 *Colds and influenza*

Symptoms	*Cold*	*Influenza*
Fever	Rare	Common (39–40°C); sudden onset
Headache	Rare	Common
General malaise	Slight	Common; often quite severe; can last several weeks
Nasal discharge	Common and abundant	Less common; usually not abundant
Sore throat	Common	Less common
Vomiting and/ or diarrhea	Rare	Common in children

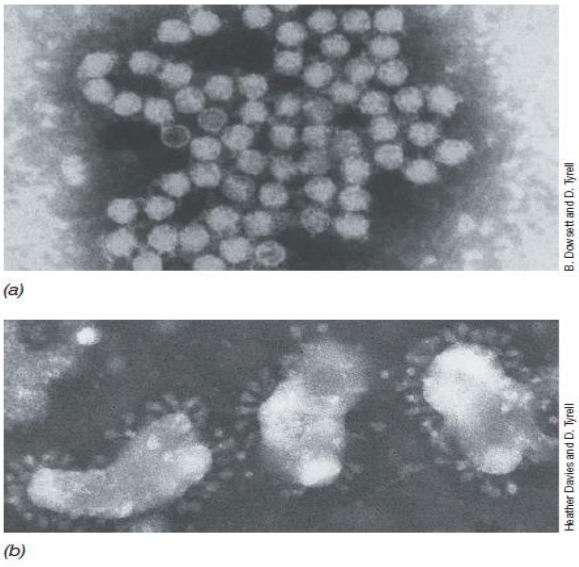


Figure 33.19 **Common cold viruses.** Transmission electron micrographs. *(a)* Human rhinovirus. Each rhinovirus virion is about 30 nm in diameter. *(b)* Human coronavirus. Each coronavirus virion is about 60 nm in diameter.

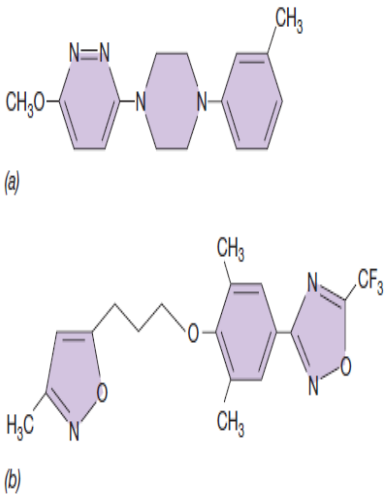


Figure 33.20 **Experimental antirhinovirus drugs.** *(a)* The structure of 3-methoxy-6-[4-(3-methylphenyl)]-1-piperazinyl. *(b)* The structure of WIN 52084, a receptor-blocking drug.

Influenza

Influenza is caused by an RNA virus of the orthomyxovirus group. Influenza virus is a single-stranded, negativesense,helical RNA genome surrounded by an envelope made up ofprotein, a lipid bilayer, and external glycoproteins (**Figure 33.21**).There are three different types of influenza viruses: influenza A,influenza B, and influenza C. Here we consider only influenza
A because it is the most important human pathogen.

Influenza Antigens and Genes

Each strain of influenza A virus can be identified by a unique setof surface glycoproteins. These glycoproteins are hemagglutinin(HA or H antigen) and neuraminidase (NA or N antigen). Each virus will have one type of HA and one type of NA on its surface.HA is important in the attachment of virus to the host cells. NAis instrumental for release of virus from host cells (Figure 33.21).Infection or immunization with an influenza strain results inproduction of IgA antibodies that are reactive with the HA andNA glycoproteins. When antibody binds to HA or NA, the virusis blocked from either attaching or releasing, and is neutralized,stopping the infection process. Over time, the HA and NA glycoproteinantigens acquire minor antigenic changes due to pointmutations in the RNA coding sequences. These changes alter oneor more amino acids in the glycoprotein, altering their ability tobe recognized by antibody. Thus, these mutations create slightlyaltered antigens, a phenomenon called **antigenic drift**. As aresult, immunity to a given virus strain diminishes as the strainmutates, and reinfection with the mutated strain can occur. The influenza A virus genome issingle-stranded RNA. The RNA genome is arranged in a highlyunusual manner; the genome is segmented, with single-stranded RNA genes found on each of eight distinct segments. During virus maturation in the host cell, the viral RNA segments are packaged randomly. To be infective,a virus must be packaged so it contains one copy of each of theeight gene segments.Occasionally more than one strain of influenza infects a singleanimal at one time. In such a case, the two strains could infect a single cell, and gene segments from both viruses would be reproduced.When packaging occurs, the segments from the twostrains may be mixed; an individual virus is likely to be a mosaic of the two infecting viruses, containing some, but not all, of thegenes from each virus. In effect, the mixed-genome virusinstantly becomes a new virus strain. This mixing of gene fragments between different strains of influenza virus is calledreassortment.Unique reassortant viruses result in **antigenic shift**, a majorchange in an antigen resulting from the total replacement of anRNA segment. Antigenic shift can immediately and completelychange one or both of the major HA and NA viral glycoproteinsand any of the other viral genes.

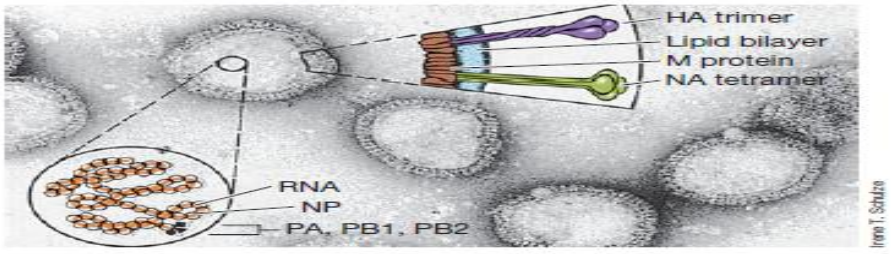


Figure 33.21 Electron micrograph of influenza virus. The photo shows the location of the major viral coat proteins and the nucleic acid. Each virion is about 100 nm in diameter. HA, hemagglutinin (three copies make up the HA coat spike); NA, neuraminidase (four copies make up the NA coat spike); M, coat protein; NP, nucleoprotein; PA, PB1, PB2, other internal proteins, some of which may have enzymatic functions.

Influenza Epidemiology

Human influenza virus is transmitted from person to personthrough the air, primarily in droplets expelled during coughingand sneezing. The virus infects the mucous membranes of theupper

respiratory tract and occasionally invades the lungs. Symptomsinclude a low-grade fever lasting 3–7 days, chills, fatigue,headache, and general aching (Table 33.1). Recovery is usuallyspontaneous and rapid. Most of the serious consequences of influenza infection occur from bacterial secondary infections inpersons whose resistance has been lowered by the influenza infection. Especially in infants and elderly people, influenza isoften followed by bacterial pneumonia; death, if it occurs, is usuallydue to the bacterial infection. Annually, influenza causes 3–5million cases of severe illness and is implicated in 250,000–500,000 deaths worldwide.Most infected individuals develop protective immunity to theinfecting virus, making it impossible for a strain of the same antigenictype to cause widespread infection—an epidemic—untilthe virus encounters another susceptible population. Immunityis dependent on the production of secretory IgA antibodies andT-cytotoxic lymphocytes directed at HA and NA glycoproteins.Influenza exists in human populations as an endemic viral disease,and severe localized influenza outbreaks occur every year from late autumn through the winter. Each year, antigenic driftresults in some reduction of immunity in the population and isresponsible for the recurrence of epidemics, severe widespreadoutbreaks, which occur in a 2- to 3-year cycle.

Table 33.2 *Influenza pandemics*

Year	*Name*	*Strain*
1889	Russian	H2N2
1900	Old Hong Kong	H3N8
1918	Spanish	H1N1
1957	Asian	H2N2
1968	Hong Kong	H3N2
2009	Swine	H1N1

Influenza Pandemics

Pandemics, worldwide epidemics, are much less frequent thanoutbreaks and epidemics, occurring from 10 to 40 years apart(**Table 33.2**). They result from antigenic shift involving reassortmentof viruses from two or more species. Virtually all of themajor pandemics resulted from reassortment of avian influenzaviruses and human influenza viruses in swine (**Figure 33.22**).Swine cells have receptors for both avian and human orthomyxovirusesand can bind and propagate both avian and humaninfluenza strains. If swine are infected with both human and avian strains at the same time, the two unrelated viruses can reassort,resulting in antigenically unique viruses (antigenic shift)that can infect many humans because of a lack of host immunity.

Reassortment with animal strains and infection into humansoccurs periodically but unpredictably, continually raising thepossibility of a rapidly emerging, highly virulent influenza strainfor which there is no preexisting immunity in the human population.Worldwide deaths due to the influenza A "Spanish flu" pandemicof 1918 was about 50 million, with some estimates as highas 100 million people worldwide; up to 2 million deaths occurredin the United States

(Figure 32.1). Although there have beenseveral pandemics during the last 130 years (Table 33.2), nonehas been as catastrophic as the 1918 flu. The virulence of the1918 influenza is not fully understood, but appears to be due tothe host response to the novel pathogen. This pathogen apparentlystimulated production and release of large amounts ofinflammatory cytokines, resulting in systemic inflammation anddisease in susceptible individuals.

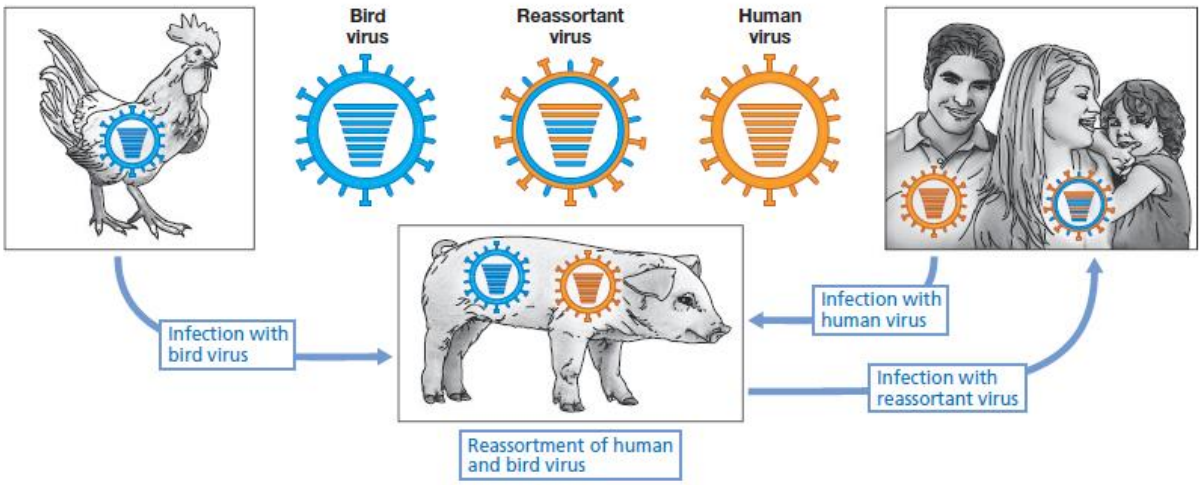


Figure 33.22 Influenza virus reassortment. Reassortments take place in swine. Influenza strains that originate in birds and humans can infect pigs. If a pig is infected at the same time with a bird virus and a human virus, the viruses can reassort. The reassortant virus may then infect humans. If the reassortant contains antigens that are unique, infections may cause pandemics.

The 1957 outbreak of the so-called Asian flu also developed into a pandemic. The pandemic strain was a virulentmutant virus, differing antigenically from all previousstrains. Immunity to this strain was not present, and the virusspread rapidly throughout the world. It first appeared in the interiorof China in February 1957 and by April had spread to HongKong. From Hong Kong, the virus infected sailors on naval shipsand emerged in San Diego, California. In May, an outbreakoccurred in Newport, Rhode Island, on a naval vessel. From thattime, outbreaks occurred continuously in various parts of theUnited States. The peak incidence occurred in October, when22 million new cases developed.Pandemic influenza A (H1N1) 2009 spread much more rapidlythan Asian flu, starting from an original focus of infection inMexico and spreading quickly to the United States, Europe, andCentral and South America. The pandemic influenza A (H1N1) 2009 virus is sometimes called “swine flu” because the reassertedvirus apparently developed in pigs (Figure 33.22). It is a reassortantvirus consisting of RNA segments derived from human andbird influenza, and reassorted in swine. From the swine reservoir,it emerged to infect humans. First recognized in March 2009, thevirus was declared a pandemic on June 11, 2009. By September2009, the virus had spread worldwide. During the flu season in the

Southern Hemisphere (May–October 2009), pandemic influenzaA (H1N1) 2009 spread rapidly, causing widespread disease.Although pandemic influenza virus did not seem to be extraordinarilyvirulent, the pandemic was widespread even during thenon-influenza-season summer months of 2009 (June–August) inNorthern Hemisphere countries in Europe and North America,demonstrating that it is fully adapted to humans and can spreadvery easily. Even though the infection was prevalent in2009–2010, the overall mortality rate for this pandemic strain wasrelatively low, an estimated 0.1–0.2%, perhaps only slightly higherthan seasonal influenza mortality. A vaccine was made available inOctober 2009 to slow the advance of the pandemic.A potentially devastating avian influenza, the influenza AH5N1 strain, also called avian influenza, appeared in Hong Kongin 1997, apparently jumping directly from the avian host tohumans without the pig intermediate. H5N1 has now beenreported in birds throughout Asia, Europe, the Middle East, andNorth Africa; it has not yet spread to birds in the Americas, Australia,or Antarctica. The H5N1 virus has reemerged severaltimes over the last decade; the most recent outbreaks occurred inEgypt and Indonesia. Since 2003, 495 cases of human H5N1infections have been confirmed worldwide, resulting in 292deaths, an overall mortality rate of almost 60%. H5N1 is spreaddirectly from avian species, usually domestic chickens or ducks, to humans through prolonged contact or the eating of infectedbirds. At this time, avian influenza can be spread human tohuman only after prolonged close contact, but some reports indicatethat H5N1 has infected swine. This event could set the stagefor reassortment with human influenza strains that also infectswine. Such a reassortment could create a new and highly infective
virus for which there is no immunity in humans, startinganother influenza pandemic. Plans are in place both nationallyand internationally to provide appropriate vaccines and supportfor potential pandemics initiated by this and other emergentinfluenza strains. A recombinant vaccine for the H5N1 virus isavailable on a limited basis.

Influenza Prevention and Treatment

Influenza epidemics can be controlled by immunization. However,the selection of appropriate strains for vaccines is complicated bythe large number of existing strains and the ability of existingstrains to undergo antigenic drift and antigenic shift. When newstrains evolve, vaccines are not immediately available, but throughcareful worldwide surveillance, samples of the major emergingstrains of influenza virus are usually obtained before there are epidemics.In the United States, immunization preparations are reformulatedannually to target current prevalent strains. The targetedstrains, chosen at the end of each influenza season, are grown inembryonated eggs and inactivated. The inactivated viral strains(two influenza A and one influenza B) are mixed to prepare a vaccineused for immunization prior to the next influenza season.

In general, influenza immunization is recommended for thoseindividuals most likely to acquire the disease and develop serioussecondary illnesses. Influenza immunization is currently recommendedfor everyone over 50 years of age, for those suffering from chronic debilitating diseases (for example, AIDS patients,chronic respiratory disease patients, and so on), and for healthcareworkers. Effective artificial immunity from the inactivatedinfluenza vaccine lasts only

a few years and is strain-specific. Anattenuated live-virus vaccine is recommended for young adults,and may confer longer-lasting immunity. Influenza A may also be controlled by use of antiviral drugs.The adamantanes, amantadine and rimantadine, are syntheticamines that inhibit viral replication. The neuraminidaseinhibitors oseltamivir (Tamiflu) and zanamivir (Relenza) block release of newly replicated virions of influenzaA and B and H5N1 avian virus. These drugs are used to treat ongoing influenza and shorten the course and severity of infection.
They are most effective when given very early in the courseof the infection. The adamantanes and oseltamivir also preventthe onset and spread of influenza.Drug resistance has already occurred in some of the most dangerousinfluenza strains. Neither pandemic influenza A (H1N1) 2009 nor the H5N1 avian influenza is susceptible to the adamantanes.Although most influenza viruses are susceptible to theneuraminidase inhibitors, a few isolates of pandemic influenza A (H1N1) 2009 are resistant to oseltamivir.Treatment of influenza symptoms with aspirin, especially inchildren, is not recommended. Aspirin treatment of influenza hasbeen linked to development of Reye's syndrome, a rare but occasionallyfatal complication involving the central nervous system.

Water borne Microbial Disease

Common-source infectious diseases are caused by microbialcontamination of materials shared by a large number of individuals.The most important common source of infectious disease is contaminated water; the failure of a single step in thedrinking water purification process may result in the exposure ofthousands or even millions of individuals to an infectious agent.Common-source waterborne diseases are significant sources of morbidity and mortality, especially in developing countries. Evenin developed countries, breakdowns in water treatment plantsor the lack of access to clean water in times of emergency cancontribute to the development of a waterborne disease outbreak.Bacteria, viruses, and protists cause waterborne infectiousdiseases. Waterborne diseases begin as infections. Contaminated water may cause infection even if only a small number of microorganismsare present. Whether or not exposure to a pathogencauses disease is a function of the virulence of the pathogen andthe general ability of the host to resist infection.

Sources of Waterborne Infection
Human pathogens can be transmitted through untreated orimproperly treated water used for drinking and cooking. Anothercommon source of disease transmission is through pathogencontaminatedwater used for swimming and bathing.

Potable Water Because everyone consumes water through drinking and cooking,water is a common source of pathogen dissemination andhas a very high potential for the catastrophic spread of epidemic disease. As we have already discussed, water supplies in developedcountries usually meet rigid quality standards, limiting thespread of waterborne diseases. Waterborne disease outbreaks, however, occasionally occur in developing countries due tolapses in water

quality. Isolated outbreaks affecting low numbersof individuals also occur from consumption of contaminated water from nonregulated sources (such as private wells) or fromconsumption of untreated water from streams or lakes. Thesesources may be contaminated by fecal material from humans or animals.Microorganisms transmitted in drinking water generally growin the intestines and leave the body in feces, which may in turnpollute water. If a new host consumes the water, the pathogenmay colonize the host's intestine and cause disease. From 1974 to2006 in the United States, 729 drinking water–associated diseaseoutbreaks occurred—an average of about 23 outbreaks per year(**Figure 35.11*a***). Bacterial, viral, and protist pathogens are occasionally transmitted in drinking water (**Table 35.1**).

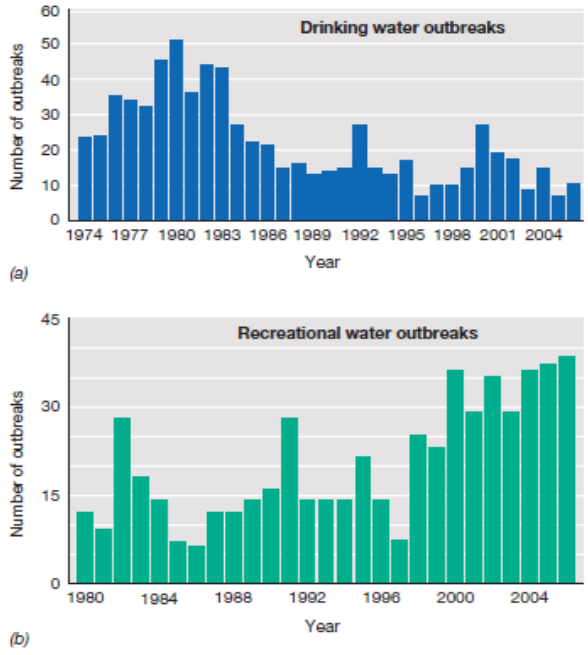


Figure 35.11 Waterborne disease outbreaks. Data were provided by the Centers for Disease Control and Prevention, Atlanta, Georgia, USA. *(a)* Reported drinking water disease outbreaks from 1974 to 2006. Of 729 outbreaks, about 90% were due to biological agents (bacteria, viruses, and protists). *(b)* Reported recreational water outbreaks from 1980 to 2006. Of 544 total outbreaks, almost all were due to biological agents.

Table 35.1 *Reported infectious disease outbreaks associated with drinking water in the United States, 2005–2006*[a]

Disease	*Agent*	*Outbreaks*	*Cases*
Cryptosporidiosis	*Cryptosporidium*	1	10
Giardiasis	*Giardia*	1	41
Legionellosis	*Legionella*	10	43
Acute gastrointestinal illness	*Escherichia coli* and *Campylobacter jejuni*	1	60
	Campylobacter jejuni	1	32
	Norovirus and *Campylobacter jejuni*	1	139
	Norovirus	2	196
	Hepatitis A	1	16
	Unknown[b]	2	75

Recreational Water Recreational waters include freshwater recreational areas such asponds, streams, and lakes, as well as public swimming and wadingpools. Recreational waters can also be sources of waterborne disease,and historically cause disease outbreaks at levels roughlycomparable to those caused by drinking water (Figure 35.11b).The operation of public swimming and wading pools is regulatedby state and local health departments. The United StatesEPA establishes limits for bacteria in recreational freshwaters(monthly geometric mean for all samples of,33/100 ml for enterococcior ,126/100 ml for E. coli) and marine waters (,35/100 mlfor enterococci). Local and state governments have the authorityto set standards above or below these guidelines, and many statesuse a single-sample maximum as well as the geometric

mean forsetting standards and defining levels of contamination thatconstitute violations. For example, the state of Indiana standardis 125 E. coli cells per 100 ml as a geometric mean, with a singlesamplemaximum of 235/100 ml. Thus, waters that exceed 235E. coli, even if their geometric mean count was not greater than125, would be in violation of Indiana's water standards. Private swimming pools, spas, and hot tubs are unregulated and areoccasional sources of outbreaks of waterborne diseases.Over a 27-year period (1980–2006), 544 waterborne diseaseoutbreaks were from recreational waters in the United States, orabout 20 outbreaks per year (Figure 35.11b). **Table 35.2** categorizesrecreational water outbreaks according to the infectiousdiseases occurring in recent years.

Waterborne Infections in Developing Countries

Worldwide, waterborne infections are a much larger problem thanin the United States and other developed countries. Developingcountries often have inadequate water and sewage treatmentfacilities, and access to safe, potable water is limited. As a result, diseases such as cholera, typhoid fever, and amebiasisare important public health problems in the developingworld.

Cholera

Cholera is a severe diarrheal disease that is now largely restrictedto the developing world. Cholera is an example of a major waterbornedisease that can be controlled by application of appropriatepublic health measures for water treatment.

Table 35.2 *Reported infectious disease outbreaks associated with recreational water in the United States, 2005–2006*[a]

Agent[b]	*Outbreaks*	*Cases*
Bacteria		
Campylobacter jejuni	1	6
Escherichia coli	3	10
Legionella	8	124
Leptospira	2	46
Pseudomonas aeruginosa	9	101
Shigella sonnei	4	41
Parasites		
Cryptosporidium	31	3751
Giardia intestinalis	1	11
Cryptosporidium and *Giardia*	1	55
Naegleria fowleri	1	2
Schistosoma	2	4
Virus		
Norovirus	5	99

[a]Data provided by the Centers for Disease Control and Prevention, Atlanta, Georgia. In all, 68 outbreaks occurred over 2 years.
[b]*Campylobacter jejuni*, *Escherichia coli*, *Shigella sonnei*, *Cryptosporidium*, *Giardia*, and norovirus outbreaks cause gastroenteritis. *Legionella* causes acute respiratory disease. *Leptospira* causes systemic infections and aseptic meningitis. *Pseudomonas aeruginosa* causes dermatitis. The amoeba *Naegleria fowleri* causes meningoencephalitis; all cases were fatal. *Schistosoma*, a helminth parasite, causes schistosomiasis, a disease characterized chiefly by parasitic infestations of venous vessels in the intestines and liver.

Biology and Epidemiology : Cholera is typically caused by ingestion of contaminated watercontaining Vibrio cholerae, a gram-negative, curved rod–shaped Proteobacterium (**Figure 35.12)**;As with many waterborne diseases, cholera can also be associated with foodconsumption.

For example, in the Americas, consumption of rawshellfish and raw vegetables has been associated with cholera.

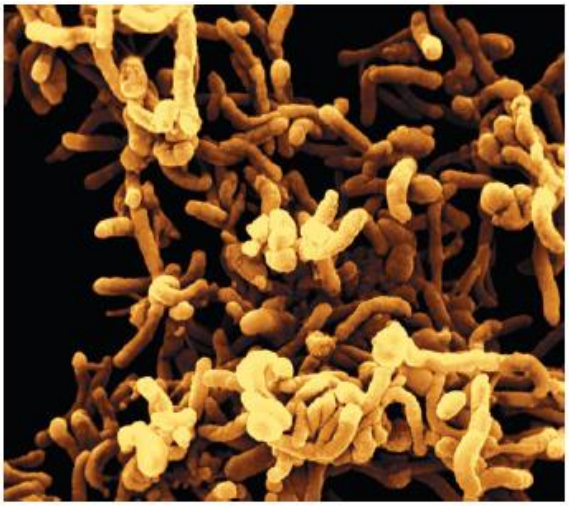


Figure 35.12 Cells of *Vibrio cholerae*. This colorized scanning electron micrograph shows a rod to curved rod morphology. The organism is about 0.3 μm in diameter and up to 2 μm in length.

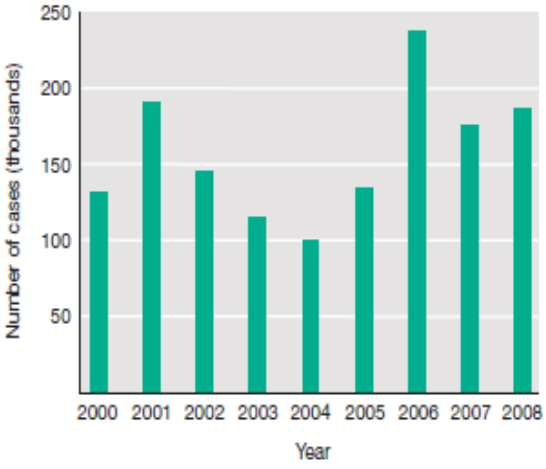


Figure 35.13 Cholera cases. The reported cholera cases from 2000 to 2008 show a generally increasing trend. Up to 90–95% of cholera cases are unreported. Over 95% of all reported cases occur in Africa. Data were provided by the World Health Organization.

Presumably, vegetables washed in contaminated water and shellfishbeds contaminated by untreated sewage transmitted thedisease.Since 1817, cholera has swept the world in seven majorpandemics. Two distinct pandemic strains of V. cholerae are recognized,known as the classic and the El Tor biotypes. TheV. cholerae O1 El Tor biotype started the seventh pandemic inIndonesia in 1961, and its spread continues to the present. Thispandemic has caused over 5 million cases of cholera and morethan 250,000 deaths and continues to be a major cause of morbidityand mortality, especially in developing countries; as istypical for infectious diseases, the highest prevalence of cholerais in developing countries, especially in Africa. In 1992, a geneticvariant known as V. cholerae O139 Bengal arose in Bangladeshand caused an extensive epidemic. V. cholerae O139 Bengal hascontinued to spread since 1992, causing several major epidemics,and may be the agent of an eighth pandemic.Cholera is endemic in Africa, Southeast Asia, the Indian subcontinent,and Central and South America. Epidemic choleraoccurs frequently in areas where sewage treatment is either inadequateor absent. Worldwide, there were 190,130 reported casesand 5143 deaths reported in 2008, with over 98% of all reportedcases occurring in Africa. About 100,000 cases or more havebeen reported annually since 2000, with a low of 95,560 cases in2004, and a high of 236,896 cases in 2006 (**Figure 35.13**). TheWorld Health Organization estimates that only 5–10% of choleracases are reported, so the total incidence of cholera exceeds1 million cases per year. Even in developed countries, the diseaseis a threat. A handful of cases are reported each year in theUnited States, rarely caused by drinking water. Many recent casesare imported, often in food. A few cases are possibly from endemic sources; raw shellfish seems to be the most commonvehicle, presumably because V. cholerae

may be free-living incoastal waters in endemic areas, where the pathogen adheres to the marine microflora ingested by the shellfish (**Figure 35.14**).

Pathogenesis

The ingestion of 10^8–10^9 cholera vibrios is generally required to cause disease. The ingested V. cholerae cells attach to epithelial cells in the small intestine where they grow and release cholera toxin, a potent enterotoxin. Studies in human volunteers have shown that stomach acidity is responsible for the large inoculum needed to initiate cholera; human volunteers given bicarbonate to neutralize gastric acidity developed cholerawhen given as few as 10^4 cells. Even lower cell numbers can initiateinfection if V. cholerae is ingested with food, presumably becausethe food protects the vibrios from destruction by stomach acidity.Cholera enterotoxin causes severe diarrhea that can resultin dehydration and death unless the patient is given fluid andelectrolyte therapy. The enterotoxin causes fluid losses of up to20 liters (20 kg or 44 lb) per day. The mortality rate from\ untreated cholera is typically 25–50% and can be much higher under conditions of severe crowding and malnutrition.

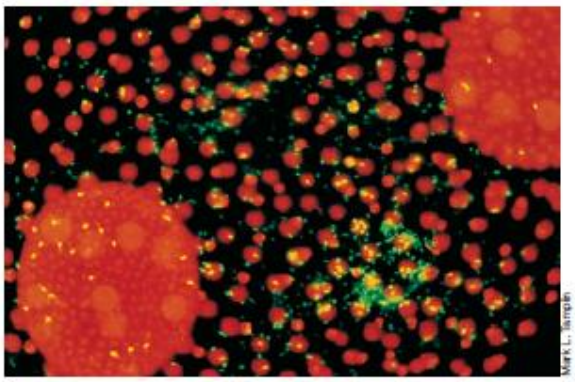


Figure 35.14 Cells of *Vibrio cholerae* attached to the surface of *Volvox*, a freshwater alga. The sample was from a cholera-endemic area in Bangladesh. The *V. cholerae* cells are stained green by a monoclonal antibody to bacterial cell surface proteins. The red color is due to the fluorescence of chlorophyll a in the algae.

Cholera is diagnosed by the presence of the gram-negative,comma-shaped V. cholerae bacilli in the “rice water” stools (nearlyliquid feces) of patients with severe diarrhea.Immunization is not normally recommended for cholera prevention,but a whole-cell oral vaccine directed against the El Torbiotype is currently available for use in high-risk situations, suchas after natural disasters that compromise water treatment andpurification systems. The vaccine, as well as natural infection,provides effective but short-lived immunity. No vaccine protectsagainst the new V. cholerae O139 Bengal serotype. Public healthmeasures such as adequate sewage treatment and a reliablesource of safe drinking water are the most important measuresfor preventing cholera. V. cholerae is eliminated from wastewaterduring proper sewage treatment and drinking water purificationprocedures. For individuals traveling in cholera-endemic areas,attention to personal hygiene and avoidance of untreated wateror ice, raw food, and raw or undercooked fish or shellfish offerprotection against contracting cholera.

Treatment of Cholera : Cholera treatment is simple, effective, and inexpensive. Intravenousor oral liquid and electrolyte replacement therapy [20 g of glucose,4.2 g of sodium chloride (NaCl), 4.0 g of sodium bicarbonate(NaHCO3), and 1.8 g of potassium chloride (KCl) dissolved in1 liter of water] is the most effective means of cholera treatment.Oral treatment is preferred because no special equipment orsterile precautions are necessary. Effective fluid and electrolytereplacement reduces mortality to about 1%. Streptomycin ortetracycline may shorten the course of infection and the sheddingof viable cells, but antibiotics are of little benefit without simultaneous fluid and electrolyte replacement.

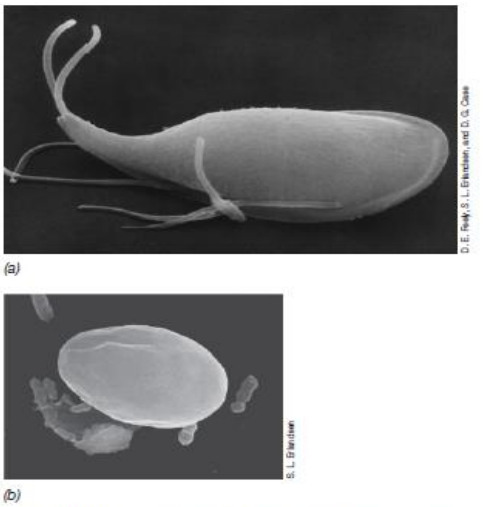
Giardiasis and Cryptosporidiosis

Giardiasis and cryptosporidiosis are diseases caused by the protists Giardia intestinalis and Cryptosporidium parvum, respectively.These organisms continue to be problematic even in well-regulatedwater supplies because they are found in nearly all surface watersand are resistant to chlorine disinfection.

Giardiasis

Giardia intestinalis, also called Giardia lamblia, is a flagellatedprotist that is usually transmitted to humans infecally contaminated water, although foodborne and sexual transmission of giardiasis have also been documented. Giardiasisis an acute gastroenteritis caused by this organism. The protest cells, called trophozoites (**Figure 35.16*a***), produce a resting stagecalled a **cyst** (Figure 35.16b). The cyst has a thick protective wallthat allows the pathogen to resist drying and chemical disinfection.After a person ingests the cysts in contaminated water, the cystsgerminate, attach to the intestinal wall, and cause the symptomsof giardiasis: an explosive, foul-smelling, watery diarrhea, intestinalcramps, flatulence, nausea, weight loss, and malaise. Symptomsmay be acute or chronic. The foul-smelling diarrhea and theabsence of blood or mucus in the stool distinguish giardiasisfrom bacterial or viral diarrheas. Many infected individualsexhibit no symptoms but act as carriers; *G. intestinalis* can establishitself in a stable, symptom-free relationship with its host.*G. intestinalis* was the infectious agent in 1 of the 20 recentdrinking water infectious disease outbreaks in the United States. The thick-walled cysts are resistant to chlorine, and most outbreaks have been associated with water systems thatused only chlorination as a means of water purification. Watersubjected to proper clarification and filtration followed by chlorinationor other disinfection is generally free ofGiardia cysts.Giardiasis can also be contracted from ingestion of water frominfected swimming pools or lakes (Table 35.2). Giardia cysthave been found in 97% of surface water sources (lakes, ponds,and streams) in the United States. Isolated cases of giardiasishave been associated with untreated drinking water in wildernessareas. Beavers and muskrats are frequent carriers of *Giardia* andmay transmit cells or cysts to water supplies, making the water apossible source of human infection. As a safety precaution, waterconsumed from rivers and streams, for example, during a campingor hiking trip, should be filtered and treated with iodine orchlorine, or filtered and boiled. Boiling is the preferred methodto ensure that water is free of pathogens.Laboratory diagnostic methods include the demonstration ofGiardia cysts in the stool or the demonstration

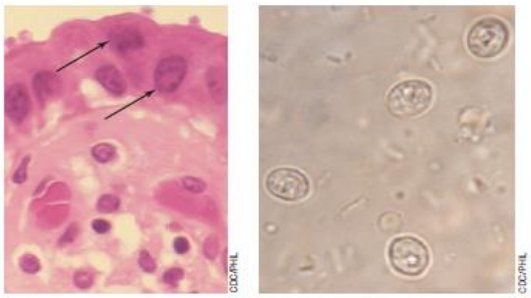
of Giardia antigensin the stool using a direct EIA (enzyme immunosorbentassay). The drugs quinacrine, furazolidone, and metronidazoleare useful for treating acute giardiasis.



(a)

(b)

Figure 35.16 **The parasite *Giardia*.** Scanning electron micrographs. *(a)* A motile trophozoite. The trophozoite is about 15 μm in length. *(b)* A giardial cyst. The cyst is about 11 μm in length.



(a) (b)

Figure 35.17 ***Cryptosporidium.*** *(a)* The arrows point to two of the many intracellular trophozoites embedded in human gastrointestinal epithelium. The trophozoites are 2–5 μm in diameter. *(b)* The thick-walled oocysts are 3–5 μm in diameter in this fecal sample.

the stomach and intestine (**Figure 35.17*a***). The protist produces thick-walled, chlorine-resistant, infective cells called *oocysts*, which are shed into water in high numbers in the feces of infected warm-blooded animals (Figure 35.17*b*). The infection is passed on when other animals consume the fecally contaminated water. *Cryptosporidium* cysts are highly resistant to chlorine (up to 14 times more resistant than chlorine-resistant *Giardia*) and

Cryptosporidiosis

The protist *Cryptosporidium parvum* lives as a parasite in warmblooded animals. The protists are small, round cellsthat invade and grow intracellularly in mucosal epithelial cells ofthe stomach and intestine (**Figure 35.17*a***). The protist producesthick-walled, chlorine-resistant, infective cells called oocysts,which are shed into water in high numbers in the feces ofinfected warm-blooded animals (Figure 35.17b). The infection ispassed on when other animals consume the fecally contaminatedwater. Cryptosporidium cysts are highly resistant to chlorine (upto 14 times more resistant than chlorine-resistant Giardia) andUV radiation disinfection. Because of this property, sedimentationand filtration methods must be used to remove *Cryptosporidium*from water supplies. From 2005 through 2006, Cryptosporidiumwas responsible for 31 of the 68 recreational waterborne diseaseoutbreaks (Table 35.2).*C. parvum* was responsible for the largest single commonsourceoutbreak of a waterborne disease ever recorded in theUnited States. In the spring of 1993 in Milwaukee, Wisconsin,USA, over 403,000 people in the population of 1.6 million developeda diarrheal illness that was traced to the municipal watersupply. Spring rains and runoff from surrounding farmland haddrained into Lake Michigan and overburdened the water purificationsystem, leading to contamination by *C. parvum*. Theprotist is a significant intestinal parasite in dairy cattle, the likelysource of the outbreak.Cryptosporidiosis is usually a self-limiting mild diarrhea thatsubsides in 2 weeks or less in normal individuals. However,individuals with impaired immunity, such as that caused by HIV/AIDS, or the very young or old can develop serious complications.In the Milwaukee outbreak, about 4400 people requiredhospital care, and 50–100 died of complications from the disease,including severe

dehydration.The Milwaukee outbreak highlights the vulnerability of waterpurification systems, the need for constant water monitoring andsurveillance, and the consequences of the failure of a large watersupply system. In addition to the toll of human morbidity andmortality, the epidemic cost an estimated $96 million in medicalcosts and lost productivity.

Laboratory diagnostic methods for cryptosporidiosis includethe demonstration of Cryptosporidium oocysts in the stool.Treatment is unnecessary for those with uncompromised immunity.For individuals undergoing immunosuppressive therapy(for example, prednisone), discontinuation of immunosuppressivedrugs is recommended. Immunocompromised individuals should be given supportive therapy such as intravenous fluids andelectrolytes.

Legionellosis (Legionnaires' Disease)

Legionella pneumophila, the bacterium that causes legionellosis,is an important waterborne pathogen normally transmitted inaerosols rather than through drinking or recreational waters.

Biology and Epidemiology

Legionella pneumophila was first discovered as the pathogen thatcaused an outbreak of pneumonia during an American Legionconvention in Philadelphia, Pennsylvania, USA, in the summer of1976. *L. pneumophila* is a thin, gram-negative, obligately aerobicrod (**Figure 35.18**) with complex nutritional requirements,including an unusually high iron requirement. The organism canbe isolated from terrestrial and aquatic habitats as well as fromlegionellosis patients.*L. pneumophila* is present in small numbers in lakes, streams,and soil. It is relatively resistant to heating and chlorination, so itcan spread through water distribution systems. It is commonlyfound in large numbers in cooling towers and evaporative condensersof large air conditioning systems. The pathogen grows inthe water and is disseminated in humidified aerosols. Humaninfection is by way of airborne droplets, but the infection is notspread from person to person. Further evidence for this is thefact that annual outbreaks of legionellosis tend to peak in mid-tolate summer months when air conditioners are extensively used.*L. pneumophila* has also been found in hot water tanks andwhirlpool spas, where it can grow to high numbers in warm(35–45°C), stagnant water. Epidemiological studies indicate that *L. pneumophila* infections occur at all times of the year, primarilyas a result of aerosols generated by heating/cooling systems andcommon practices such as showering or bathing. Overall, theincidence of reported cases of legionellosis had been about 4–6cases per million in the United States, but in the last several yearsthe incidence has risen to nearly 8 cases per million. In 2007,there were 2716 reported cases (**Figure 35.19**). The increase inreported cases may be a result of an actual increase in infectionsor an increase in recognition and reporting; formerly, up to90% of actual cases were probably not diagnosed or properlyreported. Prevention of legionellosis can be accomplished byimproving the maintenance and design of water-dependent coolingand heating systems and water delivery systems. The pathogencan be eliminated from water supplies by hyperchlorination or byheating water to greater than 63°C.

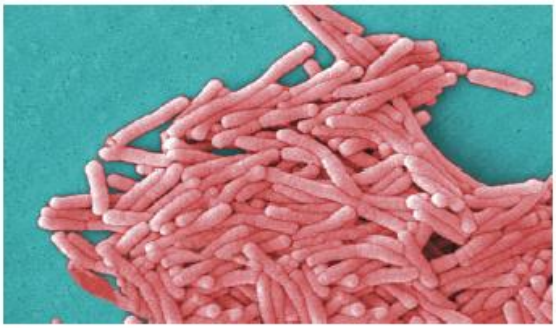


Figure 35.18 *Legionella pneumophila*. Colorized scanning electron micrograph of *L. pneumophila* cells. Cells are 0.3–0.6 μm in diameter and up to 2 μm in length.

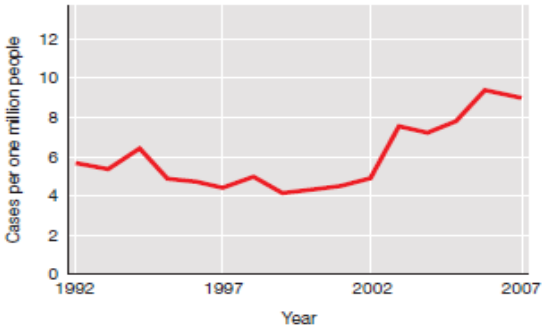


Figure 35.19 Incidence of Legionnaire's disease in the United States. In 2007, there were 2716 reported cases. Data are from the Centers for Disease Control and Prevention, Atlanta, Georgia.

Pathogenesis

In the body, *L. pneumophila* invades and grows in alveolar macrophages and monocytes as an intracellular parasite. Infectionsare often asymptomatic or produce a mild cough, sorethroat, mild headache, and fever. These mild, self-limiting cases,called Pontiac fever, are generally not treated and resolve in 2–5days. Elderly individuals whose resistance has been previouslycompromised, however, often acquire more serious infectionsresulting in pneumonia. Certain serotypes of *L. pneumophila*(more than 10 are known) are strongly associated with the pneumonicform of the infection. Prior to the onset of pneumonia,intestinal disorders are common, followed by high fever, chills,and muscle aches. These symptoms precede the dry cough and chest and abdominal pains typical of legionellosis. Death,usually due to respiratory failure, occurs in up to 10% of pneumoniacases.

Diagnosis and Treatment

Clinical detection of *L. pneumophila* is usually done by culturefrom bronchial washings, pleural fluid, or other body fluids.Serological (antibody) tests are used as retrospective evidence for Legionella infection. As an aid in diagnosis, *L. pneumophila*antigens can sometimes be detected in patient urine. *Legionella pneumoph*ila can be treated with the antibiotics rifampin anderythromycin. Intravenous administration of erythromycin is thetreatment of choice.

Typhoid Fever and Other Waterborne Diseases

Various bacteria, viruses, and protists can transmit commonsourcewaterborne diseases. These diseases are a significant sourceof morbidity, especially in developing countries.

Typhoid Fever

On a global scale, probably the most important pathogenicbacteria transmitted by the water route are **Salmonella** enteric serovar Typhi, the organism causing typhoid fever, and *Vibrio cholerae*, the organism causing cholera, which we discussedpreviously.Although *S. enterica ser. typhi* may also be transmitted bycontaminated food and by direct contact frominfected individuals, the most common and serious means oftransmission worldwide is through water. Typhoid fever has beenvirtually eliminated in developed countries, primarily due toeffective water treatment

procedures. In the United States, thereare fewer than 400 cases in most years, but, typhoid fever was a major public health threatbefore drinking water was routinely filtered and chlorinated. However, breakdown of water treatment methods,contamination of water during floods, earthquakes, and otherdisasters, or cross-contamination of water supply pipes fromleaking sewer lines can propagate epidemics of typhoid fever,even in developed countries.

Viruses

Viruses can also be transmitted in water and cause human disease.Quite commonly, enteroviruses such as poliovirus, norovirus,and hepatitis A virus are shed into the water in fecal material.The most serious of these is poliovirus, but wild poliovirus hasbeen eliminated from the Western Hemisphere and is endemiconly in Nigeria, Afghanistan, Pakistan, and India. Althoughviruses can survive in water for relatively long periods, they areinactivated by disinfection with agents such as chlorine.

Amebiasis

Certain amoebae inhabit the tissues of humans and other vertebrates,usually in the oral cavity or intestinal tract, and someof these are pathogenic. We discussed the general properties of amoeboid protists. Worldwide, *Entamoeba histolytica* is a common pathogenicprotist transmitted to humans, primarily by contaminated waterand occasionally through contaminated food. *E. histolytica* isan anaerobic amoeba; the trophozoites lack mitochondria(**Figure 35.20**). Like *Giardi*a, the trophozoites of *E. histolytica*produce cysts. Cysts ingested by humans germinate in the intestine,where amoebic cells grow both on and in intestinal mucosalcells. Many infections are asymptomatic, but continued growthmay lead to invasion and ulceration of the intestinal mucosa,causing diarrhea and severe intestinal cramps. With furthergrowth the amoebae can invade the intestinal wall, a conditioncalled dysentery, characterized by intestinal inflammation, fever,and the passage of intestinal exudates, including blood and mucus.If not treated, invasive trophozoites of *E. histolytica* can invadethe liver and occasionally the lung and brain. Growth in thesetissues can cause severe abscesses and death. Worldwide, up to100,000 individuals die each year from invasive amebic dysentery.The disease is extremely common in tropical and subtropicalcountries worldwide, with at least 50 million people developingsymptomatic diarrhea annually and up to 10-fold more havingasymptomatic disease. In the United States, several hundredcases occur each year, mostly near international borders in the Southwest.*E. histolytica* amebiasis can be treated with the drugs dehydroemetinefor invasive disease and diloxanide furoate for certainasymptomatic cases, as in immunocompromised individuals, butamoebicidal drugs are not universally effective. Spontaneouscures do occur, suggesting that the host immune system plays arole in ending the infection. However, protective immunity is not an outcome of primary infection, and reinfection is common. The disease is kept at very low incidence in regions that practice adequate sewage treatment. Amoebic infestation due to exposure to improperly treated sewage and the use of untreated surfacewaters for drinking purposes are the usual causes of amebiasis.

Demonstration of *E. histolytica* cysts in the stool, trophozoites in tissue, or the positive results for antibodies to *E. histolytica* in the blood from an EIA (enzyme immunoassay) are used for the laboratory diagnosis of amebiasis.

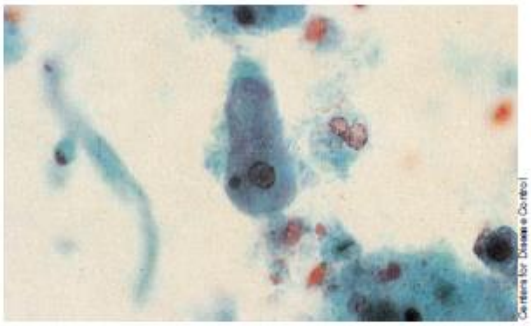


Figure 35.20 The trophozoite of *Entamoeba histolytica*, the amoeba that causes amebiasis. Note the discrete, darkly stained nucleus. The small red structures are red blood cells. The trophozoites range from 12 to 60 μm in length.

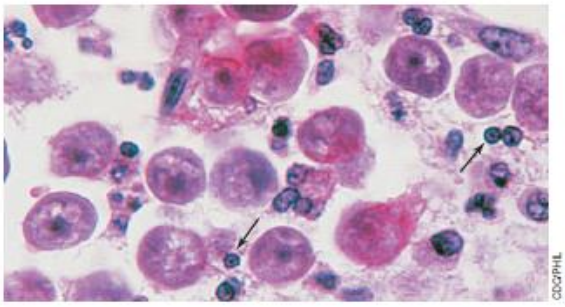


Figure 35.21 Trophozoites of *Naegleria fowleri* in brain tissue. This amoeba causes meningoencephalitis. Oval to round and amoeboid (irregularly shaped) trophozoites (arrows) are present as dark-stained structures with densely stained nuclei. There is extensive destruction of the surrounding brain tissue. Individual trophozoites are 10–35 μm long.

Naegleria fowleri can also cause amebiasis, but in a very differentform. *N. fowleri* is a free-living amoeba found in soil and inwater runoff. N. fowleri infections usually result from swimmingor bathing in warm, soil-contaminated water sources such as hotsprings or lakes and streams in the summer. This free-livingamoeba enters the body through the nose and burrows directlyinto the brain. Here, the organism propagates, causing extensivehemorrhage and brain damage (**Figure 35.21**). This condition iscalled **meningoencephalitis**. Death usually results within aweek. From 1999 to 2003, there were 12 outbreaks, each a singleindividual who was infected by swimming or wading in a lake,pond, or stream in summer. All cases resulted in death.Prevention can be accomplished by avoiding swimming inshallow, warm freshwater, such as farm ponds and shallow lakesand rivers in summer. Swimmers are advised to avoid stirring upbottom sediments, the natural habitat of the pathogen. Diagnosisof *N. fowleri* infection requires observation of the amoebae in thecerebrospinal fluid. If a definitive diagnosis can be done quickly,the drug amphotericin B is used to treat infections.

Foodborne Disease

If food is not decontaminated or preserved, pathogens may growin it and cause foodborne diseases with significant morbidityand mortality. Like waterborne diseases, foodborne illnesses arecommon-source diseases. A single contaminated food source from a food-processing plant or a restaurant may affect a large numberof people. In 2010, chicken feed contaminated with Salmonellaused at two egg production farms in Iowa infected eggs distributed nationally, and caused over 1500 infections. Each year in theUnited States, there are an estimated 25,000 foodborne disease outbreaks. As many as 76 million Americans are affected, an estimated 13 million acquire significant illnesses, 325,000 are hospitalized, and 5000 people die from

foodborne diseases each year. Most outbreaks are due to improper food handling and preparation by consumers and affect small numbers of individuals, usually in the home. Occasional outbreaks affect large numbers of individuals because they are caused by breakdowns in safe food handling and preparation at food-processing and distribution plants. Most foodborne illnesses are unreported because the connection between food and illness is not made. Foodborne illness is largely preventable; appropriate monitoring of food sources and disease outbreaks provides the basis for protecting consumers. The food industry and the government set standards and monitor food sources to control and preventfoodborne disease.

Foodborne Disease and Microbial Sampling The most prevalent foodborne diseases in the United Statesare classified as food poisonings (FP) or food infections (FI); somediseases fall into both categories. **Table 36.6** lists the microorganismsthat cause these diseases. Special microbial samplingtechniques are necessary to isolate and identify the pathogensand toxins responsible for foodborne diseases, and a variety ofgrowth-dependent, immunological, and molecular techniquesare used. Foodborne illnesses and outbreaks are reported to theCenters for Disease Control and Prevention through PulseNet and FoodNet reporting systems.

Foodborne Diseases

Food poisoning, also called **food intoxication**, is disease thatresults from ingestion of foods containing preformed microbialtoxins. The microorganisms that produced the toxins do not have to grow in the host and are often not alive at the time the contaminated food is consumed; the ingestion and action of a bioactive toxin causes the illness. We previously discussed some of these toxins, notably the exotoxin of *Clostridium botulinum*. and the superantigen toxins of *Staphylococcus*and *Streptococcus*. **Food infection** is ingestionof food containing sufficient numbers of viable pathogens tocause infection and disease in the host. We discuss major foodborneinfections.

Microbial Sampling for Foodborne Disease

Along with nonpathogenic microorganisms that cause spoilage,pathogenic microorganisms may be present in fresh foods. Rapiddiagnostic methods that do not require pathogen growth or culture have been developed to detect important food pathogenssuch as *Escherichia coli* O157:H7, *Salmonella*, *Staphylococcu*s,and *Clostridium botulinum*. Molecular and immunology-basedtests are used to identify both toxin and pathogen contamination of foods and other products such as drugs and cosmetics. The presence of afoodborne pathogen or toxin is not sufficient to link a particularfood to a specific foodborne disease outbreak; the suspectpathogen or toxin must be isolated and identified to establish itsrole in a foodborne illness.Isolation and growth of pathogens from nonliquid foods usuallyrequire preliminary treatment to suspend microorganismsembedded or entrapped within the food. A standard methoduses a specialized blender called a stomacher (**Figure 36.10**). Thestomacher processes a wide variety of solid and semisolid samplessuch as fresh and processed meat, dry fruits, cereals, grains, seeds,cheese, cosmetics, and for biomedical applications, pharmaceuticalproducts and tissue samples. The sample is sealed in a sterilebag. Paddles in the stomacher crush, blend, and homogenize

thesamples under conditions that prevent contamination by otherorganisms. Although a traditional blender could also be used toprocess samples, the sealed bag stomacher system prevents contaminationfrom outside sources, eliminates cleanup betweeneach sample run, and eliminates generation of aerosols. Thehomogenized samples can then be analyzed in various ways.Foods sampled for microorganisms or toxins should be examinedas soon after processing as possible; if examination cannotbegin within 1 hour of sampling, the food should be refrigerated.Frozen food should be thawed in the original container in arefrigerator and examined or cultured as soon as thawing iscomplete. In addition to identifying pathogens in food, diseaseinvestigators must obtain foodborne pathogens from the diseaseoutbreak patients to establish a cause-and-effect relationshipbetween the pathogen and the illness. In many cases, fecal samplescan be cultured to recover suspected foodborne pathogens.Food or patient samples can be inoculated onto enriched media,followed by transfer to differential or selective media for isolationand identification, as described for the isolation of human pathogens. Final identification of foodbornepathogens is based on growth characteristics and biochemicalreaction patterns. The use of molecular and genetic methods. such as the polymerase chain reaction, enzyme immunoassays, nucleic acid probes, nucleic acid sequencing, pulsed-field gel electrophoresis (PFGE), and ribotyping may be used to identify specific organisms.

Figure 36.10 A stomacher. Paddles in this specialized blender homogenize the solid food sample in a sealed, sterile bag. The sample is suspended in a sterile solution.

Foodborne Disease Epidemiology

There are often clusters of cases of a foodborne disease in aparticular place because microorganisms from a single commoncontaminated food, such as salads or hamburgers served from ahome, school cafeteria, college dining hall, restaurant, or mess hall,are ingested by many individuals. In addition, central processingplants and central food distribution centers provide opportunitiesfor contaminated foods to cause multiple disease outbreaksin far-flung locations, as when contaminated spinach grown inCalifornia caused outbreaks across the United States. We shall seehow the food epidemiologist tracks outbreaks and determinestheir source, often down to the field, processing plant, or pointof-preparation facility in which the food was contaminated.

Spinach and *Escherichia coli* O157:H7

In 2006 an outbreak of illness associated with *Escherichia coli*O157:H7 occurred in the United States and was linked to the consumption of ready-to-eat packaged fresh spinach. The outbreak was quickly traced to a food-processing facility in California.First linked to the spinach product in September, the outbreakcaused at least 199 infections. Of these, 102 individuals were hospitalizedand 31 developed hemolytic uremic syndrome. At leastthree deaths were attributed to the outbreak.The remarkably short duration and rapid end to this epidemic—the first case was confirmed in late August and the last reportedin early October—is a testament to efficiency and cooperationamong public health facilities across the country. In this case, two of these networks—FoodNet and PulseNet—were used to define the source and stop the outbreak. The contaminatedspinach was distributed nationwide from the Californiaprocessing plant, but most disease cases were not in the West. Thetwo states affected most were Wisconsin, with 49 cases, and Ohio,with 25 cases; there were only 2 cases in California.Because *E. coli* O157:H7 (**Figure 36.11**) has been well studied,public health officials were able to identify the strain found in thebagged spinach and determine its origin. They conclusively linkedthe outbreak to the bagged spinach, traced it back to the processingplant, and eventually traced it to an agricultural field in thevicinity of the processing plant. DNA from the organisms isolatedfrom regional outbreaks was typed using pulsed-field gel electrophoresis(PFGE), a form of gel electrophoresis that betterdistinguishes between large molecules and is used in pathogenidentification. The patterns obtained were then compared; theresults showed that the same strain was responsible for the diseasein various parts of the country. The common thread in the geographicallyisolated outbreaks was consumption of the suspectedlots of bagged spinach originating from a single California facility.The precise source of the outbreak, although it has been tracedto a field near the processing plant, remains unknown. Feral pigsand domestic cattle are present in the vicinity of the identifiedfield, and contaminated wells or surface waters used for irrigationmay have introduced the pathogen into the fields and eventuallyinto the spinach. The original source was almost certainly animalin origin, as E. coli is an enteric organism found naturally only inthe intestine of animals.The spinach epidemic, although serious and even deadly forsome, was discovered, contained, and stopped very quickly. However, this incident also shows how centralized food-processingfacilities can quickly spread disease to large and distant populations.

Food hygiene standards and surveillance must be maintainedat the highest possible level in central food-processing anddistribution facilities.

Food Disease Reporting

In the United States foodborne outbreaks are reportable to theCenters for Disease Control and Prevention through FoodNet.Identification of particular organisms responsible for foodbornedisease outbreaks is particularly important. A reporting systemcalled PulseNet International is an international molecular subtypingnetwork for foodborne disease surveillance. It consists ofnational and regional PulseNet organizations from the UnitedStates, Canada, Europe, Asia, Latin America, the Caribbean, andthe Middle East. The organization collects and shares molecularsubtyping data from PFGE DNA fingerprints of organisms implicatedin foodborne disease outbreaks.

Figure 36.11 ***Escherichia coli*** **O157:H7.** The cell, about 1 μm in diameter, as it appears in a colorized transmission electron micrograph showing peritrichous flagella.

Food Infection

Food infection results from ingestion of food containing sufficientnumbers of viable pathogens to cause infection anddisease in the host. Food infection is very common, and we begin with a common bacterial cause, Salmonella. Manyfood infection agents can also cause waterborne diseases.

Salmonellosis

Salmonellosis is a gastrointestinal disease typically caused by foodborne*Salmonella* infection. Symptoms begin after the pathogencolonizes the intestinal epithelium. *Salmonella* are gram-negative,facultatively aerobic, motile rods related to *Escherichia coli* and other enteric bacteria. Salmonella normallyinhabits the animal intestine and is thus found in sewage.The nomenclature of the Salmonella spp. is based on taxonomicthat differentiate strains by virtue of biochemical, serological,and molecular (nucleic acid–based) characteristics. Theaccepted species name for the pathogenic members of the genusis *Salmonella enterica*. Based on nucleic acid analyses, there areseven evolutionary groups or subspecies of *S. enterica*. Mosthuman pathogens fall into group

I, designated as a single subspecies,*S. enterica* subspecies *enterica*. Finally, each subspeciesmay be divided into serovars (serological variations, also calledserotypes). Thus, the organism formally named *Salmonella enteric*subspecies *enterica* serovar *Typhi* is usually called *Salmonella enterica* serovar *Typhi* and is often abbreviated to *Salmonella Typhi. S. enterica ser. Typhi* causes the serious human diseasetyphoid fever but is rare in the United States. Most of the 500 orso annual foodborne cases caused by *S. enterica ser. Typhi* areacquired outside the United States.A number of other S. enterica serovars also cause foodborne gastroenteritis.

In all, over 1400 Salmonella serovars cause disease inhumans. *S. enterica serovarsTyphimurium* and *Enteritidis* arethe most common agents of foodborne salmonellosis in humans.

Epidemiology and Pathogenesis

The incidence of salmonellosis has been steady over the lastdecade, with about 40,000–45,000 documented cases each year(**Figure 36.15**). However, less than 4% of salmonellosis cases are probably reported, so the incidence of salmonellosis is probablyover 1 million cases every year. The ultimate sources of the foodborne salmonellas are theintestinal tracts of humans and other warm-blooded animals,and there are several routes by which these bacteria may enterthe food supply. The bacteria may reach food through fecal contaminationfrom food handlers. Food production animals such aschickens, pigs, and cattle may harbor *Salmonella* serovars that arepathogenic to humans, and the bacteria may be carried throughto finished fresh foods such as eggs, meat, and dairy products.Salmonella food infections are often traced to products such as custards, cream cakes, meringues, pies, and eggnog made withuncooked eggs. Other foods commonly implicated in salmonellosisoutbreaks are meats and meat products such as meat pies,cured but uncooked sausages and meats, poultry, milk, and milkproducts.

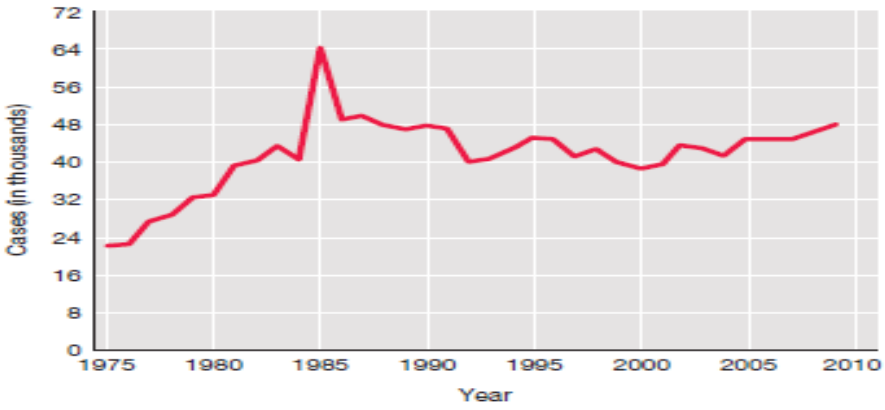


Figure 36.15 Reported cases of salmonellosis in the United States. Most cases of salmonellosis are foodborne. The total number of reported cases in 2007 was 47,995. The high incidence in 1985 was caused by contamination of pasteurized milk that was mixed with raw (unprocessed) milk in a dairy processing plant in Illinois. Data are from the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

The most common *salmonellosis* is enterocolitis. Ingestion offood containing viable Salmonella results in colonization of the small and large intestine. Onset of the disease occurs 8–48 hours after ingestion. Symptoms include the sudden onset of headache, chills, vomiting, and diarrhea, followed by a fever that lasts a fewdays. The disease normally resolves without intervention in

2–5days. After recovery, however, patients may shed *Salmonella* infeces for several weeks. Some patients recover and remainasymptomatic, but shed organisms for months or even years;they are chronic carriers. A few serovars ofSalmonella may also cause septicemia (a blood infection) andenteric or typhoid fever, a disease characterized by systemicinfection and high fever lasting several weeks. Mortality canapproach 15% in untreated typhoid fever.The pathogenesis of *Salmonella* infections starts with uptakeof the organisms from the gut. Salmonella ingested in food orwater invades phagocytes and grows as an intracellular pathogen,spreading to adjacent cells as host cells die. After invasion, pathogenicSalmonella uses a combination of endotoxins, enterotoxins,and cytotoxins to damage and kill host cellsMicrobial Sidebar "Virulence in *Salmonella*"), leading to the classicsymptoms of salmonellosis.

Diagnosis, Treatment, and Prevention

Foodborne salmonellosis is diagnosed from observation of clinicalsymptoms, history of recent food consumption, and culturing theorganism from feces. Selective and differential media are used toidentify *Salmonella* and discriminate it from other gram-negative Rods. Tests for the presence of Salmonella arecommonly used on animal food products, such as raw meat,poultry, eggs, and powdered milk. Salmonella has also beenfound, however, in nonmeat and nondairy food, including produce(cantaloupes and tomatoes) and peanut butter. Tests forSalmonella in food include several rapid tests, but even rapidtests rely on culture-based enrichment procedures to increaseSalmonella numbers to testable levels. The established standardused by PulseNet for epidemiological investigations is pulsedfieldgel electrophoresis. This moleculartyping technique can discriminate between various Salmonellaserovars.For enterocolitis, treatment is usually unnecessary, and antibiotictreatment does not shorten the course of the disease or eliminate the carrier state. Antibiotic treatment, however, significantlyreduces the length and severity of septicemia and typhoidfever. Mortality due to typhoid fever can be reduced to less than1% with appropriate antibiotic therapy. Multi-drug-resistantSalmonella are a significant clinical problem.Properly cooked foods heated to at least 70 ^{0}C are generally safeif consumed immediately, held at 50 ^{0}C, or stored immediately at48°C. Any foods that become contaminated by an infected foodhandler can support the growth of *Salmonella* if the foods areheld for long periods of time, especially without heating or refrigeration.Salmonella infections are more common in summerthan in winter, probably because warm environmental conditionsgenerally favor the growth of microorganisms in foods.Although local laws and enforcement vary, because of thelengthy carrier state, infected individuals are often banned fromwork as food handlers until their feces are negative for Salmonellain three successive cultures.

Pathogenic *Escherichia coli*

Most strains of *Escherichia coli* are common members of theenteric microflora in the human colon and are not pathogenic. A few strains, however, are potential foodborne pathogens. There are about 200 known pathogenic *E. coli* strains, all of which acton the intestine. Several are characterized by their production ofpotent enterotoxins and may cause life-threatening diarrheal

diseaseand urinary tract infections. The pathogenic strains aredivided based on the type of toxin they produce and the specificdiseases they cause.

Shiga Toxin–Producing *Escherichia coli* (STEC)

Shiga toxin–producing *Escherichia coli* (STEC) produce verotoxin,an enterotoxin similar to the Shiga toxin produced by Shigella dysenteriae. Formerly known as enterohemorrhagicE. coli (EHEC), the most widely distributed STEC is E. coliO157:H7 (Figure 36.11). Up to 90% of all STEC infections arecaused by E. coli O157:H7. After a person ingests food or water containing STEC, the bacteria grow in the small intestine andproduce verotoxin. Verotoxin causes both hemorrhagic (bloody)diarrhea and kidney failure. *E. coli* O157:H7 causes an estimated60,000 infections and 50 deaths from foodborne disease in the United States each year. STEC strains are the leadingcause of hemolytic uremic syndrome and kidney failure, with 292cases reported in 2007, about half in children under 5 years of age.About 40% of STEC infections are caused by the consumptionof contaminated uncooked or undercooked meat, particularlymass-processed ground beef. *E. coli* O157:H7 is a member of thenormal microbiome in healthy cattle; it can enter the human foodchain if meat is contaminated with intestinal contents duringslaughter and processing. In several major outbreaks in theUnited States caused by *E. coli* O157:H7, infected ground beeffrom regional distribution centers was the source of contamination. Infected meat products caused disease in several states. Anotheroutbreak was caused by processed and cured, but uncooked beefin ready-to-eat sausages. The source of contamination was thebeef, and the E. coli O157:H7 probably originated from slaughteredbeef carcasses.In 2003, the Food Safety and Inspection Service of the UnitedStates Department of Agriculture reported 20 positive results of 6584 samples (0.03%) of ground beef analyzed for *E. coli* O157:H7. *E. coli* O157:H7 has also been implicated in food infectionoutbreaks from dairy products, fresh fruit, and raw vegetables. Contamination of the fresh foods by fecal material, typically fromcattle carrying the *E. coli* O157:H7 strain, has been implicated inseveral of these cases.

Because *E. coli* O157:H7 grows in the intestines and is found in fecal material, it is also a potential source of waterborne gastrointestinal disease. Several outbreaks have also occurred in day-care facilities, where the presumed route of exposure is oral–fecal contamination.

Other Pathogenic *Escherichia coli*

Children in developing countries often contract diarrheal diseasecaused by *E. coli. E. coli* can also be the cause of “traveler’s diarrhea,”a common enteric infection causing watery diarrhea in travelers to developing countries. The primary causal agents arethe enterotoxigenic *E. coli* (ETEC). The ETEC strains usuallyproduce one of two heat-labile, diarrhea-producing enterotoxins.In studies of United States citizens traveling in Mexico, the infectionrate with ETEC is often greater than 50%. The prime vehiclesare foods such as fresh vegetables (for example, lettuce in salads)and water. The very high infection rate in travelers is due to contaminationof local public water supplies. The local population is usually resistant to the infecting strains, presumably becausethey have acquired resistance to the endemic ETEC strains.

Secretory IgA antibodies in the bowel prevent colonization ofthe pathogen in local residents, but the organism readily infectsthe nonimmune travelers and causes disease.Enteropathogenic E. coli (EPEC) strains cause diarrheal diseasesin infants and small children but do not cause invasive disease orproduce toxins. Enteroinvasive E. coli (EIEC) strains cause invasivedisease in the colon, producing watery, sometimes bloody diarrhea.The EIEC strains are taken up by phagocytes, but escape lysisin the phagolysosomes, grow in the cytoplasm, and move intoother cells in much the same way as pathogenic Salmonella strains.This invasive disease causes diarrhea and is common in developingcountries.

Diagnosis and Treatment

Illness from *E. coli* O157:H7 and other STEC strains is areportable infectious disease in the United States. The generalpattern established for diagnosis, treatment, and prevention ofinfection by *E. coli* O157:H7 reflects current procedures used forall of the pathogenic E. coli strains. Laboratory diagnosis requiresculture from the feces and identification of the O (lipopolysaccharide)and H (flagellar) antigens and toxins by serology. Identificationof strains is also done using DNA analyses such asrestriction fragment length polymorphism and PFGE. *E. coli*O157:H7 outbreaks are reported through FoodNet and PulseNetto the Centers for Disease Control and Prevention.Treatment of *E. coli* O157:H7 and other STEC infectionsincludes supportive care and monitoring of renal function, bloodhemoglobin, and platelets. Antibiotics may be harmful becausethey may cause the release of large amounts of verotoxin fromdying *E. coli* cells. For other pathogenic *E. coli* infections, treatmentusually includes supportive therapy and, for severe cases and invasivedisease, antimicrobial drugs to shorten and eliminate infection.

Prevention

The most effective way to prevent infection with foodborneSTEC is to make sure that meat is cooked thoroughly, whichmeans that it should appear gray or brown and juices should be clear. As we discussed above, the United Stateshas approved the irradiation of ground meat as an acceptablemeans of eliminating or reducing food infection bacteria,largely because *E. coli* O157:H7 has been implicated in severalfoodborne epidemics. To process foods such as ground beef,large-scale production plants may mix and grind meat fromhundreds or even thousands of animals together; the grindingprocess could distribute the pathogens from a single infectedanimal throughout the meat. Short of cooking, penetratingradiation is considered the only effective means to ensuredecontamination.In general, proper food handling, water purification, andappropriate hygiene prevent the spread of pathogenic *E. coli*.Raw foods should be washed thoroughly. Traveler’s diarrhea canbe prevented by avoiding consumption of local water and uncooked foods.

Campylobacter

Species of *Campylobacter* are the most common reported causeof bacterial foodborne infections in the United States. Cells of*Campylobacter* species are gram-negative, motile, curved rods to spiral-shaped bacteria that grow at reduced oxygen tension asmicroaerophiles. Several

pathogenic species,*Campylobacter jejuni* (**Figure 36.16**), *C. coli, and C. fetus, are recognized. C. jejuni* and *C. coli* account for almost 2 million annualcases of bacterial diarrhea (Table 36.6). C. fetus is a major cause ofsterility and spontaneous abortion in cattle and sheep.

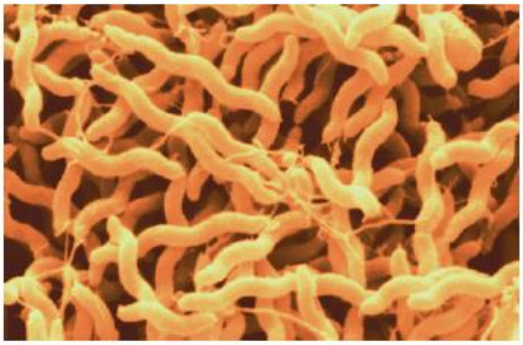


Figure 36.16 ***Campylobacter jejuni.*** The gram-negative curved rods shown in this colorized scanning electron micrograph are about 1 μm in diameter.

Epidemiology and Pathology

Campylobacter is transmitted to humans via contaminated food,most frequently in poultry, pork, raw shellfish, or in surfacewaters. *C. jejuni* is a normal resident in the intestinal tract ofpoultry; virtually all chickens and turkeys are normally colonizedwith this organism. According to the United States Departmentof Agriculture, up to 90% of turkey and chicken carcasses and over30% of hog carcasses may be contaminated with Campylobacter.Beef, on the other hand, is rarely a vehicle for this pathogen.*Campylobacter* species also infect domestic animals such asdogs, causing a milder form of diarrhea than that observed inhumans. Campylobacter infections in infants are frequentlytraced to infected domestic animals, especially dogs.After a person ingests cells of Campylobacter, the organismmultiplies in the small intestine, invades the epithelium, andcauses inflammation. Because *C. jejuni* is sensitive to gastric acid,cell numbers as high as 104 may be required to initiate infection.However, this number may be reduced to less than 500 if thebacteria are ingested in food, or are ingested by a person takingmedication to reduce stomach acid production. Campylobacterinfection causes a high fever (usually greater than 104°F or40°C), headache, malaise, nausea, abdominal cramps, and profusediarrhea with watery, frequently bloody, stools. The disease subsides in about 7–10 days. Spontaneous recovery from Campylobacter infections is often complete, but relapses occur inup to 25% of cases.

Diagnosis, Treatment, and Prevention

Diagnosis of *Campylobacter* food infection requires isolation ofthe organism from stool samples and identification by growthdependenttests, immunological assays, or molecular tests. Serious *C. jejuni* infections are often seen in infants. In these cases, diagnosisis important; selective media and specific immunologicalmethods have been developed for positive identification of

thisorganism. Erythromycin and quinolone treatment may be usefulearly in severe diarrheal disease. Adequate personal hygiene, properwashing of uncooked poultry (and any kitchenware coming incontact with uncooked poultry), and thorough cooking of meateliminate *Campylobacter* contamination.As with other foodborne infections, epidemiologic investigations are based on PFGE analysis of recovered organisms. Data sharedon PulseNet are used to track the spread of Campylobacter anddetermine its origin.

Listeriosis

Listeria monocytogenes causes **listeriosis**, a gastrointestinal foodinfection that may lead to bacteremia and meningitis. L. monocytogenesis a short, gram-positive, nonsporulating coccobacillus that is acid-, salt- and cold-tolerant and facultatively aerobic(**Figure 36.17**).

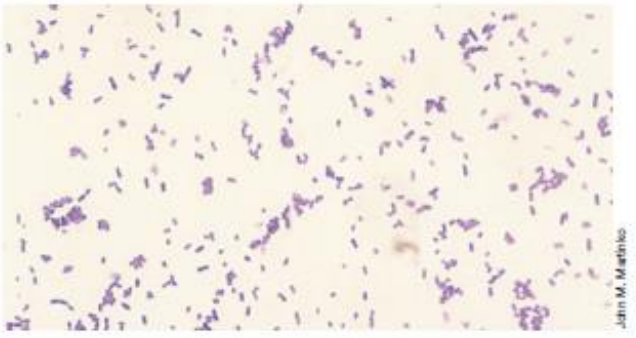


Figure 36.17 ***Listeria monocytogenes.*** This Gram stain shows gram-positive coccobacilli, about 0.5 μm in diameter.

Epidemiology and Pathology

L. monocytogenes is found widely in soil and water; virtually nofood source is safe from possible *L. monocytogenes* contamination.Food can become contaminated at any stage during foodproduction or processing. Food preservation by refrigeration,which ordinarily slows microbial growth, is ineffective in limitinggrowth of this psychrotolerant organism. Ready-to-eat meats,fresh soft cheeses, unpasteurized dairy products, and inadequatelypasteurized milk are the major food vehicles for thispathogen, even when foods are properly stored at refrigerator temperature (48°C).*L. monocytogenes* is an intracellular pathogen. It enters thebody through the gastrointestinal tract in contaminated food.Phagocytes take up the pathogen in a phagolysosome. Thistriggers production of listeriolysin O, which lyses the phagolysosome and releases *L. monocytogenes* into the cytoplasm. Here itmultiplies and produces ActA, a surface protein that induceshost cell actin polymerization, which moves the pathogen to the cytoplasmic membrane. At the cytoplasmic membrane, the complexpushes out, forming protrusions called filopods. The filopodsare then ingested by surrounding cells and the cycle starts again.This mechanism allows *L. monocytogenes* to move from cell tocell without exposure to antibodies, complement, or neutrophils.Specific immunity to *L. monocytogenes* is through cell-mediatedTH1 inflammatory cells. Particularly susceptiblepopulations include the elderly, pregnant women, newborns, andimmunosuppressed individuals [for example, transplant patientsundergoing steroid therapy and acquired immunodeficiencysyndrome (AIDS) patients]. Although exposure to *L.*

monocytogenes is undoubtedly verycommon, there are only about 2500 estimated cases of clinicallisteriosis each year, and fewer than 1000 are reported. Nearly all diagnosed cases require hospitalization. Acute listeriosis is rareand is characterized by septicemia, often leading to meningitis,with a mortality rate of 20% or higher. About 30–40 listeriosisdeaths are reported annually in the United States.

Diagnosis, Treatment, and Prevention

Listeriosis is diagnosed by culturing *L. monocytogenes* from theblood or spinal fluid. *L. monocytogenes* can be identified in foodby direct culture or by molecular methods such as ribotyping andthe polymerase chain reaction. Clinical isolates are analyzed byPFGE to determine molecular subtypes. The subtype patternsare reported to PulseNet at the Centers for Disease Controland Prevention. Intravenous antibiotic treatment with penicillin,ampicillin, or trimethoprim plus sulfamethoxazole is recommendedfor invasive disease.

Prevention measures include recalling contaminated foodand taking steps to limit L. monocytogenes contamination at thefood-processing site. Because *L. monocytogenes* is susceptible toheat and radiation, raw food and food-handling equipment can bereadily decontaminated. However, without pasteurizing or cookingthe finished food product, the risk of contamination cannot beeliminated because of the widespread distribution of the pathogen.

Individuals who are immunocompromised should avoid unpasteurizeddairy products and ready-to-eat processed meats.Pregnant women should also avoid foods that may transmit*L. monocytogenes* because spontaneous abortion is a frequentoutcome of listeriosis.

Other FoodborneInfectious Diseases

Over 200 other microorganisms, viruses, and other infectiousagents such as prions contribute to foodborne diseases.

Bacteria

*Yersinia enterocolitica*is commonly found in the intestines of domestic animalsand causes foodborne infections due to contaminated meat anddairy products. The most serious consequence of *Y. enterocolitica*infection is enteric fever, a severe life-threatening infection. Bacilluscereus produces two enterotoxins that cause diarrhea and vomiting.The organism grows in foods such as rice, pasta, meats, orsauces that are cooked and left at room temperature to coolslowly. Endospores of this gram-positive rod germinate and toxinis produced. Reheating may kill the B. cereus, but the toxin mayremain active. B. cereus may also cause a food infection similar tothat caused by *Clostridium perfringens*. *Shigella* species can causesevere invasive gastroenteritis called shigellosis. About 20,000cases of shigellosis are reported each year in the United States,with up to 150 million cases worldwide. Most *Shigella* infectionsare the result of fecal–oral contamination, but food and waterare occasional vehicles. Several members of the *Vibrio* genuscause food poisoning in persons who consume contaminatedshellfish.

Viruses : The largest number of annual foodborne infections is thought tobe caused by viruses. In general, viral foodborne illness consistsof gastroenteritis characterized by diarrhea, often

accompanied by nausea and vomiting. Recovery is spontaneous and rapid,usually within 24–48 hours (“24-hour bug”). Noroviruses (**Figure36.18**) are responsible for most of these mild foodborne infections in the United States, accounting for over 9 millionof the estimated 13 million annual cases of foodborne disease.Rotavirus, astrovirus, and hepatitis A collectively cause 100,000 cases of foodborne disease each year. These viruses inhabit thegut and are often transmitted to food or water with fecal matter.As with many foodborne infections, proper food handling, handwashing,and a source of clean water to prepare fresh foods areessential to prevent infection.

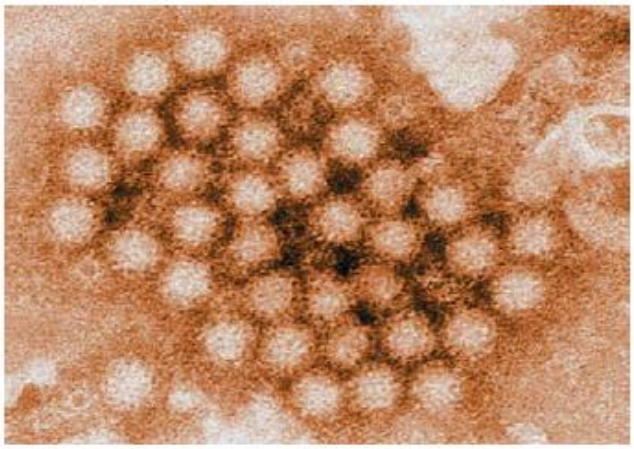
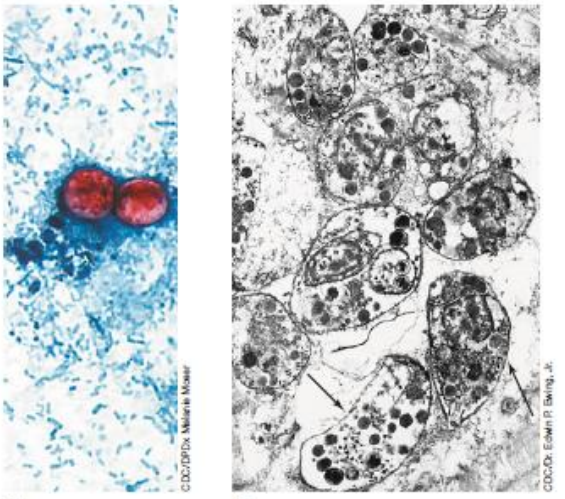


Figure 36.18 Human norovirus. The virus was isolated from a patient with diarrhea. Individual norovirus particles have an indistinct rough outer edge and are about 27 nm in diameter.

Protists Important foodborne protist diseases are listed in Table 36.6. Protists including *Giardia intestinalis*, *Cryptosporidium parvum* and *Cyclospora cayetanensis* (**Figure 36.19*a***) can be spread in foods contaminated by fecal matter in untreated water used to wash, irrigate, or spray crops. Fresh foods such as fruits are often implicated as the source of these protists. Cyclosporiasis is an acute gastroenteritis and is an important emerging disease. In the United States, most cases are acquired by eating fresh produce imported from other countries. *Toxoplasma gondii* is a protist spread through cat feces, but is also found in raw or undercooked meat. In most individuals, toxoplasmosis is a mild, self-limiting gastroenteritis. However, prenatal infection of the fetus can lead to serious acute toxoplasmosisresulting in tissue involvement, cyst formation, andcomplications such as myocarditis, blindness, and stillbirth.
Immunocompromised individuals such as people with acquiredimmunodeficiency syndrome (AIDS) may develop acute toxoplasmosis.*T. gondii* grows intracellularly and forms structures called tachyzoites (Figure 36.19b) that eventually lyse the cell andinfect nearby cells, resulting in tissue destruction. Tachyzoitescan cross the placenta and infect the fetus. Toxoplasma infectionsin compromised hosts can be treated with the antiprotistdrug pyrimethamine.



(a) (b)

Figure 36.19 Protists transmitted in food. *(a) Cyclospora cayetanensis* oocysts in a stool sample from an affected patient. The oocysts, stained red with safranin, are about 8–10 μm in diameter. *(b)* Tachyzoites of *Toxoplasma gondii*, an intracellular parasite. In this transmission electron micrograph, the tachyzoites (arrows) are in a cystlike structure in a cardiac myocyte. Tachyzoites are generally elongated to crescent in form, about 4–7 μm long by 2–4 μm wide.

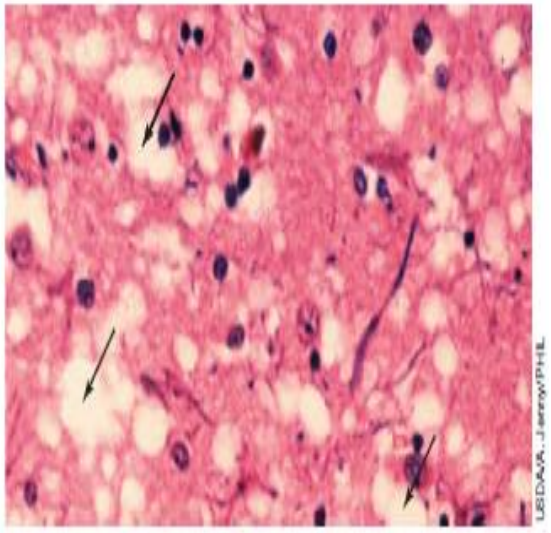


Figure 36.20 A brain section from a cow with bovine spongiform encephalopathy (BSE). The vacuoles, appearing as holes (arrows), giv the brains of infected animals a distinct spongelike appearance.

Prions, BSE, and nvCJD : Prions are proteins, presumably of host origin, that adopt novelconformations, inhibiting normal protein function and causing degeneration of neural tissue. Human priondiseases are characterized by neurological symptoms includingprogressive depression, loss of motor coordination, and dementia.A foodborne prion disease in humans known as new variantCreutzfeldt–Jakob Disease (nvCJD) has been linked to consumptionof meat products from cattle afflicted with bovine spongiformencephalopathy (BSE), a prion disease commonly called "mad
cow disease." A slow-acting degenerative nervous system disorder,nvCJD has a latent period that may extend for years after exposureto the BSE prion. Nearly 200 people in Great Britain andother European countries have acquired nvCJD. However, nvCJDlinked to domestic meat consumption has not been observedin the United States. BSE prions consumed in meat products
from affected cattle trigger human protein analogs to assume analtered conformation, resulting in protein dysfunction and disease. The terminal stages of both BSE andnvCJD are characterized by large vacuoles in brain tissue, givingthe brain a "spongy" appearance, from which BSE derives itsname (**Figure 36.20**).

In the United Kingdom and Europe, about 180,000 cattle werediagnosed with BSE and destroyed in the 1990s. Brains of slaughteredanimals are routinely tested for BSE in the United States,and several cattle with BSE have been found in Canadian andU.S. herds. In Europe and North America, all cattle known orsuspected to have BSE have been destroyed. Bans on cattle feedscontaining cattle meat and bone meal appear to have stopped thedevelopment of new cases of BSE in Europe and have kept theincidence of this disease very low in North America. The

infectingprions were probably transferred to food production animalsthrough meat and bone meal feed derived from infected cattle orother animals not approved for human consumption.
Diagnosis of BSE is done by testing using a prion-susceptiblemouse strain or by immune histochemical or micrographic analysisof biopsied neural tissue.

Soil-borne disease:

All gardens have a range of permanent soil-borne disease organisms which are usually contained in a balanced environment where organisms, soil conditions, and hosts interact in a complex system. Garden plants only show symptoms of disease when this balance is disrupted and pathogen organisms become dominant.
Gardeners can overcome disease by improving plant conditions, creating a hostile environment for the disease and by stimulating the growth of beneficial soil organisms.

Types of soil-borne diseases

Soil-borne diseases in the garden include pre and post-emergence damping-off, like *Fusarium*, *Pythium* and *Rhizoctonia* species, root rot, including *Phytophthora*, vascular wilts caused by fungi including *Verticillium* and nematodes.
Pre-emergence damping-off is where young seedlings decay in the soil before they appear above the soil surface. This occurs when conditions for seed germination are poor, such as cold, hot or very wet soil, poorly-drained soil, compacted soil or in the presence of undecayed organic matter.
Post-emergence damping-off is where stems and roots of tender seedlings are attacked at the soil line and the seedlings fall over. High salt concentrations in the soil also cause damping-off.
Root rots can affect plants beyond the seedling stage when the fungi invades internal root tissue, interfering with the supply of water and nutrients. Aboveground symptoms include loss of vigour, leaf yellowing, leaf drop, wilting starting at the growing tip, twig dieback, and sudden death.
Vascular wilts are characterised by plant wilting and discolouration of the vascular system at stems or trunks and branches.
Nematodes are microscopic, unsegmented worms. They include pest nematodes like the root knot nematode which invades the roots and causes them to form gall-like lesions that restrict water and nutrient uptake which causes wilting.

Hygiene

To prevent disease spread plant material, including cuttings, transplants, and seeds, should come from reliable sources. Research the disease history of gardens before transplanting plants from them.
Sterilise second-hand tools including pots, trellises and support material before using or reusing them in your garden. Use sodium hypochlorite at 1.2 % of available chlorine to disinfect materials.
Dispose of diseased plant material by burning or composting. Do not use the material for mulching until it is well broken down.

Preventing disease

Plant stresses

High temperatures, low soil moisture or continued flooding predispose plants to disease.
To reduce plant stress transplant during cool, moist weather when continued mild conditions are forecast.
Shade and mulch soil using medium to coarse grade bark mulches which allow rain or irrigation water to penetrate the soil. Grow transplants in well-drained soil and protect them from drying winds. Products like anti transpirants, which are sprayed onto the leaves, can help reduce moisture loss from young seedlings. Do not fertilise plants in hot, dry weather.
Seedlings should be transplanted into their permanent positions when they are sufficiently hardened but still young. Plants are generally more susceptible to transplanting injury as they age. Soil organisms will use these injuries to invade the plant.

Organic matter

Opportunistic soil pathogens thrive on rotting organic matter, which also depletes soil nitrogen, so only work well-rotted organic matter into the soil near young, growing roots. Give organic matter two to three months to break down before planting a new crop, especially in soils with a history of fungal diseases.

Salt, fertiliser and waterlogging

Salty water causes general loss of vigour in the plant and associated stunting, yellowing, wilting, leaf-loss and damping-off due to fungal infection.
Over fertilisation can also cause root injury and subsequent fungal infection.Seeds and roots decay in anaerobic conditions, which are caused by poorly aerated soils with insufficient drainage. These conditions encourage attacks by fungi such as *Pythium* and *Phytophthora* spp. In waterlogged conditions avoid over watering, improve the drainage in clay soils with gypsum and compost or choose plants that tolerate ‘wet feet’.

Soil disinfestation

If there is a history of fungal diseases in garden beds the soil can be solarised during hot weather. Moisten the soil and work to a fine tilth to 25cm deep. Then cover the soil with a thin, transparent polyethylene sheet and bury the edges 25cm or more deep. Leave the sheeting in place for at least four weeks. Make sure the polyethylene sheet is sealed so that no air can blow under it. Solar heating is less effective at the edge of the sheeting, so treated areas should not be long and narrow. This treatment kills most pathogens, while normal soil microorganisms survive in sufficient numbers to prevent recolonisation by the disease pathogens. Solarisation also kills weed seeds and insect pests.

Crop rotation

Rotate crops of annual vegetable plants and plant crops from different plant families to break the disease cycle. If diseases occur wait two to five years before replanting the crop in the same position.

VENEREAL DISEASE

Sexually transmitted infections (**STIs**), also referred to as **sexually transmitted diseases** (**STDs**), are infections that are commonly spread by sexual activity, especially vaginal intercourse, anal sex and oral sex. Many times STIs initially do not cause symptoms. This results in a greater risk of passing the disease on to others. Symptoms and signs of disease may include vaginal discharge, penile discharge, ulcers on or around the genitals, and pelvic pain. STIs can be transmitted to an infant before or during childbirth and may result in poor outcomes for the baby. Some STIs may cause problems with the ability to get pregnant.

More than 30 different bacteria, viruses, and parasites can be transmitted through sexual activity. Bacterial STIs include chlamydia, gonorrhea, and syphilis.] Viral STIs include genital herpes, HIV/AIDS, and genital warts. Parasitic STIs include trichomoniasisWhile usually spread by sex, some STIs can be spread by non-sexual contact with donor tissue, blood, breastfeeding, or during childbirth. STI diagnostic tests are usually easily available in the developed world, but this is often not the case in the developing world.

The most effective way of preventing STIs is by not having sex. Some vaccinations may also decrease the risk of certain infections including hepatitis B and some types of HPV. Safer sex practices such as use of condoms, having a smaller number of sexual partners, and being in a relationship where each person only has sex with the other also decreases the risk. Circumcision in adult males may be effective to prevent some infections. During school, comprehensive sex education may also be useful. Most STIs are treatable or curable. Of the most common infections, syphilis, gonorrhea, chlamydia, and trichomoniasis are curable, while herpes, hepatitis B, HIV/AIDS, and HPV are treatable but not curable. Resistance to certain antibiotics is developing among some organisms such as gonorrhea

In 2015, about 1.1 billion people had STIs other than HIV/AIDSAbout 500 million were infected with either syphilis, gonorrhea, chlamydia or trichomoniasisAt least an additional 530 million people have genital herpes and 290 million women have human papillomavirus STIs other than HIV resulted in 108,000 deaths in 2015 In the United States there were 19 million new cases of sexually transmitted infections in 2010. Historical documentation of STIs date back to at least the Ebers papyrus around 1550 BC and the Old Testament. There is often shame and stigma associated with these infections. The term *sexually transmitted infection* is generally preferred over *sexually transmitted disease* or *venereal disease*, as it includes those who do not have symptomatic disease.

SIGN AND SYMPTOMS : Not all STIs are symptomatic, and symptoms may not appear immediately after infection. In some instances a disease can be carried with no symptoms, which

leaves a greater risk of passing the disease on to others. Depending on the disease, some untreated STIs can lead to infertility, chronic pain or death.

The presence of an STI in prepubescent children may indicate sexual abuse.

Transmission

A sexually transmitted infection present in a pregnant woman may be passed on to the infant before or after birth.

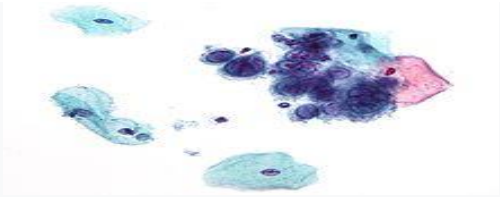
	Known risks	Possible
Performing oral sex on a man	• Throat chlamydia[17] • Throat gonorrhea[17] (25–30%) • Herpes (rare) • HPV[18] • Syphilis[17] (1%)[19]	• Hepatitis B (low risk)[20] • HIV (0.01%)[21] • Hepatitis C (unknown)
Performing oral sex on a woman	• Herpes • HPV[18]	• Throat gonorrhea[17] • Throat chlamydia[17]
Receiving oral sex—man	• Chlamydia • Gonorrhea[17] • Herpes • Syphilis[17] (1%)[19]	• HPV
Receiving oral sex—woman	• Herpes	• HPV • Bacterial vaginosis[17] • Gonorrhea[17]
Vaginal sex—man	• Chlamydia (30–50%)[20] • Crabs • Scabies • Gonorrhea (22%)[22] • Hepatitis B • Herpes (0.07% for HSV-2)[23] • HIV (0.05%)[21][23] • HPV (high: around 40–50%)[24] • Mycoplasma hominis infection[30][31][32][33][34] • *Mycoplasma genitalium*[35][36][37] • Syphilis • Trichomoniasis • *Ureaplasma infection*[38][39][34]	• Hepatitis C
Vaginal sex—woman	• Chlamydia (30–50%)[20] • Crabs • Scabies • Gonorrhea (47%)[25] • Hepatitis B (50–70%) • Herpes • HIV (0.1%)[21] • HPV (high;[20] around 40–50%)[24] • Mycoplasma hominis infection[30][31][34] • Syphilis • Trichomoniasis • Ureaplasma infection[38][39][34]	• Hepatitis C

Bacterial

- Chancroid (*Haemophilus ducreyi*)
- Chlamydia (*Chlamydia trachomatis*)
- Gonorrhea (*Neisseria gonorrhoeae*), colloquially known as "the clap"
- Granuloma inguinale or (*Klebsiella granulomatis*)
- *Mycoplasma genitalium*
- *Mycoplasma hominis*
- Syphilis (*Treponema pallidum*)
- Ureaplasma infection

Fungal

- Candidiasis (yeast infection)
- **Viral**



Micrograph showing the viral cytopathic effect of herpes (ground glass nuclear inclusions, multi-nucleation). Pap test. Pap stain.

- Viral hepatitis (Hepatitis B virus)—saliva, venereal fluids. (Note: Hepatitis A and Hepatitis E are transmitted via the fecal-oral route; Hepatitis C is rarely sexually transmittable, and the route of transmission of Hepatitis D (only if infected with B) is uncertain, but may include sexual transmission.
- Herpes simplex (Herpes simplex virus 1, 2) skin and mucosal, transmissible with or without visible blisters
- HIV (*Human Immunodeficiency Virus*)—venereal fluids, semen, breast milk, blood
- HPV (*Human Papillomavirus*)—skin and mucosal contact. 'High risk' types of HPV cause almost all cervical cancers, as well as some anal, penile, and vulvar cancer. Some other types of HPV cause genital warts.
- Molluscum contagiosum (molluscum contagiosum virus MCV)—close contact

Parasites

- Crab louse, colloquially known as "crabs" or "pubic lice" (*Pthirus pubis*). The infestation and accompanying inflammation is Pediculosis pubis
- Scabies (*Sarcoptes scabiei*)
- Trichomoniasis (*Trichomonas vaginalis*), colloquially known as "trich"

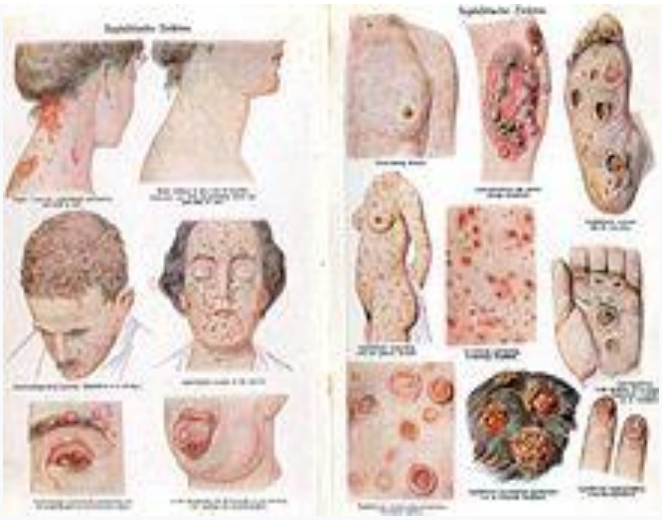
Main types:

Sexually transmitted infections include: Chlamydia is a sexually transmitted infection caused by the bacterium *Chlamydia trachomatis*. In women, symptoms may include abnormal vaginal

discharge, burning during urination, and bleeding in between periods, although most women do not experience any symptoms. Symptoms in men include pain when urinating, and abnormal discharge from their penis. If left untreated in both men and women, Chlamydia can infect the urinary tract and potentially lead to pelvic inflammatory disease (PID). PID can cause serious problems during pregnancy and even has the potential to cause infertility. It can cause a woman to have a potentially deadly ectopic pregnancy, in which the egg implants outside of the uterus. However, Chlamydia can be cured with antibiotics.

- The two most common forms of herpes are caused by infection with herpes simplex virus (HSV). HSV-1 is typically acquired orally and causes cold sores, HSV-2 is usually acquired during sexual contact and affects the genitals, and however either strain may affect either site. Some people are asymptomatic or have very mild symptoms. Those that do experience symptoms usually notice them 2 to 20 days after exposure which last 2 to 4 weeks. Symptoms can include small fluid-filled blisters, headaches, backaches, itching or tingling sensations in the genital or anal area, pain during urination, Flu like symptoms, swollen glands, or fever. Herpes is spread through skin contact with a person infected with the virus. The virus affects the areas where it entered the body. This can occur through kissing, vaginal intercourse, oral sex or anal sex. The virus is most infectious during times when there are visible symptoms, however those who are asymptomatic can still spread the virus through skin contact. The initial infection and symptoms are usually the most severe because the body does not have any antibodies built up. After the primary attack, one might have recurring attacks that are milder or might not even have future attacks. There is no cure for the disease but there are antiviral medications that treat its symptoms and lower the risk of transmission (Valtrex). Although HSV-1 is typically the "oral" version of the virus, and HSV-2 is typically the "genital" version of the virus, a person with HSV-1 orally CAN transmit that virus to their partner genitally. The virus, either type, will settle into a nerve bundle either at the top of the spine, producing the "oral" outbreak, or a second nerve bundle at the base of the spine, producing the genital outbreak.
- The human papillomavirus (HPV) is the most common STI in the United States. There are more than 40 different strands of HPV and many do not cause any health problems. In 90% of cases the body's immune system clears the infection naturally within 2 years. Some cases may not be cleared and can lead to genital warts (bumps around the genitals that can be small or large, raised or flat, or shaped like cauliflower) or cervical cancer and other HPV related cancers. Symptoms might not show up until advanced stages. It is important for women to get pap smears in order to check for and treat cancers. There are also two vaccines available for women (Cervarix and Gardasil) that protect against the types of HPV that cause cervical cancer. HPV can be passed through genital-to-genital contact as well as during oral sex. It is important to remember that the infected partner might not have any symptoms.
- Gonorrhea is caused by bacterium that lives on moist mucous membranes in the urethra, vagina, rectum, mouth, throat, and eyes. The infection can spread through contact with the penis, vagina, mouth or anus. Symptoms of gonorrhea usually appear 2 to 5 days after

contact with an infected partner however, some men might not notice symptoms for up to a month. Symptoms in men include burning and pain while urinating, increased urinary frequency, discharge from the penis (white, green, or yellow in color), red or swollen urethra, swollen or tender testicles, or sore throat. Symptoms in women may include vaginal discharge, burning or itching while urinating, painful sexual intercourse, severe pain in lower abdomen (if infection spreads to fallopian tubes), or fever (if infection spreads to fallopian tubes); however, many women do not show any symptoms. There are some antibiotic resistant strains for Gonorrhea but most cases can be cured with antibiotics.



Secondary syphilis

- Syphilis is an STI caused by a bacterium. Untreated, it can lead to complications and death. Clinical manifestations of syphilis include the ulceration of the urogenital tract, mouth or rectum; if left untreated the symptoms worsen. In recent years, the prevalence of syphilis has declined in Western Europe, but it has increased in Eastern Europe (former Soviet states). A high incidence of syphilis can be found in places such as Cameroon, Cambodia, Papua New Guinea. Syphilis infections are increasing in the United States.
- Trichomoniasis is a common STI that is caused by infection with a protozoan parasite called *Trichomonas vaginalis*. Trichomoniasis affects both women and men, but symptoms are more common in women. Most patients are treated with an antibiotic called metronidazole, which is very effective.
- HIV (human immunodeficiency virus) damages the body's immune system, which interferes with its ability to fight off disease-causing agents. The virus kills CD4 cells, which are white blood cells that help fight off various infections. HIV is carried in body fluids, and is spread by sexual activity. It can also be spread by contact with infected blood, breast feeding, childbirth, and from mother to child during pregnancy. When HIV is at its most advanced stage, an individual is said to have AIDS (acquired immunodeficiency syndrome). There are different stages of the progression of and HIV infection. The stages include primary infection, asymptomatic infection, symptomatic infection, and AIDS. In the primary

infection stage, an individual will have flu like symptoms (headache, fatigue, fever, muscle aches) for about 2 weeks. In the asymptomatic stage, symptoms usually disappear, and the patient can remain asymptomatic for years. When HIV progresses to the symptomatic stage, the immune system is weakened, and has a low cell count of CD4+ T Cells. When the HIV infection becomes life-threatening, it is called AIDS. People with AIDS fall prey to opportunistic infections and die as a result. When the disease was first discovered in the 1980s, those who had AIDS were not likely to live longer than a few years. There are now antiretroviral drugs (ARVs) available to treat HIV infections. There is no known cure for HIV or AIDS but the drugs help suppress the virus. By suppressing the amount of virus in the body, people can lead longer and healthier lives. Even though their virus levels may be low they can still spread the virus to others.

Viruses in semen

Twenty-seven different viruses have been identified in semen. Information on whether or not transmission occurs or whether the viruses cause disease is uncertain. Some of these microbes are known to be sexually transmitted. Those found in semen are listed by the CDC.

Microbes known to be sexually transmissible (but not generally considered STIs) include:

- Marburg virus – Virus in semen for seven weeks after clinical recovery.
- HTLV (both types 1 and 2) – Sexually transmissible, consumption of breast milk breastfeeding, and once mistaken as a HIV, risk of leukemia.

Pathophysiology

Many STIs are (more easily) transmitted through the mucous membranes of the penis, vulva, rectum, urinary tract and (less often—depending on type of infection) the mouth, throat, respiratory tract and eyes. The visible membrane covering the head of the penis is a mucous membrane, though it produces no mucus (similar to the lips of the mouth). Mucous membranes differ from skin in that they allow certain pathogens into the body. The amount of contact with infective sources which causes infection varies with each pathogen but in all cases, a disease may result from even light contact from fluid carriers like venereal fluids onto a mucous membrane.

Some STIs such as HIV can be transmitted from mother to child either during pregnancy or breastfeeding.

Healthcare professionals suggest safer sex, such as the use of condoms, as a reliable way of decreasing the risk of contracting sexually transmitted diseases during sexual activity, but safer sex cannot be considered to provide complete protection from an STI. The transfer of and exposure to bodily fluids, such as blood transfusions and other blood products, sharing injection needles, needle-stick injuries (when medical staff are inadvertently jabbed or pricked with needles during medical procedures), sharing tattoo needles, and childbirth are other avenues of transmission. These different means put certain groups, such as medical workers, and haemophiliacs and drug users, particularly at risk.

It is possible to be an asymptomatic carrier of sexually transmitted diseases. In particular, sexually transmitted diseases in women often cause the serious condition of pelvic inflammatory disease.

Diagnosis

Testing may be for a single infection, or consist of a number of tests for a range of STIs, including tests for Syphilis, Trichomonas, Gonorrhea, Chlamydia, herpes, hepatitis and HIV. No procedure tests for all infectious agents.

STI tests may be used for a number of reasons:

- as a diagnostic test to determine the cause of symptoms or illness
- as a screening test to detect asymptomatic or presymptomatic infections
- as a check that prospective sexual partners are free of disease before they engage in sex without safer sex precautions (for example, when starting a long term mutually monogamous sexual relationship, in fluid bonding, or for procreation).
- as a check prior to or during pregnancy, to prevent harm to the baby
- as a check after birth, to check that the baby has not caught an STI from the mother
- to prevent the use of infected donated blood or organs
- as part of the process of contact tracing from a known infected individual
- as part of mass epidemiological surveillance

Early identification and treatment results in less chance to spread disease, and for some conditions may improve the outcomes of treatment. There is often a window period after initial infection during which an STI test will be negative. During this period, the infection may be transmissible. The duration of this period varies depending on the infection and the test. Diagnosis may also be delayed by reluctance of the infected person to seek a medical professional. One report indicated that people turn to the Internet rather than to a medical professional for information on STIs to a higher degree than for other sexual problems.

PREVENTION

Strategies for reducing STI risk include: vaccination, mutual monogamy, reducing the number of sexual partners, and abstinence. Behavioral counseling for all sexually active adolescents and for adults who are at increased risk. Such interactive counseling, which can be resource intensive, is directed at a person's risk, the situations in which risk occurs, and the use of personalized goal-setting strategies.

The most effective way to prevent sexual transmission of STIs is to avoid contact of body parts or fluids which can lead to transfer with an infected partner. Not all sexual activities involve contact: cybersex, phonesex or masturbation from a distance are methods of avoiding contact.

Proper use of condoms reduces contact and risk. Although a condom is effective in limiting exposure, some disease transmission may occur even with a condom.

Both partners can get tested for STIs before initiating sexual contact, or before resuming contact if a partner engaged in contact with someone else. Many infections are not detectable immediately after exposure, so enough time must be allowed between possible exposures and testing for the tests to be accurate. Certain STIs, particularly certain persistent viruses like HPV, may be impossible to detect.

Some treatment facilities utilize in-home test kits and have the person return the test for follow-up. Other facilities strongly encourage that those previously infected return to ensure that the infection has been eliminated. Novel strategies to foster re-testing have been the use of text messaging and email as reminders. These types of reminders are now used in addition to phone calls and letters. After obtaining a sexual history, a healthcare provider can encourage risk reduction by providing prevention counseling. Prevention counseling is most effective if provided in a nonjudgmental and empathetic manner appropriate to the person's culture, language, gender, sexual orientation, age, and developmental level. Prevention counseling for STIs is usually offered to all sexually active adolescents and to all adults who have received a diagnosis, have had an STI in the past year, or have multiple sex partners.

Vaccines

Vaccines are available that protect against some viral STIs, such as Hepatitis A, Hepatitis B, and some types of HPV. Vaccination before initiation of sexual contact is advised to assure maximal protection. The development of vaccines to protect against gonorrhea is ongoing.

Condoms

Condoms and female condoms only provide protection when used properly as a barrier, and only to and from the area that they cover. Uncovered areas are still susceptible to many STIs.

In the case of HIV, sexual transmission routes almost always involve the penis, as HIV cannot spread through unbroken skin; therefore, properly shielding the penis with a properly worn condom from the vagina or anus effectively stops HIV transmission. An infected fluid to broken skin borne direct transmission of HIV would not be considered "sexually transmitted", but can still theoretically occur during sexual contact. This can be avoided simply by not engaging in sexual contact when presenting open, bleeding wounds Other STIs, even viral infections, can be prevented with the use of latex, polyurethane or polyisoprene condoms as a barrier. Some microorganisms and viruses are small enough to pass through the pores in natural skin condoms, but are still too large to pass through latex or synthetic condoms.

Proper male condom usage entails:

- Not putting the condom on too tight at the tip by leaving 1.5 centimetres (0.6 in) room for ejaculation. Putting the condom on too tightly can and often does lead to failure.

- Wearing a condom too loose can defeat the barrier
- Avoiding inverting or spilling a condom once worn, whether it has ejaculate in it or not
- If a user attempts to unroll the condom, but realizes they have it on the wrong side, then this condom may not be effective
- Being careful with the condom if handling it with long nails
- Avoiding the use of oil-based lubricants (or anything with oil in it) with latex condoms, as oil can eat holes into them
- Using flavored condoms for oral sex only, as the sugar in the flavoring can lead to yeast infections if used to penetrate

In order to best protect oneself and the partner from STIs, the old condom and its contents are to be treated as infectious and properly disposed of. A new condom is used for each act of intercourse, as multiple usage increases the chance of breakage, defeating the effectiveness as a barrier. In case of female condoms, the device consists of two rings, one in each terminal portion. The larger ring should fit snugly over the cervix and the smaller ring remains outside the vagina, covering the vulva. This system provides some protection of the external genitalia.

Other

The cap was developed after the cervical diaphragm. Both cover the cervix and the main difference between the diaphragm and the cap is that the latter must be used only once, using a new one in each sexual act. The diaphragm, however, can be used more than once. These two devices partially protect against STIs (they do not protect against HIV).

Researchers had hoped that nonoxynol-9, a vaginal microbicide would help decrease STI risk. Trials, however, have found it ineffectiveand it may put women at a higher risk of HIV infection.

Epidemiology

In 2008, it was estimated that 500 million people were infected with either syphilis, gonorrhea, chlamydia or trichomoniasis. At least an additional 530 million people have genital herpes and 290 million women have human papillomavirus. STIs other than HIV resulted in 142,000 deaths in 2013. In the United States there were 19 million new cases of sexually transmitted infections in 2010.

In 2010, 19 million new cases of sexually transmitted infections occurred in women in the United States. A 2008 CDC study found that 25–40% of U.S. teenage girls has a sexually transmitted disease. Out of a population of almost 295,270,000 peoplethere was were 110 million new and existing cases of eight sexually transmitted infections.

Over 400,000 sexually transmitted infections were reported in England in 2017, about the same as in 2016, but there were more than 20% increases in confirmed cases of gonorrhoea and syphilis. Since 2008 syphilis cases have risen by 148%, from 2,874 to 7,137, mostly among men

who have sex with men. The number of first cases of genital warts in 2017 among girls aged 15–17 years was just 441, 90% less than in 2009 – attributed to the national human papilloma virus immunisation programme.

AIDS is among the leading causes of death in present-day Sub-Saharan Africa. HIV/AIDS is transmitted primarily via unprotected sexual intercourse. More than 1.1 million persons are living with HIV/AIDS in the United States, and it disproportionately impacts African Americans. Hepatitis B is also considered a sexually transmitted disease because it can be spread through sexual contact. The highest rates are found in Asia and Africa and lower rates are in the Americas and Europe. Approximately two billion people worldwide have been infected with the hepatitis B virus.

4. FOOD MICROBIOLOGY

Humans are constantly exposed to bacteria, fungi, and virusesin food as well as in air and water. The foods we eat, whetherthey are fresh, prepared, or even preserved, are seldom sterileand may be contaminated with spoilage microorganisms or occasionallywith pathogens. On the other hand, microbial activityis important for the production of some foods. For example,cheese, buttermilk, sour cream, and yogurt are all produced bymicrobial fermentation. Sauerkraut is a fermented vegetable food.Certain sausages, pâtés, and liver spreads are produced by microbialfermentation. Cider vinegar is produced by the activities ofthe acetic acid bacteria, and alcoholic beverages are produced byfermentation. Some foods contain living microorganisms thoughtto confer health benefits. We discussed these foods, called probioticfoods, in the context of replacing or augmenting the normalmicrobial flora in the human gut.

Here we examine food preservation methods that limitunwanted microbial growth and food spoilage. We also look atmicrobial processes that aid in food preservation and, not incidentally,create a variety of fermented foods. Finally, we discussmicrobial products and microorganisms that cause food poisoningand food infection.

Microorganisms are important spoilage agents in foods, causingfood shortages and economic loss. Various methods,some utilizing desirable microbial growth, are used for controllingspoilage organisms.

- **Microbial Growth and Food Spoilage**

Microorganisms, including a few human pathogens, colonize andgrow on common foods. Foods provide a suitable medium for thegrowth of various microorganisms, and microbial growth oftenreduces food quality and availability.

Food Spoilage

Food spoilage is any change in the appearance, smell, or taste ofa food product that makes it unacceptable to the consumer.Spoiled food may still be safe to eat, but is generally regarded asunpalatable and will not be purchased or readily consumed. Foodspoilage causes losses to producers, distributors, and consumersin the form of reduced quality and quantity, and inevitably leadsto higher prices.Foods consist of organic materials that can be nutrients forthe growth of chemoorganotrophic bacteria. The physical andchemical characteristics of the food determine its degree of susceptibilityto microbial activity. With respect to spoilage, foods areclassified into

three major categories: (1) **perishable food**, includingmany fresh food items; (2) **semiperishable food**, such aspotatoes and nuts; and (3) **stable** or **nonperishable food**, suchas flour and sugar (**Table 36.1**).

The three food categories differ greatly with regard to theirmoisture content, which is related to **water activity.**

Table 36.1 *Food classification by storage potential*

Food classification	*Examples*
Perishable	Meats, fish, poultry, eggs, milk, most fruits and vegetables
Semiperishable	Potatoes, some apples, and nuts
Nonperishable	Sugar, flour, rice, and dry beans

Water activity is a measure of the availability of water for usein metabolic processes. Nonperishable foods have low water activityand can generally be stored for considerable lengths of timewithout spoilage. Perishable and semiperishable foods, by contrast,typically have higher water activities. Thus, these foods must bestored under conditions that inhibit microbial growth.Fresh foods are spoiled by many different bacteria and fungi.The chemical properties of foods vary widely, and each food ischaracterized by the nutrients it contains as well as other factorssuch as acidity or alkalinity. As a result, each fresh food is typicallycolonized and spoiled by a relatively restricted group of microorganisms;the spoilage organisms are those that can gain access tothe food and use the available nutrients (**Table 36.2**).

For example, enteric bacteria such as *Salmonella*, *Shigella*, and *Escherichia*, all potential pathogens sometimes found in the gut ofanimals, often contaminate meat. At slaughter, intestinal contentscontaining live bacteria may be accidentally spilled during removalof the intestines and result in contamination of the carcass. Theseorganisms can also contaminate produce through fecal contaminationof water supplies. Likewise, lactic acid bacteria, the most commonmicroorganisms in dairy products, are the major spoilers ofmilk and milk products. Pseudomonas species are found in bothsoil and animals and cause the spoilage of fresh foods of all types.

Table 36.2 *Microbial spoilage of fresh food*[a]

Food product	*Type of microorganism*	*Common spoilage organisms, by genus*
Fruits and vegetables	Bacteria	*Erwinia, Pseudomonas, Corynebacterium* (mainly vegetable pathogens; rarely spoil fruit)
	Fungi	*Aspergillus, Botrytis, Geotrichum, Rhizopus, Penicillium, Cladosporium, Alternaria, Phytophthora*, various yeasts
Fresh meat, poultry, eggs, and seafood	Bacteria	*Acinetobacter, Aeromonas, Pseudomonas, Micrococcus, Achromobacter, Flavobacterium, Proteus, Salmonella, Escherichia, Campylobacter, Listeria*
	Fungi	*Cladosporium, Mucor, Rhizopus, Penicillium, Geotrichum, Sporotrichum, Candida, Torula, Rhodotorula*
Milk	Bacteria	*Streptococcus, Leuconostoc, Lactococcus, Lactobacillus, Pseudomonas, Proteus*
High-sugar foods	Bacteria	*Clostridium, Bacillus, Flavobacterium*
	Fungi	*Saccharomyces, Torula, Penicillium*

Growth of Microorganisms in Foods

Microbial growth in foods follows the normal pattern for bacterialGrowth. The length of the lag phase depends on the properties of the contaminating microorganism and the food substrate. The time required for the population density to reach a significant level in a given food product depends on both the size of the initial inoculum and the rate of growth during the exponential phase. The rate of growth during the exponential phase depends on the temperature, the nutrient value of the food, and the suitability of other growth conditions.

Throughout much of the exponential growth phase, microbial numbers in a food product may be so low that no measurable effect can be observed, with only the last few population doublings leading to observable spoilage. Thus, for much of the period of microbial growth in a food there is no visible or easily detectable change in food quality; spoilage is usually observed only when the microbial population density is high.

- **Food Preservation**

Food storage and preservation methods slow the growth ofmicroorganisms that spoil food and cause foodborne disease.

1. Cold:

A crucial factor affecting microbial growth is temperature. In general, a lower storage temperature results inless microbial growth and slower spoilage. However, a number ofpsychrotolerant (cold-tolerant) microorganisms can grow, albeitslowly, at refrigerator temperatures (3–5^{0}C). Therefore, storageof perishable food products for long periods of time (more thanseveral days) is possible only at temperatures below freezing.Freezing and subsequent thawing, however, alter the physicalstructure, taste, and appearance of many foods such as leafygreen vegetables like spinach and lettuce, making them unacceptableto the consumer. Freezing is widely used, however, forthe preservation of solid foods such as meats and many fruits andvegetables. Freezers providing a temperature of –20^{0}C are mostcommonly used. Storage for weeks or months is possible at–20^{0}C, but microorganisms can still grow in pockets of

liquidwater trapped within the frozen food. For long-term storage,temperatures of -80^0C [the temperature of solid carbon dioxide(CO_2), "dry ice"] are necessary. Because of the high equipmentand energy costs necessary to maintain such low temperatures,ultracold freezing is not used for routine food storage.

2. Pickling and Acidity:

Another factor affecting microbial growth in food is pH. Foodsvary somewhat in pH, but most are neutral or acidic. Microorganismsdiffer in their ability to grow under acidic conditions,but conditions of pH 5 or less inhibit the growth of most spoilageorganisms. Therefore, weak acids are often used for food preservation,a process called **pickling**. Vinegar, a dilute acetic acidfermentation product of the acetic acid bacteria, is usually addedin the pickling process. Pickling methods usually mix the vinegarwith large amounts of salt or sugar to decrease water availabilityand further inhibit microbial growth. Common pickled foods include cucumbers (sweet, sour, and dillpickles), peppers, meats, fish, and fruits.

3. Drying and Dehydration:

As we mentioned, water activity, or aw, is a measure of the availabilityof water for use by microorganisms in metabolicprocesses. The a_w of pure water is 1.00; the molecules in purewater are loosely ordered and rearrange freely. When solute is added, the a_w decreases. As water molecules reorder around the solute, the free rearrangement of the solute-bound water molecules becomes energetically unfavorable. The microbial cells must then compete with solute for the reduced amount of free water. In general, bacteria are poor competitors for the remaining free water, but fungi are good competitors. In practice this meansthat high concentrations of solutes such as sugars or salts, which greatly reduce aw, typically inhibit bacterial growth. For example, most bacteria are inhibited by a concentration of 7.5% sodium chloride (NaCl) (a_w of 0.957), with the exception of some gram positive cocci, such as Staphylococcus. On the other hand, molds compete well for free water under conditions of low aw and often grow well in high-sugar foods such as syrups.

Some commercially important foods are preserved by the addition of salt or sugar. Sugar is used mainly in fruits (jams, jellies, and preserves). Salted products are primarily meats and fish. Sausage and ham are preserved using various curing salts, including NaCl. Some meats also undergo a smoking process. Preserved meat products vary widely in aw, depending on how much salt is added and how much the meat has been dried. Some cured meat products such as country ham or jerky can be kept at room temperature for extended periods of time. Others with higher aw require refrigeration for long-term storage.

Microbial growth in foods can also be controlled by drying, which lowers water content and availability. Drying is used to preserve highly perishable foods such as meat, fish, milk, vegetables, fruit, and eggs. The least damaging physical method used todry foods is the process of **lyophilization (freeze-drying)** in which foods are frozen and water is then removed under vacuum.This method is very expensive, however, and is used mainly forspecialized applications such as preparation of military rationsthat may need to be stored for long periods even under wet orwarm conditions.Spray drying is the process of spraying, or atomizing, liquidssuch as milk in a

heated atmosphere. The atomization producessmall droplets, increasing the surface-to-volume ratio of the liquid,promoting rapid drying without destroying the food. Thistechnology is widely used in the production of powdered milk,certain concentrated liquid dairy products such as evaporatedmilk, and concentrated food ingredients such as liquid flavorings(**Figure 36.1**).

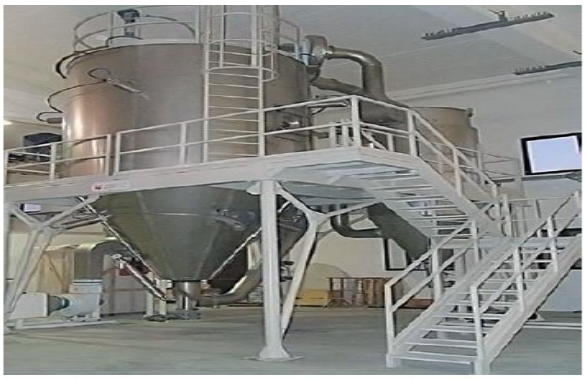


Figure 36.1 Spray dryer. Industrial spray dryers are used to dry or concentrate large volumes of high-value liquid foods.

4. Heating:

Heat is used to reduce the bacterial load or even sterilize a foodproduct; it is especially useful for the preservation of liquids andwet foods. Pasteurization, a process in which liquids are heatedto a specified temperature for a precise time, was described in. Pasteurization does not sterilize liquids, butreduces the bacterial load of spoilage organisms and pathogens, significantly extending the shelf life of the liquid. Pasteurizationcan be done at 638C (145^0F) for 30 seconds or at 71^0C (1608F) for15 seconds. Typically, perishable liquids such as milk, fruit juices,and beer are pasteurized. Ultrahigh-temperature (UHT) processing, sometimes called ultrapasteurization, can be used to preservethe same liquids. UHT processing heats the liquid to 138^0C(238^0F) for 2 to 4 seconds. This treatment kills all microorganisms,extending the shelf life of liquids like milk to 6 months orlonger without the need for refrigeration. UHT-processed milk is common in Europe, but is not easily found in the United States.

Canning is a process in which food is sealed in a containersuch as a can or glass jar and then heated. In theory, canningshould sterilize the food product, but this requires processing atthe correct temperature for the correct length of time. However,when properly sealed and heated, most canned food shouldremain stable and unspoiled indefinitely at any temperature.

The temperature–time relationships for canning depend on thetype of food, its pH, the size of the container, and the consistencyor density of the food. Because heat must completely penetratethe food within the container, effective heating times must belonger for large containers or very dense foods. Acidic foods canoften be canned effectively by heating just to boiling, 100^0C,whereas nonacidic foods must be heated to autoclave temperatures(121^0C). For some foods in large containers, times of 20–50minutes must be used. Heating times long enough to guaranteeabsolute sterility of every container would make most foodsunpalatable and could also reduce nutritional value. Even properlycanned foods, therefore, may not be sterile.

The process usedfor commercial canning, called retort canning, employs equipmentsimilar to an autoclave to apply steam under pressureIf live microorganisms remain in a can, growth of

organisms can produce extensive amounts of gas and build pressure, resultingin bulges or, in extreme cases, explosion (**Figure 36.2**). Theenvironment inside a can is anoxic, and some of the anaerobicbacteria that grow in canned foods are toxin producers of thegenus Clostridium. Food from a bulging can,therefore, should never be eaten. On the other hand, the lack of obvious gas production is not an absolute guarantee that cannedfood is safe to consume.

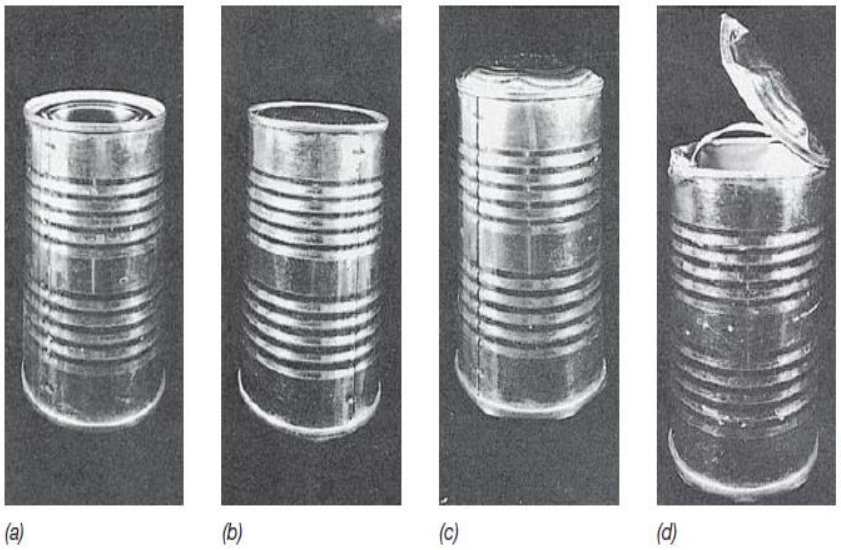


Figure 36.2 Changes in sealed tin cans as a result of microbial spoilage. *(a)* A normal can. The top of the can is pulled in slightly due to the negative pressure (vacuum) inside. *(b)* Swelling resulting from minimal gas production. The top of the can bulges slightly. *(c)* Severe swelling due to extensive gas production. *(d)* The can shown in part c was dropped, and the gas pressure resulted in a violent explosion, tearing the lid apart.

5. High-Pressure Processing:

High-pressure processing (HPP) is a technology that uses veryhigh hydrostatic pressure (up to 100,000 lb/in2) to kill mostpathogens and spoilage organisms in packaged foods. Applicationsinclude several food types. Fruits and vegetables such asavocado products, salsas, chopped onions, applesauce, ready-toeatmeats, and juices can all be processed in bulk or in consumerpackaging. The packaged foods are loaded into a vessel that isflooded with water and placed under pressure (**Figure 36.3**).Pressure treatment kills most foodborne pathogens, but does notkill endospores; the products are not absolutely sterile, but shelflife is increased from days to months.

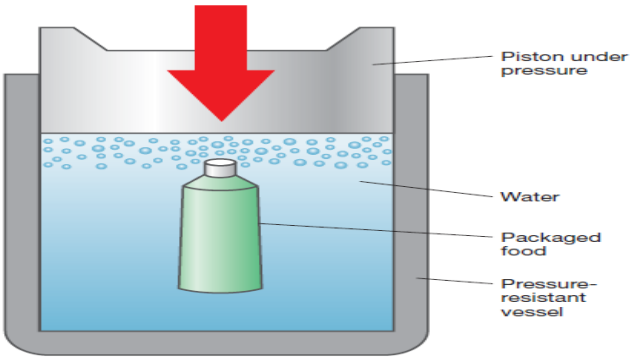


Figure 36.3 High-pressure processing (HPP) of food. Packaged foods are loaded into a vessel that is flooded with water and placed under very high hydrostatic pressure (up to 100,000 lb/in^2) to kill most pathogens and spoilage organisms in packaged foods.

6. Chemical Preservation: Over 3000 different compounds are used as food additives. These chemical additives are classified by the United States Food andDrug Administration as "generally recognized as safe" (GRAS)and find wide application in the food industry for enhancing orpreserving texture, color, freshness, or flavor. A small number ofthese compounds are used to control microbial growth in food(**Table 36.3**). Many of these microbial growth inhibitors, such as sodium propionate and sodium benzoate, have been used formany years with no evidence of human toxicity. Others, such asnitrites (a carcinogen precursor) and ethylene oxide and propyleneoxide (mutagens), are more controversial food additives becausethese compounds may adversely affect human health. The use ofspoilage-retarding additives, however, significantly extends theuseful shelf life of finished foods. Chemical food additivescontribute significantly to an increase in quantity and in theperceived quality of available food items.

Table 36.3 *Chemical food preservatives*

Chemical	*Food*
Sodium or calcium propionate	Bread
Sodium benzoate	Carbonated beverages, fruit, fruit juices, pickles, margarine, preserves
Sorbic acid	Citrus products, cheese, pickles, salads
Sulfur dioxide, sulfites, bisulfites	Dried fruits and vegetables, wine
Formaldehyde (from food-smoking process)	Meat, fish
Ethylene and propylene oxides	Spices, dried fruits, nuts
Sodium nitrite	Smoked ham, bacon

7. Irradiation:

Irradiation of food with ionizing radiation is an effectivemethod for reducing contamination by bacteria, fungi, and even insects. **Table 36.4** lists foods for which radiationtreatment has been approved in the United States. Foodsincluding herbs, spices, and grains are routinely irradiated. Freshmeats and fish can be irradiated to limit contamination by*Escherichia coli* O157:H7 (ground beef), Campylobacter jejuni(poultry), and Vibrio spp. (seafood). In an attempt to limit foodbornedisease outbreaks in fresh produce, irradiation was approved in 2008 to control foodborne pathogens in iceberg lettuceand spinach. In many countries throughout the world,spices, seafood, vegetables, grains, potatoes, sterilized meals,and meats are irradiated.

For food irradiation, gamma rays generated from radioactivecobalt (^{60}Co) or cesium (^{136}Cs), or from high-energy electronsproduced by linear accelerators, are used as radiation sources.Alternatively, beta rays can be generated from an electron gun,analogous to but significantly more powerful than the electronbeam generated by the cathode ray gun formerly

used in televisionsets. In addition, X-rays can be generated with electron beams focused on metal foil. X-rays have much greater penetratingpower than beta rays and are therefore useful for treatinglarge-volume food preparations. Beta ray and X-ray sources canbe switched on and off at will and do not require a radioactivesource.

Table 36.4 *Irradiated foods by category, dose, and purpose*

Food category	*Dose[a] (kGy)*	*Purpose*
Fresh meat: ground beef	4.50	Reduce bacterial pathogens
Herbs, spices, enzymes, and flavorings	30.00	Sterilize
Pork	1.00	Reduce *Trichinella spiralis* protist
Meats used in NASA[b] space flight program	44.0	Sterilize
Poultry	3.00	Reduce bacterial pathogens
Wheat flour	0.50	Inhibit mold
White flour	0.15	Inhibit mold

- **Food Poisoning**

Food poisoning can be caused by various bacteria and fungi.Here we consider Staphylococcus and Clostridium, the twogenera responsible for the highest numbers of microbial foodpoisoning cases.

Staphylococcal Food Poisoning

Food poisoning is often caused by staphylococcal enterotoxin(SE) produced by the bacterium Staphylococcus aureus. Staphylococciare small, gram-positive cocci (**Figure 36.12**) and, they are normal membersof the flora of the skin and upper respiratory tract of at least20–30% of all humans, and are often opportunistic pathogens.S. aureus is frequently associated with food poisoning because itcan grow in many common foods, and some strains produce several heat stable enterotoxins. SE consumed in food producesgastroenteritis characterized by nausea, vomiting, and diarrhea,usually within 1–6 hours.

Epidemiology

Each year there are an estimated 185,000 cases of staphylococcalfood poisoning in the United States. The foods mostcommonly responsible are custard- and cream-filled baked goods, poultry, eggs, raw and processed meat, puddings, and creamysalad dressings. Salads prepared with mayonnaise-based dressingssuch as those containing shellfish, chicken, pasta, tuna, potato, egg, or meat are also commonly implicated. If these foods arerefrigerated immediately after preparation, they usually remain safe because S. aureus grows poorly at low temperatures.

However,foods kept at room temperature in kitchens or outdoorsat picnics can support rapid bacterial growth and enterotoxinproduction if contaminated with S. aureus. Even if the toxincontainingfoods are heated before eating, the heat-stable toxinmay remain active. Some SEs are stable for over 16 hours at 608C,a temperature that would kill S. aureus. Live S. aureus need notbe present in foods causing illness: The illness is solely due to thepreformed SE.

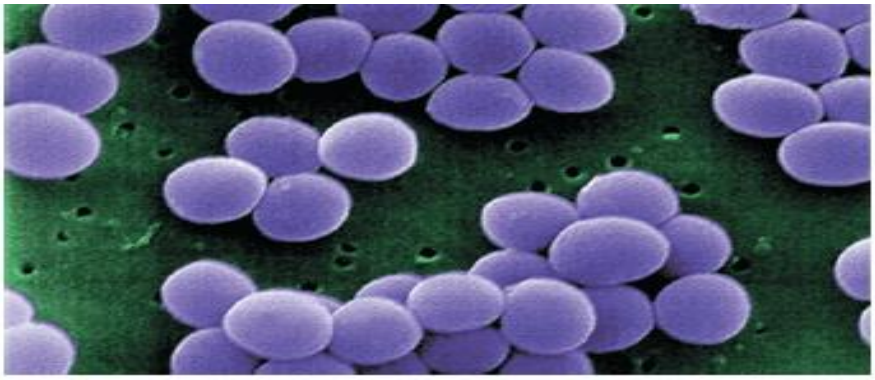


Figure 36.12 ***Staphylococcus aureus.*** In this colorized scanning electron micrograph, the individual gram-positive cocci are about 0.8 μm in diameter. Staphylococci divide in multiple planes, producing the appearance that gives the genus its name (from the Greek *staphyle*, bunch of grapes).

Staphylococcal Enterotoxins

S. aureus strains produce up to 20 different but related SEs. Moststrains of S. aureus produce only one or two of these toxins, andsome strains are nonproducers. However, any one of the toxinscan cause staphylococcal food poisoning. These enterotoxins arefurther classified as superantigens. Superantigens stimulate largenumbers of T cells, which in turn release intercellular mediatorscalled cytokines. In the intestine, superantigens activate a generalinflammatory response that causes gastroenteritis and significantfluid loss due to diarrhea and vomiting. The S. aureus enterotoxins are called SEA, SEB, SEC, and SEDand are encoded by the genes SEA, SEB, SEC, and SED. SEBand SEC are on the bacterial chromosome, SEA is on a lysogenicbacteriophage, and SED is on a plasmid. The S. aureus SE genesare genetically related. The phage- and plasmid-encodedgenes are movable genetic elements that can transfer toxin productionto nontoxigenic strains of Staphylocccus by horizontal gene transfer.

Diagnosis, Treatment, and Prevention

Certain assays detect SEs in food, and other assays detect S. aureusexonuclease, an enzyme that degrades DNA, as a metabolite infood. These qualitative tests confirm that S. aureus is or has beenpresent. To obtain quantitative data and determine the extent ofbacterial contamination, bacterial plate counts are required. Forstaphylococcal counts, a high-salt medium (either sodium chlorideor lithium chloride at a final concentration of 7.5%) is used. Comparedto most bacteria present in foods, staphylococci thrive inhabitats with a high salt content and low water activity.

The symptoms of S. aureus food poisoning can be quite severe,but are typically self-limiting, usually resolving within 48 hoursas the toxin passes from the body. Severe cases may require treatmentfor dehydration. Treatment with antibiotics is not usefulbecause staphylococcal food poisoning is caused by a preformedtoxin, not an active bacterial infection. Staphylococcal food poisoning can be prevented by proper sanitation and hygiene infood production, food preparation, and food storage. As a rule,foods susceptible to colonization by S. aureus and kept for severalhours at temperatures above 48°C should be discarded rather than eaten.

Clostridial Food Poisoning

Clostridium perfringens and Clostridium botulinum cause seriousfood poisoning. Members of the genus Clostridium are anaerobicendospore-forming rods. Canning and cookingprocedures kill living organisms but do not necessarily kill all endospores.Under appropriate anaerobic conditions, the endosporesin food can germinate and produce toxin.

***Clostridium perfringens* Food Poisoning** C. perfringens is an anaerobic, gram-positive, endospore-formingrod commonly found in soil (**Figure 36.13**). C. perfringens is alsofound in sewage, primarily because it lives in small numbers inthe intestinal tract of many humans and animals. *C. perfringens* is the most often reported cause of food poisoning in the UnitedStates, with an estimated 248,000 annual cases (Table 36.6).Perfringens food poisoning requires the ingestion of a largedose of *C. perfringens* in contaminated cooked oruncooked foods, usually high-protein foods such as meat, poultry,and fish. Large numbers of *C. perfringens* can grow in meatdishes cooked in bulk where heat penetration is often insufficient.Surviving *C. perfringens* endospores germinate under

anoxic conditions, as in sealed containers such as jars or cans.

The C. perfringens grows quickly in the food, especially if left tocool at 20–408C for short time periods. However, the toxin is notyet present at this stage.Ingested with contaminated food, the living *C. perfringensbegin* to sporulate and produce toxin in the consumer's intestine. The perfringens enterotoxin alters the permeabilityof the intestinal epithelium, leading to nausea, diarrhea, andintestinal cramps, usually with no fever. The onset of perfringensfood poisoning begins about 7–15 hours after consumption ofthe contaminated food, but usually resolves within 24 hours.Fatalities are rare.

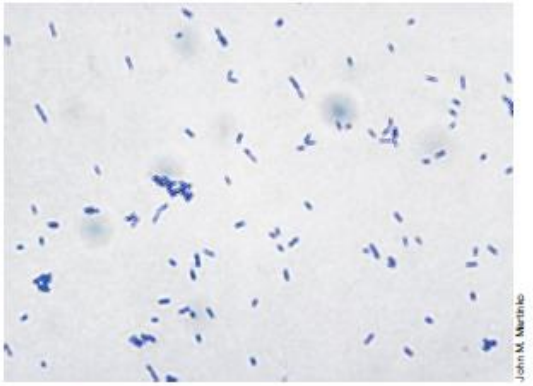


Figure 36.13 *Clostridium perfringens.* The Gram stain shows individual gram-positive rods about 1 μm in diameter.

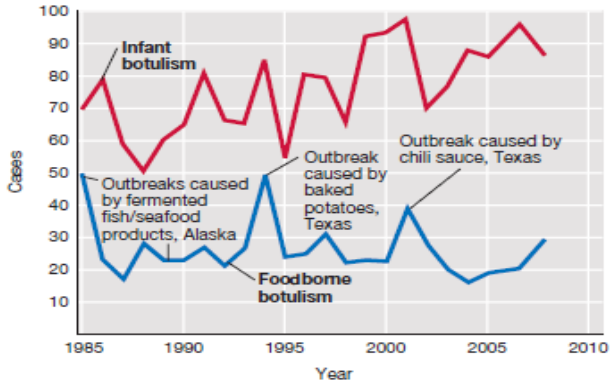


Figure 36.14 **Botulism in the United States.** Both foodborne and infant botulism are shown. In years with high numbers of cases, major outbreaks that account for the increase are indicated. Data are from the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

Diagnosis, Treatment, and Prevention

Diagnosis of perfringens food poisoning is made by isolation of *C. perfringens* from the feces or, more reliably, by a direct enzymeimmunoassay to detect *C. perfringens* enterotoxin in feces. BecauseC. perfringens food poisoning is self-limiting, antibiotic treatmentis not indicated. Supportive therapy—fluids and electrolytereplacement—may be used in serious cases. Prevention of perfringensfood poisoning requires preventing contamination of raw andcooked foods and proper heating of all foods during cooking andcanning. Cooked foods should be refrigerated as soon as possible to rapidly lower temperatures and inhibit C. perfringens growth.

Botulism

Botulism is a severe, often fatal, food poisoning caused by theconsumption of food containing the exotoxin produced byC. botulinum. This bacterium normally inhabits soil or water, butits endospores may contaminate raw foods. If the foods are properlyprocessed so that the C. botulinum endospores are removedor killed, no problem arises; however, if viable endospores remain in the food, they may germinate and produce botulinumtoxin. Ingesting even a small amount of this neurotoxin can bedangerous.

Botulinum toxin is a neurotoxinthat causes flaccid paralysis, usually affecting the autonomic nervesthat control body functions such as respiration and heartbeat. Atleast seven distinct botulinum toxins are known. Because thetoxins are destroyed by heat (808C for 10 minutes), thoroughlycooked food, even though contaminated with toxin, is totallyharmless.Most cases of foodborne botulism are caused by eating processedfoods contaminated with C. botulinum endospores. Typically,such foods are consumed without cooking after processing.

For example, nonacid, home-canned vegetables such as corn andbeans are often used without cooking when making cold salads.Smoked and fresh fish, vacuum-packed in plastic, are also ofteneaten without cooking. Under such conditions, viable C. botulinum endospores may germinate, and the vegetative cells may producesufficient toxin to cause severe food poisoning. An average of25 cases of foodborne botulism, about 18% of all botulism,occurred annually in the United States between 2000 and 2007. The majority of botulism cases occur following infection withC. botulinum. For example, infant botulism occurs after newbornsingest endospores of C. botulinum (Figure 36.14). In mostcases, the source cannot be identified because C. botulinumendospores are widespread. If the infant's normal flora is notdeveloped or if the infant is undergoing antibiotic therapy, ingestedendospores can germinate in the infant's intestine, triggeringC. botulinum growth and toxin production. Most cases of infantbotulism occur between the first week of life and 2 months of age,rarely occurring in children older than 6 months, presumablybecause the normal intestinal flora is more developed. Over 60%of all botulism cases in the United States are in infants. An averageof 86 cases of infant botulism occurred annually in the UnitedStates from 2000 to 2007 (Figure 36.14). Wound botulism can also occur from infection, presumably from endospores in contaminatingmaterial introduced via a parenteral route. Woundbotulism is most commonly associated with illicit injectable drug use; in the United States an average of about 29 cases occurredannually from 2000 to 2007.

All forms of botulism are quite rare, with at most six casesoccurring per 10 million individuals per year in the UnitedStates. Botulism, however, is a very serious disease because ofthe high mortality associated with the disease; about 16% of allfoodborne cases are fatal. Death occurs from respiratory paralysisor cardiac arrest due to the paralyzing action of the botulinumneurotoxin.

Diagnosis, Treatment, and Prevention

Botulism is diagnosed when botulinum toxin is found in patientserum or when toxin or live C. botulinum is found in food thepatient has ingested. Laboratory findings are coupled with clinicalobservations, including neurological signs of localized paralysis(impaired vision and speech) beginning 18–24 hours after ingestionof contaminated food. Treatment is by administration of

botulinum antitoxin if the diagnosis is early, and mechanicalventilation for flaccid respiratory paralysis. In infant botulism,C. botulinum and toxin are often found in bowel contents. Infantbotulism is usually self-limiting, and most infants recover withonly supportive therapy, such as assisted ventilation. Antitoxinadministration is not recommended. Respiratory failure causes

occasional deaths.Prevention of botulism requires careful control of canning andpreservation methods. Susceptible foods should be heated todestroy endospores; boiling for 20 minutes destroys the toxin.Home-prepared foods are the most common source of foodbornebotulism outbreaks.

Concept of probiotics

The growth of microorganisms in and onthe body is important for normal humandevelopment. These organisms, as wehave discussed in this chapter, are part of the normal microflora; they grow on and inthe body and are no doubt essential to thewell-being of all higher organisms.

The microorganisms we acquire andretain constitute our normal microflora andcompete at various sites in the body withpathogens, inhibiting colonization by theseorganisms. Commensals that reside in thegut are active participants in the digestionof food and manufacture essential nutrients.This leads us to the intriguing possibility thathumans could manipulate their commensalbacteria, perhaps altering, regulating, orenhancing our normal flora to increase thepositive benefits of certain selected bacteria.

In theory, the ingestion of selected microorganismsmight be used to change or reestablish our gastrointestinal microflorato promote health, especially in individualswho experience major changes in theirnormal microflora due to disease, surgery,or other medical treatments, or whosenormal microflora changes for other reasons,such as poor diet. Intentionally ingestedmicroorganisms used for this purpose arecalled *probiotics* (Figure 1).

Figure 1 Probiotic foods. Some probiotic foods widely available in the United States.

As defined by the United Nations Foodand Agricultural Organization and the WorldHealth Organization and adopted by theAmerican Academy of Microbiology, probioticsare suspensions of live microorganisms that, when administered in adequate amounts,confer a perceived health benefit on the host.

Are probiotics really useful? There isno reproducible scientific evidence thatconclusively shows that the alteration ofcommensal populations in normal healthyadults has major, long-lasting, positive healtheffects. For example, the products shownin Figure 1 are directed toward replacingor reconstituting the intestinal microfloraof humans by ingesting live, concentratedmicrobial cultures. As with the animalapplications we discuss below, the productsare directed at prevention or correction ofdigestive problems. While these productsmay confer short-term benefits, conclusive evidence for long-lasting establishment or reestablishment of an altered microflora islacking.

Probiotics are routinely used in productionfarm animals to prevent digestive problems.The focus here is on the *preventative* natureof the treatments. Probiotics are not used as a cure in these animals; the probiotics arefed on a continual basis to the animals aspart of their ordinary diets. For example,strains of *Lactobacillus, Propionibacterium,Bacillus,* and *Saccharomyces* have beensuccessfully used for this purpose.

Conceivably,similar treatments in humans couldhave similar effects.A number of human ailments respondpositively to probiotics administration, althoughthe mechanisms by which this occurs areunclear. For example, the watery diarrheachildren experience from rotavirus infectioncan be shortened by administration of severalprobiotics preparations. *Saccharomcyes* (yeast) may reduce the recurrence of diarrheaand shorten infections due to *Clostridiumdifficile*. Probiotic lactobacilli have alsobeen used to treat urogenital infectionsin humans.

The composition of the gut microfloracan change rapidly when probiotics are administered. In many cases, the makersof probiotics suggest that the microbialsupplements should be consumed on aregular basis over a long period of time toachieve the intended result; if consumptionis stopped, the gut microflora returns to itsoriginal state, indicating that the effects ofprobiotics are likely only short term. Thus,while probiotics may offer several benefits,especially for reestablishing the gut's normalmicroflora following catastrophic events suchas severe diarrheal disease, evidence forpositive and lasting benefits is not wellestablished. Carefully designed andscientifically controlled studies mustbe conducted to document the outcomesof probiotic treatment. The studies mustuse standardized preparations of probioticscontaining known organisms and administeredin precise doses to test efficacy.

6. Suggested reading

1. Brock biology of microorganism, 10 edition Madigan,Martinko,Bender, Buckley and Stahl. Pearson, New York, San francisco USA.
2. Microbiology, 5th edition, Lancin M. Prescott, ISBN-0-07-282905-2
3. Microbiology, 5h edition, M. Pelezar, ISBN: 9780074623206, 0074623206
4. https://www.wikipedia.org

7. Assignments

1. What are the General feature of the Archaea?
2. Define Amensalism.
3. Describe Atmospheric Cycles
4. Pollutants in wastewater
5. Role of Microbes in environmental management
6. What is Microbial Leaching?
7. Describe Wastewater Treatment Processes
8. What is canning?
9. Salmonellosis
10. Discuss about Botulism
11. Staphylococcal Food Poisoning
12. Discuss about Bioremediation
13. Clostridial Food Poisoning
14. Details about Biopesticides
15. Describe the process of Tuberculosis Prevention and Treatment

All the materials are self written and collected from eBooks, journals and websites.

BOTANY

POST GRADUATE DEGREE PROGRAMME
(CBCS CURRICULUM)

SEMESTER: IV

PAPER: BOET 4.4

Microbiology Course - II

Directorate of Open and Distance Learning
UNIVERSITY OF KALYANI
Kalyani, Nadia
West Bengal

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Open & Distance Learning, University of Kalyani
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Phone : (033) 2502 2212, 2502 2213
Website : www.klyuniv.ac.in

POST GRADUATE DEGREE PROGRAMME
(CBCS)
IN
BOTANY

SEMESTER - IV

Course: BOET4.4

(Microbiology Course – II)

Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI
KALYANI - 741 235,
WEST BENGAL, INDIA

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMS). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMS for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMS, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMS, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks are also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMS. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

SYLLABUS

COURSE - BOET4.4

Microbiology (Course – II)

(Full Marks – 80)

Course	Group	Details Contents Structure		Study hour
BOET4.4	Microbiology (Course – II)	**Unit1. Microbial genetics**	Genomic structure and organization of virus and bacteria; Bacterial genome replication and cell cycle; Strategies of cell division in bacteria	1
		Unit2. Gene regulation in bacteria	Gene regulation in bacteria; Transformation,transduction and conjugation in bacteria.	1
		Unit3. Molecular basis of Lytic and Lysogenic Pathway in virus	Molecular basis of regulation of lytic and lysogenic pathway in virus	1
		Unit4. Virus induced cell transformation	Virus induced cell transformation and cancer; Gene knockout and knockdown in bacteria.	1
		Unit5. Industrial microbiology	Industrially important microbial strains and its method of improvement; Bioreactors-stirred tank, bubble column,Airlift and photobioreacter.	1
		Unit6. Industrial production of ethanol	Industrial production of ethanol, Acetone-butanol	1
		Unit7. Vinegar,Penecillin, Vitamin B12	Vinegar, Penecillin, Vitamin B12.	1

Course	Group	Details Contents Structure		Study hour
BOET4.4	Microbiology (Course – II)	**Unit8.Amylase,Lysine, Cheese,Interferon**	Amylase,Lysine,Cheese,Interferon	1
		Unit9. Immunology	Organs and tissue associated with immune system; Cell mediated and humoral immunity,MHC,Cytokines	1
		Unit10. Antigen, Antibody generation	Antigen,Immunoglobulin,Antibody generation,Adjuvant ; Monoclonal and polyclonal antibodies	1
		Unit11. Hypersensitivity	Hypersensitivity,Vaccines, Immune response during Tuberculosis and AIDS.	1
		Unit12.ELISA	ELISA, RIA,Immunoflorescence & Immunoprecipitation	1

Content

COURSE - BOET4.4
Microbiology (Course – II)

Elective Paper **Credit:4**

Content Structure

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1. Introduction
2. Course Objective
3. Microbial genetics: Genomic structure and organization of virus and bacteria; Bacterial genome replication and cell cycle; Strategies of cell division in bacteria
4. Gene regulation in bacteria:Gene regulation in bacteria; Transformation,transduction and conjugation in bacteria
5. Molecular basis of Lytic and Lysogenic Pathway in virus: Molecular basis of regulation of lytic and lysogenic pathway in virus.
6. Virus induced cell transformation and Cancer : Virus induced cell transformation and cancer; Gene knockout and knockdown in bacteria
7. Industrial microbiology: Industrially important microbial strains and its method of improvement; Bioreactors-stirred tank, bubble column,Airlift and photobioreacter.
8. Industrial production of
   I. Ethanol
   II. Acetone-butanol
   III. Vinegar
   IV. Penicillium
   V. Vitamin B12

9. Amylase,Lysine,Cheese,Interferon : Amylase,Lysine,Cheese,Interferon
10. Immunology : Organs and tissue associated with immune system; Cell mediated and humoral immunity,MHC,Cytokines
11. Antigen and antibody generation: Antigen,Immunoglobulin,Antibody generation,Adjuvant ; Monoclonal and polyclonal antibodies
12. Hypersensitivity: Hypersensitivity,Vaccines, Immune response during Tuberculosis and AIDS.
13. ELLISA : EllISA, RIA,Immunoflorescence & Immunoprecipitation
14. Let us sum up
15. Suggested reading
16. Assignment

1. Introduction

A microbe, or microorganism, is a microscopic organism that comprises either a single cell (unicellular); cell clusters; or multicellular, relatively complex organisms.The study of microorganisms is called microbiology, a subject that began with Anton van Leeuwenhoek's discovery of microorganisms in 1675, using a microscope of his own design.

Microorganisms are very diverse; they include bacteria, fungi, algae, and protozoa; microscopic plants (green algae); and animals such as rotifers and planarians. Some microbiologists also include viruses, but others consider these as nonliving. Most microorganisms are unicellular, but this is not universal, since some multicellular organisms are microscopic. Some unicellular protists and bacteria, like *Thiomargarita namibiensis*, are macroscopic and visible to the naked eye.

Microorganisms live in all parts of the biosphere where there is liquid water, including soil, hot springs, on the ocean floor, high in the atmosphere, and deep inside rocks within the Earth's crust. Most importantly, these organisms are vital to humans and the environment, as they participate in the Earth's element cycles, such as the carbon cycle and the nitrogen cycle.

Microorganisms also fulfill other vital roles in virtually all ecosystems, such as recycling other organisms' dead remains and waste products through decomposition. Microbes have an important place in most higher-order multicellular organisms as symbionts, and they are also exploited by people in biotechnology, both in traditional food and beverage preparation, and in modern technologies based on genetic engineering. Pathogenic microbes are harmful, however, since they invade and grow within other organisms, causing diseases that kill humans, animals, and plants.

2. Course Objective

Recognize and describe the characteristics of important pathogens and spoilage microorganisms in foods. Upon successful completion of this course the student will be able to: This course provides learning opportunities in the basic principles of medical microbiology and infectious disease

The course is designed to develop your ability to apply the techniques used in the different phases of industrial microbiology: discovery, production(including fermentation and scale-up),bioprocessing and cell banking. It includes the principles and practices in the main applications of micro-organisms to the industrial production of foods, pure chemicals, proteins and other useful products, including the use of genetically modified organisms. This course aims

to enable graduates to enter industry with an appropriate level of understanding of the need for both the science and business aspects to be achievable to make a viable product.

3.Microbial Genetics

Genomic structure and organization of virus and bacteria

Genomic structure and organization of virus

Viruses are small obligate intracellular parasites, which by definition contain either a RNA or DNA genome surrounded by a protective, virus-coded protein coat. Viruses may be viewed as mobile genetic elements, most probably of cellular origin and characterized by a long co-evolution of virus and host. For propagation viruses depend on specialized host cells supplying the complex metabolic and biosynthetic machinery of eukaryotic or prokaryotic cells. A complete virus particle is called a virion. The main function of the virion is to deliver its DNA or RNA genome into the host cell so that the genome can be expressed (transcribed and translated) by the host cell. The viral genome, often with associated basic proteins, is packaged inside a symmetric protein capsid. The nucleic acid-associated protein, called nucleoprotein, together with the genome, forms the nucleocapsid. In enveloped viruses, the nucleocapsid is surrounded by a lipid bilayer derived from the modified host cell membrane and studded with an outer layer of virus envelope glycoproteins.

Structure and Function

Viruses are inert outside the host cell. Small viruses, e.g., polio and tobacco mosaic virus, can even be crystallized. Viruses are unable to generate energy. As obligate intracellular parasites, during replication, they fully depend on the complicated biochemical machinery of eukaryotic or prokaryotic cells. The main purpose of a virus is to deliver its genome into the host cell to allow its expression (transcription and translation) by the host cell.

A fully assembled infectious virus is called a virion. The simplest virions consist of two basic components: nucleic acid (single- or double-stranded RNA or DNA) and a protein coat, the capsid, which functions as a shell to protect the viral genome from nucleases and which during infection attaches the virion to specific receptors exposed on the prospective host cell. Capsid proteins are coded for by the virus genome. Because of its limited size (Table 41-1) the genome codes for only a few structural proteins (besides non-structural regulatory proteins involved in virus replication). Capsids are formed as single or double protein shells and consist of only one or a few structural protein species. Therefore, multiple protein copies must self assemble to form the continuous three-dimensional capsid structure. Self assembly of virus capsids follows two basic patterns: helical symmetry, in which the protein subunits and the nucleic acid are arranged in a helix, and icosahedral symmetry, in which the protein subunits assemble into a symmetric shell that covers the nucleic acid-containing core.

TABLE 41-1 Chemical and Morphologic Properties of Animal Virus Families Relevant to Human Disease (continued)

Family	Viral Genome: Type, Configuration[a] and Number of Bases per strand (X 10^3)	Shape[b]	Diameter (nm)	Enveloped[c]	Capsid Symmetry	Number of Capsomeres[d]	Site of Capsid Assembly	Enzymes, e.g. Transcriptase present in Virion
Picornaviridae	ssRNA, linear; 7-8.5	s	22-30	0	Icosahedral	32	Cytoplasm	None
Astroviridae	ssRNA, linear, sense 6.8-7.9	s	28-30	0	Icosahedral	32?	Cytoplasm	None
Caliciviridae	dsRNA, linear; sense 7.4-7.7	s	35-39	0	Icosahedral	90	Cytoplasm	None
Togaviridae	dsRNA, linear, sense 9.7-11.8	s	70	+	Icosahedral	?	Cytoplasm	None
Flaviviridae	dsRNA, linear, sense 10-12	s	45-50	+	Icosahedral	unknown	Cytoplasm	None
Reoviridae	dsRNA, linear, 10-12 segments; 18-23	s	60-80	0	Icosahedral	32 or 92	Cytoplasm	RNA-dependent RNA polymerase
Orthomyxoviridae	dsRNA, linear, 8 molecules, antisense; 10-13.6	s-pleom[e]	80-120	+	Helical	–	Cytoplasm	RNA-dependent RNA polymerase
Paramyxoviridae	dsRNA, linear, antisense; 15	s-pleom	150-300	+	Helical	–	Cytoplasm	RNA-dependent RNA polymerase
Rhabdoviridae	ssRNA, linear, antisense;11-15	u	60x180	+	Helical	–	Cytoplasm	RNA-dependent RNA polymerase
Bunyaviridae	ssRNA, linear, 3 molecules, antisense; 11-20	s-pleom	90-120	+	Helical	–	Cytoplasm	RNA dependent RNA polymerase
Coronaviridae	ssRNA, linear, sense; 30	s-pleom	120-160	+	Helical	–	Cytoplasm	None
Arenaviridae	ssRNA, linear, 2 species + ribosomal RNA; 3.4	s-pleom	110-130	+	Helical	–	Cytoplasm	RNA-dependent RNA polymerase
Retroviridae	ssRNA, linear, inverted dimer of sense strand; 7-11	s-pleom	90-120	+	Icosahedral (type C)	–	Cytoplasm	RNA-dependent DNA polymerase Protease, Integrase
Filoviridae	ssRNA, linear, antisense; 19.1	Bacilli-form[f]	80x800-2,500	+	Helical	–	Cytoplasm	RNA-transcriptase/polymerase

[a]ss = single stranded; ds = double stranded. [b]S = spherical; X = brickshaped or ovoid; U = elongated with parallel sides and a round end; pleom = pleomorphic. [c]Most enveloped viruses are sensitive to lipid solvents. [d]Applicable to viruses with icosahedral symmetry. [e]Filamentous forms also occur.

Some virus families have an additional covering, called the envelope, which is usually derived in part from modified host cell membranes. Viral envelopes consist of a lipid bilayer that closely surrounds a shell of virus-encoded membrane-associated proteins. The exterior of the bilayer is studded with virus-coded, glycosylated (trans-) membrane proteins. Therefore, enveloped viruses often exhibit a fringe of glycoprotein spikes or knobs, also called peplomers. In viruses that acquire their envelope by budding through the plasma or another intracellular cell membrane, the lipid composition of the viral envelope closely reflects that of the particular host membrane. The outer capsid and the envelope proteins of viruses are glycosylated and important in determining the host range and antigenic composition of the virion. In addition to virus-specified envelope proteins, budding viruses carry also certain host cell proteins as integral constituents of the viral envelope. Virus envelopes can be considered an additional protective coat. Larger viruses often have a complex architecture consisting of both helical and isometric symmetries confined to different structural components. Small viruses, e.g., hepatitis B virus or the members of the picornavirus or parvovirus family, are orders of magnitude more resistant than are the larger complex viruses, e.g. members of the herpes or retrovirus families.

Classification of Viruses

Viruses are classified on the basis of morphology, chemical composition, and mode of replication. The viruses that infect humans are currently grouped into 21 families, reflecting only a small part of the spectrum of the multitude of different viruses whose host ranges extend from vertebrates to protozoa and from plants and fungi to bacteria.

Morphology

Helical Symmetry

In the replication of viruses with helical symmetry, identical protein subunits (protomers) self-assemble into a helical array surrounding the nucleic acid, which follows a similar spiral path. Such nucleocapsids form rigid, highly elongated rods or flexible filaments; in either case, details of the capsid structure are often discernible by electron microscopy. In addition to classification as flexible or rigid and as naked or enveloped, helical nucleocapsids are characterized by length, width, pitch of the helix, and number of protomers per helical turn. The most extensively studied helical virus is tobacco mosaic virus (Fig. 41-1). Many important structural features of this plant virus have been detected by x-ray diffraction studies. Figure 41-2 shows Sendai virus, an enveloped virus with helical nucleocapsid symmetry, a member of the paramyxovirus family.

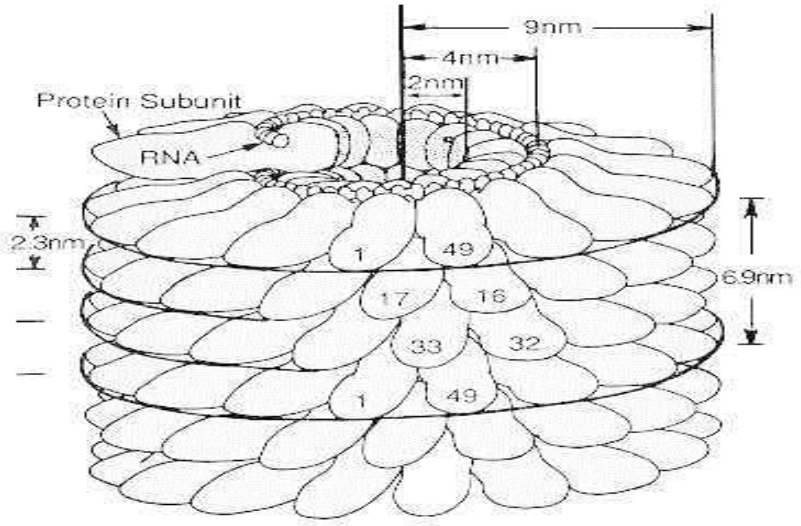


Figure 41-1The helical structure of the rigid tobacco mosaic virus rod

About 5 percent of the length of the virion is depicted. Individual 17,400-Da protein subunits (protomers) assemble in a helix with an axial repeat of 6.9 nm (49 subunits per three turns). Each turn contains a nonintegral number of subunits (16-1/3), producing a pitch of 2.3 nm. The RNA ($2\times1O^6$ Da) is sandwiched internally between adjacent turns of capsid protein, forming a RNA helix of the same pitch, 8 nm in diameter, that extends the length of virus, with three nucleotide bases in contact with each subunit. Some 2,130 protomers per virion cover and protect the RNA. The complete virus is 300 nm long and 18 nm in diameter with a hollow cylindrical core 4 nm in diameter. (From Mattern CFT: Symmetry in virus architecture.

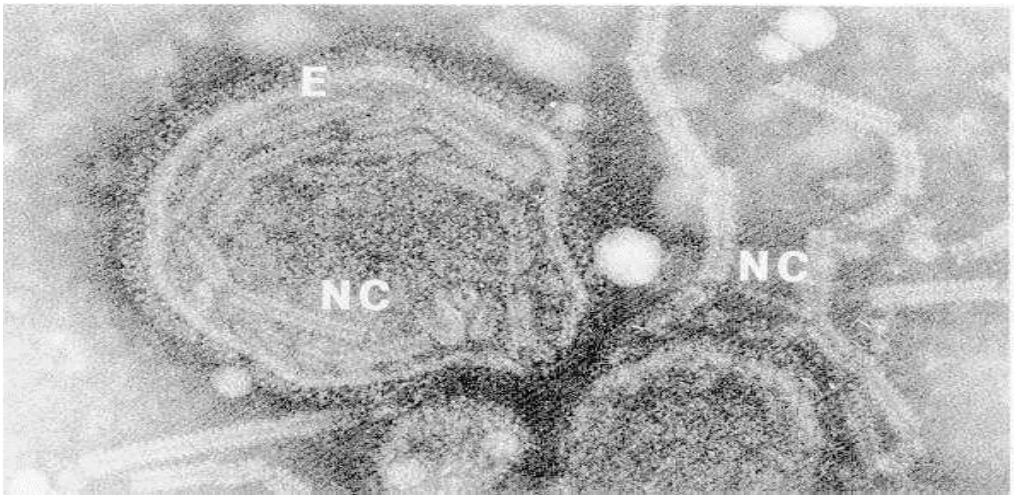


Figure 41-2: Fragments of flexible helical nucleocapsids (NC) of Sendai virus, a paramyxovirus, are seen either within the protective envelope (E) or free, after rupture of the envelope.

Icosahedral Symmetry

An icosahedron is a polyhedron having 20 equilateral triangular faces and 12 vertices (Fig. 41-3). Lines through opposite vertices define axes of fivefold rotational symmetry: all structural features of the polyhedron repeat five times within each 360° of rotation about any of the fivefold axes. Lines through the centers of opposite triangular faces form axes of threefold rotational symmetry; twofold rotational symmetry axes are formed by lines through midpoints of opposite edges. An icosaheron (polyhedral or spherical) with fivefold, threefold, and twofold axes of rotational symmetry (Fig. 41-3) is defined as having 532 symmetry (read as 5,3,2).

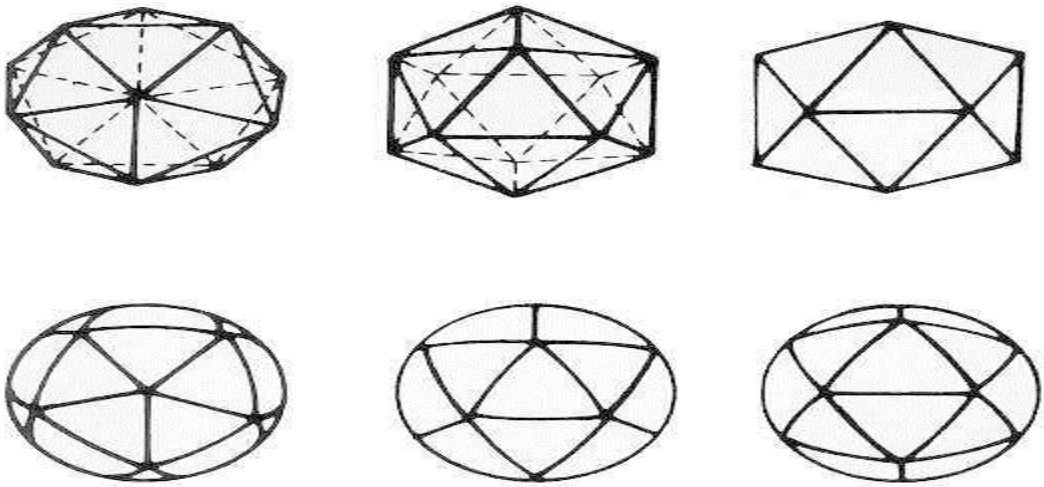


Figure 41-3: Icosahedral models seen, left to right, on fivefold, threefold, and twofold axes of rotational symmetry. These axes are perpendicular to the plane of the page and pass through the centers of each figure. Both polyhedral (upper) and spherical (lower) forms are represented by different virus families.

Viruses were first found to have 532 symmetry by x-ray diffraction studies and subsequently by electron microscopy with negative-staining techniques. In most icosahedral viruses, the protomers, i.e. the structural polypeptide chains, are arranged in oligomeric clusters called capsomeres, which are readily delineated by negative staining electron microscopy and form the closed capsid shell (Fig. 41-4 a/b). The arrangement of capsomeres into an icosahedral shell (compare Fig. 41-4 with the upper right model in Fig. 41-3) permits the classification of such viruses by capsomere number and pattern. This requires the identification of the nearest pair of vertex capsomeres (called penton: those through which the fivefold symmetry axes pass) and the distribution of capsomeres between them.

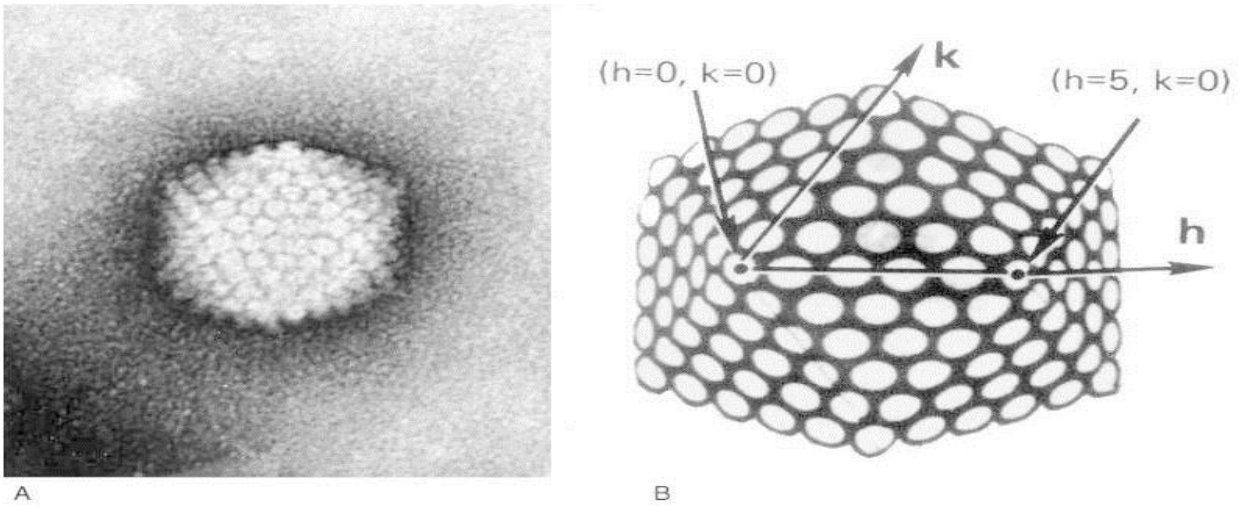


Figure 41-4: Adenovirus after negative stain electron microscopy.

(A) The capsid reveals the typical isometric shell made up from 20 equilateral triangular faces. The 252 capsomeres, 12 pentons and the 240 hollow hexon capsomeres are arranged in a T = 25 symmetry pattern vetite (x 400,000).

(B) **Adenovirus model.** Capsomeres are depicted as circles surrounded by an electron dense stain. The inclined axes, h and k, are indicated. The second vertex has indices h = 5, k = 0. The total number of capsomeres $C = 10(h^2 + hk + k^2) + 2 = 252$. Capsomere organization is also expressed by the triangulation number, T, the number of unit triangles on each of the 20 faces of the icosahedron. A unit triangle is formed by lines joining the centers of three adjacent capsomeres. $T = (h^2 + hk + k^2) = 25$ for adenoviruses, and C = 1OT + 2. The 12 vertex capsomeres are surrounded by 5 other capsomeres each, therefore called penton and show 5-fold rotational symmetry. The penton base consists of 5 identical 85 kD polypeptide chains and extrudes a long antenna-like fiber protein. The 240 hexon capsomeres are trimers of the 120 kD hexon protomere polypeptide

In the adenovirus model in Figure 41-4, one of the penton capsomeres is arbitrarily assigned the indices h = 0, k = 0 (origin), where h and k are the indicated axes of the inclined (60°) net of capsomeres. The net axes are formed by lines of the closest-packed neighboring capsomeres. In adenoviruses, the h and k axes also coincide with the edges of the triangular faces. Any second neighboring vertex capsomere has indices h = 5, k = 0 (or h = 0, k = 5). The capsomere number

(C) can be determined to be 252 from the h and k indices and the equation: $C = 10(h^2 + hk + k^2) + 2$. This symmetry and number of capsomeres is typical of all members of the adenovirus family.

Virus Core Structure

Except in helical nucleocapsids, little is known about the packaging or organization of the viral genome within the core. Small virions are simple nucleocapsids containing 1 to 2 protein species. The larger viruses contain in a core the nucleic acid genome complexed with basic protein(s) and protected by a single- or double layered capsid (consisting of more than one species of protein) or by an envelope (Fig. 41-5).

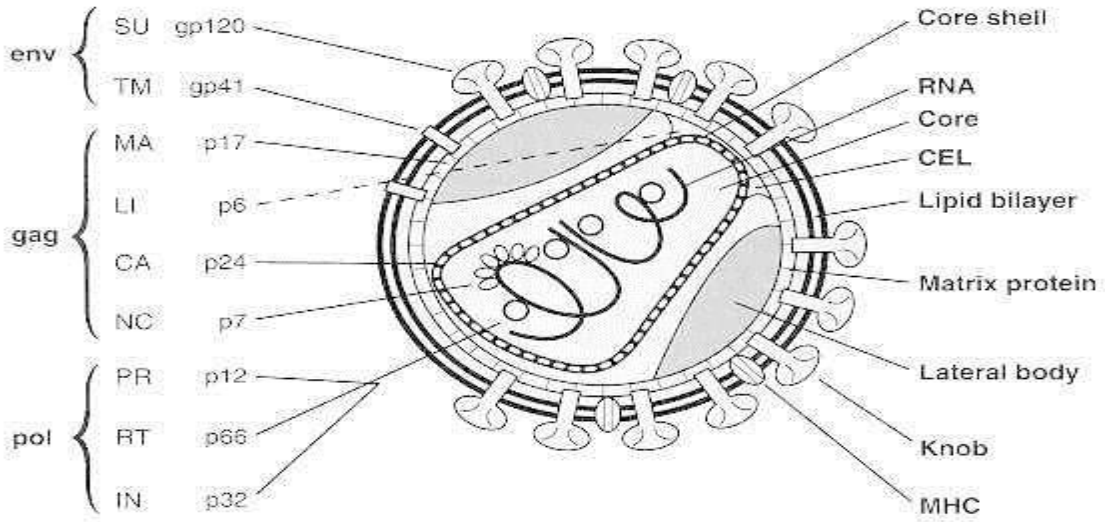


Figure 41-5Two-dimensional diagram of HIV-1 correlating (immuno-) electron microscopic findings with the recent nomenclature for the structural components in a 2-letter code and with the molecular weights of the virus structural (glyco-) proteins.

SU stands for outer surface glycoprotein, TM for transmembrane gp, MA for membrane associated or matrix protein, LI for core-envelope-link, CA for major capsid, NC for nucleocapsid protein, respectively. PR, RT and IN represent the virus-coded enzymes protease, reverse transcriptase and integrase that are functional during the life cycle of a retrovirus (from Gelderblom, HR, AIDS 5, 1991).

Chemical Composition and Mode of Replication

RNA Virus Genomes

RNA viruses, comprising 70% of all viruses, vary remarkably in genome structure (Fig. 41-6). Because of the error rate of the enzymes involved in RNA replication, these viruses usually show much higher mutation rates than do the DNA viruses. Mutation rates of 10^{-4} lead to the continuous generation of virus variants which show great adaptability to new hosts. The viral RNA may be single-stranded (ss) or double-stranded (ds), and the genome may occupy a single RNA segment or be distributed on two or more separate segments (segmented genomes). In

addition, the RNA strand of a single-stranded genome may be either a sense strand (plus strand), which can function as messenger RNA (mRNA), or an antisense strand (minus strand), which is complementary to the sense strand and cannot function as mRNA protein translation. Sense viral RNA alone can replicate if injected into cells, since it can function as mRNA and initiate translation of virus-encoded proteins. Antisense RNA, on the other hand, has no translational function and cannot per se produce viral components.

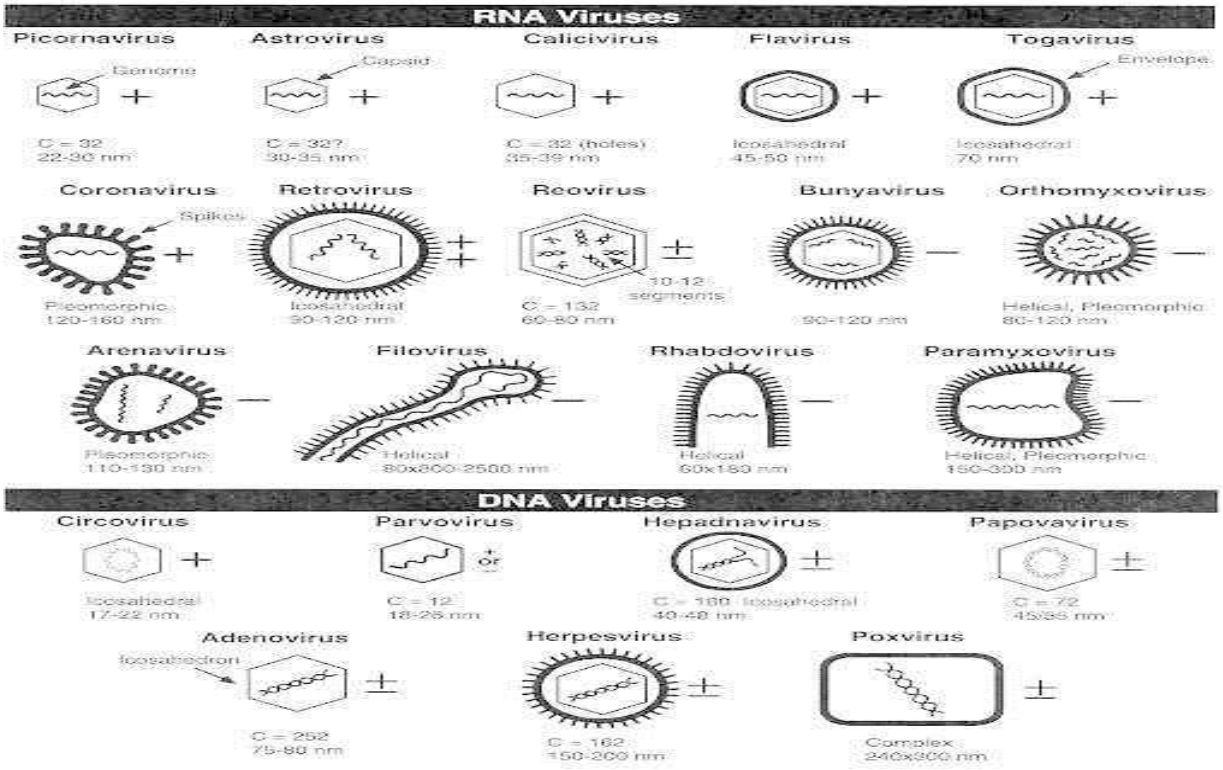


Figure 41-6: Schemes of 21 virus families infecting humans showing a number of distinctive criteria: presence of an envelope or (double-) capsid and internal nucleic acid genome

+, Sense strand; -, antisense strand; ±, dsRNA or DNA; 0, circular DNA; C, number of capsomeres or holes, where known; nm, dimensions of capsid, or envelope when present; the hexagon designates the presence of an isometric or icosahedral outline.

DsRNA viruses, e.g., members of the reovirus family, contain 10, 11 or 12 separate genome segments coding for 3 enzymes involved in RNA replication, 3 major capsid proteins and a number of smaller structural proteins. Each segment consists of a complementary sense and antisense strand that is hydrogen bonded into a linear ds molecule. The replication of these viruses is complex; only the sense RNA strands are released from the infecting virion to initiate replication.

The retrovirus genome comprises two identical, plus-sense ssRNA molecules, each monomer 7–11 kb in size, that are noncovalently linked over a short terminal region. Retroviruses contain 2 envelope proteins encoded by the env-gene, 4–6 nonglycosylated core proteins and 3 non-structural functional proteins (reverse transcriptase, integrase, protease: RT, IN, PR) specified by the gag-gene (Fig. 41-5). The RT transcribes the viral ssRNA into double-stranded, circular

proviral DNA. This DNA, mediated by the viral integrase, becomes covalently bonded into the DNA of the host cell to make possible the subsequent transcription of the sense strands that eventually give rise to retrovirus progeny. After assembly and budding, retroviruses show structural and functional maturation. In immature virions the structural proteins of the core are present as a large precursor protein shell. After proteolytic processing by the viral protease the proteins of the mature virion are rearranged and form the dense isometric or cone-shaped core typical of the mature virion, and the particle becomes infectious.

DNA Virus Genomes

Most DNA viruses (Fig. 41-6) contain a single genome of linear dsDNA. The papovaviruses, comprising the polyoma- and papillomaviruses, however, have circular DNA genomes, about 5.1 and 7.8 kb pairs in size. DsDNA serves as a template both for mRNA and for self-transcription. Three or 2 structural proteins make up the papovavirus capsid: in addition, 5-6 nonstructural proteins are encoded that are functional in virus transcription, DNA replication and cell transformation.

Single-stranded linear DNA, 4–6 kb in size, is found with the members of the Parvovirus family that comprises the parvo-, the erythro- and the dependoviruses. The virion contains 2–4 structural protein species which are differently derived from the same gene product. The adeno-associated virus (AAV, a dependovirus) is incapable of producing progeny virions except in the presence of helper viruses (adenovirus or herpesvirus). It is therefore said to be replication defective.

Circular single-stranded DNA of only 1.7 to 2.3 kb is found in members of the Circovirus family which comprise the smallest autonomously propagated viruses. The isometric capsid measures 17 nm and is composed of 2 protein species only.

Genomic structure and organization of bacteria

Bacterial genomes are generally smaller and less variant in size among species when compared with genomes of eukaryotes. Bacterial genomes can range in size anywhere from about 130 kbp to over 14 Mbp. A study that included, but was not limited to, 478 bacterial genomes, concluded that as genome size increases, the number of genes increases at a disproportionately slower rate in eukaryotes than in non-eukaryotes. Thus, the proportion of non-coding DNA goes up with genome size more quickly in non-bacteria than in bacteria. This is consistent with the fact that most eukaryotic nuclear DNA is non-gene coding, while the majority of prokaryotic, viral, and organellar genes are coding. Right now, we have genome sequences from 50 different bacterial phyla and 11 different archaeal phyla. Second-generation sequencing has yielded many draft genomes (close to 90% of bacterial genomes in GenBank are currently not complete); third-generation sequencing might eventually yield a complete genome in a few hours. The genome sequences reveal much diversity in bacteria. Analysis of over 2000 *Escherichia coli* genomes reveals an *E. coli* core genome of about 3100 gene families and a total of about 89,000 different gene families. Genome sequences show that parasitic bacteria have 500–1200 genes, free-living bacteria have 1500–7500 genes, and archaea have 1500–2700 genes. A striking discovery by Cole et al. described massive amounts of gene decay when comparing Leprosy bacillus to ancestral bacteria. Studies have since shown that several bacteria have smaller genome sizes than

their ancestors did. Over the years, researchers have proposed several theories to explain the general trend of bacterial genome decay and the relatively small size of bacterial genomes. Compelling evidence indicates that the apparent degradation of bacterial genomes is owed to a deletional bias.

Bacteria possess a compact genome architecture distinct from eukaryotes in two important ways: bacteria show a strong correlation between genome size and number of functional genes in a genome, and those genes are structured into operons. The main reason for the relative density of bacterial genomes compared to eukaryotic genomes (especially multicellular eukaryotes) is the presence of noncoding DNA in the form of intergenic regions and introns. Some notable exceptions include recently formed pathogenic bacteria. This was initially described in a study by Cole *et al*. in which *Mycobacterium leprae* was discovered to have a significantly higher percentage of pseudogenes to functional genes (~40%) than its free-living ancestors.

The general trends of bacterial evolution indicate that bacteria started as free-living organisms. Evolutionary paths led some bacteria to become pathogens and symbionts. The lifestyles of bacteria play an integral role in their respective genome sizes. Free-living bacteria have the largest genomes out of the three types of bacteria; however, they have fewer pseudogenes than bacteria that have recently acquired pathogenicity.

Facultative and recently evolved pathogenic bacteria exhibit a smaller genome size than free-living bacteria, yet they have more pseudogenes than any other form of bacteria.

Obligate bacterial symbionts or pathogens have the smallest genomes and the fewest pseudogenes of the three groups. The relationship between life-styles of bacteria and genome size raises questions as to the mechanisms of bacterial genome evolution. Researchers have developed several theories to explain the patterns of genome size evolution amongst bacteria.

Genome comparisons and phylogeny

As single-gene comparisons have largely given way to genome comparisons, phylogeny of bacterial genomes have improved in accuracy. The Average Nucleotide Identity method quantifies genetic distance between entire genomes by taking advantage of regions of about 10,000 bp. With enough data from genomes of one genus, algorithms are executed to categorize species. This has been done for the *Pseudomonas avellanae* species in 2013.

To extract information about bacterial genomes, core- and pan-genome sizes have been assessed for several strains of bacteria. In 2012, the number of core gene families was about 3000. However, by 2015, with an over tenfold increased in available genomes, the pan-genome has increased as well. There is roughly a positive correlation between the number of genomes added and the growth of the pan-genome. On the other hand, the core genome has remain static since 2012. Currently, the *E. coli* pan-genome is composed of about 90,000 gene families. About one-third of these exist only in a single genome. Many of these, however, are merely gene fragments and the result of calling errors. Still, there are probably over 60,000 unique gene families in *E. coli*.

Theories of bacterial genome evolution

Bacteria lose a large amount of genes as they transition from free-living or facultatively parasitic life cycles to permanent host-dependent life. Towards the lower end of the scale of bacterial genome size are the mycoplasmas and related bacteria. Early molecular phylogenetic studies

revealed that mycoplasmas represented an evolutionary derived state, contrary to prior hypotheses. Furthermore, it is now known that mycoplasmas are just one instance of many of genome shrinkage in obligately host-associated bacteria. Other examples are *Rickettsia*, *Buchnera aphidicola,* and *Borrelia burgdorferi*.

Small genome size in such species is associated with certain particularities, such as rapid evolution of polypeptide sequences and low GC content in the genome. The convergent evolution of these qualities in unrelated bacteria suggests that an obligate association with a host promotes genome reduction.

Given that over 80% of almost all of the fully sequenced bacterial genomes consist of intact ORFs, and that gene length is nearly constant at ~1 kb per gene, it is inferred that small genomes have few metabolic capabilities. While free-living bacteria, such as *E. coli*, *Salmonella* species, or *Bacillus* species, usually have 1500 to 6000 proteins encoded in their DNA, obligately pathogenic bacteria often have as few as 500 to 1000 such proteins.

One candidate explanation is that reduced genomes maintain genes that are necessary for vital processes pertaining to cellular growth and replication, in addition to those genes that are required to survive in the bacteria's ecological niche. However, sequence data contradicts this hypothesis. The set of universal orthologs amongst eubacteria comprises only 15% of each genome. Thus, each lineage has taken a different evolutionary path to reduced size. Because universal cellular processes require over 80 genes, variation in genes imply that the same functions can be achieved by exploitation of nonhomologous genes.

Host-dependent bacteria are able to secure many compounds required for metabolism from the host's cytoplasm or tissue. They can, in turn, discard their own biosynthetic pathways and associated genes. This removal explains many of the specific gene losses. For example, the *Rickettsia* species, which relies on specific energy substrate from its host, has lost many of its native energy metabolism genes. Similarly, most small genomes have lost their amino acid biosynthesizing genes, as these are found in the host instead. One exception is the *Buchnera*, an obligate maternally transmitted symbiont of aphids. It retains 54 genes for biosynthesis of crucial amino acids, but no longer has pathways for those amino acids that the host can synthesize. Pathways for nucleotide biosynthesis are gone from many reduced genomes. Those anabolic pathways that evolved through niche adaptation remain in particular genomes.

The hypothesis that unused genes are eventually removed does not explain why many of the removed genes would indeed remain helpful in obligate pathogens. For example, many eliminated genes code for products that are involved in universal cellular processes, including replication, transcription, and translation. Even genes supporting DNA recombination and repair are deleted from every small genome. In addition, small genomes have fewer tRNAs, utilizing one for several amino acids. So, a single codon pairs with multiple codons, which likely yields less-than-optimal translation machinery. It is unknown why obligate intracellular pathogens would benefit by retaining fewer tRNAs and fewer DNA repair enzymes.

Another factor to consider is the change in population that corresponds to an evolution towards an obligately pathogenic life. Such a shift in lifestyle often results in a reduction in the genetic population size of a lineage, since there is a finite number of hosts to occupy. This genetic drift may result in fixation of mutations that inactivate otherwise beneficial genes, or otherwise may decrease the efficiency of gene products. Hence, not will only useless genes be lost (as mutations

disrupt them once the bacteria has settled into host dependency), but also beneficial genes may be lost if genetic drift enforces ineffective purifying selection.

The very small genome of *M. genitalium* possesses dispensable genes. In a study in which single genes of this organism were inactivated using transposon-mediated mutagenesis, at least 129 of its 484 ORGs were not required for growth. A much smaller genome than that of the *M. genitalium* is therefore feasible.

Doubling time

One theory predicts that bacteria have smaller genomes due to a selective pressure on genome size to ensure faster replication. The theory is based upon the logical premise that smaller bacterial genomes will take less time to replicate. Subsequently, smaller genomes will be selected preferentially due to enhanced fitness. A study done by Mira et al. indicated little to no correlation between genome size and doubling time. The data indicates that selection is not a suitable explanation for the small sizes of bacterial genomes. Still, many researchers believe there is some selective pressure on bacteria to maintain small genome size.

Deletional bias

Selection is but one process involved in evolution. Two other major processes (mutation and genetic drift) can account for the genome sizes of various types of bacteria. A study done by Mira et al. examined the size of insertions and deletions in bacterial pseudogenes. Results indicated that mutational deletions tend to be larger than insertions in bacteria in the absence of gene transfer or gene duplication. Insertions caused by horizontal or lateral gene transfer and gene duplication tend to involve transfer of large amounts of genetic material. Assuming a lack of these processes, genomes will tend to reduce in size in the absence of selective constraint. Evidence of a deletional bias is present in the respective genome sizes of free-living bacteria, facultative and recently derived parasites and obligate parasites and symbionts.

Free-living bacteria tend to have large population-sizes and are subject to more opportunity for gene transfer. As such, selection can effectively operate on free-living bacteria to remove deleterious sequences resulting in a relatively small number of pseudogenes. Continually, further selective pressure is evident as free-living bacteria must produce all gene-products independent of a host. Given that there is sufficient opportunity for gene transfer to occur and there are selective pressures against even slightly deleterious deletions, it is intuitive that free-living bacteria should have the largest bacterial genomes of all bacteria types.

Recently-formed parasites undergo severe bottlenecks and can rely on host environments to provide gene products. As such, in recently-formed and facultative parasites, there is an accumulation of pseudogenes and transposable elements due to a lack of selective pressure against deletions. The population bottlenecks reduce gene transfer and as such, deletional bias ensures the reduction of genome size in parasitic bacteria.

Obligatory parasites and symbionts have the smallest genome sizes due to prolonged effects of deletional bias. Parasites which have evolved to occupy specific niches are not exposed to much selective pressure. As such, genetic drift dominates the evolution of niche-specific bacteria.

Extended exposure to deletional bias ensures the removal of most superfluous sequences. Symbionts occur in drastically lower numbers and undergo the most severe bottlenecks of any bacterial type. There is almost no opportunity for gene transfer for endosymbiotic bacteria, and thus genome compaction can be extreme. One of the smallest bacterial genomes ever to be sequenced is that of the endosymbiont *Carsonella rudii*. At 160 kbp, the genome of *Carsonella* is one of the most streamlined examples of a genome examined to date.

Genomic reduction

Molecular phylogenetics has revealed that every clade of bacteria with genome sizes under 2 Mb was derived from ancestors with much larger genomes, thus refuting the hypothesis that bacteriaevolved by the successive doubling of small-genomed ancestors. Recent studies performed by Nilsson et al. examined the rates of bacterial genome reduction of obligate bacteria. Bacteria were cultured introducing frequent bottlenecks and growing cells in serial passage to reduce gene transfer so as to mimic conditions of endosymbiotic bacteria. This is not to suggest that all bacterial genomes are reducing in size and complexity. While many types of bacteria have reduced in genome size from an ancestral state, there are still a huge number of bacteria that maintained or increased genome size over ancestral states. Free-living bacteria experience huge population sizes, fast generation times and a relatively high potential for gene transfer. While deletional bias tends to remove unnecessary sequences, selection can operate significantly amongst free-living bacteria resulting in evolution of new genes and processes.

Horizontal gene transfer

Unlike eukaryotes, which evolve mainly through the modification of existing genetic information, bacteria have acquired a large percentage of their genetic diversity by the horizontal transfer of genes. This creates quite dynamic genomes, in which DNA can be introduced into and removed from the chromosome.

Bacteria have more variation in their metabolic properties, cellular structures, and lifestyles than can be accounted for by point mutations alone. For example, none of the phenotypic traits that distinguish *E. coli* from *Salmonella enterica* can be attributed to point mutation. On the contrary, evidence suggests that horizontal gene transfer has bolstered the diversification and speciation of many bacteria.

Horizontal gene transfer is often detected via DNA sequence information. DNA segments obtained by this mechanism often reveal a narrow phylogenetic distribution between related species. Furthermore, these regions sometimes display an unexpected level of similarity to genes from taxa that are assumed to be quite divergent.

Although gene comparisons and phylogenetic studies are helpful in investigating horizontal gene transfer, the DNA sequences of genes are even more revelatory of their origin and ancestry within a genome. Bacterial species differ widely in overall GC content, although the genes in any one species' genome are roughly identical with respect to base composition, patterns of codon usage, and frequencies of di- and trinucleotides. As a result, sequences that are newly acquired through lateral transfer can be identified via their characteristics, which remains that of the donor.

In some species, a large proportion of laterally transferred genes originate from plasmid-, phage-, or transposon-related sequences.Although sequence-based methods reveal the prevalence of

horizontal gene transfer in bacteria, the results tend to be underestimates of the magnitude of this mechanism, since sequences obtained from donors whose sequence characteristics are similar to those of the recipient will avoid detection.

Comparisons of completely sequenced genomes confirm that bacterial chromosomes are amalgams of ancestral and laterally acquired sequences. The hyperthermophilic Eubacteria *Aquifex aeolicus* and *Thermotoga maritima* each has many genes that are similar in protein sequence to homologues in thermophilic Archaea. 24% of *Thermotoga's* 1,877 ORFs and 16% of *Aquifex's* 1,512 ORFs show high matches to an Archaeal protein, while mesophiles such as *E. coli* and *B. subtilis* have far lesser proportions of genes that are most like Archaeal homologues.

Mechanisms of lateral transfer

The genesis of new abilities due to horizontal gene transfer has three requirements. First, there must exist a possible route for the donor DNA to be accepted by the recipient cell. Additionally, the obtained sequence must be integrated with the rest of the genome. Finally, these integrated genes must benefit the recipient bacterial organism. The first two steps can be achieved via three mechanisms: transformation, transduction and conjugation.[18]

Transformation involves the uptake of named DNA from the environment. Through transformation, DNA can be transmitted between distantly related organisms. Some bacterial species, such as *Haemophilus influenzae* and *Neisseria gonorrhoeae*, are continuously competent to accept DNA. Other species, such as *Bacillus subtilis* and *Streptococcus pneumoniae*, become competent when they enter a particular phase in their lifecycle.

New genes may be introduced into bacteria by a bacteriophage that has replicated within a donor through generalized transduction or specialized transduction. The amount of DNA that can be transmitted in one event is constrained by the size of the phage capsid (although the upper limit is about 100 kilobases). While phages are numerous in the environment, the range of microorganisms that can be transduced depends on receptor recognition by the bacteriophage. Transduction does not require both donor and recipient cells to be present simultaneously in time nor space. Phage-encoded proteins both mediate the transfer of DNA into the recipient cytoplasm and assist integration of DNA into the chromosome.

Conjugation involves physical contact between donor and recipient cells and is able to mediate transfers of genes between domains, such as between bacteria and yeast. DNA is transmitted from donor to recipient either by self-transmissible or mobilizable plasmid. Conjugation may mediate the transfer of chromosomal sequences by plasmids that integrate into the chromosome.

Despite the multitude of mechanisms mediating gene transfer among bacteria, the process's success is not guaranteed unless the received sequence is stably maintained in the recipient. DNA integration can be sustained through one of many processes. One is persistence as an episome, another is homologous recombination, and still another is illegitimate incorporation through lucky double-strand break repair.

Traits introduced through lateral gene transfer

Antimicrobial resistance genes grant an organism the ability to grow its ecological niche, since it can now survive in the presence of previously lethal compounds. As the benefit to a bacterium earned from receiving such genes are time- and space-independent, those sequences that are

highly mobile are selected for. Plasmids are quite mobilizable between taxa and are the most frequent way by which bacteria acquire antibiotic resistance genes.

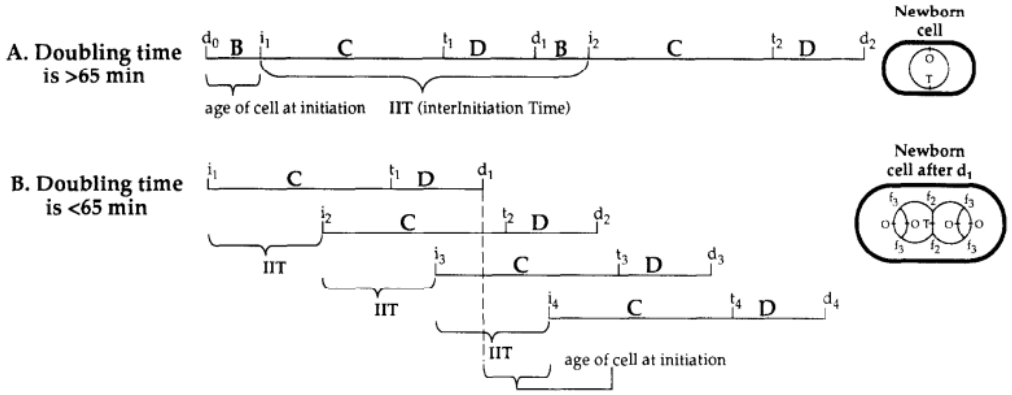
Adoption of a pathogenic lifestyle often yields a fundamental shift in an organism's ecological niche. The erratic phylogenetic distribution of pathogenic organisms implies that bacterial virulence is a consequence of the presence, or obtainment of, genes that are missing in avirulent forms. Evidence of this includes the discovery of large 'virulence' plasmids in pathogenic *Shigella* and *Yersinia*, as well as the ability to bestow pathogenic properties onto *E. coli* via experimental exposure to genes from other species.

Bacterial genome replication and cell cycle:

Three cellular processes occur during conversion of amother bacterial cell into daughter cells: cell growth, DNAreplication, and cell division. These processes are coupled, but the mechanisms that coordinate them are unknown. Bacterial cells rarely make errors in these processes during their cell cycle-less than 0.03% of thedaughter cells contain no DNA. In prokaryotic cells, therate of replication is controlled not by the rate of chainelongation, but by the rate of initiation at the origin of replication, Ori C. Thus, the coupling of replication to cell growthrequires that the frequency of initiation from oriC be a function of growth rate.

Our understanding of the bacterial cell cycle (see figure)is based on the classic work of Cooper and Helmstetter. The time in the cellcycle dedicated to replication (C) is the time between initiation (i) and termination (t) of a round of replication. Exceptin slow-growing cells, the C period (approximately 42 min)varies only slightly with growth rate. D, which is the timebetween termination (t) and cell division (d), is also invariant with growth rate (22-25 min). If the sum of C and D isless than the doubling time (7) agap between cell divisionand initi'ation (B) occurs (part A of figure). If, however, thesum of C and D is greater than T, replication reinitiatesbefore completion of the previous round of replication (part B of figure), leading to overlapping C+D periods in thesame cell. For T = 27 min (part B of figure), newborn cellscontain four origins (0) and one terminus(T) of replication.

Two replication forks (f2) are assembled at time i2, and fourof the replication forks (f,) are assembled at time i3.Independent of the length of C+D, the critical period incoordinating replication with cell growth is the interinitiation time, which is always equal to the doubling time 5.This part discusses three ways that the length of interinitiation time is likely to be regulated during cell growth; tworegulate the time at which initiation events occur in the cellcycle, and a third responds to changes in cell growth.



Initiation of Replication Is Coupled to the Cell Cycleby the Concentration of DnaA

The concentration of DnaA is critical in determining thetiming of initiation, the first step of which is the formationof a complex of 20-40 molecules of DnaA with oriC (Funnell et al., 1987). This interaction involves binding to fourrepeats called dnaA boxes, for which a new consensussequence has been derived (Schaefer and Messer, 1991).DnaA then opens the DNA helix at the AT-rich 13-merrepeats in oriC, prior to loading of DnaB helicase by DnaC. Increased synthesis ofDnaA stimulates initiation of replication. When dnaA expression is induced, the number of origins and the DNAcontent per mass unit increase.De novo protein synthesis is required for reinitiation.Experimental results support the possibility that it is atleast additional

DnaA that must be synthesized. Blockageof additional DnaA synthesis in cfnaA amber mutants by
heat inactivation of a temperature-sensitive suppressortRNAcausescessation of DNA accumulation and progressive inhibition of the rate of DNA synthesis. The kineticsof DNA pulse-labeling in these experiments are those predicted for inhibition of initiation with ompletion of existingrounds of replication (Schaus et al., 1981).
These results indicate either that there is insufficientDnaA for another initiation event or that DnaA, once having participated in initiation, cannot be reused. The latter explanation may be correct, on the basis of an interestingsituation in which initiation of replication from OriC can occur in the absence of continued DnaA synthesis (Katayama and Nagata, 1991). The temperature-sensitive phenotype of a dnaA46 mutant is suppressed when the chaperone proteins GroEL and GroES are overproduced;however, the strain then becomes cold sensitive.
Either hypothesis may explain why new DnaA has to besynthesized during each interinitiation period before a newround of replication can occur. A third hypothesis is thatall DnaA is active and that new DnaA synthesis is requiredto reach a critical mass of DnaA necessary before initiationcan occur (Hansen et al., 1991). Whichever hypothesis iscorrect, this requirement for de novo synthesis of DnaAmeans that the cell can change the length of interinitiationtime very rapidly during cell growth by changing the rateof dnaA gene expression.

Initiation of Replication is Coupled to the CellCycle by Delayed Remethylation of GATC Sitesin oriC and in the dnaA Promoter Region.

A second key control element for determining interinitiation time is the state of methylation of GATC sites in ornear oriC and in the dnaA promoter region. The E. coliminimal origin contains 11 GATC sites, within which adenine residues are methylated by the product of the damgene. In addition, 6 GATC sites are found just outside theminimal origin, to the mioC side. DNA bending occurs inthis region (Kimura et al., 1989) and the extent of bendingis greatly enhanced when these sites are methylated.This methylation control element is dispensable for cellgrowth, because insertion and deletion mutations that inactivate dam are not lethal. However, CsCl density transferexperiments demonstrate that timing of initiation occursat random throughout the cell cycle in such mutants; this can account for the abnormalnumbers of chromosomes observed in these cells (Boyeand Ldbner-Olesen, 1990). Timing is also imprecise andrandom in cells that contain an excess amount of Dammethyltransferase (Smith et al., 1992). In addition, in damcells with a plasmid-borne dam+ gene expressed from the phageh PL promoter, normal numbers of chromosomesper cell are seen only in cells grown near 37%. Thus, timing from OriC is preciseonly when the cells contain Dam methyltransferase withina limited concentration range; timing is random when theconcentration is either below or above this range.

How does GATC methylation function in precise timing?Immediately after initiation, the GATC sites in this regionare hemimethylated-the template strand is methylated and the newly replicated strand is unmethylated. Hemimethylated molecules accumulate when dam mutants are
Transformed with methylated plasmid DNA, indicating thatthese hemimethylated molecules, presumed products ofone round of replication, are unable to replicate. It wouldappear that one or more GATC sites in the origin region must be fully methylated for the origin to function ininitiation.
Thetime required for methylation of all oriC GATC sites is300/o-40% of the cell cycle (Campbell and Kleckner,1990). Therefore, other factors must necessarily be involved in determining interinitiation time.

However, initiation in vitro is dramaticallyinhibited by formation of this cell surface complex (Landoulsi et al., 1990). This sequestering of newly replicated,hemimethylated origins into a complex within the cell surface could render specific GATC sites inaccessible to Dammethyltransferase (and/or oriC inaccessible to DnaA protein).Alternatively, a factor that specifically inhibits GATCmethylation might bind to these critical GATC sites. Thisfactor could be found in the cell surface, although Dammethyltransferase at high concentrations renders hemimethylated oriC associated with an outer membrane fraction active as template for DNA synthesis in vitro (Landoulsi et al., 1990). These findings are consistent with the following hypothesis Dam methylation exerts its effects on initiation timingvia formation of a complex between hemimethylated oriCand the outer membrane. This complex prevents DnaAfrom forming interactions with oriC required for initiation.Initiation can occur again only after methylation of with dissociation from the outer membrane and subsequent formation of an initiation-competent DnaA-oriCcomplex. This hypothesis is consistent with the randomtiming observations. At low Dam methyltransferase concentrations, e.g., in darn- mutants, oriC cannot bind to theouter membrane, and DnaA can now form a functionalInitiation complex at a low but equal probability per unittime. At high Dam methyltransferase concentrations, hemimethylated GATC site(s) are methylated too quickly for outer membrane complex to stabilize, and DnaA canagain form a functional initiation complex at a low but equalprobability per unit time.

This general model can permit several alternative mechanisms. Most likely, membrane dissociation occurs onlyafter methylation of the GATC sites is completed, and relative inaccessibility of oriC in the outer membrane complexto cytoplasmic Dam methyltransferase decreases the rateof GATC methylation. The DNA bend that is dependenton methylated GATC sites (Kimura et al., 1989) may beinvolved in this dissociation. Another possibility, however,
is that the oriC-outer membrane complex is accessible toDam methyltransferase, but the complex harbors a factorthat inhibits Dam methyltransferase. This factor might simply decrease the rate of GATC methylation, or the factormight be unstable, permitting methylation to occur overtime, with subsequent dissociation of the complex.This factor could be a form ofDnaA. Methylation would then occur in the cytoplasm, priorto formation of the initial DnaA-oriC initiation complex.

lniliation of Replication Is Coupled to Growth Rate

A third control element that determines interinitiation timeinvolves transcriptional regulation. Transcription from thegid and mioC promoters, which flank oriC, can activatereplication of oriC plasmids (Ogawa and Okazaki, 1991).Using the fraction II in vitro replication system that is sensitive to rifampicin, no replication occurs when both promoters are inactive. RNA polymerase is required for initiationof replication from oriC in vivo and in the fraction II system,but not in the purified replication systems, except underconditions that inhibit oriC melting by DnaA. The oriC DNA duplexopening mediated by DnaA can be facilitated by an R loopformed by an RNA transcript hybridized to the template,even when the R loop is far from oriC (Skarstad et al.,1990). Whether transcripts from Pgid and PmioC create Rloops is not known. However, these transcripts could alsoactivate oriC by creating negative supercoils behind RNApolymerase, although in the case of mioC, this would haveto occur after transcription had passed through oriC.Transcription from both Pgid and PmioC is inhibited invitro by guanosine tetraphosphate (ppGpp)(Ogawa andOkazaki, 1991). In response to a variety of stress conditions, this nucleotide, implicated as both an alarmone anda growth rate regulator, is produced by transfer of thep,y-phosphates of ATP to the 3’ hydroxyl of GDP. Theconcentration of ppGpp is inversely proportional to growthrate. The two(p)ppGpp synthetases in E. coli are encoded by the re/Aand spoTgenes. In addition, SpoT is the only protein in the cell withppGpp degradative activity. The

growth rate of E. coli canbe manipulated by expressing the re/A gene under thecontrol of an inducible promoter.RelA synthesizes (p)ppGpp only when it is associated withribosomes that contain bound mANA and unchargedtRNA at the acceptor site.Regulation of thednaA operon is affected by the concentration of (p)ppGpp in the cell. The activity of the dnaA P2promoter (which is the stronger of the two promoters) isreduced greatly after amino acid starvation in a wild-typebut not in a re/A~ strain.

Replication and Cell Division

Cell division is a process fundamental to the cell cycle,and invariance of the D period with growth rate suggeststhat cell division is triggered by replication terminationevents. These events most likely begin when a replicationfork encounters a lersite bound by Tus in the chromosometerminus region. Decatenation of the daughter chromosomes then occurs, mostlikely mediated by DNA gyrase and topoisomerase IV. Recombinationat the dif site in the terminus region, mediated in a RecAindependent manner by the XerC recombinase. could resolve chromosomal multimers that may form by actionof RecA protein during termination (Louarn et al., 1991).Neither the dif site (Kuempel et al., 1991) nor RecAmediated recombination is required for cell viability. Decatenated chromosomes are then partitioned into regionsof the cell destined to become daughter cells.

Properties of *E. coli* partitioning mutants suggest thatthe MukA and MukB mediate such partitioning. The temporal order and detailed mechanisms ofthese chromosome termination events are not understood, but the time appears ripe for rapid advances in thisarea. Cell division occurs subsequent to or simultaneouslywith partitioning. Evidence directly linking replicationevents to cell division is lacking, however.

Strategies of cell division in bacteria

Eukaryotic microbes differ dramatically from bacteria and archaea in their reproductive strategies. Many eukaryotic microbes exhibit both asexual reproduction, involving mitosis, and sexual reproduction, involving meiosis to produce gametes or gamete like cells. Furthermore, eukaryotic microbes often alternate between haploid and diploid stages in their life cycles. Here our focus is on the types of cell division observed in bacterial and archaeal cells. Unlike in multicellular organisms, increases in cell size (cell growth) and reproduction by cell division are tightly linked in unicellular organisms. Bacteria grow to a fixed size and then reproduce through binary fission, a form of asexual reproduction. Under optimal conditions, bacteria can grow and divide extremely rapidly, and bacterial populations can double as quickly as every 9.8 minutes. In cell division, two identical clone daughter cells are produced. Some bacteria, while still reproducing asexually, form more complex reproductive structures that help disperse the newly formed daughter cells. Examples include fruiting body formation by *Myxobacteria* and aerial hyphae formation by *Streptomyces*, or budding. Budding involves a cell forming a protrusion that breaks away and produces a daughter cell.

The progeny cells, called baeocytes, are held within the cell wall of the parent cell until they are released. Other bacteria, such as members of the genus Streptomyces, form multinucleoid filaments that eventually divide to form uninucleoid spores. These spores are readily dispersed, much like the dispersal spores formed by filamentous fungi. Despite the diversity of bacterial reproductive strategies, they share certain features. In all cases, the genome of the cell must be replicated and segregated to form distinct nucleoids. At some point during reproduction, each nucleoid and its surrounding cytoplasm becomes enclosed within its own plasma membrane. These processes are the major steps of the cell cycle.

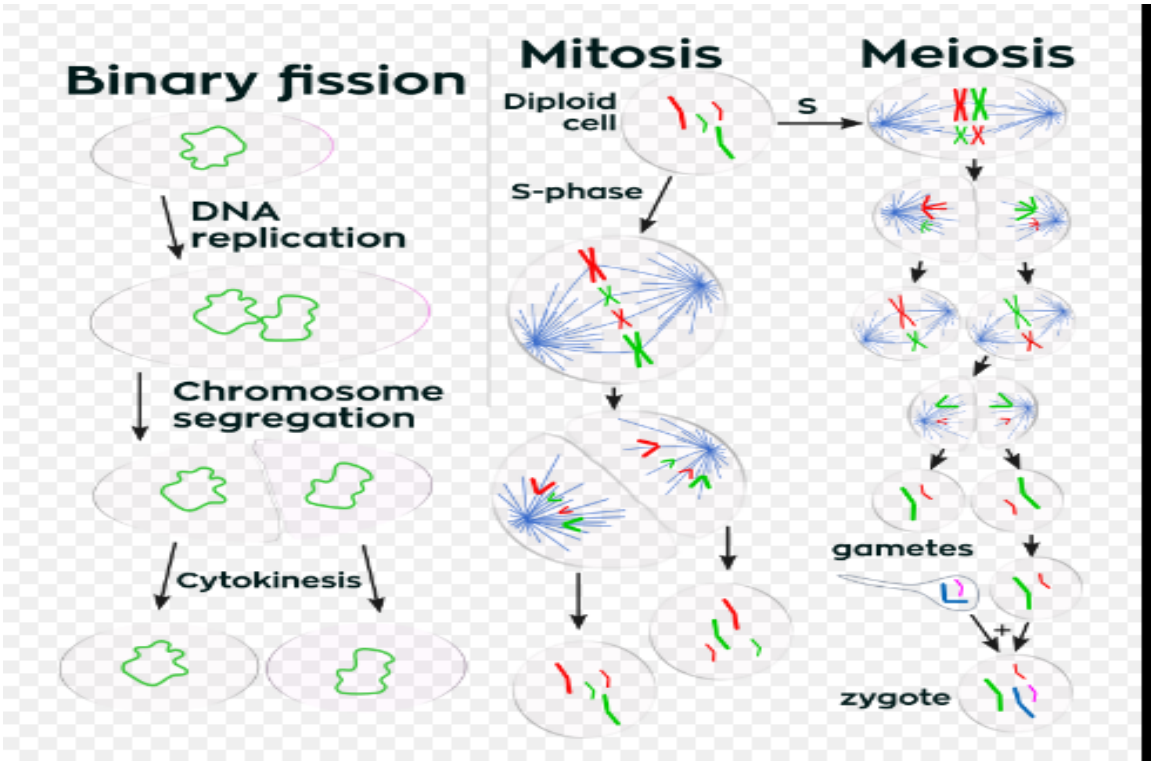


Fig: Many bacteria reproduce through binary fission, which is compared to mitosis and meiosis in this image.

Bacterial growth follows four phases. When a population of bacteria first enter a high-nutrient environment that allows growth, the cells need to adapt to their new environment. The first phase of growth is the lag phase, a period of slow growth when the cells are adapting to the high-nutrient environment and preparing for fast growth. The lag phase has high biosynthesis rates, as proteins necessary for rapid growth are produced. The second phase of growth is the logarithmic phase, also known as the exponential phase. The log phase is marked by rapid exponential growth. The rate at which cells grow during this phase is known as the *growth rate* (*k*), and the time it takes the cells to double is known as the *generation time* (*g*). During log phase, nutrients are metabolised at maximum speed until one of the nutrients is depleted and starts limiting growth. The third phase of growth is the *stationary phase* and is caused by depleted nutrients. The cells reduce their metabolic activity and consume non-essential cellular proteins. The stationary phase is a transition from rapid growth to a stress response state and there is increased expression of genes involved in DNA repair, antioxidant metabolism and nutrient transport.The final phase is the death phase where the bacteria run out of nutrients and die.

Gene Regulation in bacteria

- Bacterial genes are often found in **operons**. Genes in an operon are transcribed as a group and have a single promoter.
- Each operon contains **regulatory DNA sequences**, which act as binding sites for **regulatory proteins** that promote or inhibit transcription.

- Regulatory proteins often bind to small molecules, which can make the protein active or inactive by changing its ability to bind DNA.
- Some operons are **inducible**, meaning that they can be turned on by the presence of a particular small molecule. Others are **repressible**, meaning that they are on by default but can be turned off by a small molecule.
- **How is gene expression regulated?**
  There are various forms of **gene regulation**, that is, mechanisms for controlling which genes get expressed and at what levels. However, a lot of gene regulation occurs at the level of transcription.

  Bacteria have specific regulatory molecules that control whether a particular gene will be transcribed into mRNA. Often, these molecules act by binding to DNA near the gene and helping or blocking the transcription enzyme, RNA polymerase. Let's take a closer look at how genes are regulated in bacteria.

  In bacteria, genes are often found in operons

  In bacteria, related genes are often found in a cluster on the chromosome, where they are transcribed from one **promoter** (RNA polymerase binding site) as a single unit. Such a cluster of genes under control of a single promoter is known as an **operon**. Operons are common in bacteria, but they are rare in eukaryotes such as humans.

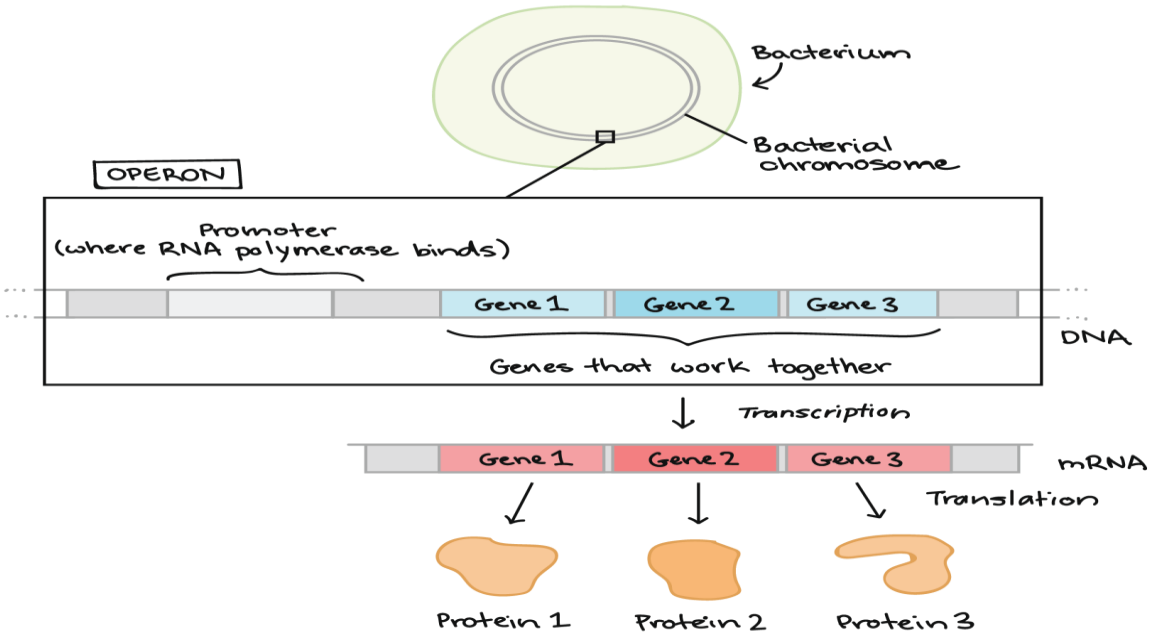


Diagram illustrating what an operon is. At the top of the diagram, we see a bacterial cell with a circular bacterial chromosome inside it.

In general, an operon will contain genes that function in the same process. For instance, a well-studied operon called the *lac* operon contains genes that encode proteins involved in uptake and metabolism of a particular sugar, lactose. Operons allow the cell to efficiently express sets of genes whose products are needed at the same time.

Anatomy of an operon

Operons aren't just made up of the coding sequences of genes. Instead, they also contain **regulatory DNA sequences** that control transcription of the operon. Typically, these sequences are binding sites for **regulatory proteins**, which control how much the operon is transcribed. The promoter, or site where RNA polymerase binds, is one example of a regulatory DNA sequence.

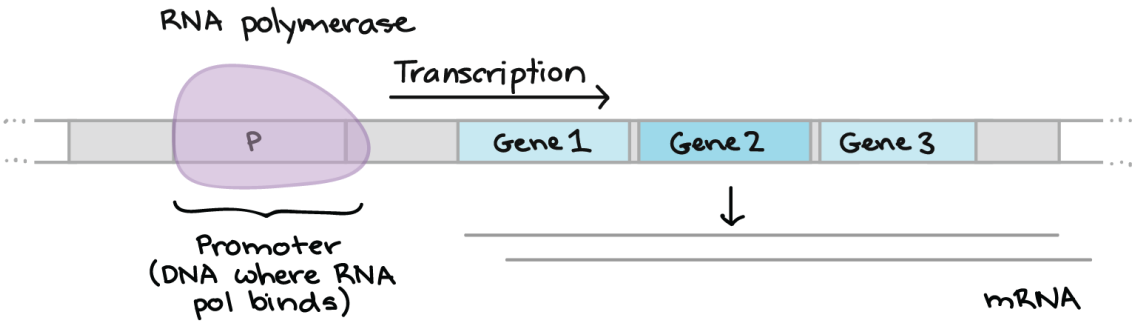


Diagram illustrating that the promoter is the site where RNA polymerase binds. The promoter is found in the DNA of the operon, upstream of (before) the genes. When the RNA polymerase binds to the promoter, it transcribes the operon and makes some mRNAs.

Most operons have other regulatory DNA sequences in addition to the promoter. These sequences are binding sites for regulatory proteins that turn expression of the operon "up" or "down."

- Some regulatory proteins are **repressors** that bind to pieces of DNA called **operators**. When bound to its operator, a repressor reduces transcription (e.g., by blocking RNA polymerase from moving forward on the DNA).

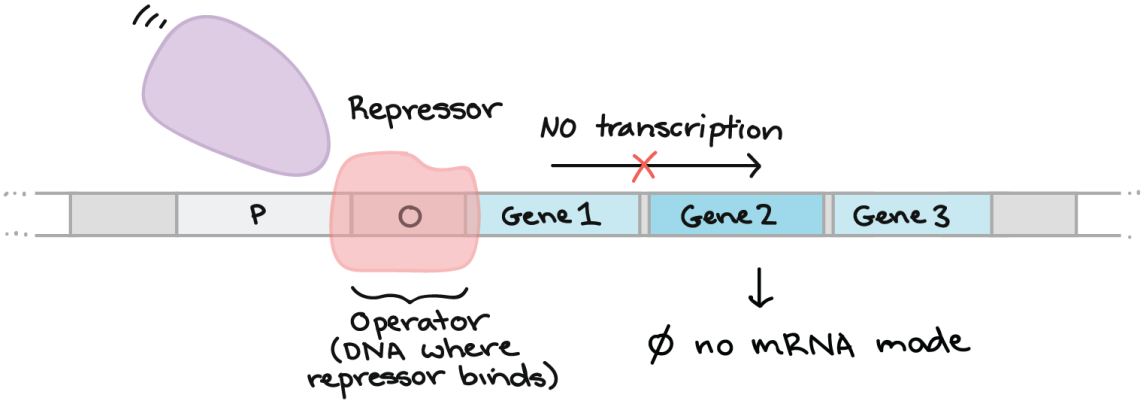


Diagram illustrating how a repressor works. A repressor protein binds to a site called on the operator. In this case (and many other cases), the operator is a region of DNA that overlaps with or lies just downstream of the RNA polymerase binding site (promoter). That is, it is in between the promoter and the genes of the operon. When the repressor binds to the operator, it prevents RNA polymerase from binding to the promoter and/or transcribing the operon. When the repressor is bound to the operator, no transcription occurs and no mRNA is made.

- Some regulatory proteins are **activators**. When an activator is bound to its DNA binding site, it increases transcription of the operon (e.g., by helping RNA polymerase bind to the promoter).

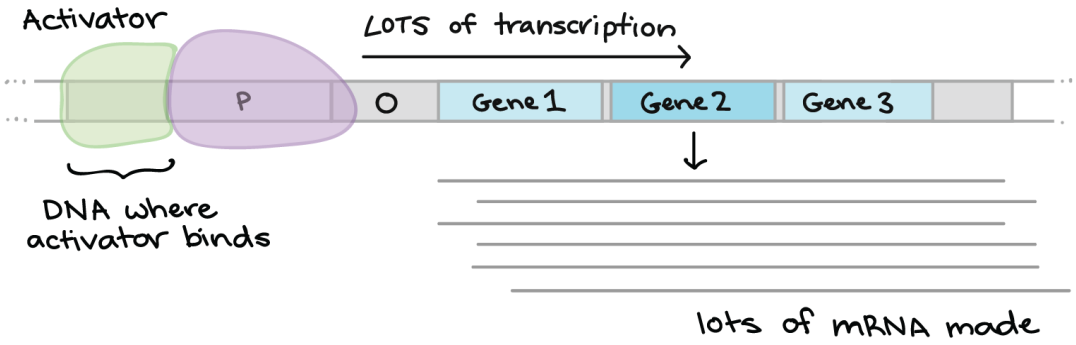


Diagram illustrating how an activator works. The activator protein binds to a specific sequence of DNA, in this case immediately upstream of (before) the promoter where RNA polymerase binds. When the activator binds, it helps the polymerase attach to the promoter (makes promoter binding more energetically favorable).

Where do the regulatory proteins come from? Like any other protein produced in an organism, they are encoded by genes in the bacterium's genome. The genes that encode regulatory proteins are sometimes called **regulatory genes**.

Are regulatory genes found in the operon they regulate?

Many regulatory proteins can themselves be turned "on" or "off" by specific small molecules. The small molecule binds to the protein, changing its shape and altering its ability to bind DNA. For instance, an activator may only become active (able to bind DNA) when it's attached to a certain small molecule.

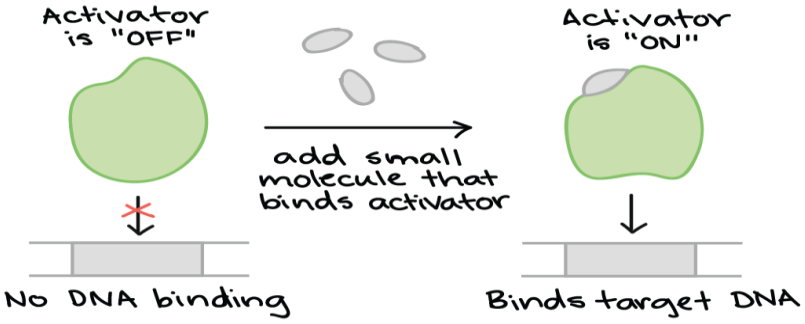


Diagram illustrating how a hypothetical activator's activity could be modulated by a small molecule. When the small molecule is absent, the activator is "off" - it takes on a shape that makes it unable to bind DNA. When the small molecule that activates the activator is added, it binds to the activator and changes its shape. This shape change makes the activator able to bind its target DNA sequence and activate transcription.

Operons may be inducible or repressible

Some operons are usually "off," but can be turned "on" by a small molecule. The molecule is called an **inducer**, and the operon is said to be **inducible**.

- For example, the *lac* operon is an inducible operon that encodes enzymes for metabolism of the sugar lactose. It turns on only when the sugar lactose is present (and other, preferred sugars are absent). The inducer in this case is allolactose, a modified form of lactose.
  Other operons are usually "on," but can be turned "off" by a small molecule. The molecule is called a **corepressor**, and the operon is said to be **repressible**.
- For example, the *trp* operon is a repressible operon that encodes enzymes for synthesis of the amino acid tryptophan. This operon is expressed by default, but can be repressed when high levels of the amino acid tryptophan are present. The corepressor in this case is tryptophan.
  These examples illustrate an important point: that gene regulation allows bacteria to respond to changes in their environment by altering gene expression (and thus, changing the set of proteins present in the cell).

Some genes and operons are expressed all the time

Many genes play specialized roles and are expressed only under certain conditions, as described above. However, there are also genes whose products are constantly needed by the cell to maintain essential functions. These **housekeeping genes** are constantly expressed under normal growth conditions ("constitutively active"). Housekeeping genes have promoters and other regulatory DNA sequences that ensure constant expression.

Transformation, transduction and conjugation in bacteria:

Key points:

- In **transformation**, a bacterium takes up a piece of DNA floating in its environment.
- In **transduction**, DNA is accidentally moved from one bacterium to another by a virus.
- In **conjugation**, DNA is transferred between bacteria through a tube between cells.

When you hear the word "clone," what do you think of? Maybe Dolly the sheep, or experiments carried out in molecular biology labs. But it's also true that the bacteria around you—on your skin, in your gut, growing on your kitchen sink—are "cloning" themselves all the time!

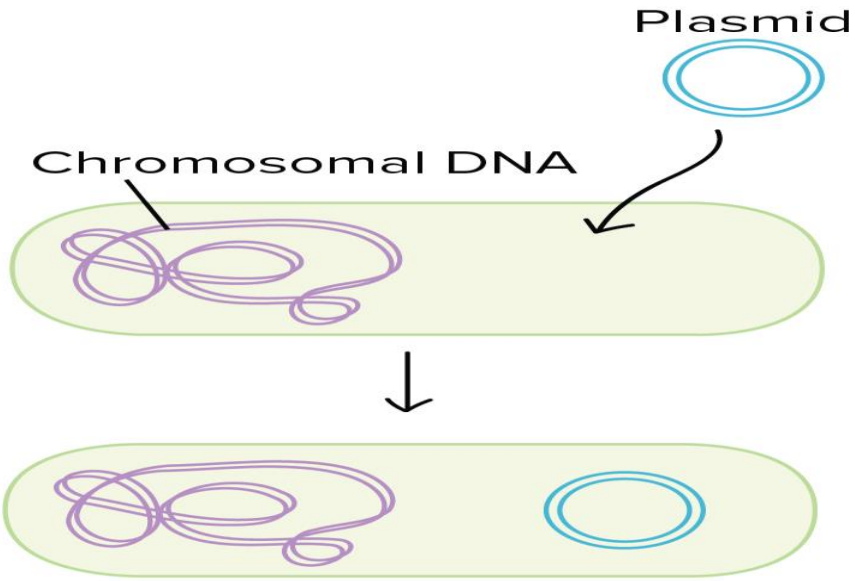
Bacteria reproduce by splitting in two via binary fission. Binary fission makes **clones**, or genetically identical copies, of the parent bacterium. Since the "child" bacteria are genetically identical to the parent, binary fission doesn't provide an opportunity for genetic recombination or genetic diversity (aside from the occasional random mutation). This contrasts with sexual reproduction.

Still, genetic variation is key to the survival of a species, allowing groups to adapt to changes in their environment by natural selection. That's true for bacteria as well as plants and animals. So it's not too surprising that prokaryotes can share genes by three other mechanisms: conjugation, transformation, and transduction.

Transformation

In **transformation**, a bacterium takes in DNA from its environment, often DNA that's been shed by other bacteria. In a laboratory, the DNA may be introduced by scientists. If the DNA is in the form of a circular DNA called a **plasmid**, it can be copied in the receiving cell and passed on to its descendants. Transformation, hence, is the **uptake of genetic material from the environment** by bacterial cells. In nature, this genetic material often comes from adjacent lysed bacteria and can include plasmid DNA or fragmented DNA released into the environment. Various factors promote natural transformation in different bacteria such as growth phase of the cellsor the presence of specific substances.

Though not all bacteria are naturally competent to take up DNA, they can be made competent through chemical manipulation in the lab. This is commonly done using calcium chloride which permeabilizes the cell membrane so the bacteria can easily uptake your plasmid of interest. Scientists can also use electroporation, the application of an electrical charge to cells, to increase cell membrane permeability and thus transformation efficiency.



Transformation in bacteria

Why would this be important? Imagine that a harmless bacterium takes up DNA for a toxin gene from a pathogenic (disease-causing) species of bacterium. If the receiving cell incorporates the new DNA into its own chromosome (which can happen by a process called homologous recombination), it too may become pathogenic.

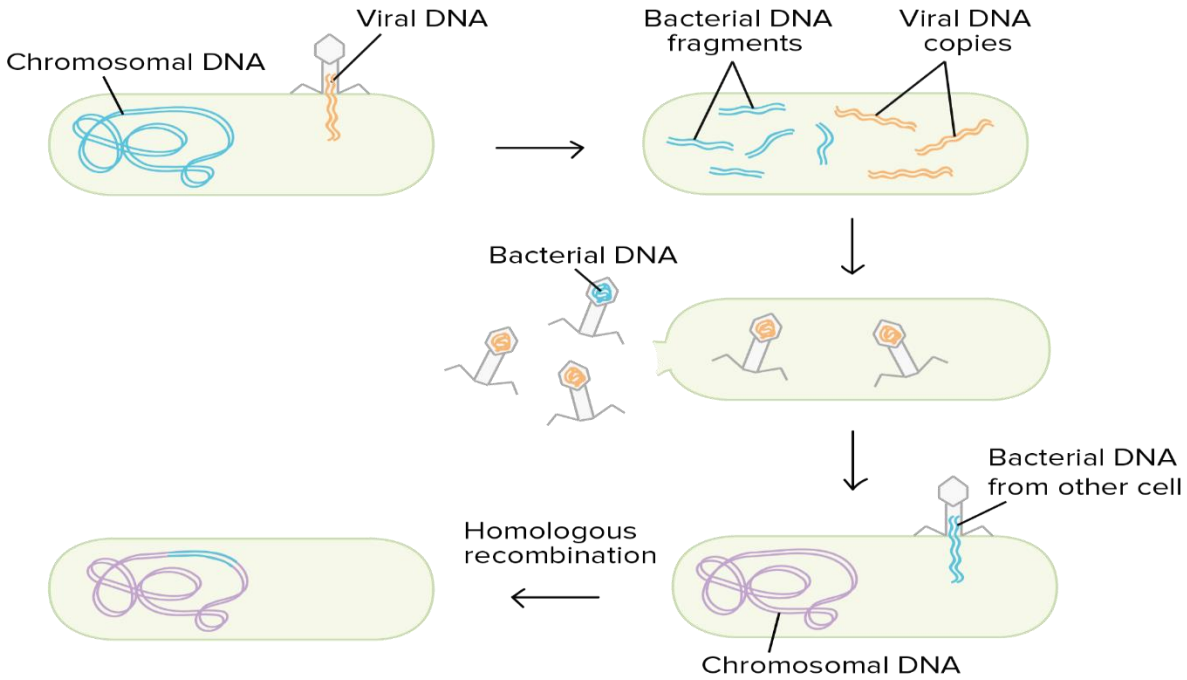
Transduction

Transduction occurs when foreign DNA or RNA is introduced into bacterial or eukaryotic cells **via a virus or viral vector**.One example are bacteriophages that attach to bacterial membranes and inject their genetic material into the cell. Once inside, phages can follow one of two different life cycles: lytic or lysogenic. Lytic phages hijack the bacterial hosts machinery to

make more viral particles. Eventually the cell lyses releasing the newly formed viral particles that can infect other bacteria. In the lysogenic cycle, the phage's genetic material is incorporated into the host's genome at a particular integration site. The integrated phage remains dormant until it is triggered to enter the lytic cycle.

During both of these life cycles bacterial DNA can be accidentally packaged into the newly created phages. Transfer of this DNA to another cell is referred to as transduction. Transferred DNA once inside the infected bacterium can either exist as transient extrachromosomal DNA, like a plasmid, or it can integrate into the host bacterium's genome through homologous or site directed recombination.Transduction is a common tool used by scientists to introduce different DNA sequences of interest into a bacterial cell or a host's genome. To do this scientists commonly use phagemids, a DNA cloning vector that contains both bacteriophage and plasmid properties. The phagemids are packaged into replication-incompetent phage particles with assistance from a 'helper' phage prior to transduction.

In **transduction**, viruses that infect bacteria move short pieces of chromosomal DNA from one bacterium to another "by accident."Yep, even bacteria can get a virus! The viruses that infect bacteria are called bacteriophages. Bacteriophages, like other viruses, are the pirates of the biological world—they commandeer a cell's resources and use them to make more bacteriophages.However, this process can be a little sloppy. Sometimes, chunks of host cell DNA get caught inside the new bacteriophage as they are made. When one of these "defective" bacteriophages infects a cell, it transfers the DNA. Some bacteriophages chop the DNA of their host cell into pieces, making this transfer process more likely^11start superscript, 1, end superscript.



Transduction in bacteria

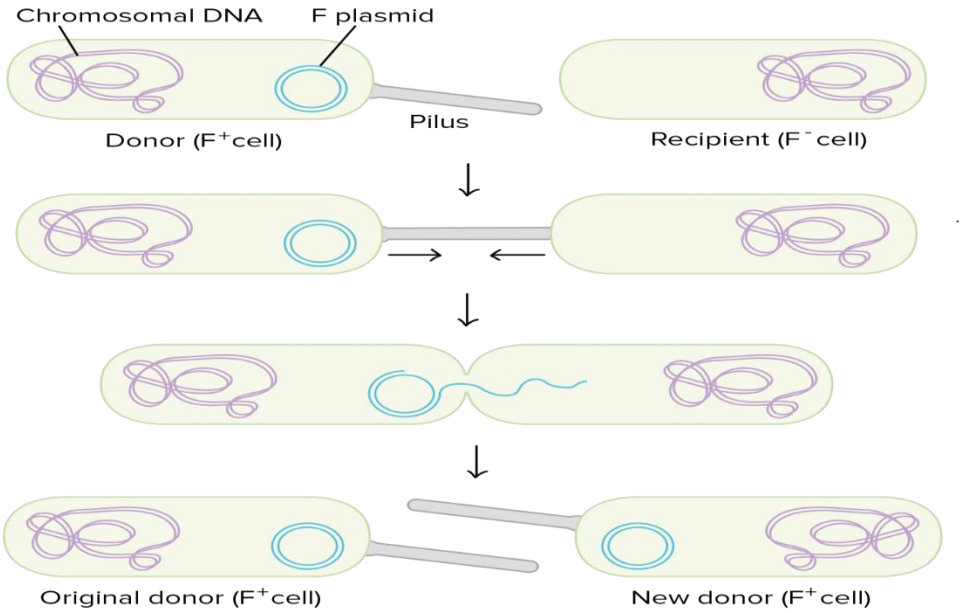
Conjugation

In **conjugation**, DNA is transferred from one bacterium to another. After the donor cell pulls itself close to the recipient using a structure called a pilus, DNA is transferred between cells. In most cases, this DNA is in the form of a plasmid. Conjugation was the first extensively studied method of gene transfer and was discovered in 1946 by Joshua Lederberg and Edward Tatum when they observed genetic recombination between two nutritional deficient *E. coli* strains that resulted in a wild type *E. coli* (Griffiths et al., 2000).

During conjugation, genetic material is transferred from a donor bacterium to a recipient bacterium through **direct contact**. This takes place through a pilus. The donor bacterium contains a DNA sequence called the Fertility factor (F-factor). The F-factor is fo und on an episome, a piece of DNA that can replicate on its own or be integrated within a bacterial chromosome and allows the donor bacterium to make a small "bridge" or sex pilus that attaches to the recipient cell drawing it close. Once in contact the donor can transfer genetic material to the recipient bacterium. The genetic material transferred is commonly a plasmid and can infer genetic advantages such as antibiotic resistance.

It is a mechanism of horizontal gene transfer as are transformation and transduction although these two other mechanisms do not involve cell-to-cell contact.Classical *E. coli* bacterial conjugation is often regarded as the bacterial equivalent of sexual reproduction or mating since it involves the exchange of genetic material. However, it is not sexual reproduction, since no exchange of gamete occurs, and indeed no generation of a new organism: instead an existing organism is transformed. During classical *E. coli* conjugation the *donor* cell provides a conjugative or mobilizable genetic element that is most often a plasmid or transposon. Most conjugative plasmids have systems ensuring that the *recipient* cell does not already contain a similar element.

The genetic information transferred is often beneficial to the recipient. Benefits may include antibiotic resistance, xenobiotic tolerance or the ability to use new metabolites. Such beneficial plasmids may be considered bacterial endosymbionts. Other elements, however, may be viewed as bacterial parasites and conjugation as a mechanism evolved by them to allow for their spread.



Schematic drawing of bacterial conjugation.

Mechanism

1. An F+ donor cell contains its chromosomal DNA and an F plasmid. It has a rodlike pilus. A recipient F- cell has only a chromosome and no F plasmid.
2. The donor cell uses its pilus to attach to the recipient cell, and the two cells are pulled together.
3. A channel forms between the cytoplasms of the two cells, and a single strand of the F plasmid is fed through.
4. Both of the cells now have an F plasmid and are F+. The former recipient cell is now a new donor and can form a pilus.

Donor cells typically act as donors because they have a chunk of DNA called the **fertility factor** (or **F factor**). This chunk of DNA codes for the proteins that make up the sex pilus. It also contains a special site where DNA transfer during conjugation begins^22squared.If the F factor is transferred during conjugation, the receiving cell turns into an F^++start superscript, plus, end superscript donor that can make its own pilus and transfer DNA to other cells. Here's one analogy: this process is sort of like how a vampire can turn other people into vampires by biting them.The F-plasmid is an episome (a plasmid that can integrate itself into the bacterial chromosome by homologous recombination) with a length of about 100 kb. It carries its own origin of replication, the *oriV*, and an origin of transfer, or *oriT*. There can only be one copy of the F-plasmid in a given bacterium, either free or integrated, and bacteria that possess a copy are called *F-positive* or *F-plus* (denoted F^+). Cells that lack F plasmids are called *F-negative* or *F-minus* (F^-) and as such can function as recipient cells.

Among other genetic information, the F-plasmid carries a *tra* and *trb* locus, which together are about 33 kb long and consist of about 40 genes. The *tra* locus includes the *pilin* gene and regulatory genes, which together form pili on the cell surface. The locus also includes the genes for the proteins that attach themselves to the surface of F^- bacteria and initiate conjugation. Though there is some debate on the exact mechanism of conjugation it seems that the pili are not the structures through which DNA exchange occurs. This has been shown in experiments where the pilus are allowed to make contact, but then are denatured with SDS and yet DNA transformation still proceeds. Several proteins coded for in the *tra* or *trb* locus seem to open a channel between the bacteria and it is thought that the traD enzyme, located at the base of the pilus, initiates membrane fusion.

When conjugation is initiated by a signal the **relaxase** enzyme creates a nick in one of the strands of the conjugative plasmid at the *oriT*. Relaxase may work alone or in a complex of over a dozen proteins known collectively as a **relaxosome**. In the F-plasmid system the relaxase enzyme is called TraI and the relaxosome consists of TraI, TraY, TraM and the integrated host factor IHF. The nicked strand, or *T-strand*, is then unwound from the unbroken strand and transferred to the recipient cell in a 5'-terminus to 3'-terminus direction. The remaining strand is replicated either independent of conjugative action (vegetative replication beginning at the *oriV*) or in concert with conjugation (conjugative replication similar to the rolling circle replication of lambda phage). Conjugative replication may require a second nick before successful transfer can occur. A recent report claims to have inhibited conjugation with chemicals that mimic an intermediate step of this second nicking event.

If the F-plasmid that is transferred has previously been integrated into the donor's genome (producing an Hfr strain ["High Frequency of Recombination"]) some of the donor's chromosomal DNA may also be transferred with the plasmid DNA.[3] The amount of chromosomal DNA that is transferred depends on how long the two conjugating bacteria remain in contact. In common laboratory strains of *E. coli* the transfer of the entire bacterial chromosome takes about 100 minutes. The transferred DNA can then be integrated into the recipient genome via homologous recombination.

A cell culture that contains in its population cells with non-integrated F-plasmids usually also contains a few cells that have accidentally integrated their plasmids. It is these cells that are responsible for the low-frequency chromosomal gene transfers that occur in such cultures. Some strains of bacteria with an integrated F-plasmid can be isolated and grown in pure culture. Because such strains transfer chromosomal genes very efficiently they are called **Hfr** (**h**igh **f**requency of **r**ecombination). The *E. coli* genome was originally mapped by interrupted mating experiments in which various Hfr cells in the process of conjugation were sheared from recipients after less than 100 minutes (initially using a Waring blender). The genes that were transferred were then investigated.

Since integration of the F-plasmid into the *E. coli* chromosome is a rare spontaneous occurrence, and since the numerous genes promoting DNA transfer are in the plasmid genome rather than in the bacterial genome, it has been argued that conjugative bacterial gene transfer, as it occurs in the *E. coli* Hfr system, is not an evolutionary adaptation of the bacterial host, nor is it likely ancestral to eukaryotic sex.

Spontaneous zygogenesis in *E. coli*

In addition to classical bacterial conjugation described above for *E. coli*, a form of conjugation referred to as spontaneous zygogenesis (Z-mating for short) is observed in certain strains of *E. coli*. In Z-mating there is complete genetic mixing, and unstable diploids are formed that throw off phenotypically haploid cells, of which some show a parental phenotype and some are true recombinants.

Molecular basis of regulation of lytic and lysogenic pathway in virus:

The lytic cycle is one of the two cycles of viral reproduction (referring to bacterial viruses or bacteriophages), the other being the lysogenic cycle. The lytic cycle results in the destruction of the infected cell and its membrane. Bacteriophages that only use the lytic cycle are called virulent phages (in contrast to temperate phages).
In the lytic cycle, the viral DNA exists as a separate free floating molecule within the bacterial cell, and replicates separately from the host bacterial DNA, whereas in the lysogenic cycle, the viral DNA is located within the host DNA. This is the key difference between the lytic and lysogenic bacteriophage cycles. However, in both cases the virus/phage replicates using the host DNA machinery.
The lytic cycle, which is also commonly referred to as the "reproductive cycle" of the bacteriaphage, is a six-stage cycle. The six stages are: attachment, penetration, transcription, biosynthesis, maturation, and lysis.

- Attachment – the phage attaches itself to the surface of the host cell in order to inject its DNA into the cell

- Penetration – the phage injects its DNA into the host cell by penetrating through the cell membrane
- Transcription – the host cell's DNA is degraded and the cell's metabolism is directed to initiate phage biosynthesis
- Biosynthesis – the phage DNA replicates inside the cell, synthesizing new phage DNA and proteins
- Maturation – the replicated material assembles into fully formed viral phages (each made up of a head, a tail and tail fibers)
- Lysis – the newly formed phages are released from the infected cell (which is itself destroyed in the process) to seek out new host cells to infect

Attachment and penetration

To infect a host cell, the virus must first inject its own nucleic acid into the cell through the plasma membrane and (if present) the cell wall. The virus does so by either attaching to a receptor on the cell's surface or by simple mechanical force. The binding is due to electrostatic interactions and is influenced by pH and the presence of ions. The virus then releases its genetic material (either single- or double-stranded RNA or DNA) into the cell. In some viruses this genetic material is circular and mimics a bacterial plasmid. At this stage the cell becomes infected and can also be targeted by the immune system. It is mostly aided by receptors in the surface of the cell.

Transcription and biosynthesis

During the transcription and biosynthesis stages, the virus hijacks the cell's replication and translation mechanisms, using them to make more viruses. The virus's nucleic acid uses the host cell's metabolic machinery to make large amounts of viral components. In the case of DNA viruses, the DNA transcribes itself into messenger RNA (mRNA) molecules that are then used to direct the cell's ribosomes. One of the first polypeptides to be translated destroys the host's DNA. In retroviruses (which inject an RNA strand), a unique enzyme called reverse transcriptase transcribes the viral RNA into DNA, which is then transcribed again into RNA. Once the viral DNA has taken control it induces the host cell's machinery to synthesize viral DNA, protein and starts multiplying. The biosynthesis is (e.g. T4) regulated in three phases of mRNA production followed by a phase of protein production.

Early phase

Enzymes modify the host's transcriptional process by RNA polymerase. Amongst other modifications, virus T4 changes the sigma factor of the host by producing an anti-sigma factor so that the host promotors are not recognized any more but now recognize T4 middle proteins. For protein synthesis Shine-Dalgarno subsequence GAGG dominates an early genes translation.

Middle phase

Virus nucleic acid (DNA or RNA depending on virus type).

Late phase

Structural proteins including those for the head and the tail.

Maturation and lysis

About 25 minutes after initial infection, approximately 200 new bacteriophages (virions) are formed. Once enough virions have matured and accumulated, specialized viral proteins are used to dissolve the bacterial cell wall. The cell bursts (i.e. it undergoes lysis) due to high internal osmotic pressure (water pressure) that can no longer be constrained by the cell wall. This releases progeny virions into the surrounding environment, where they can go on to infect other

cells and another lytic cycle begins. The phage that causes lysis of the host is called a lytic or virulent phage.

Gene regulation biochemistry

There are three classes of genes in the phage genome that regulate whether the lytic or lysogenic cycles will emerge. The first class is the immediate early genes, the second is the delayed early genes and the third is the late genes. The following refers to the well-studied temperate phage lambda of E. coli. Immediate early genes: These genes are expressed from promoters recognized by the host RNA polymerase, and include Cro, cII, and N. CII is a transcription factor that stimulates expression of the main lysogenic repressor gene, cI, whereas Cro is a repressor for cI expression. The lysis-lysogeny decision is mainly influenced by the competition between Cro and CII, resulting in the determination of whether or not sufficient CI repressor is made. If so, CI represses the early promoters and the infection is shunted into the lysogenic pathway. N is an anti-termination factor that is needed for the transcription of the delayed early genes.

Delayed early genes: These include the replication genes O and P and also Q, which encodes the anti-terminator responsible for transcription of all the late genes.

Late genes:

Q-mediated turn-on of late transcription begins about 6-8 min after infection if the lytic pathway is chosen. More than 25 genes are expressed from the single late promoter, resulting in four parallel biosynthetic pathways. Three of the pathways are for production of the three components of the virion: the DNA-filled head, the tail, and the side tail fibers. The virions self-assemble from these components, with the first virion appearing at about 20 min after infection. The fourth pathway is for lysis. In lambda 5 proteins are involved in lysis: the holin and antiholin from gene S, the endolysin from gene R and the spanin proteins from genes Rz and Rz1. In wild-type lambda, lysis occurs at about 50 min, releasing approximately 100 completed virions. The timing of lysis is determined by the holin and antiholin proteins, with the latter inhibiting the former. In overview, the holin protein accumulates in the cytoplasmic membrane until suddenly forming micron-scale holes, which triggers lysis. The endolysin R is released to the periplasm, where it attacks the peptidoglycan. The spanin proteins Rz and Rz1 accumulate in the cytoplasmic and outer membranes, respectively, and form a complex spaning the periplasm through the meshwork of the peptidoglycan. When the endolysin degrades the peptidoglycan, the spanin complexes are liberated and cause disruption of the outer membrane. Destruction of the peptidoglycan by the endolysin and disruption of the outer membrane by the spanin complex are both required for lysis in lambda infections.

Lysis inhibition: T4-like phages have two genes, rI and rIII, that inhibit the T4 holin, if the infected cell undergoes super-infection by another T4 (or closely related) virion. Repeated super-infection can cause the T4 infection to continue without lysis for hours, leading to accumulation of virions to levels 10-fold higher than normal.

Lysogeny, or the lysogenic cycle, is one of two cycles of viral reproduction (the lytic cycle being the other). Lysogeny is characterized by integration of the bacteriophage nucleic acid into the host bacterium's genome or formation of a circular replicon in the bacterial cytoplasm. In this condition the bacterium continues to live and reproduce normally. The genetic material of the bacteriophage, called a prophage, can be transmitted to daughter cells at each subsequent cell division, and at later events (such as UV radiation or the presence of certain chemicals) can release it, causing proliferation of new phages via the lytic cycle. Lysogenic cycles can also occur in eukaryotes, although the method of DNA incorporation is not fully understood.

The difference between lysogenic and lytic cycles is that, in lysogenic cycles, the spread of the viral DNA occurs through the usual prokaryotic reproduction, whereas a lytic cycle is more immediate in that it results in many copies of the virus being created very quickly and the cell is destroyed. One key difference between the lytic cycle and the lysogenic cycle is that the lysogenic cycle does not lyse the host cell straight away. Phages that replicate only via the lytic cycle are known as virulent phages while phages that replicate using both lytic and lysogenic cycles are known as temperate phages.
In the lysogenic cycle, the phage DNA first integrates into the bacterial chromosome to produce the prophage. When the bacterium reproduces, the prophage is also copied and is present in each of the daughter cells. The daughter cells can continue to replicate with the prophage present or the prophage can exit the bacterial chromosome to initiate the lytic cycle. In lysogenic cycle the host DNA is not hydrolysed but in lytic cycle the host DNA is hydrolysed in the lytic phase.

Cancer:

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. These contrast with benign tumors, which do not spread. Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss, and a change in bowel movements.While these symptoms may indicate cancer, they can also have other causes. Over 100 types of cancers affect humans.

In the developing world, 15% of cancers are due to infections such as *Helicobacter pylori*, hepatitis B, hepatitis C, human papillomavirus infection, Epstein–Barr virus and human immunodeficiency virus (HIV). These factors act, at least partly, by changing the genes of a cell. Typically, many genetic changes are required before cancer develops. Approximately 5–10% of cancers are due to inherited genetic defects from a person's parents. Cancer can be detected by certain signs and symptoms or screening tests. It is then typically further investigated by medical imaging and confirmed by biopsy.

In 2015, about 90.5 million people had cancer. About 14.1 million new cases occur a year (not including skin cancer other than melanoma). It caused about 8.8 million deaths (15.7% of deaths). Rates are increasing as more people live to an old age and as lifestyle changes occur in the developing world. The financial costs of cancer were estimated at $1.16 trillion USD per year as of 2010.

Definitions

Cancers are a large family of diseases that involve abnormal cell growth with the potential to invade or spread to other parts of the body. They form a subset of neoplasms. A neoplasm or tumor is a group of cells that have undergone unregulated growth and will often form a mass or lump, but may be distributed diffusely.
All tumor cells show the six hallmarks of cancer. These characteristics are required to produce a malignant tumor. They include:

- Cell growth and division absent the proper signals
- Continuous growth and division even given contrary signals
- Avoidance of programmed cell death
- Limitless number of cell divisions
- Promoting blood vessel construction

- Invasion of tissue and formation of metastases

The progression from normal cells to cells that can form a detectable mass to outright cancer involves multiple steps known as malignant progression.

Metastasis

Cancer can spread from its original site by local spread, lymphatic spread to regional lymph nodes or by hematogenous spread via the blood to distant sites, known as metastasis. When cancer spreads through the blood, it may spread through the body but is more likely to travel to certain areas depending on the cancer type. The symptoms of metastatic cancers depend on the tumor location and can include enlarged lymph nodes (which can be felt or sometimes seen under the skin and are typically hard), enlarged liver or enlarged spleen, which can be felt in the abdomen, pain or fracture of affected bones and neurological symptoms.

Causes

The majority of cancers, some 90–95% of cases, are due to genetic mutations from environmental and lifestyle factors. The remaining 5–10% are due to inherited genetics. *Environmental* refers to any cause that is not inherited genetically, such as lifestyle, economic, and behavioral factors and not merely pollution. Common environmental factors that contribute to cancer death include tobacco (25–30%), diet and obesity (30–35%), infections (15–20%), radiation (both ionizing and non-ionizing, up to 10%), lack of physical activity, and pollution. Psychological stress does not appear to be a risk factor for the onset of cancer, though it may worsen outcomes in those who already have cancer.

Chemicals

Exposure to particular substances have been linked to specific types of cancer. These substances are called *carcinogens*.

Tobacco smoke, for example, causes 90% of lung cancer.It also causes cancer in the larynx, head, neck, stomach, bladder, kidney, esophagus and pancreas. Tobacco smoke contains over fifty known carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons.

In Western Europe, 10% of cancers in males and 3% of cancers in females are attributed to alcohol exposure, especially liver and digestive tract cancers.Cancer from work-related substance exposures may cause between 2 and 20% of cases, causing at least 200,000 deaths. Cancers such as lung cancer and mesothelioma can come from inhaling tobacco smoke or asbestos fibers, or leukemia from exposure to benzene.

Diet and exercise

Diet, physical inactivity and obesity are related to up to 30–35% of cancer deaths.[3][48] In the United States, excess body weight is associated with the development of many types of cancer and is a factor in 14–20% of cancer deaths. A UK study including data on over 5 million people showed higher body mass index to be related to at least 10 types of cancer and responsible for around 12,000 cases each year in that country.Some specific foods are linked to specific cancers. A high-salt diet is linked to gastric cancer. Aflatoxin B1, a frequent food contaminant, causes liver cancer. Betel nut chewing can cause oral cancer. National differences in dietary practices may partly explain differences in cancer incidence. For example, gastric cancer is more common

in Japan due to its high-salt diet while colon cancer is more common in the United States. Immigrant cancer profiles mirror those of their new country, often within one generation.

Infection

Worldwide approximately 18% of cancer deaths are related to infectious diseases. This proportion ranges from a high of 25% in Africa to less than 10% in the developed world. Viruses are the usual infectious agents that cause cancer but cancer bacteria and parasites may also play a role.

Oncoviruses (viruses that can cause cancer) include human papillomavirus (cervical cancer), Epstein–Barr virus (B-cell lymphoproliferative disease and nasopharyngeal carcinoma), Kaposi's sarcoma herpesvirus (Kaposi's sarcoma and primary effusion lymphomas), hepatitis B and hepatitis C viruses (hepatocellular carcinoma) and human T-cell leukemia virus-1 (T-cell leukemias). Bacterial infection may also increase the risk of cancer, as seen in *Helicobacter pylori*-induced gastric carcinoma. Parasitic infections associated with cancer include *Schistosoma haematobium* (squamous cell carcinoma of the bladder) and the liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis* (cholangiocarcinoma).

Radiation

Radiation exposure such as ultraviolet radiation and radioactive material is a risk factor for cancer. Many non-melanoma skin cancers are due to ultraviolet radiation, mostly from sunlight.Sources of ionizing radiation include medical imaging and radon gas.

Ionizing radiation is not a particularly strong mutagen. Residential exposure to radon gas, for example, has similar cancer risks as passive smoking.Radiation is a more potent source of cancer when combined with other cancer-causing agents, such as radon plus tobacco smoke.Radiation can cause cancer in most parts of the body, in all animals and at any age. Children are twice as likely to develop radiation-induced leukemia as adults; radiation exposure before birth has ten times the effect.

Non-ionizing radio frequency radiation from mobile phones, electric power transmission and other similar sources has been described as a possible carcinogen by the World Health Organization's International Agency for Research on Cancer. Evidence, however, has not supported a concern. This includes that studies have not found a consistent link between mobile phone radiation and cancer risk.

Heredity

The vast majority of cancers are non-hereditary (sporadic). Hereditary cancers are primarily caused by an inherited genetic defect. Less than 0.3% of the population are carriers of a genetic mutation that has a large effect on cancer risk and these cause less than 3–10% of cancer. Some of these syndromes include: certain inherited mutations in the genes *BRCA1* and *BRCA2* with a more than 75% risk of breast cancer and ovarian cancer, and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome), which is present in about 3% of people with colorectal cancer, among others.

Taller people have an increased risk of cancer because they have more cells than shorter people. Since height is genetically determined to a large extent, taller people have a heritable increase of cancer risk.

Physical agents

Some substances cause cancer primarily through their physical, rather than chemical, effects. A prominent example of this is prolonged exposure to asbestos, naturally occurring mineral fibers that are a major cause of mesothelioma (cancer of the serous membrane) usually the serousmembrane surrounding the lungs. Other substances in this category, including both naturally occurring and synthetic asbestos-like fibers, such as wollastonite, attapulgite, glass wool and rock wool, are believed to have similar effects. Non-fibrous particulate materials that cause cancer include powdered metallic cobalt and nickel and crystalline silica (quartz, cristobalite and tridymite). Usually, physical carcinogens must get inside the body (such as through inhalation) and require years of exposure to produce cancer.

Chronic inflammation has been hypothesized to directly cause mutation. Inflammation can contribute to proliferation, survival, angiogenesis and migration of cancer cells by influencing the tumor microenvironment. Oncogenes build up an inflammatory pro-tumorigenic microenvironment.

Hormones

Some hormones play a role in the development of cancer by promoting cell proliferation. Insulin-like growth factors and their binding proteins play a key role in cancer cell proliferation, differentiation and apoptosis, suggesting possible involvement in carcinogenesis.

Hormones are important agents in sex-related cancers, such as cancer of the breast, endometrium, prostate, ovary and testis and also of thyroid cancer and bone cancer. For example, the daughters of women who have breast cancer have significantly higher levels of estrogen and progesterone than the daughters of women without breast cancer. These higher hormone levels may explain their higher risk of breast cancer, even in the absence of a breast-cancer gene. Similarly, men of African ancestry have significantly higher levels of testosterone than men of European ancestry and have a correspondingly higher level of prostate cancer. Men of Asian ancestry, with the lowest levels of testosterone-activating androstanediol glucuronide, have the lowest levels of prostate cancer.

Autoimmune diseases

There is an association between celiac disease and an increased risk of all cancers. People with untreated celiac disease have a higher risk, but this risk decreases with time after diagnosis and strict treatment, probably due to the adoption of a gluten-free diet, which seems to have a protective role against development of malignancy in people with celiac disease. However, the delay in diagnosis and initiation of a gluten-free diet seems to increase the risk of malignancies. Rates of gastrointestinal cancers are increased in people with Crohn's disease and ulcerative colitis, due to chronic inflammation. Also, immunomodulators and biologic agents used to treat these diseases may promote developing extra-intestinal malignancies.

Pathophysiology

Genetics

Genetic changes can occur at different levels and by different mechanisms. The gain or loss of an entire chromosome can occur through errors in mitosis. More common are mutations, which are changes in the nucleotide sequence of genomic DNA.

Large-scale mutations involve the deletion or gain of a portion of a chromosome. Genomic amplification occurs when a cell gains copies (often 20 or more) of a small chromosomal locus, usually containing one or more oncogenes and adjacent genetic material. Translocation occurs when two separate chromosomal regions become abnormally fused, often at a characteristic location. A well-known example of this is the Philadelphia chromosome, or translocation ofchromosomes 9 and 22, which occurs in chronic myelogenous leukemia and results in production of the BCR-abl fusion protein, an oncogenic tyrosine kinase.

Small-scale mutations include point mutations, deletions, and insertions, which may occur in the promoter region of a gene and affect its expression, or may occur in the gene's coding sequence and alter the function or stability of its protein product. Disruption of a single gene may also result from integration of genomic material from a DNA virus or retrovirus, leading to the expression of *viral* oncogenes in the affected cell and its descendants.

Some environments make errors more likely to arise and propagate. Such environments can include the presence of disruptive substances called carcinogens, repeated physical injury, heat, ionising radiation or hypoxia.

The errors that cause cancer are self-amplifying and compounding, for example:

- A mutation in the error-correcting machinery of a cell might cause that cell and its children to accumulate errors more rapidly.
- A further mutation in an oncogene might cause the cell to reproduce more rapidly and more frequently than its normal counterparts.
- A further mutation may cause loss of a tumor suppressor gene, disrupting the apoptosis signaling pathway and immortalizing the cell.
- A further mutation in the signaling machinery of the cell might send error-causing signals to nearby cells.

The transformation of a normal cell into cancer is akin to a chain reaction caused by initial errors, which compound into more severe errors, each progressively allowing the cell to escape more controls that limit normal tissue growth. This rebellion-like scenario is an undesirable survival of the fittest, where the driving forces of evolution work against the body's design and enforcement of order. Once cancer has begun to develop, this ongoing process, termed *clonal evolution*, drives progression towards more invasive stages. Clonal evolution leads to intra-tumour heterogeneity (cancer cells with heterogeneous mutations) that complicates designing effective treatment strategies.

Epigenetics

Epigenetic alterations are functionally relevant modifications to the genome that do not change the nucleotide sequence. Examples of such modifications are changes in DNA methylation (hypermethylation and hypomethylation), histone modification and changes in chromosomal architecture (caused by inappropriate expression of proteins such as HMGA2 or HMGA1). Each of these alterations regulates gene expression without altering the underlying DNA sequence. These changes may remain through cell divisions, last for multiple generations and can be considered to be epimutations (equivalent to mutations).

Epigenetic alterations occur frequently in cancers. As an example, one study listed protein coding genes that were frequently altered in their methylation in association with colon cancer. These included 147 hypermethylated and 27 hypomethylated genes. Of the hypermethylated genes, 10 were hypermethylated in 100% of colon cancers and many others were hypermethylated in more than 50% of colon cancers.

While epigenetic alterations are found in cancers, the epigenetic alterations in DNA repair genes, causing reduced expression of DNA repair proteins, may be of particular importance. Such alterations are thought to occur early in progression to cancer and to be a likely cause of the genetic instability characteristic of cancers.

Metastasis

Metastasis is the spread of cancer to other locations in the body. The dispersed tumors are called metastatic tumors, while the original is called the primary tumor. Almost all cancers can metastasize. Most cancer deaths are due to cancer that has metastasized.

Metastasis is common in the late stages of cancer and it can occur via the blood or the lymphatic system or both. The typical steps in metastasis are local invasion, intravasation into the blood or lymph, circulation through the body, extravasation into the new tissue, proliferation and angiogenesis. Different types of cancers tend to metastasize to particular organs, but overall the most common places for metastases to occur are the lungs, liver, brain and the bones.

Diagnosis

Most cancers are initially recognized either because of the appearance of signs or symptoms or through screening. Neither of these leads to a definitive diagnosis, which requires the examination of a tissue sample by a pathologist. People with suspected cancer are investigated with medical tests. These commonly include blood tests, X-rays, (contrast) CT scans and endoscopy.

The tissue diagnosis from the biopsy indicates the type of cell that is proliferating, its histological grade, genetic abnormalities and other features. Together, this information is useful to evaluate the prognosis and to choose the best treatment.

Cytogenetics and immunohistochemistry are other types of tissue tests. These tests provide information about molecular changes (such as mutations, fusion genes and numerical chromosome changes) and may thus also indicate the prognosis and best treatment.

Prevention

Cancer prevention is defined as active measures to decrease cancer risk. The vast majority of cancer cases are due to environmental risk factors. Many of these environmental factors are controllable lifestyle choices. Thus, cancer is generally preventable. Between 70% and 90% of common cancers are due to environmental factors and therefore potentially preventable.

Greater than 30% of cancer deaths could be prevented by avoiding risk factors including: tobacco, excess weight/obesity, poor diet, physical inactivity, alcohol, sexually transmitted infections and air pollution. Not all environmental causes are controllable, such as naturally occurring background radiation and cancers caused through hereditary genetic disorders and thus are not preventable via personal behavior.

Dietary

While many dietary recommendations have been proposed to reduce cancer risks, the evidence to support them is not definitive. The primary dietary factors that increase risk are obesity and alcohol consumption. Diets low in fruits and vegetables and high in red meat have been implicated but reviews and meta-analyses do not come to a consistent conclusion. A 2014 meta-analysis found no relationship between fruits and vegetables and cancer. Coffee is associated with a reduced risk of liver cancer. Studies have linked excess consumption of red or processed meat to an increased risk of breast cancer, colon cancer and pancreatic cancer, a phenomenon that could be due to thepresence of carcinogens in meats cooked at high temperatures. In 2015 the IARC reported that eating processed meat (e.g., bacon, ham, hot dogs, sausages) and, to a lesser degree, red meat was linked to some cancers.

Dietary recommendations for cancer prevention typically include an emphasis on vegetables, fruit, whole grains and fish and an avoidance of processed and red meat (beef, pork, lamb), animal fats, pickled foods and refined carbohydrates.

Medication

Medications can be used to prevent cancer in a few circumstances. In the general population, NSAIDs reduce the risk of colorectal cancer; however, due to cardiovascular and gastrointestinal side effects, they cause overall harm when used for prevention. Aspirin has been found to reduce the risk of death from cancer by about 7%. COX-2 inhibitors may decrease the rate of polyp formation in people with familial adenomatous polyposis; however, it is associated with the same adverse effects as NSAIDs. Daily use of tamoxifen or raloxifene reduce the risk of breast cancer in high-risk women. The benefit versus harm for 5-alpha-reductase inhibitor such as finasteride is not clear.

Vitamin supplementation does not appear to be effective at preventing cancer. While low blood levels of vitamin D are correlated with increased cancer risk. Beta-carotene supplementation increases lung cancer rates in those who are high risk. Folic acid supplementation is not effective in preventing colon cancer and may increase colon polyps. Selenium supplementation has not been shown to reduce the risk of cancer.

Vaccination

Vaccines have been developed that prevent infection by some carcinogenic viruses. Human papillomavirus vaccine (Gardasil and Cervarix) decrease the risk of developing cervical cancer. The hepatitis B vaccine prevents infection with hepatitis B virus and thus decreases the risk of liver cancer. The administration of human papillomavirus and hepatitis B vaccinations is recommended where resources allow.

Gene	Cancer types
BRCA1, BRCA2	Breast, ovarian, pancreatic

HNPCC, MLH1, MSH2, MSH6, PMS1, PMS2	Colon, uterine, small bowel, stomach, urinary tract

Management

Many treatment options for cancer exist. The primary ones include surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy and palliative care. Which treatments are used depends on the type, location and grade of the cancer as well as the patient's health and preferences. The treatment intent may or may not be curative.

Chemotherapy

Chemotherapy is the treatment of cancer with one or more cytotoxic anti-neoplastic drugs (chemotherapeutic agents) as part of a standardized regimen. The term encompasses a variety of drugs, which are divided into broad categories such as alkylating agents and antimetabolites. Traditional chemotherapeutic agents act by killing cells that divide rapidly, a critical property of most cancer cells.The effectiveness of chemotherapy is often limited by its toxicity to other tissues in the body. Even when chemotherapy does not provide a permanent cure, it may be useful to reduce symptoms such as pain or to reduce the size of an inoperable tumor in the hope that surgery will become possible in the future.

Radiation

Radiation therapy involves the use of ionizing radiation in an attempt to either cure or improve symptoms. It works by damaging the DNA of cancerous tissue, killing it. To spare normal tissues (such as skin or organs, which radiation must pass through to treat the tumor), shaped radiation beams are aimed from multiple exposure angles to intersect at the tumor, providing a much larger dose there than in the surrounding, healthy tissue.

Surgery

Surgery is the primary method of treatment for most isolated, solid cancers and may play a role in palliation and prolongation of survival. It is typically an important part of definitive diagnosis and staging of tumors, as biopsies are usually required. In localized cancer, surgery typically attempts to remove the entire mass along with, in certain cases, the lymph nodes in the area. For some types of cancer this is sufficient to eliminate the cancer.

Palliative care

Palliative care is treatment that attempts to help the patient feel better and may be combined with an attempt to treat the cancer. Palliative care includes action to reduce physical, emotional, spiritual and psycho-social distress. Unlike treatment that is aimed at directly killing cancer cells, the primary goal of palliative care is to improve quality of life.Palliative care may be confused with hospice and therefore only indicated when people

Immunotherapy

A variety of therapies using immunotherapy, stimulating or helping the immune system to fight cancer, have come into use since 1997. Approaches include antibodies, checkpoint therapy, and adoptive cell transfer.

Laser therapy

Laser therapy uses high-intensity light to treat cancer by shrinking or destroying tumors or precancerous growths. Lasers are most commonly used to treat superficial cancers that are on the surface of the body or the lining of internal organs. It is used to treat basal cell skin cancer and the very early stages of others like cervical, penile, vaginal, vulvar, and non-small cell lung cancer. It is often combined with other treatments, such as surgery, chemotherapy, or radiation therapy. Laser-induced interstitial thermotherapy (LITT), or interstitial laser photocoagulation, uses lasers to treat some cancers using hyperthermia, which uses heat to shrink tumors bydamaging or killing cancer cells. Laser are more precise than surgery and cause less damage, pain, bleeding, swelling, and scarring. A disadvantage is surgeons must have specialized training. It may be more expensive than other treatments.

Alternative medicine

Complementary and alternative cancer treatments are a diverse group of therapies, practices and products that are not part of conventional medicine. Cancer researcher Andrew J. Vickers stated, "The label 'unproven' is inappropriate for such therapies; it is time to assert that many alternative cancer therapies have been 'disproven'."

Prognosis

Survival rates vary by cancer type and by the stage at which it is diagnosed, ranging from majority survival to complete mortality five years after diagnosis. Once a cancer has metastasized, prognosis normally becomes much worse. About half of patients receiving treatment for invasive cancer (excluding carcinoma *in situ* and non-melanoma skin cancers) die from that cancer or its treatment.

Survival is worse in the developing world, partly because the types of cancer that are most common there are harder to treat than those associated with developed countries.

People with cancer have an increased risk of blood clots in their veins which can be life-threatening. The use of blood thinners such as heparin decrease the risk of blood clots but have not been shown to increase survival in people with cancer. People who take blood thinners also have an increased risk of bleeding.

Economic effect

The total health care expenditure on cancer in the US was estimated to be $80.2 billion in 2015. Even though cancer-related health care expenditure have increased in absolute terms during recent decades, the share of health expenditure devoted to cancer treatment has remained close to 5% between the 1960s and 2004. A similar pattern has been observed in Europe where about 6% of all health care expenditure are spent on cancer treatment. In addition to health care expenditure, cancer causes indirect costs in the form of productivity losses due to sick days, permanent incapacity and disability as well as premature death during working age. Cancer causes also costs for informal care. Indirect costs and informal care costs are typically estimated to exceed or equal the health care costs of cancer.

Research

Because cancer is a class of diseases, it is unlikely that there will ever be a single "cure for cancer" any more than there will be a single treatment for all infectious diseases.Angiogenesis

inhibitors were once incorrectly thought to have potential as a "silver bullet" treatment applicable to many types of cancer. Angiogenesis inhibitors and other cancer therapeutics are used in combination to reduce cancer morbidity and mortality.

Experimental cancer treatments are studied in clinical trials to compare the proposed treatment to the best existing treatment. Treatments that succeeded in one cancer type can be tested against other types. Diagnostic tests are under development to better target the right therapies to the right patients, based on their individual biology.

The improved understanding of molecular biology and cellular biology due to cancer research has led to new treatments for cancer since US President Richard Nixon declared the "War on Cancer" in 1971. Since then, the country has spent over $200 billion on cancer research, including resources from public and private sectors. The cancer death rate (adjusting for size and age of the population) declined by five percent between 1950 and 2005.

Virus-inuced cell transformation:

Viral transformation is the change in growth, phenotype, or indefinite reproduction of cells caused by the introduction of inheritable material. Viral transformation can occur both naturally and medically. Natural transformations can include viral cancers, such as human papillomavirus (HPV) and T-cell Leukemia virus type I. Hepatitis B and C are also the result of natural viral transformation of the host cells. Viral transformation can also be induced for use in medical treatments.

Type

Malignant transformation

There are three types of viral infections that can be considered under the topic of viral transformation. These are cytocidal, persistent, and transforming infections. Cytocidal infections can cause fusion of adjacent cells, disruption of transport pathways including ions and other cell signals, disruption of DNA, RNA and protein synthesis, and nearly always leads to cell death. Persistent infections involve viral material that lays dormant within a cell until activated by some stimulus. This type of infection usually causes few obvious changes within the cell but can lead to long chronic diseases.

Table 1: Cellular effects of viral infections

		Genetic	Cell Fate	Morphological	Biochemical	Physiological
Cytocidal	Productive ---------- Abortive	DNA degradation ---------- Possible mutation	Death ---------- Usually death	Rounding of the cell Fusion with adjacent cells Appearance of inclusion bodies	Inhibit DNA, RNA, and protein synthesis Interfere with sub-cellular interactions	Insufficient movement of ions secondary messengers Activation of cellular cascades
Persistent	Latent ---------- Chronic ---------- Slow	Possible Mutation ---------- Possible Mutation ---------- Possible Mutation	Survival ---------- Variable ---------- Variable	Fusion with adjacent cells Appearance of inclusion bodies Budding	Immune responses limit viral spread Antigen-antibody complexes can incorporate viral antigens cau sing inflammation	Rare until stimulated
Transforming	DNA viruses ---------- RNA viruses	Mutation ---------- Mutation	Survival ---------- Survival	Unlimited cell replication	Inactivates tumor suppressor proteins Impairs cell cycle regulation	Unlimited cell replication

Cytocidal infections:
Cytocidal infections are often associated with changes in cell morphology, physiology and are thus important for the complete viral replication and transformation. *Cytopathic Effects*, often include a change in cell's morphology such as fusion with adjacent cells to form polykaryocytes as well as the synthesis of nuclear and cytoplasmic inclusion bodies. *Physiological changes* include the insufficient movement of ions, formation of secondary messengers, and activation of cellular cascades to continue cellular activity. *Biochemically*, many viruses inhibit the synthesis of host DNA, RNA, proteins directly or even interfere with protein-protein, DNA-protein, RNA-protein interactions at the subcellular level. *Genotoxicity* involves breaking, fragmenting, or rearranging chromosomes of the host. Lastly, *biologic effects* include the viruses' ability to affect the activity of antigens and immunologlobulins in the host cell.

There are two types of cytocidal infections, productive and abortive. In productive infections, additional infectious viruses are produced. Abortive infections do not produce infectious viruses. One example of a productive cytocidal infection is the herpes virus.

Persistent infections:
There are three types of persistent infections, latent, chronic and slow, in which the virus stays inside the host cell for prolonged periods of time. During *latent infections* there is minimal to no expression of infected viral genome. The genome remains within the host cell until the virus is ready for replication. *Chronic infections* have similar cellular effects as acute cytocidal infections but there is a limited number of progeny and viruses involved in transformation.

Transforming infections
Transformation infections is limited to abortive or restrictive infections. This constitutes the broadest category of infections as it can include both cytocidal and persistent infection. Viral transformation is most commonly understood as transforming infections, so the remainder of the article focuses on detailing transforming infections.

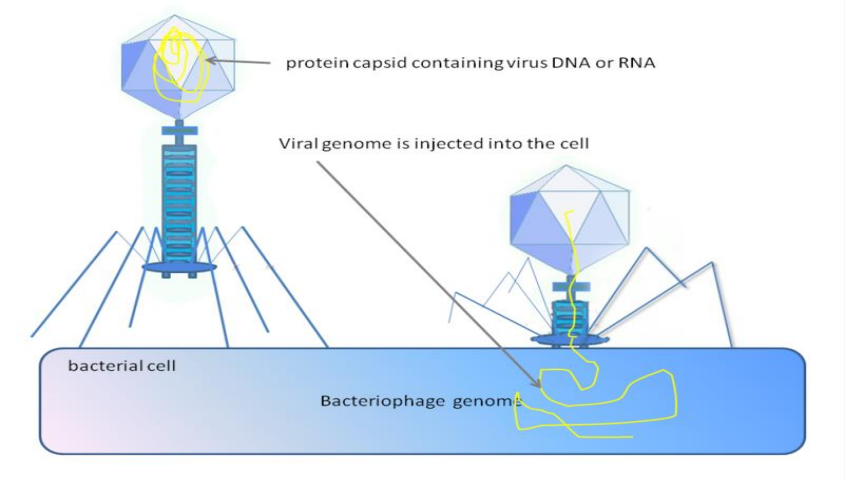


Figure 2: Phage injecting its genome into bacterial cell

As shown in Figure 2, a bacteriophage lands on a cell and pins itself to the cell. The phage can then penetrate the cell membrane and inject the viral DNA into the host cell. The viral DNA can then either lay dormant until stimulated by a source such as UV light or it can be immediately taken up by the host's genome.

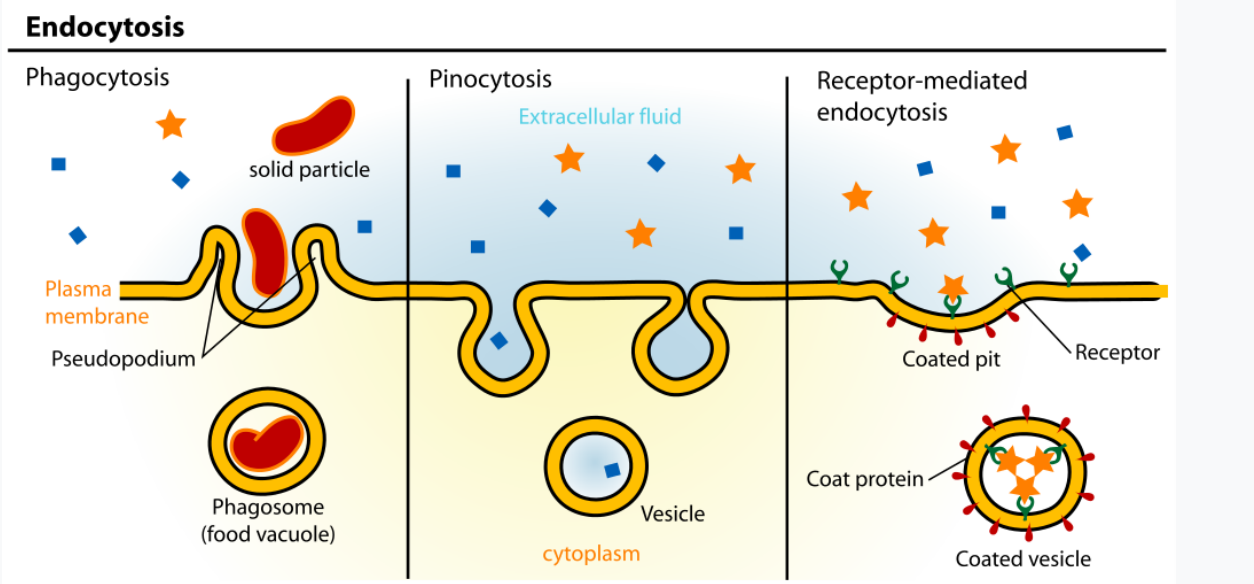


Figure 3: Examples of endocytosis

The process is similar in animal cells. In most cases, rather than viral DNA being injected into an animal cell, a section of the membrane encases the virus and the cell then absorbs both the virus and the encasing section of the membrane into the cell. This process, called endocytosis, is shown in Figure 3.

Assays

Virus quantification

An assay is an analytic tool often used in a laboratory setting in order to assess or measure some quality of a target entity. In virology, assays can be used to differentiate between transformed and non-transformed cells. Varying the assay used, changes the selective pressure on the cells and therefore can change what properties are selected in the transformed cells.

Three common assays used are the focus forming assay, the Anchorage independent growth assay, and the reduced serum assay.

The focus forming assay (FFA) is used to grow cells containing a transforming oncogene on a monolayer of non-transformed cells. The transformed cells will form raised, dense spots on the sample as they grow without contact inhibition.This assay is highly sensitive compared to other assays used for viral analysis, such as the yield reduction assay.

An example of the Anchorage independent growth assay is the soft agar assay. The assay is assessing the cells' ability to grow in a gel or viscous fluid. Transformed cells can grow in this environment and are considered anchorage independent. Cells that can only grow when attached to a solid surface are anchorage dependent untransformed cells. This assay is considered one of the most stringent for detection of malignant transformation

In a reduced serum assay, cells are assayed by exploiting the changes in cell serum requirements. Non-transformed cells require at least a 5% serum medium in order to grow; however, transformed cells can grow in an environment with significantly less serum.

Gene knockout

A **gene knockout** (abbreviation: **KO**) is a genetic technique in which one of an organism's genes is made inoperative ("knocked out" of the organism). However, KO can also refer to the gene that is knocked out or the organism that carries the gene knockout. The KO technique is essentially the opposite of a gene knock-in. Knocking out two genes simultaneously in an organism is known as a **double knockout** (**DKO**). Similarly the terms **triple knockout** (**TKO**) and **quadruple knockouts** (**QKO**) are used to describe three or four knocked out genes, respectively. However, one needs to distinguish between heterozygous and homozygous KOs. In the former, only one of two gene copies (alleles) is knocked out, in the latter both are knocked out.

Methods

Knockouts are accomplished through a variety of techniques. Originally, **naturally occurring mutations** were identified and then gene loss or inactivation had to be established by DNA sequencing or other methods.

Homologous recombination

Traditionally, homologous recombination was the main method for causing a gene knockout. This method involves creating a DNA construct containing the desired mutation. For knockout purposes, this typically involves a drug resistance marker in place of the desired knockout gene. The construct will also contain a minimum of 2kb of homology to the target sequence. The construct can be delivered to stem cells either through microinjection or electroporation. This method then relies on the cell's own repair mechanisms to recombine the DNA construct into the existing DNA. This results in the sequence of the gene being altered, and most cases the gene will be translated into a nonfunctional protein, if it is translated at all. In diploid organisms, which contain two alleles for most genes, and may as well contain several related genes that collaborate in the same role, additional rounds of transformation and selection are performed until every targeted gene is knocked out. Selective breeding may be required to produce homozygous knockout animals.

Site-specific nucleases:

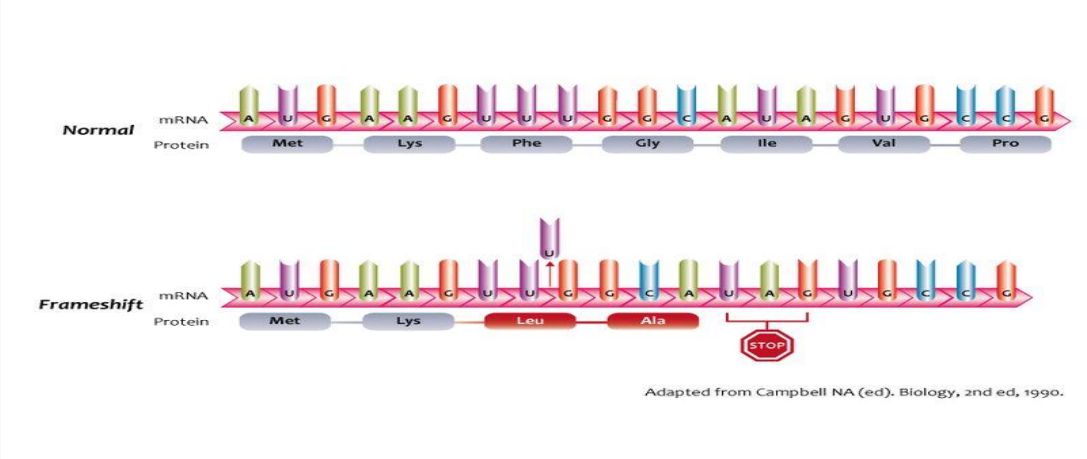


Fig 1. Frameshift mutation resulting from a single base pair deletion, causing altered amino acid sequence and premature stop codon.

Once this occurs, the cell's repair mechanisms will attempt to repair this double stranded break, often through non-homologous end joining (NHEJ), which involves directly ligating the two cut ends together. This may be done imperfectly, therefore sometimes causing insertions or deletions of base pairs, which cause frameshift mutations. These mutations can render the gene in which they occur nonfunctional, thus creating a knockout of that gene. This process is more efficient than homologous recombination, and therefore can be more easily used to create biallelic knockouts.[3]

Conditional knockouts:

A **conditional knockout** allows gene deletion in a tissue in a time specific manner. This is required in place of a gene knockout if the null mutation would lead to embryonic death. This is done by introducing short sequences called loxP sites around the gene. These sequences will be introduced into the germ-line via the same mechanism as a knock-out. This germ-line can then be crossed to another germline containing Cre-recombinase which is a viral enzyme that can recognize these sequences, recombines them and deletes the gene flanked by these sites.

Use

Knockouts are primarily used to understand the role of a specific gene or DNA region by comparing the knockout organism to a wildtype with a similar genetic background.

Knockout organisms are also used as screening tools in the development of drugs, to target specific biological processes or deficiencies by using a specific knockout, or to understand the mechanism of action of a drug by using a library of knockout organisms spanning the entire genome, such as in *Saccharomyces cerevisiae*.

Gene knockdown

Gene knockdown is an experimental technique by which the expression of one or more of an organism's genes is reduced. The reduction can occur either through genetic modification or by treatment with a reagent such as a short DNA or RNA oligonucleotide that has a sequence complementary to either gene or an mRNA transcript.

Versus transient knockdown

If a DNA of an organism is genetically modified, the resulting organism is called a "knockdown organism." If the change in gene expression is caused by an oligonucleotide binding to an mRNA or temporarily binding to a gene, this leads to a temporary change in gene expression that does not modify the chromosomal DNA, and the result is referred to as a "transient knockdown".

In a transient knockdown, the binding of this oligonucleotide to the active gene or its transcripts causes decreased expression through a variety of processes. Binding can occur either through the blocking of transcription (in the case of gene-binding), the degradation of the mRNA transcript (e.g. by small interfering RNA (siRNA)) or RNase-H dependent antisense, or through the blocking of either mRNA translation, pre-mRNA splicing sites, or nuclease cleavage sites used for maturation of other functional RNAs, including miRNA (e.g. by morpholino oligos or other RNase-H independent antisense).

The most direct use of transient knockdowns is for learning about a gene that has been sequenced, but has an unknown or incompletely known function. This experimental approach is known as reverse genetics. Researchers draw inferences from how the knockdown differs from individuals in which the gene of interest is operational. Transient knockdowns are often used in developmental biology because oligos can be injected into single-celled zygotes and will be present in the daughter cells of the injected cell through embryonic development.

RNA interference

RNA interference (RNAi) is a means of silencing genes by way of mRNA degradation. Gene knockdown by this method is achieved by introducing small double-stranded interfering RNAs (siRNA) into the cytoplasm. Small interfering RNAs can originate from inside the cell or can be exogenously introduced into the cell. Once introduced into the cell, exogenous siRNAs are processed by the RNA-induced silencing complex (RISC). The siRNA is complementary to the target mRNA to be silenced, and the RISC uses the siRNA as a template for locating the target mRNA. After the RISC localizes to the target mRNA, the RNA is cleaved by a ribonuclease.

RNAi is widely used as a laboratory technique for genetic functional analysis. RNAi in organisms such as *C. elegans* and *Drosophila melanogaster* provides a quick and inexpensive means of investigating gene function. In *C. elegans* research, the availability of tools such as the Ahringer RNAi Library give laboratories a way of testing many genes in a variety of experimental backgrounds. Insights gained from experimental RNAi use may be useful in identifying potential therapeutic targets, drug development, or other applications. RNA interference is a very useful research tool, allowing investigators to carry out large genetic screens in an effort to identify targets for further research related to a particular pathway, drug, or phenotype.

4. Industrial microbiology Industrially Important Microbes and Their Products

'**Industrial microbiology** is a branch of biotechnology that applies microbial sciences to create industrial products in mass quantities. There are multiple ways to manipulate a microorganism in order to increase maximum product yields. Introduction of mutations into an organism may be accomplished by introducing them to mutagens. Another way to increase production is by gene amplification, this is done by the use of plasmids, and vectors. The plasmids and/ or vectors are used to incorporate multiple copies of a specific gene that would allow more enzymes to be produced that eventually cause more product yield. The manipulation of organisms in order to yield a specific product has many applications to the real world like the production of some antibiotics, vitamins, enzymes, amino acids, solvents, alcohol and daily products. Microorganisms play a big role in the industry, with multiple ways to be used. Medicinally, microbes can be used for creating antibiotics in order to treat antibiotics. Microbes can also be used for the food industry as well. Microbes are very useful in creating some of the mass produced products that are consumed by people. The chemical industry also uses microorganisms in order to synthesis amino acids and organic solvents. Microbes can also be used in an agricultural application for use as a biopesticide instead of using dangerous chemicals and or inoculants to help plant proliferation.

List of microorganisms used in food and beverage preparation

Microorganism	Type Of Microorganism	Food or Beverage
Acetobacter aceti	bacterium	chocolate
Acetobacter aceti	bacterium	vinegar
Acetobacter cerevisiae	bacterium	Beer
Acetobacter fabarum	bacterium	chocolate
Acetobacter fabarum	bacterium	coffee
Acetobacter lovaniensis	bacterium	vegetables
Acetobacter malorum	bacterium	vinegar
Acetobacter orientalis	bacterium	vegetables
Acetobacter pasteurianus	bacterium	chocolate
Acetobacter pasteurianus	bacterium	vinegar
Acetobacter pomorum	bacterium	vinegar
Acetobacter syzygii	bacterium	chocolate

Microorganism	Type Of Microorganism	Food or Beverage
Acetobacter syzygii	bacterium	vinegar
Acetobacter tropicalis	bacterium	chocolate
Acetobacter tropicalis	bacterium	coffee
Arthrobacter arilaitensis	bacterium	smear-ripened cheese
Arthrobacter bergerei	bacterium	smear-ripened cheese
Arthrobacter globiformis	bacterium	smear-ripened cheese
Arthrobacter nicotianae	bacterium	surface-ripened cheese
Arthrobacter nicotianae	bacterium	Tilsit cheese
Arthrobacter variabilis	bacterium	smear-ripened cheese
Aspergillus acidus	fungus	tea
Aspergillus niger	fungus	awamori
Aspergillus fumigatus	fungus	chocolate
Aspergillus oryzae	fungus	miso
Aspergillus oryzae	fungus	sake
Aspergillus oryzae	fungus	soy sauce
Aspergillus sojae	fungus	miso
Aspergillus sojae	fungus	soy sauce
Bacillus cereus	bacterium	chocolate
Bacillus coagulans	bacterium	chocolate
Bacillus licheniformis	bacterium	chocolate
Bacillus pumilus	bacterium	chocolate
Bacillus sphaericus	bacterium	stinky tofu
Bacillus stearothermophilus	bacterium	chocolate
Bacillus subtilis	bacterium	chocolate
Bacillus subtilis	bacterium	natto
Bifidobacterium adolescentis	bacterium	yogurt
Bifidobacterium animalis	bacterium	dairy
Bifidobacterium bifidum	bacterium	dairy
Bifidobacterium breve	bacterium	dairy
Bifidobacterium breve	bacterium	soy
Bifidobacterium infantis	bacterium	dairy

Microorganism	Type Of Microorganism	Food or Beverage
Bifidobacterium lactis	bacterium	dairy
Bifidobacterium longum	bacterium	dairy
Bifidobacterium pseudolongum	bacterium	dairy
Bifidobacterium thermophilum	bacterium	dairy
Brachybacterium alimentarium	bacterium	Beaufort cheese
Brachybacterium alimentarium	bacterium	Gruyère cheese
Brachybacterium tyrofermentans	bacterium	Beaufort cheese
Brachybacterium tyrofermentans	bacterium	Gruyère cheese
Brevibacterium aurantiacum	bacterium	cheese
Brevibacterium casei	bacterium	smear-ripened cheese
Brevibacterium linens	bacterium	smear-ripened cheese
Candida colliculosa	fungus	cheese
Candida colliculosa	fungus	kefir
Candida exiguus	fungus	sourdough bread
Candida humicola	fungus	chocolate
Candida kefyr	fungus	surface-ripened cheese
Candida krusei	fungus	surface-ripened cheese
Candida milleri	fungus	sourdough bread
Candida mycoderma	fungus	Limburger cheese

Bioreactor

A **bioreactor** refers to any manufactured device or system that supports a biologically active environment. In one case, a bioreactor is a vessel in which a chemical process is carried out which involves organisms or biochemically active substances derived from such organisms. This process can either be aerobic or anaerobic. These bioreactors are commonly cylindrical, ranging in size from litres to cubic metres, and are often made of stainless steel.

It may also refer to a device or system designed to grow cells or tissues in the context of cell culture. These devices are being developed for use in tissue engineering or biochemical/bioprocess engineering.

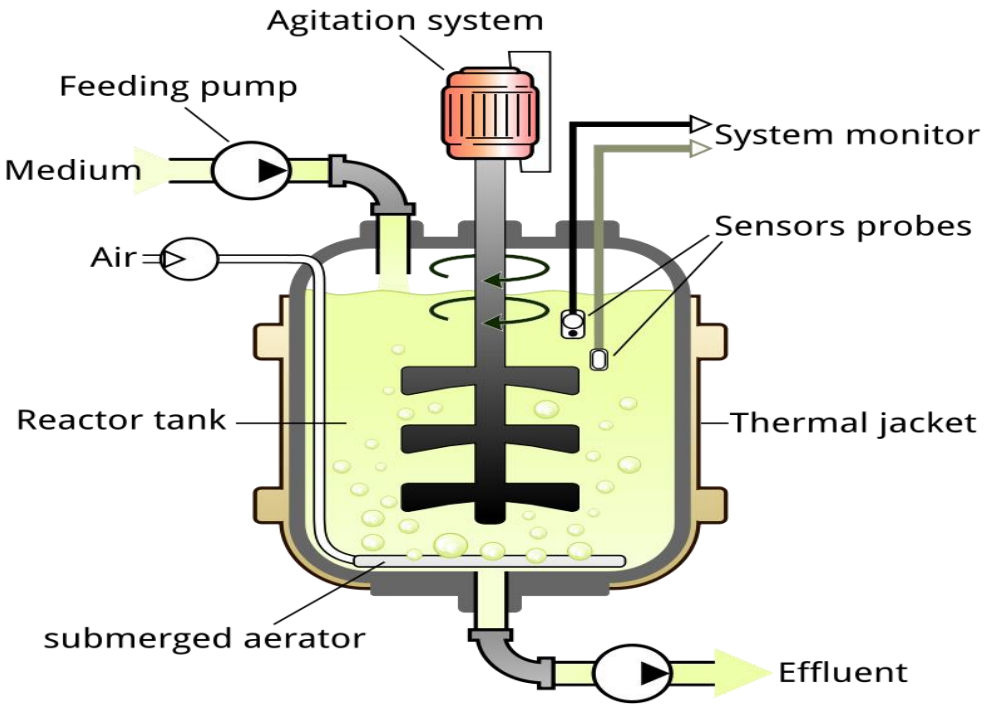
On the basis of **mode of operation**, a bioreactor may be classified as batch, fed batch or continuous (e.g. a continuous stirred-tank reactor model). An example of a continuous bioreactor is the chemostat.

Organisms growing in bioreactors may be submerged in liquid medium or may be attached to the surface of a solid medium. Submerged cultures may be suspended or immobilized. Suspension bioreactors can use a wider variety of organisms, since special attachment surfaces are not

needed, and can operate at a much larger scale than immobilized cultures. However, in a continuously operated process the organisms will be removed from the reactor with the effluent. Immobilization is a general term describing a wide variety of methods for cell or particle attachment or entrapment. It can be applied to basically all types of biocatalysis including enzymes, cellular organelles, animal and plant cells. Immobilization is useful for continuously operated processes, since the organisms will not be removed with the reactor effluent, but is limited in scale because the microbes are only present on the surfaces of the vessel.

Large scale immobilized cell bioreactors are:

- moving media, also known as moving bed biofilm reactor (MBBR)
- packed bed
- fibrous bed
- Membrane



General structure of a continuous stirred-tank type bioreactor

Design

Bioreactor design is a relatively complex engineering task, which is studied in the discipline of biochemical/bioprocess engineering. Under optimum conditions, the microorganisms or cells are able to perform their desired function with limited production of impurities. The environmental conditions inside the bioreactor, such as temperature, nutrient concentrations, pH, and dissolved gases (especially oxygen for aerobic fermentations) affect the growth and productivity of the organisms. The temperature of the fermentation medium is maintained by a cooling jacket, coils, or both. Particularly exothermic fermentations may require the use of external heat exchangers. Nutrients may be continuously added to the fermenter, as in a fed-batch system, or may be

charged into the reactor at the beginning of fermentation. The pH of the medium is measured and adjusted with small amounts of acid or base, depending upon the fermentation. For aerobic (and some anaerobic) fermentations, reactant gases (especially oxygen) must be added to the fermentation. Since oxygen is relatively insoluble in water (the basis of nearly all fermentation media), air (or purified oxygen) must be added continuously. The action of the rising bubbles helps mix the fermentation medium and also "strips" out waste gases, such as carbon dioxide. In practice, bioreactors are often pressurized; this increases the solubility of oxygen in water. In an aerobic process, optimal oxygen transfer is sometimes the rate limiting step. Oxygen is poorly soluble in water—even less in warm fermentation broths—and is relatively scarce in air (20.95%). Oxygen transfer is usually helped by agitation, which is also needed to mix nutrients and to keep the fermentation homogeneous. Gas dispersing agitators are used to break up air bubbles and circulate them throughout the vessel.Fouling can harm the overall efficiency of the bioreactor, especially the heat exchangers. To avoid it, the bioreactor must be easily cleaned. Interior surfaces are typically made of stainless steel for easy cleaning and sanitation. Typically bioreactors are cleaned between batches, or are designed to reduce fouling as much as possible when operated continuously. Heat transfer is an important part of bioreactor design; small vessels can be cooled with a cooling jacket, but larger vessels may require coils or an external heat exchanger.

Types

1) **Photobioreactor:**

A photobioreactor (PBR) is a bioreactor which incorporates some type of light source (that may be natural sunlight or artificial illumination). Virtually any translucent container could be called a PBR, however the term is more commonly used to define a closed system, as opposed to an open storage tank or pond. Photobioreactors are used to grow small phototrophic organisms such as cyanobacteria, algae, or moss plants. These organisms use light through photosynthesis as their energy source and do not require sugars or lipids as energy source. Consequently, risk of contamination with other organisms like bacteria or fungi is lower in photobioreactors when compared to bioreactors for heterotroph organisms.

2) Sewage treatment

Conventional sewage treatment utilises bioreactors to undertake the main purification processes. In some of these systems, a chemically inert medium with very high surface area is provided as a substrate for the growth of biological film. Separation of excess biological film takes place in settling tanks or cyclones. In other systems aerators supply oxygen to the sewage and biota to create activated sludge in which the biological component is freely mixed in the liquor in "flocs". In these processes, the liquid's Biochemical Oxygen Demand (BOD) is reduced sufficiently to render the contaminated water fit for reuse. The biosolids can be collected for further processing, or dried and used as fertilizer. An extremely simple version of a sewage bioreactor is a septic tank whereby the sewage is left in situ, with or without additional media to house bacteria. In this instance, the biosludge itself is the primary host for the bacteria.

3) Bioreactors for specialized tissues

Many cells and tissues, especially mammalian ones, must have a surface or other structural support in order to grow, and agitated environments are often destructive to these cell types and tissues. Higher organisms, being auxotrophic, also require highly specialized growth media. This poses a challenge when the goal is to culture larger quantities of cells for therapeutic production purposes, and a significantly different design is needed compared to industrial bioreactors used for growing protein expression systems such as yeast and bacteria.

Many research groups have developed novel bioreactors for growing specialized tissues and cells on a structural scaffold, in attempt to recreate organ-like tissue structures in-vitro. Among these include tissue bioreactors that can grow heart tissue, skeletal muscle tissue, ligaments, cancer tissue models, and others. Currently, scaling production of these specialized bioreactors for industrial use remains challenging and is an active area of research.

Modelling

Mathematical models act as an important tool in various bio-reactor applications including wastewater treatment. These models are useful for planning efficient process control strategies and predicting the future plant performance. Moreover, these models are beneficial in education and research areas.Bioreactors are generally used in those industries which are concerned with food, beverages and pharmaceuticals. The emergence of Biochemical engineering is of recent origin. Processing of biological materials using biological agents such as cells, enzymes or antibodies are the major pillars of biochemical engineering.

Operational stages in a bio-process

A bioprocess is composed mainly of three stages — upstream processing, bioreaction, and downstream processing — to convert raw material to finished product. The raw material can be of biological or non-biological origin. It is first converted to more suitable form for processing. This is done in upstream processing step which involves chemical hydrolysis, preparation of liquid medium, separation of particulate, air purification and many other preparatory operations.After upstream processing step, the resulting feed is transferred to one or more Bioreaction stages. The Biochemical reactors or bioreactors form the base of the Bioreaction step. This step mainly consists of three operations, namely, production of biomass, metabolite biosynthesis and biotransformation.Finally, the material produced in the bioreactor must be further processed in the downstream section to convert it into more useful form. The downstream process mainly consists of physical separation operations which includes, solid liquid separation, adsorption, liquid-liquid extraction, distillation, drying etc.

Specifications

A typical bioreactor consists of following parts:

- Agitator – used for the mixing of the contents of the reactor which keeps the "cells" in the perfect homogenous condition for better transport of nutrients and oxygen to the desired product(s).
- Baffle – used to break the vortex formation in the vessel, which is usually highly undesirable as it changes the center of gravity of the system and consumes additional power.
- Sparger – In aerobic cultivation process, the purpose of the sparger is to supply adequate oxygen to the growing cells.
- Jacket – The jacket provides the annular area for circulation of constant temperature of water which keeps the temperature of the bioreactor at a constant value.

Development of modelling equations for bioreactors

Assumptions:

- The reactor contents are perfectly mixed together.
- The reactor is operating at a constant temperature (i.e., it is isothermal).
- The feed should be clean and pure (i.e., no biomass in the feed stream).
- The feed stream and reactor contents have equal and constant density (ρ).
- The feed and product streams have the same flow rate (F).

Continuous stirred-tank reactor

The continuous stirred-tank reactor (CSTR), also known as vat- or backmix reactor, or a continuous-*flow* stirred-tank reactor (C*F*STR), is a common model for a chemical reactor in chemical engineering and environmental engineering. A CSTR often refers to a model used to estimate the key unit operation variables when using a continuous agitated-tank reactor to reach a specified output. The mathematical model works for all fluids: liquids, gases, and slurries.

The behavior of a CSTR is often approximated or modeled by that of a Continuous Ideally Stirred-Tank Reactor (CISTR). All calculations performed with CISTRs assume perfect mixing. In a perfectly mixed reactor, the output composition is identical to composition of the material inside the reactor, which is a function of residence time and rate of reaction. If the residence time is 5-10 times the mixing time, this approximation is valid for engineering purposes. The CISTR model is often used to simplify engineering calculations and can be used to describe research reactors. In practice it can only be approached, in particular in industrial size reactors.

Applications

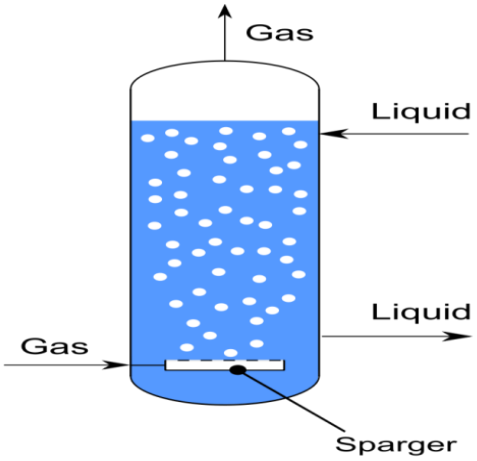
Within the field of environmental engineering, CSTRs are usually applied in wastewater treatment processes. CSTRs are commonly used in the activated sludge treatment process during wastewater treatment. Anaerobic digesters for the stabilization of biosolids produced during biological treatment of wastewater are also designed as CSTRs.

CSTRs facilitate rapid dilution rates which make them resistant to both high pH and low pH volatile fatty acid wastes. CSTRs are less efficient compared to other types of reactors as they require larger reactor volumes to achieve the same reaction rate as other reactor models such as Plug Flow Reactors (PFR).

CSTRs are also a useful tool for modeling non-ideal flow reactors. Combining ideal CSTRs and PFRs in series and in parallel with one another can achieve a variety of flow regimes. For examples, an infinite series of ideal CSTRs is equivalent hydraulically to an ideal PFR.

Bubble column reactor

A **bubble column reactor** is an apparatus used to generate and control gas-liquid chemical reactions. It consists of a vertically-arranged cylindrical column filled with liquid, at the bottom of which gas is inserted.



Representation of a bubble column reactor

Principle

The introduction of gas takes place at the bottom of the column and causes a turbulent stream to enable an optimum gas exchange. Numerous forms of construction exist. The mixing is done by the gas sparging and it requires less energy than mechanical stirring. The liquid can be in parallel flow or counter-current.

Bubble column reactors are characterized by a high liquid content and a moderate phase boundary surface. The bubble column is particularly useful in reactions where the gas-liquid reaction is slow in relation to the absorption rate. This is the case for gas-liquid reactions with a Hatta number Ha <0.3.

Bubble column reactors are used in various types of chemical reactions like wet oxidation, or as algae bioreactor. Since the computerized design of bubble columns is restricted to a few partial processes, experience in the choice of a particular type column still plays an important role.

Literature

Bubble column reactors belong to the general class of multiphase reactors which consist of three main categories namely, the trickle bed reactor (fixed or packed bed), fluidized bed reactor, and the bubble column reactor. Bubble columns are the devices in which gas, in the form of bubbles, come in contact with the liquid. The purpose may be simply to mix the two phases or substances are transferred from one phase to another i.e. when the gaseous reactants are dissolved in liquid or when liquid reactant products are stripped. The bubble column in which the gas is fed into the column at the bottom and rises in the liquid escaping from it at the upper surface; the gas is consumed to a greater or lesser extent depending on the intensity of mass transfer and chemical reaction.

Airlift reactor

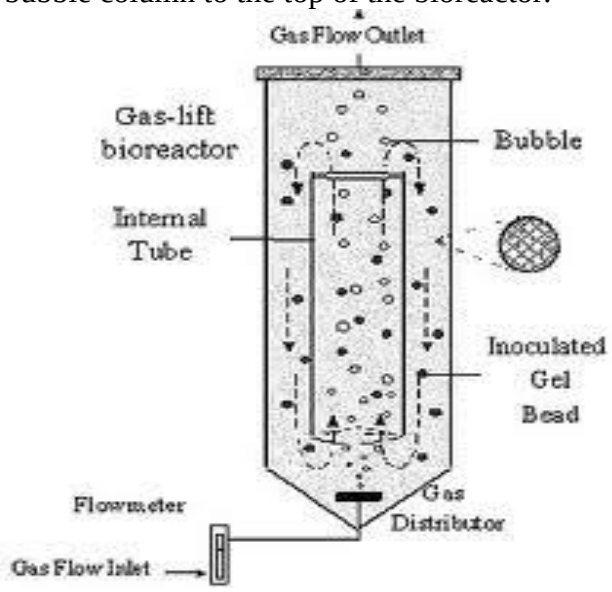
Bioreactors of the airlift type are a promising design for aerobic fermentations. The basic knowledge required for understanding and predicting the performance of these reactors is only now beginning to emerge. In this review we present our observations and those of other investigators in an attempt to build up a coherent picture of airlift devices. All the major aspects—mixing and hydrodynamics, mass and heat transfer—in these reactors are considered. Comparisons between bubble columns and airlift systems are made where analogies, similarities

and/or differences between them provide insight into airlift systems. Throughout, the areas of particular concern and those in need of further research in this field are mentioned. Extensive work on all forms of airlift reactors, particularly in non-Newtonian media—homogeneous and suspensions—remains to be done. Current knowledge does not permit airlift reactor design with a high degree of confidence. However, the technical feasibility of all types of fermentations—plant cell, tissue culture, bacterial, fungal, and those utilizing yeasts—in airlift vessels has been demonstrated.

Airlift bioreactors are used for cell culturing, pallet form fermentation, and immobilized enzyme reactions. Typically, airlift bioreactors are used when the desired reactants and/or final products are in a gaseous state and for aerobic cell cultures.

- **How airlift bioreactors work**

An airlift bioreactor works by agitating the contents of the bioreactor pneumatically using gas. The gas used for agitation can act to either, introduce new molecules to the mixture inside of the bioreactor, or remove specific metabolic molecules produced by microorganisms. Airlift bioreactors have a built in bubble column designed to release gas into the bioreactor. Gas is usually injected into the bubble column at the bottom of the bioreactor. Mixing occurs as the bubbles rise through the bubble column to the top of the bioreactor.



Simple diagram of an airlift bioreactor

The pattern of of fluid circulation inside of airlift bioreactors can be customized through the design of it's bubble column and shape. There are two separate channels within an airlift bioreactors; one channel for gas/liquid up-flow and one channel for gas/liquid down-flow. Both channels create a closed gas/liquid circuit, and has a mechanism for removing gaseous substances at the top of the bioreactor called the gas separator.

- **The two types of airlift bioreactors**

There are two types of airlifts bioreactors: 1) external loop vessels, 2) baffled vessels. External loop vessels have distinct and separate conduits for circulation of gases and liquids. Baffled vessels draw upon spider diffraction techniques to customize gas and liquid through the bioreactor flow by including channels, vanes, and/or other methods of obstruction. Baffled vessels allow for greater control of gas and liquid circulation patterns within bioreactors through the creation of customized bubble disengagement and gas/liquid flow rates.

- **Advantages of airlift bioreactors**

The primary advantage of airlifts bioreactors over other bioreactors is due to the nature of how the contents inside airlift bioreactors are mixed. They have no focal points of energy dissipation, and have homogenous shear and stress forces throughout the entire bioreactor. Making airlift bioreactors ideal for culturing shear sensitive cells.

Photobioreactor

A photobioreactor (PBR) is a bioreactor that utilizes a light source to cultivate phototrophic microorganisms. These organisms use photosynthesis to generate biomass from light and carbon dioxide and include plants, mosses, macroalgae, microalgae, cyanobacteria and purple bacteria. Within the artificial environment of a photobioreactor, specific conditions are carefully controlled for respective species. Thus, a photobioreactor allows much higher growth rates and purity levels than anywhere in nature or habitats similar to nature. Hypothetically, phototropic biomass could be derived from nutrient-rich wastewater and flue gas carbon dioxide in a photobioreactor.

Open systems

The first approach for the controlled production of phototrophic organisms was a natural open pond or artificial raceway pond. Therein, the culture suspension, which contains all necessary nutrients and carbon dioxide, is pumped around in a cycle, being directly illuminated from sunlight via the liquid's surface. This construction principle is the simplest way of production for phototrophic organisms. But due to their depth (up to 0.3 m) and the related reduced average light supply, open systems only reach limited areal productivity rates. In addition, the consumption of energy is relatively high, as high amounts of water containing low product concentration have to be processed. Open space is expensive in areas with a dense population, while water is rare in others. Using open technologies causes high losses of water due to evaporation into the atmosphere.

Closed systems

Since the 1950s several approaches have been conducted to develop closed systems, which theoretically provide higher cell densities of phototrophic organisms and therefore a lower demand of water to be pumped than open systems. In addition, closed construction avoids system-related water losses and the risk of contamination through landing water birds or dust is minimized. All modern photobioreactors have tried to balance between a thin layer of culture suspension, optimized light application, low pumping energy consumption, capital expenditure and microbial purity. Many different systems have been tested, but only a few approaches were able to perform at an industrial scale.

Redesigned laboratory fermenters

The simplest approach is the redesign of the well-known glass fermenters, which are state of the art in many biotechnological research and production facilities worldwide. The moss reactor for

example shows a standard glass vessel, which is externally supplied with light. The existing head nozzles are used for sensor installation and for gas exchange. This type is quite common in laboratory scale, but it has never been established in bigger scale, due to its limited vessel size.

Tubular photobioreactors

Made from glass or plastic tubes, this photobioreactor type has succeeded within production scale. The tubes are oriented horizontally or vertically and are supplied from a central utilities installation with pump, sensors, nutrients and CO_2. Tubular photobioreactors are established worldwide from laboratory up to production scale, e.g. for the production of the carotenoid Astaxanthine from the green algae *Haematococcus pluvialis* or for the production of food supplement from the green algae *Chlorella vulgaris*. These photobioreactors take advantage from the high purity levels and their efficient outputs. The biomass production can be done at a high quality level and the high biomass concentration at the end of the production allows energy efficient downstream processing. Due to the recent prices of the photobioreactors, economically feasible concepts today can only be found within high-value markets, e.g. food supplement or cosmetics.

The advantages of tubular photobioreactors at production scale are also transferred to laboratory scale. A combination of the mentioned glass vessel with a thin tube coil allows relevant biomass production rates a laboratory research scale. Being controlled by a complex process control system the regulation of the environmental conditions reaches a high level.

Christmas tree photobioreactor

An alternative approach is shown by a photobioreactor, which is built in a tapered geometry and which carries a helically attached, translucent double hose circuit system. The result is a layout similar to a Christmas tree. The tubular system is constructed in modules and can theoretically be scaled outdoors up to agricultural scale. A dedicated location is not crucial, similar to other closed systems, and therefore non-arable land is suitable as well. The material choice should prevent biofouling and ensure high final biomass concentrations. The combination of turbulence and the closed concept should allow a clean operation and a high operational availability.

Plate photobioreactor

Another development approach can be seen with the construction based on plastic or glass plates. Plates with different technical design are mounted to form a small layer of culture suspension, which provides an optimized light supply. In addition, the simpler construction compared to tubular reactors allows the use of less expensive plastic materials. From the pool of different concepts e.g. meandering flow designs or bottom gassed systems have been realized and shown good output results. Some unsolved issues are material life time stability or the biofilm forming. Applications at industrial scale are limited by the scalability of plate systems.

Horizontal photobioreactor

This photobioreactor type consists of a plate-shaped basic geometry with peaks and valleys arranged in regular distance. This geometry causes the distribution of incident light over a larger surface which corresponds to a dilution effect. This also helps solving a basic problem in phototrophic cultivation, because most microalgae species react sensitively to high light intensities. Most microalgae experience light saturation already at light intensities, ranging substantially below the maximum daylight intensity of approximately 2000 W/m^2. Simultaneously, a larger light quantity can be exploited in order to improve photoconversion efficiency.

Foil photobioreactor

The pressure of market prices has led the development of foil-based photobioreactor types. Inexpensive PVC or PE foils are mounted to form bags or vessels which cover algae suspensions and expose them to light. The pricing ranges of photobioreactor types have been enlarged with the foil systems. It has to be kept in mind, that these systems have a limited sustainability as the foils have to be replaced from time to time. For full balances, the investment for required support systems has to be calculated as well.

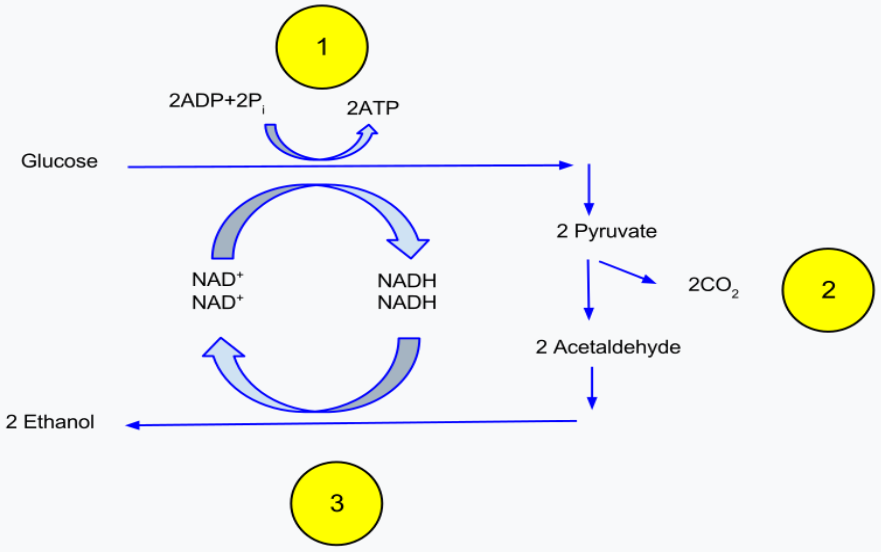
Porous Substrate Bioreactor

Porous Substrate Bioreactor (PSBR), being developed at University of Cologne, also known as the twin-layer system, uses a new principle to separate the algae from a nutrient solution by means of a porous reactor surface on which the microalgae are trapped in biofilms. This new procedure reduces by a factor of up to one hundred the amount of liquid needed for operation compared to the current technology, which cultivates algae in suspensions.

Outlook

The discussion around microalgae and their potentials in carbon dioxide sequestration and biofuel production has caused high pressure on developers and manufacturers of photobioreactors.Today, none of the mentioned systems is able to produce phototrophic microalgae biomass at a price which is able to compete with crude oil. New approaches test e.g. dripping methods to produce ultra-thin layers for maximal growth with application of flue gas and waste water. Further on, much research is done worldwide on genetically modified and optimized microalgae.

Industrial production of Ethanol



In ethanol fermentation, (1) one glucose molecule breaks down into two pyruvates. The energy from this exothermic reaction is used to bind the inorganic phosphates to ADP and convert NAD+ to NADH. (2) The two pyruvates are then broken down into two acetaldehydes and give off two CO2 as a by-product. (3) The two acetaldehydes are then converted to two ethanol by using the H- ions from NADH, converting NADH back into NAD+.

Ethanol fermentation, also called **alcoholic fermentation**, is a biological process which converts sugars such as glucose, fructose, and sucrose into cellular energy, producing ethanol and carbon dioxide as by-products. Because yeasts perform this conversion in the absence of oxygen, alcoholic fermentation is considered an anaerobic process. It also takes place in some species of fish (including goldfish and carp) where (along with lactic acid fermentation) it provides energy when oxygen is scarce.[1]
Ethanol fermentation has many uses, including the production of alcoholic beverages, the production of ethanol fuel, and bread cooking.

Biochemical process of fermentation of sucrose

The chemical equations below summarize the fermentation of sucrose ($C_{12}H_{22}O_{11}$) into ethanol (C_2H_5OH). Alcoholic fermentation converts one mole of glucose into two moles of ethanol and two moles of carbon dioxide, producing two moles of ATP in the process.
The overall chemical formula for alcoholic fermentation is:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

Sucrose is a dimer of glucose and fructose molecules. In the first step of alcoholic fermentation, the enzyme invertase cleaves the glycosidic linkage between the glucose and fructose molecules.

$$C_{12}H_{22}O_{11} + H_2O + \text{invertase} \rightarrow 2\ C_6H_{12}O_6$$

Next, each glucose molecule is broken down into two pyruvate molecules in a process known as glycolysis.[2] Glycolysis is summarized by the equation:

$$C_6H_{12}O_6 + 2\ ADP + 2\ P_i + 2\ NAD^+ \rightarrow 2\ CH_3COCOO^- + 2\ ATP + 2\ NADH + 2\ H_2O + 2\ H^+$$

CH_3COCOO^- is pyruvate, and P_i is inorganic phosphate. Finally, pyruvate is converted to ethanol and CO_2 in two steps, regenerating oxidized NAD+ needed for glycolysis:

1. $CH_3COCOO^- + H^+ \rightarrow CH_3CHO + CO_2$catalyzed by pyruvate decarboxylase
2. $CH_3CHO + NADH + H^+ \rightarrow C_2H_5OH + NAD^+$

This reaction is catalyzed by alcohol dehydrogenase (ADH1 in baker's yeast).
As shown by the reaction equation, glycolysis causes the reduction of two molecules of NAD^+ to NADH. Two ADP molecules are also converted to two ATP and two water molecules via substrate-level phosphorylation.

Related processes

Fermentation of sugar to ethanol and CO_2 can also be done by *Zymomonas mobilis*, however the path is slightly different since formation of pyruvate does not happen by glycolysis but instead by the Entner–Doudoroff pathway. Other microorganisms can produce ethanol from sugars by fermentation but often only as a side product. Examples are

- Heterolactic acid fermentation in which Leuconostoc bacterias produce Lactate + Ethanol + CO_2
- Mixed acid fermentation where Escherichia produce ethanol mixed with lactate, acetate, succinate, formate, CO_2, and H_2
- 2,3-butanediol fermentation by Enterobacter producing ethanol, butanediol, lactate, formate, CO_2, and H_2

Effect of oxygen

Fermentation does not require oxygen. If oxygen is present, some species of yeast (e.g., *Kluyveromyces lactis* or *Kluyveromyces lipolytica*) will oxidize pyruvate completely to carbon dioxide and water in a process called cellular respiration, hence these species of yeast will produce ethanol only in an anaerobic environment (not cellular respiration). This phenomenon is known as the Pasteur effect.
However, many yeasts such as the commonly used baker's yeast *Saccharomyces cerevisiae* or fission yeast *Schizosaccharomyces pombe* under certain conditions, ferment rather than respire even in the presence of oxygen. In wine making this is known as the counter-Pasteur effect. These yeasts will produce ethanol even under aerobic conditions, if they are provided with the right kind of nutrition. During batch fermentation, the rate of ethanol production per milligram of cell protein is maximal for a brief period early in this process and declines progressively as ethanol accumulates in the surrounding broth. Studies demonstrate that the removal of this accumulated ethanol does not immediately restore fermentative activity, and they provide evidence that the decline in metabolic rate is due to physiological changes (including possible ethanol damage) rather than to the presence of ethanol. Several potential causes for the decline in fermentative activity have been investigated. Viability remained at or above 90%, internal pH remained near neutrality, and the specific activities of the glycolytic and alcohologenic enzymes (measured in vitro) remained high throughout batch fermentation. None of these factors appears to be causally related to the fall in fermentative activity during batch fermentation.

Bread baking

Ethanol fermentation causes bread dough to rise. Yeast organisms consume sugars in the dough and produce ethanol and carbon dioxide as waste products. The carbon dioxide forms bubbles in the dough, expanding it to a foam. Less than 2% ethanol remains after baking.

Alcoholic beverages

All ethanol contained in alcoholic beverages (including ethanol produced by carbonic maceration) is produced by means of fermentation induced by yeast.

- Wine is produced by fermentation of the natural sugars present in grapes; cider and perry are produced by similar fermentation of natural sugar in apples and pears, respectively; and other fruit wines are produced from the fermentation of the sugars in any other kinds of

fruit. Brandy and eaux de vie (e.g. slivovitz) are produced by distillation of these fruit-fermented beverages.

- Mead is produced by fermentation of the natural sugars present in honey.
- Beer, whiskey, and vodka are produced by fermentation of grain starches that have been converted to sugar by the enzyme amylase, which is present in grain kernels that have been malted (i.e. germinated). Other sources of starch (e.g. potatoes and unmalted grain) may be added to the mixture, as the amylase will act on those starches as well. It it also amylase-induce fermented with saliva in a few countries. Whiskey and vodka are also distilled; gin and related beverages are produced by the addition of flavoring agents to a vodka-like feedstock during distillation.
- Rice wines (including sake) are produced by the fermentation of grain starches converted to sugar by the mold *Aspergillus oryzae*. *Baijiu*, *soju*, and *shōchū* are distilled from the product of such fermentation.
- Rum and some other beverages are produced by fermentation and distillation of sugarcane. Rum is usually produced from the sugarcane product molasses.

In all cases, fermentation must take place in a vessel that allows carbon dioxide to escape but prevents outside air from coming in. This is to reduce risk of contamination of the brew by unwanted bacteria or mold and because a buildup of carbon dioxide creates a risk the vessel will rupture or fail, possibly causing injury or property damage.

Feedstocks for fuel production

Yeast fermentation of various carbohydrate products is also used to produce the ethanol that is added to gasoline.

The dominant ethanol feedstock in warmer regions is sugarcane. In temperate regions, corn or sugar beets are used.

In the United States, the main feedstock for the production of ethanol is currently corn. Approximately 2.8 gallons of ethanol are produced from one bushel of corn (0.42 liter per kilogram). While much of the corn turns into ethanol, some of the corn also yields by-products such as DDGS (distillers dried grains with solubles) that can be used as feed for livestock. A bushel of corn produces about 18 pounds of DDGS (320 kilograms of DDGS per metric ton of maize). Although most of the fermentation plants have been built in corn-producing regions, sorghum is also an important feedstock for ethanol production in the Plains states. Pearl millet is showing promise as an ethanol feedstock for the southeastern U.S. and the potential of duckweed is being studied.

In some parts of Europe, particularly France and Italy, grapes have become a *de facto* feedstock for fuel ethanol by the distillation of surplus wine. Surplus sugary drinks may also be used. In Japan, it has been proposed to use rice normally made into sake as an ethanol source.

Cassava as ethanol feedstock

Ethanol can be made from mineral oil or from sugars or starches. Starches are cheapest. The starchy crop with highest energy content per acre is cassava, which grows in tropical countries.

Thailand already had a large cassava industry in the 1990s, for use as cattle feed and as a cheap admixture to wheat flour. Nigeria and Ghana are already establishing cassava-to-ethanol plants. Production of ethanol from cassava is currently economically feasible when crude oil prices are above US$120 per barrel.

New varieties of cassava are being developed, so the future situation remains uncertain. Currently, cassava can yield between 25-40 tonnes per hectare (with irrigation and fertilizer), and from a tonne of cassava roots, circa 200 liters of ethanol can be produced (assuming cassava with 22% starch content). A liter of ethanol contains circa 21.46. MJ of energy. The overall energy efficiency of cassava-root to ethanol conversion is circa 32%.
The yeast used for processing cassava is *Endomycopsis fibuligera*, sometimes used together with bacterium *Zymomonas mobilis*.

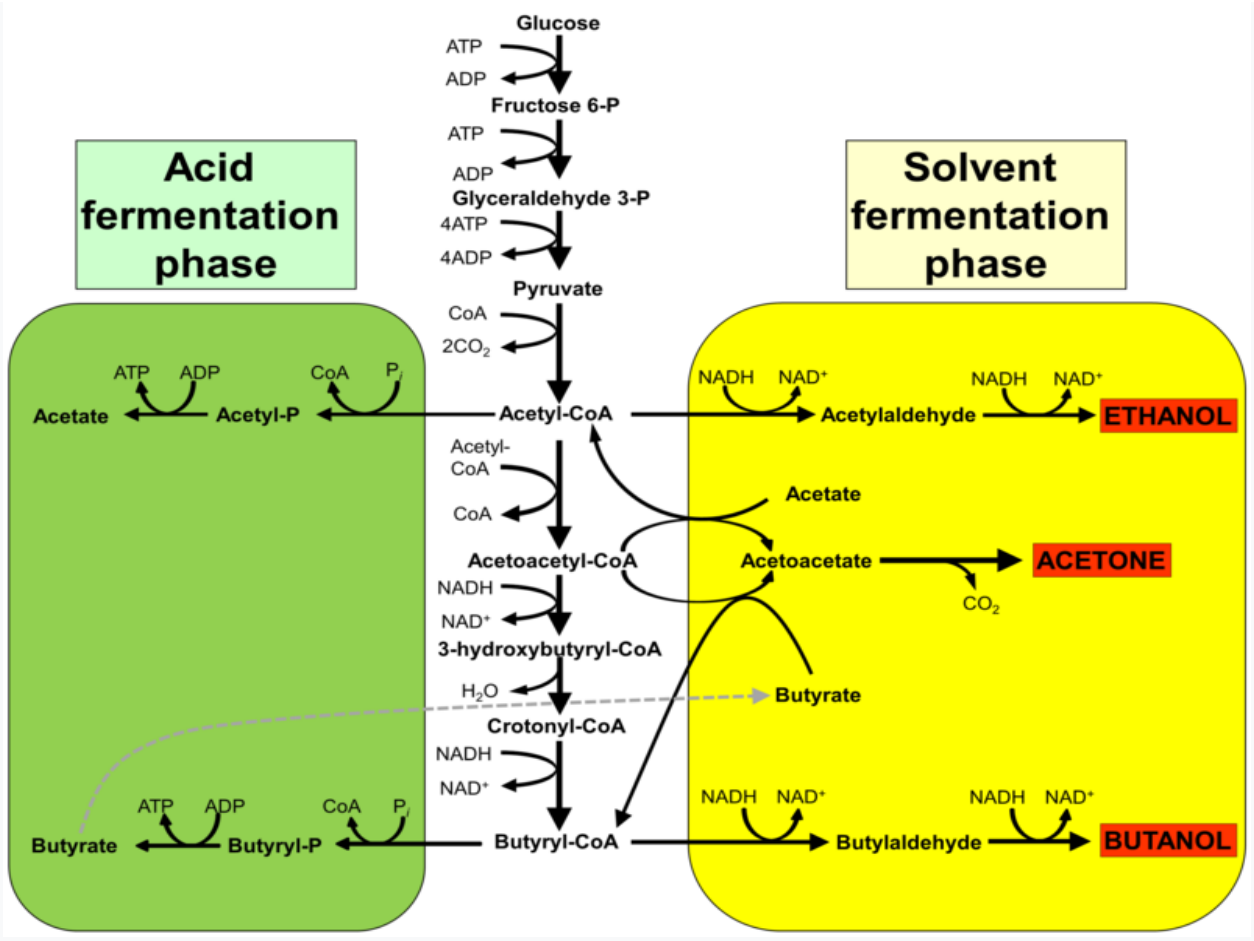
Byproducts of fermentation

Ethanol fermentation produces unharvested byproducts such as heat, carbon dioxide, food for livestock, water, methanol, fuels, fertilizer and alcohols. The cereal unfermented solid residues from the fermentation process, which can be used as livestock feed or in the production of biogas, are referred to as Distillers grains and sold as WDG, *Wet Distiller's grains*, and DDGS, *Dried Distiller's Grains with Solubles*, respectively.

Microbes used in ethanol fermentation

Yeast, Saccharomyces cerevisiaeSchizosaccharomycesZymomonas mobilis (a bacterium)

Acetone–butanol–ethanol fermentation



This figure shows pathway of acetone–butanol–ethanol fermentation by clostridia.

Acetone–butanol–ethanol (ABE) fermentation is a process that uses bacterial fermentation to produce acetone, n-Butanol, and ethanol from carbohydrates such as starch and glucose. It was developed by the chemist Chaim Weizmann and was the primary process used to make acetone during World War I, such as to produce cordite, a substance essential for the British war industry.

The process

The process may be likened to how yeast ferments sugars to produce ethanol for wine, beer, or fuel, but the organisms that carry out the ABE fermentation are strictly anaerobic (obligate anaerobes). The ABE fermentation produces solvents in a ratio of 3 parts acetone, 6 parts butanol to 1 part ethanol. It usually uses a strain of bacteria from the Class Clostridia (Family Clostridiaceae). *Clostridium acetobutylicum* is the most well-studied and widely used species, although *Clostridium beijerinckii* has also been used with good results.

For gas stripping, the most common gases used are the off-gases from the fermentation itself, a mixture of carbon dioxide and hydrogen gas.

History

The production of butanol by biological means was first performed by Louis Pasteur in 1861.
In 1905, Austrian biochemist Franz Schardinger found that acetone could similarly be produced.
In 1910 Auguste Fernbach (1860-1939) developed a bacterial fermentation process using potato starch as a feedstock in the production of butanol.
Industrial exploitation of ABE fermentation started in 1916, during World War I, with Chaim Weizmann's isolation of Clostridium acetobutylicum, as described in U.S. patent 1315585.
The Weizmann process was operated by Commercial Solvents Corporation from about 1920 to 1964 with plants in the US (Terre Haute, IN, and Peoria, IL), and Liverpool, England. The Peoria plant was the largest of the three; it used molasses as feedstock and had 96 50,000-gallon fermenters.After World War II, ABE fermentation became generally non-profitable, compared to the production of the same three solvents (acetone, butanol, ethanol) from petroleum.
During the 1950s and 1960s, ABE fermentation was replaced by petroleum chemical plants. Due to different raw material costs, ABE fermentation was viable in South Africa until the early 1980s, with the last plant closing in 1983. The last operational plant was operated by Green Biologics Ltd in Minnesota until it shut down in June 2019.

Improvement attempts

In order to make ABE fermentation profitable, many in-situ product recovery systems have been developed. These include gas stripping, pervaporation, membrane distillation, adsorption, and reverse osmosis. Green Biologics Ltd has implemented this at an industrial scale.

Current perspectives

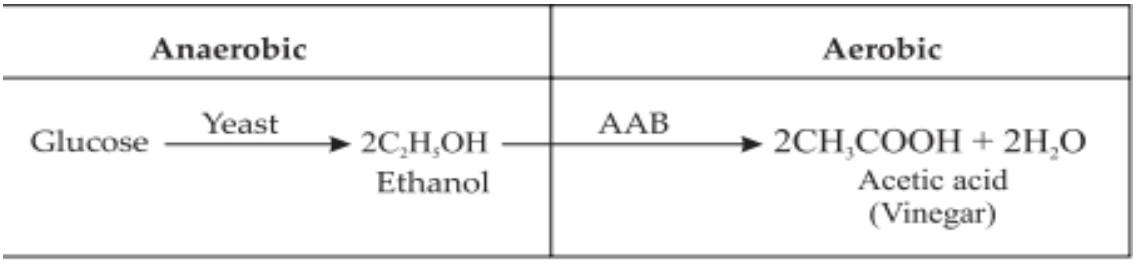
ABE fermentation is attracting renewed interest with an eye on butanol as a renewable biofuel.

Vinegar

Vinegar contains about 5% acetic acid in water, varying amounts of fixed fruit acids, colouring matter, salts and a few other fermentation products which impart characteristic flavour and aroma to the product. Vinegar traditionally has been used as a food preservative. Vinegar production methods could range from traditional methods employing wooden casks (Orleans Process) and surface culture (Generator Process) to submerged fermentation. Vinegar is the product made from the conversion of ethyl alcohol to acetic acid by a genus of bacteria Acetobacter. Many technical devices have been developed to improve the industrial production of vinegar. Generally, these improvements increase the speed of the transformation of ethanol into acetic acid in the presence of acetic acid bacteria. In this review a detailed description on vinegar production, methods of production, different substrates and microorganisms used for its production, and the chemistry of vinegar is presented.

Vinegar production

Vinegar is the product made from the conversion of ethyl alcohol to acetic acid by a genus of bacteria, *Acetobacter*. Therefore, vinegar can be produced from any alcoholic material from alcohol-water mixtures to various fruit wines (Peppler and Beaman 1967). Vinegar bacteria, also called acetic acid bacteria (AAB), are members of the genus *Acetobacter* and characterised by their ability to convert ethyl alcohol (C2H5OH) into acetic acid(CH3COOH) by oxidation as shown below:



Vinegar is a solution of acetic acid produced by a two-step bioprocess. In the first step, fermentable sugars are transformed into ethanol by the action of yeast. In the second step, AAB oxidize the ethanol into acetic acid in an aerobic process. AAB are well known for their ability to spoil wines because they can produce large amounts of acetic acid from ethanol and other compounds present in wines.

Substrates used in vinegar production

One of the critical steps in vinegar production is the preparation of the raw material (Solieri and Giudici 2009). This step is required to obtainthe fermentable sugar and juice solution to be acetified. The processing differs depending on the raw material used. In general, fruits require less preparation than seeds; however, seeds are more easily stored and

preserved after harvest. Fruits are highly perishable, rich in water, and need to be processed very quickly. Therefore, basic safe food handling practices, storage, and processing are essential to prevent the growth of pathogenic microorganisms. These microorganisms could alter the quality of the final product or even produce dangerous toxins such as aflatoxin. Processing has expanded the market of both fruit and vegetable products (Singh and Verma 1995). The seasonal gluts are avoided by the utilization of fruits/vegetables in processing industries for the preparation of various value added products. In advanced countries, 70-75% of perishables are processed before reaching the consumer's table, whereas in India only 1-2% of the total produce is processed utilizing only 40% of the installed processing capacity.

Penicillin

Penicillin (**PCN** or **pen**) is a group of antibiotics, derived originally from common moulds known as Penicillium moulds; which includes penicillin G (intravenous use), penicillin V (use by mouth), procaine penicillin, and benzathine penicillin (intramuscular use). Penicillin antibiotics were among the first medications to be effective against many bacterial infections caused by staphylococci and streptococci. They are still widely used today, though many types of bacteria have developed resistance following extensive use.

About 10% of people report that they are allergic to penicillin; however, up to 90% of this group may not actually be allergic. Serious allergies only occur in about 0.03%. Those who are allergic to penicillin are most often given cephalosporin C because of its functional groups. All penicillins are β-lactam antibiotics, which are some of the most powerful and successful achievements in modern science.

Penicillin was discovered in 1928 by Scottish scientist Alexander Fleming. People began using it to treat infections in 1942. There are several enhanced penicillin families which are effective against additional bacteria; these include the antistaphylococcal penicillins, aminopenicillins and the antipseudomonal penicillins. They are derived from *Penicillium* fungi.

Natural penicillins: Penicillin G, Penicillin K, Penicillin N, Penicillin O, Penicillin V

β-lactamase-resistant: Methicillin, Nafcillin, Oxacillin, Cloxacillin, Dicloxacillin, Flucloxacillin

History

Starting in the late 19th century there had been many accounts by scientists and physicians on the antibacterial properties of the different types of moulds including the mould penicillium but they were unable to discern what process was causing the effect. The effects of penicillium mould were finally isolated in 1928 by Scottish scientist Alexander Fleming, in work that seems to have been independent of those earlier observations. Fleming recounted that the date of his discovery of penicillin was on the morning of Friday 28 September 1928. The traditional version of this story describes the discovery as a serendipitous accident: in his laboratory in the basement of St Mary's Hospital in London (now part of Imperial College), Fleming noticed a Petri dish

containing Staphylococci that had been mistakenly left open was contaminated by blue-green mould from an open window, which formed a visible growth. There was a halo of inhibited bacterial growth around the mould. Fleming concluded that the mould released a substance that repressed the growth and caused lysing of the bacteria.

Once Fleming made his discovery he grew a pure culture and discovered it was a *Penicillium* mould, now known as *Penicillium chrysogenum*. Fleming coined the term "penicillin" to describe the filtrate of a broth culture of the *Penicillium* mould. Fleming asked C. J. La Touche to help identify the mould, which he incorrectly identified as *Penicillium rubrum* (later corrected by Charles Thom). He expressed initial optimism that penicillin would be a useful disinfectant, because of its high potency and minimal toxicity in comparison to antiseptics of the day, and noted its laboratory value in the isolation of *Bacillus influenzae* (now called *Haemophilus influenzae*).

Fleming was a famously poor communicator and orator, which meant his findings were not initially given much attention. He was unable to convince a chemist to help him extract and stabilize the antibacterial compound found in the broth filtrate. Despite this, he remained interested in the potential use of penicillin and presented a paper entitled "A Medium for the Isolation of Pfeiffer's Bacillus" to the Medical Research Club of London, which was met with little interest and even less enthusiasm by his peers. Had Fleming been more successful at making other scientists interested in his work, penicillin for medicinal use would possibly have been developed years earlier.

Despite the lack of interest of his fellow scientists, he did conduct several experiments on the antibiotic substance he discovered. The most important result proved it was nontoxic in humans by first performing toxicity tests in animals and then on humans. His subsequent experiments on penicillin's response to heat and pH allowed Fleming to increase the stability of the compound. The one test that modern scientists would find missing from his work was the test of penicillin on an infected animal, the results of which would likely have sparked great interest in penicillin and sped its development by almost a decade. The importance of his work has been recognized by the placement of an International Historic Chemical Landmark at the Alexander Fleming Laboratory Museum in London on November 19, 1999.

Medical application

In 1930, Cecil George Paine, a pathologist at the Royal Infirmary in Sheffield, attempted to use penicillin to treat sycosis barbae, eruptions in beard follicles, but was unsuccessful. Moving on to ophthalmia neonatorum, a gonococcal infection in infants, he achieved the first recorded cure with penicillin, on November 25, 1930. He then cured four additional patients (one adult and three infants) of eye infections, and failed to cure a fifth.

In 1940, Australian scientist Howard Florey (later Baron Florey) and a team of researchers (Ernst Boris Chain, Edward Abraham, Arthur Duncan Gardner, Norman Heatley, Margaret Jennings, J. Orr-Ewing and G. Sanders) at the Sir William Dunn School of Pathology, University of Oxford made progress in showing the *in vivo* bactericidal action of penicillin. In 1940, they showed that penicillin effectively cured bacterial infection in mice. In 1941, they treated a policeman, Albert Alexander, with a severe face infection; his condition improved, but then supplies of penicillin ran out and he died. Subsequently, several other patients were treated

successfully. In December 1942, survivors of the Cocoanut Grove fire in Boston were the first burn patients to be successfully treated with penicillin.

Mass production

By late 1940, the Oxford team under Howard Florey had devised a method of mass-producing the drug, but yields remained low. In 1941, Florey and Heatley travelled to the US in order to interest pharmaceutical companies in producing the drug and inform them about their process.

Florey and Chain shared the 1945 Nobel Prize in Medicine with Fleming for their work.

The challenge of mass-producing this drug was daunting. On March 14, 1942, the first patient was treated for streptococcal sepsis with US-made penicillin produced by Merck & Co. Half of the total supply produced at the time was used on that one patient, Anne Miller. By June 1942, just enough US penicillin was available to treat ten patients. In July 1943, the War Production Board drew up a plan for the mass distribution of penicillin stocks to Allied troops fighting in Europe. The results of fermentation research on corn steep liquor at the Northern Regional Research Laboratory at Peoria, Illinois, allowed the United States to produce 2.3 million doses in time for the invasion of Normandy in the spring of 1944. After a worldwide search in 1943, a mouldy cantaloupe in a Peoria, Illinois market was found to contain the best strain of mould for production using the corn steep liquor process. Pfizer scientist Jasper H. Kane suggested using a deep-tank fermentation method for producing large quantities of pharmaceutical-grade penicillin. Large-scale production resulted from the development of a deep-tank fermentation plant by chemical engineer Margaret Hutchinson Rousseau. As a direct result of the war and the War Production Board, by June 1945, over 646 billion units per year were being produced.

G. Raymond Rettew made a significant contribution to the American war effort by his techniques to produce commercial quantities of penicillin, wherein he combined his knowledge of mushroom spawn with the function of the Sharples Cream Separator. By 1943, Rettew's lab was producing most of the world's penicillin. During World War II, penicillin made a major difference in the number of deaths and amputations caused by infected wounds among Allied forces, saving an estimated 12%–15% of lives. Availability was severely limited, however, by the difficulty of manufacturing large quantities of penicillin and by the rapid renal clearance of the drug, necessitating frequent dosing. Methods for mass production of penicillin were patented by Andrew Jackson Moyer in 1945.Florey had not patented penicillin, having been advised by Sir Henry Dale that doing so would be unethical.

Penicillin is actively excreted, and about 80% of a penicillin dose is cleared from the body within three to four hours of administration. Indeed, during the early penicillin era, the drug was so scarce and so highly valued that it became common to collect the urine from patients being treated, so that the penicillin in the urine could be isolated and reused. This was not a satisfactory solution, so researchers looked for a way to slow penicillin excretion. They hoped to find a molecule that could compete with penicillin for the organic acid transporter responsible for excretion, such that the transporter would preferentially excrete the competing molecule and the penicillin would be retained. The uricosuric agent probenecid proved to be suitable. When probenecid and penicillin are administered together, probenecid competitively inhibits the excretion of penicillin, increasing penicillin's concentration and prolonging its activity. Eventually, the advent of mass-production techniques and semi-synthetic penicillins resolved the

supply issues, so this use of probenecid declined. Probenecid is still useful, however, for certain infections requiring particularly high concentrations of penicillins.

After World War II, Australia was the first country to make the drug available for civilian use. In the U.S., penicillin was made available to the general public on March 15, 1945.

Structure determination and total synthesis

The chemical structure of penicillin was first proposed by Edward Abraham in 1942 and was later confirmed in 1945 using X-ray crystallography by Dorothy Crowfoot Hodgkin, who was also working at Oxford.[54] She later received the Nobel prize for this and other structure determinations.

Chemist John C. Sheehan at the Massachusetts Institute of Technology (MIT) completed the first chemical synthesis of penicillin in 1957. Sheehan had started his studies into penicillin synthesis in 1948, and during these investigations developed new methods for the synthesis of peptides, as well as new protecting groups—groups that mask the reactivity of certain functional groups. Although the initial synthesis developed by Sheehan was not appropriate for mass production of penicillins, one of the intermediate compounds in Sheehan's synthesis was 6-aminopenicillanic acid (6-APA), the nucleus of penicillin. Attaching different groups to the 6-APA 'nucleus' of penicillin allowed the creation of new forms of penicillin.

Developments from penicillin

The narrow range of treatable diseases or "spectrum of activity" of the penicillins, along with the poor activity of the orally active phenoxymethylpenicillin, led to the search for derivatives of penicillin that could treat a wider range of infections. The isolation of 6-APA, the nucleus of penicillin, allowed for the preparation of semisynthetic penicillins, with various improvements over benzylpenicillin (bioavailability, spectrum, stability, tolerance).

The first major development was ampicillin in 1961. It offered a broader spectrum of activity than either of the original penicillins. Further development yielded β-lactamase-resistant penicillins, including flucloxacillin, dicloxacillin, and methicillin. These were significant for their activity against β-lactamase-producing bacterial species, but were ineffective against the methicillin-resistant *Staphylococcus aureus* (MRSA) strains that subsequently emerged.

Another development of the line of true penicillins was the antipseudomonal penicillins, such as carbenicillin, ticarcillin, and piperacillin, useful for their activity against Gram-negative bacteria. However, the usefulness of the β-lactam ring was such that related antibiotics, including the mecillinams, the carbapenems and, most important, the cephalosporins, still retain it at the center of their structures.

Production

Penicillin is a secondary metabolite of certain species of *Penicillium* and is produced when growth of the fungus is inhibited by stress. It is not produced during active growth. Production is also limited by feedback in the synthesis pathway of penicillin.

α-ketoglutarate + AcCoA → homocitrate → L-α-aminoadipic acid → L-lysine + β-lactam

The by-product, L-lysine, inhibits the production of homocitrate, so the presence of exogenous lysine should be avoided in penicillin production.

The *Penicillium* cells are grown using a technique called fed-batch culture, in which the cells are constantly subject to stress, which is required for induction of penicillin production. The available carbon sources are also important: glucose inhibits penicillin production, whereas lactose does not. The pH and the levels of nitrogen, lysine, phosphate, and oxygen of the batches must also be carefully controlled.

The biotechnological method of directed evolution has been applied to produce by mutation a large number of *Penicillium* strains. These techniques include error-prone PCR, DNA shuffling, ITCHY, and strand-overlap PCR.

Semisynthetic penicillins are prepared starting from the penicillin nucleus 6-APA.

Biosynthesis

Overall, there are three main and important steps to the biosynthesis of penicillin G (benzylpenicillin).

- The first step is the condensation of three amino acids—L-α-aminoadipic acid, L-cysteine, L-valine into a tripeptide. Before condensing into the tripeptide, the amino acid L-valine must undergo epimerization to become D-valine. The condensed tripeptide is named δ-(L-α-aminoadipyl)-L-cysteine-D-valine (ACV). The condensation reaction and epimerization are both catalyzed by the enzyme δ-(L-α-aminoadipyl)-L-cysteine-D-valine synthetase (ACVS), a nonribosomal peptide synthetase or NRPS.
- The second step in the biosynthesis of penicillin G is the oxidative conversion of linear ACV into the bicyclic intermediate isopenicillin N by isopenicillin N synthase (IPNS), which is encoded by the gene *pcbC*. Isopenicillin N is a very weak intermediate, because it does not show strong antibiotic activity.
- The final step is a transamidation by isopenicillin N N-acyltransferase, in which the α-aminoadipyl side-chain of isopenicillin N is removed and exchanged for a phenylacetyl side-chain. This reaction is encoded by the gene *penDE*, which is unique in the process of obtaining penicillins.

Vitamin B_{12}

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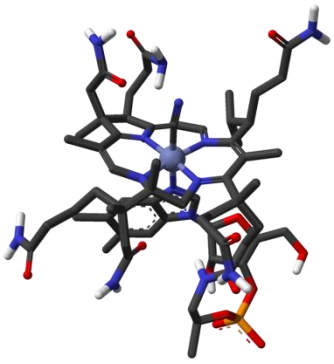
Vitamin B_{12}, also known as **cobalamin**, is a water-soluble vitamin involved in the metabolism of every cell of the human body: it is a cofactor in DNA synthesis, and in both fatty acid and amino acid metabolism. It is particularly important in the normal functioning of the nervous system via its role in the synthesis of myelin, and in the maturation of developing red blood cells in the bone marrow.

Vitamin B_{12} is one of eight B vitamins; it is the largest and most structurally complex vitamin.[2] It consists of a class of chemically related compounds (vitamers), all of which show physiological activity. It contains the biochemically rare element cobalt (chemical symbol **Co**) positioned in the center of a corrin ring. The only organisms to produce vitamin B_{12} are certain bacteria, and archaea. Some of these bacteria are found on plants that herbivores eat; they are taken into the animal, proliferate and form part of their permanent gut flora, producing vitamin B_{12} internally.



Most omnivorous people in developed countries obtain enough vitamin B_{12} from consuming animal products, including meat, milk, eggs, and fish. Grain-based foods are often fortified by having the vitamin added to them. Vitamin B_{12} supplements are available in single agent or multivitamin tablets. Pharmaceutical preparations may be given by intramuscular injection. Because there are few non-animal sources of the vitamin, vegans are advised to consume a dietary supplement or fortified foods for B_{12} intake, or risk serious health consequences. Children in some regions of developing countries are at particular risk due to increased requirements during growth coupled with diets low in animal-sourced foods.

The most common cause of vitamin B_{12} deficiency in developed countries is impaired absorption due to a loss of gastric intrinsic factor, which must be bound to food-source B_{12} in order for absorption to occur.[2] A second major cause is age-related decline in stomach acid production (achlorhydria), because acid exposure frees protein-bound vitamin. For the same reason, people on long-term antacid therapy, using proton-pump inhibitors, H2 blockers or other antacids are at increased risk. Deficiency may be characterised by limb neuropathy or a blood disorder called pernicious anemia, a type of megaloblastic anemia. Folate levels in the individual may affect the course of pathological changes and symptomatology of vitamin B_{12} deficiency.

Production

Biosynthesis

Vitamin B_{12} is derived from a tetrapyrrolic structural framework created by the enzymes deaminase and cosynthetase which transform aminolevulinic acid via porphobilinogen and hydroxymethylbilane to uroporphyrinogen III. The latter is the first macrocyclic intermediate common to haem, chlorophyll, sirohaem and B_{12} itself. Later steps, especially the incorporation of the additional methyl groups of its structure, were investigated using methyl-labelled S-adenosyl methionine. It was not until a genetically-engineered strain of *Pseudomonas denitrificans* was used, in which eight of the genes involved in the biosynthesis of the vitamin had been overexpressed, that the complete sequence of methylation and other steps could be determined, thus fully establishing all the intermediates in the pathway.

Industrial:

Industrial production of B_{12} is achieved through fermentation of selected microorganisms. *Streptomyces griseus*, a bacterium once thought to be a fungus, was the commercial source of vitamin B_{12} for many years. The species *Pseudomonas denitrificans* and *Propionibacterium freudenreichii* subsp. *shermanii* are more commonly used today. These are grown under special conditions to enhance yield. Rhone-Poulenc improved yield via genetic engineering *P. denitrificans*. The company was acquired by Aventis, later merged to Sanofi-Aventis, and is now produced by a Sanofi division.Propionibacterium, the other commonly used bacterial species, produce no exotoxins or endotoxins and are generally recognized as safe (have been granted GRAS status) by the Food and Drug Administration of the United States.

The total world production of vitamin B_{12}, by four companies (the French Sanofi-Aventis and three Chinese companies) in 2008 was 35,000 kg (77,175 lb).

Laboratory

The complete laboratory synthesis of B_{12} was achieved by Robert Burns Woodward and Albert Eschenmoser in 1972, and remains one of the classic feats of organic synthesis, requiring the effort of 91 postdoctoral fellows (mostly at Harvard) and 12 PhD students (at ETH Zurich) from 19 nations. The synthesis constitutes a formal total synthesis, since the research groups only prepared the known intermediate cobyric acid, whose chemical conversion to vitamin B_{12} was previously reported. Though it constitutes an intellectual achievement of the highest caliber, the Eschenmoser–Woodward synthesis of vitamin B_{12} is of no practical consequence due to its length, taking 72 chemical steps and giving an overall chemical yield well under 0.01%. And although there have been sporadic synthetic efforts since 1972, the Eschenmoser–Woodward synthesis remains the only completed (formal) total synthesis.

Species from the following genera and the following individual species are known to synthesize B_{12}: *Propionibacterium shermanii, Pseudomonas denitrificans, Streptomyces griseus, Acetobacterium, Aerobacter, Agrobacterium, Alcaligenes, Azotobacter, Bacillus, Clostridium, Corynebacterium, Flavobacterium, Lactobacillus, Micromonospora, Mycobacterium, Nocardia, Proteus, Rhizobium, Salmonella, Serratia, Streptococcus* and *Xanthomonas*.

Amylase

Amylase (/ˈæmɪleɪz/) is an enzyme that catalyses the hydrolysis of starch (Latin amylum) into sugars. Amylase is present in the saliva of humans and some other mammals, where it begins the chemical process of digestion. Foods that contain large amounts of starch but little sugar, such as rice and potatoes, may acquire a slightly sweet taste as they are chewed because amylase degrades some of their starch into sugar. The pancreas and salivary gland make amylase (alpha amylase) to hydrolyse dietary starch into disaccharides and trisaccharides which are converted by other enzymes to glucose to supply the body with energy. Plants and some bacteria also produce amylase. Specific amylase proteins are designated by different Greek letters. All amylases are glycoside hydrolases and act on α-1,4-glycosidic bonds.

Classification

	α-amylase	**β-amylase**	**γ-amylase**
Source	Animals, plants, microbes	Plants, microbes	Animals, microbes
Tissue	Saliva, pancreas	Seeds, fruits	Small intestine
Cleavage site	Random α-1,4 glycosidic bond	Second α-1,4 glycosidic bond	Last α-1,4 glycosidic bond
Reaction products	Maltose, dextrin, etc	Maltose	Glucose
Optimum pH	6.7–7.0	4.0–5.0	3.0
Optimum temperature in brewing	63–70 °C	55–65 °C	

α-Amylase:
The α-amylases (EC 3.2.1.1) (CAS 9014-71-5) (alternative names: 1,4-α-D-glucan glucanohydrolase; glycogenase) are calcium metalloenzymes. By acting at random locations along the starch chain, α-amylase breaks down long-chain saccharides, ultimately yielding either maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. They belong to glycoside hydrolase family 13.
Because it can act anywhere on the substrate, α-amylase tends to be faster-acting than β-amylase. In animals, it is a major digestive enzyme, and its optimum pH is 6.7–7.0.
In human physiology, both the salivary and pancreatic amylases are α-amylases.
The α-amylase form is also found in plants, fungi (ascomycetes and basidiomycetes) and bacteria (*Bacillus*).
β-Amylase:
Another form of amylase, β-amylase (EC 3.2.1.2) (alternative names: 1,4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase) is also synthesized by bacteria, fungi, and plants. Working from the non-reducing end, β-amylase catalyzes the hydrolysis of the second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruit, β-amylase breaks starch into maltose, resulting in the sweet flavor of ripe fruit. They belong to glycoside hydrolase family 14.
Both α-amylase and β-amylase are present in seeds; β-amylase is present in an inactive form prior to germination, whereas α-amylase and proteases appear once germination has begun. Many microbes also produce amylase to degrade extracellular starches. Animal tissues do not

contain β-amylase, although it may be present in microorganisms contained within the digestive tract. The optimum pH for β-amylase is 4.0–5.0.

γ-Amylase:

γ-Amylase (EC 3.2.1.3) (alternative names: glucan 1,4-α-glucosidase; amyloglucosidase; *exo*-1,4-α-glucosidase; glucoamylase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase) will cleave α(1–6) glycosidic linkages, as well as the last α-1,4 glycosidic bond at the nonreducing end of amylose and amylopectin, yielding glucose. The γ-amylase has most acidic optimum pH of all amylases because it is most active around pH 3. They belong to a variety of different GH families, such as glycoside hydrolase family 15 in fungi, glycoside hydrolase family 31 of human MGAM, and glycoside hydrolase family 97 of bacterial forms.

Uses

Fermentation

α- and β-amylases are important in brewing beer and liquor made from sugars derived from starch. In fermentation, yeast ingests sugars and excretes ethanol. In beer and some liquors, the sugars present at the beginning of fermentation have been produced by "mashing" grains or other starch sources (such as potatoes). In traditional beer brewing, malted barley is mixed with hot water to create a "mash", which is held at a given temperature to allow the amylases in the malted grain to convert the barley's starch into sugars. Different temperatures optimize the activity of alpha or beta amylase, resulting in different mixtures of fermentable and unfermentable sugars. In selecting mash temperature and grain-to-water ratio, a brewer can change the alcohol content, mouthfeel, aroma, and flavor of the finished beer.

In some historic methods of producing alcoholic beverages, the conversion of starch to sugar starts with the brewer chewing grain to mix it with saliva.This practice is no longer widely in use.

Flour additive

Amylases are used in breadmaking and to break down complex sugars, such as starch (found in flour), into simple sugars. Yeast then feeds on these simple sugars and converts it into the waste products of ethanol and carbon dioxide. This imparts flavour and causes the bread to rise. While amylases are found naturally in yeast cells, it takes time for the yeast to produce enough of these enzymes to break down significant quantities of starch in the bread. This is the reason for long fermented doughs such as sourdough. Modern breadmaking techniques have included amylases (often in the form of malted barley) into bread improver, thereby making the process faster and more practical for commercial use.

α-Amylase is often listed as an ingredient on commercially package-milled flour. Bakers with long exposure to amylase-enriched flour are at risk of developing dermatitisor asthma.

Molecular biology

In molecular biology, the presence of amylase can serve as an additional method of selecting for successful integration of a reporter construct in addition to antibiotic resistance. As reporter genes are flanked by homologous regions of the structural gene for amylase, successful integration will disrupt the amylase gene and prevent starch degradation, which is easily detectable through iodine staining.

Medical uses

Amylase also has medical applications in the use of pancreatic enzyme replacement therapy (PERT). It is one of the components in Sollpura (liprotamase) to help in the breakdown of saccharides into simple sugars.

Other uses

An inhibitor of alpha-amylase, called phaseolamin, has been tested as a potential diet aid.

When used as a food additive, amylase has E number E1100, and may be derived from pig pancreas or mold fungi.

Bacilliary amylase is also used in clothing and dishwasher detergents to dissolve starches from fabrics and dishes.

Factory workers who work with amylase for any of the above uses are at increased risk of occupational asthma. Five to nine percent of bakers have a positive skin test, and a fourth to a third of bakers with breathing problems are hypersensitive to amylase.

History

In 1831, Erhard Friedrich Leuchs (1800–1837) described the hydrolysis of starch by saliva, due to the presence of an enzyme in saliva, "ptyalin", an amylase. it was named after the Ancient Greek name for saliva: - ptyalon.

The modern history of enzymes began in 1833, when French chemists Anselme Payen and Jean-François Persoz isolated an amylase complex from germinating barley and named it "diastase". It is from this term that all subsequent enzyme names tend to end in the suffix -ase.

In 1862, Alexander Jakulowitsch Danilewsky (1838–1923) separated pancreatic amylase from trypsin.

Evolution

Salivary amylase

Saccharides are a food source rich in energy. Large polymers such as starch are partially hydrolyzed in the mouth by the enzyme amylase before being cleaved further into sugars. Many mammals have seen great expansions in the copy number of the amylase gene. These duplications allow for the pancreatic amylase *AMY2* to re-target to the salivary glands, allowing animals to detect starch by taste and to digest starch more efficiently and in higher quantities. This has happened independently in mice, rats, dogs, pigs, and most importantly, humans after the agricultural revolution.

Following the agricultural revolution 12,000 years ago, human diet began to shift more to plant and animal domestication in place of gathering and hunting. Starch has become a staple of the human diet.

Despite the obvious benefits, early humans did not possess salivary amylase, a trend that is also seen in evolutionary relatives of the human, such as chimpanzees and bonobos, who possess either one or no copies of the gene responsible for producing salivary amylase.

Like in other manuals, the pancreatic alpha-amylase *AMY2* was duplicated multiple times. One event allowed it to evolve salivary specificity, leading to the production of amylase in the saliva (named in humans as *AMY1*). The 1p21.1 region of human chromosome 1 contains many copies of these genes, variously named *AMY1A*, *AMY1B*, *AMY1C*, *AMY2A*, *AMY2B*, and so on.

However, not all humans possess the same number of copies of the *AMY1* gene. Populations known to rely more on saccharides have a higher number of AMY1 copies than human populations that, by comparison, consume little starch. The number of *AMY1* gene copies in humans can range from six copies in agricultural groups such as European-American and Japanese (two high starch populations) to only 2–3 copies in hunter-gatherer societies such as the Biaka, Datog, and Yakuts.

The correlation that exists between starch consumption and number of *AMY1* copies specific to population suggest that more AMY1 copies in high starch populations has been selected for by natural selection and considered the favorable phenotype for those individuals. Therefore, it is most likely that the benefit of an individual possessing more copies of *AMY1* in a high starch population increases fitness and produces healthier, fitter offspring.

This fact is especially apparent when comparing geographically close populations with different eating habits that possess a different number of copies of the *AMY1* gene. Such is the case for some Asian populations that have been shown to possess few *AMY1* copies relative to some agricultural population in Asia. This offers strong evidence that natural selection has acted on this gene as opposed to the possibility that the gene has spread through genetic drift.

Variations of amylase copy number in dogs mirrors that of human populations, suggesting they acquired the extra copies as they followed humans around. Unlike humans whose amylase levels depend on starch content in diet, wild animals eating a broad range of foods tend to have more copies of amylase. This may have to do with mainly detection of starch as opposed to digestion.

Cheese:

Cheese is a dairy product derived from milk that is produced in a wide range of flavors, textures, and forms by coagulation of the milk protein casein. It comprises proteins and fat from milk, usually the milk of cows, buffalo, goats, or sheep. During production, the milk is usually acidified, and adding the enzyme rennet causes coagulation. The solids are separated and pressed into final form. Some cheeses have molds on the rind, the outer layer, or throughout. Most cheeses melt at cooking temperature.

Over a thousand types of cheese exist and are currently produced in various countries. Their styles, textures and flavors depend on the origin of the milk (including the animal's diet), whether they have been pasteurized, the butterfat content, the bacteria and mold, the processing, and how long they have been aged for. Herbs, spices, or wood smoke may be used as flavoring agents. The yellow to red color of many cheeses is produced by adding annatto. Other ingredients may be added to some cheeses, such as black pepper, garlic, chives or cranberries. A *cheesemonger*, or specialist seller of cheeses, may have expertise with selecting the cheeses, purchasing, receiving, storing and ripening them.

For a few cheeses, the milk is curdled by adding acids such as vinegar or lemon juice. Most cheeses are acidified to a lesser degree by bacteria, which turn milk sugars into lactic acid, then the addition of rennet completes the curdling. Vegetarian alternatives to rennet are available; most are produced by fermentation of the fungus *Mucor miehei*, but others have been extracted from various species of the *Cynara* thistle family. Cheesemakers near a dairy region may benefit from fresher, lower-priced milk, and lower shipping costs.

Cheese is valued for its portability, long life, and high content of fat, protein, calcium, and phosphorus. Cheese is more compact and has a longer shelf life than milk, although how long a cheese will keep depends on the type of cheese. Hard cheeses, such as Parmesan, last longer than soft cheeses, such as Brie or goat's milk cheese. The long storage life of some cheeses, especially when encased in a protective rind, allows selling when markets are favorable. Vacuum packaging of block-shaped cheeses and gas-flushing of plastic bags with mixtures of carbon dioxide and nitrogen are used for storage and mass distribution of cheeses in the 21st century.

History

Cheese is an ancient food whose origins predate recorded history. There is no conclusive evidence indicating where cheesemaking originated, whether in Europe, Central Asia or the Middle East, but the practice had spread within Europe prior to Roman times and, according to Pliny the Elder, had become a sophisticated enterprise by the time the Roman Empire came into being.

Earliest proposed dates for the origin of cheesemaking range from around 8000 BCE, when sheep were first domesticated. Since animal skins and inflated internal organs have, since ancient times, provided storage vessels for a range of foodstuffs, it is probable that the process of cheese making was discovered accidentally by storing milk in a container made from the stomach of an animal, resulting in the milk being turned to curd and whey by the rennet from the stomach. There is a legend—with variations—about the discovery of cheese by an Arab trader who used this method of storing milk.

The earliest evidence of cheesemaking in the archaeological record dates back to 5500 BCE and is found in what is now Kuyavia, Poland, where strainers coated with milk-fat molecules have been found.

Cheesemaking may have begun independently of this by the pressing and salting of curdled milk to preserve it. Observation that the effect of making cheese in an animal stomach gave more solid and better-textured curds may have led to the deliberate addition of rennet. Early archeological evidence of Egyptian cheese has been found in Egyptian tomb murals, dating to about 2000 BCE.

The earliest cheeses were likely quite sour and salty, similar in texture to rustic cottage cheese or feta, a crumbly, flavorful Greek cheese. Cheese produced in Europe, where climates are cooler than the Middle East, required less salt for preservation. With less salt and acidity, the cheese became a suitable environment for useful microbes and molds, giving aged cheeses their respective flavors. The earliest ever discovered preserved cheese was found in the Taklamakan Desert in Xinjiang, China, and it dates back as early as 1615 BCE.

A 2018 paper published in *Analytical Chemistry* stated that the world's oldest cheese, dating to approximately 3200 years before present, was found in ancient Egyptian tombs.

Modern era

Until its modern spread along with European culture, cheese was nearly unheard of in east Asian cultures, in the pre-Columbian Americas, and only had limited use in sub-Mediterranean Africa, mainly being widespread and popular only in Europe, the Middle East, the Indian subcontinent, and areas influenced by those cultures. But with the spread, first of European imperialism, and later of Euro-American culture and food, cheese has gradually become known and increasingly popular worldwide.

The first factory for the industrial production of cheese opened in Switzerland in 1815, but large-scale production first found real success in the United States. Credit usually goes to Jesse Williams, a dairy farmer from Rome, New York, who in 1851 started making cheese in an assembly-line fashion using the milk from neighboring farms. Within decades, hundreds of such dairy associations existed.

The 1860s saw the beginnings of mass-produced rennet, and by the turn of the century scientists were producing pure microbial cultures. Before then, bacteria in cheesemaking had come from the environment or from recycling an earlier batch's whey; the pure cultures meant a more standardized cheese could be produced.

Factory-made cheese overtook traditional cheesemaking in the World War II era, and factories have been the source of most cheese in America and Europe ever since.

Production of cheese – 2014
From whole cow milk

Country	Production (millions of tonnes)
European Union	9
United States	5.4
Germany	1.9
France	1.8
Italy	1.2
Netherlands	0.8
World	**18.7**

Production

In 2014, world production of cheese from whole cow milk was 18.7 million tonnes, with the United States accounting for 29% (5.4 million tonnes) of the world total followed by Germany, France and Italy as major producers.

Other 2014 world totals for processed cheese include:

- from skimmed cow milk, 2.4 million tonnes (leading country, Germany, 845,500 tonnes)
- from goat milk, 523,040 tonnes (leading country, South Sudan, 110,750 tonnes)
- from sheep milk, 680,302 tonnes (leading country, Greece, 125,000 tonnes)
- from buffalo milk, 282,127 tonnes (leading country, Egypt, 254,000 tonnes)

During 2015, Germany, France, Netherlands and Italy exported 10-14% of their produced cheese. The United States was a marginal exporter (5.3% of total cow milk production), as most of its output was for the domestic market.

Consumption

France, Iceland, Finland, Denmark and Germany were the highest consumers of cheese in 2014, averaging 25 kg (55 lb) per person.

Processing

A required step in cheesemaking is separating the milk into solid curds and liquid whey. Usually this is done by acidifying (souring) the milk and adding rennet. The acidification can be accomplished directly by the addition of an acid, such as vinegar, in a few cases (paneer, queso fresco). More commonly starter bacteria are employed instead which convert milk sugars into lactic acid. The same bacteria (and the enzymes they produce) also play a large role in the eventual flavor of aged cheeses. Most cheeses are made with starter bacteria from the *Lactococcus*, *Lactobacillus*, or *Streptococcus* families. Swiss starter cultures also include *Propionibacter shermani*, which produces carbon dioxide gas bubbles during aging, giving Swiss cheese or Emmental its holes (called "eyes").

Some fresh cheeses are curdled only by acidity, but most cheeses also use rennet. Rennet sets the cheese into a strong and rubbery gel compared to the fragile curds produced by acidic coagulation alone. It also allows curdling at a lower acidity—important because flavor-making bacteria are inhibited in high-acidity environments. In general, softer, smaller, fresher cheeses are curdled with a greater proportion of acid to rennet than harder, larger, longer-aged varieties.

While rennet was traditionally produced via extraction from the inner mucosa of the fourth stomach chamber of slaughtered young, unweaned calves, most rennet used today in cheesemaking is produced recombinantly. The majority of the applied chymosin is retained in the whey and, at most, may be present in cheese in trace quantities. In ripe cheese, the type and provenance of chymosin used in production cannot be determined.

Curd processing

At this point, the cheese has set into a very moist gel. Some soft cheeses are now essentially complete: they are drained, salted, and packaged. For most of the rest, the curd is cut into small cubes. This allows water to drain from the individual pieces of curd.

Some hard cheeses are then heated to temperatures in the range of 35–55 °C (95–131 °F). This forces more whey from the cut curd. It also changes the taste of the finished cheese, affecting both the bacterial culture and the milk chemistry. Cheeses that are heated to the higher temperatures are usually made with thermophilic starter bacteria that survive this step—either *Lactobacilli* or *Streptococci*.

Salt has roles in cheese besides adding a salty flavor. It preserves cheese from spoiling, draws moisture from the curd, and firms cheese's texture in an interaction with its proteins. Some cheeses are salted from the outside with dry salt or brine washes. Most cheeses have the salt mixed directly into the curds.

Other techniques influence a cheese's texture and flavor. Some examples are :

- Stretching: (Mozzarella, Provolone) The curd is stretched and kneaded in hot water, developing a stringy, fibrous body.
- Cheddaring: (Cheddar, other English cheeses) The cut curd is repeatedly piled up, pushing more moisture away. The curd is also mixed (or *milled*) for a long time, taking the sharp edges off the cut curd pieces and influencing the final product's texture.
- Washing: (Edam, Gouda, Colby) The curd is washed in warm water, lowering its acidity and making for a milder-tasting cheese.

Most cheeses achieve their final shape when the curds are pressed into a mold or form. The harder the cheese, the more pressure is applied. The pressure drives out moisture—the molds are designed to allow water to escape—and unifies the curds into a single solid body.

Ripening

A newborn cheese is usually salty yet bland in flavor and, for harder varieties, rubbery in texture. These qualities are sometimes enjoyed—cheese curds are eaten on their own—but normally cheeses are left to rest under controlled conditions. This aging period (also called ripening, or, from the French, *affinage*) lasts from a few days to several years. As a cheese ages, microbes and enzymes transform texture and intensify flavor. This transformation is largely a result of the breakdown of casein proteins and milkfat into a complex mix of amino acids, amines, and fatty acids.

Some cheeses have additional bacteria or molds intentionally introduced before or during aging. In traditional cheesemaking, these microbes might be already present in the aging room; they are simply allowed to settle and grow on the stored cheeses. More often today, prepared cultures are used, giving more consistent results and putting fewer constraints on the environment where the cheese ages. These cheeses include soft ripened cheeses such as Brie and Camembert, blue cheeses such as Roquefort, Stilton, Gorgonzola, and rind-washed cheeses such as Limburger.

Types

There are many types of cheese, with around 500 different varieties recognized by the International Dairy Federation, more than 400 identified by Walter and Hargrove, more than 500 by Burkhalter, and more than 1,000 by Sandine and Elliker. The varieties may be grouped or classified into types according to criteria such as length of ageing, texture, methods of making, fat content, animal milk, country or region of origin, etc.—with these criteria either being used singly or in combination, but with no single method being universally used. The method most commonly and traditionally used is based on moisture content, which is then further discriminated by fat content and curing or ripening methods. Some attempts have been made to rationalise the classification of cheese—a scheme was proposed by Pieter Walstra which uses the primary and secondary starter combined with moisture content, and Walter and Hargrove suggested classifying by production methods which produces 18 types, which are then further grouped by moisture content.

Cooking and eating

At refrigerator temperatures, the fat in a piece of cheese is as hard as unsoftened butter, and its protein structure is stiff as well. Flavor and odor compounds are less easily liberated when cold. For improvements in flavor and texture, it is widely advised that cheeses be allowed to warm up to room temperature before eating. If the cheese is further warmed, to 26–32°C (79–90°F), the fats will begin to "sweat out" as they go beyond soft to fully liquid.

Above room temperatures, most hard cheeses melt. Rennet-curdled cheeses have a gel-like protein matrix that is broken down by heat. When enough protein bonds are broken, the cheese itself turns from a solid to a viscous liquid. Soft, high-moisture cheeses will melt at around 55°C (131°F), while hard, low-moisture cheeses such as Parmesan remain solid until they reach about 82°C (180 °F).Acid-set cheeses, including halloumi, paneer, some whey cheeses and many varieties of fresh goat cheese, have a protein structure that remains intact at high temperatures. When cooked, these cheeses just get firmer as water evaporates.

Cheeseboard

A cheeseboard (or cheese course) may be served at the end of a meal, either replacing, before or following dessert. The British tradition is to have cheese after dessert, accompanied by sweet wines like Port. In France, cheese is consumed before dessert, with robust red wine. A cheeseboard typically has contrasting cheeses with accompaniments, such as crackers, biscuits, grapes, nuts, celery or chutney. A cheeseboard 70 feet (21 m) long was used to feature the variety of cheeses manufactured in Wisconsin, where the state legislature recognizes a "cheesehead" hat as a state symbol.

Nutrition and health

The nutritional value of cheese varies widely. Cottage cheese may consist of 4% fat and 11% protein while some whey cheeses are 15% fat and 11% protein, and triple-crème cheeses are 36% fat and 7% protein. In general, cheese is a rich source (20% or more of the Daily Value, DV)

of calcium, protein, phosphorus, sodium and saturated fat. A 28-gram (one ounce) serving of cheddar cheese contains about 7 grams (0.25 oz) of protein and 202 milligrams of calcium. Nutritionally, cheese is essentially concentrated milk, but altered by the culturing and aging processes: it takes about 200 grams (7.1 oz) of milk to provide that much protein, and 150 grams (5.3 oz) to equal the calcium.

MacroNutrients (g) of common cheeses per 100 g

Cheese	Water	Protein	Fat	Carbs
Swiss	37.1	26.9	27.8	5.4
Feta	55.2	14.2	21.3	4.1
Cheddar	36.8	24.9	33.1	1.3
Mozzarella	50	22.2	22.4	2.2
Cottage	80	11.1	4.3	3.4

Vitamin contents in %DV of common cheeses per 100 g

Cheese	A	B1	B2	B3	B5	B6	B9	B12	Ch.	C	D	E	K
Swiss	17	4	17	0	4	4	1	**56**	2.8	0	11	2	3
Feta	8	10	50	5	10	21	8	28	2.2	0	0	1	2
Cheddar	20	2	22	0	4	4	5	14	3	0	3	1	3
Mozzarella	14	2	17	1	1	2	2	38	2.8	0	0	1	3
Cottage	3	2	10	0	6	2	3	7	3.3	0	0	0	0

Mineral contents in %DV of common cheeses per 100 g

Cheese	Ca	Fe	Mg	P	K	Na	Zn	Cu	Mn	Se
Swiss	**79**	10	1	**57**	2	8	29	2	0	26
Feta	**49**	4	5	**34**	2	46	19	2	1	21
Cheddar	72	4	7	51	3	26	21	2	1	20
Mozzarella	**51**	2	5	**35**	2	26	19	1	1	24
Cottage	8	0	2	16	3	15	3	1	0	14

Ch. = Choline; Ca = Calcium; Fe = Iron; Mg = Magnesium; P = Phosphorus; K = Potassium; Na = Sodium; Zn = Zinc; Cu = Copper; Mn = Manganese; Se = Selenium;

Pasteurization

A number of food safety agencies around the world have warned of the risks of raw-milk cheeses. The U.S. Food and Drug Administration states that soft raw-milk cheeses can cause "serious infectious diseases including listeriosis, brucellosis, salmonellosis and tuberculosis". It is U.S. law since 1944 that all raw-milk cheeses (including imports since 1951) must be aged at least 60 days. Australia has a wide ban on raw-milk cheeses as well, though in recent years exceptions have been made for Swiss Gruyère, Emmental and Sbrinz, and for French Roquefort. There is a trend for cheeses to be pasteurized even when not required by law.

Pregnant women may face an additional risk from cheese; the U.S. Centers for Disease Control has warned pregnant women against eating soft-ripened cheeses and blue-veined cheeses, due to the listeria risk, which can cause miscarriage or harm the fetus.

Cultural attitudes

Although cheese is a vital source of nutrition in many regions of the world and it is extensively consumed in others, its use is not universal.
Cheese is rarely found in Southeast and East Asian cuisines, presumably for historical reasons as dairy farming has historically been rare in these regions. However, Asian sentiment against cheese is not universal. In Nepal, the Dairy Development Corporation commercially manufactures cheese made from yak milk and a hard cheese made from either cow or yak milk known as chhurpi.The national dish of Bhutan, ema datshi, is made from homemade yak or mare milk cheese and hot peppers. In Yunnan, China, several ethnic minority groups produce Rushan and Rubing from cow's milk. Cheese consumption may be increasing in China, with annual sales doubling from 1996 to 2003 (to a still small 30 million U.S. dollars a year). Certain kinds of Chinese preserved bean curd are sometimes misleadingly referred to in English as "Chinese cheese" because of their texture and strong flavor.
Strict followers of the dietary laws of Islam and Judaism must avoid cheeses made with rennet from animals not slaughtered in a manner adhering to halal or kosher laws. Both faiths allow cheese made with vegetable-based rennet or with rennet made from animals that were processed in a halal or kosher manner. Many less orthodox Jews also believe that rennet undergoes enough processing to change its nature entirely and do not consider it to ever violate kosher law. As cheese is a dairy food, under kosher rules it cannot be eaten in the same meal with any meat.
Rennet derived from animal slaughter, and thus cheese made with animal-derived rennet, is not vegetarian. Most widely available vegetarian cheeses are made using rennet produced by fermentation of the fungus *Mucor miehei*. Vegans and other dairy-avoiding vegetarians do not eat conventional cheese, but some vegetable-based cheese substitutes (soy or almond) are used as substitutes.
Even in cultures with long cheese traditions, consumers may perceive some cheeses that are especially pungent-smelling, or mold-bearing varieties such as Limburger or Roquefort, as unpalatable. Such cheeses are an acquired taste because they are processed using molds or microbiological cultures, allowing odor and flavor molecules to resemble those in rotten foods. One author stated: "An aversion to the odor of decay has the obvious biological value of steering us away from possible food poisoning, so it is no wonder that an animal food that gives off whiffs of shoes and soil and the stable takes some getting used to."
Collecting cheese labels is called "tyrosemiophilia".

Interferon

Interferons (**IFN**s) are a group of signaling proteins made and released by host cells in response to the presence of several viruses. In a typical scenario, a virus-infected cell will release interferons causing nearby cells to heighten their anti-viral defenses.
IFNs belong to the large class of proteins known as cytokines, molecules used for communication between cells to trigger the protective defenses of the immune system that help eradicate pathogens. Interferons are named for their ability to "interfere" with viral replication by protecting cells from virus infections. IFNs also have various other functions: they activate immune cells, such as natural killer cells and macrophages; they increase host defenses by up-regulating antigen presentation by virtue of increasing the expression of major histocompatibility complex (MHC) antigens. Certain symptoms of infections, such as fever, muscle pain and "flu-like symptoms", are also caused by the production of IFNs and other cytokines.
More than twenty distinct IFN genes and proteins have been identified in animals, including humans. They are typically divided among three classes: Type I IFN, Type II IFN, and Type III IFN. IFNs belonging to all three classes are important for fighting viral infections and for the regulation of the immune system.

Types

Based on the type of receptor through which they signal, human interferons have been classified into three major types.

- Interferon type I: All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α/β receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. The type I interferons present in humans are IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω. In general, type I interferons are produced when the body recognizes a virus that has invaded it. They are produced by fibroblasts and monocytes. However, the production of type I IFN-α is prohibited by another cytokine known as Interleukin-10.
- Interferon type II (IFN-γ in humans): This is also known as immune interferon and is activated by Interleukin-12. Furthermore, type II interferons are released by Cytotoxic T cells and T helper cells, type 1 specifically.
- Interferon type III: Signal through a receptor complex consisting of IL10R2 (also called CRF2-4) and IFNLR1 (also called CRF2-12). Although discovered more recently than type I and type II IFNs, recent information demonstrates the importance of Type III IFNs in some types of virus or fungal infections.

Induction of interferons

Production of interferons occurs mainly in response to microbes, such as viruses and bacteria, and their products. Binding of molecules uniquely found in microbes—viral glycoproteins, viral RNA, bacterial endotoxin (lipopolysaccharide), bacterial flagella, CpG motifs—by pattern recognition receptors, such as membrane bound Toll like receptors or the cytoplasmic receptors RIG-I or MDA5, can trigger release of IFNs. Toll Like Receptor 3 (TLR3) is important for inducing interferons in response to the presence of double-stranded RNA viruses; the ligand for this receptor is double-stranded RNA (dsRNA). After binding dsRNA, this receptor activates the transcription factors IRF3 and NF-κB, which are important for initiating synthesis of many inflammatory proteins. RNA interference technology tools such as siRNA or vector-based reagents can either silence or stimulate interferon pathways. Release of IFN from cells (specifically IFN-γ in lymphoid cells) is also induced by mitogens. Other cytokines, such as interleukin 1, interleukin 2, interleukin-12, tumor necrosis factor and colony-stimulating factor, can also enhance interferon production.

Downstream signaling

By interacting with their specific receptors, IFNs activate *signal transducer and activator of transcription* (STAT) complexes; STATs are a family of transcription factors that regulate the expression of certain immune system genes. Some STATs are activated by both type I and type II IFNs. However each IFN type can also activate unique STATs.

STAT activation initiates the most well-defined cell signaling pathway for all IFNs, the classical Janus kinase-STAT (JAK-STAT) signaling pathway. In this pathway, JAKs associate with IFN receptors and, following receptor engagement with IFN, phosphorylate both STAT1 and STAT2. As a result, an IFN-stimulated gene factor 3 (ISGF3) complex forms—this contains STAT1, STAT2 and a third transcription factor called IRF9—and moves into the cell nucleus. Inside the nucleus, the ISGF3 complex binds to specific nucleotide sequences called *IFN-stimulated response elements* (ISREs) in the promoters of certain genes, known as IFN stimulated genes ISGs. Binding of ISGF3 and other transcriptional complexes activated by IFN signaling to these specific regulatory elements induces transcription of those genes. A collection of known ISGs is available on Interferome, a curated online database of ISGs (www.interferome.org); Additionally, STAT homodimers or heterodimers form from different combinations of STAT-1, -3, -4, -5, or -6 during IFN signaling; these dimers initiate gene transcription by binding to IFN-activated site (GAS) elements in gene promoters. Type I IFNs can induce expression of genes with either ISRE or GAS elements, but gene induction by type II IFN can occur only in the presence of a GAS element.

In addition to the JAK-STAT pathway, IFNs can activate several other signaling cascades. For instance, both type I and type II IFNs activate a member of the CRK family of adaptor

proteins called CRKL, a nuclear adaptor for STAT5 that also regulates signaling through the C3G/Rap1 pathway. Type I IFNs further activate *p38 mitogen-activated protein kinase* (MAP kinase) to induce gene transcription.[21] Antiviral and antiproliferative effects specific to type I IFNs result from p38 MAP kinase signaling. The *phosphatidylinositol 3-kinase* (PI3K) signaling pathway is also regulated by both type I and type II IFNs. PI3K activates P70-S6 Kinase 1, an enzyme that increases protein synthesis and cell proliferation; phosphorylates of ribosomal protein s6, which is involved in protein synthesis; and phosphorylates a translational repressor protein called *eukaryotic translation-initiation factor 4E-binding protein 1* (EIF4EBP1) in order to deactivate it.

Interferons can disrupt signaling by other stimuli. For example, Interferon alpha induces RIG-G, which disrupts the CSN5-containing COP9 signalosome (CSN), a highly conserved multiprotein complex implicated in protein deneddylation, deubiquitination, and phosphorylation. RIG-G has shown the capacity to inhibit NF-κB and STAT3 signaling in lung cancer cells, which demonstrates the potential of type I IFNs.

Interferon therapy

Diseases

Interferon beta-1a and interferon beta-1b are used to treat and control multiple sclerosis, an autoimmune disorder. This treatment may help in reducing attacks in relapsing-remitting multiple sclerosis and slowing disease progression and activity in secondary progressive multiple sclerosis.

Interferon therapy is used (in combination with chemotherapy and radiation) as a treatment for some cancers. This treatment can be used in hematological malignancy, such as in leukemia and lymphomas including hairy cell leukemia, chronic myeloid leukemia, nodular lymphoma, and cutaneous T-cell lymphoma. Patients with recurrent melanomas receive recombinant IFN-α2b.Both hepatitis B and hepatitis C are treated with IFN-α, often in combination with other antiviral drugs. Some of those treated with interferon have a sustained virological response and can eliminate hepatitis virus. The most harmful strain—hepatitis C genotype I virus—can be treated with a 60-80% success rate with the current standard-of-care treatment of interferon-α, ribavirin and recently approved protease inhibitors such as Telaprevir (Incivek) May 2011, Boceprevir (Victrelis) May 2011 or the nucleotide analog polymerase inhibitor Sofosbuvir (Sovaldi) December 2013. Biopsies of patients given the treatment show reductions in liver damage and cirrhosis. Some evidence shows giving interferon immediately following infection can prevent chronic hepatitis C, although diagnosis early in infection is difficult since physical symptoms are sparse in early hepatitis C infection. Control of chronic hepatitis C by IFN is associated with reduced hepatocellular carcinoma.

Unconfirmed results suggested that interferon eye drops may be an effective treatment for people who have herpes simplex virus epithelial keratitis, a type of eye infection. There is no clear evidence to suggest that removing the infected tissue (debridement) followed by interferon drops is an effective treatment approach for these types of eye infections. Unconfirmed results suggested that the combination of interferon and an antiviral agent may speed the healing process compared to antiviral therapy alone.

When used in systemic therapy, IFNs are mostly administered by an intramuscular injection. The injection of IFNs in the muscle or under the skin is generally well tolerated. The most frequent adverse effects are flu-like symptoms: increased body temperature, feeling ill, fatigue, headache, muscle pain, convulsion, dizziness, hair thinning, and depression. Erythema, pain, and hardness at the site of injection are also frequently observed. IFN therapy causes immunosuppression, in particular through neutropenia and can result in some infections manifesting in unusual ways.

Drug formulations:

Pharmaceutical forms of interferons	
Generic name	**Trade name**

Interferon alfa	Multiferon
Interferon alpha 2a	Roferon A
Interferon alpha 2b	Intron A/Reliferon/Uniferon
Human leukocyte Interferon-alpha (HuIFN-alpha-Le)	Multiferon
Interferon beta 1a, liquid form	Rebif
Interferon beta 1a, lyophilized	Avonex
Interferon beta 1a, biogeneric (Iran)	Cinnovex
Interferon beta 1b	Betaseron / Betaferon
Interferon gamma 1b	Actimmune
PEGylated interferon alpha 2a	Pegasys
PEGylated interferon alpha 2a (Egypt)	Reiferon Retard
PEGylated interferon alpha 2b	PegIntron
PEGylated interferon alpha 2b (Europe)	Besremi
PEGylated interferon alpha 2b plus ribavirin (Canada)	Pegetron

Several different types of interferons are approved for use in humans. One was first approved for medical use in 1986. For example, in January 2001, the Food and Drug Administration (FDA) approved the use of PEGylated interferon-alpha in the USA; in this formulation, PEGylated interferon-alpha-2b (*Pegintron*), polyethylene glycol is linked to the interferon molecule to make the interferon last longer in the body.

5. Immunology:

Organs and tissues associated with Immune System

The immune system is consisting of different organs and tissues that are found throughout the body. These organs can be classified functionally into two main groups. The primary lymphoid organs provide appropriate microenvironments for the development and maturation of lymphocytes. The secondary lymphoid organs trap antigen from defined tissues or vascular spaces and are sites where mature lymphocytes can interact effectively with that antigen. Blood vessels and lymphatic systems connect these organs, uniting them into a functional whole.

Carried within the blood and lymph and populating the lymphoid organs are various white blood cells, or leukocytes, that participate in the immune response. Of these cells,only the lymphocytes possess the attributes of diversity, specificity, memory, and self/non-self recognition, the hallmarks of an adaptive immune response. All the other cells play accessory roles in adaptive immunity,serving to activate lymphocytes, to increase the effectiveness of antigen clearance by phagocytosis, or to secrete various immune-effector molecules. Some leukocytes, especially T lymphocytes, secrete various protein molecules called cytokines. These molecules act as immune regulatory hormones and play important roles in the regulation of immune responses. This chapter describes the formation of blood cells,the properties of the various immune-system cells,and the functions of the lymphoid organs.The immune system includes primary lymphoid organs, secondary lymphatic tissues and various cells in the innate and adaptive immune systems.

Key Points

- The key primary lymphoid organs of the immune system are the thymus and bone marrow, and secondary lymphatic tissues such as spleen, tonsils, lymph vessels, lymph nodes, adenoids, and skin and liver.
- Leukocytes (white blood cells) act like independent, single-celled organisms and are the second arm of the innate immune system.
- The innate leukocytes include the phagocytes (macrophages, neutrophils, and dendritic cells), mast cells, eosinophils, basophils, and natural killer cells. These cells identify and eliminate pathogens and are also important mediators in the activation of the adaptive immune system.
- The cells of the adaptive immune system are special types of leukocytes, called lymphocytes. B cells and T cells are the major types of lymphocytes and are derived from hematopoietic stem cells in the bone marrow.
- The lymphatic system is a part of the circulatory system, comprising a network of conduits called lymphatic vessels. The lymphatic system has multiple functions such as the transportation of white blood cells to and from the lymph nodes into the bones.

Key Terms

- **lymphocytes**: A lymphocyte is a type of white blood cell in the vertebrate immune system. The three major types of lymphocyte are T cells, B cells and natural killer (NK) cells. T cells

(thymus cells) and B cells (bursa-derived cells) are the major cellular components of the adaptive immune response.

- **Leukocytes**: Cells of the immune system involved in defending the body against both infectious disease and foreign materials. Five different and diverse types of leukocytes exist.

Immune System Organs

The key primary lymphoid organs of the immune system include the thymus and bone marrow, as well as secondary lymphatic tissues including spleen, tonsils, lymph vessels, lymph nodes, adenoids, skin, and liver.

The thymus "educates" T cells and provides an inductive environment for the development of T cells from hematopoietic progenitor cells. The thymus is largest and most active during the neonatal and pre-adolescent periods of development. By the early teens, the thymus begins to atrophy and thymic stroma is replaced by adipose tissue. Nevertheless, residual T-lymphopoiesis continues throughout adult life.

Bone marrow is the flexible tissue found in the interior of bones. In humans, red blood cells are produced in the heads of long bones. The red bone marrow is a key element of the lymphatic system, being one of the primary lymphoid organs that generate lymphocytes from immature hematopoietic progenitor cells. Bone marrow and thymus constitute the primary lymphoid tissues involved in the production and early selection of lymphocytes.

The lymphatic system is a part of the circulatory system, comprising a network of conduits called lymphatic vessels that carry a clear fluid, called lymph, unidirectionally towards the heart. The lymphatic system has multiple interrelated functions including the transportation of white blood cells to and from the lymph nodes into the bones, and the transportation of antigen -presenting cells (such as dendritic cells) to the lymph nodes where an immune response is stimulated. Lymphoid tissue is found in many organs, particularly the lymph nodes.

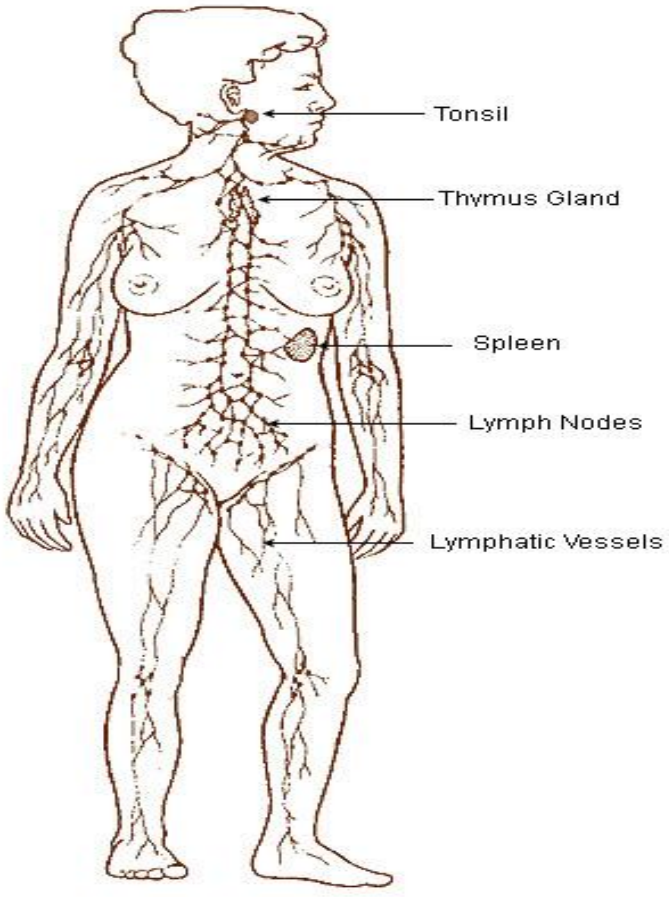


Figure: **The Lymph Nodes and Lymph Vessels in Human Beings**:

The lymphatic system is a part of the circulatory system, comprising a network of conduits called lymphatic vessels that carry a clear fluid called lymph.The spleen is similar in structure to a large lymph node and acts primarily as a blood filter. It synthesizes antibodies in its white pulp and removes antibody-coated bacteria along with antibody-coated blood cells by way of blood and lymph node circulation.The palatine tonsils and the nasopharyngeal tonsil are lymphoepithelial tissues located near the oropharynx and nasopharynx. These immunocompetent tissues are the immune system's first line of defense against ingested or inhaled foreign pathogens. The fundamental immunological roles of tonsils aren't yet understood.

Lymph nodes are distributed widely throughout areas of the body, including the armpit and stomach, and linked by lymphatic vessels. Lymph nodes are garrisons of B, T and other immune cells. Lymph nodes act as filters or traps for foreign particles and are important in the proper functioning of the immune system. They are packed tightly with the white blood cells, called lymphocytes and macrophages.

The skin is one of the most important parts of the body because it interfaces with the environment, and is the first line of defense from external factors, acting as an anatomical barrier from pathogens and damage between the internal and external environment in bodily defense. Langerhans cells in the skin are part of the adaptive immune system.

The liver has a wide range of functions, including immunological effects—the reticuloendothelial system of the liver contains many immunologically active cells, acting as a "sieve" for antigens carried to it via the portal system.

Immune System Cells

Leukocytes (white blood cells) are immune system cells involved in defending the body against infectious disease and foreign materials. Five different types of leukocytes exist, all produced and derived from a multipotent cell in the bone marrow known as a hematopoietic stem cell. The innate leukocytes include the phagocytes, mast cells, eosinophils, basophils, and natural killer cells. These cells identify and eliminate pathogens and are important mediators in the activation of the adaptive immune system.

Neutrophils and macrophages are phagocytes that travel throughout the body in pursuit of invading pathogens. Neutrophils are normally found in the bloodstream and are the most abundant type of phagocyte. During the acute phase of inflammation neutrophils migrate toward the site of inflammation and are usually the first cells to arrive at the scene of infection. Macrophages reside within tissues and produce a wide array of chemicals. They also act as scavengers, ridding the body of worn-out cells and other debris, and as antigen-presenting cells that activate the adaptive immune system. Dendritic cells are phagocytes in tissues that are in contact with the external environment, and are located mainly in the skin, nose, lungs, stomach, and intestines. These cells serve as a link between the bodily tissues and the innate and adaptive immune systems, as they present antigen to T-cells, one of the key cell types of the adaptive immune system.

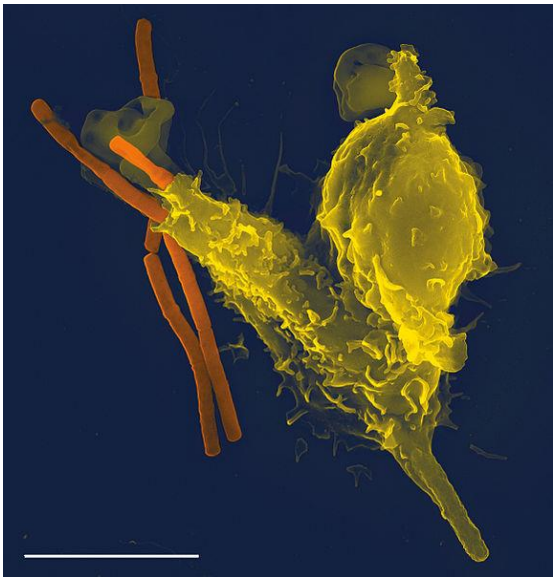


Figure: **A Phagocyte in Action**: Neutrophil engulfing anthrax bacteria. Taken with a Leo 1550 scanning electron microscope. Scale bar is 5 micrometers.

Mast cells reside in connective tissues and mucous membranes, and regulate the inflammatory response. They are most often associated with allergy and anaphylaxis.

Basophils and eosinophils are related to neutrophils. They secrete chemical mediators that are involved in defending against parasites, and play a role in allergic reactions, such as asthma.

Natural killer cells are leukocytes that attack and destroy tumor cells, or cells that have been infected by viruses.

The cells of the adaptive immune system are special types of leukocytes, called lymphocytes. B cells and T cells are the major types of lymphocytes and are derived from hematopoietic stem cells in the bone marrow.

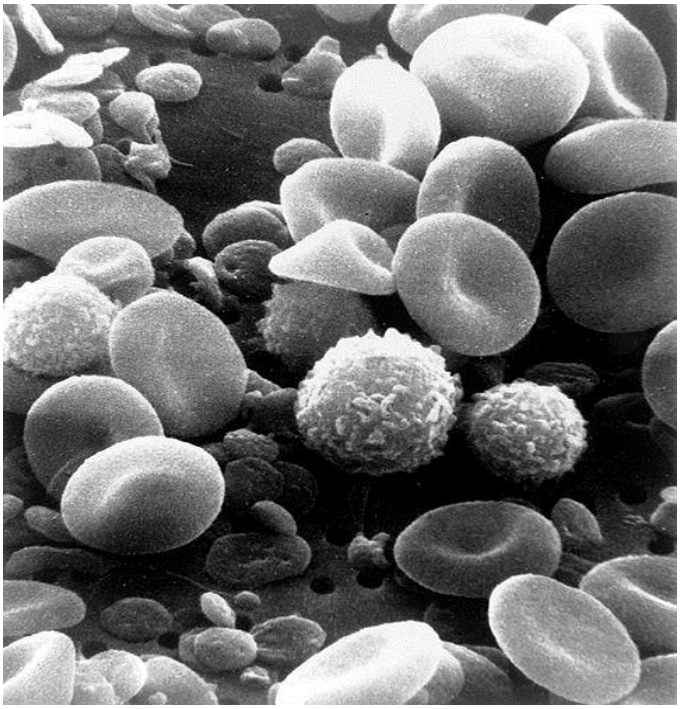


Figure: **Blood Cells**: Red blood cells, several white blood cells including lymphocytes, a monocyte, a neutrophil, and many small disc-shaped platelets.

T cells recognize a "non-self" target, such as a pathogen, only after antigens have been processed and presented in combination with a "self" receptor, called a major histocompatibility complex (MHC) molecule. There are two major subtypes of T cells: the killer T cell, which kills cells that are infected

with viruses (and other pathogens) or are otherwise damaged or dysfunctional, and the helper T cell, which regulates both innate and adaptive immune responses and helps determine which immune responses the body makes to a particular pathogen. These cells have no cytotoxic activity and do not kill infected cells or clear pathogens directly. A third, minor subtype are the γ T cells that recognize intact antigens not bound to MHC receptors.

In contrast, the B cell antigen-specific receptor is an antibody molecule on the B cell surface, which recognizes whole pathogens without any need for antigen processing. Each lineage of B cell expresses a different antibody, so the complete set of B cell antigen receptors represent all the antibodies that the body can manufacture.

Hematopoiesis

All blood cells arise from a type of cell called the hematopoietic stem cell (HSC).Stem cells are cells that can differentiate into other cell types; they are self-renewing—they maintain their population level by cell division. In humans, hematopoiesis, the formation and development of red and white blood cells,begins in the embryonic yolk sac during the first weeks of development.Here,yolk-sac stem cells differentiate into primitive erythroid cells that contain embryonic hemoglobin. In the third month of gestation, hematopoietic stem cells migrate from the yolk sac to the fetal liver and then to the spleen; these two organs have major roles in hematopoiesis from the third to the seventh months of gestation. After that, the differentiation of HSCs in the bone marrow becomes the major factor in hematopoiesis, and by birth there is little or no hematopoiesis in the liver and spleen.It is remarkable that every functionally specialized, mature blood cell is derived from the same type of stem cell. In contrast to a unipotent cell, which differentiates into a single cell type, a hematopoietic stem cell is multipotent, or pluripotent, able to differentiate in various ways and thereby generate erythrocytes, granulocytes, monocytes, mast cells, lymphocytes, and megakaryocytes. These stem cells are few, normally fewer than one HSC per 5 of 104 cells in the bone marrow.

The study of hematopoietic stem cells is difficult both because of their scarcity and because they are hard to grow in vitro.As a result,little is known about how their proliferation and differentiation are regulated. By virtue of their capacity for self-renewal, hematopoietic stem cells are maintained at stable levels throughout adult life; however, when there is an increased demand for hematopoiesis, HSCs display an enormous proliferative capacity. This can be demonstrated in mice whose hematopoietic systems have been completely destroyed by a lethal dose of x-rays (950 rads; one rad represents the absorption by an irradiated target of an amount of radiation corresponding to 100 ergs/gram of target). Such irradiated mice will die within 10 days unless they are infused with normal bone-marrow cells from a syngeneic (genetically identical) mouse. Although a normal mouse has 3 108 bone-marrow cells, infusion of only 104–105 bone-marrow cells (i.e., 0.01%–0.1% of the normal amount) from a donor is sufficient to completely restore the hematopoietic system, which demonstrates the enormous proliferative and differentiative capacity of the stem cells.Early in hematopoiesis, a multipotent stem cell differentiates along one of two pathways, giving rise to either a common lymphoid progenitor cell or a common myeloidprogenitor cell (Figure 2-1). The types and amounts of growth factors in the microenvironment of a particular stem cell or progenitor cell control its differentiation. During the development of the lymphoid and myeloid lineages, stem cells differentiate into progenitor cells, which have lost thecapacity for self-renewal and are committed to a particular cell lineage. Common lymphoid progenitor cells give rise to B, T, and NK (natural killer) cells and some dendritic cells.Myeloid stem cells generate progenitors of red blood cells (erythrocytes), many of the various white blood cells (neutrophils, eosinophils, basophils, monocytes, mast cells, dendritic cells), and platelets.Progenitor commitment depends on the acquisition of responsiveness to particular growth factors and cytokines. When the appropriate factors and cytokines are present, progenitor cells proliferate and differentiate into the corresponding cell type, either a mature erythrocyte, a particular type of leukocyte, or a platelet-generating cell (the megakaryocyte). Red and white blood cells pass into bonemarrow channels, from which they enter the circulation.

In bone marrow, hematopoietic cells grow and mature on a meshwork of stromal cells, which are nonhematopoietic cells that support the growth and differentiation of hematopoietic cells.Stromal cells include fat cells,endothelial cells, fibroblasts, and macrophages. Stromal cells influence the differentiation of hematopoietic stem cells by providing a hematopoietic-inducing microenvironment (HIM) consisting of a cellular matrix and factors that promote growth and differentiation. Many of these hematopoietic growth factors are soluble agents that arrive at their target cells by diffusion, others are membrane-bound molecules on the surface of stromal cells that require cell-to-cell contact between the responding cells and the stromal cells. During infection, hematopoiesis is stimulated by the production of hematopoietic growth factors by activated macrophages and T cells.

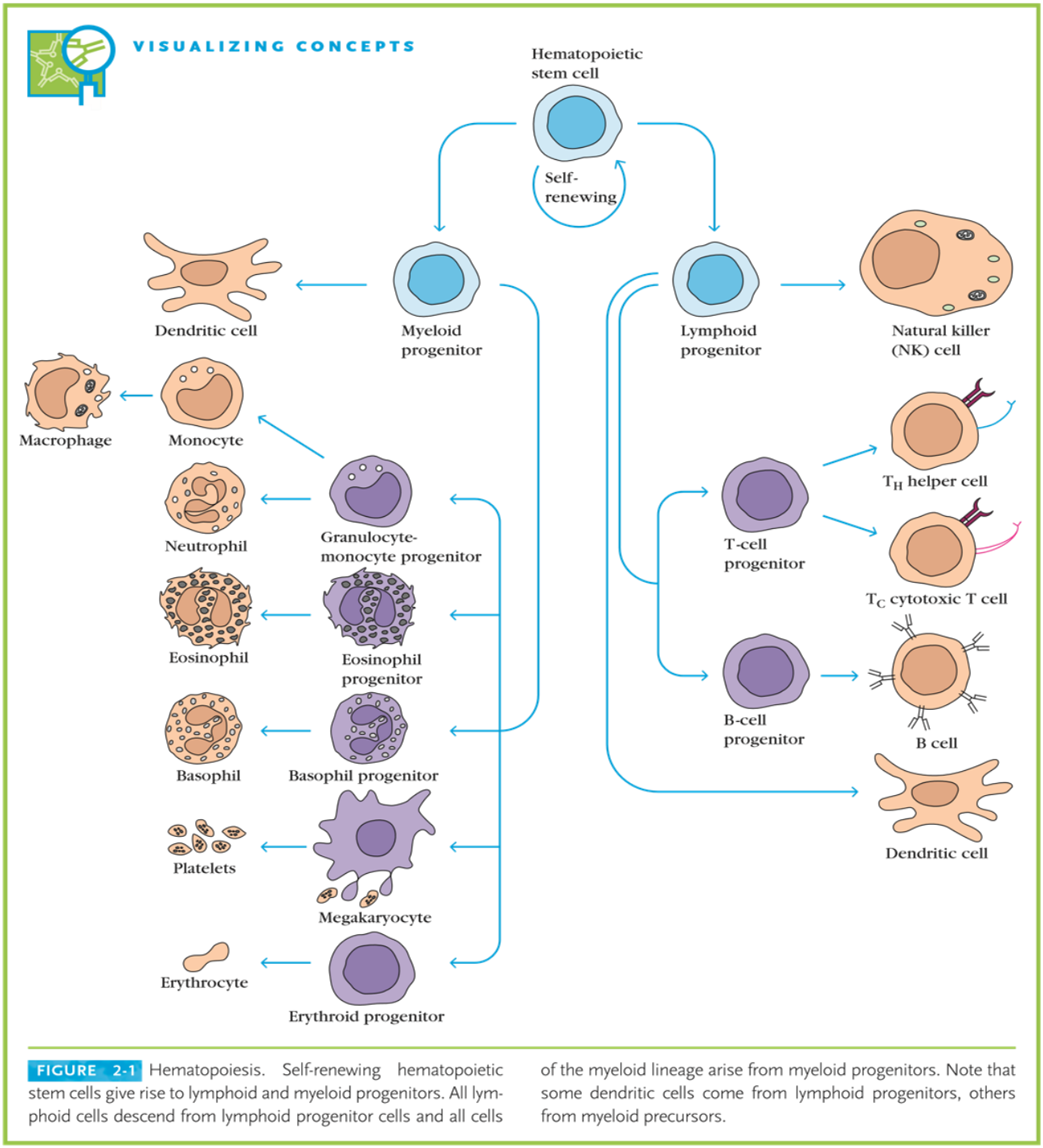


FIGURE 2-1 Hematopoiesis. Self-renewing hematopoietic stem cells give rise to lymphoid and myeloid progenitors. All lymphoid cells descend from lymphoid progenitor cells and all cells of the myeloid lineage arise from myeloid progenitors. Note that some dendritic cells come from lymphoid progenitors, others from myeloid precursors.

Cells of the Immune System

Lymphocytes are the central cells of the immune system, responsible for adaptive immunity and the immunologic attributes of diversity, specificity, memory, and self/nonself tant roles, engulfing and destroying microorganisms, presenting antigens, and secreting cytokines.

Lymphoid Cells

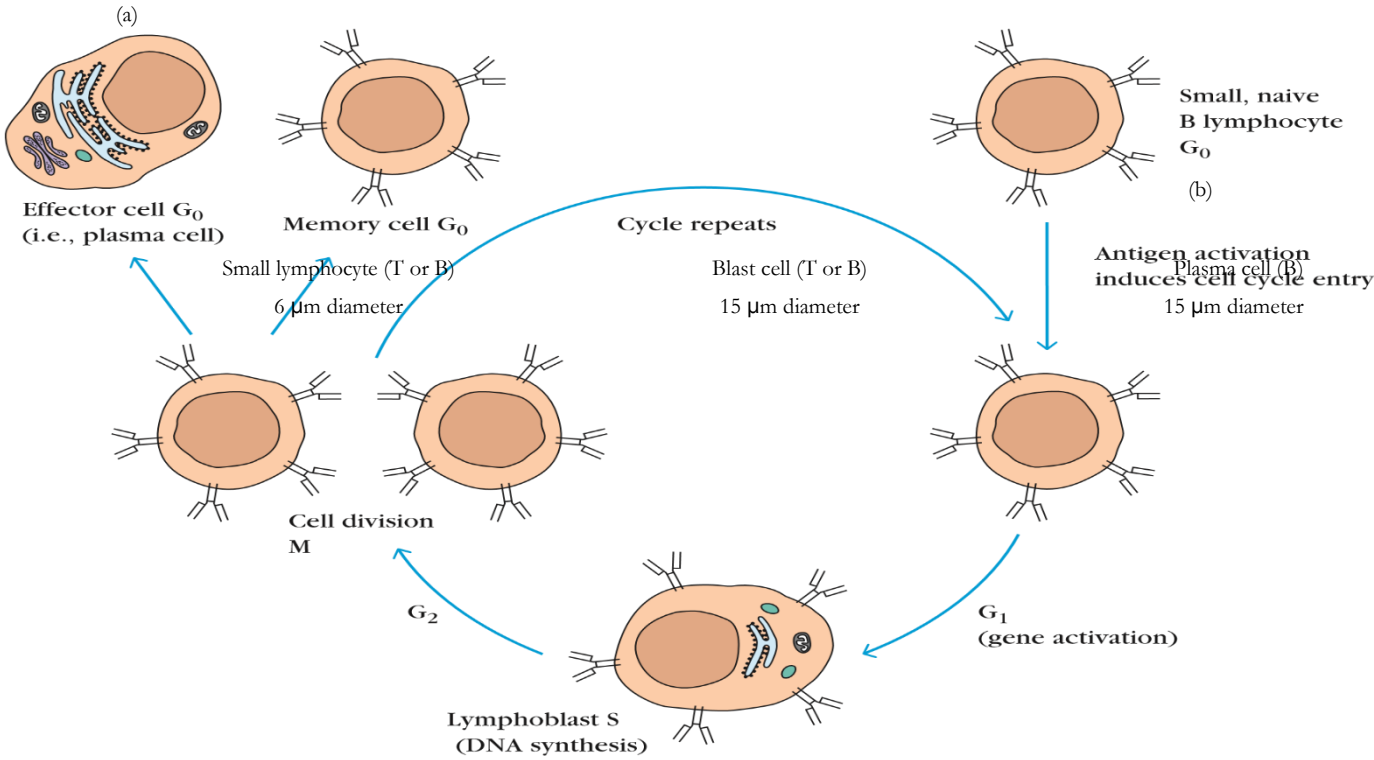
Lymphocytes constitute 20%–40% of the body's white blood cells and 99% of the cells in the lymph (Table 2-4). There are approximately 1011 (range depending on body size and age: ~1010–1012) lymphocytes in the human body. These lymphocytes continually circulate in the blood and lymph and are capable of migrating into the tissue spaces and lymphoid organs, thereby integrating the immune system to a high degree.

The lymphocytes can be broadly subdivided into three populations—B cells, T cells, and natural killer cells—on the basis of function and cell-membrane components. Natural killer cells (NK cells) are large,granular lymphocytes that do not express the set of surface markers typical of B or T cells. Resting B and T lymphocytes are small, motile, nonphagocytic cells, which cannot be distinguished morphologically. B and T lymphocytes that have not interacted with antigen— referred to as naive, or unprimed—are resting cells in the G0 phase of the cell cycle. Known as small lymphocytes, these cells are only about 6 m in diameter; their cytoplasm forms a barely discernible rim around the nucleus. Small lymphocytes have densely packed chromatin, few mitochondria, and a poorly developed endoplasmic reticulum and Golgi apparatus. The naive lymphocyte is generally thought to have a short life span. Interaction of small lymphocytes with antigen, in the presence of certain cytokines discussed later, induces these cells to enter the cell cycle by progressing from G0 into G1 and subsequently into S, G2, and M (Figure 2-7a). As they progress through the cell cycle, lymphocytes enlarge into 15 m-diameter blast cells, called lymphoblasts; these cells have a higher cytoplasm:nucleus ratio and more organellar complexity than small lymphocytes.Lymphoblasts proliferate and eventually differentiate into effector cells or into memory cells. Effector cells function in various ways to eliminate antigen. These cells have short lifespans, generally ranging from a few days to a few weeks. Plasma cells—the antibody-secreting effector cells of the Bcell lineage—have a characteristic cytoplasm that contains abundant endoplasmic reticulum (to support their high rate of protein synthesis) arranged in concentric layers and also many Golgi vesicles (see Figure 2-7). The effector cells of the T-cell lineage include the cytokine-secreting T helper cell (TH cell) and the T cytotoxic lymphocyte (TC cell). Some of the progeny of B and T lymphoblasts differentiate into memory cells.The persistence of this population of cells is responsible for life-long immunity to many pathogens. Memory cells look like small lymphocytes but can be distinguished from naive cells by the presence or absence of certain cellmembrane molecules.

Different lineages or maturational stages of lymphocytes can be distinguished by their expression of membrane molecules recognized by particular monoclonal antibodies (antibodies that are specific for a single epitope of an antigen; see Chapter 4 for a description of monoclonal antibodies).All of the monoclonal antibodies that react with a particular membrane molecule are grouped together as a cluster of differentiation (CD). Each new monoclonal antibody that recognizes a leukocyte membrane molecule is analyzed for whether it falls within a recognized CD designation; if it does not, it is given a new CD designation reflecting a new membrane molecule. Although the CD nomenclature was originally developed for the membrane molecules of human leukocytes, the homologous membrane molecules of other species, such as mice, are commonly referred to by the same CD designations. Table 2-5 lists some common CD molecules (often referred to as CD markers) found on human lymphocytes. However, this is only a partial listing of the more than 200 CD markers that have been described.A complete list and description of known CD markers is in the appendix at the end of this book.

TABLE 2-4 Normal adult blood-cell counts

Cell type	Cells/mm^3	%
Red blood cells	5.010^6	
Platelets	2.510^5	
Leukocytes	7.310^3	
Neutrophil		50–70
Lymphocyte		20–40
Monocyte		1–6
Eosinophil		1–3
Basophil		1



B LYMPHOCYTES

The B lymphocyte derived its letter designation from its site of maturation, in the bursa of Fabricius in birds; the name turned out to be apt,for bone marrow is its major site of maturation in a number of mammalian species, including humans and mice. Mature B cells are definitively distinguished from other lymphocytes by their synthesis and display of membrane-bound immunoglobulin (antibody) molecules,which serve as receptors for antigen. Each of the approximately 1.5 x 105 molecules of antibody on the membrane of a single B cell has an identical binding site for antigen. Among the other molecules expressed on the membrane of mature B cells are the following:

B220 (a form of CD45) is frequently used as a marker for B cells and their precursors. However, unlike antibody, it is not expressed uniquely by B-lineage cells.

Class II MHC molecules permit the B cell to function as an antigen-presenting cell (APC).

CR1 (CD35) and CR2 (CD21) are receptors for certain complement products.

FcRII (CD32) is a receptor for IgG, a type of antibody.

B7-1 (CD80) and B7-2 (CD86) are molecules that interact with CD28 and CTLA-4, important regulatory molecules on the surface of different types of T cells, including TH cells.

CD40 is a molecule that interacts with CD40 ligand on the surface of helper T cells. In most cases this interaction is critical for the survival of antigenstimulated B cells and for their development into antibody-secreting plasma cells or memory B cells.

Interaction between antigen and the membrane-bound antibody on a mature naive B cell, as well as interactions with T cells and macrophages, selectively induces the activation and differentiation of B-cell clones of corresponding specificity. In this process,the B cell divides repeatedly and differentiates over a 4- to 5-day period, generating a population of plasma cells and memory cells. Plasma cells, which have lower levels of membrane-bound antibody than B cells, synthesize and secrete antibody. All clonal progeny from a given B cell secrete antibody molecules with the same antigen-binding specificity. Plasma cells are terminally differentiated cells, and many die in 1 or 2 weeks.

T LYMPHOCYTES

T lymphocytes derive their name from their site of maturation in the thymus. Like B lymphocytes, these cells have membrane receptors for antigen. Although the antigenbinding T-cell receptor is structurally distinct from immunoglobulin, it does share some common structural features with the immunoglobulin molecule,most notably in the structure of its antigen-binding site. Unlike the membrane-bound antibody on B cells, though, the T-cell receptor (TCR) does not recognize free antigen. Instead the TCR recognizes only antigen that is bound to particular classes of self-molecules. Most T cells recognize antigen only when it is bound to a self-molecule encoded by genes within the major histocompatibility complex (MHC). Thus, as explained in Chapter 1, a fundamental difference between the humoral and cell-mediated branches of the immune system is that the B cell is capable of binding soluble antigen,whereas the T cell is restricted to binding antigen displayed on self-cells. To be recognized by most T cells,this antigen must be displayed together with MHC molecules on the surface of antigen-presenting cells or on virus-infected cells, cancer cells, and grafts. The T-cell system has developed to eliminate these altered self-cells,which pose a threat to the normal functioning of

the body.Like B cells, T cells express distinctive membrane molecules. All T-cell subpopulations express the T-cell receptor, a complex of polypeptides that includes CD3; and most can be distinguished by the presence of one or the other of two membrane molecules, CD4 and CD8. In addition, most mature T cells express the following membrane molecules: CD28, a receptor for the co-stimulatory B7 family of molecules present on B cells and other antigenpresenting cells CD45, a signal-transduction molecule. T cells that express the membrane glycoprotein molecule CD4 are restricted to recognizing antigen bound to class II MHC molecules, whereas T cells expressing CD8, a dimeric membrane glycoprotein,are restricted to recognition of antigen bound to class I MHC molecules. Thus the expression of CD4 versus CD8 corresponds to the MHC restriction of the T cell. In general, expression of CD4 and of CD8 also defines two major functional subpopulations of T lymphocytes. CD4 T cells generally function as T helper (TH) cells and are class-II restricted; CD8 T cells generally function as T cytotoxic (TC) cells and are class-I restricted.Thus the ratio of TH to TC cells in a sample can be approximated by assaying the number of CD4 and CD8 T cells. This ratio is approximately 2:1 in normal human peripheral blood, but it may be significantly altered by immunodeficiency diseases, autoimmune diseases, and other disorders.The classification of CD4 class II–restricted cells as TH cells and CD8 class I–restricted cells as TC cells is not absolute. Some CD4 cells can act as killer cells. Also, some TC cells have been shown to secrete a variety of cytokines and exert an effect on other cells comparable to that exerted by TH cells. The distinction between TH and TC cells, then, is not always clear; there can be ambiguous functional activities. However, because these ambiguities are the exception and not the rule,the generalization of T helper (TH) cells as being CD4 and class-II restricted and of T cytotoxic cells (TC) as being CD8 and class-I restricted is assumed throughout this text, unless otherwise specified.

CDdesignation*	Function	Bcell	T_H	T_C	NKcell
CD2	Adhesion molecule; signal transduction				
CD3	Signal-transduction element of T-cell receptor				
CD4	Adhesion molecule that binds to class II MHC molecules; signal transduction		(usually)	(usually)	
CD5	Unknown		(subset)		
CD8	Adhesion molecule that binds to class I MHC molecules; signal transduction		(usually)	(usually)	(variable)
CD16 (FcRIII)	Low-affinity receptor for Fc region of IgG				
CD21 (CR2)	Receptor for complement (C3d) and Epstein-Barr virus				
CD28	Receptor for co-stimulatory B7 molecule on antigen-presenting cells				
CD32 (FcRII)	Receptor for Fc region of IgG				
CD35 (CR1)	Receptor for complement (C3b)				
CD40	Signal transduction				
CD45	Signal transduction				
CD56	Adhesion molecule				

TABLE :Common CD markers used to distinguish functional lymphocyte subpopulations

TH cells are activated by recognition of an antigen–class II MHC complex on an antigen-presenting cell. After activation, the TH cell begins to divide and gives rise to a clone of effector cells, each specific for the same antigen–class II MHC complex. These TH cells secrete various cytokines, which play a central role in the activation of B cells, T cells, and other cells that participate in the immune response. Changes in the pattern of cytokines produced by TH cells can change the type of immune response that develops amongother leukocytes. The TH1 response produces a cytokine profile that supports inflammation and activates mainly certain T cells and macrophages, whereas the TH2 response activates mainly B cells and immune responses that depend upon antibodies. TC cells are activated when they interact with an antigen–class I MHC complex on the surface of an altered self-cell (e.g., a virus-infected cell or a tumor cell) in the presence of appropriate cytokines. This activation, which results in proliferation, causes the TC cell to differentiate into an effector cell called a cytotoxic T lymphocyte (CTL). In contrast to TH cells, most CTLs secrete few cytokines. Instead, CTLs acquire the ability to recognize and eliminate altered self-cells.

Another subpopulation of T lymphocytes—called T suppressor (TS) cells—has been postulated. It is clear that some T cells help to suppress the humoral and the cell-mediated branches of the immune system, but the actual isolation and cloning of normal TS cells is a matter of controversy and dispute among immunologists. For this reason, it is uncertain whether TS cells do indeed constitute a separate functional subpopulation of T cells. Some immunologists believe that the suppression mediated by T cells observed in some systems is simply the consequence of activities of TH or TC subpopulations whose end results are suppressive.

NATURAL KILLER CELLS

The natural killer cell was first described in 1976, when it was shown that the body contains a small population of large, granular lymphocytes that display cytotoxic activity against a wide range of tumor cells in the absence of any previous immunization with the tumor. NK cells were subsequently shown to play an important role in host defense both against tumor cells and against cells infected with some, though not all, viruses. These cells, which constitute 5%–10% of lymphocytes in human peripheral blood, do not express the membrane molecules and receptors that distinguish T- and B-cell lineages. Although NK cells do not have T-cell receptors or immunoglobulin incorporated in their plasma membranes, they can recognize potential target cells in two different ways. In some cases, an NK cell employs NK cell receptors to distinguish abnormalities, notably a reduction in the display of class I MHC molecules and the unusual profile of surface antigens displayed by some tumor cells and cells infected by some viruses.Another way in which NK cells recognize potential target cells depends upon the fact that some tumor cells and cells infected by certain viruses display antigens against which the immune system has made an antibody response, so that antitumor or antiviral antibodies are bound to their surfaces. Because NK cells express CD16, a membrane receptor for the carboxyl-terminal end of the IgG molecule, called the Fc region, they can attach to theseantibodies and subsequently destroy the targeted cells. This is an example of a process known as antibody-dependent cellmediated cytotoxicity (ADCC).

Several observations suggest that NK cells play an important role in host defense against tumors. For example, in humans the Chediak-Higashi syndrome—an autosomal recessive disorder—is associated with impairment in neutrophils, macrophages, and NK cells and an increased incidence of lymphomas. Likewise, mice with an autosomal mutation called beige lack NKcells; these mutants are more susceptible than normal mice to tumor growth following injection with live tumor cells.

There has been growing recognition of a cell type, the NK1-T cell, that has some of the characteristics of both T cells and NK cells. Like T cells, NK1-T cells have T cell receptors (TCRs).Unlike most T cells,the TCRs of NK1-T cells interact with MHC-like molecules called CD1 rather than with class I or class II MHC molecules. Like NK cells, they have variable levels of CD16 and other receptors typical of NK cells, and they can kill cells.A population of triggered NK1-T cells can rapidly secrete large amounts of the cytokines needed to support antibody production by B cells as well as inflammation and the development and expansion of cytotoxic T cells. Some immunologists view this cell type as a kind of rapid response system that has evolved to provide early help while conventional TH responses are still developing.

Mononuclear Phagocytes

The mononuclear phagocytic system consists of monocytes circulating in the blood and macrophages in the tissues (Figure 2-8). During hematopoiesis in the bone marrow, granulocyte-monocyte progenitor cells differentiate into promonocytes, which leave the bone marrow and enter the blood, where they further differentiate into mature monocytes. Monocytes circulate in the bloodstream for about 8 h, during which they enlarge; they then migrate into the tissues and differentiate into specific tissue macrophages or, as discussed later, into dendritic cells.

Differentiation of a monocyte into a tissue macrophage involves a number of changes: The cell enlarges five- to tenfold; its intracellular organelles increase in both number and complexity; and it acquires increased phagocytic ability, produces higher levels of hydrolytic enzymes, and begins to secrete a variety of soluble factors. Macrophages are dispersed throughout the body. Some take up residence in particular tissues, becoming fixed macrophages, whereas others remain motile and are called free, or wandering, macrophages. Free macrophages travel by amoeboid movement throughout the tissues. Macrophage-like cells serve different functions in different tissues and are named according to their tissue location:

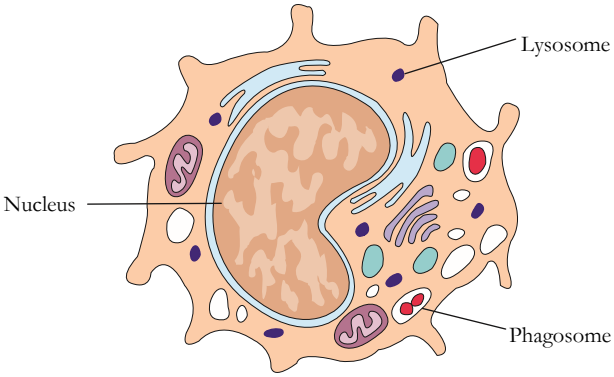
Alveolar macrophages in the lung
Histiocytes in connective tissues
Kupffer cells in the liver
Mesangial cells in the kidney
Microglial cells in the brain
Osteoclasts in bone

Although normally in a resting state, macrophages are activated by a variety of stimuli in the course of an immune response. Phagocytosis of particulate antigens serves as an initial activating stimulus. However, macrophage activity can be further enhanced by cytokines secreted by activated TH cells, by mediators of the inflammatory response, and by components of bacterial

cell walls. One of the most potent activators of macrophages is interferon gamma (IFN-) secreted by activated TH cells.

Activated macrophages are more effective than resting ones in eliminating potential pathogens,because they exhibit greater phagocytic activity, an increased ability to kill ingested microbes, increased secretion of inflammatory mediators, and an increased ability to activate T cells. In addition,activated macrophages, but not resting ones, secrete various cytotoxic proteins that help them eliminate a broad range ofpathogens,including virus-infected cells,tumor cells,and intracellular bacteria. Activated macrophages also express higher levels of class II MHC molecules, allowing them to function more effectively as antigen-presenting cells. Thus, macrophages and TH cells facilitate each other's activation during the immune response.

(a) Monocyte



(b) Macrophage

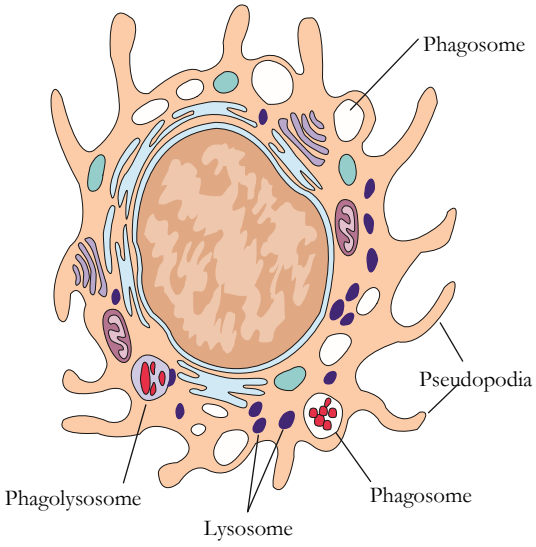


FIGURE 8

Typicalmorphologyofamonocyte

Macrophages are five- to tenfold larger than monocytes and contain more organelles, especially lysosomes.

PHAGOCYTOSIS

Macrophages are capable of ingesting and digesting exogenous antigens, such as whole microorganisms and insoluble particles, and endogenous matter, such as injured or dead host cells,cellular debris,and activated clotting factors.In the first step in phagocytosis, macrophages are attracted by and move toward a variety of substances generated in an immune response; this process is called chemotaxis. The next step in phagocytosis is adherence of the antigen to the macrophage cell membrane. Complex antigens, such as whole bacterial cells or viral particles, tend to adhere well and are readily phagocytosed; isolated proteins and encapsulated bacteria tend to adhere poorly and are less readily phagocytosed. Adherence induces membrane protrusions, called pseudopodia, to extend around the attached material (Figure 2-9a). Fusion of the pseudopodia encloses the material within a membrane-bounded structure called a phagosome, which then enters the endocytic processing pathway (Figure 2-9b). In this pathway, a phagosome moves toward the cell interior, where it fuses with a lysosome to form a phagolysosome. Lysosomes contain lysozyme and a variety of other hydrolytic enzymes that digest the ingested material. The digested contents of the phagolysosome are then eliminated in a process called exocytosis (see Figure 2-9b).

The macrophage membrane has receptors for certain classes of antibody. If an antigen (e.g., a bacterium) is coated with the appropriate antibody, the complex of antigen and antibody binds to antibody receptors on the macrophage membrane more readily than antigen alone and phagocytosis is enhanced. In one study, for example, the rate of phagocytosis of an antigen was 4000-fold higher in the presence of specific antibody to the antigen than in its absence. Thus, antibody functions as an opsonin, a molecule that binds to both antigen and macrophage and enhances phagocytosis. The process by which particulate antigens are rendered more susceptible to phagocytosis is called opsonization.

ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES

A number of antimicrobial and cytotoxic substances produced by activated macrophages can destroy phagocytosed microorganisms (Table 2-6). Many of the mediators of cytotoxicity listed in Table 2-6 are reactive forms of oxygen.

OXYGEN-DEPENDENT KILLING MECHANISMS

Activated phagocytes produce a number of reactive oxygenintermediates (ROIs) and reactive nitrogen intermediates that have potent antimicrobial activity. During phagocytosis, a metabolic process known as the **respiratory burst** occurs in activated macrophages. This process results in the activation of a membrane-bound oxidase that catalyzes the reduction of oxygen to superoxide anion, a reactive oxygen intermediate that is extremely toxic to ingested microorganisms. The superoxide anion also generates other powerful oxidizing agents, including hydroxyl radicals and hydrogen peroxide. As the lysosome fuses with the phagosome, the activity of myeloperoxidase produces hypochlorite from hydrogen per-oxide and chloride ions. Hypochlorite, the active agent of household bleach, is toxic to ingested microbes. When macrophages are activated with bacterial cell-wall components such as lipopolysaccharide (LPS) or, in the case of mycobacteria, muramyl dipeptide (MDP), together with a T-cell–derived cytokine (IFN-), they begin to express

high levels of nitric oxide synthetase (NOS), an enzyme that oxidizes L-arginine to yield L-citrulline and nitric oxide (NO), a gas:
L-arginine+ O2+NADPH → NO+ L-citrulline + NADP
Nitric oxide has potent antimicrobial activity; it also can combine with the superoxide anion to yield even more potent antimicrobial substances. Recent evidence suggests that much of the antimicrobial activity of macrophages against bacteria, fungi, parasitic worms, andprotozoa is due to nitric oxide and substances derived from it.

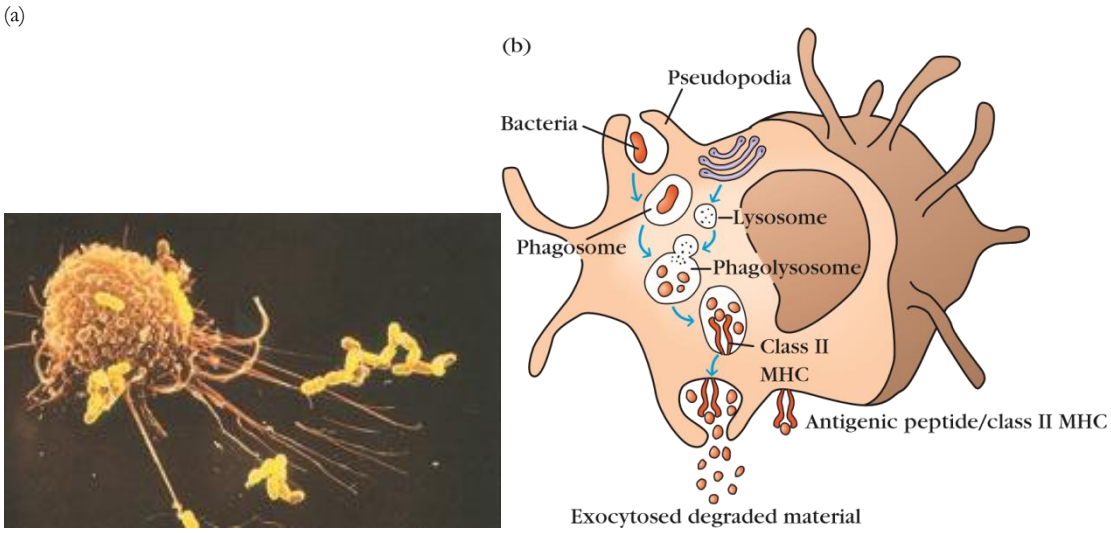


FIGURE 2-9 Macrophages can ingest and degrade particulate antigens, including bacteria. (a) Scanning electron micrograph of a macrophage. Note the long pseudopodia extending toward and making contact with bacterial cells, an early step in phagocytosis. (b) Phagocytosis and processing of exogenous antigen by macrophages.Most of the products resulting from digestion of ingested material are exocytosed, but some peptide products may interact with class II MHC molecules, forming complexes that move to the cell surface, where they are presented to T_H cells. [*Photograph by L. Nilsson, © Boehringer Ingelheim International GmbH.*]

OXYGEN-INDEPENDENT KILLING MECHANISMS Activated macrophages also synthesize lysozyme and various hydrolytic enzymes whose degradative activities do not require oxygen. In addition, activated macrophages produce a group of antimicrobial and cytotoxic peptides, commonly known as defensins. These molecules are cysteine-rich cationic peptides containing 29–35 amino-acid residues. Each peptide, which contains six invariant cysteines, forms a circular molecule that is stabilized by intramolecular disulfide bonds. These circularized defensin peptides have been shown to form ion-permeable channels in bacterial cell membranes. Defensins can kill a variety of bacteria, including Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli, Pseudomonas aeruginosa, and Haemophilus influenzae. Activated macrophages also secrete tumor necrosis factor (TNF-), a cytokine that has a variety of effects and is cytotoxic for some tumor cells.
ANTIGEN PROCESSING AND PRESENTATION
Although most of the antigen ingested by macrophages is degraded and eliminated, experiments with radiolabeled antigens have demonstrated the presence of antigen peptides on the macrophage membrane. As depicted in Figure 2-9b, phagocytosed antigen is digested within the

endocytic processing pathway into peptides that associate with class II MHC molecules; these peptide–class II MHC complexes then move to the macrophage membrane. Activation of macrophages induces increased expression of both class II MHC molecules and the co-stimulatory B7 family of membrane molecules, thereby rendering the macrophages more effective in activating TH cells. This processing and presentation of antigen, are critical to TH-cell activation,a central event in the development of both humoral and cell-mediated immune responses.

SECRETION OF FACTORS

A number of important proteins central to development of immune responses are secreted by activated macrophages (Table 2-7). These include a collection of cytokines, such as interleukin 1 (IL-1), TNF- and interleukin 6 (IL-6), that promote inflammatory responses. Typically, each of these agents has a variety of effects. For example, IL-1 activates lymphocytes; andIL-1,IL-6,and TNF- promote fever by affecting the thermoregulatory center in the hypothalamus.

Activated macrophages secrete a variety of factors involved in the development of an inflammatory response.The complement proteins are a group of proteins that assist in eliminating foreign pathogens and in promoting the ensuing inflammatory reaction. The major site of synthesis of complement proteins is the liver, although these proteins are also produced in macrophages. The hydrolytic enzymes contained within the lysosomes of macrophages also can be secreted when the cells are activated. The buildup of these enzymes within the tissues contributes to the inflammatory response and can, in some cases, contribute to extensive tissue damage. Activated macrophages also secrete soluble factors, such as TNF-, that can kill a variety of cells. The secretion of these cytotoxic factors has been shown to contribute to tumor destruction by macrophages. Finally, as mentioned earlier, activated macrophages secrete a number of cytokines that stimulate inducible hematopoiesis.

Granulocytic Cells

The granulocytes are classified as neutrophils, eosinophils, or basophils on the basis of cellular morphology and cytoplasmic staining characteristics (Figure 2-10). The neutrophil has a multilobed nucleus and a granulated cytoplasm that stains with both acid and basic dyes; it is often called a polymorphonuclear leukocyte (PMN) for its multilobed nucleus. The eosinophil has a bilobed nucleus and a granulated cytoplasm that stains with the acid dye eosin red (hence its name). The basophil has a lobed nucleus and heavily granulated cytoplasm that stains with the basic dye methylene blue. Both neutrophils and eosinophils are phagocytic, whereas basophils are not. Neutrophils, which constitute 50%–70%of the circulating white blood cells, are much more numerous than eosinophils (1%–3%) or basophils (1%).

NEUTROPHILS

Neutrophils are produced by hematopoiesis in the bone marrow.They are released into the peripheral blood and circulate for 7–10 h before migrating into the tissues, where they have a life span of only a few days.In response to many types of infections,the bone marrow releases more than the usual number of neutrophils and these cells generally are the first to arrive at a site of inflammation. The resulting transient increase in the number of circulating neutrophils,called leukocytosis, is used medically as an indication of infection.

Movement of circulating neutrophils into tissues, called extravasation, takes several steps: the cell first adheres to the vascular endothelium, then penetrates the gap between adjacent endothelial cells lining the vessel wall, and finally penetrates the vascular basement membrane, moving out into the tissue spaces. A number of substances generated in an inflammatory reaction serve as chemotactic factors that promote accumulation of neutrophils at an

inflammatory site.Among these chemotactic factors are some of the complementcomponents,components of the blood-clotting system,and several cytokines secreted by activated TH cells and macrophages.

Like macrophages, neutrophils are active phagocytic cells. Phagocytosis by neutrophils is similar to that described for macrophages, except that the lytic enzymes and bactericidal substances in neutrophils are contained within primary and secondary granules (see Figure 2-10a).The larger,denser primary granules are a type of lysosome containing peroxidase, lysozyme, and various hydrolytic enzymes. The smaller secondary granules contain collagenase, lactoferrin, and lysozyme. Both primary and secondary granules fuse with phagosomes, whose contents are then digested and eliminated much as they are in macrophages.

Neutrophils also employ both oxygen-dependent and oxygen-independent pathways to generate antimicrobial substances. Neutrophils are in fact much more likely than macrophages to kill ingested microorganisms. Neutrophils exhibit a larger respiratory burst than macrophages and consequently are able to generate more reactive oxygen intermediates and reactive nitrogen intermediates. In addition, neutrophils express higher levels of defensins than macrophages do.

EOSINOPHILS

Eosinophils, like neutrophils, are motile phagocytic cells that can migrate from the blood into the tissue spaces. Their phagocytic role is significantly less important than that of neutrophils, and it is thought that they play a role in the defense against parasitic organisms. The secreted contents of eosinophilic granules may damage the parasite membrane.

BASOPHILS

Basophils are nonphagocytic granulocytes that function by releasing pharmacologically active substances from their cytoplasmic granules.These substances play a major role in certain allergic responses.

MAST CELLS

Mast-cell precursors, which are formed in the bone marrow by hematopoiesis, are released into the blood as undifferentiated cells; they do not differentiate until they leave the blood and enter the tissues. Mast cells can be found in a wide variety of tissues,including the skin,connective tissues of various organs, and mucosal epithelial tissue of the respiratory, genitourinary, and digestive tracts. Like circulating basophils, these cells have large numbers of cytoplasmic granules that contain histamine and other pharmacologically active substances.Mast cells,together with blood basophils,play an important role in the development of allergies.

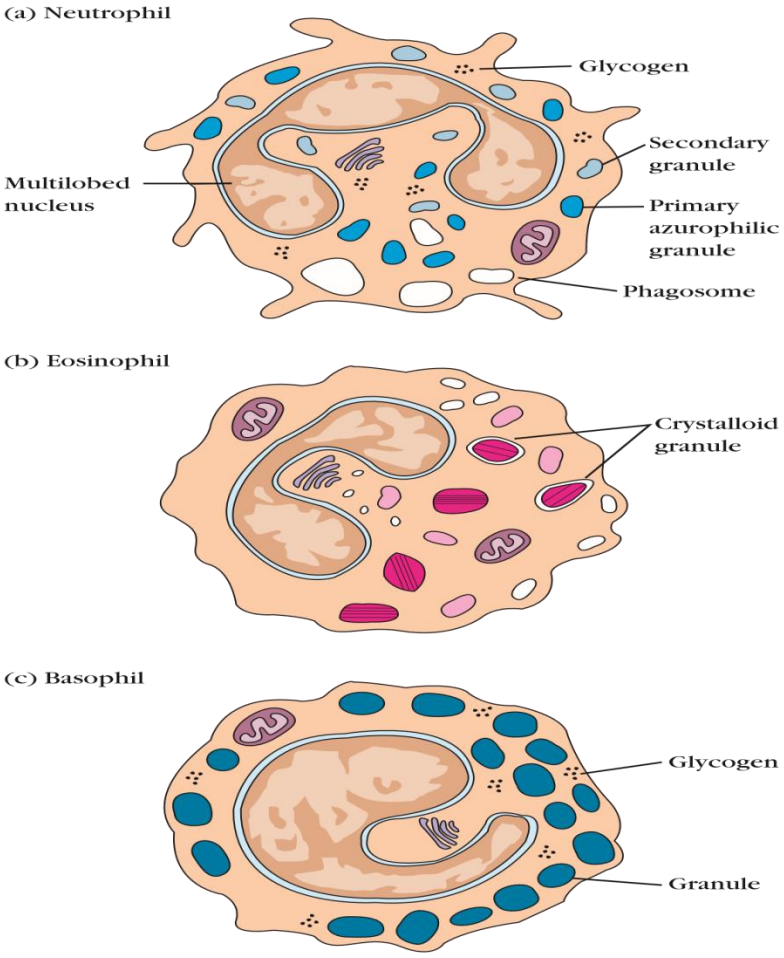


FIGURE 2-10 Drawings showing typical morphology of granulo-

DENDRITIC CELLS

The dendritic cell (DC) acquired its name because it is covered with long membrane extensions that resemble the dendrites of nerve cells. Dendritic cells can be difficult to isolate because the conventional procedures for cell isolation tend to damage their long extensions. The development of isolation techniques that employ enzymes and gentler dispersion has facilitated isolation of these cells for study in vitro. There are many types of dendritic cells, although most mature dendritic cells have the same major function, the presentation of antigen to TH cells. Four types of dendritic cells are known: Langerhans cells, interstitial dendritic cells, myeloid cells, and lymphoid dendritic cells. Each arises from hematopoietic stem cells via different pathways and in different locations. Figure 2-11 shows that they descend through both the myeloid and lymphoid lineages. Despite their differences,they all constitutively express high levels of both class II MHC molecules and members of the co-stimulatory B7 family. For this reason, they are more potent antigen-presenting cells than macrophages and B cells, both of which need to be activated before they can function as antigen-presenting cells (APCs). Immature or precursor forms of each of these types of dendritic cells acquire antigen by phagocytosis or endocytosis; the antigen is processed,and mature dendritic cells present it to TH cells. Following microbial

invasion or during inflammation, mature and immature forms of Langerhans cells and interstitial dendritic cells migrate into draining lymph nodes, where they make the critical presentation of antigen to TH cells that is required for the initiation of responses by those key cells.

Another type of dendritic cell,the follicular dendritic cell (Figure 2-12), does not arise in bone marrow and has a different function from the antigen-presenting dendritic cells described above.Follicular dendritic cells do not express class II MHC molecules and therefore do not function as antigenpresenting cells for TH-cell activation. These dendritic cells were named for their exclusive location in organized structures of the lymph node called lymph follicles, which are rich in B cells. Although they do not express class II molecules, follicular dendritic cells express high levels of membrane receptors for antibody,which allows the binding of antigen-antibody complexes. The interaction of B cells with this bound antigen can have important effects on B cell responses.

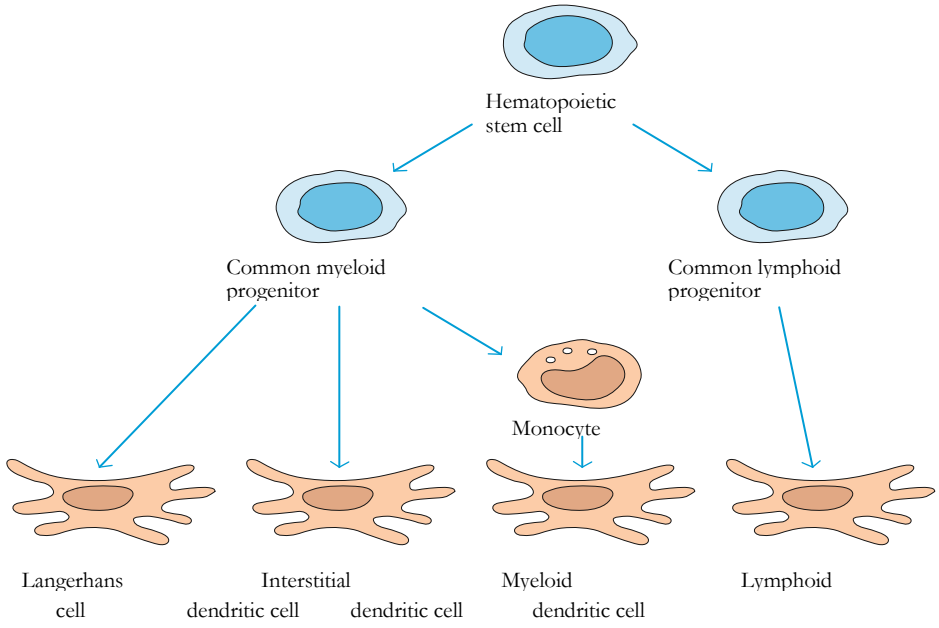


FIGURE 2-11 Dendritic cells arise from both the myeloid and lymphoid lineages.

Organs of the Immune System

A number of morphologically and functionally diverse organs and tissues have various functions in the development of immune responses. These can be distinguished by function as the primary and secondary lymphoid organs (Figure 2-13). The thymus and bone marrow are the primary (or central) lymphoid organs, where maturation of lymphocytes takes place. The lymph nodes, spleen, and various mucosalassociated lymphoid tissues (MALT) such as gut-associated lymphoid tissue (GALT) are the secondary (or peripheral) lymphoid organs, which trap antigen and provide sites for mature lymphocytes to interact with that antigen. In addition, tertiary lymphoid tissues, which normally contain fewer lymphoid cells than secondary lymphoid organs, can import lymphoid cells during an inflammatory response. Most prominent of these are cutaneous-associated lymphoid tissues.Once mature lymphocytes have been generated in the

primary lymphoid organs, they circulate in the blood and lymphatic system, a network of vessels that collect fluid that has escaped into the tissues from capillaries of the circulatory system and ultimately return it to the blood.

Primary Lymphoid Organs

Immature lymphocytes generated in hematopoiesis mature and become committed to a particular antigenic specificity within the primary lymphoid organs. Only after a lympho-cyte has matured within a primary lymphoid organ is the cell immunocompetent (capable of mounting an immune response). T cells arise in the thymus, and in many mammals—humans and mice for example—B cells originate in bone marrow.

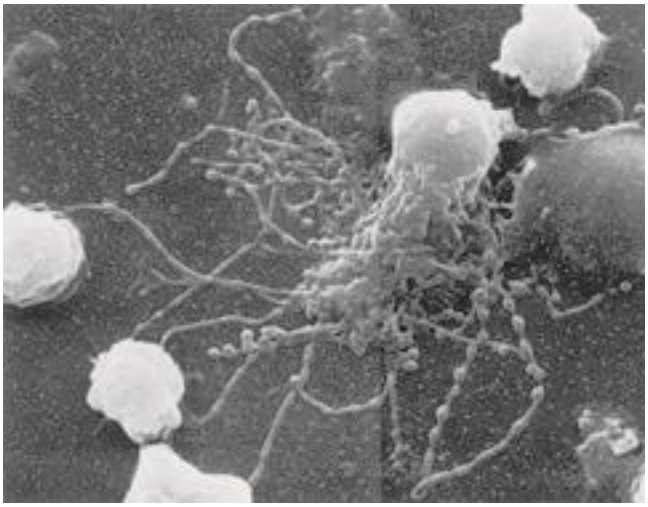


FIGURE 2-12

Scanning electron micrograph of follicular dendritic cells showing long, beaded dendrites. The beads are coated with antigen-antibody complexes. The dendrites emanate from the cell body. [*From A. K. Szakal et al., 1985,* J. Immunol. *134:1353; © 1996 by American Association of Immunologists, reprinted with permission.*]

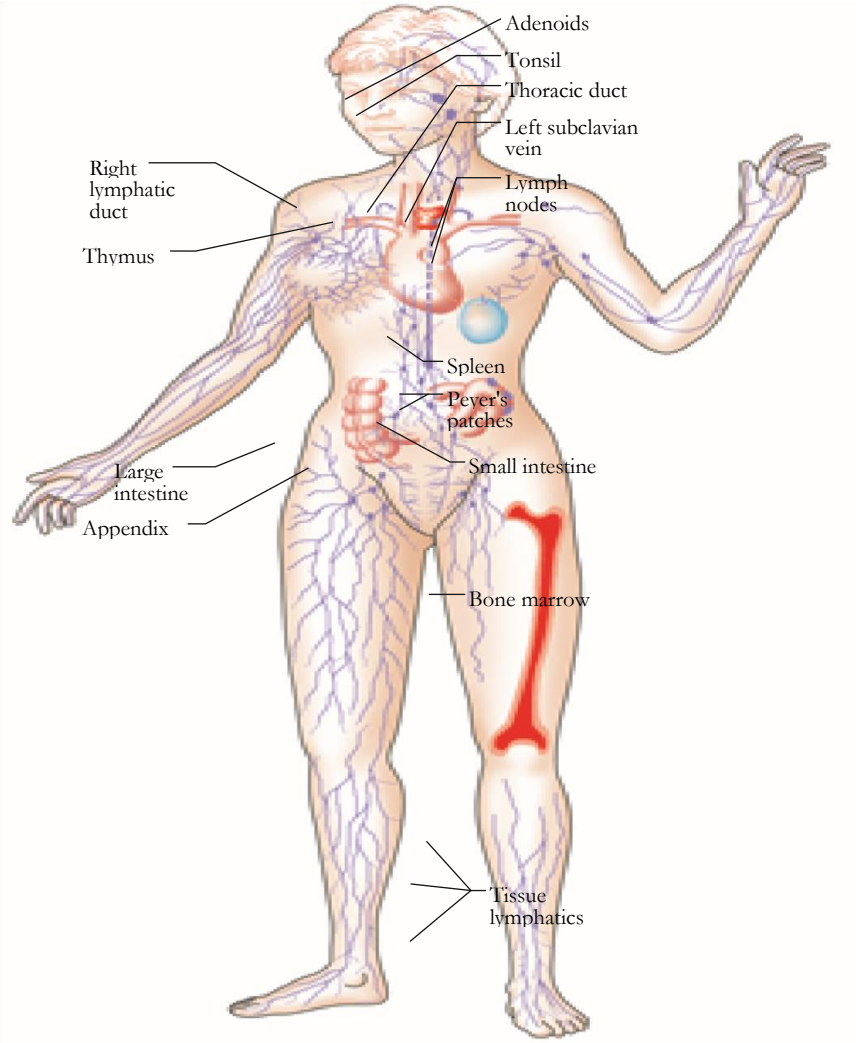


FIGURE 2-13

The human lymphoid system. The primary organs (bone marrow and thymus) are shown in red; secondary organs and tissues, in blue. These structurally and functionally diverse lymphoid organs and tissues are interconnected by the blood vessels (not shown) and lymphatic vessels (purple) through which lymphocytes circulate. Only one bone is shown, but all major bones contain marrow and thus are part of the lymphoid system. [*Adapted from H. Lodish et al., 1995,* Molecular Cell Biology, *3rd ed., Scientific American Books.*]

THYMUS

The thymus is the site of T-cell development and maturation. It is a flat, bilobed organ situated above the heart. Each lobe is surrounded by a capsule and is divided into lobules, which are separated from each other by strands of connective tissue called trabeculae.Each lobule is organized into two compartments: the outer compartment, or cortex, is densely packed with immature T cells, called thymocytes, whereas the inner compartment, or medulla, is sparsely populated with thymocytes.

Both the cortex and medulla of the thymus are crisscrossed by a three-dimensional stromal-cell network composed of epithelial cells, dendritic cells, and macrophages, which make up the framework of the organ and contribute to the growth and maturation of thymocytes. Many of these stromal cells interact physically with the developing thymocytes (Figure 2-14). Some thymic epithelial cells in the outer cortex, called nurse cells, have long membrane extensions that surround as many as 50 thymocytes, forming large multicellular complexes. Other cortical epithelial cells have long interconnecting cytoplasmic extensions that form a network and have been shown to interact with numerous thymocytes as they traverse the cortex.

The function of the thymus is to generate and select a repertoire of T cells that will protect the body from infection. As thymocytes develop, an enormous diversity of T-cell receptors is generated by a random process that produces some T cells with receptors capable of recognizing antigen-MHC complexes. However, most of the T-cell receptors produced by this random process are incapable of recognizing antigen-MHC complexes and a small portion react with combinations of self antigen-MHC complexes. Using mechanisms that are discussed in Chapter 10, the thymus induces the death of those T cells that cannot recognize antigen-MHC complexes and those that react with self-antigen– MHC and pose a danger of causing autoimmune disease. More than 95% of all thymocytes die by apoptosis in the thymus without ever reaching maturity.

THE THYMUS AND IMMUNE FUNCTION

The role of the thymus in immune function can be studied in mice by examining the effects of neonatal thymectomy, a procedure in which the thymus is surgically removed from newborn mice. These thymectomized mice show a dramatic decrease in circulating lymphocytes of the T-cell lineage and an absence of cell-mediated immunity. Other evidence of the importance of the thymus comesfrom studies of a congenital birth defect in humans (DiGeorge's syndrome) and in certain mice (nude mice) in which the thymus fails to develop. In both cases,there is an absence of circulating T cells and of cell-mediated immunity and an increase in infectious disease.

Aging is accompanied by a decline in thymic function. This decline may play some role in the decline in immune function during aging in humans and mice. The thymus reaches its maximal size at puberty and then atrophies, with a significant decrease in both cortical and medullary cells andan increase in the total fat content of the organ. Whereas the average weight of the thymus is 70 g in infants,its age-dependent involution leaves an organ with an average weight of only 3 g in the elderly (Figure 2-15).

A number of experiments have been designed to look at the effect of age on the immune function of the thymus. In one experiment, the thymus from a 1-day-oldor 33-monthold mouse was grafted into thymectomized adults. (For most laboratory mice, 33 months is very old.) Mice receiving the newborn thymus graft showed a significantly larger improvement in immune function than mice receiving the 33month-old thymus.

BONE MARROW

In humans and mice, bone marrow is the site of B-cell origin and development. Arising from lymphoid progenitors, immature B cells proliferate and differentiate within the bone marrow, and

stromal cells within the bonemarrow interact directly with the B cells and secrete various cytokines that are required for development. Like thymic selection during Tcell maturation, a selection process within the bone marrow eliminates B cells with self-reactive antibody receptors. This process is explained in detail in Chapter 11. Bone marrow is not the site of B-cell development in all species. In birds, a lymphoid organ called the bursa of Fabricius, a lymphoidtissue associated with the gut, is the primary site of B-cell maturation.In mammals such as primates and rodents,there is no bursa and no single counterpart to it as a primary lymphoid organ. In cattle and sheep, the primary lymphoid tissue hosting the maturation, proliferation, and diversification of B cells early in gestation is the fetal spleen. Later in gestation, this function is assumed by a patch of tissue embeddedin the wall of the intestine called the ileal Peyer's patch,which contains a large number (10^{10}) B cells. The rabbit, too, usesgut-associated tissues such as the appendix as primary lymphoid tissue for important steps in the proliferation and diversification of B cells.

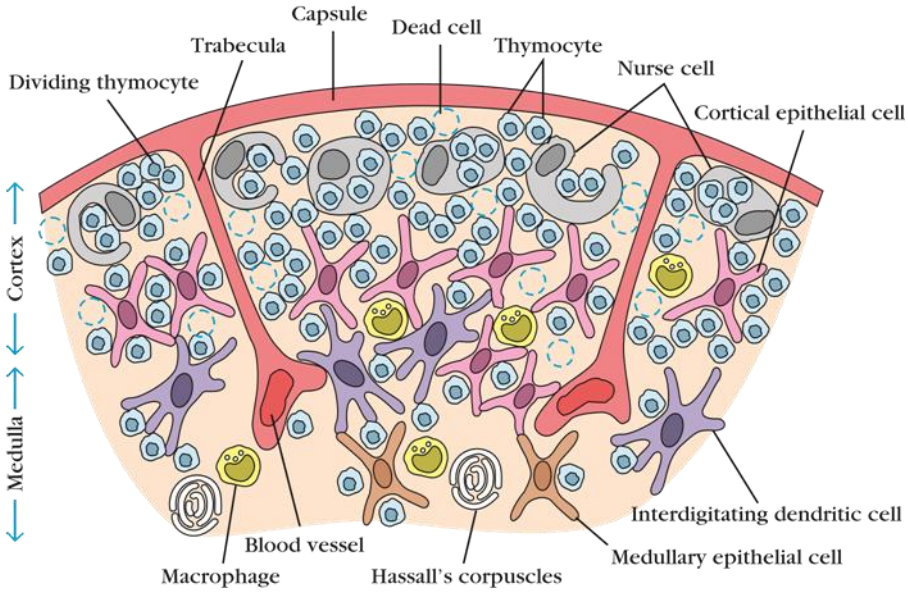


FIGURE: Diagrammatic cross section of a portion of the thymus, showing several lobules separated by connective tissue strands (trabeculae). The densely populated outer cortex is thought to contain many immature thymocytes (blue), which undergo rapid proliferation coupled with an enormous rate of cell death. Also present in the outer cortex are thymic nurse cells (gray), which are specialized epithelial cells with long membrane extensions that surround as many as 50 thymocytes. The medulla is sparsely populated and is thought to contain thymocytes that are more mature. During their stay within the thymus, thymocytes interact with various stromal cells, including cortical epithelial cells (light red), medullary epithelial cells (tan), interdigitating dendritic cells (purple), and macrophages (yellow). These cells produce thymic hormones and express high levels of class I and class II MHC molecules. Hassalls corpuscles, found in the medulla, contain concentric layers of degenerating epithelial cells. [*Adapted, with permission, from W. van Ewijk, 1991,* Annu. Rev. Immunol. ***9:****591, © 1991 by Annual Reviews.*]

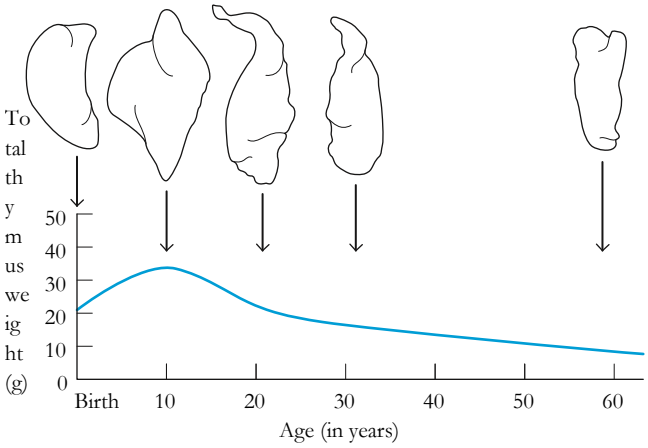


FIGURE 2-15 Changesinthethymuswithage.T

Lymphatic System: As blood circulates under pressure, its fluid component (plasma) seeps through the thin wall of the capillaries into the surrounding tissue. Much of this fluid, called interstitial fluid, returns to the blood through the capillary membranes. The remainder of the interstitial fluid, now called lymph, flows from the spaces in connective tissue into a network of tiny open lymphatic capillaries and then into a series of progressively larger collecting vessels called lymphatic vessels (Figure 2-16).The largest lymphatic vessel, the thoracic duct, empties into the left subclavian vein near the heart (see Figure 2-13). In this way, the lymphatic system captures fluid lost from the blood and returns it to the blood, thus ensuring steady-state levels of fluid within the circulatory system. The heart does not pump the lymph through the lymphatic system; instead the flow of lymph is achieved as the lymph vessels are squeezed by movements of the body's muscles. A series of one-way valves along the lymphatic vessels ensures that lymphflows only in one direction. When a foreign antigen gains entrance to the tissues, it is picked up by the lymphatic system (which drains all the tissues of the body) and is carried to various organizedlymphoid tissues such as lymph nodes, which trap the foreign antigen. As lymph passes from the tissues to lymphatic vessels, it becomes progressively enriched in lymphocytes. Thus, the lymphatic system also serves as a means of transporting lymphocytes and antigen from the connective tissues to organized lymphoid tissues where the lymphocytes may interact with the trapped antigen and undergo activation.

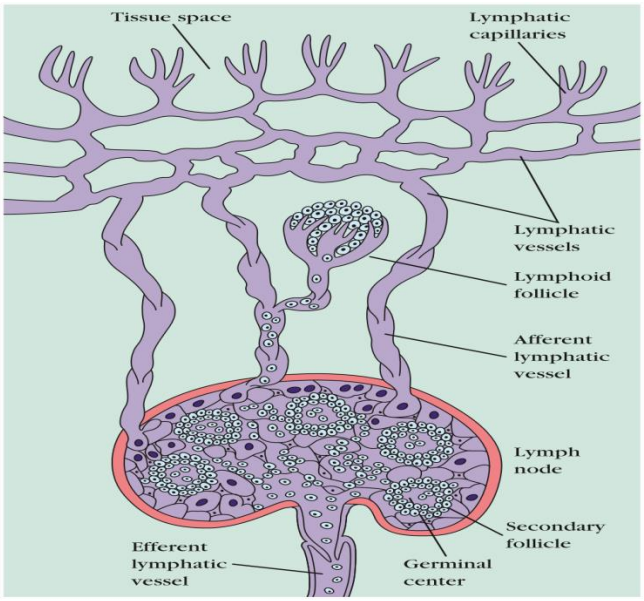


FIGURE 2-16

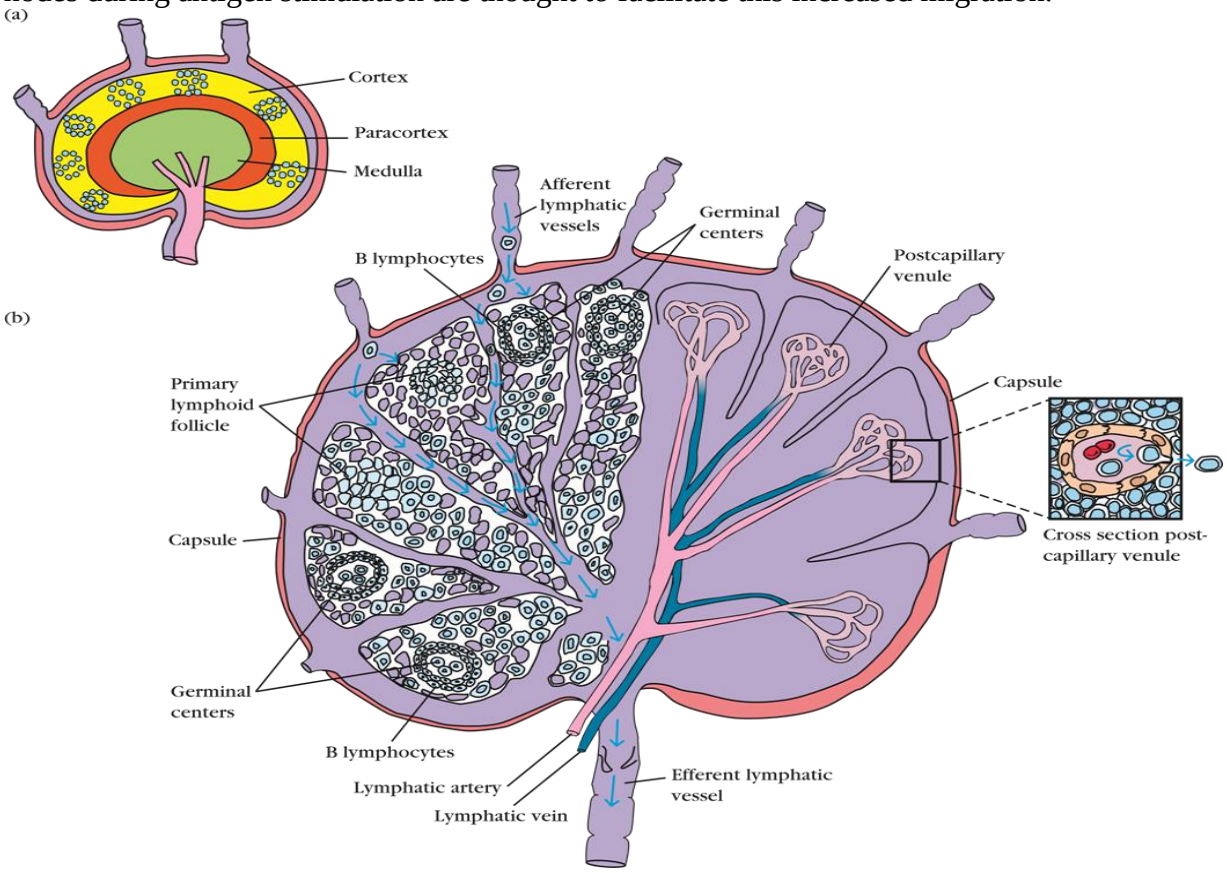
Lymphatic vessels. Small lymphatic capillaries opening into the tissue spaces pick up interstitial tissue fluid and carry it into progressively larger lymphatic vessels, which carry the fluid, now called lymph, into regional lymph nodes. As lymph leaves the nodes, it is carried through larger efferent lymphatic vessels, which eventually drain into the circulatory system at the thoracic duct or right lymph duct (see Figure 2-13).

Secondary Lymphoid Organs: Various types of organized lymphoid tissues are located along the vessels of the lymphatic system. Some lymphoid tissue in the lung and lamina propria of the intestinal wall consists of diffuse collections of lymphocytes and macrophages. Other lymphoid tissue is organized into structures called lymphoid follicles, which consist of aggregates of lymphoid and nonlymphoid cells surrounded by a network of draininglymphatic capillaries. Until it is activated by antigen, a lymphoid follicle—called a primary follicle—comprises a network of follicular dendritic cells and small resting B cells. After an antigenic challenge, a primary follicle becomes a larger secondary follicle—a ring of concentrically packed B lymphocytes surrounding a center (the germinal center) in which one finds a focus of proliferating B lymphocytes and an area that contains nondividing B cells, and some helper T cells interspersed with macrophages and follicular dendritic cells. Most antigen-activated B cells divide and differentiate into antibody-producing plasma cells in lymphoid follicles, but only a few B cells in the antigen-activated population find their way into germinal centers. Those that do undergo one or more rounds of cell division, during which the genes that encode their antibodies mutate at an unusually high rate. Following the period of division and mutation, there is a rigorous selection process in which more than 90% of these B cells die by apoptosis. In general, those B cells producing antibodies that bind antigen more strongly have a much better chance of surviving than do their weaker companions. The small number of B cells that survive the germinal center's rigorous selection differentiate into plasma cells or memorycells and emerge. Lymph nodes and the spleen are the most highly organized of the secondary lymphoid organs; they comprise not only lymphoid follicles, but additional distinct regions of T-cell and B-cell activity, and they are surrounded by a fibrous capsule. Less-organized lymphoid tissue, collectively called mucosal-associated lymphoid tissue (MALT), is found in various body sites. MALT includes Peyer's patches (in the small intestine), the tonsils, and the appendix, as well as

numerous lymphoid follicles within the lamina propria of the intestines and in the mucous membranes lining the upper airways, bronchi, and genital tract.

LYMPH NODES: Lymph nodes are the sites where immune responses are mounted to antigens in lymph. They are encapsulated bean shaped structures containing a reticular network packed with lymphocytes, macrophages, and dendritic cells. Clustered at junctions of the lymphatic vessels, lymph nodes arethe first organized lymphoid structure to encounter antigens that enter the tissue spaces. As lymph percolates through a node, any particulate antigen that is brought in with the lymph will be trapped by the cellular network of phagocytic cells and dendritic cells (follicular and interdigitating). The overall architecture of a lymph node supports an ideal microenvironment for lymphocytes to effectively encounter and respond to trapped antigens.Morphologically, a lymph node can be divided into three roughly concentric regions: the cortex, the paracortex, and the medulla, each of which supports a distinct microenvironment (Figure 2-18). The outermost layer, the cortex, contains lymphocytes (mostly B cells), macrophages, and follicular dendritic cells arranged in primary follicles. After antigenic challenge, the primary follicles enlarge into secondary follicles, each containing a germinal center.In children with B-cell deficiencies, the cortex lacks primary follicles and germinal centers. Beneath the cortex is the paracortex, which is populated largely by T lymphocytes and also contains interdigitating dendritic cells thought to have migrated from tissues to the node. These interdigitating dendritic cells expressof class II MHC molecules, which are necessary for presenting antigen to TH cells. Lymph nodes taken from neonatally thymectomized mice have unusually few cells in the paracortical region; the paracortex is therefore sometimes referred to as a thymus-dependent area in contrast to the cortex, which is a thymus-independent area. The innermost layer of a lymph node, the medulla, is more sparsely populated with lymphoid-lineage cells; of those present, many are plasma cells actively secreting antibody molecules. As antigen is carried into a regional node by the lymph, it is trapped, processed, and presented together with class II MHC molecules by interdigitating dendritic cells in the paracortex, resulting in the activation of TH cells. The initial activation of B cells is also thought to take place within the T-cell-rich paracortex. Once activated, TH and B cells form small foci consisting largely of proliferating B cells at the edges of the paracortex. Some B cells within the foci differentiate into plasma cells secreting IgM and IgG. These foci reach maximum size within 4–6 days of antigen challenge. Within 4–7 days of antigen challenge, a few B cells and TH cells migrate to the primary follicles of the cortex. It is not known what causes this migration.Within a primary follicle, cellular interactions between follicular dendritic cells, B cells, and TH cells take place, leading to development of a secondary follicle with a central germinal center. Some of the plasma cells generated in the germinal center move to the medullary areas of the lymph node, and many migrate to bone marrow.Afferent lymphatic vessels pierce the capsule of a lymph node at numerous sites and empty lymph into the subcapsular sinus (see Figure 2-18b). Lymph coming from the tissues percolates slowly inward through the cortex, paracortex, and medulla, allowing phagocytic cells and dendritic cells to trap any bacteria or particulate material (e.g., antigen-antibody complexes) carried by the lymph. After infection or the introduction of other antigens into the body, the lymph leaving a node through its single efferent lymphatic vessel is enriched with antibodies newly secreted by medullary plasma cells and also has a fiftyfold higher concentration of lymphocytes than the afferent lymph.The increase in lymphocytes in lymph leaving a node is due in part to lymphocyte proliferation within the node in response to antigen. Most of the increase, however, represents blood-borne lymphocytes that migrate into the node by passing between specialized endothelial cells that line the **postcapillary venules** of the

node. Estimates are that 25% of the lymphocytes leaving a lymph node have migrated across this endothelial layer and entered the node from the blood. Because antigenic stimulation within a node can increase this migration tenfold, the concentration of lymphocytes in a node that is actively responding can increase greatly, and the node swells visibly. Factors released in lymph nodes during antigen stimulation are thought to facilitate this increased migration.



Structure of a lymph node. (a) The three layers of a lymph node support distinct microenvironments. (b) The left side depicts the arrangement of reticulum and lymphocytes within the various regions of a lymph node. Macrophages and dendritic cells, which trap antigen, are present in the cortex and paracortex. T_H cells are concentrated in the paracortex; B cells are located primarily in the cortex, within follicles and germinal centers. The medulla is populated largely by antibody-producing plasma cells.

SPLEEN: The spleen plays a major role in mounting immune responses to antigens in the blood stream. It is a large, ovoid secondary lymphoid organ situated high in the left abdominal cavity. While lymph nodes are specialized for trapping antigen from local tissues, the spleen specializes in filtering blood and trapping blood-borne antigens; thus, it can respond to systemic infections. Unlike the lymph nodes, the spleen is not supplied by lymphatic vessels. Instead, bloodborne antigens and lymphocytes are carried into the spleen through the splenic artery. Experiments with radioactively labeled lymphocytes show that more recirculating lymphocytes pass daily through the spleen than through all the lymph nodes combined.The spleen is surrounded by a capsule that extends a number of projections (trabeculae) into the interior to form a compartmentalized structure. The compartments are of two types, the red pulp and white pulp, which are separated by a diffuse marginal zone (Figure 2-19). The splenic red pulp consists of a

network of sinusoids populated by macrophages and numerous red blood cells (erythrocytes) and few lymphocytes; it is the site where old and defective red blood cells are destroyed and removed. Many of the macrophages within the red pulp contain engulfed red blood cells or iron pigments from degraded hemoglobin. The splenic white pulp surrounds the branches of the splenic artery, forming a periarteriolar lymphoid sheath (PALS) populated mainly by T lymphocytes. Primary lymphoid follicles are attached to thePALS.These follicles are rich in B cells and some of them contain germinal centers. The marginal zone, located peripheral to the PALS, is populated by lymphocytes and macrophages. Blood-borne antigens and lymphocytes enter the spleen through the splenic artery, which empties into the marginal zone. In the marginal zone, antigen is trapped by interdigitating dendritic cells, which carry it to the PALS. Lymphocytes in the blood also enter sinuses in the marginal zone and migrate to the PALS. The initial activation of B and T cells takes place in the Tcell-rich PALS. Here interdigitating dendritic cells capture antigen and present it combined with class II MHC molecules to TH cells. Once activated, these TH cells can then activate B cells.The activated B cells,together with some TH cells, then migrate to primary follicles in the marginal zone. Upon antigenic challenge,these primary follicles develop into characteristic secondary follicles containing germinal centers (like those in the lymph nodes), where rapidly dividing B cells (centroblasts) and plasma cells are surrounded by dense clusters of concentrically arranged lymphocytes.The effects of splenectomy on the immune response depends on the age at which the spleen is removed. In children, splenectomy often leads to an increased incidence of bacterial sepsis caused primarily by Streptococcus pneumoniae, Neisseria meningitidis, and Haemophilus influenzae. Splenectomy in adults has less adverse effects, although it leads to some increase in blood-borne bacterial infections (bacteremia).

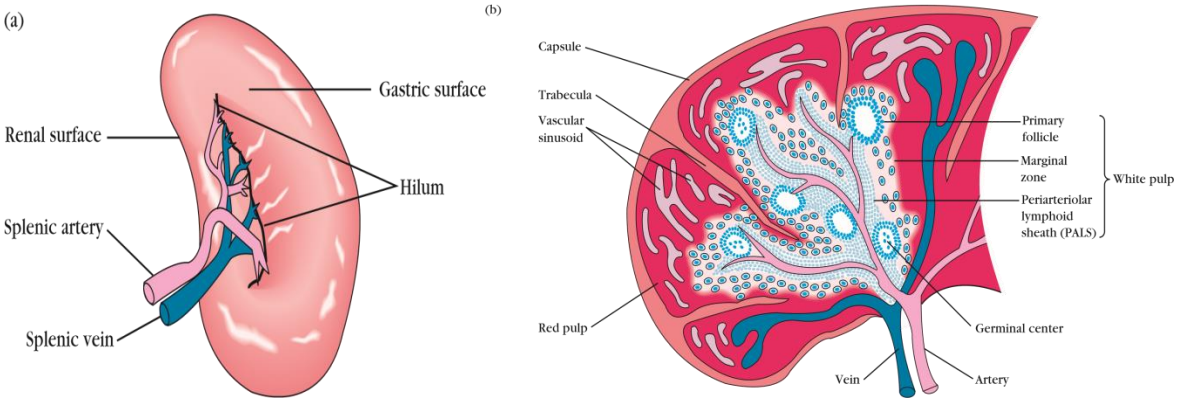


FIGURE 2-19 Structure of the spleen. (a) The spleen, which is cyte-filled red pulp surrounds the sinusoids. (b) Diagrammatic cross section of the spleen.

MUCOSAL-ASSOCIATED LYMPHOID TISSUE

The mucous membranes lining the digestive,respiratory,and urogenital systems have a combined surface area of about 400 m2 (nearly the size of a basketball court) and are the major sites of entry for most pathogens. These vulnerable membrane surfaces are defended by a group of

organized lymphoid tissues mentioned earlier and known collectively as mucosal-associated lymphoid tissue (MALT). Structurally, these tissues range from loose, barely organized clusters of lymphoid cells in the lamina propria of intestinal villi to well-organized structures such as the familiar tonsils and appendix, as well as Peyer's patches, which are found within the submucosal layer of the intestinal lining. The functional importance of MALT in the body's defense is attested to by its large population of antibody-producing plasma cells, whose number far exceedsthat of plasma cells in the spleen, lymph nodes, and bone marrow combined.

The tonsils are found in three locations: lingual at the base of the tongue; palatine at the sides of the back of the mouth; and pharyngeal (adenoids) in the roof of the nasopharynx (Figure 2-20). All three tonsil groups are nodular structures consisting of a meshwork of reticular cells and fibers interspersed with lymphocytes,macrophages,granulocytes, and mast cells. The B cells are organized into follicles and germinal centers; the latter are surrounded by regions showing T-cell activity. The tonsils defend against antigens entering through the nasal and oral epithelial routes.The best studied of the mucous membranes is the one that lines the gastrointestinal tract.This tissue,like that of the respiratory and urogenital tracts, has the capacity to endocytose antigen from the lumen. Immune reactions are initiated against pathogens and antibody can be generated andexported to the lumen to combat the invading organisms. As shown in Figures 2-21 and 2-22, lymphoid cells are found in various regions within this tissue. The outer mucosal epithe-lial layer contains so-called intraepithelial lymphocytes (IELs). Many of these lymphocytes are T cells that express unusual receptors (T-cell receptors, or TCRs), which exhibit limited diversity for antigen. Although this population of T cells is well situated toencounter antigens that enter through the intestinal mucous epithelium, their actualfunction remains largely unknown The lamina propria, which lies under the epithelial layer, contains large numbers of B cells, plasma cells, activated TH cells, and macrophages in loose clusters. Histologic sections have revealed more than 15,000 lymphoid follicles within the intestinal lamina propria of a healthy child. The submucosal layer beneath the lamina propria contains Peyer's patches, nodules of 30–40 lymphoid follicles. Like lymphoid follicles in other sites, those that compose Peyer's patches can develop into secondary follicles with germinal centers.

The epithelial cells of mucous membranes play an important role in promoting the immune response by delivering small samples of foreign antigen from the lumina of the respiratory, digestive, and urogenital tracts to the underlying mucosal-associated lymphoid tissue. This antigen transport is carried out by specialized M cells. The structure of the M cell is striking: these are flattened epithelial cells lacking the microvilli that characterize the rest of the mucous epithelium. In addition, M cells have a deep invagination, or pocket, in the basolateral plasma membrane; this pocket is filled with a cluster of B cells, T cells, and macrophages (Figure 2-22a). Luminal antigens are endocytosed into vesicles that are transported from the luminalmembrane to the underlying pocket membrane. The vesicles then fuse with the pocket membrane, delivering the potentially response-activating antigens to the clusters of lymphocytes contained within the pocket.

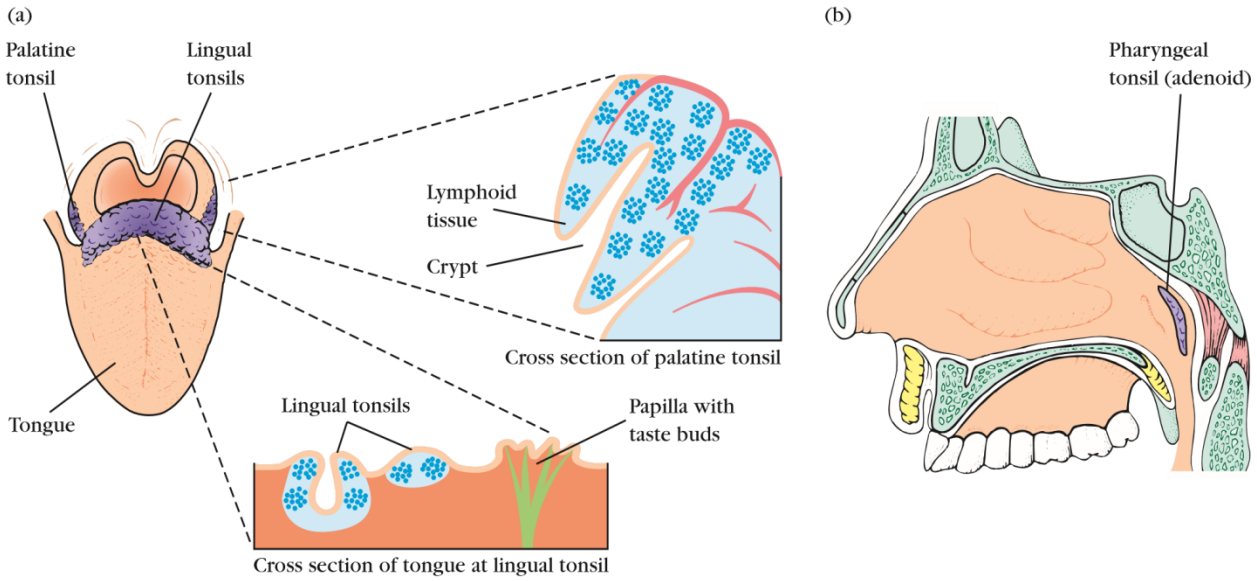


FIGURE 2-20

Three types of tonsils. (a) The position and internal features of the palatine and lingual tonsils; (b) a view of the position of the nasopharyngeal tonsils (adenoids). [Part b adapted from J. Klein, 1982, Immunology, The Science of Self-Nonself Discrimination, © 1982 by John Wiley and Sons, Inc.]

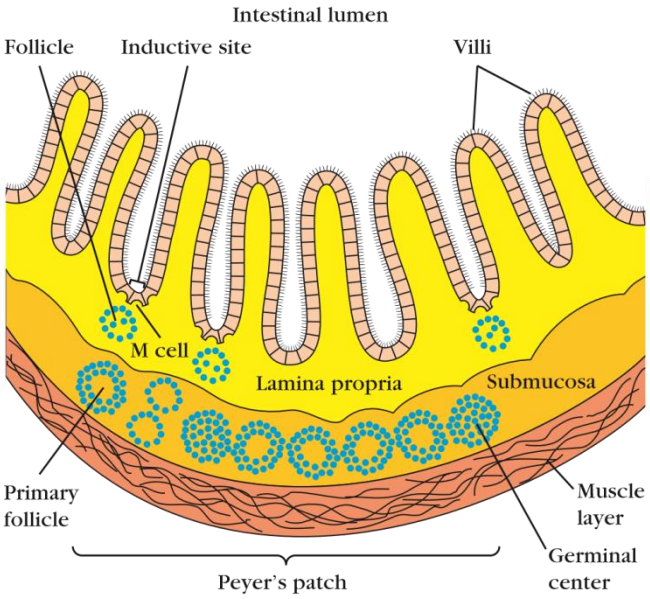


FIGURE 2-21

Cross-sectional diagram of the mucous membrane lining the intestine showing a nodule of lymphoid follicles that constitutes a Peyer's patch in the submucosa. The intestinal lamina propria contains loose clusters of lymphoid cells and diffuse follicles.

M cells are located in so-called inductive sites—small regions of a mucous membrane that lie over organized lymphoid follicles (Figure 2-22b). Antigens transported across the mucous membrane by M cells can activate B cellswithinlymphoid follicles. The activated B cells differentiate into plasma cells, which leave the follicles and secrete the IgA class of antibodies. These antibodies then are transported across the epithelial cells and released as secretory IgA into the lumen, where they can interact with antigens.As described, mucous membranes are an effective barrier to the entrance of most pathogens, which thereby contributes to nonspecific immunity. One reason for this is that the mucosal epithelial cells are cemented to one another by tight junctions that make it difficult for pathogens to penetrate. Interestingly, some enteric pathogens, including both bacteria and viruses, have exploited the M cell as an entry route through the mucous-membrane barrier. In some cases, the pathogen is internalized by the M cell and transported into the pocket. In other cases, the pathogen binds to the M cell and disrupts the cell, thus allowing entry of the pathogen. Among the pathogens that use M cells in these ways are several invasive Salmonella species, *Vibrio cholerae*, and the polio virus.

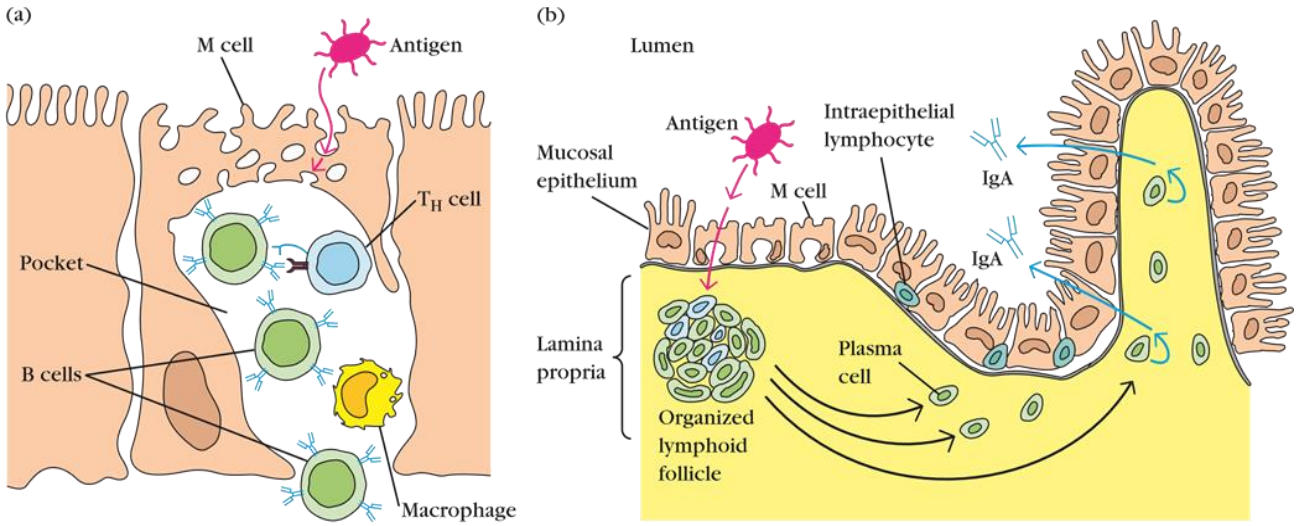


Figure: Structure of M cells and production of IgA at inductive sites. (a) M cells, located in mucous membranes, endocytose antigen from the lumen of the digestive, respiratory, and urogenital tracts. The antigen is transported across the cell and released into the large basolateral pocket. (b) Antigen transported across the epithelial layer by M cells at an inductive site activates B cells in the underlying lymphoid follicles. The activated B cells differentiate into IgA-producing plasma cells, which migrate along the submucosa. The outer mucosal epithelial layer contains intraepithelial lymphocytes, of which many are CD8_ T cells that express _ TCRs with limited receptor diversity for antigen.

Cutaneous-Associated Lymphoid Tissue

The skin is an important anatomic barrier to the external environment, and its large surface area makes this tissue important in nonspecific (innate) defenses. The epidermal(outer) layer of the skin is composed largely of specialized epithelial cells called keratinocytes. These cells secrete a number of cytokines that may function to induce a local inflammatory reaction. In addition, keratinocytes can be induced to express class II MHC molecules and may function as antigen-

presenting cells. Scattered among the epithelial-cell matrix of the epidermis are Langerhans cells, a type of dendritic cell, which internalize antigen by phagocytosis or endocytosis. The Langerhans cells then migrate from the epidermis toregional lymph nodes, where they differentiate into interdigitating dendritic cells. These cells express high levels of class II MHC molecules and function as potent activators of naive TH cells.
The epidermis also contains so-called intraepidermal lymphocytes. These are similar to the intraepithelial lymphocytes of MALT in that most of them are CD8 T cells, many of which express T-cell receptors, which have limited diversity for antigen. These intraepidermal T cells are well situated to encounter antigens that enter through the skin and some immunologists believe that they may play a role in combating antigens that enter through the skin. The underlying dermal layer of the skin contains scattered CD4 and CD8 T cells and macrophages. Most of these dermal T cells were either previously activated cells or are memory cells.

Cell- mediated and humoral immunity

Cell-mediated immunity is an immune response that does not involve antibodies. Rather, cell mediated immunity is the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to antigen. Historically, the immune system was separated into two branches: humoral immunity, for which the protective function of immunization could be found in the humor (cell-free bodily fluid or serum) and **cellular immunity**, for which the protective function of immunization was associated with cells. CD4 cells or helper T cells provide protection against different pathogens. Naive T cells, which are mature T cells that have yet to encounter an antigen, are converted into activated effector T cells after encountering antigen-presenting cells (APCs). These APCs, such as macrophages, dendritic cells, and B cells in some circumstances, load antigenic peptides onto the MHC of the cell, in turn presenting the peptide to receptors on T cells. The most important of these APCs are highly specialized dendritic cells; conceivably operating solely to ingest and present antigens. [1]

Activated Effector T cells can be placed into three functioning classes, detecting peptide antigens originating from various types of pathogen: The first class being 1) Cytotoxic T cells, which kill infected target cells by apoptosis without using cytokines, 2) TH1 cells, which primarily function to activate macrophages, and 3) TH2 cells, which primarily function to stimulate B cells into producing antibodies.

The innate immune system and the adaptive immune system each comprise both humoral and cell-mediated components.

Cellular immunity protects the body through:

- T-cell mediated immunity or T-cell immunity: activating antigen-specific cytotoxic T cells that are able to induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens;
- Macrophage and natural killer cell action: enabling the destruction of pathogens via recognition and secretion of cytotoxic granules (for natural killer cells) and phagocytosis (for macrophages); and

- Stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses.

Cell-mediated immunity is directed primarily at microbes that survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in defending against fungi, protozoans, cancers, and intracellular bacteria. It also plays a major role in transplant rejection.

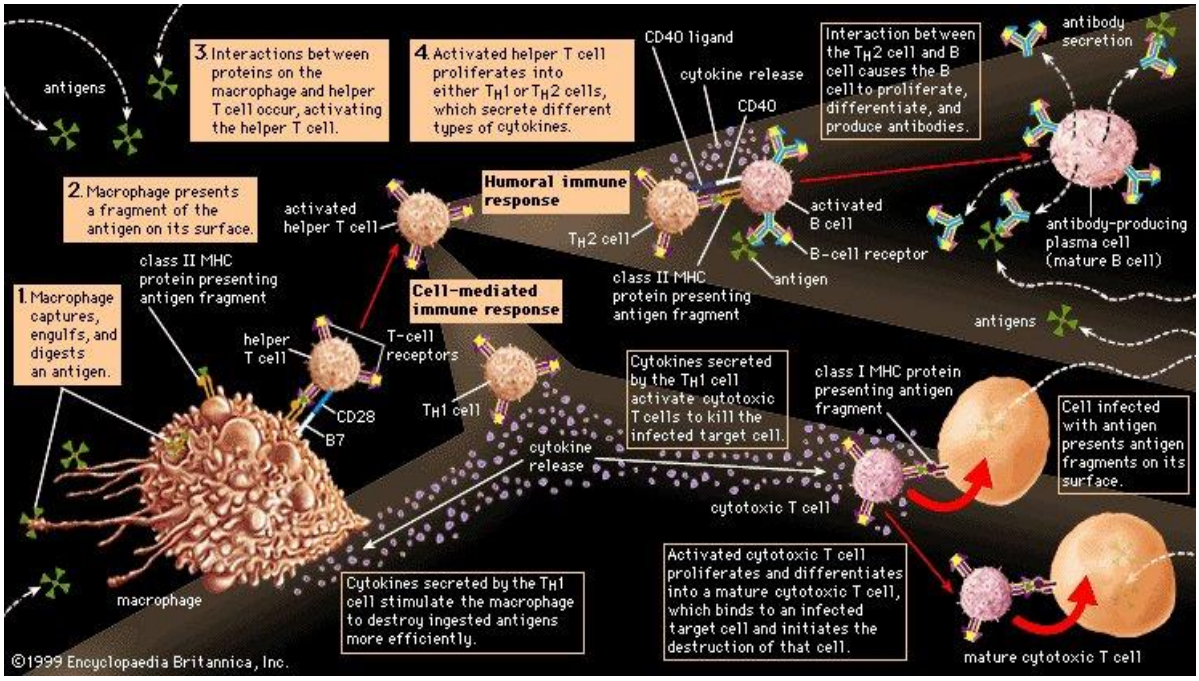


Figure: Stimulation of immune response by activated helper T cells

Activated by complex interaction with molecules on the surface of a macrophage or some other antigen-presenting cell, a helper T cell proliferates into two general subtypes, T_H1 and T_H2. These in turn stimulate the complex pathways of the cell-mediated immune response and the humoral immune response, respectively.

Humoral immunity is the aspect of immunity that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins, and certain antimicrobial peptides. Humoral immunity is so named because it involves substances found in the humors, or body fluids. It contrasts with cell-mediated immunity. Its aspects involving antibodies are often called antibody-mediated immunity.The study of the molecular and cellular components that form the immune system, including their function and interaction, is the central science of immunology. The immune system is divided into a more primitive innate immune system, and acquired or adaptive immune system of vertebrates, each of which contains humoral and cellular components.Humoral immunity refers to antibody production and the accessory processes that accompany it, including: Th2 activation and cytokine production, germinal center formation and isotype switching, affinity maturation and memory cell generation. It also refers to the effector functions of antibodies, which include pathogen and toxin neutralization, classical complement activation, and opsonin promotion of phagocytosis and pathogen elimination.The concept of humoral immunity developed based on analysis of antibacterial activity of the serum

components. Hans Buchner is credited with the development of the humoral theory. In 1890 he described alexins, or "protective substances", which exist in the blood serum and other bodily fluid and are capable of killing microorganisms. Alexins, later redefined "complement" by Paul Ehrlich, were shown to be the soluble components of the innate response that lead to a combination of cellular and humoral immunity, and bridged the features of innate and acquired immunity. Following the 1888 discovery of the bacteria that cause diphtheria and tetanus, Emil von Behring and Kitasato Shibasaburō showed that disease need not be caused by microorganisms themselves. They discovered that cell-free filtrates were sufficient to cause disease. In 1890, filtrates of diphtheria, later named diphtheria toxins, were used to vaccinate animals in an attempt to demonstrate that immunized serum contained an antitoxin that could neutralize the activity of the toxin and could transfer immunity to non-immune animals. In 1897, Paul Ehrlich showed that antibodies form against the plant toxins ricin and abrin, and proposed that these antibodies are responsible for immunity. Ehrlich, with his friend Emil von Behring, went on to develop the diphtheria antitoxin, which became the first major success of modern immunotherapy. The presence and specificity of compatibility antibodies became the major tool for standardizing the state of immunity and identifying the presence of previous infections.

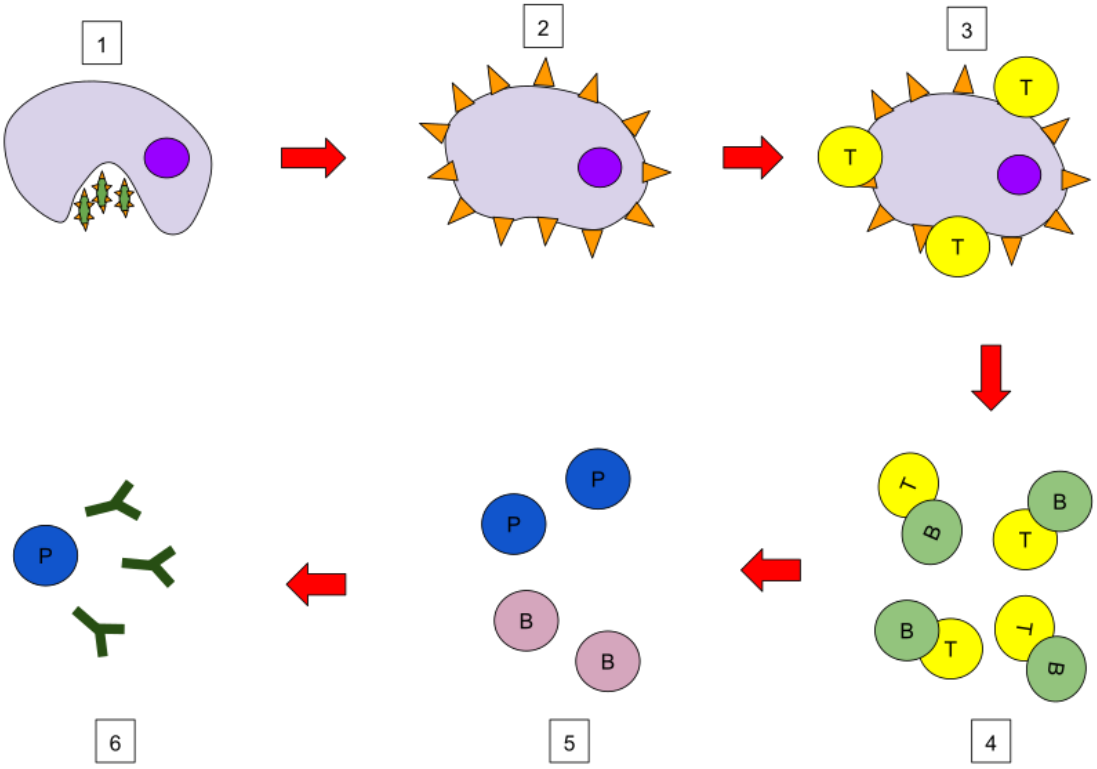
Major discoveries in the study of humoral immunity

Substance	Activity	Discovery
Alexin(s) Complement	Soluble components in the serum that are capable of killing microorganisms	Buchner (1890), Ehrlich (1892)
Antitoxins	Substances in the serum that can neutralize the activity of toxins, enabling passive immunization	von Behring and Kitasato (1890)
Bacteriolysins	Serum substances that work with the complement proteins to induce bacterial lysis	Richard Pfeiffer (1895)
Bacterial agglutinins and precipitins	Serum substances that agglutinate bacteria and precipitate bacterial toxins	von Gruber and Durham (1896), Kraus (1897)
Hemolysins	Serum substances that work with complementto lyse red blood cells	Belfanti and Carbone (1898) Jules Bordet (1899)
Opsonins	Serum substances that coat the outer membraneof foreign substances and enhance the rate of phagocytosis by macrophages	Wright and Douglas (1903)
Antibody	Formation (1900), antigen-antibody binding hypothesis (1938), produced by B cells (1948), structure (1972), immunoglobulin genes (1976)	Founder: P Ehrlich

Mechanism:

In humoral immune response, first the B cells mature in the bone marrow and gain B-cell receptors (BCR's) which are displayed in large number on the cell surface.These membrane-

bound protein complexes have antibodies which are specific for antigen detection. Each B cell has a unique antibody that binds with an antigen. The mature B cells migrate from the bone marrow to the lymph nodes or other lymphatic organs, where they begin to encounter pathogens.



Step 1: A macrophage engulfs the pathogen. Step 2: The macrophage then digests the bacterium and presents the pathogen's antigens. Step 3: A T helper cell binds to the macrophage and becomes an activated T helper cell. Step 4: The activated T helper cell binds to a B cell in order to activate the B cell. Step 5: When the B cells are activated, some B cells turn into plasma cells and are released in the blood, while other B cells become B memory cells that quicken response for a second exposure. Step 6: Plasma cells then secrete antibodies, which bind to antigens to fight the invading pathogens.

B cell activation

When a B cell encounters an antigen, the antigen is bound to the receptor and taken inside the B cell by endocytosis. The antigen is processed and presented on the B cell's surface again by MHC-II proteins.

B cell proliferation

The B cell waits for a helper T cell (TH) to bind to the complex. This binding will activate the TH cell, which then releases cytokines that induce B cells to divide rapidly, making thousands of identical clones of the B cell. These daughter cells either become plasma cells or memory cells. The memory B cells remain inactive here; later when these memory B cells encounter the same

antigen due to reinfection, they divide and form plasma cells. On the other hand, the plasma cells produce a large number of antibodies which are released free into the circulatory system.

Antibody-antigen reaction

Now these antibodies will encounter antigens and bind with them. This will either interfere with the chemical interaction between host and foreign cells, or they may form bridges between their antigenic sites hindering their proper functioning, or their presence will attract macrophages or killer cells to attack and phagocytose them.

Complement system

The complement system is a biochemical cascade of the innate immune system that helps clear pathogens from an organism. It is derived from many small blood plasma proteins that work together to disrupt the target cell's plasma membrane leading to cytolysis of the cell. The complement system consists of more than 35 soluble and cell-bound proteins, 12 of which are directly involved in the complement pathways. The complement system is involved in the activities of both innate immunity and acquired immunity.

Activation of this system leads to cytolysis, chemotaxis, opsonization, immune clearance, and inflammation, as well as the marking of pathogens for phagocytosis. The proteins account for 5% of the serum globulin fraction. Most of these proteins circulate as zymogens, which are inactive until proteolytic cleavage.

Three biochemical pathways activate the complement system: the classical complement pathway, the alternate complement pathway, and the mannose-binding lectin pathway. The classical complement pathway typically requires antibodies for activation and is a specific immune response, while the alternate pathway can be activated without the presence of antibodies and is considered a non-specific immune response. Antibodies, in particular the IgG1 class, can also "fix" complement.

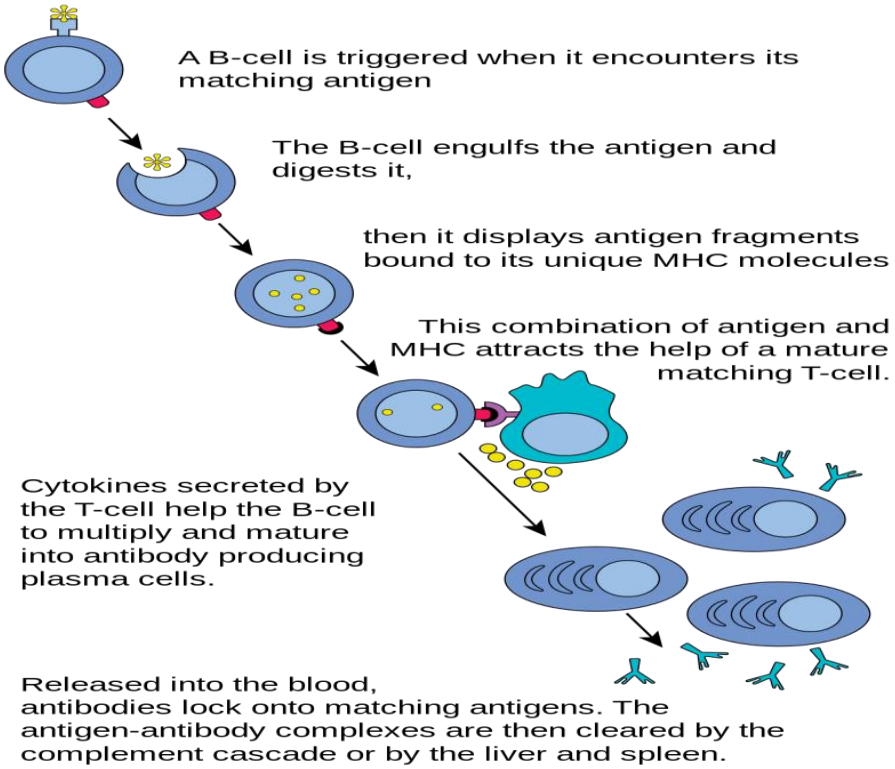


Fig: B cell activation is a large part of the humoral immune response.

Antibodies

Immunoglobulins are glycoproteins in the immunoglobulin superfamily that function as antibodies. The terms antibody and immunoglobulin are often used interchangeably. They are found in the blood and tissue fluids, as well as many secretions. In structure, they are large Y-shaped globular proteins. In mammals there are five types of antibody: IgA, IgD, IgE, IgG, and IgM. Each immunoglobulin class differs in its biological properties and has evolved to deal with different antigens. Antibodies are synthesized and secreted by plasma cells that are derived from the B cells of the immune system.

An antibody is used by the acquired immune system to identify and neutralize foreign objects like bacteria and viruses. Each antibody recognizes a specific antigen unique to its target. By binding their specific antigens, antibodies can cause agglutination and precipitation of antibody-antigen products, prime for phagocytosis by macrophages and other cells, block viral receptors, and stimulate other immune responses, such as the complement pathway.

An incompatible blood transfusion causes a transfusion reaction, which is mediated by the humoral immune response. This type of reaction, called an acute hemolytic reaction, results in the rapid destruction (hemolysis) of the donor red blood cells by host antibodies. The cause is

usually a clerical error, such as the wrong unit of blood being given to the wrong patient. The symptoms are fever and chills, sometimes with back pain and pink or red urine (hemoglobinuria). The major complication is that hemoglobin released by the destruction of red blood cells can cause acute kidney failure.

MHC (Major histocompatibility complex)

The major histocompatibility complex (MHC) is a large locus on vertebrate's DNA containing a set of closely linked polymorphic genes that code for cell surface proteins essential for the acquired immune system. This locus got its name because it was discovered in the study of tissue compatibility upon transplantation. Later studies revealed that tissues rejection due to incompatibility is an experimental artifact masking the real function of MHC molecules - binding an antigen derived from self-proteins or from pathogen and the antigen presentation on the cell surface for recognition by the appropriate T-cells. MHC molecules mediate interactions of leukocytes, also called white blood cells (WBCs), which are immune cells, with other leukocytes or with body cells. The MHC determines compatibility of donors for organ transplant, as well as one's susceptibility to an autoimmune disease via cross-reacting immunization.

In a cell, protein molecules of the host's own phenotype or of other biologic entities are continually synthesized and degraded. Each MHC molecule on the cell surface displays a small peptide, molecular fraction of a protein, called an epitope. The presented self-antigens prevent an organism's immune system targeting its own cells. Presentation of pathogen-derived proteins results in the elimination of the infected cell by the immune system.

Diversity of antigen presentation, mediated by MHC antigens, is attained in at least three ways: **(1)** an organism's MHC repertoire is polygenic (via multiple, interacting genes); **(2)** MHC expression is codominant (from both sets of inherited alleles); **(3)** MHC gene variants are highly polymorphic (diversely varying from organism to organism within a species) Major histocompatibility complex and sexual selection has been observed in male mice making mate choices of females with different MHCs and thus demonstrating sexual selection. Also, at least for MHC I presentation, there has been evidence of antigenic peptide splicing which can combine peptides from different proteins, vastly increasing antigen diversity.Discovery

The first descriptions of the MHC were made by British immunologist Peter Gorer in 1936. MHC genes were first identified in inbred mice strains. Clarence Little transplanted tumors across differing strains and found rejection of transplanted tumors according to strains of host versus donor. George Snell selectively bred two mouse strains, attained a new strain nearly identical to one of the progenitor strains, but differing crucially in histocompatibility—that is, tissue compatibility upon transplantation—and thereupon identified an MHC locus. Later Jean Dausset demonstrated the existence of MHC genes in human and described the first human leucocyte antigen, the protein which we call now HLA-A2. Some years later Baruj Benacerraf showed that polymorphic MHC genes not only determine an individual’s unique constitution of antigens but also regulate the interaction among the various cells of the immunological system. These three scientists have been awarded the Nobel Prize in Physiology or Medicine for 1980 for their discoveries concerning “genetically determined structures on the cell surface that regulate immunological reactions”.

The first fully sequenced and annotated MHC was published for human in 1999 by the consortium of sequencing centers from UK, USA and Japan in Nature. It was "virtual MHC" since it was mosaic from different individuals. Much shorter MHC locus from chicken was published in the same issue of Nature. Many other species have been sequenced and the evolution of MHC was studied, e.g. in the gray short-tailed opossum (Monodelphis domestica), a marsupial, MHC spans 3.95 Mb, yielding 114 genes, 87 shared with humans. Marsupial MHC

genotypic variation lies between eutherian mammals and birds, taken as minimal MHC encoding, but is closer in organization to that of nonmammals. The special database IPD-MHC Database was created which provides a centralised repository for sequences of the Major Histocompatibility Complex (MHC) from a number of different species. The database contains 77 species for the release from 2019-12-19.

Genes

MHC locus is present in all jawed vertebrates, it is assumed to have arisen about 450 million years ago. Despite the difference in the number of genes included in MHC of different species, the overall organization of the locus is rather similar. Usual MHC contains about hundred genes and pseudogenes, not all of them are involved in the immunity. In humans, the MHC region occurs on chromosome 6, between the flanking genetic markers MOG and COL11A2 (from 6p22.1 to 6p21.3 about 29Mb to 33Mb on the hg38 assembly), and contains 224 genes spanning 3.6 megabase pairs (3 600 000 bases). About half have known immune functions. The human MHC is also called the HLA (human leukocyte antigen) complex (often just the HLA). Similarly, there is SLA (Swine leukocyte antigens), BoLA (Bovine leukocyte antigens), DLA for dogs, etc. However, historically, the MHC in mice is called the Histocompatibility system 2 or just the H-2, in rats - RT1, and in chicken - B-locus.

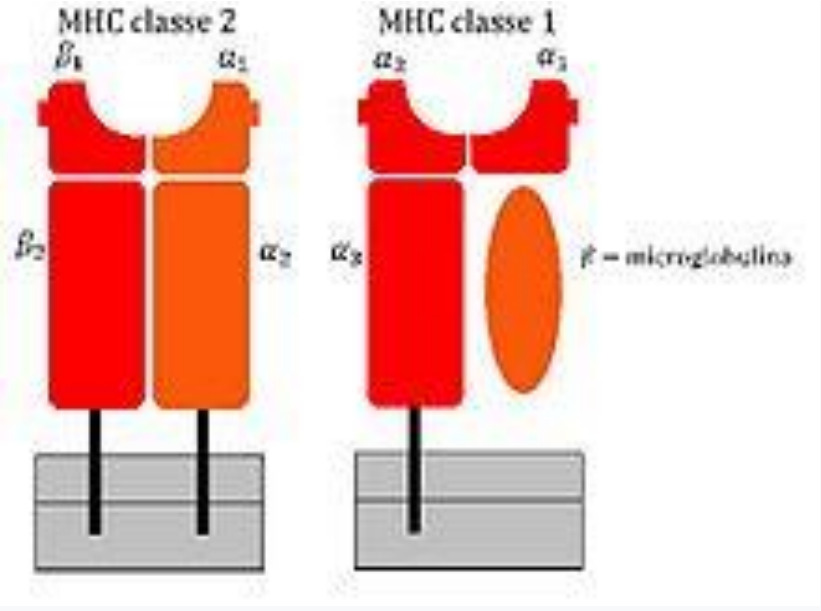
The MHC gene family is divided into three subgroups: MHC class I, MHC class II, and MHC class III. Among all those genes present in MHC, there are two types of genes coding for the proteins MHC class I molecules and MHC class II molecules that directly involved in the antigen presentation.

Class	Encoding	Expression
I	(1) peptide-binding proteins, which select short sequences of amino acids for antigen presentation, as well as (2) molecules aiding antigen-processing (such as TAP and tapasin).	One chain, called α, whose ligands are the CD8 receptor—borne notably by cytotoxic T cells—and inhibitory receptors borne by NK cells
II	(1) peptide-binding proteins and (2) proteins assisting antigen loading onto MHC class II's peptide-binding proteins (such as MHC II DM, MHC II DQ, MHC II DR, and MHC II DP).	Two chains, called α & β, whose ligands are the CD4 receptors borne by helper T cells.
III	Other immune proteins, outside antigen processing and presentation, such as components of the complement cascade (e.g., C2, C4, factor B), the cytokines of immune signaling (e.g., TNF-α), and heat shock proteins buffering cells from stresses	Various

MHC class I

MHC class I molecules are expressed in all nucleated cells and also in platelets—in essence all cells but red blood cells. It presents epitopes to killer T cells, also called cytotoxic T lymphocytes (CTLs). A CTL expresses CD8 receptors, in addition to TCRs. When a CTL's CD8 receptor docks to a MHC class I molecule, if the CTL's TCR fits the epitope within the MHC class I molecule, the CTL triggers the cell to undergo programmed cell death by apoptosis. Thus, MHC class I helps mediate cellular immunity, a primary means to address intracellular pathogens, such as viruses and some bacteria, including bacterial L forms, bacterial genus *Mycoplasma*, and bacterial genus *Rickettsia*. In humans, MHC class I comprises HLA-A, HLA-B, and HLA-C molecules.

The first crystal structure of Class I MHC molecule, human HLA-A2, was published in 1989.[14] The structure revealed that MHC-I molecules are heterodimers, they have polymorphic heavy α-subunit which gene occurs in the MHC locus and small invariant β_2 microglobulin subunit which gene is located usually outside MHC. Polymorphic heavy chain of MHC-I molecule contains N-terminal extra-cellular region composed by three domains, α1, α2, and α3, transmembrane helix to hold MHC-I molecule on the cell surface and short cytoplasmic tail. Two domains, α1 and α2 form deep peptide-binding groove between two long α-helices and the floor of the groove formed by eight β-strands. Immunoglobulin-like domain α3 involved in the interaction with CD8 co-receptor. β_2 microglobulin provides stability of the complex and participates in the recognition of peptide-MHC class I complex by CD8 co-receptor. The peptide is non-covalently bound to MHC-I, it is held by the several pockets on the floor of the peptide-binding groove. Amino acid side-chains that are most polymorphic in human alleles fill up the central and widest portion of the binding groove, while conserved side-chains are clustered at the narrower ends of the groove.



Schematic view of MHC class I and MHC class II molecules

Classical MHC molecules present epitopes to the TCRs of CD8+ T lymphocytes. **Nonclassical molecules** (MHC class IB) exhibit limited polymorphism, expression patterns, and presented antigens; this group is subdivided into a group encoded within MHC loci (e.g., HLA-E, -F, -G), as well as those not (e.g., stress ligands such as ULBPs, Rae1, and H60); the antigen/ligand for many of these molecules remain unknown, but they can interact with each of CD8+ T cells, NKT cells, and NK cells.

MHC Class II

MHC class II can be conditionally expressed by all cell types, but normally occurs only on "professional" antigen-presenting cells (APCs): macrophages, B cells, and especially dendritic cells (DCs). An APC takes up an antigenic protein, performs antigen processing, and returns a molecular fraction of it—a fraction termed the epitope—and displays it on the APC's surface coupled within an MHC class II molecule (antigen presentation). On the cell's surface, the epitope can be recognized by immunologic structures like T cell receptors (TCRs). The molecular region which binds to the epitope is the paratope.On surfaces of helper T cells are CD4 receptors, as well as TCRs. When a naive helper T cell's CD4 molecule docks to an APC's MHC class II molecule, its TCR can meet and bind the epitope coupled within the MHC class II. This event primes the naive T cell. According to the local milieu, that is, the balance of cytokines secreted by APCs in the microenvironment, the naive helper T cell (Th0) polarizes into either a memory Th cell or an effector Th cell of phenotype either type 1 (Th1), type 2 (Th2), type 17 (Th17), or regulatory/suppressor (Treg), as so far identified, the Th cell's terminal differentiation.

MHC class II thus mediates immunization to—or, if APCs polarize Th0 cells principally to Treg cells, immune tolerance of—an antigen. The polarization during primary exposure to an antigen is key in determining a number chronic diseases, such as inflammatory bowel diseases and asthma, by skewing the immune response that memory Th cells coordinate when their memory recall is triggered upon secondary exposure to similar antigens. B cells express MHC class II to present antigens to Th0, but when their B cell receptors bind matching epitopes, interactions which are not mediated by MHC, these activated B cells secrete soluble immunoglobulins: antibody molecules mediating humoral immunity.

Class II MHC molecules are also heterodimers, genes for both α and β subunits are polymorphic and located within MHC class II subregion. Peptide-binding groove of MHC-II molecules is forms by N-terminal domains of both subunits of the heterodimer, α1 and β1, unlike MHC-I molecules, where two domains of the same chain are involved. In addition, both subunits of MHC-II contain transmembrane helix and immunoglobulin domains α2 or β2 that can be recognized by CD4 co-receptors. In this way MHC molecules chaperone which type of lymphocytes may bind to the given antigen with high affinity, since different lymphocytes express different T-Cell Receptor (TCR) co-receptors.MHC class II molecules in humans have five to six isotypes. Classic molecules present peptides to CD4+ lymphocytes. Nonclassic molecules, accessories, with intracellular functions, are not exposed on cell membranes, but in internal membranes in lysosomes, normally loading the antigenic peptides onto classic MHC class II molecules.

Sr.No	Feature	Class I MHC	Class II MHC
1	Constituting polypeptide chains	α chain (45KDa in humans) β_2 chain (12 KDa in humans)	α chain (30-34 KDa in humans) β chain (26-29 KDa in humans)
2	Antigen binding domain	α1and α2 domains	α_1 and β_1 domains
3	Binds protein antigens of	8-10 amino acids residues	13-18 amino acids residues
4	Peptide bending cleft	Floor formed by β sheets and sides by a helices, blocked at both the ends	Floor formed by β sheets and sides by a helices, opened at both the ends
5	Antigenic peptide motifs involved in binding	Anchor residues located at amino and carbon terminal ends	Anchor residues located almost uniformly along the peptide
6	Presents antigenic peptide to	CD8+ T cells	CD4+ T cells

MHC class III

Class III molecules have physiologic roles unlike classes I and II, but are encoded between them in the short arm of human chromosome 6. Class III molecules include several secreted proteins with immune functions: components of the complement system (such as C2, C4, and B factor), cytokines (such as TNF-α, LTA, and LTB), and heat shock proteins.

Function

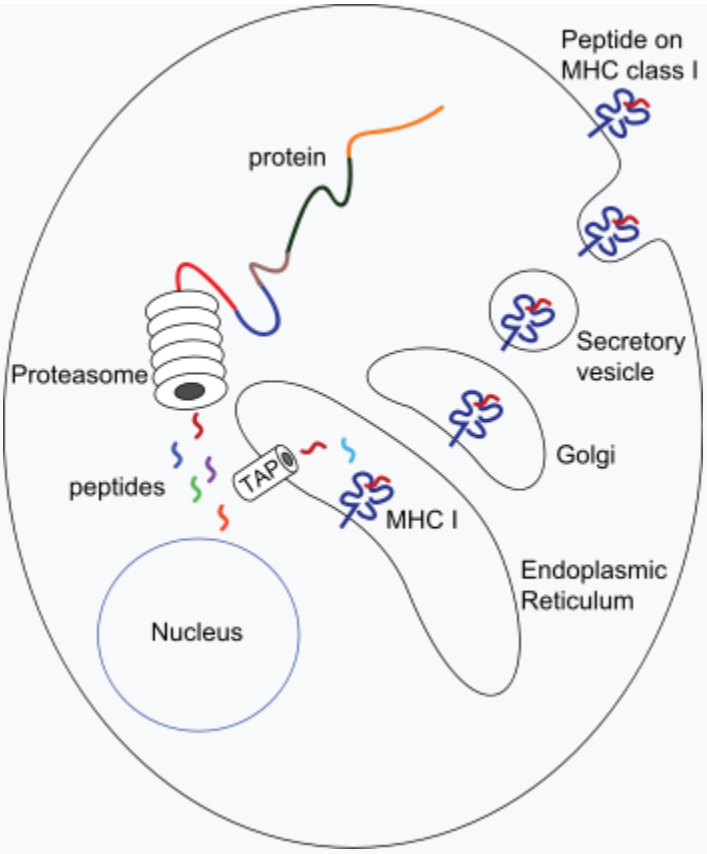
MHC is the tissue-antigen that allows the immune system (more specifically T cells) to bind to, recognize, and tolerate itself (autorecognition). MHC is also the chaperone for intracellular peptides that are complexed with MHCs and presented to T cell receptors (TCRs) as potential foreign antigens. MHC interacts with TCR and its co-receptors to optimize binding conditions for the TCR-antigen interaction, in terms of antigen binding affinity and specificity, and signal transduction effectiveness.Essentially, the MHC-peptide complex is a complex of auto-antigen/allo-antigen. Upon binding, T cells should in principle tolerate the auto-antigen, but activate when exposed to the allo-antigen. Disease states occur when this principle is disrupted.Antigen presentation: MHC molecules bind to both T cell receptor and CD4/CD8 co-receptors on T lymphocytes, and the antigen epitope held in the peptide-binding groove of the MHC molecule interacts with the variable Ig-Like domain of the TCR to trigger T-cell activation.

Autoimmune reaction: Having some MHC molecules increases the risk of autoimmune diseases more than having others. HLA-B27 is an example. It is unclear how exactly having the HLA-B27 tissue type increases the risk of ankylosing spondylitis and other associated

inflammatory diseases, but mechanisms involving aberrant antigen presentation or T cell activation have been hypothesized.

Tissue allorecognition: MHC molecules in complex with peptide epitopes are essentially ligands for TCRs. T cells become activated by binding to the peptide-binding grooves of any MHC molecule that they were not trained to recognize during positive selection in the thymus.

Antigen processing and presentation

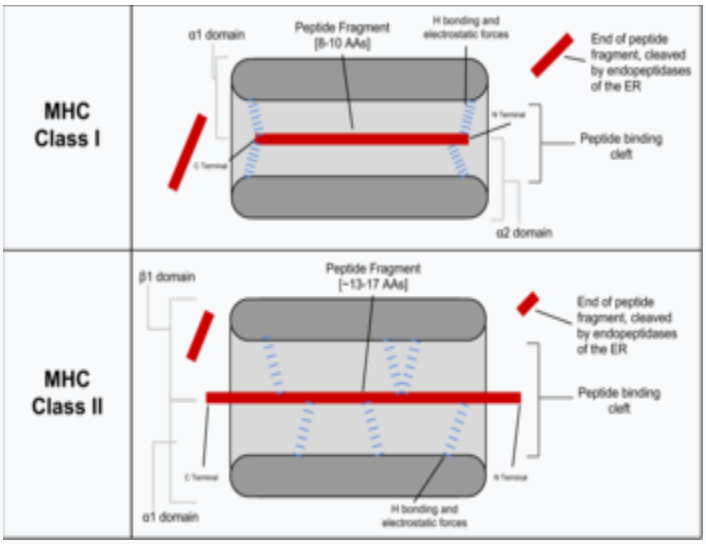


MHC class I pathway: Proteins in the cytosol are degraded by the proteasome, liberating peptides internalized by TAP channel in the endoplasmic reticulum, there associating with MHC-I molecules freshly synthesized. MHC-I/peptide complexes enter Golgi apparatus, are glycosylated, enter secretory vesicles, fuse with the cell membrane, and externalize on the cell membrane interacting with T lymphocytes.

Peptides are processed and presented by two classical pathways:

- In **MHC class II**, phagocytes such as macrophages and immature dendritic cells take up entities by phagocytosis into phagosomes—though B cells exhibit the more general endocytosis into endosomes—which fuse with lysosomes whose acidic enzymes cleave the uptaken protein into many different peptides. Via physicochemical dynamics in molecular interaction with the particular MHC class II variants borne by the host, encoded in the host's genome, a particular peptide exhibits immunodominance and loads onto MHC class II molecules. These are trafficked to and externalized on the cell surface.

- In **MHC class I**, any nucleated cell normally presents cytosolic peptides, mostly self peptides derived from protein turnover and defective ribosomal products. During viral infection, intracellular microorganism infection, or cancerous transformation, such proteins degraded in the proteosome are as well loaded onto MHC class I molecules and displayed on the cell surface. T lymphocytes can detect a peptide displayed at 0.1%-1% of the MHC molecules.



Peptide binding for Class I and Class II MHC molecules, showing the binding of peptides between the alpha-helix walls, upon a beta-sheet base. The difference in binding positions is shown. Class I primarily makes contact with backbone residues at the Carboxy and amino terminal regions, while Class II primarily makes contacts along the length of the residue backbone. The precise location of binding residues is determined by the MHC allele.

Table 2. Characteristics of the antigen processing pathways

Characteristic	**MHC-I pathway**	**MHC-II pathway**
Composition of the stable peptide-MHC complex	Polymorphic chain α and β_2 microglobulin, peptide bound to α chain	Polymorphic chains α and β, peptide binds to both
Types of antigen-presenting cells (APC)	All nucleated cells	Dendritic cells, mononuclear phagocytes, B lymphocytes, some endothelial cells, epithelium of thymus
T lymphocytes able	Cytotoxic T	Helper T lymphocytes (CD4+)

to respond	lymphocytes (CD8+)	
Origin of antigenic proteins	cytosolic proteins (mostly synthetized by the cell; may also enter from the extracellular medium via phagosomes)	Proteins present in endosomes or lysosomes (mostly internalized from extracellular medium)
Enzymes responsible for peptide generation	Cytosolic proteasome	Proteases from endosomes and lysosomes (for instance, cathepsin)
Location of loading the peptide on the MHC molecule	Endoplasmic reticulum	Specialized vesicular compartment
Molecules implicated in transporting the peptides and loading them on the MHC molecules	TAP (transporter associated with antigen processing)	DM, invariant chain

T lymphocyte recognition restrictions

In their development in the thymus, T lymphocytes are selected to recognize MHC molecules of the host, but not recognize other self antigens. Following selection, each T lymphocyte shows dual specificity: The TCR recognizes self MHC, but only non-self antigens.

MHC restriction occurs during lymphocyte development in the thymus through a process known as positive selection. T cells that do not receive a positive survival signal — mediated mainly by thymic epithelial cells presenting self peptides bound to MHC molecules — to their TCR undergo apoptosis. Positive selection ensures that mature T cells can functionally recognize MHC molecules in the periphery (i.e. elsewhere in the body).

The TCRs of T lymphocytes recognise only sequential epitopes, also called linear epitopes, of only peptides and only if coupled within an MHC molecule. (Antibody molecules secreted by activated B cells, though, recognize diverse epitopes—peptide, lipid, carbohydrate, and nucleic acid—and recognize conformational epitopes, which have three-dimensional structure.)

Cytokine

Cytokines are a broad and loose category of small proteins (~5–20 kDa) that are important in cell signaling. Cytokines are peptides, and cannot cross the lipid bilayer of cells to enter the cytoplasm. Cytokines have been shown to be involved in autocrine, paracrine and endocrine signaling as immunomodulating agents. Their definite distinction from hormones is still part of ongoing research. Cytokines include chemokines, interferons, interleukins, lymphokines, and tumour necrosis factors, but generally not hormones or growth factors (despite some overlap in the terminology). Cytokines are produced by a broad range of cells, including immune cells like macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells; a given cytokine may be produced by more than one type of cell. They act through receptors, and are especially important in the immune system; cytokines modulate the balance between humoral and cell-based immune responses, and they regulate the maturation, growth, and responsiveness of particular cell populations. Some cytokines enhance or inhibit the action of other cytokines in complex ways.

They are different from hormones, which are also important cell signaling molecules, in that hormones circulate in higher concentrations and tend to be made by specific kinds of cells.They are important in health and disease, specifically in host responses to infection, immune responses, inflammation, trauma, sepsis, cancer, and reproduction.

Discovery of cytokines

Interferon-alpha, an interferon type I, was identified in 1957 as a protein that interfered with viral replication.[3] The activity of interferon-gamma (the sole member of the interferon type II class) was described in 1965; this was the first identified lymphocyte-derived mediator. Macrophage migration inhibitory factor (MIF) was identified simultaneously in 1966 by John David and Barry Bloom.

In 1969 Dudley Dumonde proposed the term "lymphokine" to describe proteins secreted from lymphocytes and later, proteins derived from macrophages and monocytes in culture were called "monokines".

ln 1974, Stanley Cohen published an article describing the production of MIF in virus-infected allantoic membrane and kidney cells, showing its production is not limited to immune cells. This led to his proposal of the term cytokine.

Ogawa described the early acting growth factors, intermediate acting growth factors and late acting growth factors.

Difference from hormones

Classic hormones circulate in nanomolar (10^{-9} M) concentrations that usually vary by less than one order of magnitude. In contrast, some cytokines (such as IL-6) circulate in picomolar (10^{-12} M) concentrations that can increase up to 1,000 times during trauma or infection. The widespread distribution of cellular sources for cytokines may be a feature that differentiates them from hormones. Virtually all nucleated cells, but especially endo/epithelial cells and resident macrophages (many near the interface with the external environment) are potent producers of IL-1, IL-6, and TNF-α. In contrast, classic hormones, such as insulin, are secreted

from discrete glands (e.g., the pancreas). The current terminology refers to cytokines as immunomodulating agents.

A contributing factor to the difficulty of distinguishing cytokines from hormones is that some immunomodulating effects of cytokines are systemic rather than local. For instance, to accurately utilize hormone terminology, cytokines may be autocrine or paracrine in nature, and chemotaxis, chemokinesis and endocrine as a pyrogen. Essentially, cytokines are not limited to their immunomodulatory status as molecules.

Nomenclature

Cytokines have been classed as lymphokines, interleukins, and chemokines, based on their presumed function, cell of secretion, or target of action. Because cytokines are characterised by considerable redundancy and pleiotropism, such distinctions, allowing for exceptions, are obsolete.

- The term *interleukin* was initially used by researchers for those cytokines whose presumed targets are principally leukocytes. It is now used largely for designation of newer cytokine molecules and bears little relation to their presumed function. The vast majority of these are produced by T-helper cells.
- *Lymphokines*: produced by lymphocytes
- *Monokines*: produced exclusively by monocytes
- *Interferons*: involved in antiviral responses
- *Colony stimulating factors*: support the growth of cells in semisolid media
- *Chemokines*: mediate chemoattraction (chemotaxis) between cells.

Classification

Structural

Structural homogeneity has been able to partially distinguish between cytokines that do not demonstrate a considerable degree of redundancy so that they can be classified into four types:

- The four-α-helix bundle family: member cytokines have three-dimensional structures with a bundle of four α-helices. This family, in turn, is divided into three sub-families:
    1. the IL-2 subfamily
        - This is the largest family. It contains several non-immunological cytokines including erythropoietin (EPO) and thrombopoietin (TPO). Some members share a gamma-chain in their receptors.
    2. the interferon (IFN) subfamily
    3. the IL-10 subfamily.
- Furthermore, four-α-helix bundle cytokines can be grouped into *long-chain* and *short-chain* cytokines by topology.
- the IL-1 family, which primarily includes IL-1 and IL-18
- the IL-17 family, which has yet to be completely characterized, though member cytokines have a specific effect in promoting proliferation of T-cells that cause cytotoxic effects.

- the cysteine-knot cytokines include members of the Transforming growth factor beta superfamily, including TGF-β1, TGF-β2 and TGF-β3.

Functional

A classification that proves more useful in clinical and experimental practice outside of structural biology divides immunological cytokines into those that enhance cellular immune responses, type 1 (TNFα, IFN-γ, etc.), and type 2 (TGF-β, IL-4, IL-10, IL-13, etc.), which favor antibody responses.

A key focus of interest has been that cytokines in one of these two sub-sets tend to inhibit the effects of those in the other. Dysregulation of this tendency is under intensive study for its possible role in the pathogenesis of autoimmune disorders.

Several inflammatory cytokines are induced by oxidative stress. The fact that cytokines themselves trigger the release of other cytokines and also lead to increased oxidative stress makes them important in chronic inflammation, as well as other immunoresponses, such as fever and acute phase proteins of the liver (IL-1,6,12, IFN-a).

Cytokines also play a role in anti-inflammatory pathways and are a possible therapeutic treatment for pathological pain from inflammation or peripheral nerve injury. There are both pro-inflammatory and anti-inflammatory cytokines that regulate this pathway.

Receptors

In recent years, the cytokine receptors have come to demand the attention of more investigators than cytokines themselves, partly because of their remarkable characteristics, and partly because a deficiency of cytokine receptors has now been directly linked to certain debilitating immunodeficiency states. In this regard, and also because the redundancy and pleomorphism of cytokines are, in fact, a consequence of their homologous receptors, many authorities think that a classification of cytokine receptors would be more clinically and experimentally useful.

A classification of cytokine receptors based on their three-dimensional structure has, therefore, been attempted. Such a classification, though seemingly cumbersome, provides several unique perspectives for attractive pharmacotherapeutic targets.

- Immunoglobulin (Ig) superfamily, which are ubiquitously present throughout several cells and tissues of the vertebrate body, and share structural homology with immunoglobulins (antibodies), cell adhesion molecules, and even some cytokines. Examples: IL-1 receptor types.
- Hemopoietic Growth Factor (type 1) family, whose members have certain conserved motifs in their extracellular amino-acid domain. The IL-2 receptor belongs to this chain, whose γ-chain (common to several other cytokines) deficiency is directly responsible for the x-linked form of Severe Combined Immunodeficiency (X-SCID).
- Interferon (type 2) family, whose members are receptors for IFN β and γ.
- Tumor necrosis factors (TNF) (type 3) family, whose members share a cysteine-rich common extracellular binding domain, and includes several other non-cytokine ligands like CD40, CD27 and CD30, besides the ligands on which the family is named (TNF).

- Seven transmembrane helix family, the ubiquitous receptor type of the animal kingdom. All G protein-coupled receptors (for hormones and neurotransmitters) belong to this family. Chemokine receptors, two of which act as binding proteins for HIV (CD4 and CCR5), also belong to this family.
- Interleukin-17 receptor (IL-17R) family, which shows little homology with any other cytokine receptor family. Structural motifs conserved between members of this family include: an extracellular fibronectin III-like domain, a transmembrane domain and a cytoplasmic SERIF domain. The known members of this family are as follows: IL-17RA, IL-17RB, IL-17RC, IL17RD and IL-17RE.

Cellular effects

Each cytokine has a matching cell-surface receptor. Subsequent cascades of intracellular signaling then alter cell functions. This may include the upregulation and/or downregulation of several genes and their transcription factors, resulting in the production of other cytokines, an increase in the number of surface receptors for other molecules, or the suppression of their own effect by feedback inhibition.

The effect of a particular cytokine on a given cell depends on the cytokine, its extracellular abundance, the presence and abundance of the complementary receptor on the cell surface, and downstream signals activated by receptor binding; these last two factors can vary by cell type. Cytokines are characterized by considerable redundancy, in that many cytokines appear to share similar functions.

It seems to be a paradox that cytokines binding to antibodies have a stronger immune effect than the cytokine alone. This may lead to lower therapeutic doses.Said et al. showed that inflammatory cytokines cause an IL-10-dependent inhibition of T-cell expansion and function by up-regulating PD-1 levels on monocytes which leads to IL-10 production by monocytes after binding of PD-1 by PD-L. Adverse reactions to cytokines are characterized by local inflammation and/or ulceration at the injection sites. Occasionally such reactions are seen with more widespread papular eruptions.

Roles of endogenous cytokines in health and disease

Cytokines are often involved in several developmental processes during embryogenesis. Cytokines are crucial for fighting off infections and in other immune responses.However, they can become dysregulated and pathological in inflammation, trauma, sepsis, and hemorrhagic stroke.

Adverse effects of cytokines have been linked to many disease states and conditions ranging from schizophrenia, major depression and Alzheimer's disease to cancer. Normal tissue integrity is preserved by feedback interactions between diverse cell types mediated by adhesion molecules and secreted cytokines; disruption of normal feedback mechanisms in cancer threatens tissue integrity. Over-secretion of cytokines can trigger a dangerous syndrome known as a cytokine storm. Cytokines storms may have been the cause of severe adverse events during a clinical trial of TGN1412. Cytokine storms are also suspected to be the main cause of death in the 1918 "Spanish Flu" pandemic. Deaths were weighted more heavily towards people with healthy immune systems, due to its ability to produce stronger immune responses, likely increasing cytokine levels.

Another important example of cytokine storm is seen in acute pancreatitis. Cytokines are integral and implicated in all angles of the cascade resulting in the systemic inflammatory response syndrome and multi organ failure associated with this intra-abdominal catastrophe.

Medical use as drugs

Some cytokines have been developed into protein therapeutics using recombinant DNA technology. Recombinant cytokines being used as drugs as of 2014 include:

- Bone morphogenetic protein (BMP), used to treat bone-related conditions
- Erythropoietin (EPO), used to treat anemia
- Granulocyte colony-stimulating factor (G-CSF), used to treat neutropenia in cancer patients
- Granulocyte macrophage colony-stimulating factor (GM-CSF), used to treat neutropenia and fungal infections in cancer patients
- Interferon alfa, used to treat hepatitis C and multiple sclerosis
- Interferon beta, used to treat multiple sclerosis
- Interleukin 2 (IL-2), used to treat cancer.
- Interleukin 11 (IL-11), used to treat thrombocytopenia in cancer patients.
- Interferon gamma is used to treat chronic granulomatous disease and osteopetrosis

Antigen

In immunology, **antigens** (**Ag**) are structures (aka substances) specifically bound by antibodies (Ab) or a cell surface version of Ab ~ B cell antigen receptor (BCR). The term antigen originally described a structural molecule that binds specifically to an antibody only in the form of native antigen. It was expanded later to refer to any molecule or a linear molecular fragment after processing the native antigen that can be recognized by T-cell receptor (TCR). BCR and TCR are both highly variable antigen receptors diversified by somatic V(D)J recombination. Both T cells and B cells are cellular components of adaptive immunity. The Ag abbreviation stands for an *antibody generator*.

Antigens are "targeted" by antibodies. Each antibody is specifically produced by the immune system to match an antigen after cells in the immune system come into *contact* with it; this allows a precise identification or matching of the antigen and the initiation of a tailored response. The antibody is said to "match" the antigen in the sense that it can bind to it due to an adaptation in a region of the antibody; because of this, many different antibodies are produced, each able to bind a different antigen while sharing the same basic structure. In most cases, an adapted antibody can only react to and bind one specific antigen; in some instances, however, antibodies may cross-react and bind more than one antigen.

Also, an antigen is a molecule that binds to Ag-specific receptors, but cannot necessarily induce an immune response in the body by itself. Antigens are usually proteins, peptides (amino acid chains) and polysaccharides (chains of monosaccharides/simple sugars) but lipids and nucleic acids become antigens only when combined with proteins and polysaccharides. In general, saccharides and lipids (as opposed to peptides) qualify as antigens but not as immunogens since they cannot elicit an immune response on their own. Furthermore, for a peptide to induce an immune response (activation of T-cells by antigen-presenting cells) it must be a large enough size, since peptides too small will also not elicit an immune response.

The antigen may originate from within the body ("self-antigen") or from the external environment ("non-self"). The immune system is supposed to identify and attack "non-self" invaders from the outside world or modified/harmful substances present in the body and usually does not react to self-antigens under normal homeostatic conditions due to negative selection of T cells in the thymus.

Vaccines are examples of antigens in an immunogenic form, which are intentionally administered to a recipient to induce the memory function of adaptive immune system toward the antigens of the pathogen invading that recipient.

Etymology

Paul Ehrlich coined the term antibody (in German *Antikörper*) in his side-chain theory at the end of the 19th century. In 1899, Ladislas Deutsch (Laszlo Detre) (1874–1939) named the hypothetical substances halfway between bacterial constituents and antibodies "substances immunogenes ou antigenes" (antigenic or immunogenic substances). He originally believed those substances to be precursors of antibodies, just as zymogen is a precursor of an enzyme. But, by 1903, he understood that an antigen induces the production of immune bodies (antibodies) and wrote that the word *antigen* is a contraction of antisomatogen

(*Immunkörperbildner*). The *Oxford English Dictionary* indicates that the logical construction should be "anti(body)-gen".

Terminology

- Epitope – The distinct surface features of an antigen, its *antigenic determinant*. Antigenic molecules, normally "large" biological polymers, usually present surface features that can act as points of interaction for specific antibodies. Any such feature constitutes an epitope. Most antigens have the potential to be bound by multiple antibodies, each of which is specific to one of the antigen's epitopes. Using the "lock and key" metaphor, the antigen can be seen as a string of keys (epitopes) each of which matches a different lock (antibody). Different antibody **idiotypes**, each have distinctly formed complementarity-determining regions.
- Allergen – A substance capable of causing an allergic reaction. The (detrimental) reaction may result after exposure via ingestion, inhalation, injection, or contact with skin.
- Superantigen – A class of antigens that cause non-specific activation of T-cells, resulting in polyclonal T-cell activation and massive cytokine release.
- Tolerogen – A substance that invokes a specific immune non-responsiveness due to its molecular form. If its molecular form is changed, a tolerogen can become an immunogen.
- Immunoglobulin-binding protein – Proteins such as protein A, protein G, and protein L that are capable of binding to antibodies at positions outside of the antigen-binding site. While antigens are the "target" of antibodies, immunoglobulin-binding proteins "attack" antibodies.
- T-dependent antigen – Antigens that require the assistance of T cells to induce the formation of specific antibodies.
- T-independent antigen – Antigens that stimulate B cells directly.
- Immunodominant antigens – Antigens that dominate (over all others from a pathogen) in their ability to produce an immune response. T cell responses typically are directed against a relatively few immunodominant epitopes, although in some cases (e.g., infection with the malaria pathogen *Plasmodium spp.*) it is dispersed over a relatively large number of parasite antigens.

Antigen-presenting cells present antigens in the form of peptides on histocompatibility molecules. The T cell selectively recognize the antigens; depending on the antigen and the type of the histocompatibility molecule, different types of T cells will be activated. For T Cell Receptor (TCR) recognition, the peptide must be processed into small fragments inside the cell and presented by a major histocompatibility complex (MHC). The antigen cannot elicit the immune response without the help of an immunologic adjuvant. Similarly, the adjuvant component of vaccines plays an essential role in the activation of the innate immune system.

An immunogen is an antigen substance (or adduct) that is able to trigger a humoral (innate) or cell-mediated immune response. It first initiates an innate immune response, which then causes the activation of the adaptive immune response. An antigen binds the highly variable immunoreceptor products (B cell receptor or T cell receptor) once these have been generated. Immunogens are those antigens, termed immunogenic, capable of inducing an immune response.

At the molecular level, an antigen can be characterized by its ability to bind to an antibody's variable Fab region. Different antibodies have the potential to discriminate among specific epitopes present on the antigen surface. A hapten is a small molecule that changes the structure of an antigenic epitope. In order to induce an immune response, it needs to be attached to a large carrier molecule such as a protein (a complex of peptides). Antigens are usually carried

by proteins and polysaccharides, and less frequently, lipids. This includes parts (coats, capsules, cell walls, flagella, fimbriae, and toxins) of bacteria, viruses, and other microorganisms. Lipids and nucleic acids are antigenic only when combined with proteins and polysaccharides. Non-microbial non-self antigens can include pollen, egg white and proteins from transplanted tissues and organs or on the surface of transfused blood cells.

Sources

Antigens can be classified according to their source.

1. Exogenous antigens

Exogenous antigens are antigens that have entered the body from the outside, for example, by inhalation, ingestion or injection. The immune system's response to exogenous antigens is often subclinical. By endocytosis or phagocytosis, exogenous antigens are taken into the antigen-presenting cells (APCs) and processed into fragments. APCs then present the fragments to T helper cells ($CD4^+$) by the use of class II histocompatibility molecules on their surface. Some T cells are specific for the peptide:MHC complex. They become activated and start to secrete cytokines, substances that activate cytotoxic T lymphocytes (CTL), antibody-secreting B cells, macrophages and other particles. Some antigens start out as exogenous, and later become endogenous (for example, intracellular viruses). Intracellular antigens can be returned to circulation upon the destruction of the infected cell.

2. Endogenous antigens

Endogenous antigens are generated within normal cells as a result of normal cell metabolism, or because of viral or intracellular bacterial infection. The fragments are then presented on the cell surface in the complex with MHC class I molecules. If activated cytotoxic $CD8^+$ T cells recognize them, the T cells secrete various toxins that cause the lysis or apoptosis of the infected cell. In order to keep the cytotoxic cells from killing cells just for presenting self-proteins, the cytotoxic cells (self-reactive T cells) are deleted as a result of tolerance (negative selection). Endogenous antigens include xenogenic (heterologous), autologous and idiotypic or allogenic (homologous) antigens. Sometimes antigens are part of the host itself in an autoimmune disease.

3. Autoantigens

An autoantigen is usually a normal protein or protein complex (and sometimes DNA or RNA) that is recognized by the immune system of patients suffering from a specific autoimmune disease. Under normal conditions, these antigens should not be the target of the immune system, but in autoimmune diseases, their associated T cells are not deleted and instead attack.

4. Neoantigens

Neoantigens are those that are entirely absent from the normal human genome. As compared with nonmutated self-antigens, neoantigens are of relevance to tumor control, as the quality of the T cell pool that is available for these antigens is not affected by central T cell tolerance. Technology to systematically analyze T cell reactivity against neoantigens became available only recently.[15] Neoantigens can be directly detected and quantified through a method called MANA-SRM developed by a molecular diagnostics company, Complete Omics Inc., through collaborating with a team in Johns Hopkins University School of Medicine.

5. Viral antigens

For virus-associated tumors, such as cervical cancer and a subset of head and neck cancers, epitopes derived from viral open reading frames contribute to the pool of neoantigens.

6. Tumor antigens

Tumor antigens are those antigens that are presented by MHC class I or MHC class II molecules on the surface of tumor cells. Antigens found only on such cells are called tumor-specific antigens (TSAs) and generally result from a tumor-specific mutation. More common are antigens that are presented by tumor cells and normal cells, called tumor-associated antigens (TAAs). Cytotoxic T lymphocytes that recognize these antigens may be able to destroy tumor cells. Tumor antigens can appear on the surface of the tumor in the form of, for example, a mutated receptor, in which case they are recognized by B cells. For human tumors without a viral etiology, novel peptides (neo-epitopes) are created by tumor-specific DNA alterations.

Process:

A large fraction of human tumor mutations are effectively patient-specific. Therefore, neoantigens may also be based on individual tumor genomes. Deep-sequencing technologies can identify mutations within the protein-coding part of the genome (the exome) and predict potential neoantigens. In mice models, for all novel protein sequences, potential MHC-binding peptides were predicted. The resulting set of potential neoantigens was used to assess T cell reactivity. Exome–based analyses were exploited in a clinical setting, to assess reactivity in patients treated by either tumor-infiltrating lymphocyte (TIL) cell therapy or checkpoint blockade. Neoantigen identification was successful for multiple experimental model systems and human malignancies.

The false-negative rate of cancer exome sequencing is low—i.e.: the majority of neoantigens occur within exonic sequence with sufficient coverage. However, the vast majority of mutations within expressed genes do not produce neoantigens that are recognized by autologous T cells.

As of 2015 mass spectrometry resolution is insufficient to exclude many false positives from the pool of peptides that may be presented by MHC molecules. Instead, algorithms are used to identify the most likely candidates. These algorithms consider factors such as the likelihood of proteasomal processing, transport into the endoplasmic reticulum, affinity for the relevant MHC class I alleles and gene expression or protein translation levels.

The majority of human neoantigens identified in unbiased screens display a high predicted MHC binding affinity. Minor histocompatibility antigens, a conceptually similar antigen class are also correctly identified by MHC binding algorithms. Another potential filter examines whether the mutation is expected to improve MHC binding. The nature of the central TCR-exposed residues of MHC-bound peptides is associated with peptide immunogenicity.

Nativity

A native antigen is an antigen that is not yet processed by an APC to smaller parts. T cells cannot bind native antigens, but require that they be processed by APCs, whereas B cells can be activated by native ones.

Antigenic specificity

Antigenic specificity is the ability of the host cells to recognize an antigen specifically as a unique molecular entity and distinguish it from another with exquisite precision. Antigen specificity is due primarily to the side-chain conformations of the antigen. It is measurable and need not be linear or of a rate-limited step or equation

Antibody

An **antibody** (**Ab**), also known as an **immunoglobulin** (**Ig**), is a large, Y-shaped protein produced mainly by plasma cells that is used by the immune system to neutralize pathogens such as pathogenic bacteria and viruses. The antibody recognizes a unique molecule of the pathogen, called an antigen, via the fragment antigen-binding (Fab) variable region. Each tip of the "Y" of an antibody contains a paratope (analogous to a lock) that is specific for one particular epitope (analogous to a key) on an antigen, allowing these two structures to bind together with precision. Using this binding mechanism, an antibody can *tag* a microbe or an infected cell for attack by other parts of the immune system, or can neutralize its target directly (for example, by inhibiting a part of a microbe that is essential for its invasion and survival). Depending on the antigen, the binding may impede the biological process causing the disease or may activate macrophages to destroy the foreign substance. The ability of an antibody to communicate with the other components of the immune system is mediated via its Fc region (located at the base of the "Y"), which contains a conserved glycosylation site involved in these interactions. The production of antibodies is the main function of the humoral immune system.

Antibodies are secreted by B cells of the adaptive immune system, mostly by differentiated B cells called plasma cells. Antibodies can occur in two physical forms, a soluble form that is secreted from the cell to be free in the blood plasma, and a membrane-bound form that is attached to the surface of a B cell and is referred to as the B-cell receptor (BCR). The BCR is found only on the surface of B cells and facilitates the activation of these cells and their subsequent differentiation into either antibody factories called plasma cells or memory B cells that will survive in the body and remember that same antigen so the B cells can respond faster upon future exposure. In most cases, interaction of the B cell with a T helper cell is necessary to produce full activation of the B cell and, therefore, antibody generation following antigen binding.Soluble antibodies are released into the blood and tissue fluids, as well as many secretions to continue to survey for invading microorganisms.

Antibodies are glycoproteins belonging to the immunoglobulin superfamily. They constitute most of the gamma globulin fraction of the blood proteins. They are typically made of basic structural units—each with two large heavy chains and two small light chains. There are several different types of antibody heavy chains that define the five different types of crystallisable fragments (Fc) that may be attached to the antigen-binding fragments. The five different types of Fc regions allow antibodies to be grouped into five *isotypes*. Each Fc region of a particular antibody isotype is able to bind to its specific Fc Receptor (FcR), except for IgD, which is essentially the BCR, thus allowing the antigen-antibody complex to mediate different roles depending on which FcR it binds. The ability of an antibody to bind to its corresponding FcR is further modulated by the structure of the glycan(s) present at conserved sites within its Fc region. The ability of antibodies to bind to FcRs helps to direct the appropriate immune response for each different type of foreign object they encounter. For example, IgE is responsible for an allergic response consisting of mast cell degranulation and histamine release. IgE's Fab paratope binds to allergic antigen, for example house dust mite particles, while its Fc region binds to Fc receptor ε. The allergen-IgE-FcRε interaction mediates allergic signal transduction to induce conditions such as asthma.

Though the general structure of all antibodies is very similar, a small region at the tip of the protein is extremely variable, allowing millions of antibodies with slightly different tip structures, or antigen-binding sites, to exist. This region is known as the *hypervariable region*. Each of these variants can bind to a different antigen. This enormous diversity of antibody paratopes on the antigen-binding fragments allows the immune system to recognize an equally wide variety of antigens.[1] The large and diverse population of antibody paratope is generated by random recombination events of a set of gene segments that encode different antigen-binding sites (or *paratopes*), followed by random mutations in this area of the antibody gene, which create further diversity. This recombinational process that produces clonal antibody paratope diversity is called V(D)J or VJ recombination. Basically, the antibody paratope is polygenic, made up of three genes, V, D, and J. Each paratope locus is also polymorphic, such that during antibody production, one allele of V, one of D, and one of J is chosen. These gene segments are then joined together using random genetic recombination to produce the paratope. The regions where the genes are randomly recombined together is the hyper variable region used to recognise different antigens on a clonal basis.

Antibody genes also re-organize in a process called class switching that changes the one type of heavy chain Fc fragment to another, creating a different isotype of the antibody that retains the antigen-specific variable region. This allows a single antibody to be used by different types of Fc receptors, expressed on different parts of the immune system.

Adjuvant

An **adjuvant** is a pharmacological or immunological agent that modifies the effect of other agents. Adjuvants may be added to a vaccine to boost the immune response to produce more antibodies and longer-lasting immunity, thus minimizing the dose of antigen needed. Adjuvants may also be used to enhance the efficacy of a vaccine by helping to modify the immune response to particular types of immune system cells: for example, by activating T cells instead of antibody-secreting B cells depending on the purpose of the vaccine. Adjuvants are also used in the production of antibodies from immunized animals. There are different classes of adjuvants that can push immune response in different directions, but the most commonly used adjuvants include aluminum hydroxide and paraffin oil.

Immunologic adjuvants

Immunologic adjuvants are added to vaccines to stimulate the immune system's response to the target antigen, but do not provide immunity themselves. Adjuvants can act in various ways in presenting an antigen to the immune system. Adjuvants can act as a depot for the antigen, presenting the antigen over a longer period of time, thus maximizing the immune response before the body clears the antigen. Examples of depot type adjuvants are oil emulsions. An adjuvant can also act as an irritant, which engages and amplifies the body's immune response.[3] A tetanus, diphtheria, and pertussis (DPT) vaccine, for example, contains small quantities of inactivated toxins produced by each of the target bacteria, but also contains some aluminium hydroxide. Such aluminium salts are common adjuvants in vaccines sold in the United States and have been used in vaccines for more than 70 years.

Mechanism

Adjuvants are needed to improve routing and adaptive immune responses to antigens. This reaction is mediated by two main types of lymphocytes, B and T lymphocytes. Adjuvants apply their effects through different mechanisms. Some adjuvants, such as alum, function as delivery systems by generating depots that trap antigens at the injection site, providing a slow release that

continues to stimulate the immune system.This is now under debate, as studies have shown that surgical removal of these depots had no impact on the magnitude of IgG1 response.

As stabilizing agents

Although immunological adjuvants have traditionally been viewed as substances that aid the immune response to the antigen, adjuvants have also evolved as substances that can aid in stabilizing formulations of antigens, especially for vaccines administered for animal health.

Types

- Analgesic adjuvants
- Inorganic compounds: alum, aluminum hydroxide, aluminum phosphate, calcium phosphate hydroxide
- Mineral oil: paraffin oil
- Bacterial products: killed bacteria *Bordetella pertussis*, *Mycobacterium bovis*, toxoids
- Nonbacterial organics: squalene
- Delivery systems: detergents (Quil A)
- Plant saponins from *Quillaja* (See Quillaia), soybean, *Polygala senega*
- Cytokines: IL-1, IL-2, IL-12
- Combination: Freund's complete adjuvant, Freund's incomplete adjuvant
- Food-based oil: Adjuvant 65, which is a product based on peanut oil.Adjuvant 65 was tested in influenza vaccines in the 1970s, but was never released commercially.

Mechanism of immune stimulation

Adjuvants can enhance the immune response to the antigen in different ways:

- Extend the presence of antigen in the blood
- Help the antigen presenting cells absorb antigen
- Activate macrophages and lymphocytes
- Support the production of cytokines

Alum as an adjuvant

Alum was the first aluminium salt used as an adjuvant, but has been almost completely replaced by aluminium hydroxide and aluminium phosphate for commercial vaccines.

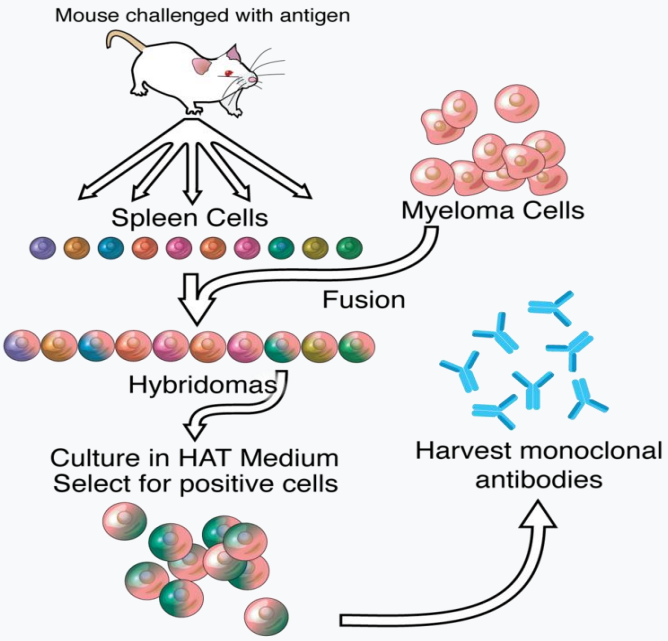
Adverse effects

Adjuvants may make vaccines too reactogenic, which often leads to fever. This is often an expected outcome upon vaccination and is usually controlled in infants by over-the-counter medication if necessary.

An increased number of narcolepsy (a chronic sleep disorder) cases in children and adolescents was observed in Scandinavian and other European countries after vaccinations to address the H1N1 “swine flu” pandemic in 2009.

Narcolepsy has previously been associated with HLA-subtype DQB1602, which has led to the prediction that it is an autoimmune process. After a series of epidemiological investigations, researchers found that the higher incidence correlated with the use of AS03-adjuvanted influenza vaccine (Pandemrix). Those vaccinated with Pandemrix have almost a 12 times higher risk of developing the disease. The adjuvant of the vaccine contained vitamin E that was no more than a day's normal dietary intake. Vitamin E increases hypocretin-specific fragments that bind to DQB1602, leading to the hypothesis that autoimmunity may arise in genetically susceptible individuals,but there is no clinical data to support this hypothesis yet.

Monoclonal antibody



A general representation of the method used to produce monoclonal antibodies.

Monoclonal antibodies (**mAb** or **moAb**) are antibodies that are made by identical immune cells that are all clones of a unique parent cell. Monoclonal antibodies can have monovalent affinity, in that they bind to the same epitope (the part of an antigen that is recognized by the antibody). In contrast, polyclonal antibodies bind to multiple epitopes and are usually made by several different plasma cell (antibody secreting immune cell) lineages. Bispecific monoclonal antibodies can also be engineered, by increasing the therapeutic targets of one single monoclonal antibody to two epitopes.

Given almost any substance, it is possible to produce monoclonal antibodies that specifically bind to that substance; they can then serve to detect or purify that substance. This has become an important tool in biochemistry, molecular biology, and medicine. When used as medications, non-proprietary drug names end in **-mab** (see "Nomenclature of monoclonal antibodies") and many immunotherapy specialists use the word **mab** anacronymically.

History

The idea of "magic bullets" was first proposed by Paul Ehrlich, who, at the beginning of the 20th century, postulated that, if a compound could be made that selectively targeted a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity. He and Élie Metchnikoff received the 1908 Nobel Prize for Physiology or Medicine for this work.

In the 1970s, the B-cell cancer multiple myeloma was known. It was understood that these cancerous B-cells all produce a single type of antibody (a paraprotein). This was used to study

the structure of antibodies, but it was not yet possible to produce identical antibodies specific to a given antigen.

Production of monoclonal antibodies involving human–mouse hybrid cells was first described by Jerrold Schwaber in 1973 and remains widely cited among those using human-derived hybridomas.

In 1975, Georges Köhler and César Milstein succeeded in making fusions of myeloma cell lines with B cells to create hybridomas that could produce antibodies, specific to known antigens and that were immortalized. They and Niels Kaj Jerne shared the Nobel Prize in Physiology or Medicine in 1984 for the discovery.

In 1988, Greg Winter and his team pioneered the techniques to humanize monoclonal antibodies, eliminating the reactions that many monoclonal antibodies caused in some patients.

In 2018, James P. Allison and Tasuku Honjo received the Nobel Prize in Physiology or Medicine for their discovery of cancer therapy by inhibition of negative immune regulation, using monoclonal antibodies that prevent inhibitory linkages.

Production

Hybridoma development

Much of the work behind production of monoclonal antibodies is rooted in the production of hybridomas, which involves identifying antigen-specific plasma/plasmablast cells (ASPCs) that produce antibodies specific to an antigen of interest and fusing these cells with myeloma cells. Rabbit B-cells can be used to form a rabbit hybridoma. Polyethylene glycol is used to fuse adjacent plasma membranes, but the success rate is low, so a selective medium in which only fused cells can grow is used. This is possible because myeloma cells have lost the ability to synthesize **hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**, an enzyme necessary for the salvage synthesis of nucleic acids. The absence of HGPRT is not a problem for these cells unless the de novo purine synthesis pathway is also disrupted. Exposing cells to aminopterin (a folic acid analogue, which inhibits dihydrofolate reductase, DHFR), makes them unable to use the de novo pathway and become fully auxotrophic for nucleic acids, thus requiring supplementation to survive.

The selective culture medium is called HAT medium because it contains hypoxanthine, aminopterin and thymidine. This medium is selective for fused (hybridoma) cells. Unfused myeloma cells cannot grow because they lack HGPRT and thus cannot replicate their DNA. Unfused spleen cells cannot grow indefinitely because of their limited life span. Only fused hybrid cells, referred to as hybridomas, are able to grow indefinitely in the medium because the spleen cell partner supplies HGPRT and the myeloma partner has traits that make it immortal (similar to a cancer cell).

This mixture of cells is then diluted and clones are grown from single parent cells on microtitre wells. The antibodies secreted by the different clones are then assayed for their ability to bind to the antigen (with a test such as ELISA or Antigen Microarray Assay) or immuno-dot blot. The most productive and stable clone is then selected for future use.

The hybridomas can be grown indefinitely in a suitable cell culture medium. They can also be injected into mice (in the peritoneal cavity, surrounding the gut). There, they produce tumors secreting an antibody-rich fluid called ascites fluid.

The medium must be enriched during *in vitro* selection to further favour hybridoma growth. This can be achieved by the use of a layer of feeder fibrocyte cells or supplement medium such as briclone. Culture-media conditioned by macrophages can be used. Production in cell culture is usually preferred as the ascites technique is painful to the animal. Where alternate techniques exist, ascites is considered unethical.

Novel mAb development technology

Several monoclonal antibody technologies had been developed recently, such as phage display, single B cell culture, single cell amplification from various B cell population and single plasma cell interrogation technologies. Different from traditional hybridoma technology, the newer technologies use molecular biology techniques to amplify the heavy and light chains of the antibody genes by PCR and produce in either bacterial or mammalian systems with recombinant technology. One of the advantages of the new technologies is applicable to multiple animals, such as rabbit, llama, chicken and other common experimental animals in the laboratory.

Purification

After obtaining either a media sample of cultured hybridomas or a sample of ascites fluid, the desired antibodies must be extracted. Cell culture sample contaminants consist primarily of media components such as growth factors, hormones and transferrins. In contrast, the *in vivo* sample is likely to have host antibodies, proteases, nucleases, nucleic acids and viruses. In both cases, other secretions by the hybridomas such as cytokines may be present. There may also be bacterial contamination and, as a result, endotoxins that are secreted by the bacteria. Depending on the complexity of the media required in cell culture and thus the contaminants, one or the other method (*in vivo* or *in vitro*) may be preferable.

The sample is first conditioned, or prepared for purification. Cells, cell debris, lipids and clotted material are first removed, typically by centrifugation followed by filtration with a 0.45 μm filter. These large particles can cause a phenomenon called membrane fouling in later purification steps. In addition, the concentration of product in the sample may not be sufficient, especially in cases where the desired antibody is produced by a low-secreting cell line. The sample is therefore concentrated by ultrafiltration or dialysis.

Most of the charged impurities are usually anions such as nucleic acids and endotoxins. These can be separated by ion exchange chromatography. Either cation exchange chromatography is used at a low enough pH that the desired antibody binds to the column while anions flow through, or anion exchange chromatography is used at a high enough pH that the desired antibody flows through the column while anions bind to it. Various proteins can also be separated along with the anions based on their isoelectric point (pI). In proteins, the isoelectric point (pI) is defined as the pH at which a protein has no net charge. When the pH > pI, a protein has a net negative charge, and when the pH < pI, a protein has a net positive charge. For example, albumin has a pI of 4.8, which is significantly lower than that of most monoclonal

antibodies, which have a pI of 6.1. Thus, at a pH between 4.8 and 6.1, the average charge of albumin molecules is likely to be more negative, while mAbs molecules are positively charged and hence it is possible to separate them. Transferrin, on the other hand, has a pI of 5.9, so it cannot be easily separated by this method. A difference in pI of at least 1 is necessary for a good separation.

Transferrin can instead be removed by size exclusion chromatography. This method is one of the more reliable chromatography techniques. Since we are dealing with proteins, properties such as charge and affinity are not consistent and vary with pH as molecules are protonated and deprotonated, while size stays relatively constant. Nonetheless, it has drawbacks such as low resolution, low capacity and low elution times.

A much quicker, single-step method of separation is protein A/G affinity chromatography. The antibody selectively binds to protein A/G, so a high level of purity (generally >80%) is obtained. However, this method may be problematic for antibodies that are easily damaged, as harsh conditions are generally used. A low pH can break the bonds to remove the antibody from the column. In addition to possibly affecting the product, low pH can cause protein A/G itself to leak off the column and appear in the eluted sample. Gentle elution buffer systems that employ high salt concentrations are available to avoid exposing sensitive antibodies to low pH. Cost is also an important consideration with this method because immobilized protein A/G is a more expensive resin.

To achieve maximum purity in a single step, affinity purification can be performed, using the antigen to provide specificity for the antibody. In this method, the antigen used to generate the antibody is covalently attached to an agarose support. If the antigen is a peptide, it is commonly synthesized with a terminal cysteine, which allows selective attachment to a carrier protein, such as KLH during development and to support purification. The antibody-containing medium is then incubated with the immobilized antigen, either in batch or as the antibody is passed through a column, where it selectively binds and can be retained while impurities are washed away. An elution with a low pH buffer or a more gentle, high salt elution buffer is then used to recover purified antibody from the support.

Antibody heterogeneity

Product heterogeneity is common in monoclonal antibodies and other recombinant biological products and is typically introduced either upstream during expression or downstream during manufacturing.

These variants are typically aggregates, deamidation products, glycosylation variants, oxidized amino acid side chains, as well as amino and carboxyl terminal amino acid additions.These seemingly minute structural changes can affect preclinical stability and process optimization as well as therapeutic product potency, bioavailability and immunogenicity. The generally accepted purification method of process streams for monoclonal antibodies includes capture of the product target with protein A, elution, acidification to inactivate potential mammalian viruses, followed by ion chromatography, first with anion beads and then with cation beads.

Displacement chromatography has been used to identify and characterize these often unseen variants in quantities that are suitable for subsequent preclinical evaluation regimens such as animal pharmacokinetic studies. Knowledge gained during the preclinical development phase is

critical for enhanced product quality understanding and provides a basis for risk management and increased regulatory flexibility. The recent Food and Drug Administration's Quality by Design initiative attempts to provide guidance on development and to facilitate design of products and processes that maximizes efficacy and safety profile while enhancing product manufacturability.

Recombinant

The production of recombinant monoclonal antibodies involves repertoire cloning, CRISPR/Cas9, or phage display/yeast display technologies. Recombinant antibody engineering involves antibody production by the use of viruses or yeast, rather than mice. These techniques rely on rapid cloning of immunoglobulin gene segments to create libraries of antibodies with slightly different amino acid sequences from which antibodies with desired specificities can be selected. The phage antibody libraries are a variant of phage antigen libraries. These techniques can be used to enhance the specificity with which antibodies recognize antigens, their stability in various environmental conditions, their therapeutic efficacy and their detectability in diagnostic applications. Fermentation chambers have been used for large scale antibody production.

Chimeric antibodies

While mouse and human antibodies are structurally similar, the differences between them were sufficient to invoke an immune response when murine monoclonal antibodies were injected into humans, resulting in their rapid removal from the blood, as well as systemic inflammatory effects and the production of human anti-mouse antibodies (HAMA).

Recombinant DNA has been explored since the late 1980s to increase residence times. In one approach, mouse DNA encoding the binding portion of a monoclonal antibody was merged with human antibody-producing DNA in living cells. The expression of this "chimeric" or "humanised" DNA through cell culture yielded part-mouse, part-human antibodies.

Human antibodies

Ever since the discovery that monoclonal antibodies could be generated, scientists have targeted the creation of *fully* human products to reduce the side effects of humanised or chimeric antibodies. Two successful approaches have been identified: transgenic mice and phage display.

As of November 2016, thirteen of the nineteen *fully* human monoclonal antibody therapeutics on the market were derived from transgenic mice technology.

Adopting organizations who market transgenic technology include:

- Medarex — which marketed the UltiMab platform. Medarex was acquired in July 2009 by Bristol Myers Squibb
- Abgenix — which marketed the Xenomouse technology. Abgenix was acquired in April 2006 by Amgen.
- Regeneron Pharmaceuticals VelocImmune technology.
- Kymab - who market their Kymouse technology.

- Open Monoclonal Technology's OmniRat™ and OmniMouse™ platform.
- TRIANNI, Inc. – who market their TRIANNI Mouse platform.
- Ablexis, LLC - who market their AlivaMab Mouse platform.

Phage display can be used to express variable antibody domains on filamentous phage coat proteins (Phage major coat protein). These phage display antibodies can be used for various research applications. ProAb was announced in December 1997 and involved high throughput screening of antibody libraries against diseased and non-diseased tissue, whilst Proximol used a free radical enzymatic reaction to label molecules in proximity to a given protein.

Monoclonal antibodies have been approved to treat cancer, cardiovascular disease, inflammatory diseases, macular degeneration, transplant rejection, multiple sclerosis and viral infection.

In August 2006, the Pharmaceutical Research and Manufacturers of America reported that U.S. companies had 160 different monoclonal antibodies in clinical trials or awaiting approval by the Food and Drug Administration.

Applications

Diagnostic tests

Once monoclonal antibodies for a given substance have been produced, they can be used to detect the presence of this substance. Proteins can be detected using the Western blot and immuno dot blot tests. In immunohistochemistry, monoclonal antibodies can be used to detect antigens in fixed tissue sections, and similarly, immunofluorescence can be used to detect a substance in either frozen tissue section or live cells.

Analytic and chemical uses

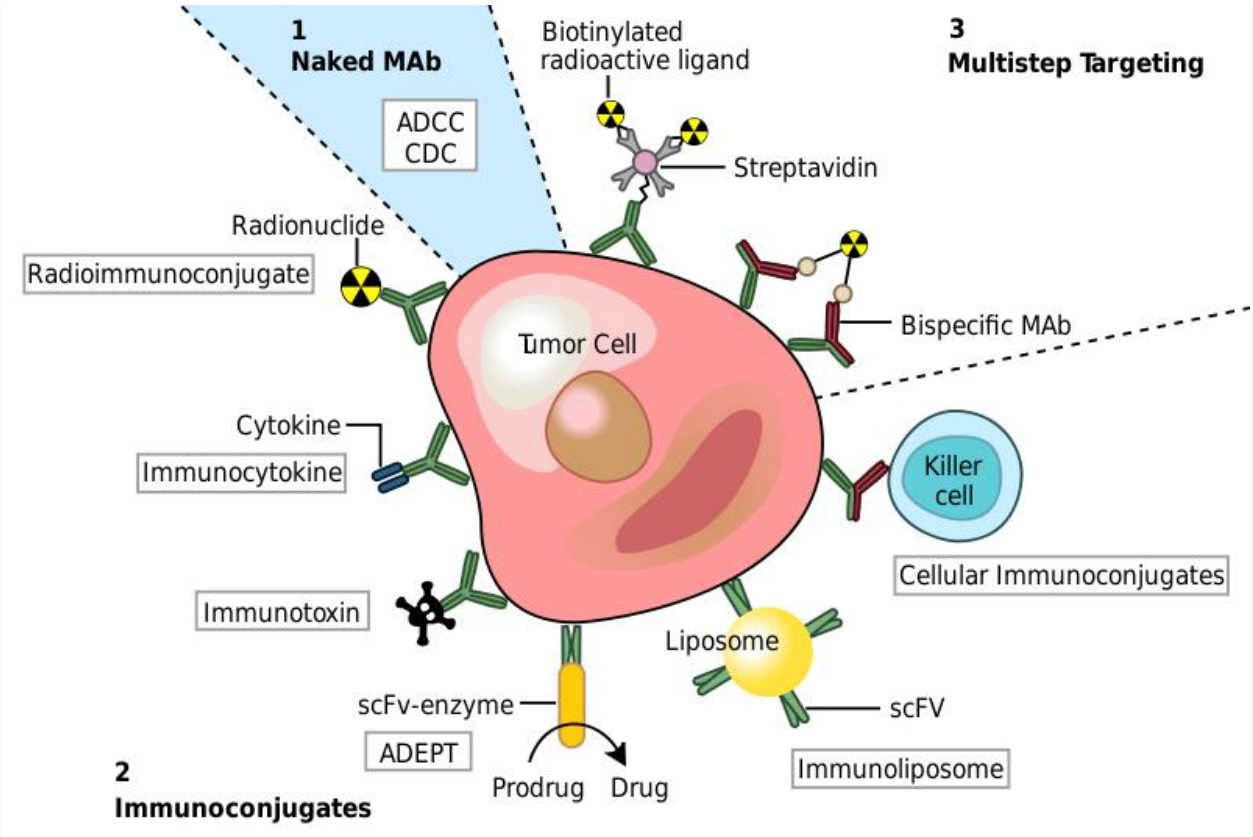
Antibodies can also be used to purify their target compounds from mixtures, using the method of immunoprecipitation.

Therapeutic uses

Therapeutic monoclonal antibodies act through multiple mechanisms, such as blocking of targeted molecule functions, inducing apoptosis in cells which express the target, or by modulating signalling pathways.

Cancer treatment

One possible treatment for cancer involves monoclonal antibodies that bind only to cancer cell-specific antigens and induce an immune response against the target cancer cell. Such mAbs can be modified for delivery of a toxin, radioisotope, cytokine or other active conjugate or to design bispecific antibodies that can bind with their Fab regions both to target antigen and to a conjugate or effector cell. Every intact antibody can bind to cell receptors or other proteins with its Fc region.



Monoclonal antibodies for cancer. ADEPT, antibody directed enzyme prodrug therapy; ADCC: antibody dependent cell-mediated cytotoxicity; CDC: complement-dependent cytotoxicity; MAb: monoclonal antibody; scFv, single-chain Fv fragment.

MAbs approved by the FDA for cancer include:

- Alemtuzumab
- Bevacizumab
- Cetuximab
- Gemtuzumab ozogamicin
- Ipilimumab
- Ofatumumab
- Panitumumab
- Pembrolizumab
- Ranibizumab
- Rituximab
- Trastuzumab

Autoimmune diseases

Monoclonal antibodies used for autoimmune diseases include infliximab and adalimumab, which are effective in rheumatoid arthritis, Crohn's disease, ulcerative colitis and ankylosing spondylitis by their ability to bind to and inhibit TNF-α. Basiliximab and daclizumab inhibit IL-2 on activated T cells and thereby help prevent acute rejection of kidney transplants. Omalizumab inhibits human immunoglobulin E (IgE) and is useful in treating moderate-to-severe allergic asthma.

Examples of therapeutic monoclonal antibodies

For a more comprehensive list, see List of monoclonal antibodies.

Monoclonal antibodies for research applications can be found directly from antibody suppliers, or through use of a specialist search engine like CiteAb. Below are examples of clinically important monoclonal antibodies.

Main category	Type	Application	Mechanism/Target	Mode
Anti-inflammatory	**infliximab**	• rheumatoid arthritis • Crohn's disease • ulcerative colitis • ankylosing spondylitis	inhibits TNF-α	chimeric
	adalimumab	• rheumatoid arthritis • Crohn's disease • ulcerative colitis • ankylosing spondylitis	inhibits TNF-α	human
	basiliximab	• acute rejection of kidney transplants	inhibits IL-2 on activated T cells	chimeric
	daclizumab	• acute rejection of kidney transplants	inhibits IL-2 on activated T cells	humanized
	omalizumab	• moderate-to-severe allergic asthma	inhibits human immunoglobulin E (IgE)	humanized
Anti-cancer	**gemtuzumab**	• relapsed acute myeloid leukemia	targets myeloid cell surface antigen CD33 on leukemia cells	humanized

	alemtuzumab	• B cell leukemia	targets an antigen CD52 on T- and B-lymphocytes	humanized
	rituximab	• non-Hodgkin's lymphoma • rheumatoid arthritis	targets phosphoprotein CD20 on B lymphocytes	chimeric
	trastuzumab	• breast cancer with HER2/neu overexpression	targets the HER2/neu (erbB2) receptor	humanized
	nimotuzumab	• approved in squamous cell carcinomas, Glioma • clinical trials for other indications underway	EGFR inhibitor	humanized
	cetuximab	• approved in squamous cell carcinomas, colorectal carcinoma	EGFR inhibitor	chimeric
	bevacizumab & ranibizumab	• Anti-angiogenic cancer therapy	inhibits VEGF	humanized
Anti-cancer and anti-viral	**bavituximab**	• cancer, hepatitis C infection	immunotherapy, targets phosphatidylserine	chimeric
Other	**palivizumab**	• RSV infections in children	inhibits an RSV fusion (F) protein	humanized
	abciximab	• prevent coagulation in coronary angioplasty	inhibits the receptor GpIIb/IIIa on platelets	chimeric

Side effects

Several monoclonal antibodies, such as Bevacizumab and Cetuximab, can cause different kinds of side effects. These side effects can be categorized into common and serious side effects.

Some common side effects include:

- Dizziness
- Headaches

- Allergies
- Diarrhea
- Cough
- Fever
- Itching
- Back pain
- General weakness
- Loss of appetite
- Insomnia
- Constipation

Among the possible serious side effects are:

- Anaphylaxis
- Bleeding
- Arterial and venous blood clots
- Autoimmune thyroiditis
- Hypothyroidism
- Hepatitis
- Heart failure
- Cancer
- Anemia
- Decrease in white blood cells
- Stomatitis
- Enterocolitis
- Gastrointestinal perforation
- Mucositis

Polyclonal antibodies

Polyclonal antibodies (pAbs) are antibodies that are secreted by different B cell lineages within the body (whereas monoclonal antibodies come from a single cell lineage). They are a collection of immunoglobulin molecules that react against a specific antigen, each identifying a different epitope.

Production

The general procedure to produce polyclonal antibodies is as follows:

1. Antigen preparation
2. Adjuvant selection and preparation
3. Animal selection
4. Injection process
5. Blood serum extraction

An antigen/adjuvant conjugate is injected into an animal of choice to initiate an amplified immune response. After a series of injections over a specific length of time, the animal is expected to have created antibodies against the conjugate. Blood is then extracted from the animal and then purified to obtain the antibody of interest.

Inoculation is performed on a suitable mammal, such as a mouse, rabbit or goat. Larger mammals are often preferred as the amount of serum that can be collected is greater. An antigen is injected into the mammal. This induces the B-lymphocytes to produce IgG immunoglobulins specific for the antigen. This polyclonal IgG is purified from the mammal's serum.

By contrast, monoclonal antibodies are derived from a single cell line

Many methodologies exist for polyclonal antibody production in laboratory animals. Institutional guidelines governing animal use and procedures relating to these methodologies are generally oriented around humane considerations and appropriate conduct for adjuvant (agents which modify the effect of other agents while having few if any direct effects when given by themselves) use. This includes adjuvant selection, routes and sites of administration, injection volumes per site and number of sites per animal. Institutional policies generally include allowable volumes of blood per collection and safety precautions including appropriate restraint and sedation or anesthesia of animals for injury prevention to animals or personnel.

The primary goal of antibody production in laboratory animals is to obtain high titer, high affinity antisera for use in experimentation or diagnostic tests. Adjuvants are used to improve or enhance an immune response to antigens. Most adjuvants provide for an injection site, antigen depot which allows for a slow release of antigen into draining lymph nodes.

Many adjuvants also contain or act directly as:

1. surfactants which promote concentration of protein antigens molecules over a large surface area, and
2. immunostimulatory molecules or properties. Adjuvants are generally used with soluble protein antigens to increase antibody titers and induce a prolonged response with accompanying memory.

Such antigens by themselves are generally poor immunogens. Most complex protein antigens induce multiple B-cell clones during the immune response, thus, the response is polyclonal. Immune responses to non-protein antigens are generally poorly or enhanced by adjuvants and there is no system memory.

Antibodies are currently also being produced from isolation of human B-lymphocytes to produce specific recombinant monoclonal antibody mixtures. The biotechnology company, Symphogen, develops this type of antibodies for therapeutic applications. They are the first research company to reach phase two trials with the monoclonal antibody mixtures that mimic the diversity of the polyclonal antibody drugs. This production prevents viral and prion transmission and this is the simple process.

Animal selection

Animals frequently used for polyclonal antibody production include chickens, goats, guinea pigs, hamsters, horses, mice, rats, and sheep. However, the rabbit is the most commonly used laboratory animal for this purpose. Animal selection should be based upon:

1. the amount of antibody needed,
2. the relationship between the donor of the antigen and the recipient antibody producer (generally the more distant the phylogenetic relationship, the greater the potential for high titer antibody response) and
3. the necessary characteristics [e.g., class, subclass (isotype), complement fixing nature] of the antibodies to be made. Immunization and phlebotomies are stress associated and, at least when using rabbits and rodents, specific pathogen free (SPF) animals are preferred. Use of such animals can dramatically reduce morbidity and mortality due to pathogenic organisms, especially *Pasteurella multocida* in rabbits.

Goats or horses are generally used when large quantities of antisera are required. Many investigators favor chickens because of their phylogenetic distance from mammals. Chickens transfer high quantities of IgY (IgG) into the egg yolk and harvesting antibodies from eggs eliminates the need for the invasive bleeding procedure. One week's eggs can contain 10 times more antibodies than the volume of rabbit blood obtained from one weekly bleeding. However, there are some disadvantages when using certain chicken derived antibodies in immunoassays. Chicken IgY does not fix mammalian complement component C1 and it does not perform as a precipitating antibody using standard solutions.

Although mice are used most frequently for monoclonal antibody production, their small size usually prevents their use for sufficient quantities of polyclonal, serum antibodies. However, polyclonal antibodies in mice can be collected from ascites fluid using any one of a number of ascites producing methodologies.

When using rabbits, young adult animals (2.5–3.0 kg or 5.5-6.5 lbs) should be used for primary immunization because of the vigorous antibody response. Immune function peaks at puberty and primary responses to new antigens decline with age. Female rabbits are generally preferred because they are more docile and are reported to mount a more vigorous immune response than males. At least two animals per antigen should be used when using outbred animals. This principle reduces potential total failure resulting from non-responsiveness to antigens of individual animals.

Antigen preparation

The size, extent of aggregation and relative nativity of protein antigens can all dramatically affect the quality and quantity of antibody produced. Small polypeptides (<10 ku) and non-protein antigens generally need to be conjugated or crosslinked to larger, immunogenic, carrier proteins to increase immunogenicity and provide T cell epitopes. Generally, the larger the immunogenic protein the better. Larger proteins, even in smaller amounts, usually result in better engagement of antigen presenting antigen processing cells for a satisfactory immune response. Injection of soluble, non-aggregated proteins has a higher probability of inducing tolerance rather than a satisfactory antibody response.

Keyhole limpet hemocyanin (KLH) and bovine serum albumin are two widely used carrier proteins. Poly-L-lysine has also been used successfully as a backbone for peptides. Although the

use of Poly-L-lysine reduces or eliminates production of antibodies to foreign proteins, it may result in failure of peptide-induced antibody production. Recently, liposomes have also been successfully used for delivery of small peptides and this technique is an alternative to delivery with oily emulsion adjuvants.

Antigen quantity

Selection of antigen quantity for immunization varies with the properties of the antigen and the adjuvant selected. In general, microgram to milligram quantities of protein in adjuvant are necessary to elicit high titer antibodies. Antigen dosage is generally species, rather than body weight, associated. The so-called "window" of immunogenicity in each species is broad but too much or too little antigen can induce tolerance, suppression or immune deviation towards cellular immunity rather than a satisfactory humoral response. Optimal and usual protein antigen levels for immunizing specific species have been reported in the following ranges:

1. rabbit, 50–1000 µg;
2. mouse, 10–50 µg;
3. guinea pig, 50–500 µg; and
4. goat, 250–5000 µg.

Optimal "priming" doses are reported to be at the low end of each range.

The affinity of serum antibodies increases with time (months) after injection of antigen-adjuvant mixtures and as antigen in the system decreases. Widely used antigen dosages for "booster" or secondary immunizations are usually one half to equal the priming dosages. Antigens should be free of preparative byproducts and chemicals such as polyacrylamide gel, SDS, urea, endotoxin, particulate matter and extremes of pH.

Peptide antibodies

When a peptide is being used to generate the antibody, it is extremely important to design the antigens properly. There are several resources that can aid in the design as well as companies that offer this service. Expasy has aggregated a set of public tools under its ProtScale page that require some degree of user knowledge to navigate. For a more simple peptide scoring tool there is a Antigen Profiler tool available that will enable you to score individual peptide sequences based upon a relation epitope mapping database of previous immunogens used to generate antibodies. Finally, as a general rule peptides should follow some basic criteria.

When examining peptides for synthesis and immunization, it is recommended that certain residues and sequences be avoided due to potential synthesis problems. This includes some of the more common characteristics:

- Extremely long repeats of the same amino acid (e.g. RRRR)
- Serine (S), Threonine (T), Alanine (A), and Valine (V) doublets
- Ending or starting a sequence with a proline (P)
- Glutamine (Q) or Asparagine (N) at the n-terminus
- Peptides over weighted with hydrophobic residues (e.g. V, A, L, I, etc....)

Reactivity

Investigators should also consider the status of nativity of protein antigens when used as immunogens and reaction with antibodies produced. Antibodies to native proteins react best with native proteins and antibodies to denatured proteins react best with denatured proteins. If elicited antibodies are to be used on membrane blots (proteins subjected to denaturing conditions) then antibodies should be made against denatured proteins. On the other hand, if antibodies are to be used to react with a native protein or block a protein active site, then antibodies should be made against the native protein. Adjuvants can often alter the nativity of the protein. Generally, absorbed protein antigens in a preformed oil-in-water emulsion adjuvant, retain greater native protein structure than those in water-in-oil emulsions.

Asepticity

Antigens should always be prepared using techniques that ensure that they are free of microbial contamination. Most protein antigen preparations can be sterilized by passage through a 0.22μm filter. Septic abscesses often occur at inoculation sites of animals when contaminated preparations are used. This can result in failure of immunization against the targeted antigen.

Adjuvants

There are many commercially available immunologic adjuvants. Selection of specific adjuvants or types varies depending upon whether they are to be used for research and antibody production or in vaccine development. Adjuvants for vaccine use only need to produce protective antibodies and good systemic memory while those for antiserum production need to rapidly induce high titer, high avidity antibodies. No single adjuvant is ideal for all purposes and all have advantages and disadvantages. Adjuvant use generally is accompanied by undesirable side effects of varying severity and duration. Research on new adjuvants focuses on substances which have minimal toxicity while retaining maximum immunostimulation. Investigators should always be aware of potential pain and distress associated with adjuvant use in laboratory animals.

The most frequently used adjuvants for antibody production are Freund's, Alum, the Ribi Adjuvant System and Titermax.

Freund’s adjuvants

There are two basic types of Freund's adjuvants: Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA). FCA is a water-in-oil emulsion that localizes antigen for release periods up to 6 months. It is formulated with mineral oil, the surfactant mannide monoleate and heat killed *Mycobacterium tuberculosis*, *Mycobacterium butyricum* or their extracts (for aggregation of macrophages at the inoculation site). This potent adjuvant stimulates both cell mediated and humoral immunity with preferential induction of antibody against epitopes of denatured proteins. Although FCA has historically been the most widely used adjuvant, it is one of the more toxic agents due to non-metabolizable mineral oil and it induces granulomatous reactions. Its use is limited to laboratory animals and it should be used only with weak antigens. It should not be used more than once in a single animal since multiple FCA inoculations can cause severe systemic reactions and decreased immune responses. Freund's Incomplete Adjuvant has the same formulation as FCA but does not contain mycobacterium or its components. FIA usually is limited to booster doses of antigen since it normally much less effective than FCA for primary antibody induction. Freund's adjuvants are normally mixed with equal parts of antigen preparations to form stable emulsions.

Ribi Adjuvant System

Ribi adjuvants are oil-in-water emulsions where antigens are mixed with small volumes of a metabolizable oil (squalene) which are then emulsified with saline containing the surfactant Polysorbate 80. This system also contains refined mycobacterial products (cord factor, cell wall skeleton) as immunostimulants and bacterial monophosphoryl lipid A. Three different species oriented formulations of the adjuvant system are available. These adjuvants interact with membranes of immune cells resulting in cytokine induction, which enhances antigen uptake, processing and presentation. This adjuvant system is much less toxic and less potent than FCA but generally induces satisfactory amounts of high avidity antibodies against protein antigens.

Titermax

Titermax represents a newer generation of adjuvants that are less toxic and contain no biologically derived materials. It is based upon mixtures of surfactant acting, linear, blocks or chains of nonionic copolymers polyoxypropylene (POP) and polyoxyethylene (POE). These copolymers are less toxic than many other surfactant materials and have potent adjuvant properties which favor chemotaxis, complement activation and antibody production. Titermax adjuvant forms a microparticulate water-in-oil emulsion with a copolymer and metabolizable squalene oil. The copolymer is coated with emulsion stabilizing silica particles which allows for incorporation of large amounts of a wide variety of antigenic materials. The adjuvant active copolymer forms hydrophilic surfaces, which activate complement, immune cells and increased expression of class II major histocompatibility molecules on macrophages. Titermax presents antigen in a highly concentrated form to the immune system, which often results in antibody titers comparable to or higher than FCA.

Specol: Specol is a water in oil adjuvant made of purified mineral oil. It has been reported to induce immune response comparable to Freund's adjuvant in rabbit and other research animal while producing fewer histological lesions.

Pharmaceutical uses

Digoxin Immune Fab is the antigen binding fragment of polyclonal antibodies raised to Digitalis derivative as a hapten bound to a protein and is used for the reversal of life-threatening digoxin or digitoxin toxicity.

Rho(D) immune globulin is made from pooled human plasma provided by Rh-negative donors with antibodies to the D antigen. It is used to provide passive immune binding of antigen, preventing a maternal active immune response which could potentially result in hemolytic disease of the newborn.Rozrolimupab is the anti-RhD recombinant human polyclonal antibody composed of 25 unique IgG1 antibodies and is used for the treatment of immune thrombocytopenia purpura and prevention of isoimmunization in Rh-negative pregnant women.

Advantages

The use of polyclonal antibodies (PAbs) over monoclonal antibodies has its advantages. They're inexpensive to make and can be generated fairly quickly, taking up to several months to produce. PAbs are heterogeneous, which allows them to bind to a wide range of antigen epitopes. Because PAbs are produced from a large number of B cell clones, they're more likely to successfully bind

to a specific antigen. PAbs remain stable in different environments, such as a change in pH or salt concentration, which allows them to be more applicable in certain procedures. Additionally, depending on the amount needed, PAbs can be made in large quantities in relation to the size of the animal used.

Hypersensitivity

Hypersensitivity (also called **hypersensitivity reaction** or **intolerance**) refers to undesirable reactions produced by the normal immune system, including allergies and autoimmunity. They are usually referred to as an over-reaction of the immune system and these reactions may be damaging, uncomfortable, or occasionally fatal. Hypersensitivity reactions require a pre-sensitized (immune) state of the host. The Gell and Coombs classification of hypersensitivity is the most widely used, and distinguishes four types of immune response which result in bystander tissue damage.

Coombs and Gell classification

Immunologic aspects of hypersensitivity reactions

Type	Alternative names	Antibodies or Cell Mediators	Immunologic Reaction	Often mentioned disorders
I	• Allergy • Immediate • Anaphylactic	• Antibody IgE	Fast response which occurs in minutes, rather than multiple hours or days. Free antigens cross link the IgE on mast cells and basophils which causes a release of vasoactive biomolecules. Testing can be done via skin test for specific IgE.	• Atopy • Anaphylaxis • Asthma • Churg-Strauss Syndrome
II	• Cytotoxic, • Antibody-dependent	• Antibody Ig M • Antibody Ig G • Complement • MAC	Antibody (IgM or IgG) binds to antigen on a target cell, which is actually a host cell that is perceived by the immune system as foreign, leading to cellular destruction via the MAC. Testing includes both the direct and indirect Coombs test.	• Autoimmune hemolytic anemia • Rheumatic heart disease • Thrombocytopenia • Erythroblastosis fetalis • Goodpasture's syndrome • Graves' disease • Myasthenia gravis

Immunologic aspects of hypersensitivity reactions

Type	Alternative names	Antibodies or Cell Mediators	Immunologic Reaction	Often mentioned disorders
III	• Immune complex	• Antibody Ig G • Complement • Neutrophils	Antibody (IgG) binds to soluble antigen, forming a circulating immune complex. This is often deposited in the vessel walls of the joints and kidney, initiating a local inflammatory reaction.[4]	• Serum sickness • Rheumatoid arthritis • Arthus reaction • Post streptococcal glomerulonephritis • Membranous nephropathy • Reactive arthritis • Lupus nephritis • Systemic lupus erythematosus • Extrinsic allergic alveolitis (hypersensitivity pneumonitis)
IV	• Delayed, • cell-mediated immune memory response, • Antibody-independent	Cells • T-cells	T helper cells (specifically T_h1 cells) are activated by an antigen presenting cell. When the antigen is presented again in the future, the memory Th1 cells will activate macrophages and cause an inflammatory response. This ultimately can lead to tissue damage.	• Contact dermatitis, including Urushiol-induced contact dermatitis (poison ivy rash). • Mantoux test • Chronic transplant rejection • Multiple sclerosis • Coeliac disease • Hashimoto's thyroiditis • Granuloma annulare
V	• Autoimmune	• IgM or IgG • Complement		• Graves' disease • Myasthenia gravis

Type V

This is an additional type that is sometimes (especially in the UK) used as a distinction from Type 2.
Instead of binding to cell surfaces, the antibodies recognise and bind to the cell surface receptors, which either prevents the intended ligand binding with the receptor or mimics the effects of the ligand, thus impairing cell signaling.
Some clinical examples are:

- Graves' disease
- Myasthenia gravis

The use of Type 5 is rare. These conditions are more frequently classified as Type 2, though sometimes they are specifically segregated into their own subcategory of Type 2.

Vaccine

A **vaccine** is a biological preparation that provides active acquired immunity to a particular infectious disease. A vaccine typically contains an agent that resembles a disease-causing microorganism and is often made from weakened or killed forms of the microbe, its toxins, or one of its surface proteins. The agent stimulates the body's immune system to recognize the agent as a threat, destroy it, and to further recognize and destroy any of the microorganisms associated with that agent that it may encounter in the future. Vaccines can be prophylactic (to prevent or ameliorate the effects of a future infection by a natural or "wild" pathogen), or therapeutic (e.g., vaccines against cancer, which are being investigated).
The administration of vaccines is called vaccination. Vaccination is the most effective method of preventing infectious diseases; widespread immunity due to vaccination is largely responsible for the worldwide eradication of smallpox and the restriction of diseases such as polio, measles, and tetanus from much of the world. The effectiveness of vaccination has been widely studied and verified; for example, vaccines that have proven effective include the influenza vaccine, the HPV vaccine, and the chicken pox vaccine. The World Health Organization (WHO) reports that licensed vaccines are currently available for twenty-five different preventable infections.
The terms *vaccine* and *vaccination* are derived from *Variolae vaccinae* (smallpox of the cow), the term devised by Edward Jenner to denote cowpox. He used it in 1798 in the long title of his *Inquiry into the Variolae vaccinae Known as the Cow Pox*, in which he described the protective effect of cowpox against smallpox. In 1881, to honor Jenner, Louis Pasteur proposed that the terms should be extended to cover the new protective inoculations then being developed.

Effectiveness

There is overwhelming scientific consensus that vaccines are a very safe and effective way to fight and eradicate infectious diseases. Limitations to their effectiveness, nevertheless, exist. Sometimes, protection fails because the host's immune system simply does not respond adequately or at all. Lack of response commonly results from clinical factors such as diabetes, steroid use, HIV infection, or age. It also might fail for genetic reasons if the host's immune system includes no strains of B cells that can generate antibodies suited to reacting effectively and binding to the antigens associated with the pathogen.
Even if the host does develop antibodies, protection might not be adequate; immunity might develop too slowly to be effective in time, the antibodies might not disable the pathogen completely, or there might be multiple strains of the pathogen, not all of which are equally

susceptible to the immune reaction. However, even a partial, late, or weak immunity, such as a one resulting from cross-immunity to a strain other than the target strain, may mitigate an infection, resulting in a lower mortality rate, lower morbidity, and faster recovery.

Adjuvants commonly are used to boost immune response, particularly for older people (50–75 years and up), whose immune response to a simple vaccine may have weakened.

The efficacy or performance of the vaccine is dependent on a number of factors:

- the disease itself (for some diseases vaccination performs better than for others)
- the strain of vaccine (some vaccines are specific to, or at least most effective against, particular strains of the disease)
- whether the vaccination schedule has been properly observed.
- idiosyncratic response to vaccination; some individuals are "non-responders" to certain vaccines, meaning that they do not generate antibodies even after being vaccinated correctly.
- assorted factors such as ethnicity, age, or genetic predisposition.

If a vaccinated individual does develop the disease vaccinated against (breakthrough infection), the disease is likely to be less virulent than in unvaccinated victims.

The following are important considerations in the effectiveness of a vaccination program:

1. careful modeling to anticipate the effect that an immunization campaign will have on the epidemiology of the disease in the medium to long term
2. ongoing surveillance for the relevant disease following introduction of a new vaccine
3. maintenance of high immunization rates, even when a disease has become rare.

In 1958, there were 763,094 cases of measles in the United States; 552 deaths resulted. After the introduction of new vaccines, the number of cases dropped to fewer than 150 per year (median of 56). In early 2008, there were 64 suspected cases of measles. Fifty-four of those infections were associated with importation from another country, although only 13% were actually acquired outside the United States; 63 of the 64 individuals either had never been vaccinated against measles or were uncertain whether they had been vaccinated.

Vaccines led to the eradication of smallpox, one of the most contagious and deadly diseases in humans. Other diseases such as rubella, polio, measles, mumps, chickenpox, and typhoid are nowhere near as common as they were a hundred years ago thanks to widespread vaccination programs. As long as the vast majority of people are vaccinated, it is much more difficult for an outbreak of disease to occur, let alone spread. This effect is called herd immunity. Polio, which is transmitted only between humans, is targeted by an extensive eradication campaign that has seen endemic polio restricted to only parts of three countries (Afghanistan, Nigeria, and Pakistan). However, the difficulty of reaching all children as well as cultural misunderstandings have caused the anticipated eradication date to be missed several times.

Vaccines also help prevent the development of antibiotic resistance. For example, by greatly reducing the incidence of pneumonia caused by *Streptococcus pneumoniae*, vaccine programs have greatly reduced the prevalence of infections resistant to penicillin or other first-line antibiotics.

The measles vaccine is estimated to prevent 1 million deaths every year.

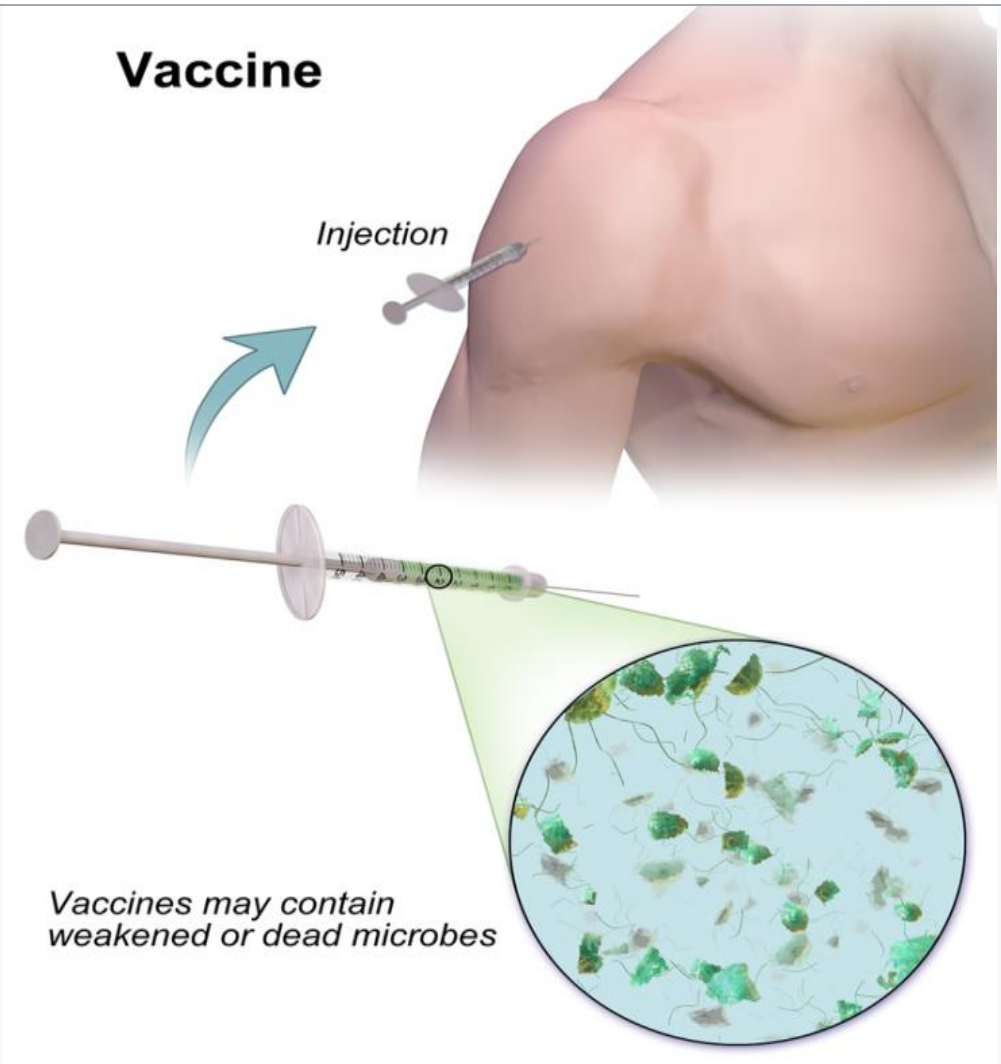
Adverse effects

Vaccination given during childhood is generally safe. Adverse effects, if any, are generally mild. The rate of side effects depends on the vaccine in question. Some common side effects include fever, pain around the injection site, and muscle aches.Additionally, some individuals may be allergic to ingredients in the vaccine. MMR vaccine is rarely associated with febrile seizures.

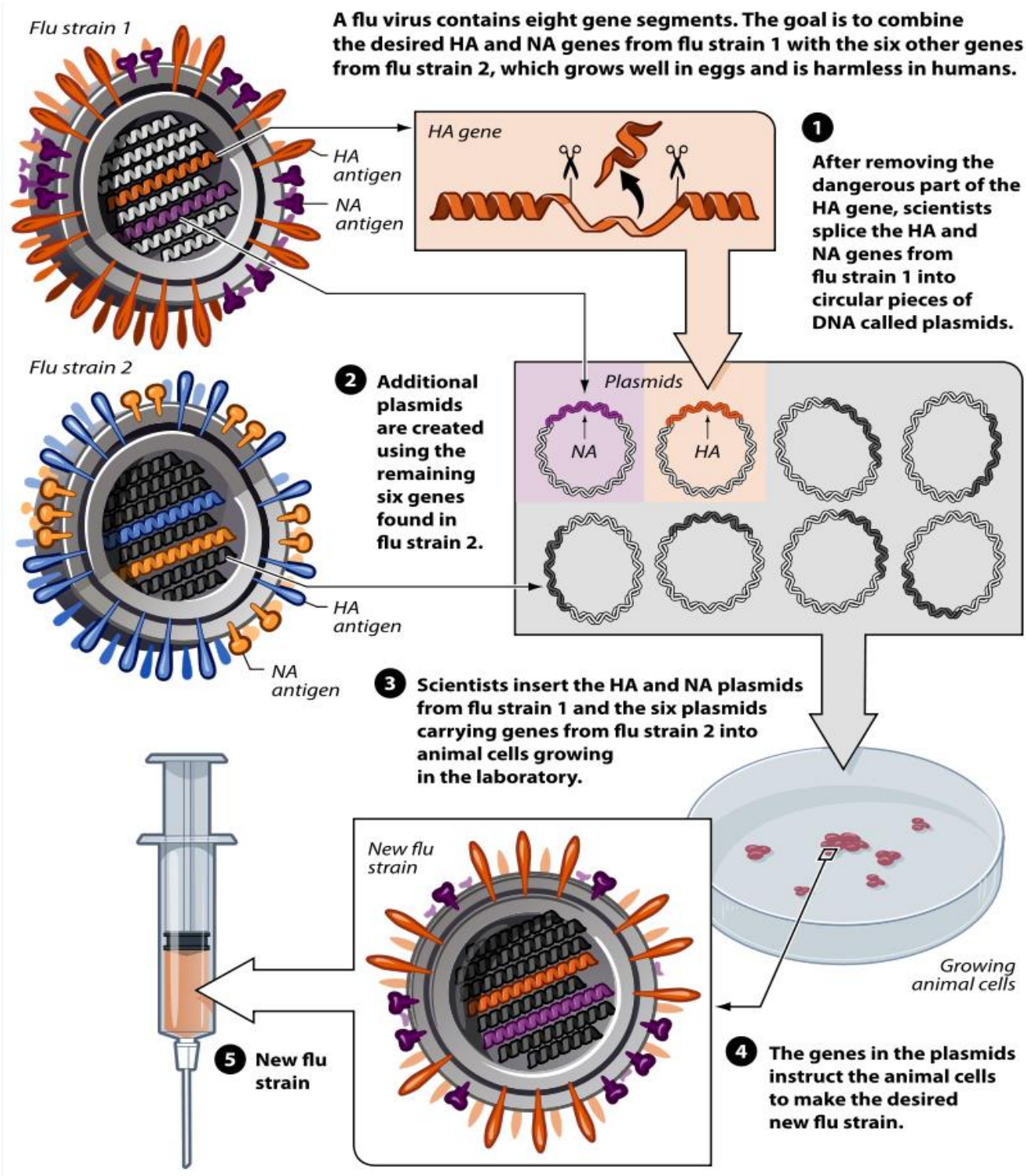
Severe side effects are extremely rare. Varicella vaccine is rarely associated with complications in immunodeficient individuals and rotavirus vaccines are moderately associated with intussusception.

At least 19 countries have no-fault compensation programs to provide compensation for those suffering severe adverse effects of vaccination. The United States' program is known as the National Childhood Vaccine Injury Act and the United Kingdom employs the Vaccine Damage Payment.

Types



Vaccine



Avian flu vaccine development by reverse genetics techniques.

Vaccines contain dead or inactivated organisms or purified products derived from them.
There are several types of vaccines in use. These represent different strategies used to try to reduce the risk of illness while retaining the ability to induce a beneficial immune response.

Inactivated

Some vaccines contain inactivated, but previously virulent, micro-organisms that have been destroyed with chemicals, heat, or radiation. Examples include the polio vaccine, hepatitis A vaccine, rabies vaccine and some influenza vaccines.

Attenuated

Some vaccines contain live, attenuated microorganisms. Many of these are active viruses that have been cultivated under conditions that disable their virulent properties, or that use closely related but less dangerous organisms to produce a broad immune response. Although most attenuated vaccines are viral, some are bacterial in nature. Examples include the viral diseases yellow fever, measles, mumps, and rubella, and the bacterial disease typhoid. The live *Mycobacterium tuberculosis* vaccine developed by Calmette and Guérin is not made of a contagious strain but contains a virulently modified strain called "BCG" used to elicit an immune response to the vaccine. The live attenuated vaccine containing strain *Yersinia pestis* EV is used for plague immunization. Attenuated vaccines have some advantages and disadvantages. They typically provoke more durable immunological responses and are the preferred type for healthy adults. But they may not be safe for use in immunocompromised individuals, and on rare occasions mutate to a virulent form and cause disease.

Toxoid

Toxoid vaccines are made from inactivated toxic compounds that cause illness rather than the micro-organism. Examples of toxoid-based vaccines include tetanus and diphtheria. Toxoid vaccines are known for their efficacy. Not all toxoids are for micro-organisms; for example, *Crotalus atrox* toxoid is used to vaccinate dogs against rattlesnake bites.

Subunit

Protein subunit—rather than introducing an inactivated or attenuated micro-organism to an immune system (which would constitute a "whole-agent" vaccine), a fragment of it can create an immune response. Examples include the subunit vaccine against Hepatitis B virus that is composed of only the surface proteins of the virus (previously extracted from the blood serum of chronically infected patients, but now produced by recombination of the viral genes into yeast) or as an edible algae vaccine, the virus-like particle (VLP) vaccine against human papillomavirus (HPV) that is omposed of the viral major capsid protein, and the hemagglutinin and neuraminidase subunits of the influenza virus. Subunit vaccine is being used for plague immunization.

Conjugate

Conjugate—certain bacteria have polysaccharide outer coats that are poorly immunogenic. By linking these outer coats to proteins (e.g., toxins), the immune system can be led to recognize the polysaccharide as if it were a protein antigen. This approach is used in the *Haemophilus influenzae* type B vaccine.

Experimental

A number of innovative vaccines are also in development and in use:

- Dendritic cell vaccines combine dendritic cells with antigens in order to present the antigens to the body's white blood cells, thus stimulating an immune reaction. These vaccines have shown some positive preliminary results for treating brain tumors and are also tested in malignant melanoma.

- Recombinant vector – by combining the physiology of one micro-organism and the DNA of another, immunity can be created against diseases that have complex infection processes. An example is the RVSV-ZEBOV vaccine licensed to Merck that is being used in 2018 to combat ebola in Congo.
- DNA vaccination – an alternative, experimental approach to vaccination called *DNA vaccination*, created from an infectious agent's DNA, is under development. The proposed mechanism is the insertion (and expression, enhanced by the use of electroporation, triggering immune system recognition) of viral or bacterial DNA into human or animal cells. Some cells of the immune system that recognize the proteins expressed will mount an attack against these proteins and cells expressing them. Because these cells live for a very long time, if the pathogen that normally expresses these proteins is encountered at a later time, they will be attacked instantly by the immune system. One potential advantage of DNA vaccines is that they are very easy to produce and store. As of 2015, DNA vaccination is still experimental and is not approved for human use.
- T-cell receptor peptide vaccines are under development for several diseases using models of Valley Fever, stomatitis, and atopic dermatitis. These peptides have been shown to modulate cytokine production and improve cell-mediated immunity.
- Targeting of identified bacterial proteins that are involved in complement inhibition would neutralize the key bacterial virulence mechanism.

While most vaccines are created using inactivated or attenuated compounds from micro-organisms, synthetic vaccines are composed mainly or wholly of synthetic peptides, carbohydrates, or antigens.

Valence

Vaccines may be *monovalent* (also called *univalent*) or *multivalent* (also called *polyvalent*). A monovalent vaccine is designed to immunize against a single antigen or single microorganism. A multivalent or polyvalent vaccine is designed to immunize against two or more strains of the same microorganism, or against two or more microorganisms. The valency of a multivalent vaccine may be denoted with a Greek or Latin prefix (e.g., *tetravalent* or *quadrivalent*). In certain cases, a monovalent vaccine may be preferable for rapidly developing a strong immune response.

Heterotypic

Also known as heterologous or "Jennerian" vaccines, these are vaccines that are pathogens of other animals that either do not cause disease or cause mild disease in the organism being treated. The classic example is Jenner's use of cowpox to protect against smallpox. A current example is the use of BCG vaccine made from Mycobacterium bovis to protect against human tuberculosis.

Nomenclature

Various fairly standardized abbreviations for vaccine names have developed, although the standardization is by no means centralized or global. For example, the vaccine names used in the United States have well-established abbreviations that are also widely known and used elsewhere. An extensive list of them provided in a sortable table and freely accessible, is available at a US Centers for Disease Control and Prevention web page. The page explains that "The abbreviations [in] this table (Column 3) were standardized jointly by staff of the Centers for Disease Control and Prevention, ACIP Work Groups, the editor of the *Morbidity and Mortality Weekly Report* (MMWR), the editor of *Epidemiology and Prevention of Vaccine-Preventable Diseases* (the Pink Book), ACIP members, and liaison organizations to the ACIP."

Some examples are "DTaP" for diphtheria and tetanus toxoids and acellular pertussis vaccine, "DT" for diphtheria and tetanus toxoids, and "Td" for tetanus and diphtheria toxoids. At its page on tetanus vaccination, the CDC further explains that "Upper-case letters in these abbreviations denote full-strength doses of diphtheria (D) and tetanus (T) toxoids and pertussis (P) vaccine. Lower-case "d" and "p" denote reduced doses of diphtheria and pertussis used in the adolescent/adult-formulations. The 'a' in DTaP and Tdap stands for 'acellular,' meaning that the pertussis component contains only a part of the pertussis organism." Another list of established vaccine abbreviations is at the CDC's page called "Vaccine Acronyms and Abbreviations", with abbreviations used on U.S. immunization records. The United States Adopted Name system has some conventions for the word order of vaccine names, placing head nouns first and adjectives postpositively. This is why the USAN for "OPV" is "poliovirus vaccine live oral" rather than "oral poliovirus vaccine".

Developing immunity

The immune system recognizes vaccine agents as foreign, destroys them, and "remembers" them. When the virulent version of an agent is encountered, the body recognizes the protein coat on the virus, and thus is prepared to respond, by (1) neutralizing the target agent before it can enter cells, and (2) recognizing and destroying infected cells before that agent can multiply to vast numbers.

When two or more vaccines are mixed together in the same formulation, the two vaccines can interfere. This most frequently occurs with live attenuated vaccines, where one of the vaccine components is more robust than the others and suppresses the growth and immune response to the other components. This phenomenon was first noted in the trivalent Sabin polio vaccine, where the amount of serotype 2 virus in the vaccine had to be reduced to stop it from interfering with the "take" of the serotype 1 and 3 viruses in the vaccine. This phenomenon has also been found to be a problem with the dengue vaccines currently being researched,where the DEN-3 serotype was found to predominate and suppress the response to DEN-1−2 and 4 serotypes.

Adjuvants and preservatives

Vaccines typically contain one or more adjuvants, used to boost the immune response. Tetanus toxoid, for instance, is usually adsorbed onto alum. This presents the antigen in such a way as to produce a greater action than the simple aqueous tetanus toxoid. People who have an adverse reaction to adsorbed tetanus toxoid may be given the simple vaccine when the time comes for a booster.

In the preparation for the 1990 Persian Gulf campaign, whole cell pertussis vaccine was used as an adjuvant for anthrax vaccine. This produces a more rapid immune response than giving only the anthrax vaccine, which is of some benefit if exposure might be imminent.

Vaccines may also contain preservatives to prevent contamination with bacteria or fungi. Until recent years, the preservative thimerosal was used in many vaccines that did not contain live virus. As of 2005, the only childhood vaccine in the U.S. that contains thimerosal in greater than trace amounts is the influenza vaccine, which is currently recommended only for children with certain risk factors. Single-dose influenza vaccines supplied in the UK do not list thiomersal (its UK name) in the ingredients. Preservatives may be used at various stages of production of vaccines, and the most sophisticated methods of measurement might detect traces of them in the finished product, as they may in the environment and population as a whole.

Schedule

Vaccination schedule

In order to provide the best protection, children are recommended to receive vaccinations as soon as their immune systems are sufficiently developed to respond to particular vaccines, with additional "booster" shots often required to achieve "full immunity". This has led to the development of complex vaccination schedules. In the United States, the Advisory Committee on Immunization Practices, which recommends schedule additions for the Centers for Disease Control and Prevention, recommends routine vaccination of children against: hepatitis A, hepatitis B, polio, mumps, measles, rubella, diphtheria, pertussis, tetanus, HiB, chickenpox, rotavirus, influenza, meningococcal disease and pneumonia. A large number of vaccines and boosters recommended (up to 24 injections by age two) has led to problems with achieving full compliance. In order to combat declining compliance rates, various notification systems have been instituted and a number of combination injections are now marketed (e.g., Pneumococcal conjugate vaccine and MMRV vaccine), which provide protection against multiple diseases.

Besides recommendations for infant vaccinations and boosters, many specific vaccines are recommended for other ages or for repeated injections throughout life—most commonly for measles, tetanus, influenza, and pneumonia. Pregnant women are often screened for continued resistance to rubella. The human papillomavirus vaccine is recommended in the U.S. (as of 2011) and UK (as of 2009). Vaccine recommendations for the elderly concentrate on pneumonia and influenza, which are more deadly to that group. In 2006, a vaccine was introduced against shingles, a disease caused by the chickenpox virus, which usually affects the elderly.

History

Edward Jenner

Prior to the introduction of vaccination with material from cases of cowpox (heterotypic immunisation), smallpox could be prevented by deliberate inoculation of smallpox virus, later referred to as variolation to distinguish it from smallpox vaccination. The earliest hints of the practice of inoculation for smallpox in China come during the 10th century. The Chinese also practiced the oldest documented use of variolation, dating back to the fifteenth century. They implemented a method of "nasal insufflation" administered by blowing powdered smallpox material, usually scabs, up the nostrils. Various insufflation techniques have been recorded throughout the sixteenth and seventeenth centuries within China. Two reports on the Chinese

practice of inoculation were received by the Royal Society in London in 1700; one by Dr. Martin Lister who received a report by an employee of the East India Company stationed in China and another by Clopton Havers.

Sometime during the late 1760s whilst serving his apprenticeship as a surgeon/apothecary Edward Jenner learned of the story, common in rural areas, that dairy workers would never have the often-fatal or disfiguring disease smallpox, because they had already contracted cowpox, which has a very mild effect in humans. In 1796, Jenner took pus from the hand of a milkmaid with cowpox, scratched it into the arm of an 8-year-old boy, James Phipps, and six weeks later inoculated (variolated) the boy with smallpox, afterwards observing that he did not catch smallpox. Jenner extended his studies and in 1798 reported that his vaccine was safe in children and adults and could be transferred from arm-to-arm reducing reliance on uncertain supplies from infected cows. Since vaccination with cowpox was much safer than smallpox inoculation, the latter, though still widely practised in England, was banned in 1840.

The second generation of vaccines was introduced in the 1880s by Louis Pasteur who developed vaccines for chicken cholera and anthrax, and from the late nineteenth century vaccines were considered a matter of national prestige, and compulsory vaccination laws were passed.

The twentieth century saw the introduction of several successful vaccines, including those against diphtheria, measles, mumps, and rubella. Major achievements included the development of the polio vaccine in the 1950s and the eradication of smallpox during the 1960s and 1970s. Maurice Hilleman was the most prolific of the developers of the vaccines in the twentieth century. As vaccines became more common, many people began taking them for granted. However, vaccines remain elusive for many important diseases, including herpes simplex, malaria, gonorrhea, and HIV.

Timeline of vaccines

Economics of development

One challenge in vaccine development is economic: Many of the diseases most demanding a vaccine, including HIV, malaria and tuberculosis, exist principally in poor countries. Pharmaceutical firms and biotechnology companies have little incentive to develop vaccines for these diseases because there is little revenue potential. Even in more affluent countries, financial returns are usually minimal and the financial and other risks are great.

Most vaccine development to date has relied on "push" funding by government, universities and non-profit organizations. Many vaccines have been highly cost effective and beneficial for public health. The number of vaccines actually administered has risen dramatically in recent decades.This increase, particularly in the number of different vaccines administered to children before entry into schools may be due to government mandates and support, rather than economic incentive.

Patents

The filing of patents on vaccine development processes can also be viewed as an obstacle to the development of new vaccines. Because of the weak protection offered through a patent on the final product, the protection of the innovation regarding vaccines is often made through the patent of processes used in the development of new vaccines as well as the protection of secrecy.

According to the World Health Organization, the biggest barrier to local vaccine production in less developed countries has not been patents, but the substantial financial, infrastructure, and workforce expertise requirements needed for market entry. Vaccines are complex mixtures of biological compounds, and unlike the case of drugs, there are no true generic vaccines. The vaccine produced by a new facility must undergo complete clinical testing for safety and efficacy similar to that undergone by that produced by the original manufacturer. For most vaccines, specific processes have been patented. These can be circumvented by alternative manufacturing methods, but this required R&D infrastructure and a suitably skilled workforce. In the case of a few relatively new vaccines such as the human papillomavirus vaccine, the patents may impose an additional barrier.

Production

Vaccine production has several stages. First, the antigen itself is generated. Viruses are grown either on primary cells such as chicken eggs (e.g., for influenza) or on continuous cell lines such as cultured human cells (e.g., for hepatitis A). Bacteria are grown in bioreactors (e.g., *Haemophilus influenzae* type b). Likewise, a recombinant protein derived from the viruses or bacteria can be generated in yeast, bacteria, or cell cultures. After the antigen is generated, it is isolated from the cells used to generate it. A virus may need to be inactivated, possibly with no further purification required. Recombinant proteins need many operations involving ultrafiltration and column chromatography. Finally, the vaccine is formulated by adding adjuvant, stabilizers, and preservatives as needed. The adjuvant enhances the immune response of the antigen, stabilizers increase the storage life, and preservatives allow the use of multidose vials. Combination vaccines are harder to develop and produce, because of potential incompatibilities and interactions among the antigens and other ingredients involved.

Vaccine production techniques are evolving. Cultured mammalian cells are expected to become increasingly important, compared to conventional options such as chicken eggs, due to greater productivity and low incidence of problems with contamination. Recombination technology that produces genetically detoxified vaccine is expected to grow in popularity for the production of bacterial vaccines that use toxoids. Combination vaccines are expected to reduce the quantities of antigens they contain, and thereby decrease undesirable interactions, by using pathogen-associated molecular patterns.

In 2010, India produced 60 percent of the world's vaccine worth about $900 million(€670 million).

Excipients

Beside the active vaccine itself, the following excipients and residual manufacturing compounds are present or may be present in vaccine preparations:

- Aluminum salts or gels are added as adjuvants. Adjuvants are added to promote an earlier, more potent response, and more persistent immune response to the vaccine; they allow for a lower vaccine dosage.
- Antibiotics are added to some vaccines to prevent the growth of bacteria during production and storage of the vaccine.

- Egg protein is present in influenza and yellow fever vaccines as they are prepared using chicken eggs. Other proteins may be present.
- Formaldehyde is used to inactivate bacterial products for toxoid vaccines. Formaldehyde is also used to inactivate unwanted viruses and kill bacteria that might contaminate the vaccine during production.
- Monosodium glutamate (MSG) and 2-phenoxyethanol are used as stabilizers in a few vaccines to help the vaccine remain unchanged when the vaccine is exposed to heat, light, acidity, or humidity.
- Thimerosal is a mercury-containing antimicrobial that is added to vials of vaccine that contain more than one dose to prevent contamination and growth of potentially harmful bacteria. Due to the controversy surrounding thimerosal it has been removed from most vaccines except multi-use influenza, where it was reduced to levels so that a single dose contained less than 1 microgram of mercury, a level similar to eating 10g of canned tuna.[86]

Role of preservatives

Many vaccines need preservatives to prevent serious adverse effects such as *Staphylococcus* infection, which in one 1928 incident killed 12 of 21 children inoculated with a diphtheria vaccine that lacked a preservative. Several preservatives are available, including thiomersal, phenoxyethanol, and formaldehyde. Thiomersal is more effective against bacteria, has a better shelf-life, and improves vaccine stability, potency, and safety; but, in the U.S., the European Union, and a few other affluent countries, it is no longer used as a preservative in childhood vaccines, as a precautionary measure due to its mercury content. Although controversial claims have been made that thiomersal contributes to autism, no convincing scientific evidence supports these claims.Furthermore, a 10–11 year study of 657,461 children found that the MMR vaccine does not cause autism and actually reduced the risk of autism by 7 percent.

Delivery systems

The development of new delivery systems raises the hope of vaccines that are safer and more efficient to deliver and administer. Lines of research include liposomes and *ISCOM* (immune stimulating complex).

Notable developments in vaccine delivery technologies have included oral vaccines. Early attempts to apply oral vaccines showed varying degrees of promise, beginning early in the 20th century, at a time when the very possibility of an effective oral antibacterial vaccine was controversial. By the 1930s there was increasing interest in the prophylactic value of an oral typhoid fever vaccine for example.

An oral polio vaccine turned out to be effective when vaccinations were administered by volunteer staff without formal training; the results also demonstrated increased ease and efficiency of administering the vaccines. Effective oral vaccines have many advantages; for example, there is no risk of blood contamination. Vaccines intended for oral administration need not be liquid, and as solids, they commonly are more stable and less prone to damage or to spoilage by freezing in transport and storage. Such stability reduces the need for a "cold chain": the resources required to keep vaccines within a restricted temperature range from the

manufacturing stage to the point of administration, which, in turn, may decrease costs of vaccines.

A microneedle approach, which is still in stages of development, uses "pointed projections fabricated into arrays that can create vaccine delivery pathways through the skin".

An experimental needle-free vaccine delivery system is undergoing animal testing. A stamp-size patch similar to an adhesive bandage contains about 20,000 microscopic projections per square cm. This dermal administration potentially increases the effectiveness of vaccination, while requiring less vaccine than injection.

Plasmids

The use of plasmids has been validated in preclinical studies as a protective vaccine strategy for cancer and infectious diseases. However, in human studies, this approach has failed to provide clinically relevant benefit. The overall efficacy of plasmid DNA immunization depends on increasing the plasmid's immunogenicity while also correcting for factors involved in the specific activation of immune effector cells.

Veterinary medicine

Goat vaccination against sheep pox and pleural pneumonia

Vaccinations of animals are used both to prevent their contracting diseases and to prevent transmission of disease to humans. Both animals kept as pets and animals raised as livestock are routinely vaccinated. In some instances, wild populations may be vaccinated. This is sometimes accomplished with vaccine-laced food spread in a disease-prone area and has been used to attempt to control rabies in raccoons.

Where rabies occurs, rabies vaccination of dogs may be required by law. Other canine vaccines include canine distemper, canine parvovirus, infectious canine hepatitis, adenovirus-2, leptospirosis, bordatella, canine parainfluenza virus, and Lyme disease, among others.

Cases of veterinary vaccines used in humans have been documented, whether intentional or accidental, with some cases of resultant illness, most notably with brucellosis. However, the reporting of such cases is rare and very little has been studied about the safety and results of such practices. With the advent of aerosol vaccination in veterinary clinics for companion animals, human exposure to pathogens that are not naturally carried in humans, such as Bordetella bronchiseptica, has likely increased in recent years. In some cases, most notably rabies, the parallel veterinary vaccine against a pathogen may be as much as orders of magnitude more economical than the human one.

DIVA vaccines

DIVA (Differentiation of Infected from Vaccinated Animals), also known as SIVA (Segregation of Infected from Vaccinated Animals), vaccines make it possible to differentiate between infected and vaccinated animals.

DIVA vaccines carry at least one epitope less than the microorganisms circulating in the field. An accompanying diagnostic test that detects antibody against that epitope allows us to actually make that differentiation.

First DIVA vaccines

The first DIVA vaccines (formerly termed marker vaccines and since 1999 coined as DIVA vaccines) and companion diagnostic tests have been developed by J.T. van Oirschot and colleagues at the Central Veterinary Institute in Lelystad, The Netherlands. They found that some existing vaccines against pseudorabies (also termed Aujeszky's disease) had deletions in their viral genome (among which was the gE gene). Monoclonal antibodies were produced against that deletion and selected to develop an ELISA that demonstrated antibodies against gE. In addition, novel genetically engineered gE-negative vaccines were constructed.Along the same lines, DIVA vaccines and companion diagnostic tests against bovine herpesvirus 1 infections have been developed.

Use in practice

The DIVA strategy has been applied in various countries and successfully eradicated pseudorabies virus. Swine populations were intensively vaccinated and monitored by the companion diagnostic test and, subsequently, the infected pigs were removed from the population. Bovine herpesvirus 1 DIVA vaccines are also widely used in practice.

Other DIVA vaccines (under development)

Scientists have put and still, are putting much effort in applying the DIVA principle to a wide range of infectious diseases, such as, for example, classical swine fever, avian influenza, Actinobacillus pleuropneumonia and Salmonella infections in pigs.

Trends

Vaccine development has several trends:

- Until recently, most vaccines were aimed at infants and children, but adolescents and adults are increasingly being targeted.
- Combinations of vaccines are becoming more common; vaccines containing five or more components are used in many parts of the world.
- New methods of administering vaccines are being developed, such as skin patches, aerosols via inhalation devices, and eating genetically engineered plants.
- Vaccines are being designed to stimulate innate immune responses, as well as adaptive.
- Attempts are being made to develop vaccines to help cure chronic infections, as opposed to preventing disease.
- Vaccines are being developed to defend against bioterrorist attacks such as anthrax, plague, and smallpox.
- Appreciation for sex and pregnancy differences in vaccine responses "might change the strategies used by public health officials".

- Scientists are now trying to develop synthetic vaccines by reconstructing the outside structure of a virus, this will help prevent vaccine resistance.

Principles that govern the immune response can now be used in tailor-made vaccines against many noninfectious human diseases, such as cancers and autoimmune disorders. For example, the experimental vaccine CYT006-AngQb has been investigated as a possible treatment for high blood pressure. Factors that affect the trends of vaccine development include progress in translatory medicine, demographics, regulatory science, political, cultural, and social responses.

Plants as bioreactors for vaccine production

Transgenic plants have been identified as promising expression systems for vaccine production. Complex plants such as tobacco, potato, tomato, and banana can have genes inserted that cause them to produce vaccines usable for humans. Bananas have been developed that produce a human vaccine against hepatitis B. Another example is the expression of a fusion protein in alfalfa transgenic plants for the selective directioning to antigen presenting cells, therefore increasing vaccine potency against Bovine Viral Diarrhea Virus (BVDV).

Immune response during Tuberculosis and AIDS

- Tuberculosis

Tuberculosis (TB), caused by Mycobacterium tuberculosis (Mtb) infection, was among the top 10 causes of death worldwide in 2017 with about 1.5 million registered deceases (1). Mtb was responsible for approximately 10.0 million incident cases of TB disease with 10% of these occurring
among children. One to five bacilli may suffice to transmit the infection by air. When inhaled, Mtb encounters a first line of defense consisting of airway epithelial cells (AECs) and "professional" phagocytes (neutrophils, monocytes and dendritic cells). If this first line succeeds in eliminating the Mtb rapidly, the infection aborts. Otherwise, phagocytes are infected and the Mtb reproduces inside the cells, initially causing few, if any, clinical manifestations. The establishment of the infection, the development of active TB (ATB) rather than latent TB infection (LTBI) and the eventual evolution of LTBI to ATB depends on the complex relation between bacterial and host factors.

METHODS

In order to perform a narrative review of the available literature,we searched the PubMed database from April 2014 through April2019, using the following key words: "immune," "immunity,""tuberculosis," "*Mycobacterium tuberculosis*." Subsequently, foreach topic, specific key words ("susceptibility," "resistance,""virulence," "airway epithelial cell," "macrophage," "neutrophil,""dendritic cell," "natural killer," "mast cell," "complement," "CD4," "CD8," "humoral," "antibody," and "granuloma") wereassociated with the word "tuberculosis" in order to accessproper specific literature. The search and the selection processwere not systematic. Articles were limited to English languageand full text availability, and they were excluded if they wereredundant or not pertinent. References of all relevant articleswere also evaluated, and studies published previously than2014 were cited if considered relevant. Results were criticallysummarized in the following paragraphs: (1) "host and bacterialdeterminants in human tuberculosis," (2) "innate immuneresponse against *Mycobacterium tuberculosis,*" and (3) "adaptiveimmune response against *Mycobacterium tuberculosis.*"

HOST AND BACTERIAL DETERMINANTS IN HUMAN TUBERCULOSIS

Several epidemiological models of family members who havelong shared the bedroom with subjects with ATB, sailors wholived in confined spaces with subjects with open TB and extensive case studies of South African miners and Norwegian or Americanstudents, have clearly demonstrated that 5 to 20% of thosewho meet subjects with ATB do not become infected (resilientindividuals or *resisters*), or become infected only transientlyand then get rid of the infection (early sterilization or *earlyclearance*). An individual can be defined resilient if after closeand prolonged contact with the index case shows simultaneous negativity of the skin reactivity test and of the IFN-γ release assay(IGRA) which persists for at least 1 year. Studies carried out onsiblings have shown that Mtb resilience is more frequent

betweentwo siblings than between two unrelated subjects, suggestingthe role of genetics in the development of Mtb resilience.Genome wide linkage analysis detected several loci like *2q21-2q24*, *5p13-5q22*, and the *TST1* on *11p14* associated with the resilientphenotype).

On the other side, the study of TB susceptibility, has shedlight onto various components of immunity to mycobacteriain humans. Different genetic polymorphisms which modulate the host immune response in favor of TB infection anddisease progression have been identified in human leukocyteantigens (HLA), toll like receptors (TLR), vitamin D receptors (VDR), cytokines with their receptors and many otherfunctional immune components. Moreover, mendeliansusceptibilities to mycobacterial disease (MSMD) have been identified as clinical conditions with selective susceptibilityto poorly virulent mycobacteria in the absence of patentimmunodeficiency. Since 1996, 11 genes which underlie21 different genetic disorders related to interferon (IFN)-γimmunity and responsible for MSMD have been identified. Furthermore, transcriptomic studies have described a TBsignature of neutrophil-driven IFN-inducible genes in ATB,including IFN-γ but also type I IFNs, reflecting diseaseextension and response to treatment and highlighting thepreviously under-appreciated role of IFNαβ signaling in TBpathogenesis.

Beyond host factors, bacterial virulence constitutes the othermajor player when evaluating the risk of TB infection. Virulenceis not merely limited to bacterial strain or burden in respiratorysecretion but takes into account the differential Mtb geneexpression in the different phases of infection. Mtb lacksclassical virulence factors such as toxins and its immuneescaping ability depends on the modulation of lipid metabolism,metal-transporter proteins, protease, proteins inhibiting theantimicrobial effectors of macrophages (M8s) and manyothers.

The study of immune response in resilient and susceptibleindividuals, together with bacterial factors, has offeredfundamental information for the understanding of TBimmunology suggesting potential improvements in diagnosticand therapeutic approaches.

INNATE IMMUNE RESPONSE AGAINST

Mycobacteriumtuberculosis

The significance of innate immunity in the defense against Mtbstands out clearly as we consider the MSMD where a disruptionof the innate axis leads to dramatic, life-threatening clinicalpresentation of TB.

M8, neutrophils, dendritic cells (DCs), natural killer cells(NK), mast cells and complement are the major players of innateimmunity. On the other hand, AECs also contribute to thedefense attempt against Mtb and could be considered as innateimmunity components.

Airway Epithelial Cells

AECs are the first cells to come in contact with Mtb. Beyondtheir major role as physical barriers,

they display severalimmunological functions albeit being traditionally considered as "non-professional" immune cells. Through pattern recognitionreceptors (PRRs), AECs can perceive the presence of Mtb andconsequently modulate the composition of the airways surface liquid improving its antimicrobial capacity. Moreover,PRRs activation leads to the production of inflammatorycytokines and to the activation of mucosal-associated invariant T cells stimulating IFN-γ and tumor necrosis factor (TNF)-αproduction

Macrophages

M8s are the first line of defense, but only if the ratio offorces lies clearly to their advantage and the intervention isimmediate they can cancel the infection. Otherwise, theyfavor its development because they become first a niche forthe slow replication of the Mtb and then the sanctuary for thepersistence of the infection inside the phagosome during thelatent infection phase. Mtb expresses an extremely wide variety ofvirulence factors that counteract M8s efforts in suppressing thepathogen. Among Mtb strategies we can include the inhibition of intracellular trafficking, the inhibition of autophagy, theacquisition of cytosol access, the induction of host cell death andthe neutralization of toxic components as reactive oxygen speciesand toxic metals.

Whilst IFN-γ is a key element in the containment of Mtbwithin the M8, it is now widely recognized that performingthis function requires the presence of vitamin D. Thanks to vitamin D, the macrophage increases phagosome maturation andthe production of antimicrobial peptides through the maximalregulation of the hCAP-18 gene encoding for cathelicidinantimicrobial peptide which activates, in turn, the transcriptionof autophagy-related genes. The 6 kDa early secretoryantigenic target (ESAT-6) protein family secretion (ESX) systemis a sophisticated secretion system that Mtb uses to exportproteins with immune-escaping activity. So that while theIFN-γ axis is struggling against the ESX-system to enhancephagolysosmal activity, vitamin D deficiency abets the Mtbreplication.

Nitric oxide (NO) within macrophages plays a less importantrole in humans than that one observed in animal models.
Although, in humans too, reactive oxygen species (ROS) playa well-documented role in the immune response to Mtb ashighlighted by the discovery of TB susceptibility in patients displaying mutations in a catalytic subunit of NADPH-oxidase2 involved in ROS production on phagolysosomal membrane. Moreover, it is demonstrated that Mtb affects NADPHoxidase activity through nucleoside diphosphate kinase (Npk)interaction with small GTPases involved in NADPH-oxidaseassembly and functioning.
The fight unfolds inside the phagosome of the M8 betweenthe cell and the Mtb with metals as a battlefield of sorts. TheM8 delivers an overload of copper and zinc, which are toxic to Mtb at high concentrations. Mtb deploys a series of protectionmechanisms that include

controlling the capture of such metals,oxidation, and an increase in efflux. The up-regulation of*ctpC* gene encoding for the P-type ATPase which regulates theintra-bacterial levels of Zinc is a clear example of how Mtbmanages to prevent heavy metal poisoning. As a countermove, the M8 then attempts to block the arrival of nutrients tothe Mtb such as iron and manganese.

Neutrophils

Neutrophil granulocytes are the most widely present cellpopulation within BAL and sputum in patients with active TB. There is evidence of their role as defense mechanismsagainst Mtb. In particular, there is a clear inverse correlationbetween the number of neutrophilic granulocytes in theperipheral blood and the hazard of developing TB aftercontact with an infectious subject. Antimicrobial peptides andapoptotic neutrophils are phagocytized by M8 and carry outan effective activity against Mtb inside these cells. This ispossible thanks to the fusion, within the M8, of neutrophilgranules with phagosomes containing Mtb. Furthermore, ETosis, extracellular traps (ET) formation, is a type of cell death that differently from apoptosis is characterized by DNArelease, consequent M8 activation and the formation of a DNA scaffold that incorporates pathogens and exposes themto antimicrobial molecules. The formation of neutrophilsETs, thus constitutes an improved killing strategy and a synergic alliance between phagocytes.

Moreover, as with many immune mechanisms, neutrophilsdo not only play a positive role, but can eventually constitutea negative element, causing tissue damage through production and subsequent release of their antimicrobial products. Tothis phenomenon, it must be added the potentially negativeinteraction with lymphocytes. Neutrophils express on their cell membrane the ligand 1 of cell death (programmed death ligand1 or PD-L1), which interacts with the lymphocyte receptorfor programmed death (programmed death receptor or PD-1), and determines, in the course of chronic infections, the loss offunction and finally the death of lymphocytes.

Neutrophils with expressed PD-L1 are present in highproportion in patients with ATB.

Dendritic Cells

DCs are functionally located in the middle between innateand adaptive immunity. These cells play a fundamental role inthe immune defense system due to antigen presentation, costimulating activity and the large cytokine production capacitywith activity on the lymphocytes cluster of differentiation (CD) 4. DCs role in TB immunity is controversial. Present evidenceis not sufficient to establish whether these cells strengthencellular immunity or if their manipulation by the Mtb can beused as a tool to diminish specific T-cell response. DCssoon become a niche for the Mtb. CD209, also called DCspecific intercellular adhesion molecule 3-grabbing non-integrinreceptor (DC-SIGN), represents the gateway of Mtb into theDC. CD209 is, under normal conditions, a receptor forCD54, the intercellular adhesion molecule 1 (ICAM1) presenton endothelial cells where it favors DCs migration. CD209 iscoupled with the lipoarabinomannan mannose (ManLAM) ofthe Mtb that penetrates into the cell. This penetration leads toa disruption

of DCs activity by prompting the production ofinterleukin (IL)-10 and reducing the production of IL-12, thus causing a suppression of T lymphocytes activity. Themanipulation of the maturation of the DCs probably representsone of the winning strategies of Mbt that, by restraining theactivity of DCs and, consequently, of T lymphocytes, allowsthe Mtb, whose speed of growth is relatively slow, to efficientlyestablish a bridgehead in the airways. Based on the above mentioned mechanism DC-SIGN has recently been proposedas a potential target for a vaccine purpose eventually able to enhance immunity against Mtb. On the other side, DCSIGN may prevent tissue pathology by maintaining a balancedinflammatory state and thus promoting host protection.

Natural Killer Cells

It is certain that NK cells enter the immunological circuitof Mtb infection both in their CD56 diminished phenotype(preferential cytotoxic activity) and in CD56 bright phenotype (preferential cytokines secreting activity). In several studiesthe percentage representation of NK cell is augmented in theperipheral blood of patients with ATB. There is a directrelationship between NK cell representation, clinical conditionand response to therapy. Nonetheless, it has not yet beenascertained exactly what the cause and the consequence is. Several components of the Mbt wall are recognized and boundby the NKp44 receptor of NK cells. In addition, Mtbinfected NKs lyse and stimulate M8s to produce IFN-γ and IL-22, which increase phagolysosomal fusion thus inhibiting Mtbreplication and stimulate the production of additional IFN-γ byCD8+ lymphocytes. This effect is mediated by the IL-15 and IL-18 production by an infected M8. As a further infection controlmechanism DCs favor the development of T lymphocytes withγδ receptor through TNF-α and IL-12 production.

Mast Cells

The role of mast cells in Mtb infection is not well-knownin humans. In mice, mast cells capture Mtb via CD48and internalize it. This process ensues the development of acytokine cascade, some of them with protective roles, includingIL-12, IL-13, IL-6, CXLL2, CCL7, CCL2, TNF-α, and consequentneutrophils recall in the site of infection. Histamine's role is ambivalent in terms of Mtb clearance as on one handit augments lung neutrophilia but on the other it seemsto impair the efficient production of a T helper 1 (Th1)response. The presence of mast cell ETs containing Mtbin humans has not been proved. However, mast cells enclosea large number of mediators known to take part in theprocess.

Complement Proteins

The role of the complement cascade on the progression of theinfection and Mtb disease is almost unknown. It is likelythat the C5 and C7 components play a defensive role. However, ithas been observed that a high expression of C1q correlates witha worse clinical condition, so as to be a

marker between latentTB and active TB but still with unclear significance in terms ofpathogenesis.

ADAPTIVE IMMUNE RESPONSE AGAINST *Mycobacterium tuberculosis*

The immune response of T lymphocytes begins at the momentthat Mtb spreads inside the lymph nodes but its arousal laysin the early activation of the innate immune system. Inside thelymph nodes, T lymphocytes undergo a process of activationand expansion of the specific populations for the Mtb antigens.
However, at this point, the largest part is done and theinfection is now established. Cellular immune response can beevidenced 2–6 weeks after Mtb infection by the development of a delayed hypersensitivity response to intradermal injectedtuberculin (DHT) or purified protein derivative. It is importantto underline that protective response to TB does not relate with DHT positivity and disease can occur in those who mountadequate DHT response.

Lymphocytes T CD4+

The *in vivo* human model of HIV-infected CD4+-depletedpatients is the most striking evidence of the pivotal role ofthese cells in TB immunity. The process of maturation of the phagosome of M8 is facilitated and increased by IFN-γ, theproduction of which is mostly dependent on the T lymphocytesCD4+ with a minor support of lymphocytes CD8+ and T lymphocytes with γδ receptor. Animal models of knockoutmice for IFN-γ clearly show that these animals suffer a verysevere course of Mtb infection exactly as it happens in humans with MSMD. It is well known that patients with mutations ingenes encoding IFN-γ or its receptors undergo disseminatedinfection by BCG or other non-tuberculous components of the*mycobacteria genus*. IFN-γ production is modest in patientswith active TB, but recovers with antitubercular treatmentwithout reaching levels similar to those of uninfected subjects. The optimal production of IFN-γ, as well as that of IL-17,is linked to an equally optimal cooperation between DCs andT lymphocytes CD4+. In its defensive strategy, Mtb markedly interferes in the CD40-CD40 ligand binding, that is essential forthe cooperation between both cell lines. The importance ofIFN-γ production by CD4+ cells is particularly relevant at theearly stages of Mtb infection as it is demonstrated that adequateIFN-γ levels can be obtained with 3 weeks of delay even in CD4- disrupted mice thanks to the compensation offered by other cell types like CD8+.
Moreover, IFN-γ cannot control infection alone and it requires the association of other molecules such as IL-6, IL-1 and the TNF-α. The chemokines CCL5, CCL9, CXCL10, and CCL2attract immunity cells at the site of infection and their production is stimulated by TNF-α and boosts the production of NO byM8.
Several studies, both in adult and pediatric patients, havedemonstrated CD4+ percentage and absolute value reduction inthe peripheral blood of patients with ATB suggesting both an augmented pooling in the site of infection but also eventuallya primary role of TB in immune modifications related with the severity of infection.

A portion of T lymphocytes are Foxp3+ and perform a control function over the activity of other T lymphocytes in fact, theyare defined as T regulators (Treg). It is only on a hypothetical level that we can imagine any positive role of this cell line on the disease progression limiting tissue damage by other immune cells; however, it has been ascertained that, by restraining theresponse of the T lymphocytes, the Tregs favor the infection development and persistence. Similarly, T lymphocytes CD4+ may deal more damage, or at least become irrelevant,rather than hinder the progress of the infection.

Lymphocytes T CD8+

For a long time, it was considered that, unlike T lymphocytesCD4+, T lymphocytes CD8+ had no role in controllingthe infection and Mtb disease. This concept stemmed fromthe modest availability of human models with T lymphocyteCD8+ defect, unlike the large human model of HIV infection.

An activity against Mtb is conceivable considering that Tlymphocytes CD8+ recognize Mtb antigens through class Imolecules of the major histocompatibility complex (MHC), and produce IL-2, IFN-γ and TNF-α, which have a well-known rolein controlling Mtb. Furthermore, T lymphocytes CD8+ exert acytolytic action against Mtb by means of perforin and granulysin,albeit not by Fas (CD95) -Fas ligand interaction. Thisdirect cell-to-cell contact determines the apoptosis of the Mtbinfected cell (especially M8) depriving Mtb from its naturalgrowth environment and at the same time reducing its viabilityby unknown mechanism. On the other hand, lymphocytesCD8+ produce IL-10 and TGF-β which instead favor thedevelopment of the Mtb infection.

Humoral Adaptive Immunity

The role of humoral adaptive immunity in TB is extremelyuncertain. Complement-mediated opsonization does notalter Mtb survival. High levels of antibody titers correlate with more serious conditions of infection and disease, and passiveimmunization with antibodies does not confer protection.Patients with a defective antibody-production mechanism and/or B lymphocyte defect are not particularly at risk of TB infection.

The role of the crystallizable fragment or Fc in the constantportion of the immunoglobulin, which binds and activatesvarious cell lines present in the granuloma (NK cells, monocytes, neutrophils) the low-affinity FcγRIIIb receptor and the highaffinity FcγRIIa receptor have shown different functional profilesand glycosylation patterns in subjects with ATB rather thanLTBI.

The loss of FcγRIIIb activity and the increase of FcγRIIamediated inhibitory function (which correlates with a high IL10production) are associated with a worse clinical profile and can distinguish ATB from LTBI and suggests a role of antibodiesin the augmented phagolysosomal maturation and Mtb killingobserved in LTBI patients.

The Ancestral Defense: Granuloma

Following the development of adaptive immunity, a complexand well-coordinated mechanism is established between bothimmunity mechanisms, i.e., innate and adaptive, which seal theMtb inside granuloma. This mechanism developsin at least 90% of the infected subjects and leads to LTBI.

During latent TB, which would be better described as nonreplicating-persistence phase (in fact, Mtb works perfectly albeitin a different way than during active TB), the subject is generallypositive for the tuberculin skin test and for the IGRA. Latent TB becomes active when, for the most various reasons,a condition of immunodepression develops. At this stage, thesubject may become capable of transmitting the infection becausethe granuloma opens in the bronchial lumen and Mtb areexpelled when coughing. At the beginning of the infection, Mtbdemands an environment with inflammatory traits to developthe granuloma; subsequently, however, its survival is linked toan environment lacking or with low inflammation. This switchis caused by ESAT-6, a well-known Mtb virulence factorinvolved in the ESX secretion system, to which it gives its name. ESAT-6 causes the transformation of M8 from phenotypeM1, which produces IL-6, IL-12 and TNF-α, into M8 withphenotype M2, which is capable of stimulating production ofIL-10. As currently known, IL-6 and TNF-α favorinflammation, whilst IL-10 curbs it. Accordingly, the formationof the granuloma is triggered by the M8 and then develops with multi-nucleated giant cells and M8 with abundant presenceof intracytoplasmic lipids, which lend these cells their frothyappearance. Around these cells, there is a ring of T lymphocytesalthough B lymphocytes, neutrophils and dendritic cells (CD) also participate in the formation of granuloma.

Inside the granuloma, cholesterol—and not glucose orglycerol—is the only carbon source. This leads to a lack of carbonand nutrients, hypoxia and a high concentration of nitric oxide (NO). The significance of cholesterol in the survival of Mtb insidethe granuloma is evidenced by the negative role that statins playagainst Mtb.

The debate remains open on whether the granuloma is purelyprotective for the host or if it promotes disease progressionand tissue damage. This uncertainty depends on the extreme heterogeneity detected in granuloma morphology at thedifferent stages of disease, on the role of inflammation, hypoxiaand differential Mtb gene expression and lipid metabolismmanipulation inside the granulomas of ATB and LTBI patients. The most likely answer is that an homeostatic interactionestablishes and the granuloma becomes a well-suited shelter forboth Mtb long-term survival and host protection

AIDS

Recent advances that enable the identification ofpatients within the first few weeks of HIV-1 infection1,2have provided researchers access to samples fromacutely infected patients earlier and in higher numbersthan previously available. This has advanced our under-standing of the nature of the transmitted virus and thefirst immune responses in the period before establish-ment of stable viraemia (the viral set point), which occurs3–6 months after infection. The first weeks

followingHIV-1 transmission are extremely dynamic: they areassociated with rapid damage to generative immune cellmicroenvironments, caused by direct viral cytopathicityand bystander effects, and with immune responses thatpartially control the virus.
In this Review, we focus our discussion on the earlyhost or viral factors that are crucial for determining theoutcome of HIV-1 infection. These include the natureof the transmitted virus, or founder virus, suppression ofthe initial infection by genetically influenced immune responses, and the rate of virus mutation and viralfitness of selected mutants. In addition, we reviewwhat is known about the nature of innate and adaptiveimmune responses during this early phase of infection,drawn from studies of humans and macaques infectedwith HIV-1 and simian immunodeficiency virus (SIV),respectively. Finally, we discuss how our knowledge ofthe events of early HIV-1 infection can improve thedesign of a preventive vaccine .

The biology of early HIV-1 infection

Transmission. Most HIV-1 infections occur by sexualexposure through the genital tract or rectal mucosa.Although it is not possible to study the very first events following HIV-1 transmission in humans *in vivo*, wehave gained some understanding from studies in which mucosal tissue explants were infected *in vitro*3–5. Further understanding of the first stages of infection *in vivo* has been obtained from studies in which macaques were inoc-ulated intrarectally or intravaginally with SIV. It is still uncertain whether HIV-1 is transmitted as a free or a cell-bound virus, but SIV can be transmitted in either form8.
In addition, the mechanism by which HIV-1 crosses the genital mucosal epithelium is unclear. Diffusion ofHIV-1 across the vaginal mucosa is slowed by cervico-vaginal mucus9. It is possible that virus that reaches themucosal epithelium crosses this barrier by transcytosis orby making direct contact with dendrites of intraepithelialdendritic cells (DCs). Preliminary unpublished findingssuggest that virions may also move through intercellular spaces in the epithelium to make initial cell contact withunderlying mucosal Langerhans cells and CD4+ T cells.
Given that multiple sexual exposures are usually neededfor infection to occur, crossing of the epithelial cell barrierby the virus is probably a rare event, although it is morecommon if the genital mucosa is damaged by physicaltrauma or co-existing genital infections.

Eclipse phase. Following transmission of the virus, thereis a period of ~10 days, known as the eclipse phase,before viral RnA becomes detectable in the plasma(FIG. 1). Single-genome amplification and sequencing of thefirst detectable virus has shown that ~80% of mucosallytransmitted HIV-1 clade B and C infections are initiatedby a single virus12–14. Infectious molecular clones derivedfrom these primary founder viruses could infect CD4+ T cells with greater efficiency than they could infect monocytes and macrophages, which differs from thevirus quasispecies that arise later in the infection andcan infect lymphoid and myeloid cell types with equalefficiency. Studies in rhesus macaques inoculated intra-rectally with a complex SIV quasispecies also showedthat productive infection arises from a

single infecting virus, which supports the use of SIV infection of rhesusmacaques as a model for HIV-1 transmission and vaccine studies. In other studies in which macaques were infected experimentally, the first cells to be infected inthe vaginal mucosa were found in foci of resident memory T cells that expressed the virus receptors CD4 andCC-chemokine receptor 5 (CCR5), which is consistentwith the cell tropism of cloned HIV-1 founder virus. Homogeneity of the founder virus indicates that theestablished infection probably arises from a single focusof infected mucosal CD4+ T cells. Virus replication atthis focus might in fact be supported by early innateimmune responses that lead to the recruitment of additional susceptible T cells to the site16. The failure of mostinfected foci to become established may be explainedby the high error rate in reverse transcription thatoccurs during HIV-1 replication and the effects of thehost antiviral apolipoprotein B mRnA-editing enzyme, catalytic polypeptide-like (APOBEC) cytidine deaminasesAPoBeC3G and APoBeC3F, which cause many virusesproduced in infected CD4+ T cells to be defective.

Peak viraemia. At the end of the eclipse phase, virusand/or virus-infected cells reach the draining lymphnode, where they meet activated CD4+CCR5+ T cells,which are targets for further infection. This processis augmented by DCs that bind and internalize virus through DC-specific ICAM3-grabbing non-integrin(DC-SIGn; also known as CD209) and carry the virus to activated T cells. B cells may also be involved in theearly spread of infection by binding the virus through the complement receptor CD21 (also known as CR2). The virus then replicates rapidly and spreads through-out the body to other lymphoid tissues, particularlygut-associated lymphoid tissue (GAlT), where activated CD4+CCR5+ memory T cells are present in high numbers. Approximately 20% of CD4+ T cells in theGAlT are infected in both humans with acute HIV-1infection and SIV-infected macaques. up to 60% ofuninfected CD4+ T cells at this site become activatedand die by apoptosis, resulting in the release of apoptotic microparticles that can suppress immune function23. Therefore, ~80% of CD4+ T cells in the GAlTcan be depleted in the first 3 weeks of HIV-1 infection.

while HIV-1 is replicating in the GAlT andother lymphoid tissues, the plasma viraemia increasesexponentially to reach a peak, usually more than a million RnA copies per ml of blood, at 14–21 days afterSIV infection in macaques and at 21–28 days after HIV-1 infection in humans (FIG. 1). CD4+ T cell numbers arelow at the time of peak viraemia but later return to nearnormal levels in the blood but not in the GAlT. Although B cells are not depleted during early HIV-1infection, B cell responses are impaired owing to thedestruction of other cell types that are important forthe development of germinal centres. up to 50% of germinal centres in the gut are lost within the first 80 days of infection.

Establishing viral set point. At the point of peak viraemiathe immune response has not affected the amino acidsequence of the virus, despite the extensive activation of innate immune cells. Thereafter, the viral loaddecreases over 12–20 weeks to reach a more stable level,known as the viral set point. Virus diversification occurs during this decrease in viral load, and multipleescape mutants are selected under the pressure of adaptive

immune responses that are first detectable just before peak viraemia. In the absence of antiretroviral drug therapy(ART), the set point is maintained by a balance betweenvirus turnover and the immune responses.

The death rate of infected cells has been calculatedfrom decay curves of viraemia after ART initiation. Formost infected memory T cells, the half-life is less than aday34. However, other cell populations have slower ratesof decay35, and cell populations other than CD4+ T cellsmaintain latent pools of HIV-1. Cells are probably latently infected within days of HIV-1 transmissionand are unlikely to be removed by natural or vaccine stimulated anti-HIV-1 immune responses, given thatthey cannot be eliminated by ART37.

Immune activation. Activation of innate cells and Band T cells is a striking feature of acute HIV-1 infection of humans and SIV infection of rhesus macaques,and it persists to a varying degree into chronic infection. The dysregulation of immune cells is not limitedto cells that are infected by, or are specific for, HIV-1. Chronic immune activation is not observedin naturally SIV-infected sooty mangabeys, in whichthe infections rarely progress to AIDS. This is despitehigh levels of virus replication and acute CD4+ T celldepletion39, suggesting a role for immune activation inAIDS development. Indeed, there is a positive correlation between markers of CD8+ T cell activation and HIVdisease progression40–42. Immune activation is associated with early and extensive apoptosis of B and T cells, leading to the release ofapoptotic microparticles into the blood (FIG. 2), andincreased expression of tumour necrosis factor (TnF) related apoptosis-inducing ligand (TRAIl; also known as TnFSF10) and FAS ligand (also known as CD95l),which kill bystander cells and are immunosuppressive.

The causes of HIV-associated immune activation established in early HIV-1 infection are not clearly defined.

Multiple related events probablycontribute to such activation, including direct viralinfection of immune cells, pro-inflammatory cytokineproduction by innate cells (which drives both directand bystander activation of other immune cells), translocation of microbial products into the blood throughdamaged intestinal epithelium, loss of virally infectedregulatory T (TReg) cells and chronic mycobacterial andviral co-infections.

Genetic control of HIV-1 set point

In contrast to other pathogens that have infected andselected humans for millennia, HIV-1 is a new pathogen to humans. Therefore, the influence of the host'sgenetics on the immune response to HIV-1 infectionmay be more evident. The most dramatic finding in thisregard is that homozygosity for a 32 base pair deletionin *CCR5*, which abrogates its expression, protects almostcompletely from HIV-1 infection48. Furthermore, theHlA alleles HlA-B*5701, HlA-B*5703, HlA-B*5801,HlA-B27 and HlA-B51 are all associated with goodcontrol of the virus and a slower progression to AIDS49,partly because the epitopes recognized by the T cells inthese individuals are focused on conserved regions ofthe viral Gag protein. A genome-wide

association study50 found a strong protective influence for a single nucleotide polymorphism (SNP) located 35 kilobasesupstream of the HlA-C locus and confirmed the association of HlA-B57 with a low viral set point. This HlA-C-linked SnP may be associated with low-level expressionof HlA-C50, which might in turn affect T cell or naturalkiller (nK) cell function during HIV-1 infection. Bycontrast, some subtypes of HlA-B35 are associated withrapid disease progression, especially if homozygous, although the mechanism is not understood.
It has been shown that the expression of the killerimmunoglobulin-like receptors KIR3DS1 and KIR3Dl1 which deliver activating and inhibitory signals to nKcells, respectively — delays progression to AIDS in individuals with HlA class I allotypes containing the 80Ile variant of the Bw4 motif52, which are thought to be ligands for these receptors. expansion of nK cells thatexpress KIR3DS1 and/or KIR3Dl1 during acute HIV-1infection has been observed but only if the HlA-B Bw480Ile motif is present55, which is supported by *in vitro* data demonstrating that nK cells expressing KIR3DS1control HIV-1 replication efficiently in HlA-B Bw480Ile-expressing target cells56. It is possible that KIR3DS1mediates specific recognition of HIV-infected cells bynK cells, although the exact nature of the ligand is elusive. These observations probably reflect an influenceof interactions between KIR3DS1 and/or KIR3Dl1 andHlA-B Bw4 80Ile on the development and/or functionsof nK cells, and possibly CD8+ T cells, which may helpto control viral set point.

Early innate immune responses to HIV-1

Acute-phase proteins and cytokines. Insight into theearliest systemic immune responses to HIV-1 infection has been gained by studying plasma donors whoacquired HIV-1 infection. Frequent samples weretaken before infection, through peak viraemia and seroconversion. Samples from different donors were alignedrelative to the time that viral RnA was first detectable(100 copies per ml) (T0). The first detectable innateimmune response, occurring sometimes just before T0, wasan increase in the levels of some acute-phase proteins, such as serum amyloid A (H. Kramer and B. Kessler, personalcommunication). A further wave of acute-phase proteinproduction coincided with a cytokine response (describedbelow) and a rapid increase in plasma viraemia. Theproduction of acute-phase proteins can be triggered by pro inflammatory cytokines (such as interleukin-1 (Il-1)) andalso by extrinsic factors such as lipopolysaccharide (lPS).
lPS is detectable in the plasma during chronic infectionwith HIV-1 or SIV and may be derived from commensalbacteria that translocate from the gut lumen followingdepletion of HIV-1-infected intestinal CCR5+ T helper cells. Immunostaining of GAlT biopsies collected from acutely infected patients showed higher levelsof pro-inflammatory cytokines than healthy tissues.
As viraemia increases, so do the levels of cytokinesand chemokines in the plasma (FIG. 3). levels of Il-15,type I interferons (IFns) and CXC-chemokine ligand 10(CXCl10) increase rapidly

but transiently. Il-18, TnF,IFnγ and Il-22 also increase rapidly but are sustained at high levels, whereas the increase in Il-10 is slightlydelayed (FIG. 3). Some of these cytokines have antiviralactivity; for example, type I IFns inhibit HIV replicationin severe combined immunodeficient mice reconstitutedwith human lymphocytes61. Also, type I IFns, Il-15 andIl-18 enhance innate and adaptive immune responses.
However, the intense cytokine response during acuteHIV infection may also promote viral replication and mediate immunopathology.
The cellular sources of the acute-phase cytokines andchemokines during early HIV-1 infection have not beendefinitively identified, but probably include infectedCD4+CCR5+ T cells, activated DCs16, monocytes, macrophages, nK cells, nKT cells and, subsequently, HIV-specific T cells. The cytokine storm observedduring early HIV-1 infection is much greater than thatobserved in acute hepatitis B and hepatitis C virus infections, indicating that a systemic cytokine response ofthis magnitude is not a pre-requisite for viral clearance.
The intense cytokine response in acute HIV infectionmay instead fuel viral replication and mediate immune pathology (discussed below). High-level systemiccytokine responses during acute infections with avianinfluenza virus and severe acute respiratory syndrome-associated coronavirus are likewise associated withimmunopathological consequences.
DCs. DCs are markedly reduced in number during acuteHIV-1 infection. This rapid decline in circulating DCs,particularly plasmacytoid DCs (pDCs), may be due to activation-induced cell death or to the migration ofactivated DCs into lymph nodes, where an increase in DC numbers is observed. *In vitro*, pDCs becomeactivated by the binding of viral envelope proteins toCD4 expressed by the pDCs followed by virion endocytosis and by the triggering of Toll-like receptor byviral RnA67. However, HIV-exposed conventional DCs do not become fully activated and show defective Il-12production68, which is consistent with the low levels ofIl-12 observed during acute HIV infection58. In addition, HIV-exposed pDCs produce IFnα, which enhancesadaptive immune responses. However, HIV-exposed pDCs also produce indoleamine 2,3-dioxygenase (IDo),which induces the differentiation of CD4+ T cells intoTReg cells that might suppress HIV-specific immuneresponses. Conventional DCs can prime virus specific CD4+ and CD8+ T cell responses following *in vitro* exposure to HIV71.
NK and NKT cells. As with most viral infections, nKcells and nKT cells become activated during acute HIVinfection. Prior to the peak in viraemia, blood nKcells proliferate and show enhanced activity when tested*ex vivo*. The nK cell population expressing KIR3DS1and/or KIR3Dl1 expands during acute infection inindividuals that also express HlA-B Bw4 80Ile. nKand nKT cells can control HIV replication throughcytolysis of virally infected cells and the production ofantiviral cytokines and chemokines. In addition, theycan interact with DCs and thereby influence T cellresponses. HIV-1 has evolved a strategy to reduce theexpression of ligands for nK cell receptors by infectedcells74. This finding, and the clear role of KIR3D molecules in determining the viral set point, supportsthe involvement of nK cells in the control of HIV-1.However, the timing of nK cell antiviral effects remainsuncertain. nK cells do not

contribute to the selection ofvirus escape mutants before peak viraemia, although it is possible, but not proved, that they account for someof the unexplained mutations that appear together withthose that are selected by early T cell responses as viraemia decreases to reach the set point. Alternatively, theantiviral effects of nK (and/or nKT) cells might have a greater influence at later time points.

Implications for vaccine design.

Can the protectivepotential of innate immune responses be harnessed byvaccination? Because nK cells share some characteristicswith memory cells after their initial activation, it maybe possible to prime their antiviral activity through vaccination. However, the activation of innate immunityshould be attempted with caution, as innate immuneresponses can also be harmful. For example, inductionof mucosal inflammatory responses by some microbicides has led to increased acquisition of HIV-1 infection. Furthermore, as discussed earlier, activated DCs can transmit virus to CD4+ T cellsand, during the eclipse phase of infection, chemokinesproduced by pDCs can recruit susceptible CD4+ T cellsto the foci of infection. Immune activation inducedby innate immune cells and the resulting production of pro-inflammatory cytokines and chemokines can promote HIV-1 replication. Type I IFns and TnF also havepro-apoptotic effects and can thereby contribute to aloss of activated DCs and the bystander destruction ofCD4+ T cells and B cells. The opposing effects of innate immune activation were highlighted in a study in whichIl-15 was administered to treat acute SIV infection inrhesus macaques: nK cell and SIV-specific CD8+ T cellnumbers were increased, resulting in fewer SIV-infectedcells in lymph nodes, but the activation and proliferationof CD4+ T cells was enhanced and a higher viral loadwas established80. Therefore, vaccine-induced activationof innate immune responses will have to be thoroughly tested in the macaque SIV model and used with cautionin humans.

Early T cell responses in HIV-1 infection

CD8+ T cell responses. A few studies have measuredHIV-1-specific CD8+ T cell responses during early HIV-1infection, before the first antibodies are detectable(Fiebig stages I or II82). Similar to SIV infection inmacaques, the first T cell responses to HIV-1 infectionarise as viraemia approaches its peak, and the T cellresponse peaks 1–2 weeks later, as viraemia declines. Thehomogeneity of the founder virus at the time of the peakof viraemia12–14 indicates that there is no immune-drivenselection of escape mutants as viraemia increases. Following the peak in the CD8+ T cell response,the virus sequence starts to change dramatically. Rapid selection of mutations occurs at discrete sitesin the virus genome as viraemia declines to the viralset point. Detailed analysis of four patientsduring the very early stages of infection indicatedthat most of the amino acid changes in the viruswere selected by CD8+ T cells that recognize epitopesexpressed by the founder virus but not by the escape mutant virus. Mutations in the viral envelope protein that were selected by neutralizing antibodiesappeared later, at ~12 weeks. A minority of virus escapemutants were not associated

with demonstrable T cellresponses: a few mutations were probably reversions from the sequence of the transmitted virus that wasselected by T cells in the patient's sexual partner; others may have been selected by antibody-dependentcell-mediated virus inhibition or by nK cells. notably,T cell- and antibody-mediated selection of viral escapemutants rarely involved a single amino acid change inthe epitope; most mutants involved multiple changes such that various mutants were 'tested' until the fittest were selecte.d The first T cell-selected mutationscould replace the original sequence of the founder viruswithin 10 days, and were then followed by sequentialselection of escape mutations at different epitopes. Thispattern continues throughout the course of HIV infection. Changes in sequence could involve amino acidsthat are upstream of the T cell epitope and are probablyimportant for antigen processing.

The earliest T cell responses are often specific for envand nef. Responses to other viral proteins, including the conserved Gag p24 and Pol proteins, tended toarise during later waves of T cell responses and may bemore important for maintaining the viral load at the set point than for controlling early viraemia. often, thefirst T cell responses decline rapidly when the escapemutations are selected, or they may decline throughexhaustion. The loss of T cells after virus mutationimplies complete loss of the epitope and no tendencyfor the virus to revert to the original sequence becauseof loss of fitness.

The finding that escape mutants appeared so rapidly raises questions regarding the effectiveness of theearly T cell response. A mathematical model has provided some answers. The rapid loss of the foundervirus sequence and its replacement by escape mutant viruses implies complete CD8+ T cell-mediated inhibition of virus production by infected cells. From therate of loss of founder virus sequence, the fraction ofcells killed per day was calculated to be 0.15–0.35 for the earliest T cell responses. As a virus-infected cell has a lifespan of 1 day *in vivo*, this means that 15–35%of infected cells must be killed prematurely by a singleT cell response, which must reduce virus production. Therefore, CD8+ T cells curb viraemia in acute HIV-1infection. However, selection of escape mutants wouldminimize this beneficial effect if the mutants were as fitas the founder virus and if the earliest responses werenot immediately succeeded by new T cell responses to new (mutated) epitopes, which in turn may select further escape mutants. ultimately, respondingT cells target epitopes that are more highly conservedand in which escape occurs at a cost to the fitness of thevirus. Such immunodominant responses to more highly conserved epitopes are more likely to result in a lowerlevel of viraemia at the set point. when a virus thathas undergone such escape mutations is transmitted, itsset point is also lower in the new host. The level of setpoint viraemia is therefore influenced by the nature ofthe transmitted virus and the specificity of early CD8+T cell responses. Immunodominant T cell responses tothe more conserved immunodominant virus epitopesare likely to result in a lower viral set point. CD8+ T cells are also important for the maintenanceof viral set point. There have been many reports of virusescape mutations from around the time the set point isreached.

Using the same mathematical models as described earlier, CD8+ T cells are thought to make only a small contribution (killing 4–6% of virus-infectedcells per day) to infected-cell death

during chronic infection, the rest being due to virus cytopathicity orinfected-cell activation. However, this may be an underestimate of the T cell contribution because of the fitnesscosts of the escape mutations on the virus, such thatmutant viruses grow more slowly than the founder virus.

Some of the epitopes that are recognized by the T cellsduring later stages of infection are so highly conservedthat the virus must undergo compensating mutationsat other sites for escape to occur, which slowsthe outgrowth of the mutant viruses. The calculation isfurther confounded by the difficulty of simultaneousvirus escape from more than one T cell.

In contrast to the earliest stages of HIV-1 infection whenthe range of epitopes recognized by the T cell responseis narrow, the later response is broad, often directed against more than 10 epitopes. Responses to conserved epitopes are probably important in the long-termcontrol of viral load, because patients with HlA-B27,HlA-B*5701, HlA-B*5703 or HlA-B*5801 that dowell clinically have CD8+ T cells that recognize less variable regions of the virus, particularly in Gag. TheHIV-1 quasispecies in these patients do escape slowlyduring long-term infection, but each escape mutantincurs a proved fitness cost to the virus. The time ittakes for the first T cell responses to become targeted toconserved epitopes might be important in determininglong-term control of viral infection. It is not clearwhat features determine which CD8+ T cell epitopes willbecome immunodominant; it is clear that HlA type isimportant, but the precursor frequency of naive T cellsthat are specific for HIV proteins is also likely to be afactor that is probably influenced both by genetics anda history of previous (cross-reactive) antigen exposure.Vaccines could influence this.

The CD4+ T cell response.

HIV-1 infects and significantlydepletes memory CD4+ T cells, and HIV-1-specific CD4+ T cells are particularly susceptible to HIV-1infection. CD4+ T cell responses to HIV proteins havealways been difficult to show, and there is a disparitybetween the measurements of CD4+ T cell responsesto antigen when observing cytokine production versusproliferation. Nevertheless, several epitopes for CD4+T cells have been identified, particularly in Gag. expansion of HIV-specific CD4+ T cell responses occursin acute HIV-1 infection, but such responses declinerapidly; although, very early administration of ART,to control viraemia and prevent the killing of CD4+ T cells,can rescue strong HIV-1 CD4+ T cell responses. However, even with the probably suboptimal help fromthe weakened CD4+ T cell repertoire, the first CD8+T cell responses are strong, although their progressioninto long-term memory cells could be impaired. Therapid decline of CD8+ T cell responses observed after the founder epitope is eliminated from the virus in theplasma, owing to escape mutations, is consistent withthe impaired long-term CD8+ T cell memory that hasbeen observed in a model in which mice were depleted of CD4+ T cells.

Implications for vaccine development.

The findingsdescribed above suggest a role for CD8+ T cells in theearliest immune control of acute HIV-1 infection. CD8+ T cells develop abnormally and become dysfunctional as HIV-1 infection progresses, butthe early HIV-1-specific CD8+ T cell response seems to

be functionally normal (G. Ferrari, personal communication). Although not all the factors that contributeto a low virus set point and good long-term prognosis(without ART) are known, it is clear that CD8+ T cellsare important components. If a vaccine cannot completely prevent infection, there should be a benefit fromstimulating appropriate CD8+ T cell responses, as shownrecently in the macaque SIV model. An effective vaccine would need to stimulate CD8+ T cell responses tomultiple epitopes, especially to those that are highlyconserved. It would also be favourable to stimulate abroad T cell response that recognizes common variantsof the founder virus epitope sequence, which would limit escape options.

Antibody responses during acute HIV-1 infection

Early neutralizing and non-neutralizing antibodyresponses. Antibodies that neutralize autologous virusdevelop slowly, arising ~12 weeks or longer after HIV-1transmission116–118. Antibodies that show some degree ofneutralization of heterologous virus eventually arise in~20% of patientsyears after infection. To determine the specificity and kinetics of antibody productionafter HIV-1 transmission and to understand why broadlyreactive neutralizing antibodies are not made duringacute HIV infection, it is important to study the earliestB cell responses to the transmitted virus. Env-specific antibody responses to autologous,consensus env epitopes were determined in the sameplasma donor cohort asdescribed earlier for innateimmunity. The first detectable B cell response wasfound to occur 8 days after T0 in the form of immunecomplexes, whereas the first free antibody in theplasma was specific for env glycoprotein (gp)41 andappeared 13 days after T0. By contrast, the appearanceof env gp120-specific antibodies was delayed an additional 14 days, as was the production of other non neutralizing env-specific antibodies (FIG. 5;TABLE 1). The first HIV-1-specific IgA responses inmucosal secretions, which were detected within thefirst 3 weeks after T0, also recognized gp41 during acuteHIV infection (n. l. yates and G.D.T., unpublishedobservations). A study that applied mathematical modelling to early viral dynamics indicated that the initialgp41-specific IgG and IgM responses did not signify cantly affect the early dynamics of plasma viral load57. These acute gp41- and gp120-specific antibodies didnot select escape mutations, indicating that these earlyarising antibodies are ineffective against HIV-1. Similaranalyses of the effect of the initial immune complexesand gp41-specific IgA responses on viral dynamics areneeded to understand the interplay between the initialhost antibody responses and virus replication. It is notknown why the initial antibody response to env is non-neutralizing; it may relate to the immune dominanceof denatured or non-functional env forms. The first antibodies to induce escape mutants are autolo-gous-virus-neutralizing antibodies that develop ~12 ormore weeks after transmission. Fc receptor(FcR)-mediated and complement-associated anti-HIV effector functions have also been reported during primary infection; however, further studiesare required to define their role and capacity to select escape mutations.

The range of epitopes bound by the first (specificto autologous virus) neutralizing antibodies in HIV-1clade C infection is narrow and epitopes are oftenrestricted to certain virus isolates. Similar to infections with clade B HIV-1, the initial autologous-virus-neutralizing antibodies induced in clade C infectionsare induced with similar kinetics and are usuallyspecific only for the initially transmitted env variant.

Although the autologous-virus-neutralizing antibodyresponse can control the virus quasispecies presentwhen these antibodies appear in infections with HIV-1clade B or C viruses, the narrowness of the responseallows rapid viral escape (K. Bar and G. Shaw, personal communication).

Broad-specificity, neutralizing antibodies to conservedEnv regions are rare.

Interestingly, antibodies specificfor the conserved regions of HIV-1 env such asthe carbohydrate epitope recognized by the uniquebroad-specificity neutralizing monoclonal antibody2G12, the CD4-binding site recognized by themonoclonal antibody 1b12 and the membrane proximal region recognized by the monoclonal antibodies2F5, Z13 and 4e10 are rarely generated during HIV-1 infection; when they do occur, theydevelop only after ~20–30 months of infection. These observations indicate that both genetic factorsand maturation of the antibody response to HIV-1 arenecessary for the generation of this rare, late, broad specificity, neutralizing antibody response. Affinity maturation through somatic hypermutation may be crucial for the generation of these neutralizing antibodies andmay be delayed because of impaired CD4+ T cell help.

Because the 2F5, 4e10 and 1b12 monoclonal antibodieshave long hydrophobic complementarity-determiningregion 3 (CDR3) sequences and show polyreactivityfor autologous molecules, it has been suggested thatB cell regulatory mechanisms such as self tolerance may control their production.

Early damage to mucosal B cell generative microenvironments.

Acute HIV-1 infection profoundlyaffects blood and tissue B cells. HIV-1 induces early class switching in polyclonal B cells and is associated withmarked increases in the number of blood and tissuememory B cells and plasma cells, as well as a decreasein the number of naive B cells. In the mucosal B cellinductive microenvironments, such as Peyer's patches, where HIV and SIV replicate at high levels during acute infection, both HIV-1 and SIV140 caninduce the lysis of follicular B cells, massive B cell apoptosis and loss of ~50% of germinal centres within thefirst 80 days of infection. Early loss of germinal centresmay result in defects in the ability to rapidly generatehigh-affinity HIV-1 antibodies and lead to a delay in theinduction of autologous-virus-neutralizing antibodies.

Implications for vaccine design

The finding that thegeneration of potentially protective antibodies is delayeduntil after initial control of viraemia ~12 weeks after transmission and then focused on only a fewepitopes impliesthat it will be important to develop a vaccine that primesa very early and broad antibody response that targetsmultiple neutralizing epitopes for effective control of early viral expansion; the natural process is too little,too late. The early perturbations to B cells

by the virussimilarly indicate the need for a vaccine that either hashigh levels of durable protective antibody responses orprimes in order to induce a rapid secondary response. The rarity of broad-specificity, neutralizing antibodyresponses to conserved epitopes in env emphasizes theneed to search for and find those small B cell subsetsthat can make broad-specificity, neutralizing antibodies:immunogens and adjuvants are needed that target thosespecific B cells.

ELISA

The **enzyme-linked immunosorbent assay** (**ELISA**) is a commonly used analytical biochemistry assay, first described by Engvall and Perlmann in 1971.[1] The assay uses a solid-phase **enzyme immunoassay** (**EIA**) to detect the presence of a ligand (commonly a protein) in a liquid sample using antibodies directed against the protein to be measured. ELISA has been used as a diagnostic tool in medicine, plant pathology, and biotechnology, as well as a quality control check in various industries.

In the most simple form of an ELISA, antigens from the sample are attached to a surface. Then, a matching antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change.

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are non-specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

Of note, ELISA can perform other forms of ligand binding assays instead of strictly "immuno" assays, though the name carried the original "immuno" because of the common use and history of development of this method. The technique essentially requires any ligating reagent that can be immobilized on the solid phase along with a detection reagent that will bind specifically and use an enzyme to generate a signal that can be properly quantified. In between the washes, only the ligand and its specific binding counterparts remain specifically bound or "immunosorbed" by antigen-antibody interactions to the solid phase, while the nonspecific or unbound components are washed away. Unlike other spectrophotometric wet lab assay formats where the same reaction well (e.g., a cuvette) can be reused after washing, the ELISA plates have the reaction products immunosorbed on the solid phase, which is part of the plate, and so are not easily reusable.

Principle

As an analytical biochemistry assay and a "wet lab" technique, ELISA involves detection of an analyte (i.e., the specific substance whose presence is being quantitatively or qualitatively analyzed) in a liquid sample by a method that continues to use liquid reagents during the analysis (i.e., controlled sequence of biochemical reactions that will generate a signal which can be easily quantified and interpreted as a measure of the amount of analyte in the sample) that stays liquid and remains inside a reaction chamber or well needed to keep the reactants contained. This is in opposition to "dry lab" techniques that use dry strips. Even if the sample is liquid (e.g., a measured small drop), the final detection step in "dry" analysis involves reading of a dried strip by methods such as reflectometry and does not need a reaction containment chamber to prevent spillover or mixing between samples.

As a heterogenous assay, ELISA separates some component of the analytical reaction mixture by adsorbing certain components onto a solid phase which is physically immobilized. In ELISA, a liquid sample is added onto a stationary solid phase with special binding properties and is followed by multiple liquid reagents that are sequentially added, incubated, and washed, followed by some optical change (e.g., color development by the product of an enzymatic reaction) in the final liquid in the well from which the quantity of the analyte is measured. The quantitative "reading" is usually based on detection of intensity of transmitted light by spectrophotometry, which involves quantitation of transmission of some specific wavelength of light through the liquid (as well as the transparent bottom of the well in the multiple-well plate format). The sensitivity of detection depends on amplification of the signal during the analytic reactions. Since enzyme reactions are very well known amplification processes, the signal is generated by enzymes which are linked to the detection reagents in fixed proportions to allow accurate quantification, and thus the name "enzyme-linked."

The analyte is also called the ligand because it will specifically bind or ligate to a detection reagent, thus ELISA falls under the bigger category of ligand binding assays.The ligand-specific binding reagent is "immobilized," i.e., usually coated and dried onto the transparent bottom and sometimes also side wall of a well (the stationary "solid phase"/"solid substrate" here as opposed to solid microparticle/beads that can be washed away), which is usually constructed as a multiple-well plate known as the "ELISA plate." Conventionally, like other forms of immunoassays, the specificity of antigen-antibody type reaction is used because it is easy to raise an antibody specifically against an antigen in bulk as a reagent. Alternatively, if the analyte itself is an antibody, its target antigen can be used as the binding reagent.

History

Before the development of the ELISA, the only option for conducting an immunoassay was radioimmunoassay, a technique using radioactively labeled antigens or antibodies. In radioimmunoassay, the radioactivity provides the signal, which indicates whether a specific antigen or antibody is present in the sample. Radioimmunoassay was first described in a scientific paper by Rosalyn Sussman Yalow and Solomon Berson published in 1960.

As radioactivity poses a potential health threat, a safer alternative was sought. A suitable alternative to radioimmunoassay would substitute a nonradioactive signal in place of the radioactive signal. When enzymes (such as horseradish peroxidase) react with appropriate substrates (such as ABTS or TMB), a change in color occurs, which is used as a signal. However, the signal has to be associated with the presence of antibody or antigen, which is why the enzyme has to be linked to an appropriate antibody. This linking process was independently developed by Stratis Avrameas and G. B. Pierce. Since it is necessary to remove any unbound antibody or antigen by washing, the antibody or antigen has to be fixed to the surface of the container; i.e., the immunosorbent must be prepared. A technique to accomplish this was published by Wide and Jerker Porath in 1966.

In 1971, Peter Perlmann and Eva Engvall at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen in the Netherlands independently published papers that synthesized this knowledge into methods to perform EIA/ELISA.

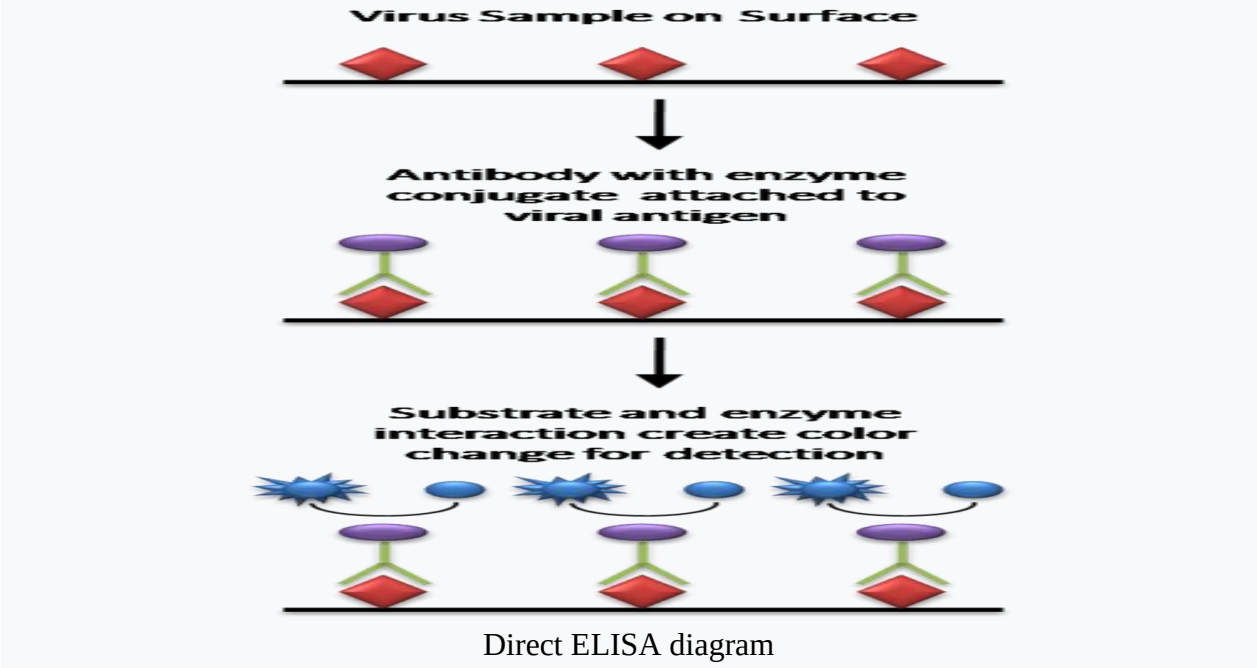
Traditional ELISA typically involves chromogenic reporters and substrates that produce some kind of observable color change to indicate the presence of antigen or analyte. Newer ELISA-like techniques use fluorogenic, electrochemiluminescent, and quantitative PCR reporters to create quantifiable signals. These new reporters can have various advantages, including higher sensitivities and multiplexing. In technical terms, newer assays of this type are not strictly ELISAs, as they are not "enzyme-linked", but are instead linked to some nonenzymatic reporter. However, given that the general principles in these assays are largely similar, they are often grouped in the same category as ELISAs.

In 2012, an ultrasensitive, enzyme-based ELISA test using nanoparticles as a chromogenic reporter was able to give a naked-eye colour signal, from the detection of mere attograms of analyte. A blue color appears for positive results and red color for negative. Note that this detection only can confirm the presence or the absence of analyte not the actual concentration.

Types

There are many ELISA tests for particular molecules that use the matching antibodies. ELISA tests are broken into several types of tests based on how the analytes and antibodies are bonded and used. The major types are described here.

- **Direct ELISA**



Direct ELISA diagram

The steps of direct ELISA follows the mechanism below:

- A buffered solution of the antigen to be tested for is added to each well (usually 96-well plates) of a microtiter plate, where it is given time to adhere to the plastic through charge interactions.
- A solution of nonreacting protein, such as bovine serum albumin or casein, is added to each well in order to cover any plastic surface in the well which remains uncoated by the antigen.
- The primary antibody with an attached (conjugated) enzyme is added, which binds specifically to the test antigen coating the well.
- A substrate for this enzyme is then added. Often, this substrate changes color upon reaction with the enzyme.
- The higher the concentration of the primary antibody present in the serum, the stronger the color change. Often, a spectrometer is used to give quantitative values for color strength.

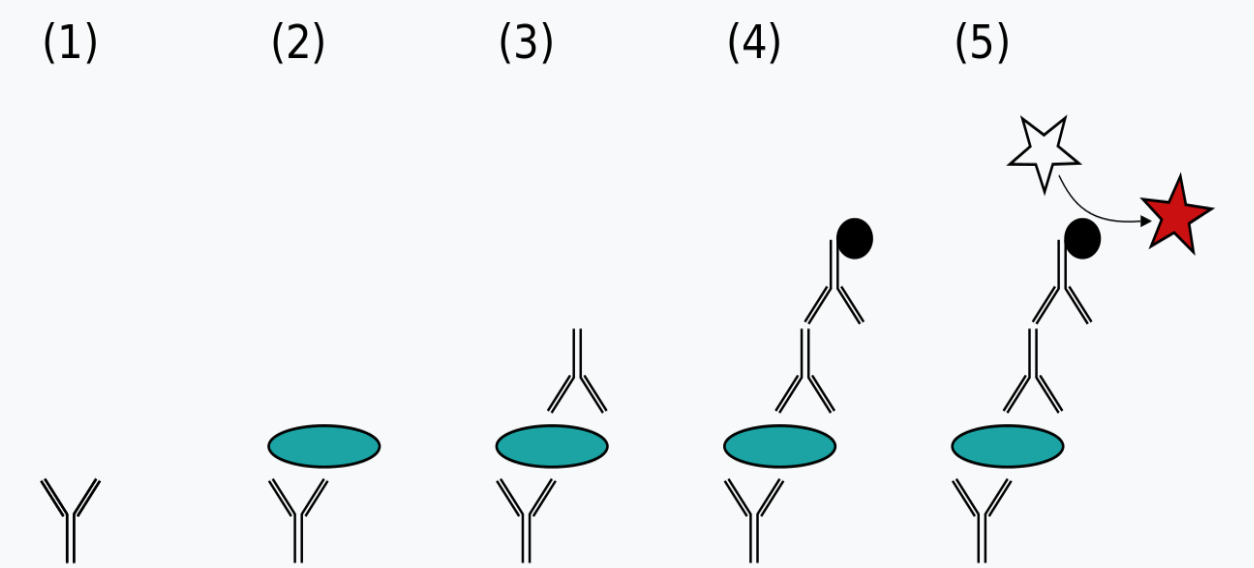
The enzyme acts as an amplifier; even if only few enzyme-linked antibodies remain bound, the enzyme molecules will produce many signal molecules. Within common-sense limitations, the enzyme can go on producing color indefinitely, but the more antibody is bound, the faster the color will develop. A major disadvantage of the direct ELISA is the method of antigen immobilization is not specific; when serum is used as the source of test antigen, all proteins in the sample may stick to the microtiter plate well, so small concentrations of analyte in serum must compete with other serum proteins when binding to the well surface. The sandwich or indirect ELISA provides a solution to this problem, by using a "capture" antibody specific for the test antigen to pull it out of the serum's molecular mixture.

ELISA may be run in a qualitative or quantitative format. Qualitative results provide a simple positive or negative result (yes or no) for a sample. The cutoff between positive and negative is determined by the analyst and may be statistical. Two or three times the standard deviation (error

inherent in a test) is often used to distinguish positive from negative samples. In quantitative ELISA, the optical density (OD) of the sample is compared to a standard curve, which is typically a serial dilution of a known-concentration solution of the target molecule. For example, if a test sample returns an OD of 1.0, the point on the standard curve that gave OD = 1.0 must be of the same analyte concentration as the sample.

The use and meaning of the names "indirect ELISA" and "direct ELISA" differs in the literature and on web sites depending on the context of the experiment. When the presence of an antigen is analyzed, the name "direct ELISA" refers to an ELISA in which only a labelled primary antibody is used, and the term "indirect ELISA" refers to an ELISA in which the antigen is bound by the primary antibody which then is detected by a labeled secondary antibody. In the latter case a sandwich ELISA is clearly distinct from an indirect ELISA. When the "primary" antibody is of interest, e.g. in the case of immunization analyses, this antibody is directly detected by the secondary antibody and the term "indirect ELISA" applies to a setting with two antibodies.

- **Sandwich ELISA**



A sandwich ELISA. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form.

A "sandwich" ELISA is used to detect sample antigen.

The steps are:

1. A surface is prepared to which a known quantity of capture antibody is bound.
2. Any nonspecific binding sites on the surface are blocked.
3. The antigen-containing sample is applied to the plate, and captured by antibody.
4. The plate is washed to remove unbound antigen.

5. A specific antibody is added, and binds to antigen (hence the 'sandwich': the antigen is stuck between two antibodies). This primary antibody could also be in the serum of a donor to be tested for reactivity towards the antigen.
6. Enzyme-linked secondary antibodies are applied as detection antibodies that also bind specifically to the antibody's Fc region (nonspecific).
7. The plate is washed to remove the unbound antibody-enzyme conjugates.
8. A chemical is added to be converted by the enzyme into a color or fluorescent or electrochemical signal.
9. The absorbance or fluorescence or electrochemical signal (e.g., current) of the plate wells is measured to determine the presence and quantity of antigen.

The image to the right includes the use of a secondary antibody conjugated to an enzyme, though, in the technical sense, this is not necessary if the primary antibody is conjugated to an enzyme (which would be direct ELISA). However, the use of a secondary-antibody conjugate avoids the expensive process of creating enzyme-linked antibodies for every antigen one might want to detect. By using an enzyme-linked antibody that binds the Fc region of other antibodies, this same enzyme-linked antibody can be used in a variety of situations. Without the first layer of "capture" antibody, any proteins in the sample (including serum proteins) may competitively adsorb to the plate surface, lowering the quantity of antigen immobilized. Use of the purified specific antibody to attach the antigen to the plastic eliminates a need to purify the antigen from complicated mixtures before the measurement, simplifying the assay, and increasing the specificity and the sensitivity of the assay. A sandwich ELISA used for research often needs validation because of the risk of false positive results.

- **Competitive ELISA**

A third use of ELISA is through competitive binding. The steps for this ELISA are somewhat different from the first two examples:

1. Unlabeled antibody is incubated in the presence of its antigen (sample).
2. These bound antibody/antigen complexes are then added to an antigen-coated well.
3. The plate is washed, so unbound antibodies are removed. (The more antigen in the sample, the more Ag-Ab complexes are formed and so there are less unbound antibodies available to bind to the antigen in the well, hence "competition".)
4. The secondary antibody, specific to the primary antibody, is added. This second antibody is coupled to the enzyme.
5. A substrate is added, and remaining enzymes elicit a chromogenic or fluorescent signal.
6. The reaction is stopped to prevent eventual saturation of the signal.

Some competitive ELISA kits include enzyme-linked antigen rather than enzyme-linked antibody. The labeled antigen competes for primary antibody binding sites with the sample antigen (unlabeled). The less antigen in the sample, the more labeled antigen is retained in the well and the stronger the signal.

Commonly, the antigen is not first positioned in the well.

For the detection of HIV antibodies, the wells of microtiter plate are coated with the HIV antigen. Two specific antibodies are used, one conjugated with enzyme and the other present in serum (if serum is positive for the antibody). Cumulative competition occurs between the two antibodies for the same antigen, causing a stronger signal to be seen. Sera to be tested are added to these wells and incubated at 37 °C, and then washed. If antibodies are present, the antigen-antibody reaction occurs. No antigen is left for the enzyme-labelled specific HIV antibodies. These antibodies remain free upon addition and are washed off during washing. Substrate is added, but there is no enzyme to act on it, so a positive result shows no color change.

- **Reverse ELISA**

A fourth ELISA test does not use the traditional wells. This test leaves the antigens suspended in the test fluid.

1. Unlabeled antibody is incubated in the presence of its antigen (sample)
2. A sufficient incubation period is provided to allow the antibodies to bind to the antigens.
3. The sample is then passed through the Scavenger container. This can be a test tube or a specifically designed flow through channel. The surface of the Scavenger container or channel has "Scavenger Antigens" bound to it. These can be identical or sufficiently similar to the primary antigens that the free antibodies will bind.
4. The Scavenger container must have sufficient surface area and sufficient time to allow the Scavenger Antigens to bind to all the excess Antibodies introduced into the sample.
5. The sample, that now contains the tagged and bound antibodies, is passed through a detector. This device can be a flow cytometer or other device that illuminates the tags and registers the response.

This test allows multiple antigens to be tagged and counted at the same time. This allows specific strains of bacteria to be identified by two (or more) different color tags. If both tags are present on a cell, then the cell is that specific strain. If only one is present, it is not.

This test is done, generally, one test at a time and cannot be done with the microtiter plate. The equipment needed is usually less complicated and can be used in the field.

Commonly used enzymatic markers

The following table lists the enzymatic markers commonly used in ELISA assays.

- OPD (*o*-phenylenediamine dihydrochloride) turns amber to detect HRP (Horseradish Peroxidase), which is often used to as a conjugated protein.
- TMB (3,3',5,5'-tetramethylbenzidine) turns blue when detecting HRP and turns yellow after the addition of sulfuric or phosphoric acid.
- ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) turns green when detecting HRP.
- PNPP (*p*-Nitrophenyl Phosphate, Disodium Salt) turns yellow when detecting alkaline phosphatase.

Applications

Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool for determining serum antibody concentrations (such

as with the HIV testor West Nile virus). It has also found applications in the food industry in detecting potential food allergens, such as milk, peanuts, walnuts, almonds, and eggs and as serological blood test for coeliac disease. ELISA can also be used in toxicology as a rapid presumptive screen for certain classes of drugs.

The ELISA was the first screening test widely used for HIV because of its high sensitivity. In an ELISA, a person's serum is diluted 400 times and applied to a plate to which HIV antigens are attached. If antibodies to HIV are present in the serum, they may bind to these HIV antigens. The plate is then washed to remove all other components of the serum. A specially prepared "secondary antibody" — an antibody that binds to other antibodies — is then applied to the plate, followed by another wash. This secondary antibody is chemically linked in advance to an enzyme.

Thus, the plate will contain enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate for the enzyme is applied, and catalysis by the enzyme leads to a change in color or fluorescence. ELISA results are reported as a number; the most controversial aspect of this test is determining the "cut-off" point between a positive and a negative result.

A cut-off point may be determined by comparing it with a known standard. If an ELISA test is used for drug screening at workplace, a cut-off concentration, 50 ng/ml, for example, is established, and a sample containing the standard concentration of analyte will be prepared. Unknowns that generate a stronger signal than the known sample are "positive." Those that generate weaker signal are "negative".

Dr Dennis E Bidwell and Alister Voller created the ELISA test to detect various kind of diseases, such as dengue, malaria, Chagas disease, Johne's disease, and others. ELISA tests also are used as in *in vitro* diagnostics in medical laboratories. The other uses of ELISA include:

- detection of *Mycobacterium* antibodies in tuberculosis
- detection of rotavirus in feces
- detection of hepatitis B markers in serum
- detection of hepatitis C markers in serum
- detection of enterotoxin of *E. coli* in feces
- detection of HIV antibodies in blood samples

Radioimmunoassay

A **radioimmunoassay** (**RIA**) is an immunoassay that uses radiolabeled molecules in a stepwise formation of immune complexes. A RIA is a very sensitive in vitro assay technique used to measure concentrations of substances, usually measuring antigen concentrations (for example, hormone levels in blood) by use of antibodies.

Although the RIA technique is extremely sensitive and extremely specific, requiring specialized equipment, it remains among the least expensive methods to perform such measurements. It requires special precautions and licensing, since radioactive substances are used.

In contrast, an immunoradiometric assay (IRMA) is an immunoassay that uses radiolabeled molecules but in an immediate rather than stepwise way.

A radioallergosorbent test (RAST) is an example of radioimmunoassay. It is used to detect the causative allergen for an allergy.

Method

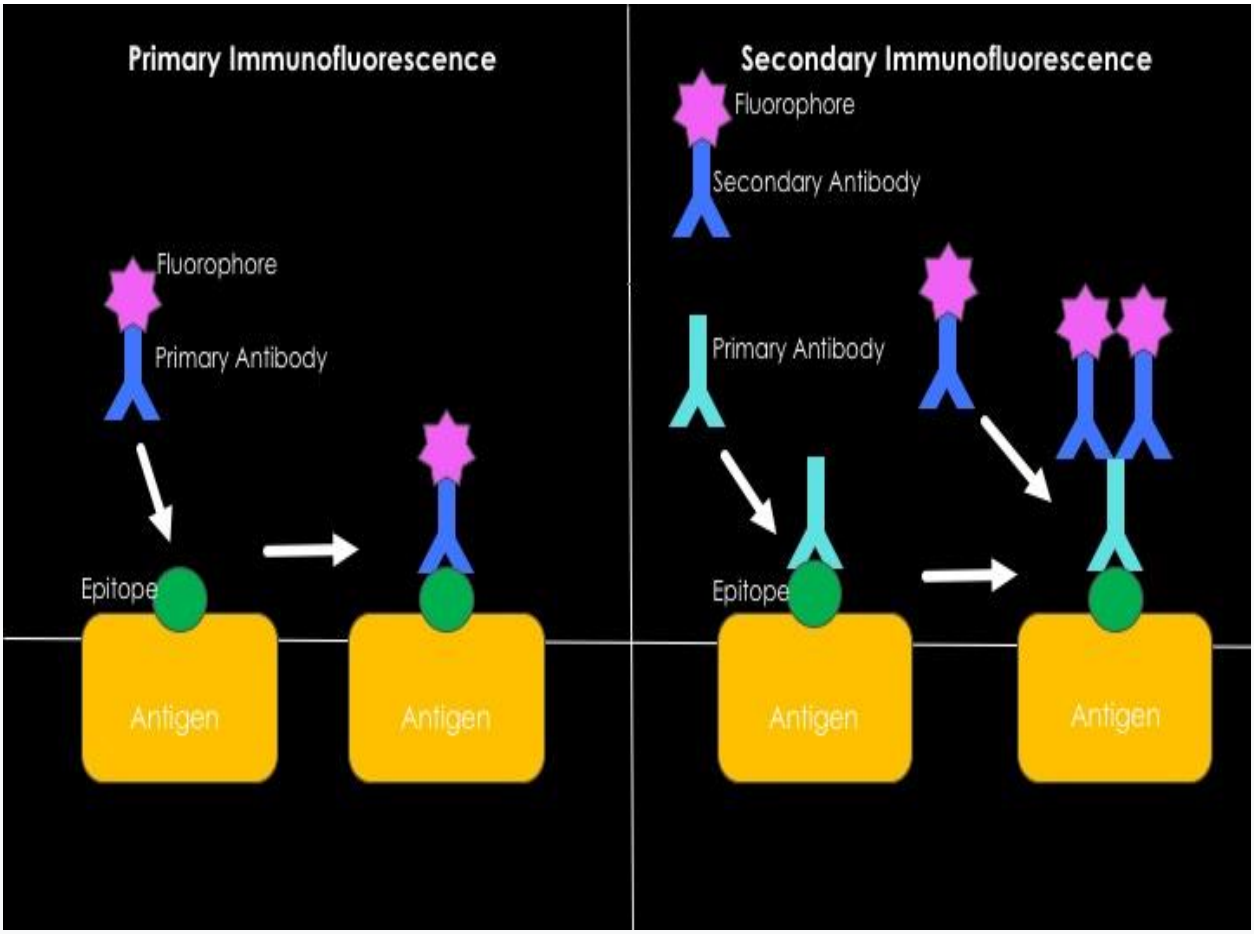
Classically, to perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by labeling it with gamma-radioactive isotopes of iodine, such as 125-I, attached to tyrosine. This radiolabeled antigen is then mixed with a known amount of antibody for that antigen, and as a result, the two specifically bind to one another. Then, a sample of serum from a patient containing an unknown quantity of that same antigen is added. This causes the unlabeled (or "cold") antigen from the serum to compete with the radiolabeled antigen ("hot") for antibody binding sites. As the concentration of "cold" antigen is increased, more of it binds to the antibody, displacing the radiolabeled variant, and reducing the ratio of antibody-bound radiolabeled antigen to free radiolabeled antigen. The bound antigens are then separated from the unbound ones, and the radioactivity of the free(unbound) antigen remaining in the supernatant is measured using a gamma counter.

This method can be used for any biological molecule in principle and is not restricted to serum antigens, nor is it required to use the indirect method of measuring the free antigen instead of directly measuring the captured antigen. For example, if it is undesirable or not possible to radiolabel the antigen or target molecule of interest, a RIA can be done if two different antibodies that recognize the target are available and the target is large enough (e.g., a protein) to present multiple epitopes to the antibodies. One antibody would be radiolabeled as above while the other would remain unmodified. The RIA would begin with the "cold" unlabeled antibody being allowed to interact and bind to the target molecule in solution. Preferably, this unlabeled antibody is immobilized in some way, such as coupled to an agarose bead, coated to a surface, etc. Next, the "hot" radiolabeled antibody is allowed to interact with the first antibody-target molecule complex. After extensive washing, the direct amount of radioactive antibody bound is measured and the amount of target molecule quantified by comparing it to a reference amount assayed at the same time. This method is similar in principle to the non-radioactive sandwich ELISA method.

History

This method was developed by Rosalyn Sussman Yalow, Roger Guillemin, and Andrew Schally at the Veterans Administration Hospital in the Bronx, New York. This revolutionary development earned Dr. Yalow the Nobel Prize for Medicine in 1977, the second woman ever to win it. In her acceptance speech, Dr. Yalow said, "The world cannot afford the loss of the talents of half its people if we are to solve the many problems which beset us."

Immunofluorescence



These figures demonstrates the basic mechanism of immunofluorescence. Primary immunofluorescence is depicted on the left, which shows an antibody with a fluorophore group bound to it directly binding to the epitope of the antigen for which it is specific. Once the antibody binds to the epitope, the sample can be viewed under fluorescent microscope to confirm the presence of the antigen in the sample. Conversely, secondary immunofluorescence is depicted to the right, which shows that first an untagged primary antibody binds to the epitope of the antigen in a mechanism similar to the one described above. However, after the primary antibodies have bound to their target, a secondary antibody (tagged with a fluorophore) comes along. This secondary antibody's binding sites are specific for the primary antibody that's already bound to the antigen, and therefore the secondary antibody binds to the primary antibody. This

method allows for more fluorophore-tagged antibodies to attach to their target, thus increasing the fluorescent signal during microscopy.

Immunofluorescence is a technique used for light microscopy with a fluorescence microscope and is used primarily on microbiological samples. This technique uses the specificity of antibodies to their antigen to target fluorescent dyes to specific biomolecule targets within a cell, and therefore allows visualization of the distribution of the target molecule through the sample. The specific region an antibody recognizes on an antigen is called an epitope.There have been efforts in epitope mapping since many antibodies can bind the same epitope and levels of binding between antibodies that recognize the same epitope can vary. Additionally, the binding of the fluorophore to the antibody itself cannot interfere with the immunological specificity of the antibody or the binding capacity of its antigen. Immunofluorescence is a widely used example of immunostaining (using antibodies to stain proteins) and is a specific example of immunohistochemistry (the use of the antibody-antigen relationship in tissues). This technique primarily makes use of fluorophores to visualise the location of the antibodies.

Immunofluorescence can be used on tissue sections, cultured cell lines, or individual cells, and may be used to analyze the distribution of proteins, glycans, and small biological and non-biological molecules. This technique can even be used to visualize structures such as intermediate-sized filaments. If the topology of a cell membrane has yet to be determined, epitope insertion into proteins can be used in conjunction with immunofluorescence to determine structures. Immunofluorescence can also be used as a "semi-quantitative" method to gain insight into the levels and localization patterns of DNA methylation since it is a more time-consuming method than true quantitative methods and there is some subjectivity in the analysis of the levels of methylation. Immunofluorescence can be used in combination with other, non-antibody methods of fluorescent staining, for example, use of DAPI to label DNA. Several microscope designs can be used for analysis of immunofluorescence samples; the simplest is the epifluorescence microscope, and the confocal microscope is also widely used. Various super-resolution microscope designs that are capable of much higher resolution can also be used.

Types

Preparation of fluorescence

To make fluorochrome-labeled antibodies, a fluorochrome must be conjugated ("tagged") to the antibody. Likewise, an antigen can also be conjugated to the antibody with a fluorescent probe in a technique called fluorescent antigen technique. Staining procedures can apply to both fixed antigen in the cytoplasm or to cell surface antigens on living cells, called "membrane immunofluorescence". It is also possible to label the complement of the antibody-antigen complex with a fluorescent probe. In addition to the element to which fluorescence probes are attached, there are two general classes of immunofluorescence techniques: primary and secondary. The following descriptions will focus primarily on these classes in terms of conjugated antibodies.

There are two classes of immunofluorescence techniques, primary (or direct) and secondary (or indirect).

Primary (direct)

Primary (direct) immunofluorescence uses a single, primary antibody, chemically linked to a fluorophore. The primary antibody recognizes the target molecule (antigen) and binds to a specific region called the epitope. This is accomplished by a process which manipulates the immune response of organism with adaptive immunity. The attached fluorophore can be detected via fluorescent microscopy, which, depending on the messenger used, will emit a specific wavelength of light once excited. Direct immunofluorescence, although somewhat less common, has notable advantages over the secondary (indirect) procedure. The direct attachment of the messenger to the antibody reduces the number of steps in the procedure, saving time and reducing non-specific background signal. However, some disadvantages do exist in this method. Since the number of fluorescent molecules that can be bound to the primary antibody is limited, direct immunofluorescence is substantially less sensitive than indirect immunofluorescence and may result in false negatives. Direct immunofluorescence also requires the use of much more primary antibody, which is extremely expensive, sometimes running up to $400.00/mL.

Secondary (indirect)

Secondary (indirect) immunofluorescence uses two antibodies; the unlabeled first (primary) antibody specifically binds the target molecule, and the secondary antibody, which carries the fluorophore, recognizes the primary antibody and binds to it. Multiple secondary antibodies can bind a single primary antibody. This provides signal amplification by increasing the number of fluorophore molecules per antigen.This protocol is more complex and time-consuming than the primary (or direct) protocol above, but allows more flexibility because a variety of different secondary antibodies and detection techniques can be used for a given primary antibody.

This protocol is possible because an antibody consists of two parts, a variable region (which recognizes the antigen) and constant region (which makes up the structure of the antibody molecule). It is important to realize that this division is artificial and in reality the antibody molecule is four polypeptide chains: two heavy chains and two light chains. A researcher can generate several primary antibodies that recognize various antigens (have different variable regions), but all share the same constant region. All these antibodies may therefore be recognized by a single secondary antibody. This saves the cost of modifying the primary antibodies to directly carry a fluorophore.

Different primary antibodies with different constant regions are typically generated by raising the antibody in different species. For example, a researcher might create primary antibodies in a goat that recognize several antigens, and then employ dye-coupled rabbit secondary antibodies that recognize the goat antibody constant region ("rabbit anti-goat" antibodies). The researcher may then create a second set of primary antibodies in a mouse that could be recognized by a separate "donkey anti-mouse" secondary antibody. This allows re-use of the difficult-to-make dye-coupled antibodies in multiple experiments.

Limitations

As with most fluorescence techniques, a significant problem with immunofluorescence is photobleaching. Loss of activity caused by photobleaching can be controlled by reducing or limiting the intensity or time-span of light exposure, by increasing the concentration of fluorophores, or by employing more robust fluorophores that are less prone to bleaching (e.g., Alexa Fluors, Seta Fluors, or DyLight Fluors). Some problems that may arise from this technique include autofluorescence, extraneous undesired specific fluorescence, and nonspecific fluorescence. Autofluorescence includes fluorescence emitted from the sample tissue or cell itself. Extraneous undesired specific fluorescence occurs when a targeted antigen is impure and contains antigenic contaminants. Nonspecific fluorescence involves the loss of a probe's specificity due to fluorophore, from improper fixation, or from a dried out specimen.

Immunofluorescence is only limited to fixed (i.e., dead) cells when structures within the cell are to be visualized because antibodies do not penetrate the cell membrane when reacting with fluorescent labels. Antigenic material must be fixed firmly on the site of its natural localization inside the cell. Intact antibodies can also be too large to dye cancer cells *in vivo*. Their size results in slow tumor penetration and long circulating half-life. Research has been done investigating the use of diabodies to get around this limitation.[11] Proteins in the supernatant or on the outside of the cell membrane can be bound by the antibodies; this allows for living cells to be stained. Depending on the fixative that is being used, proteins of interest might become cross-linked and this could result in either false positive or false negative signals due to non-specific binding.

An alternative approach is using recombinant proteins containing fluorescent protein domains, e.g., green fluorescent protein (GFP). Use of such "tagged" proteins allows determination of their localization in live cells. Even though this seems to be an elegant alternative to immunofluorescence, the cells have to be transfected or transduced with the GFP-tag, and as a consequence they become at least S1 or above organisms that require stricter security standards in a laboratory. This technique involves altering the genetic information of cells.

Advances

Many improvements to this method lie in the improvement of fluorescent microscopes and fluorophores. Super-resolution methods generally refer to a microscope's ability to produce resolution below the Abbe limit (a limit placed on light due to its wavelength). This diffraction limit is about 200-300 nm in the lateral direction and 500-700 nm in the axial direction. This limit is comparable or larger than some structures in the cell, and consequently, this limit prevented scientists from determining details in their structure. Super-resolution in fluorescence, more specifically, refers to the ability of a microscope to prevent the simultaneous fluorescence of adjacent spectrally identical fluorophores. This process effectively sharpens the point-spread function of the microscope. Examples of recently developed super-resolution fluorescent microscope methods include stimulated emission depletion (STED) microscopy, saturated

structured-illumination microscopy (SSIM), fluorescence photoactivation localization microscopy (FPALM), and stochastic optical reconstruction microscopy (STORM).

Immunoprecipitation

Immunoprecipitation (**IP**) is the technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. This process can be used to isolate and concentrate a particular protein from a sample containing many thousands of different proteins. Immunoprecipitation requires that the antibody be coupled to a solid substrate at some point in the procedure.

Types

Individual protein immunoprecipitation (IP)

Involves using an antibody that is specific for a known protein to isolate that particular protein out of a solution containing many different proteins. These solutions will often be in the form of a crude lysate of a plant or animal tissue. Other sample types could be body fluids or other samples of biological origin.

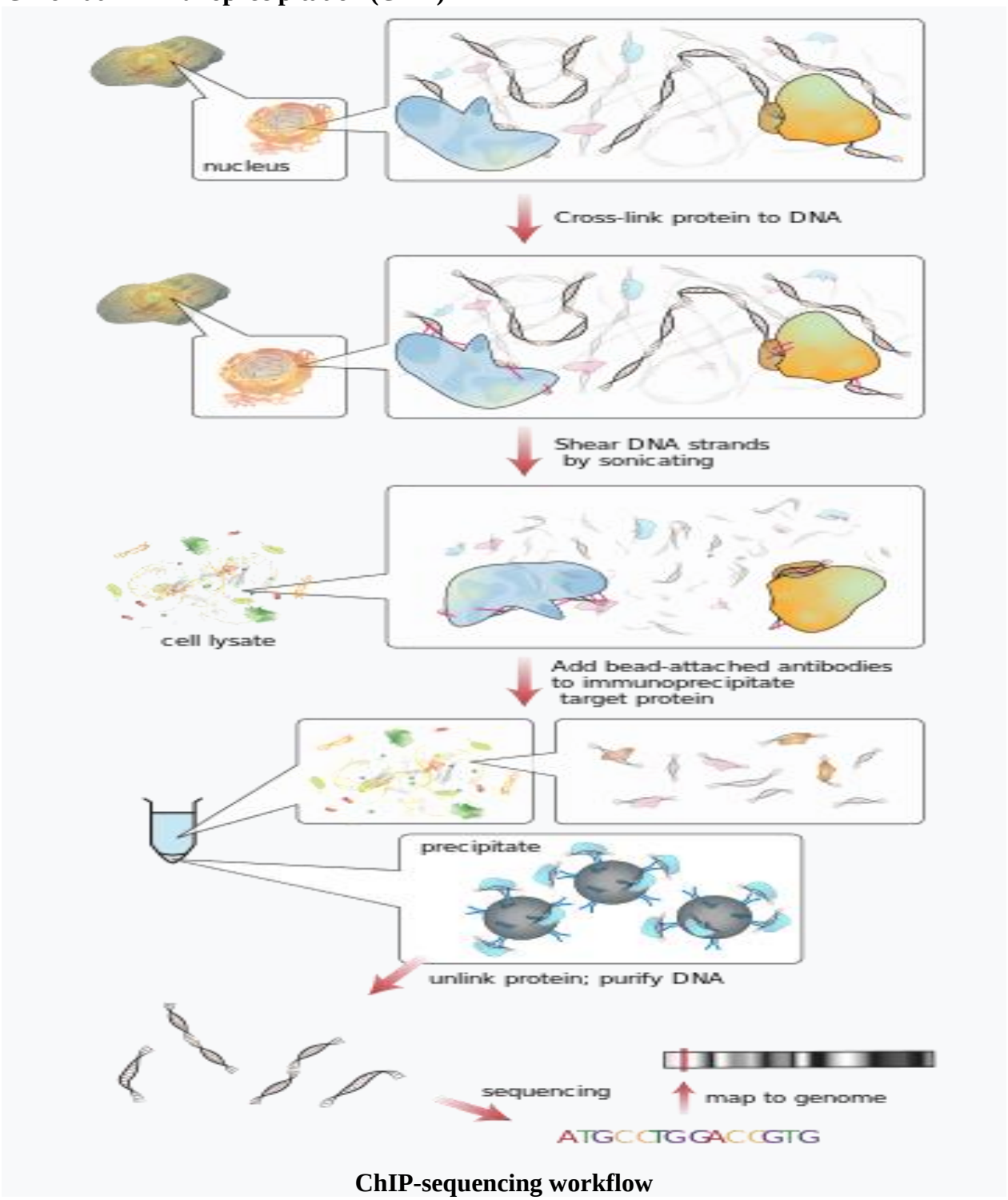
Protein complex immunoprecipitation (Co-IP)

Immunoprecipitation of intact protein complexes (i.e. antigen along with any proteins or ligands that are bound to it) is known as co-immunoprecipitation (Co-IP). Co-IP works by selecting an antibody that targets a known protein that is believed to be a member of a larger complex of proteins. By targeting this *known* member with an antibody it may become possible to pull the entire protein complex out of solution and thereby identify *unknown* members of the complex.

This works when the proteins involved in the complex bind to each other tightly, making it possible to pull multiple members of the complex out of solution by latching onto one member with an antibody. This concept of pulling protein complexes out of solution is sometimes referred to as a "pull-down". Co-IP is a powerful technique that is used regularly by molecular biologists to analyze protein–protein interactions.

- A particular antibody often selects for a subpopulation of its target protein that has the epitope exposed, thus failing to identify any proteins in complexes that hide the epitope. This can be seen in that it is rarely possible to precipitate even half of a given protein from a sample with a single antibody, even when a large excess of antibody is used.
- As successive rounds of targeting and immunoprecipitations take place, the number of identified proteins may continue to grow. The identified proteins may not ever exist in a single complex at a given time, but may instead represent a network of proteins interacting with one another at different times for different purposes.
- Repeating the experiment by targeting different members of the protein complex allows the researcher to double-check the result. Each round of pull-downs should result in the recovery of both the original known protein as well as other previously identified members of the complex (and even new additional members). By repeating the immunoprecipitation in this way, the researcher verifies that each identified member of the protein complex was a valid identification. If a particular protein can only be recovered by targeting one of the known members but not by targeting other of the known members then that protein's status as a member of the complex may be subject to question.

Chromatin immunoprecipitation (ChIP)



ChIP-sequencing workflow

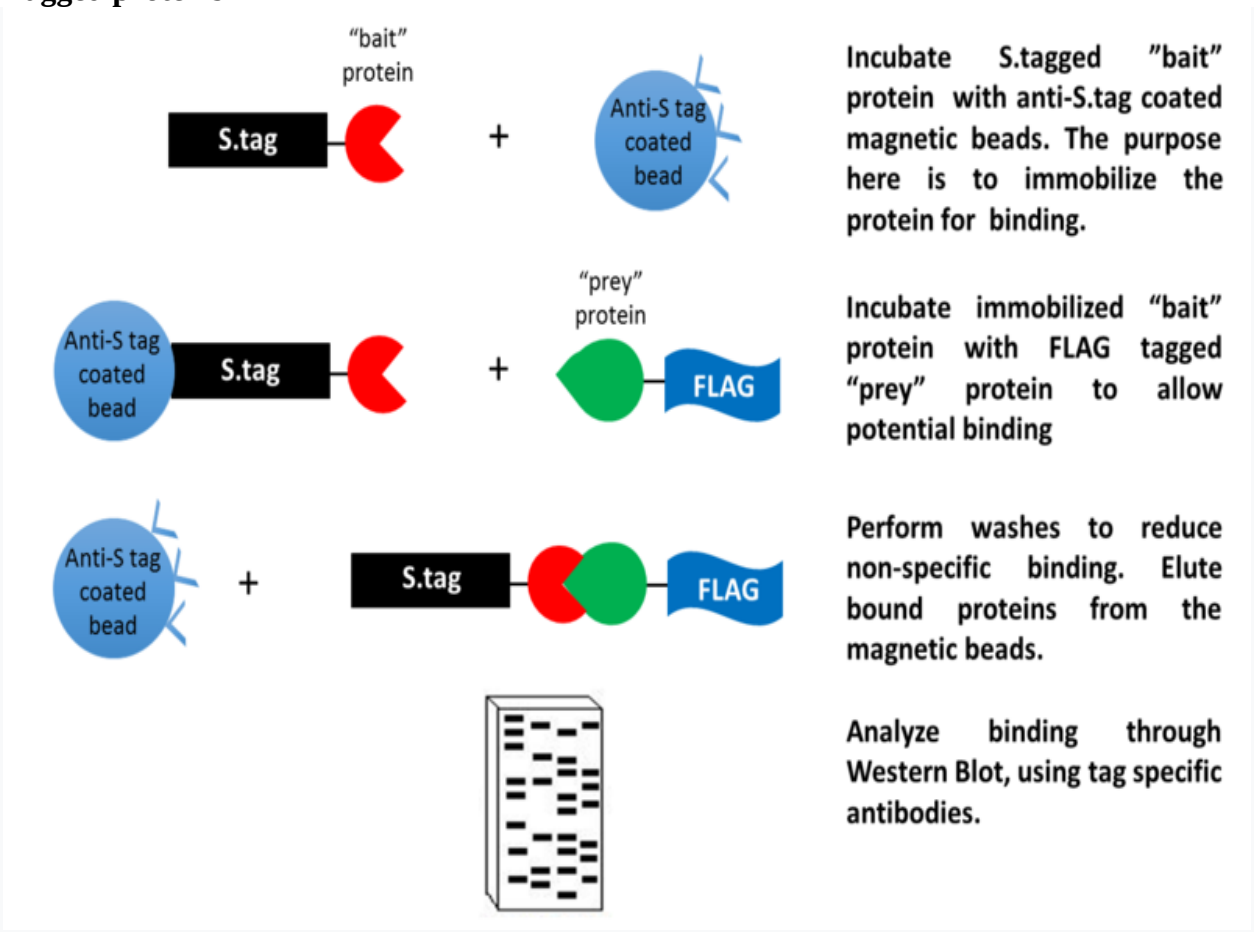
Chromatin immunoprecipitation (ChIP) is a method used to determine the location of DNA binding sites on the genome for a particular protein of interest. This technique gives a picture of the protein–DNA interactions that occur inside the nucleus of living cells or tissues. The *in vivo* nature of this method is in contrast to other approaches traditionally employed to answer the same questions.

The principle underpinning this assay is that DNA-binding proteins (including transcription factors and histones) in living cells can be cross-linked to the DNA that they are binding. By using an antibody that is specific to a putative DNA binding protein, one can immunoprecipitate the protein–DNA complex out of cellular lysates. The crosslinking is often accomplished by applying formaldehyde to the cells (or tissue), although it is sometimes advantageous to use a more defined and consistent crosslinker such as DTBP. Following crosslinking, the cells are lysed and the DNA is broken into pieces 0.2–1.0 kb in length by sonication. At this point the immunoprecipitation is performed resulting in the purification of protein–DNA complexes. The purified protein–DNA complexes are then heated to reverse the formaldehyde cross-linking of the protein and DNA complexes, allowing the DNA to be separated from the proteins. The identity and quantity of the DNA fragments isolated can then be determined by PCR. The limitation of performing PCR on the isolated fragments is that one must have an idea which genomic region is being targeted in order to generate the correct PCR primers. Sometimes this limitation circumvented simply by cloning the isolated genomic DNA into a plasmid vector and then using primers that are specific to the cloning region of that vector. Alternatively, when one wants to find where the protein binds on a genome-wide scale, ChIP-Sequencing is used and has recently emerged as a standard technology that can localize protein binding sites in a high-throughput, cost-effective fashion, allowing also for the characterization of the cistrome. Previously, DNA microarray was also used (ChIP-on-chip or ChIP-chip).

RNP Immunoprecipitation (RIP)

Similar to chromatin immunoprecipitation (ChIP) outlined above, but rather than targeting DNA binding proteins as in ChIP, an RNP immunoprecipitation targets ribonucleoproteins (RNPs). Live cells are first lysed and then the target protein and associated RNA are immunoprecipitated using an antibody targeting the protein of interest. The purified RNA-protein complexes can be separated by performing an RNA extraction and the identity of the RNA can be determined by cDNA sequencing or RT-PCR. Some variants of RIP, such as PAR-CLIP include cross-linking steps, which then require less careful lysis conditions.

Tagged proteins



Pull down assay using tagged proteins

One of the major technical hurdles with immunoprecipitation is the great difficulty in generating an antibody that specifically targets a single known protein. To get around this obstacle, many groups will engineer **tags** onto either the C- or N- terminal end of the protein of interest. The advantage here is that the same tag can be used time and again on many different proteins and the researcher can use the same antibody each time. The advantages with using tagged proteins are so great that this technique has become commonplace for all types of immunoprecipitation including all of the types of IP detailed above. Examples of tags in use are the Green Fluorescent Protein (GFP) tag, Glutathione-S-transferase (GST) tag and the FLAG-tag tag. While the use of a tag to enable pull-downs is convenient, it raises some concerns regarding biological relevance because the tag itself may either obscure native interactions or introduce new and unnatural interactions.

Methods

The two general methods for immunoprecipitation are the direct capture method and the indirect capture method.

Direct

Antibodies that are specific for a particular protein (or group of proteins) are immobilized on a solid-phase substrate such as superparamagnetic microbeads or on microscopic agarose (non-magnetic) beads. The beads with bound antibodies are then added to the protein mixture, and the proteins that are targeted by the antibodies are captured onto the beads via the antibodies; in other words, they become immunoprecipitated.

Indirect

Antibodies that are specific for a particular protein, or a group of proteins, are added directly to the mixture of protein. The antibodies have not been attached to a solid-phase support yet. The antibodies are free to float around the protein mixture and bind their targets. As time passes, the beads coated in protein A/G are added to the mixture of antibody and protein. At this point, the antibodies, which are now bound to their targets, will stick to the beads.

From this point on, the direct and indirect protocols converge because the samples now have the same ingredients. Both methods gives the same end-result with the protein or protein complexes bound to the antibodies which themselves are immobilized onto the beads.

Selection

An indirect approach is sometimes preferred when the concentration of the protein target is low or when the specific affinity of the antibody for the protein is weak. The indirect method is also used when the binding kinetics of the antibody to the protein is slow for a variety of reasons. In most situations, the direct method is the default, and the preferred, choice.

Technological advances

Agarose

Historically the solid-phase support for immunoprecipitation used by the majority of scientists has been highly-porous **agarose beads** (also known as agarose resins or slurries). The advantage of this technology is a very high potential binding capacity, as virtually the entire sponge-like structure of the agarose particle (50 to 150μm in size) is available for binding antibodies (which will in turn bind the target proteins) and the use of standard laboratory equipment for all aspects of the IP protocol without the need for any specialized equipment. The advantage of an extremely high binding capacity must be carefully balanced with the quantity of antibody that the researcher is prepared to use to coat the agarose beads. Because antibodies can be a cost-limiting factor, it is best to calculate backward ***from*** the amount of protein that needs to be captured (depending upon the analysis to be performed downstream), ***to*** the amount of antibody that is required to bind that quantity of protein (with a small excess added in order to account for inefficiencies of the system), and back still further ***to*** the quantity of agarose that is needed to bind that particular quantity of antibody. In cases where antibody saturation is not required, this technology is unmatched in its ability to capture extremely large quantities of captured target proteins. The caveat here is that the ***"high capacity advantage"*** can become a ***"high capacity disadvantage"*** that is manifested when the enormous binding capacity of the sepharose/agarose beads is not completely saturated with antibodies. It often happens that the amount of antibody available to the researcher for their immunoprecipitation experiment is less than sufficient to

saturate the agarose beads to be used in the immunoprecipitation. In these cases the researcher can end up with agarose particles that are only partially coated with antibodies, and the portion of the binding capacity of the agarose beads that is not coated with antibody is then free to bind anything that will stick, resulting in an elevated background signal due to non-specific binding of lysate components to the beads, which can make data interpretation difficult. While some may argue that for these reasons it is prudent to match the quantity of agarose (in terms of binding capacity) to the quantity of antibody that one wishes to be bound for the immunoprecipitation, a simple way to reduce the issue of non-specific binding to agarose beads and increase specificity is to preclear the lysate, which for any immunoprecipitation is highly recommended.

Preclearing

Lysates are complex mixtures of proteins, lipids, carbohydrates and nucleic acids, and one must assume that some amount of non-specific binding to the IP antibody, Protein A/G or the beaded support will occur and negatively affect the detection of the immunoprecipitated target(s). In most cases, ***preclearing*** the lysate at the start of each immunoprecipitation experiment (see step 2 in the "protocol" section below) is a way to remove potentially reactive components from the cell lysate prior to the immunoprecipitation to prevent the non-specific binding of these components to the IP beads or antibody. The basic preclearing procedure is described below, wherein the lysate is incubated with beads alone, which are then removed and discarded prior to the immunoprecipitation. This approach, though, does not account for non-specific binding to the IP antibody, which can be considerable. Therefore, an alternative method of preclearing is to incubate the protein mixture with exactly the same components that will be used in the immunoprecipitation, except that a non-target, irrelevant antibody of the same antibody subclass as the IP antibody is used instead of the IP antibody itself.[4] This approach attempts to use as close to the exact IP conditions and components as the actual immunoprecipitation to remove any non-specific cell constituent without capturing the target protein (unless, of course, the target protein non-specifically binds to some other IP component, which should be properly controlled for by analyzing the discarded beads used to preclear the lysate). The target protein can then be immunoprecipitated with the reduced risk of non-specific binding interfering with data interpretation.

Superparamagnetic beads

While the vast majority of immunoprecipitations are performed with agarose beads, the use of superparamagnetic beads for immunoprecipitation is a much newer approach that is only recently gaining in popularity as an alternative to agarose beads for IP applications. Unlike agarose, magnetic beads are solid and can be spherical, depending on the type of bead, and antibody binding is limited to the surface of each bead. While these beads do not have the advantage of a porous center to increase the binding capacity, magnetic beads are significantly smaller than agarose beads (1 to 4μm), and the greater number of magnetic beads per volume than agarose beads collectively gives magnetic beads an effective surface area-to-volume ratio for optimum antibody binding.

Commercially available magnetic beads can be separated based by size uniformity into monodisperse and polydisperse beads. Monodisperse beads, also called microbeads, exhibit exact uniformity, and therefore all beads exhibit identical physical characteristics, including the binding capacity and the level of attraction to magnets. Polydisperse beads, while similar in size to monodisperse beads, show a wide range in size variability (1 to 4μm) that can influence their

binding capacity and magnetic capture. Although both types of beads are commercially available for immunoprecipitation applications, the higher quality monodisperse superparamagnetic beads are more ideal for automatic protocols because of their consistent size, shape and performance. Monodisperse and polydisperse superparamagnetic beads are offered by many companies, including Invitrogen, Thermo Scientific, and Millipore.

Agarose vs. magnetic beads

Proponents of magnetic beads claim that the beads exhibit a faster rate of protein bindingover agarose beads for immunoprecipitation applications, although standard agarose bead-based immunoprecipitations have been performed in 1 hour. Claims have also been made that magnetic beads are better for immunoprecipitating extremely large protein complexes because of the complete lack of an upper size limit for such complexes, although there is no unbiased evidence stating this claim. The nature of magnetic bead technology does result in less sample handling due to the reduced physical stress on samples of magnetic separation versus repeated centrifugation when using agarose, which may contribute greatly to increasing the yield of labile (fragile) protein complexes.Additional factors, though, such as the binding capacity, cost of the reagent, the requirement of extra equipment and the capability to automate IP processes should be considered in the selection of an immunoprecipitation support.

Binding capacity

Proponents of both agarose and magnetic beads can argue whether the vast difference in the binding capacities of the two beads favors one particular type of bead. In a bead-to-bead comparison, agarose beads have significantly greater surface area and therefore a greater binding capacity than magnetic beads due to the large bead size and sponge-like structure. But the variable pore size of the agarose causes a potential upper size limit that may affect the binding of extremely large proteins or protein complexes to internal binding sites, and therefore magnetic beads may be better suited for immunoprecipitating large proteins or protein complexes than agarose beads, although there is a lack of independent comparative evidence that proves either case.

Some argue that the significantly greater binding capacity of agarose beads may be a disadvantage because of the larger capacity of non-specific binding. Others may argue for the use of magnetic beads because of the greater quantity of antibody required to saturate the total binding capacity of agarose beads, which would obviously be an economical disadvantage of using agarose. While these arguments are correct outside the context of their practical use, these lines of reasoning ignore two key aspects of the principle of immunoprecipitation that demonstrates that the decision to use agarose or magnetic beads is not simply determined by binding capacity.

First, non-specific binding is not limited to the antibody-binding sites on the immobilized support; any surface of the antibody or component of the immunoprecipitation reaction can bind to nonspecific lysate constituents, and therefore nonspecific binding will still occur even when completely saturated beads are used. This is why it is important to preclear the sample before the immunoprecipitation is performed.

Second, the ability to capture the target protein is directly dependent upon the amount of immobilized antibody used, and therefore, in a side-by-side comparison of agarose and magnetic bead immunoprecipitation, the most protein that either support can capture is limited by the

amount of antibody added. So the decision to saturate any type of support depends on the amount of protein required, as described above in the Agarose section of this page.

Cost

The price of using either type of support is a key determining factor in using agarose or magnetic beads for immunoprecipitation applications. A typical first-glance calculation on the cost of magnetic beads compared to sepharose beads may make the sepharose beads appear less expensive. But magnetic beads may be competitively priced compared to agarose for analytical-scale immunoprecipitations depending on the IP method used and the volume of beads required per IP reaction.

Using the traditional batch method of immunoprecipitation as listed below, where all components are added to a tube during the IP reaction, the physical handling characteristics of agarose beads necessitate a minimum quantity of beads for each IP experiment (typically in the range of 25 to 50µl beads per IP). This is because sepharose beads must be concentrated at the bottom of the tube by centrifugation and the supernatant removed after each incubation, wash, etc. This imposes absolute physical limitations on the process, as pellets of agarose beads less than 25 to 50µl are difficult if not impossible to visually identify at the bottom of the tube. With magnetic beads, there is no minimum quantity of beads required due to magnetic handling, and therefore, depending on the target antigen and IP antibody, it is possible to use considerably less magnetic beads.

Conversely, spin columns may be employed instead of normal microfuge tubes to significantly reduce the amount of agarose beads required per reaction. Spin columns contain a filter that allows all IP components except the beads to flow through using a brief centrifugation and therefore provide a method to use significantly less agarose beads with minimal loss.

Equipment

As mentioned above, only standard laboratory equipment is required for the use of agarose beads in immunoprecipitation applications, while high-power magnets are required for magnetic bead-based IP reactions. While the magnetic capture equipment may be cost-prohibitive, the rapid completion of immunoprecipitations using magnetic beads may be a financially beneficial approach when grants are due, because a 30-minute protocol with magnetic beads compared to overnight incubation at 4 °C with agarose beads may result in more data generated in a shorter length of time.

Automation

An added benefit of using magnetic beads is that automated immunoprecipitation devices are becoming more readily available. These devices not only reduce the amount of work and time to perform an IP, but they can also be used for high-throughput applications.

Summary

While clear benefits of using magnetic beads include the increased reaction speed, more gentle sample handling and the potential for automation, the choice of using agarose or magnetic beads based on the binding capacity of the support medium and the cost of the product may depend on the protein of interest and the IP method used. As with all assays, empirical testing is required to determine which method is optimal for a given application.

Protocol

Background

Once the solid substrate bead technology has been chosen, antibodies are coupled to the beads and the antibody-coated-beads can be added to the heterogeneous protein sample (e.g. homogenized tissue). At this point, antibodies that are immobilized to the beads will bind to the proteins that they specifically recognize. Once this has occurred the immunoprecipitation portion of the protocol is actually complete, as the specific proteins of interest are bound to the antibodies that are themselves immobilized to the beads. Separation of the immunocomplexes from the lysate is an extremely important series of steps, because the protein(s) must remain bound to each other (in the case of co-IP) and bound to the antibody during the wash steps to remove non-bound proteins and reduce background.

When working with agarose beads, the beads must be pelleted out of the sample by briefly spinning in a centrifuge with forces between 600–3,000 x g (times the standard gravitational force). This step may be performed in a standard microcentrifuge tube, but for faster separation, greater consistency and higher recoveries, the process is often performed in small spin columns with a pore size that allows liquid, but not agarose beads, to pass through. After centrifugation, the agarose beads will form a very loose fluffy pellet at the bottom of the tube. The supernatant containing contaminants can be carefully removed so as not to disturb the beads. The wash buffer can then be added to the beads and after mixing, the beads are again separated by centrifugation.

With superparamagnetic beads, the sample is placed in a magnetic field so that the beads can collect on the side of the tube. This procedure is generally complete in approximately 30 seconds, and the remaining (unwanted) liquid is pipetted away. Washes are accomplished by resuspending the beads (off the magnet) with the washing solution and then concentrating the beads back on the tube wall (by placing the tube back on the magnet). The washing is generally repeated several times to ensure adequate removal of contaminants. If the superparamagnetic beads are homogeneous in size and the magnet has been designed properly, the beads will concentrate uniformly on the side of the tube and the washing solution can be easily and completely removed.

After washing, the precipitated protein(s) are eluted and analyzed by gel electrophoresis, mass spectrometry, western blotting, or any number of other methods for identifying constituents in the complex. Protocol times for immunoprecipitation vary greatly due to a variety of factors, with protocol times increasing with the number of washes necessary or with the slower reaction kinetics of porous agarose beads.

Steps

1. Lyse cells and prepare sample for immunoprecipitation.
2. Pre-clear the sample by passing the sample over beads alone or bound to an irrelevant antibody to soak up any proteins that non-specifically bind to the IP components.
3. Incubate solution with antibody against the protein of interest. Antibody can be attached to solid support before this step (direct method) or after this step (indirect method). Continue the incubation to allow antibody-antigen complexes to form.
4. Precipitate the complex of interest, removing it from bulk solution.

5. Wash precipitated complex several times. Spin each time between washes when using agarose beads or place tube on magnet when using superparamagnetic beads and then remove the supernatant. After the final wash, remove as much supernatant as possible.
6. Elute proteins from the solid support using low-pH or SDS sample loading buffer.
7. Analyze complexes or antigens of interest. This can be done in a variety of ways:
    1. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) followed by gel staining.
    2. SDS-PAGE followed by: gel staining, cutting out individual stained protein bands, and sequencing the proteins in the bands by MALDI-Mass Spectrometry
    3. Transfer and Western Blot using another antibody for proteins that were interacting with the antigen, followed by detection using a chemiluminescent or fluorescent secondary antibody.

15.Suggested reading

1. Brock biology of microorganism, 10 edition Madigan,Martinko,Bender, Buckley and Stahl. Pearson, New York, San francisco USA.

2. Microbiology, 5th edition, Lancin M. Prescott, ISBN-0-07-282905-2

3. Microbiology, 5h edition, M. Pelezar, ISBN: 9780074623206, 0074623206

4. https://www.wikipedia.org

16.Assignment

1. Discuss the strategies of cell division in bacteria
2. Diffrenciate between lytic and lysogenic cycle
3. Regulation of lytic and lysogenic cycle
4. Briefly describe the industrial production of Penicillin
5. Describe the Structure of different types of Immunoglobulins
6. Define antigen.state the charecteristics of antigen
7. Illustrate different types of ELISA
8. How Ethanol is produced? What are the two methods of producing ethanol?
9. Does cancer have symptoms? What is the solution of cancer?
10. Does vitamin B12 fight cancer? Symptoms of vitamin B12 deficiency
11. What are the mechanisms of gene regulation?
12. How does bacterial conjugation occur? What is the purposeof bacterial conjugation?

All the materials are self written and collected from eBooks, journals and Websites

BOTANY

POST GRADUATE DEGREE PROGRAMME
(CBCS CURRICULUM)

SEMESTER: IV

PAPER: BOSCT 4.1

Evolution

Directorate of Open and Distance Learning
UNIVERSITY OF KALYANI
Kalyani, Nadia
West Bengal

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POST GRADUATE DEGREE PROGRAMME (CBCS)
IN
BOTANY

SEMESTER - IV

Course: BOSCT 4.1

(Evolution)

Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMs). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMs for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMs, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMs, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks are also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMs. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and Distance Learning
University of Kalyani

SYLLABUS
COURSE – BOSCT 4.1
Evolution
(Full Marks – 50)

Course	Group	Details Contents Structure		Study hour
BOSCT 4.1	**Evolution**	**Unit 1. Emergence of evolutionary thoughts**	1. Emergence of evolutionary thoughts: Lamarck; Darwin – concepts of variation, Neo-Darwinism, adaptation, struggle, fitness and natural selection. .	1
		Unit 2. Origin of cells and unicellular evolution-I	2. Origin of cells and unicellular evolution: Origin of basic biological molecules; abiotic synthesis of organic monomers and polymers; concept of Oparin and Haldane; experiment of Urey-Miller (1953). .	1
		Unit 3. Origin of cells and unicellular evolution-II	Origin of cells and unicellular evolution: The first cell; evolution of prokaryotes; origin of eukaryotic cells; evolution of unicellular eukaryotes; anaerobic metabolism, photosynthesis and aerobic metabolism.	1
		Unit 4. Evolutionary timescale & diversification of plant life	Evolutionary timescale & diversification of plant life: The evolutionary time scale; eras, periods and epoch; major events in the evolutionary time scale; origins of unicellular and multicellular organisms; major groups of plants.	1
		Unit 5. Genetic variations-I	Genetic variations: Origin of genetic variation; Mendelian genetics; polygenic traits, linkage and recombination.	1
		Unit 6. Genetic variations-II	Genetic variations: Epistasis, gene - environment interaction; heritability; population genetics; molecular evolution; molecular clocks.	1

Content

COURSE – BOSCT 4.1

(Evolution)

Soft Core Theory Paper **Credit = 2**

Content Structure

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1. Introduction
2. Course Objectives
3. Emergence of evolutionary thoughts: Lamarck; Darwin – concepts of variation, Neo-Darwinism,adaptation, struggle, fitness and natural selection.
4. Origin of cells and unicellular evolution: Origin of basic biological molecules; abiotic synthesis oforganic monomers and polymers; concept of Oparin and Haldane; experiment of Urey-Miller(1953); the first cell; evolution of prokaryotes; origin of eukaryotic cells; evolution of unicellulareukaryotes; anaerobic metabolism, photosynthesis and aerobic metabolism.
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6. Genetic variations: Origin of genetic variation; Mendelian genetics; polygenic traits, linkage and recombination; epistasis, gene - environment interaction; heritability; population genetics; molecular evolution; molecular clocks.
7. Let’s sum up
8. Suggested Reading
9. Assignment

1. Introduction

The thirst for knowledge, to understand the past and predict the future, and to organize our world. Evolution is not just about fossils. It is also about molecules, genes, mutations, populations, and sex in living organisms. All of these things are primary sources of data about evolutionary processes that occur when organisms try to survive and reproduce. Evolution also is about rigorous analyses — what we must do with the data to say something that is scientifically defendable. So if you thought that evolutionary biology was limited to dusty old curators in dusty old museums, think again. Scientists at universities, research centers, and museums are conducting some of the most sophistocated analyses of any kind today, using some of the best prepared specimens, most advanced techniques, and fastest computers available. Nothing in biology can be truly understood without first understanding evolution. In these ways, knowledge of evolution can improve the quality of human life.

2. Course Objectives

A student who has completed the course should have solid knowledge of:

1. Explain the ideas of Lamarck and Malthus as they relate to Darwin's concept of evolution.
2. Explain how the discovery of DNA revolutionized the understanding of evolution.
3. a deeper insight into the evolutionary processes - both selective and random - which can explain the genetic composition of populations, form, behaviour and distribution of organisms
4. Define the four forces of evolution and provide examples for each.
5. Natural selection as key to understanding the natural world; how natural selection produces adaptation; the origins of genetic variation; fitness.
6. how new species arise;
7. To know the history of life and the origin and evolution of life

3. Emergence of evolutionary thoughts: Lamarck; Darwin – concepts of variation, Neo Darwinism, adaptation, struggle, fitness and natural selection.

Evolution is change in the heritable characteristics of biological populations over successive generations. Evolutionary processes give rise to biodiversity at every level of biological organisation, including the levels of species, individual organisms, and molecules.

In the mid-19th century, **Charles Darwin** formulated the scientific theory of evolution by natural selection, published in his book On the **Origin of Species** (1859). Evolution by natural selection is a process first demonstrated by the observation that often, more offspring are produced than can possibly survive. This is followed by three observable facts about living organisms: 1) traits vary among individuals with respect to morphology, physiology, and behaviour (phenotypic variation), 2) different traits confer different rates of survival and reproduction (differential fitness), and 3) traits can be passed from generation to generation (heritability of fitness). Thus, in successive generations members of a population are more likely to be replaced by progeny of parents better adapted to survive and reproduce in the biophysical environment in which natural selection takes place.

History of evolutionary thought

Classical times

The proposal that one type of organism could descend from another type goes back to some of the first pre-Socratic Greek philosophers, such as **Anaximander** and **Empedocles**.

Medieval

In contrast to these materialistic views, Aristotelianism considered all natural things as actualisations of fixed natural possibilities, known as forms. This was part of a medieval teleological understanding of nature in which all things have an intended role to play in a divine cosmic order.

Pre-Darwinian

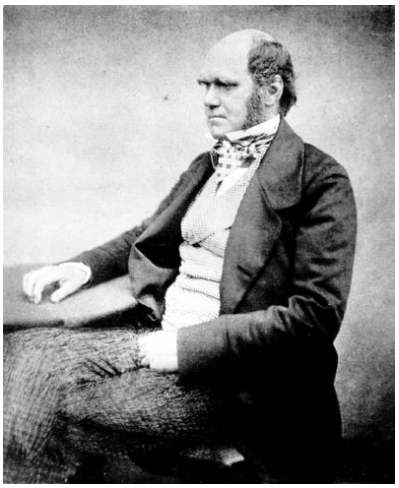
In the 17th century, John Ray applied one of the previously more general terms for fixed natural types, "species," to plant and animal types. The biological classification introduced by **Carl Linnaeus** in 1735 explicitly recognised the hierarchical nature of species relationships, but still viewed species as fixed according to a divine plan.

Other naturalists of this time speculated on the evolutionary change of species over time according to natural laws. In 1751, **Pierre Louis Maupertuis** wrote of natural modifications occurring during reproduction and accumulating over many generations to produce new species. **Georges-Louis Leclerc, Comte de Buffon** suggested that species could degenerate into different organisms, and Erasmus Darwin proposed that all warm-blooded animals could have descended from a single microorganism (or "filament"). The first full-fledged evolutionary scheme was **Jean-Baptiste Lamarck's** "transmutation" theory of 1809, which envisaged spontaneous generation continually producing simple forms of life that developed greater complexity in parallel lineages with an inherent progressive tendency, and postulated that on a local level these lineages adapted to the environment by inheriting changes caused by their use or disuse in parents. (The latter process was later called **Lamarckism**.) These ideas were condemned by established naturalists as speculation lacking empirical support

Darwinian revolution

The crucial break from the concept of constant typological classes or types in biology came with the theory of evolution through natural selection, which was formulated by Charles Darwin in terms of variable populations. Partly influenced by An Essay on the Principle of Population (1798) by **Thomas**

Robert Malthus, Darwin noted that population growth would lead to a "struggle for existence" in which favorable variations prevailed as others perished. In each generation, many offspring fail to survive to an age of reproduction because of limited resources. This could explain the diversity of plants and animals from a common ancestry through the working of natural laws in the same way for all types of organism. Darwin developed his theory of "natural selection" from 1838 onwards and was writing up his "big book" on the subject when **Alfred Russel Wallace** sent him a version of virtually the same theory in 1858. Their separate papers were presented together at an 1858 meeting of theLinnean Society of London.At the end of 1859, Darwin's publication of his "abstract" as On the Origin of Species explained natural selection in detail and in a way that led to an increasingly wide acceptance of Darwin's concepts of evolution at the expense of alternative theories. Thomas Henry Huxley applied Darwin's ideas to humans, using paleontology and comparative anatomy to provide strong evidence that humans and apes shared a common ancestry. Some were disturbed by this since it implied that humans did not have a special place in the universe.



Pangenesis and heredity

The mechanisms of reproductive heritability and the origin of new traits remained a mystery. Towards this end, Darwin developed his provisional theory of pangenesis. In 1865, **Gregor Mendel** reported that traits were inherited in a predictable manner through the independent assortment and segregation of elements (later known as genes). Mendel's laws of inheritance eventually supplanted most of Darwin's pangenesis theory. August Weismann made the important distinction between germ cells that give rise to gametes (such as sperm and egg cells) and the somatic cells of the body, demonstrating that heredity passes through the germ line only. **Hugo de Vries** connected Darwin's pangenesis theory to Weismann's germ/soma cell distinction and proposed that Darwin's pangenes were concentrated in the cell nucleus and when expressed they could move into the cytoplasm to change the cells structure. De Vries was also one of the researchers who made Mendel's work well-known, believing that Mendelian traits corresponded to the transfer of heritable variations along the germline. To explain how new variants originate, de Vries developed a mutation theory that led to a temporary rift between those who accepted Darwinian evolution and biometricians who allied with de Vries.

The 'modern synthesis'

In the 1920s and 1930s the so-called modern synthesis connected natural selection and population genetics, based on Mendelian inheritance, into a unified theory that applied generally to any branch of biology. The modern synthesis explained patterns observed across species in populations, through fossil transitions in palaeontology, and complex cellular mechanisms in developmental biology.The publication of the structure of DNA by James Watson and Francis Crick with contribution of **Rosalind Franklin** in 1953 demonstrated a physical mechanism for inheritance. Molecular biology improved our understanding of the relationship between genotype and phenotype. Advancements were also made in phylogenetic systematics, mapping the transition of traits into a comparative and testable framework through the publication and use of evolutionary trees. In 1973, evolutionary biologist **Theodosius Dobzhansky** penned that "nothing in biology makes sense except in the light of evolution," because it has brought to light the relations of what first seemed disjointed facts in natural history into a coherent explanatory body of knowledge that describes and predicts many observable facts about life on this planet.

Further syntheses

Since then, the modern synthesis has been further extended to explain biological phenomena across the full and integrative scale of the biological hierarchy, from genes to species. One extension, known as evolutionary developmental biology and informally called "evo-devo," emphasises how changes between generations (evolution) acts on patterns of change within individual organisms (development). Since the beginning of the 21st century and in light of discoveries made in recent decades, some biologists have argued for an extended evolutionary synthesis, which would account for the effects of non-genetic inheritance modes, such as epigenetics, parental effects, ecological inheritance and cultural inheritance, and evolvability.

Lamarckism:

Lamarckism (or Lamarckian inheritance) is the hypothesis that an organism can pass on characteristics that it has acquired through use or disuse during its lifetime to its offspring. It is also known as the inheritance of acquired characteristics or soft inheritance. It is inaccuratelynamed after the **French biologist Jean-Baptiste Lamarck** (1744–1829), who incorporated the action of soft inheritance into his evolutionary theories as a supplement to his concept of orthogenesis, a drive towards complexity. On the Origin of Species (1859), Charles Darwin supported the idea of "use and disuse inheritance", though rejecting other aspects of Lamarck's theory.

Lamarck's evolutionary framework

Lamarck's two-factor theory involves 1) a complexifying force that drives animal body plans towards higher levels (orthogenesis) creating a ladder of phyla, and 2) an adaptive force that causes animals with a given body plan to adapt to circumstances (use and disuse, inheritance of acquired characteristics), creating a diversity of species and genera. Popular views of Lamarckism only consider an aspect of the adaptive force.

Lamarck proposed a systematic theoretical framework for understanding evolution. He saw evolution as comprising four laws:

First law

"Life by its own force tends to increase the volume of all organs which possess the force of life, and the force of life extends the dimensions of those parts up to an extent that those parts bring to themselves;"

Second law

"The production of a new organ in an animal body, results from a new requirement arising. and which continutes to make itself felt, and a new movement which that requirement gives birth to, and it's upkeep/maintenance;"

Third Law

"The development of the organs, and their ability, are constantly a result of the use of those organs."

Fourth Law

"All that has been acquired, traced, or changed, in the physiology of individuals, during their life, is conserved through the genesis, reproduction, and transmitted to new individuals who are related to those who have undergone those changes."

Lamarck's discussion of heredity

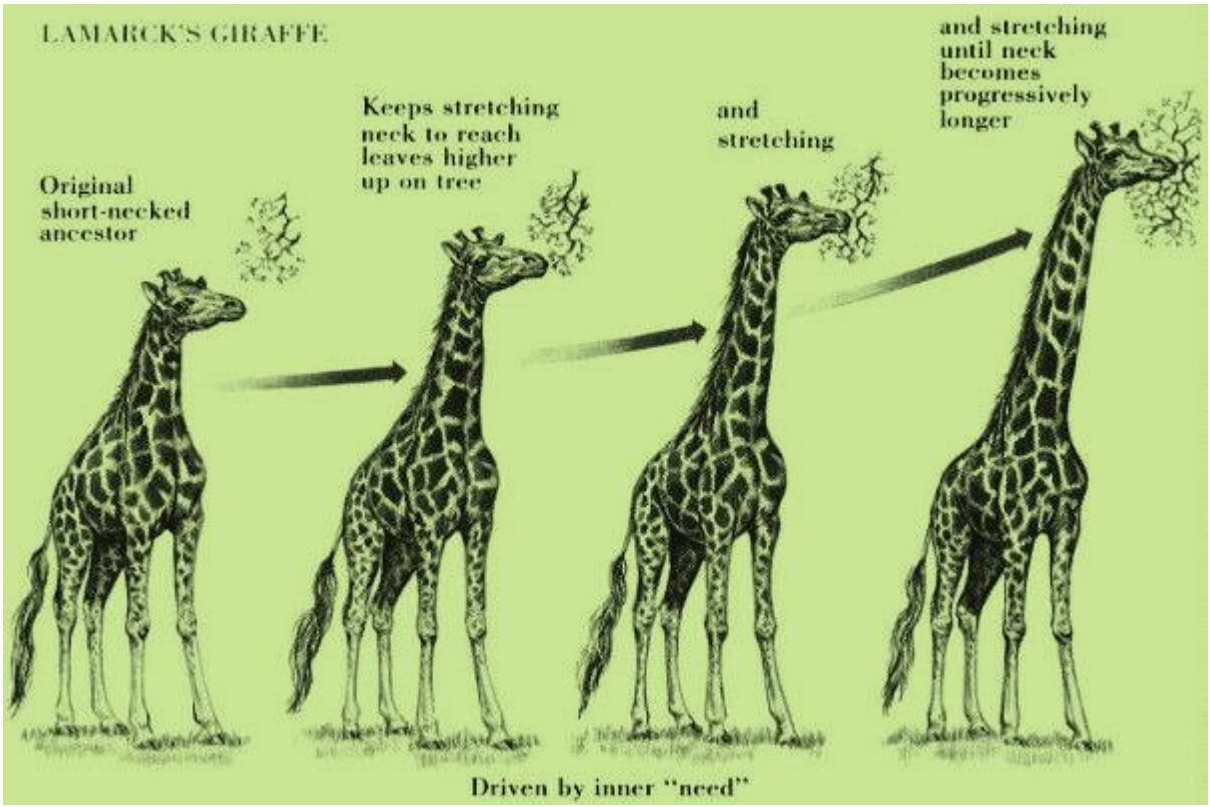
In an aside from his evolutionary framework, Lamarck briefly mentioned two traditional ideas in his discussion of heredity, in his day considered to be generally true.

- The first was the idea of use versus disuse; he theorized that individuals lose characteristics they do not require, or use, and develop characteristics that are useful.
- The second was to argue that the acquired traits were heritable. He gave as an imagined illustration the idea that when giraffes stretch their necks to reach leaves high in trees, they

would strengthen and gradually lengthen their necks. These giraffes would then have offspring with slightly longer necks.

First Law [Use and Disuse]: In every animal which has not passed the limit of its development, a more frequent and continuous use of any organ gradually strengthens, develops and enlarges that organ, and gives it a power proportional to the length of time it has been so used; while the permanent disuse of any organ imperceptibly weakens and deteriorates it, and progressively diminishes its functional capacity, until it finally disappears.

Second Law [Soft Inheritance]: All the acquisitions or losses wrought by nature on individuals, through the influence of the environment in which their race has long been placed, and hence through the influence of the predominant use or permanent disuse of any organ; all these are preserved by reproduction to the new individuals which arise, provided that the acquired modifications are common to both sexes, or at least to the individuals which produce the young.



Criticism of Lamarckism:

Lamarck's theory was subject to severe criticism. Two scientists Cuvier and Weismann were great critics of Lamarck.

Some objections raised against Lamarckism are as follows:

a. Though the tendency to increase in size has been shown in many forms, there are also instances where there is reduction in size. For example, trees that are primitive, are large in size, while the shrubs, herbs and grasses that evolved later are smaller in size.

b. If new organs were to develop in response to a new need, then man should have developed wings by now.

c. Changes acquired during the lifetime of an organism cannot be inherited by the offspring. For example, if a man loses his arm in war, he does not produce children without an arm. According to August Weismann, somatic changes acquired during the lifetime of the organisms are non- heritable, whereas, changes in the germplasm or reproductive cells are inheritable by the offspring.

Neo-Lamarckism:

Although Lamarck's doctrine of inheritance of acquired characters was strongly refuted and rejected, yet a few biologists accepted the theory in modified way. The prominent among them were cope (1840 -1897), Giard (1846 -1908), Packard Spencer and Mc Bridge.

According to Neo-Lamarckism the adaptation is the universal feature of living beings. It arises as a result of interaction between the structure, function and environment. The changed or fluctuating environmental conditions can alter the habit and structure of the organisms.

So, the organisms acquire new adaptation in response to new environmental conditions and consequently variations among plants and animals may result. These variations become established gradually in the heredity of the race.

This is the modified version of Lamarckism or Neo-Lamarckism because it does not follow the general perfecting tendency in evolution and stresses mainly on the direct action of environment on organic structure. According to some Neo Lamarckians, the fur development on the skin of some animals as adaptation against cold weather is the consequence of changed environment from warmer to colder state.

On the other hand, if the environment comes to the normal state again, the fur would also disappear. Neo-Lamarckians have discarded natural selection as the sole mechanism of evolution. They are of the opinion that the interaction between the structure, function and environment is the only cause of evolution. Today no evolutionist supports the Neo-Lamarckism.

Darwinism:

Darwinism is a theory of biological evolution developed by the **English naturalist Charles Darwin** (1809–1882) and others, stating that all species of organisms arise and develop through the natural selection of small, inherited variations that increase the individual's ability to compete, survive, and reproduce. Also called Darwinian theory, it originally included the broad concepts of transmutation of species or of evolution which gained general scientific acceptance after Darwin published **On the Origin of Species** in 1859, including concepts which predated Darwin's theories

In 1831, Charles Darwin on a voyage on HMS Beagle for five years noted the flora, fauna and geology of the islands of the South Pacific and collected numerous living and fossil specimens. He also sailed to the Galapagos Islands about 600 miles from the west coast of America

He observed a number of variations or differences among the organisms that lived on these islands. The common birds of the Galapagos Islands were the finches that were remarkably different from the finches of the mainland. These closely related species of finches had beaks of different shapes and sizes and were adapted for feeding on completely different diets.

The main points of the Theory of Natural Selection are as follows:

a. Over Production or Enormous Fertility:

Living organisms have an innate capacity to produce more individuals to ensure continuity of the race. For example, an oyster may produce over 60-80 million eggs per year. A rabbit produces six young ones in a litter and four litters in a year and the young rabbit becomes reproductively active in six months from birth. A single female salmon produces 28,000,000 eggs in a season.

b. Struggle for Existence:

Organisms multiply in a geometric ratio, while the food supply increases in an arithmetic ratio. This leads to intense competition between organisms to ensure living to obtain maximum amount of food and shelter.

Struggle exists at three levels:

i. Intraspecific struggle is the competition among individuals of the same species or closely related forms. This type of struggle is very severe as the need of the population is the same.

ii. Interspecific struggle is the struggle between organisms of different species living together. Individuals of one species compete with other species for similar requirements.

Struggle with the environment means the various hazards of the nature like extreme heat or cold, excess moisture or drought, storms, earthquakes, volcanoes eruptions, etc. also affect the survival of various organisms.

c. Variations amongst Organisms:

Differences that exist among organisms are called variations. Variations may be harmful, neutral or useful. Variations that are passed on from generation to generation are called heritable variations and these form the raw material for evolution. These variations arise due to changes in the genes or the chromosomes.

d. Survival of the Fittest:

During the struggle for existence, the individuals that exhibit variations beneficial in facing the environment will survive, while those that cannot face the hardship will be eliminated. Those organisms best able to survive and reproduce will leave more offspring than those unsuccessful individuals. This is referred to as survival of the fittest.

According to Darwin, the giraffe exhibited variations in the length of the neck and legs. When the grass on the ground became scarce, giraffes with long necks and legs had an advantage over those with shorter neck and legs, as they could feed on the tall trees. So these forms survived and reproduced and became abundant. Over a period of time, giraffes with short necks starved and became extinct.

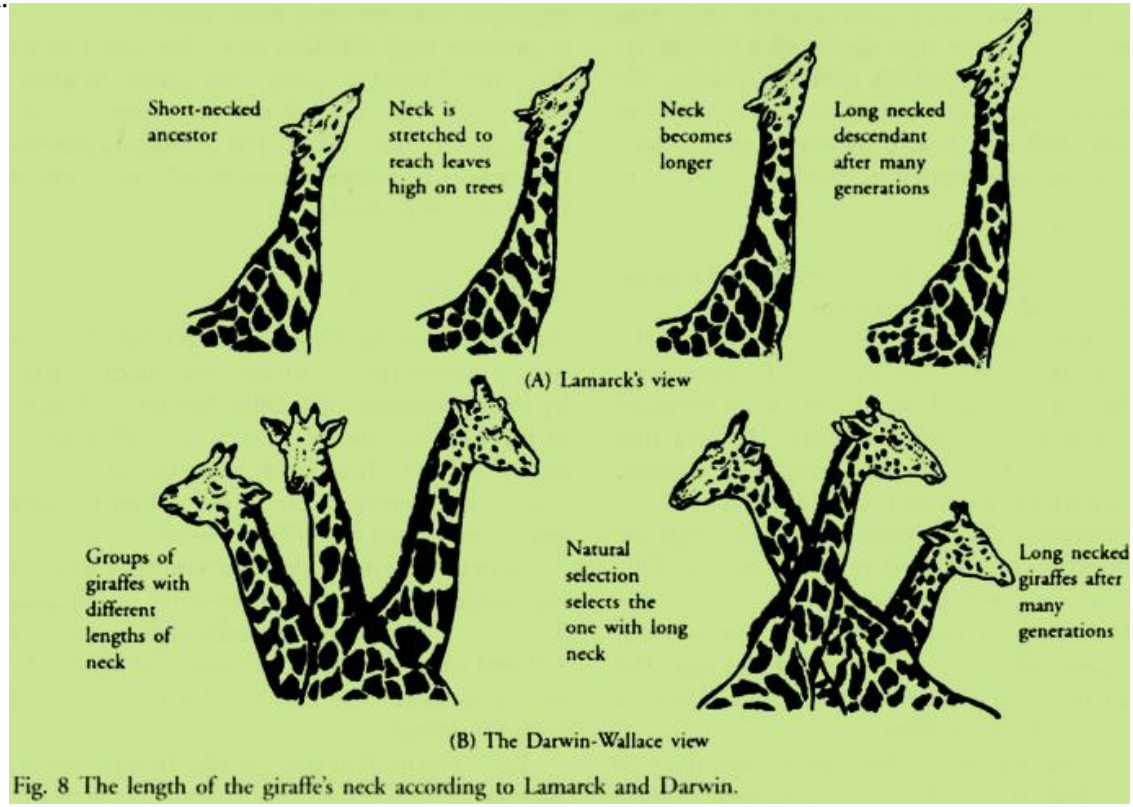


Fig. 8 The length of the giraffe's neck according to Lamarck and Darwin.

e. Origin of Species:

As a result of struggle for existence, variability and inheritance, individuals that are better adapted, survived and became abundant. Slowly over a period of time, this group, which was remarkably different from the original population, becomes established as a new species. This group is also subject to the same forces of change as their ancestors were and this process continues to give rise to new species.

Criticism of Darwinism:

The following points have been raised against the theory of natural selection:

a. Darwin was unable to explain the mechanism of inheritance of characters. Darwin proposed the theory of pangenesis to explain this phenomenon. He said that every cell or organ produces minute hereditary particles called **pangenes or gemmules**. These were carried through the blood and deposited in the gametes. This theory was not accepted.

b. According to natural selection, only useful organs are favoured by natural selection. The existence of vestigial organs in organisms could not be explained.

c. In some species of deer, the antlers develop beyond the stage of usefulness. These structures are of no functional significance to the animal.

d. Darwin was unable to explain the source of variations in organisms.

Artificial Selection:

Artificial selection is the isolation of natural population and the selective breeding of organisms with characteristic which are useful to humans. In this method, human exert a directional selection pressure that leads to changes in allele and genotype frequencies within the population. This is an evolutionary mechanism which gives rise to new breeds, strains, varieties, races and subspecies.

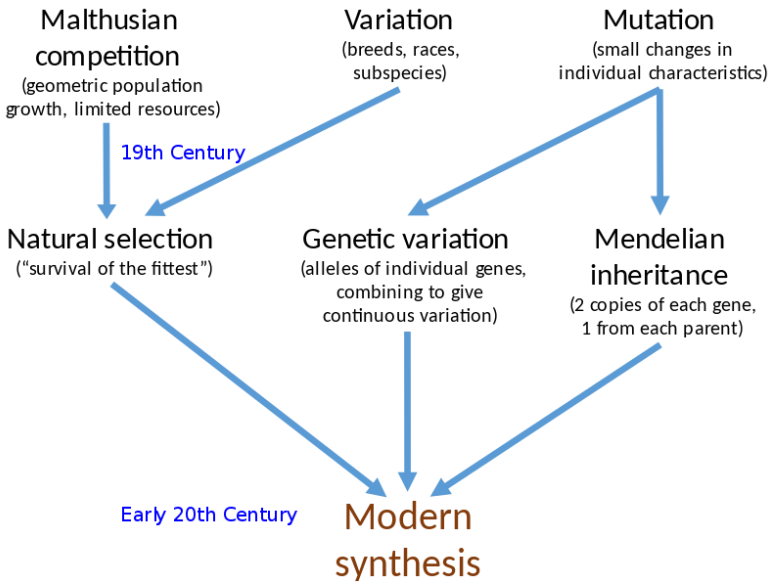
Darwin studied domestication in plants and animals in detail. He concluded that by artificial selection different varieties of plants and animals could be produced. Cabbage, cauliflower, broccoli, kale, kohlrabi, brussels sprouts could be produced from the common wild mustard (Fig. 9). Similarly, Darwin also raised several types of pigeons from the rock pigeon by artificial selection.

Neo-Darwinism:

Neo-Darwinism is the interpretation of Darwinian evolution through natural selection as it has variously been modified since it was first proposed. It was early on used to name Charles Darwin's ideas of natural selection separated from his hypothesis of pangenesis as a Lamarckian source of variation involving blending inheritance.

In the early 20th century, the concept became associated with the modern synthesis of natural selection and Mendelian genetics that took place at that time.

In the late 20th century and into the 21st century, neo-Darwinism denoted any strong advocacy of Darwin's thinking, such as the gene-centered view of evolution.



This is modification of Darwinism in light of cytogenetics. The main contributors of this theory are T.H. Huxley, S. Wright, Th. Dobjhansky, Karl Gegenbaur, Ernst Haeckel, Weismann and G.L. Stebbins Darwin's theory was criticised for several defects. It did not explain the origin of variations and could not distinguish somatic (non-heritable) from germinal (heritable) variations.

Darwin failed to explain why even harmful variations were selected or preserved leading ultimately to extinction of the species e.g., horns of Irish elks. The supporters of this theory believed that natural selection has accounted every-thing that has undergone evolution.

Weismann and his supporters discarded Darwin's theory except its principal element of natural selection. This made clear-cut distinction between somatic and germinal variations in their theory of germplasm but they could not appreciate the role of mutations in evolution. Darwin was of the opinion that the mutation resulted mainly by single force, i.e., natural selection.

According to Neo-Darwinism adaptations result from multiple forces and the natural selection is one of them. The theory also holds that the characters are not inherited as such but there are character determinant which control the development of the particular characters.

The ultimate character results due to the interaction of the determinants as well as the interaction between organisms and the environment during development.

This theory gives better mechanism of evolution and states that:

(i) The sources of variations are mutations and gene recombinations through sexual reproduction.

(ii) Chromosomal organization and variation affect genetic linkage and produce variations in gene pool of cross fertilizing populations.

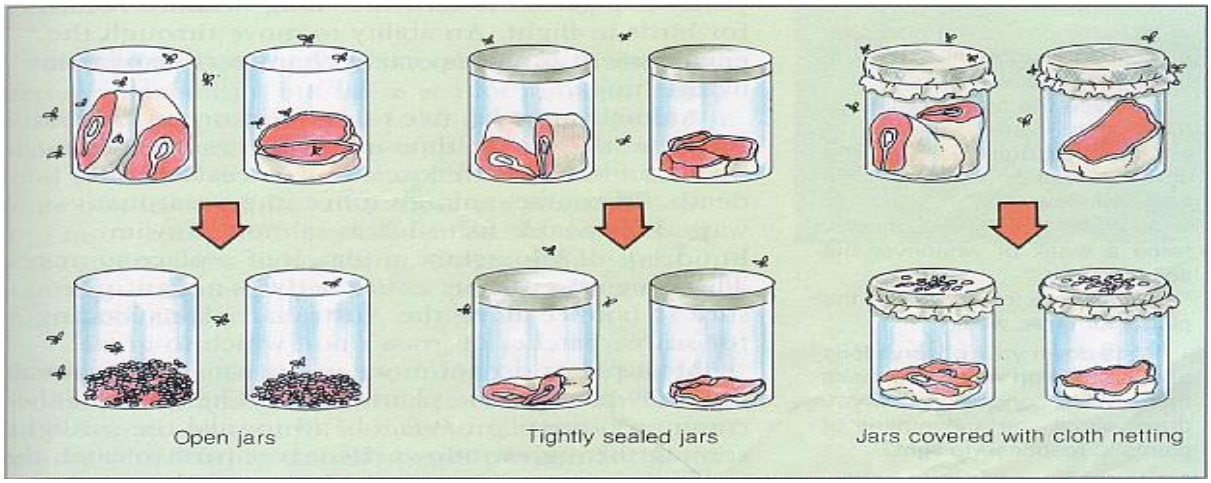
It is remarkable that variations only provide the raw material and not the direction of evolution which is the function of natural selection. The limits to which the natural selection can guide the population are set by reproductive isolation only. Neo-Darwinism has received great support from recent studies of population genetics.

4. Origin of cells and unicellular evolution: Origin of basic biological molecules; abiotic synthesis of organic monomers and polymers; concept of Oparin and Haldane; experiment of Urey-Miller (1953); the first cell; evolution of prokaryotes; origin of eukaryotic cells; evolution of unicellular eukaryotes; anaerobic metabolism, photosynthesis and aerobic metabolism.

Abiogenesis

Abiogenesis is the generation of life from non-living matter, now more precisely known as spontaneous generation. Spontaneous generation was a concept proposed by Aristotle around mid-300 B.C. This theory states that complex living organisms are generated from decaying organic substances e.g., organism like mice spontaneously appears in stored grain or maggots spontaneously appear in meat.

Francesco Redi, an Italian physician, was the first who disproved the Theory of spontaneous Generation by performing a controlled experiment. In l668, Francesco Redi performed an experiment to check whether maggots really came from decaying meat. He did this by placing meat in a number or Jars and covering half of them with fine gauze while leaving the others uncovered. Maggots developed only on the meat in the uncovered jars. From this, Redi concluded that the maggots did not come from the meat, but from tiny eggs that flies had laid on the meat. Since flies could not land on the meat in the covered jars, they could not lay eggs on that meat, and no maggots formed. Therefore, decaying meat could not produce maggots.



Later, Lazzaro Spallanzani (an Italian naturalist) performed a similar experiment with broth. He put broth into two glass flasks and sterilized them by boiling the flask containing broth. One of the flasks was left open to the air. The other flask was sealed up to keep out any organisms that might be floating in the air. Microorganisms developed only in the uncovered flask. From this, Spallanzani concluded that the microorganisms did not come from the broth, but were in the air that entered the flask.

Unfortunately, many scientists were not convinced by his experiment. Louis Pasteur, a French chemist who finally disproved the theory of Spontaneous Generation in the mid 1800's. He performed the same type of experiment as Spallanzani. Louis Pasteur, however, allowed air to enter into the flask of sterile broth.

He performed experiments with two flasks-one with a straight neck and other with S-shaped neck. Flasks with a straight neck allowed both air and microorganisms to enter. Whereas the other flask with S-shaped neck allowed only air to enter but not microorganisms. The broth in the straight neck flask contaminated with microorganisms but the broth in the flask with an S-shaped neck did not become contaminated. Therefore, Louis Pasteur showed that even though air could get in the flask, the broth did not produce microorganisms.

Scientists finally were convinced that living things, no matter how small, do not come from nonliving things. The present theory of where living things come from is called biogenesis. This theory states that living things come only from other iliving things. for example, mice come only from mice, and microorganisms such as bacteria can only come from other bacteria. Since spontaneous generation was now proved incorrect, many scientists began to wonder how life started on Earth. Oparin and Haldane attempted to answer this question. They proposed that life had arisen from simpler molecules on the lifeless earth under much different atmospheric conditions that exist today. However, instead of life arising suddenly, as previous spontaneous generation theories proposed, Oparin and Haldane believed that it ocurred over a very long period of time.

Chmical evolution:

Oparin-Haldane Hypothesis (1920s)

Oparin (Russia) & J.B.S. Haldane (Britain) proposed an explanation for the chemical evolution of life. **Primordial soup**, or **prebiotic soup**, is a hypothetical condition of the Earth's atmosphere before the emergence of life. It is a chemical environment in which the first biological molecules (organic compounds) were formed under natural forces. According to the theory, simple organic compounds were created from non-living inorganic molecules (abiogenesis) through physical and chemical reactions on the Earth's surface. The so formed organic molecules accumulate into a rich organic ocean, or a "soup". In this soup, simple organic molecules reacted with each other (polymerise) to form more complex molecules, including nucleic acids and proteins, which are the central structural and functional components of all organisms. These molecules then aggregate to become the first forms of life.

The British naturalist Charles Darwin had vaguely imagined the primordial soup as a "warm little pond" in 1871. A coherent scientific argument was introduced by a Soviet biochemist Alexander Oparin in 1924. According to Oparin, in the primitive Earth's surface, carbon, hydrogen, water vapour, and ammonia reacted to form the first organic compounds. Unbeknownst to Oparin, whose writing was circulated only in Russian, an English scientist John Burdon Sanderson Haldane independently arrived at similar conclusion in 1929. It was Haldane who gave the name "soup" to the theory.

The theory is variously known as "primordial soup theory", "prebiotic soup theory", and "Oparin-Haldane hypothesis". Biochemist Robert Shapiro has summarized the theory in its "mature form" as follows:

- Early Earth had a chemically reducing atmosphere.
- This atmosphere, exposed to energy in various forms, produced simple organic compounds ("monomers").
- These compounds accumulated in a "soup", which may have been concentrated at various locations (shorelines, oceanic vents etc.).
- By further transformation, more complex organic polymers – and ultimately life – developed in the soup.

Coacervates are submicroscopic structures of colloidal solution of proteins and carbohydrates which show osmotic activity and could give rise to larger aggregates. They sustain more rapid reaction

bringing about oxidation, reduction and polymerization. These molecular aggregates could have been easily surrounded by a boundary layer, akin to membrane. These coacervates have interesting properties.

In one experiment, enzyme phosphorylase, which was trapped inside coacervates, releases the phosphate form glucose-1-phosphate and polymerizes the glucose units into starch deriving energy from phosphate bond.

The coacervates, therefore, grow and eventually divide into two coacervates. In another experiment, the red-ox enzyme NADH dehydrogenase was trapped inside the coacervates, catalyses the reduction of methyl red coupled to oxidation of NADH, analogous to electron transfer system.

Thus coacervates have the interesting properties:

(a) Spatial organisation of simple bio-energetic process;

(b) Maintain a certain size and, therefore, divide if their volume increases.

(c) A spatial structure in which replicators could reside.

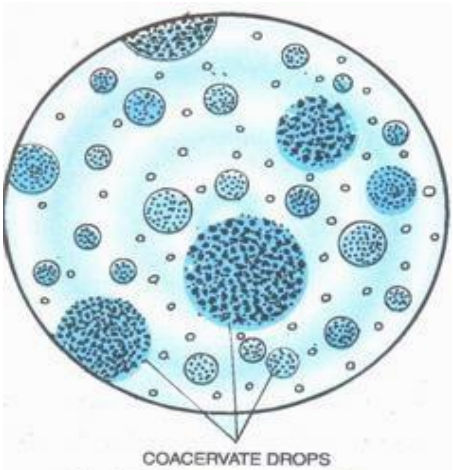


Fig. 7.4. Coacervate particles.

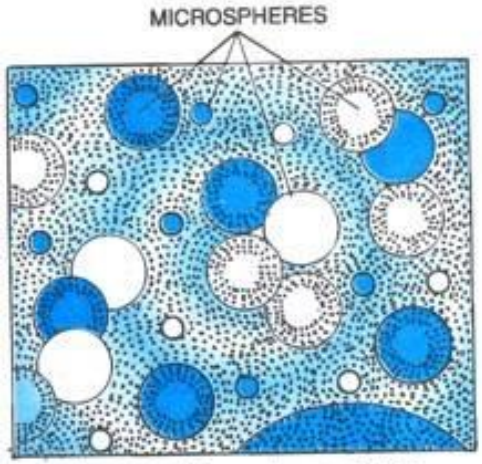


Fig. 7.10. Microspheres of Fox.

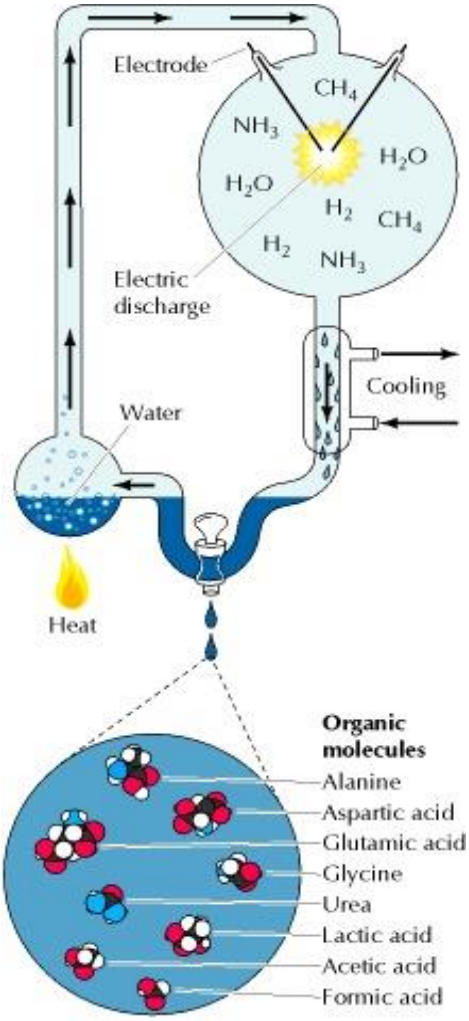
Proteinoid microspheres

In trying to uncover the intermediate stages of abiogenesis mentioned by Bernal, Sidney W. Fox in the 1950s and 1960s studied the spontaneous formation of peptide structures (small chains of amino acids) under conditions that might plausibly have existed early in Earth's history. In one of his experiments, he allowed amino acids to dry out as if puddled in a warm, dry spot in prebiotic conditions. He found that, as they dried, the amino acids formed long, often cross-linked, thread-like, submicroscopic polypeptide molecules now named "proteinoid microspheres".

Miller–Urey experiment:

The Miller–Urey experiment (or Miller experiment) was a chemical experiment that simulated the conditions thought at the time to be present on the early Earth, and tested the chemical origin of life under those conditions. The experiment supported Alexander Oparin's and J. B. S. Haldane's hypothesis that putative conditions on the primitive Earth favoured chemical reactions that synthesized more complex organic compounds from simpler inorganic precursors. Considered to be the classic experiment investigating abiogenesis, it was conducted in 1952

The experiment used water (H_2O), methane (CH_4), ammonia (NH_3), and hydrogen (H_2). The chemicals were all sealed inside a sterile 5-liter glass flask connected to a 500 ml flask half-full of liquid water. The liquid water in the smaller flask was heated to induce evaporation, and the water vapour was allowed to enter the larger flask. Continuous electrical sparks were fired between the electrodes to simulate lightning in the water vapour and gaseous mixture, and then the simulated atmosphere was cooled again so that the water condensed and trickled into a U-shaped trap at the bottom of the apparatus.



After a day, the solution collected at the trap had turned pink in colour.At the end of one week of continuous operation, the boiling flask was removed, and mercuric chloride was added to prevent microbial contamination. The reaction was stopped by adding barium hydroxide and sulfuric acid, and evaporated to remove impurities. Using paper chromatography, Miller identified five amino acids present in the solution: glycine, α-alanine and β-alanine were positively identified, while aspartic acid and α-aminobutyric acid (AABA) were less certain, due to the spots being faint.

One-step reactions among the mixture components can produce hydrogen cyanide (HCN), formaldehyde (CH_2O), and other active intermediate compounds (acetylene, cyanoacetylene, etc.).

$CO_2 \rightarrow CO + [O]$ (atomic oxygen)

$CH_4 + 2[O] \rightarrow CH_2O + H_2O$

$CO + NH_3 \rightarrow HCN + H_2O$

$CH_4 + NH_3 \rightarrow HCN + 3H_2$ (BMA process)

The formaldehyde, ammonia, and HCN then react by Strecker synthesis to form amino acids and other biomolecules:

$CH_2O + HCN + NH_3 \rightarrow NH_2\text{-}CH_2\text{-}CN + H_2O$

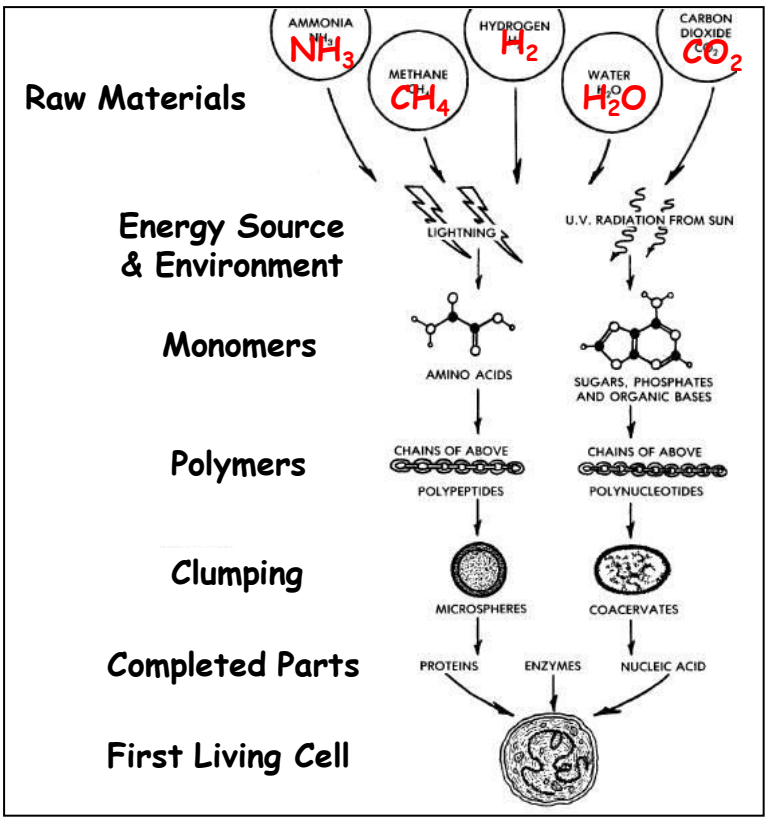
$NH_2\text{-}CH_2\text{-}CN + 2H_2O \rightarrow NH_3 + NH_2\text{-}CH_2\text{-}COOH$ (glycine)

Furthermore, water and formaldehyde can react, via Butlerov's reaction to produce various sugars like ribose.

First cells may have originated by chemical evolution involving 4 steps (Fig. 55):

- Abiotic (Non-biological) synthesis & accumulation of small organic molecules (monomers) → C + H = organic molecule
- Monomers joined together to form polymers (proteins, nucleic acids)
- Origin of self-replicating hereditary molecules (inheritance of traits) → proteins and polynucleic acids
- Packaging of these organic molecules into cell like membrane bound droplets - protobionts (precursor to prokaryotic cell) → Aggregates of abiotically produced molecules that had an internal chemical environment differing from the external chemical environment and exhibit some of the properties associated with life (i.e. metabolism, excitability, heredity).

Fig. 55: Likely steps in the origin of Life



Evidence that supports the four-stage hypothesis for the origin of life

In 1920s, I. Oparin (Russia) and J. B. S. Haldane (Great Britain) postulated that abiotic synthesis of organic molecules is testable in the laboratory.

Hypothesis: Conditions on primitive earth favored chemical reactions that synthesized organic compounds from inorganic precursors. These conditions were different from present atmosphere.

As we know that earth atmosphere was

- No Free Oxygen
- More Reducing Environment than present (no free oxygen, only in form of H_2O, CH_4, NH_4, and H_2) = lots of free electrons that could be used to reduce carbon and produce organic molecules.
- Energy from lots of Lightning, UV radiation (no O_2 to block UV rays from the sun) and Volcanic Activity (heat).

This early environment was recreated in the laboratory by **Stanley Miller & Harold Urey (Fig. 56) in 1950s and tested the Oparin-Haldane hypothesis.**

Results of this experiment is that they were able to create Amino Acids, Urea, Simple Fatty Acids as in a week which were resultant of 15% of the conversion of carbon in the mixture to organic compounds. They concluded that **Life may have evolved in "primordial soup" of biological molecules formed in early Earth's oceans.**

The initial Miller-Urey experiment and various similar experiments succeeded in producing:

- All 20 amino acids
- Several sugars

- Lipids
- Purines and Pyrimidines
- ATP (when phosphate was added)

More recent experiments subjecting a reducing mixture of gases to a violent energy source produces:

- Formaldehyde
- Hydrogen Cyanide
- Cyanoacetylene
- All highly reactive intermediate molecules

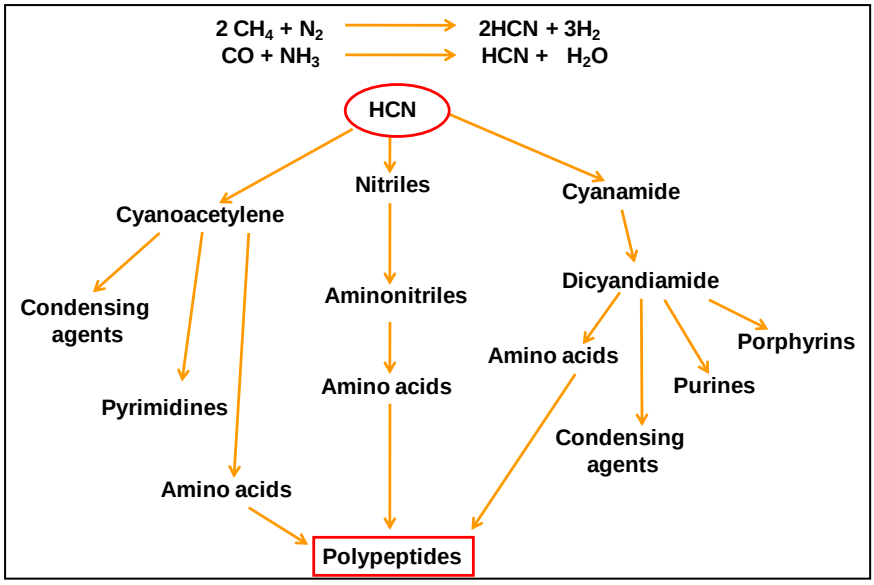
All react with water and NH_3 or N_2 to produce a variety of organic compounds:

- Amino Acids, Fatty Acids, Urea, Sugars,
- Aldehydes, Purine and Pyrimidine Bases

And it signifies the subunits for complex organic compounds.

Chemical evolution of biomolecules is supposed to produce in following pathway (Fig. 57).

Fig. 57: Chemical evolution of biomolecules



Nowadays DNA needs proteins in order to form, and proteins require DNA to form, so how could these have formed without each other? But was it the same situation during the origin of life?

Which came first- RNA?

The answer may be RNA, which can store information like DNA, serve as an enzyme like proteins, and help create both DNA and proteins. Later DNA and proteins succeeded this "RNA world," because they are more stable & efficient.

For life to originate and perpetuate, we need to have a molecule that can be used to store information and also can catalyze the synthesis of other molecules. Thomas Czech (1980) proved that

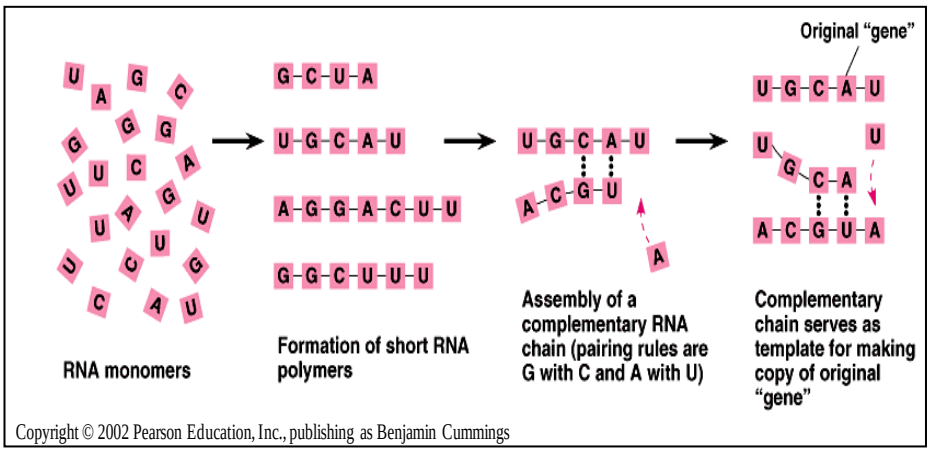
- RNA can catalyze simple reactions leading to the belief that RNA was probably the first genetic molecule to start life.
- RNA can help as a template for protein synthesis and for more RNA synthesis.
- Once proteins (enzymes) are made, they can make carbohydrates and lipids. Later on DNA evolved to be more stable molecule and proteins evolved to be more efficient enzymes.
- RNA with catalytic activity is referred to as ribozyme.

So, RNA was probably the first hereditary material which is termed as RNA World

Today some organisms such as viruses use RNA to store information.

- Short polymers of ribonucleotides can be synthesized abiotically in the laboratory (Fig. 58).
- If these polymers are added to a solution of ribonucleotide monomers, sequences up to 10 based long are copied from the template according to the base-pairing rules.
- If zinc is added, the copied sequences may reach 40 nucleotides with less than 1% error.

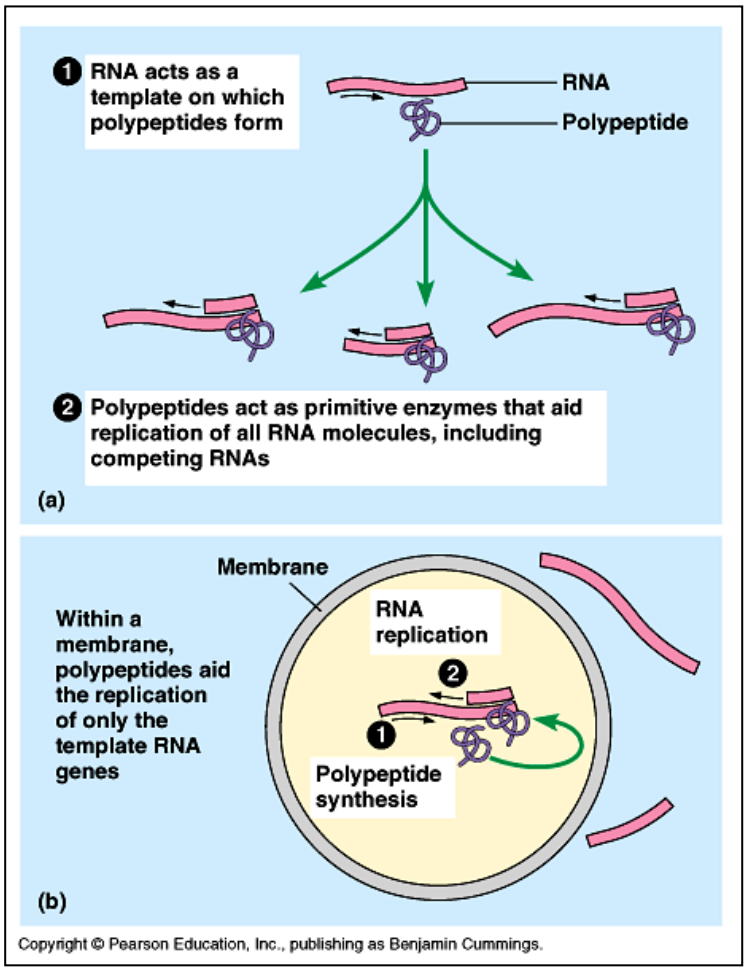
Fig. 58: Short polymer of RNA synthesis



Natural selection could refine protobionts containing hereditary information

- Once primitive RNA genes and their polypeptide products were packaged within a membrane, the protobionts could have evolved as units (Fig. 59).
- Molecular cooperation could be refined because favorable components were concentrated together, rather than spread throughout the surroundings.

Fig. 59: Protobionts containing hereditary information

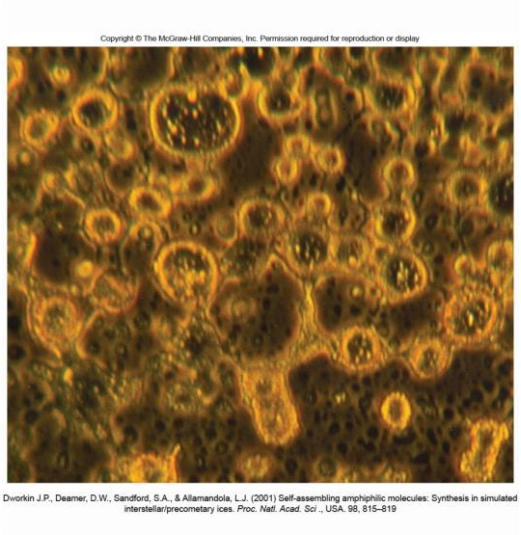


How did the membrane form?

The strongest current hypothesis for pre-biotic assembly of biologically important polymers suggests that they occurred within the boundaries of semi-permeable membranes (Fig. 60).

- Membranes were formed by aggregation of amphiphilic molecules.
- Meteorites are common sources of organic amphiphiles.

Fig. 60: Cell membrane formation



Evidences for the origin of life - prokaryotes recovered from Precambrian strata

The definite evidences of earliest life forms in the Precambrian strata were come to existence by the landmark publication of Tyler and Barghoorn (1954, 1965). A. C. Seward described the Precambrian "as an age of algae – algae with doubtful credentials." Fossil evidences of anaerobic chemo-heterotrophic bacteria, unicellular cyanobacteria are identified in the 3500 million (3.5 b.y.) year's old rocks in the Western Australia (Warrawoona Group) as the oldest structurally preserved forms of life so far recorded. Precambrian lifeforms occur principally in two types of sedimentary deposits namely 'cherts' and 'shales'.

Cherts: These rocks are composed of minute interlocking grains of silica, occurring as the mineral quartz (SiO_2), that have been deposited chemically, petrifying microscopic organisms in the place in which they live. Fossils preserved on these rocks are generally unflattened, composed of three-dimensionally preserved organic-walled cells that are thoroughly embedded in, and in filled by, the petrifying fine-grained quartz in the layers of stromalites. Stromatolites are extensive mats of microorganisms mostly comprising of cyanobacteria and green algae

Shales: This type of rocks is formed by consolidation of layers of clay or mud disseminated, along with phytoplankton and other debris, at the bottom of lakes or ocean basins. Carbonaceous microfossils of shales have been preserved by compression and flattened between thin layers of consolidated silt.

Evidences supporting the earliest life forms in the Precambrian strata are mostly recovered from the fossils sites of Onverwacht Group (Swaziland Supergroup), and Fig Tree Group of South Africa and Warrawoona Group (Pilbara Supergroup) of Western Australia

Prokaryotes dominated from 3.5 to 2 billion years ago. During this time, the first divergence occurred: Bacteria and Archaea Earliest fossil prokaryotes are mainly representative of two types of living organisms:

Blue-Green Algae (Cyanobacteria)

Bacteria

The oldest unequivocal remains of a diversity of microorganisms occur in the 2.0 BYO or 2000 my Gunflint Cherts of the Canadian Shield. It includes not only bacteria and cyanobacteria but also ammonia consuming *Kakabekia* and some things that resemble green algae and fungus-like organisms

Origin and Evolution of First Cell:

Early living cells were RNA life forms, self- replicating RNA covered by lipoprotein vesicles were the pre-prokaryotes, with time the proteins replaced the catalytic function of RNA, and DNA replaced the coding function of RNA, the progenitors of modern prokaryotes with DNA-RNA-protein functioning types evolved.

Evolution of first primitive cell from RNA world represents a huge gap. Primitive bacterial cell represents an immensely complicated struc-ture with at least 1000 genes in comparison with our ideas about RNA world.

1. Dominating role of protein as enzymes over ribozymes.
2. Differentiation of different types of RNA.
3. The shift from RNA to DNA as carrier of genetic information.
4. Origin of genetic code.
5. Formation of chromosome.
6. Increasing genetic information.
7. Phenotypic expression of a genotype.
8. Origin of cell-membrane.
9. Evolution of metabolic process.

Proteins as Enzymes:

Introduction of pro-teins as enzymes resulted in more specific cata-lysis. Enzymatic capability of the RNA strands could be improved if individual amino acids were attached as in tRNA, i.e., amino acids acted as co-enzymes for the ribozymes. The next step is the specialization of RNA so that '+' strand had the role as mRNA and '-'strand functioned as tRNA and attached to the '+' strand with an anti-codon triplet.

Finally, the amino acids could be coupled together as a polypeptide strand that would further improve catalytic activity. This idea is supported by the fact that tRNAs from different organisms with similar function are more closely related and thus tRNA can be traced back to the origin of the genetic code and to a RNA world.

Differentiation of Different Types of RNA:

Functional specialization of different RNAs was adaptive in increasing the efficiency within proto-cells. Some kinds of RNA (tRNA) specialized in collecting amino acids and others (rRNA) in cou-pling them together are the basis of the code in a third kind of RNA (mRNA).

From RNA to DNA:

Emergence of complex organisms requires the transition from RNA to DNA as genetic material. Double stranded DNA is much more stable than RNA and allows enzy-matic proof-reading and correction in connection with replication and thus reduces the rate of mutation.

The genetic information in RNA organisms corresponds to a maximum of 10 thousand base pairs in comparison to 10 million base pairs in bacterial chromosome.

Replacement of ribose by deoxyribose in the carbohydrate backbone of RNA and replacement of uracil base by thymine resulted in DNA. Deoxyribose is formed in cells through an enzymatically con-trolled reduction of ribose. Enzymatic synthesis of DNA from RNA by reverse transcriptase in RNA virus is well known today.

Origin of Code in First Cell:

Genetic code based on four bases expressed in triplets with redundancy for twenty amino acids is almost universal. Though there is no chemical relationship between the mRNA codon or anticodon of tRNA and the chemical structure of amino acid, but the speci-ficity of given tRNA to a particular

amino acid has developed. All these features minimize the risk of replication errors and rate of point mutations.

Formation of Chromosome:

Free floating RNA molecules once enclosed in a membrane would become adaptive to have genes linked together in a single chromosome. Different kinds of free-floating RNA molecules replicated inside their proto-cells undergo unequal distribution in daughter cells after division of proto-cell with reduced fitness.

This might be overcome by con-necting the RNA molecules into a single strand combined with simultaneous replication which results in equal distribution of genome between daughter cells.

Increasing Genetic Information:

Genome size gets increased with increasing complexity from a couple of genes in virus to 1000 in bacte-ria, 5000 in fruit-fly, and 30 000 in human or higher plants, but not associated with a drastic increase in the number of translatable genes.

The important mechanism of increasing genetic information is gene doubling followed by muta-tion and selection leading to production of new enzyme and biomolecules. Natural horizontal gene transfer as found in bacteria (transformation, conjugation, and transduction) could lead to increase in genetic information.

Phenotypic Expression of a Genotype:

Though genes are often correlated to certain phenotypic traits but genes only specify proteins/ enzymes. Variations of a gene (alleles) can have effects on the phenotype through variations in the specified protein. Actually the production of a given phenotype is the result of network of interactions between genes and enzymes and between different enzymes which is far too com-plex to be un-ravelled.

Origin of Cell Membrane in First Cell:

Spontaneous for-mation of molecular double layer on the water surfaces by lipids served as a model for the ori-gin of double layer phospholipid cell membrane. This is due to hydrophobic (mutually attracted) and hydrophilic (attracted to water) end of linear-molecules.

If the lipid films form spheres, the hydrophobic ends are hidden inside the film attaining lowest energy state. Phospho-lipids are easily formed in the presence of lipids, glycerol and phosphate and such spheres can be made experimentally through shaking and sonication.

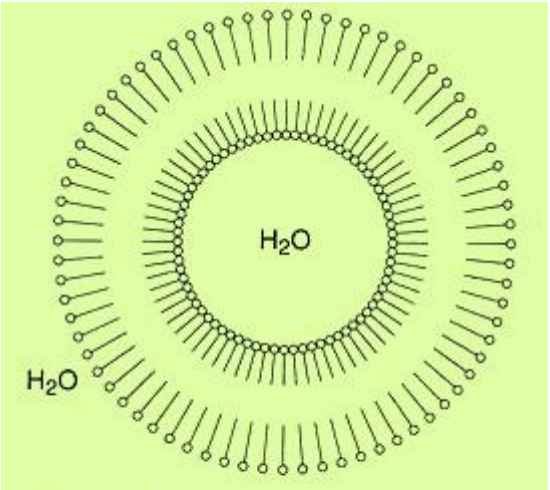


Fig. 2.11 : A spherical shell consisting of a double layer of lipid molecules. The hydrophilic ends (with circles) point outwards and towards the inside of the sphere while the hydrophobic ends are hidden inside the membrane (from Frenchel)

Constant addition of mass to the contents of the spheres and to the membrane results in budding and division of cells. Residence of most vital functions (energy metabolism, transport channels) of the cell in the cell membrane is based on a variety of embedded protein.

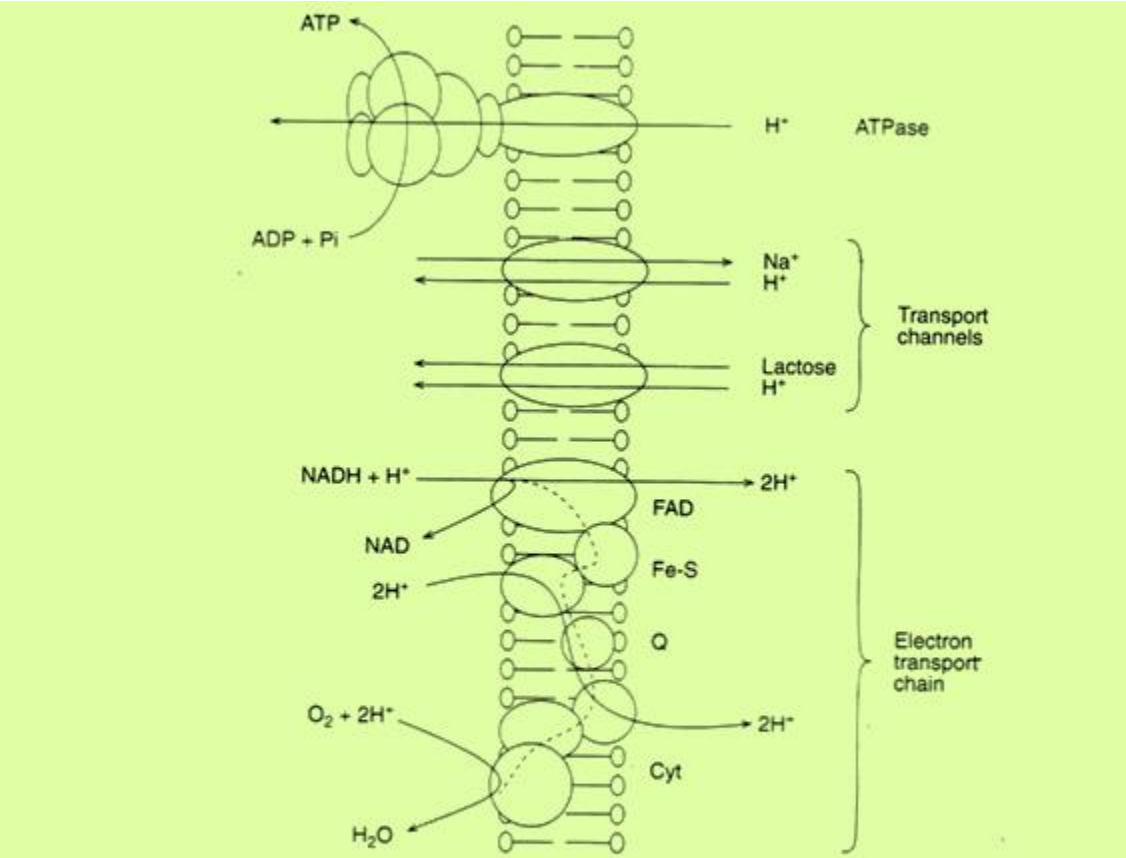


Fig. 2.12 :The inner cell membrane of a bacterium, the inside is to the left. Different proteins (including ATPase and transport channels) are embedded in the membrane. The electron transport chain functions as a proton pump that expels protons from the cell. The path of the electron is indicated by the stippled line. FAD, flavoprotein; Fe-S, iron-sulphur protein; Q, quinone; Cyt, cytochromes among which the last one, cytochrome c oxidase catalyses the reduction of O_2 to H_2O (From Frenchel)

Evolution of Metabolism in First Cell:

The fundamental types of energy metabolism are photo-trophy, respiration, fermentation, metanogenesis; all of which are represented among the bacteria. Dissimilatory energy metabolism (catabolic) refers to the mechanism to generate ATP with high energy rich phosphate bonds.

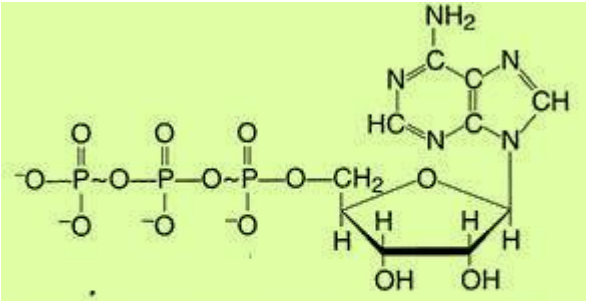


Fig. 2.13 :An adenosine triphosphate molecule (ATP). Energy is released through hydrolysis of the high energy phosphate bonds (indicated by ~)

Adenosine Triphosphate Molecule (ATP)

Assimilatory metabolism (anabolic) refers to metabolic processes that serve to build the com-ponents of the cell from chemical compounds of environment through phototrophic (photosynthe-sis), chemotrophic (chemosynthesis) or hetero-trophic modes. The process of energy meta-bolism are based on coupled redox processes of the type AH2 -1- B BH2 + A.

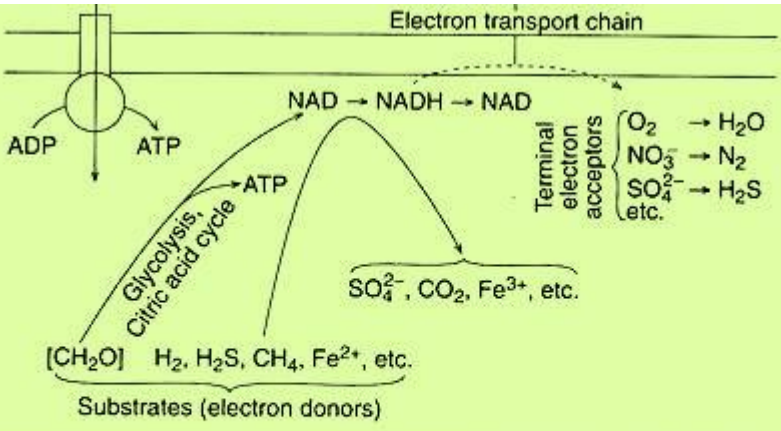


Fig. 2.14 :Respiration processes. Substrates reduce NAD to NADH that again is re-oxidized by the electron transport chain. The electron transport chain reduces an external electron acceptor and it functions as a proton pump. The return flux of the protons is coupled to ATP synthesis. When the substrate is carbohydrates these are first degraded by a complex series of processes (the glycolytic pathway and the citric acid cycle) into CO_2 and NADH

The important hydrogen carrier found in cell is NADVNADFH or its phosphorylated version NADP/NADPH.

Respiration Processes

Fermentation represented the most primitive form of energy metabolism whose biochem-istry is simple and does not require an external oxidant (electron acceptor) and independent of O_2. Well known fermentation processes include lactic acid fermentation, ethanol fermentation, butyric acid fermentation. Respiratory carbohy-drate metabolism is initiated by an anaerobic fer-mentation.

First membrane bound electron trans-port mechanism was based on simple functional molecules but without the protein component. The protein component developed later which improved efficiency and specificity.

Such naked molecules like quinine, metal containing por-phyrins, inorganic FeS common in anoxic prebi-otic earth, could have incorporated into primitive cell membrane that can be photo-activated and responsible for a primitive electron transport system or a kind of photochemical energy trans-duction.

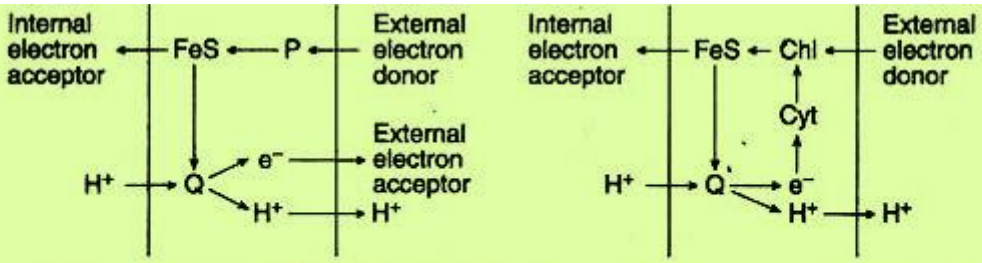


Fig. 2.15 :Theoretical models for a primitive (photic or respiratory) energy transduction apparatus built into the cell mem-brane. FeS, iron sulphide; P, a porphyrin molecule; Q, quinone; Chl, a protochlorophyll (a photo-excitable porphyrin); Cyt, cytochrome. Idea after B. K. Pierson and J. M. Olson (1989)

Photosensitive porphyrin has become protochlorophyll and cytochrome. Respiring organisms are derived from phototrophic one through secondary loss of chloro-phylls and dependent on external chemical reductants. The photosynthetic purple non- sulphur bacteria has electron transport system almost identical to that of mitochondria .

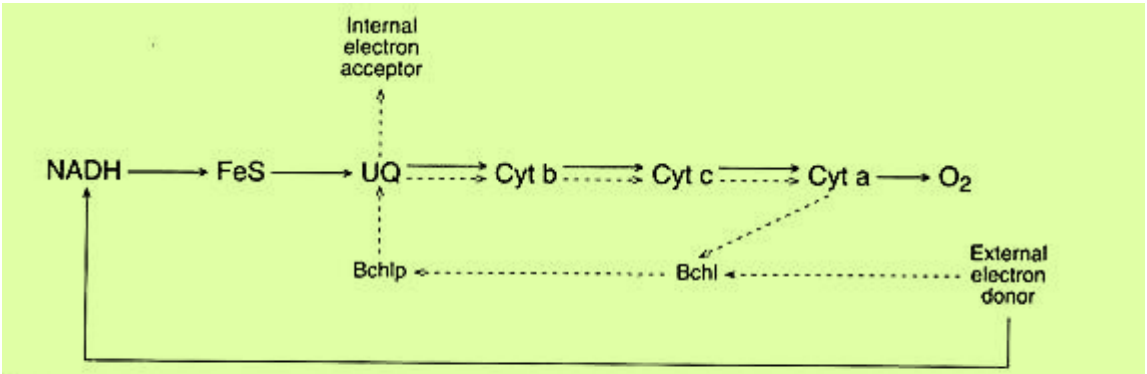


Fig. 2.16 :The photosynthetic and respiratory system in a purple non-sulphur bacterium. Stippled line, path of electrons in light (cyclic photophosphorylation, photosynthesis); uninterrupted line, in the dark (respiration), FeS, iron-sulphur protein; UQ, ubiquinone; Cyt, cytochromes; Bchl, bacteriochlorophyll a; Bchlp, bacteriophaeophytin. Loss of bacteriochlorophyll results in a non-photosynthetic, respiring organism

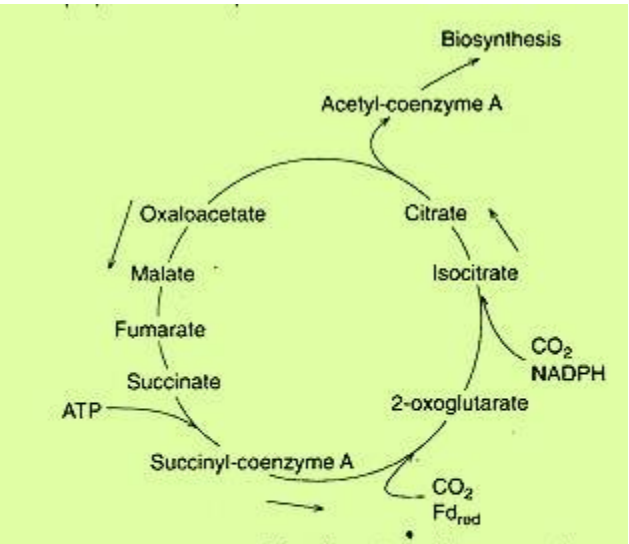


Fig. 2.18 :In green sulphur bacteria and in some archae-bacteria a reverse citric acid cycle is used for the assimilation of CO_2. It must be assumed that this was the original function of the citric acid cycle, that only secondarily took over the role as a dissimilatory and oxidative process for the degradation of organic matter

Photosynthetic and Respiratory System

Green Sulphur Bacteria

Mechanism to explain the origin of compli-cated biochemical processes involving many steps and cycles is the fact that these pathways are mostly reversible, catalysing the process in either direction. Assimilatory reduction of CO_2 with the help of $NADFH_2$ and energy (ATP) may run in a opposite way and become dissimilatory and oxidative pathway, degrading and oxidizing organic matter into CO_2 and release ATP through respiratory glycolytic pathway and citric acid cycle.

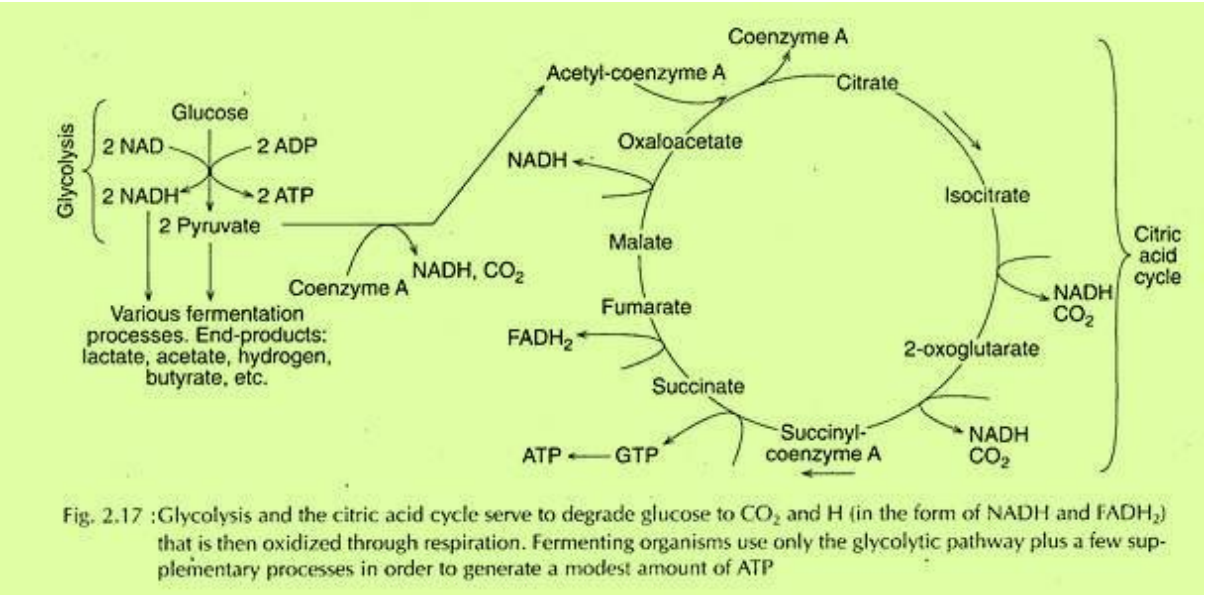


Fig. 2.17 :Glycolysis and the citric acid cycle serve to degrade glucose to CO_2 and H (in the form of NADH and $FADH_2$) that is then oxidized through respiration. Fermenting organisms use only the glycolytic pathway plus a few supplementary processes in order to generate a modest amount of ATP

Glycosis and the Citric Acid Cycle

CO_2 assimilated through Calvin cycle into organic matter undergoes oxidation through glycolytic pathway which is actually a reverse process of Calvin cycle. The origin of citric acid cycle can be traced by the fact that green sulphur bacteria assimilate CO_2 through a reverse citric acid cycle which is reductive and requires ATP.

Prokaryotic Cell:

From the above discussion it is crystal clear that the chemical evolution on prebiotic earth gave rise to organic molecules which included protein, nucleic acid, etc.; establishment of tem-plate system evolved enzyme systems and a sur-rounding lipid membrane; an energy transfer mechanism involving ATP has evolved.

This may have been the beginning of a stable structural and functional organisation having resemblance of a biological cell. These cells are called prokaryotic because of the absence of membrane bound nucleus and organelles.

Primitive prokaryotic cells were essentially anaerobic cells (anaerobic bacteria) because the early earth was devoid of oxygen. Depletion of organic compounds in the primaeval soup resulted in the appearance of photosynthetic cells (blue green algae) which can fix CO_2 and probably nitrogen also.

Photosynthetic cells were responsible for production of oxygen in atmosphere which resulted in the ori-gin of aerobic cells (aerobic bacteria) with metabolic pathways for aerobic respiration.

Origin of eukaryotic cells:

The eukaryotic cell seems to have evolved from a symbiotic community of prokaryotic cells. DNA-bearing organelles like the mitochondria and the chloroplasts are descended from ancient symbiotic oxygen-breathing proteobacteria and cyanobacteria, respectively, which were endosymbiosed by an ancestral archaean prokaryote.

There is still considerable debate about whether organelles like the hydrogenosome predated the origin of mitochondria, or vice versa: see the hydrogen hypothesis for the origin of eukaryotic cells.

Endosymbiotic Theory:

The more well documented and generally accepted theory for the origin of eukaryotic organelles is endosymbiotic theory. Recent evidences justify that organelles have originated from the endosymbiotic association of ingested aerobic and photosynthetic prokaryotes, the precursors of mitochondria and chloroplast respectively.

Molecular data have played an important role in supporting xenogenous origin (from outside of cell) rather than autogenous origin (from within the cell) of organelles. Recent phylogenetic analyses reveal that many eukary-otic organellar and nuclear genes whose prokaryotic ancestry can be pinned down are of bacterial origin.

Phylogenetic analyses reveal that many eukaryotic orgnallear and nuclear genes whose prokaryotic ancestry can be pinned down are of bacterial origin. In the case of endosymbiosis one type of cell (symbiont) entered into another type of cell (host) through phagocytosis.

The ingested cell under some circumstances could survive and repro-duce within cytoplasm of the host cell. The rela-tionship is stabilized by their mutual benefits of metabolic symbiosis and becomes obligatory.

Horizontal gene transfer from symbiont to host genome causes the loss of corresponding protein synthesizing ability of the symbiont and is likely to be selectively favoured. The development from symbiont to organelle is completed by the loss of its independent survival ability.

This idea is based on the fact that organelles like mitochondria and chloroplasts:

(i) Are replicators, i.e., can divide indepen-dently.

(ii) Carry genetic information, i.e., DNA.

(iii) With protein synthesizing machinery, i.e., ability of transcription and translation.

(iv) Have own ribosomes of prokaryotic type, i.e., 70S type.

The evidences supporting bacterial origin of mitochondria and chloroplasts are convincing.

a. Mitochondria and Chloroplasts contain their own DNA

(i) DNA simple, closed circular supercoiled dsDNA with single origin point.

(ii) DNA controls the synthesis of their rRNA 2 and tRNA, ribosomal proteins and certain proteins of respiratory chain (mitochon-dria) and similar genes for PSI, PSIl, cytochrome of complex, ATP synthase and ribulose bisphosphate carboxylase of choroplastids.

b. They contain their own ribosomes:

(i) 30S + 50S = 70S

{16S rRNA + 21 PP}{5S & 23S rRNA + 34 PP}

(ii) Shine-Dalgarno sequence on 16S rRNA.

c. Antibiotic specificity:

Ribosomes are sensi-tive to chloramphenicol (SOS), streptomycin and tetracycline (30S) like bacteria but eukaryotic ribosomes are insensitive to these antibiotics.

d. Molecular phylogeny:

16S rRNA and tRNA sequencing have shown that chloroplasts and mitochondria are evolutionarily related to bacteria.

Serial Endosymbiotic Theory:

Serial Endosymbiotic Theory, supported by Taylor 1974, Gray 1983, Doolittle and Daniels 1988, Margulis 1995, proposes the following steps of evolutionary origin of eukaryotic cell (Fig. 2.23).

SE I (Origin of Flagella):

A thermo acidophil, fermenting, Gram(-ve) bacterium merged with Spirochaete through phagocytosis to develop so- called undulipodium flagellated cells.

SE II (Origin of Nucleus):

The resulting pre- eukaryote went through secondary endosymbiosis by engulfing archaebacterium with membra-nous folds. The archaebacterium becomes nucle-us, losing cell membrane, while the membra-nous folds develop nuclear envelope and endo-plasmic reticulum. The genome of bacterium is transferred to the nucleus through membrane pores. Classical example of such eukaryote is *Giardia lamblia***.**

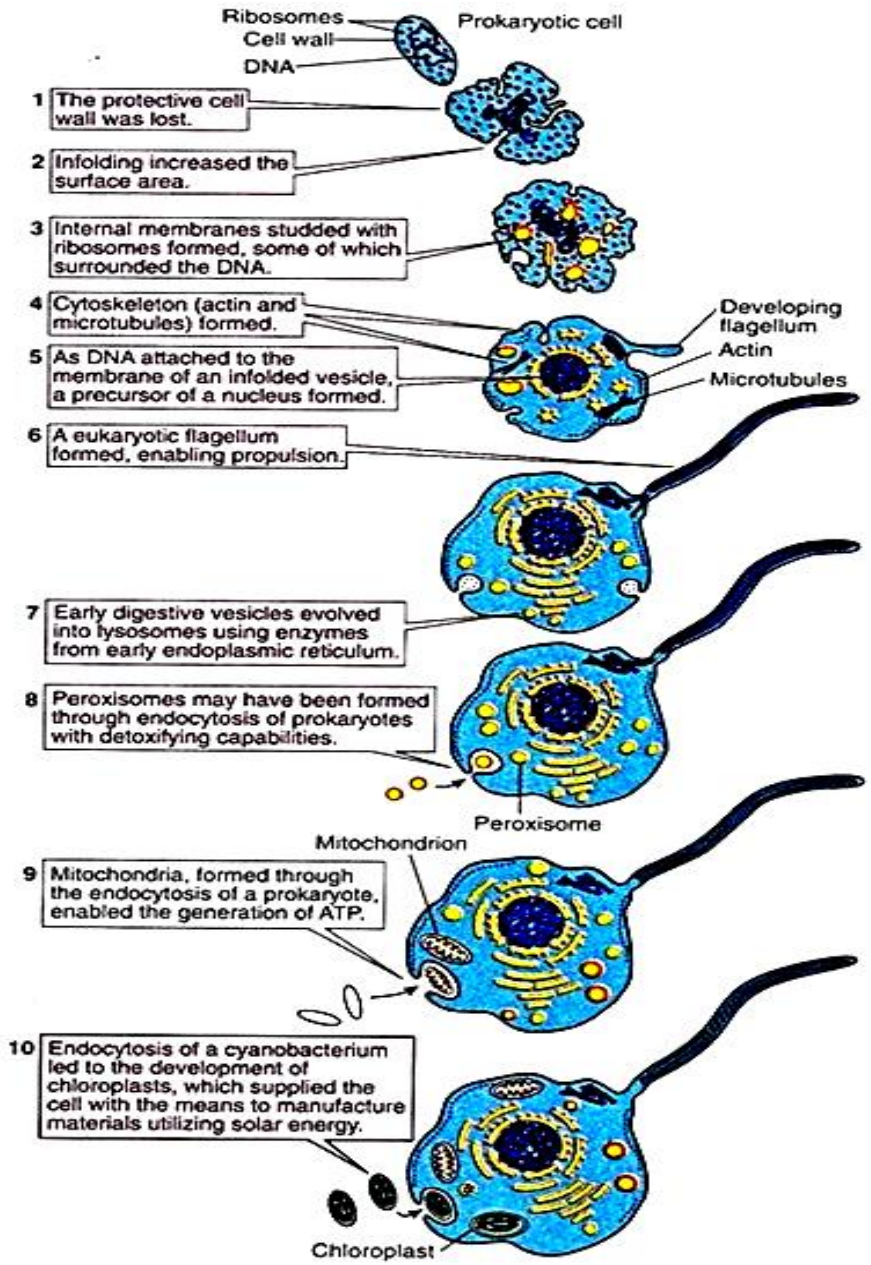


Fig. 2.23: Steps of evolutionary origin of eukaryotic cell
(http://bes.whfreeman.com/thelifewire/content/chp28/f28003.gif)

SE III (Origin of Mitochondria):

Mitochondria are surrounded by a double membrane repre-senting outer and inner membrane of bacteria. The inner membrane is invaginated forming tubular or discoid cristae. The biochemistry of energy metabolism in mitochondria is very much similar to that of purple non-sulphur bacteria.

The theory implies that the aerobic bacterium established itself as a symbiont within an anae-robic fermenting proto-eukaryote and lost the ability of photosynthesis and become mitochon-drion. Strombidium purpureum is an example, where mitochondrial rRNA sequence shows analogy to eubacterial rRNA.

The serial endosymbiotic theory postulated that the capture of an proteobacterial endosymbiont by a nucleus containing eukaryotic host resembling extant amitochondriate protists, results in the origin of mitochondria.

Giardiai like anaerobic primitive eukaryotes by engulfment of an aerobic Gram(-ve) eubacterium like Paracoccus denitrificans resulted pro-tista (unicellular eukaryote) with mitochondria; classical example is Pelomyxa palustris.

SE IV (Origin of Chloroplast):

Chloroplasts in mitochondria containing eukaryotic cell evolved by association of photosynthetic endosymbionts like photosynthetic bacteria or cyanobacteria (Mereschowsky). Plastid genes are strikingly similar to cyanobacteria in sequence organization and mode ‘of expression. Phylo-genetic analysis of rRNA and tufA sequences indicates cyanobacterial origin of all plastids.

Anaerobic Ciliate Strombidium Purpureum Harbours Symbiotic Purple Non-Surlphur Bacteria

A well-studied example of endosymbiotic cyanobacteria (cyanelles) is Cyanophora paradoxum. In cryptomonad flagellates and dinoflagellates chloroplasts represent a second generation endosymbiont. This type of secondary/tertiary endosymbiosis results in several sets of membranes around the chloroplast in which the outermost membrane represents the cell mem-brane of the latest endosymbiont.

Level of Endosymbiosis

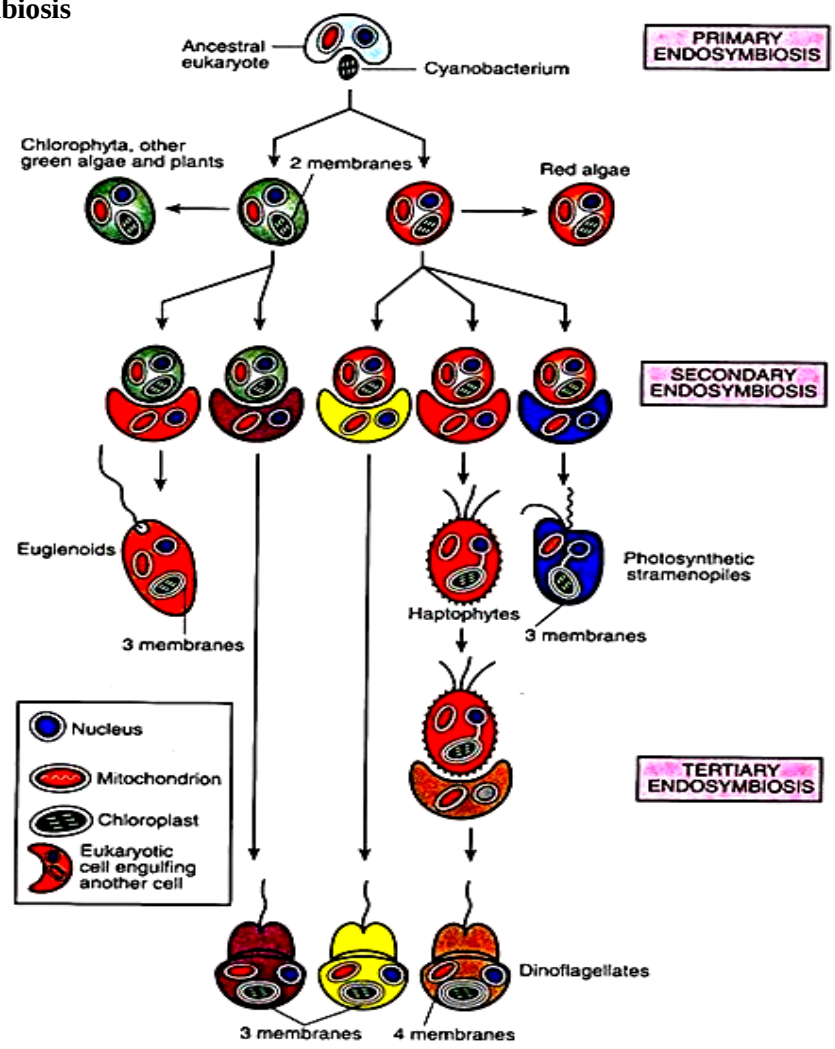


Fig. 2.25: Level of Endosymbiosis

Origin of Peroxisomes:

Peroxisomes may have been formed through endocytosis of prokaryotes with detoxifying capabilities.

Origin of CERL system:

Lysosomes are developed from invaginated vesicles with enzymes. Further extension of invaginations into the cytoplasm formed tubular network to form Golgi bodies and endoplasmic reticulum.

5. Evolutionary timescale & diversification of plant life: The evolutionary time scale; eras, periods and epoch; major events in the evolutionary time scale; origins of unicellular and multicellular organisms; major groups of plants.

Geologic time scale:

The **geologic time scale** (GTS) is a system of chronological dating that relates geological strata (stratigraphy) to time. It is used by geologists, paleontologists, and other Earth scientists to describe the timing and relationships of events that have occurred during Earth's history. The table of geologic time spans, presented here, agree with the nomenclature, dates and standard color codes set forth by the International Commission on Stratigraphy (ICS).

The geological history of Earth follows the major events in Earth's past based on the geologic time scale, a system of chronological measurement based on the study of the planet's rock layers (stratigraphy). Earth formed about 4.54 billion years ago by accretion from the solar nebula, a disk-shaped mass of dust and gas left over from the formation of the Sun, which also created the rest of the Solar System.

Why is it necessary?

- for establishing the history of evolutionary events
- for determining the rates of evolutionary processes

The earliest era is Precambrian, which began about 4600 million years ago. It has few fossils. Abundant fossils have been reported in the succeeding eras viz. Palaeozoic, Mesozoic and Cenozoic.

Eons:

Geologic time is divided into four large segments called Eons:

Hadean, Archean, Proterozoic and Phanerozoic

Eon is divided into four Eras

Geological Eras:

A geological era is a very long division of geological time, lasting tens of millions of years. Its beginning and end are recognized by major changes in layers of rocks and fossils in the earth. As mentioned above, the earliest era is Precambrian It began about 4600 millions of years ago. Its duration has been 4030 millions of years.

Palaeozoic era began about 570 millions of years ago. Mesozoic era began about 225 millions of years ago. Cenozoic is the most recent era. It began about 65 million years ago, after the Mesozoic era, and it is still continuing.

Eras are divided into periods and epochs.

Geological Periods:

A geological era is divided further into several subdivisions called periods. So a period is a major subdivision of a geological era. Major periods of Palaeozoic era are Permian, Carboniferous, Devonian, Silurian, Ordovician and Cambrian. Mesozoic era is divided into three periods viz. Cretaceous, Jurassic and Triassic.

Similarly Cenozoic era is divided into two periods viz. Quarternary and Tertiary. The oldest known period of geological time scale is Cambrian (began about 570 million years ago) while the most recent period is Quaternary (began about 2.5 million years ago).

The divisions among Eras reflect major changes in the fossil records, including the extinction and appearance of new life forms. Three major Era boundaries (two of which are defined by mass extinctions in the animal record and the 3rd by the rapid expansion of multicellular animals):

Mesozoic-Cenozoic (c. 65 Ma, dinosaur died out along with 75% of marine invertebrates)

Palaeozoic-Mesozoic (c. 248 Ma, extinction of up to 96% of marine species)

Precambrian-Palaeozoic (c. 543 Ma, major increase in numbers of multicellular animals with hard parts soon after the boundary-Cambrian Explosion)

Periods are divided into epochs.

Geological Epochs:

A subdivision of a geological period is called a geological epoch. Recent, Pleistocene, Pliocene, Miocene, Oligocene, Eocene and Paleocene are the epochs of Periods Quaternary and Tertiary of Cenozoic era.

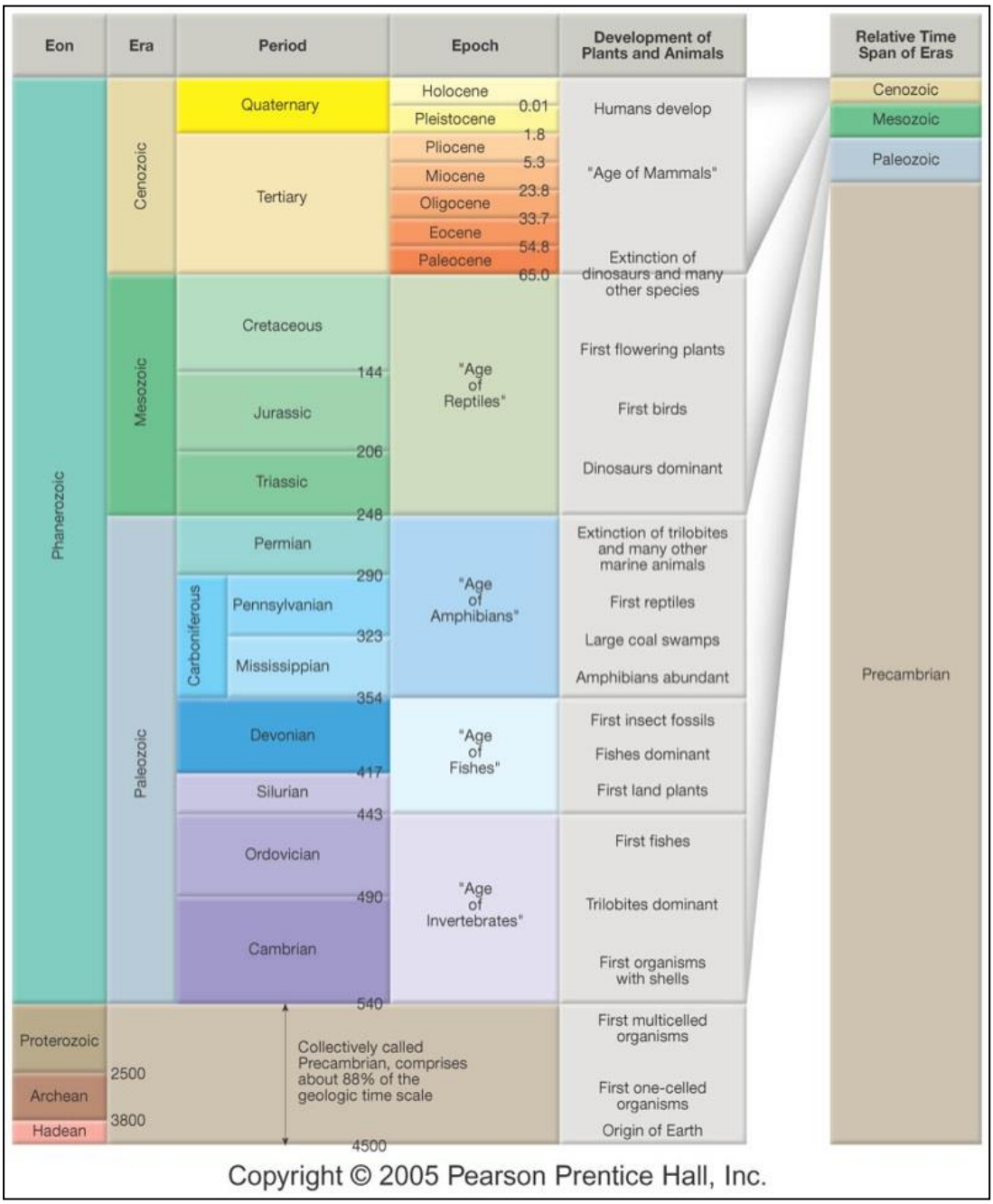
Geologists and earth scientists have used the relationship between layers and types of rocks, presence of plant and animal fossils, and radioactive dating to assemble a sequence of evolutionary events that have occurred over geologic time.

OUTLINE OF GEOLOGICAL TIME SCALE

Eon	Era	Sub-Era	Period	Epoch	Age (my)
Phanerozoic	Cenozoic	Quaternary		Holocene	
				Pleistocene	1.8
		Tertiary	Neogene	Pliocene	5.3
				Miocene	23.8
			Palaeogene	Oligocene	33.7
				Eocene	54.8
				Palaeocene	65
	Mesozoic		Cretaceous	late	
				early	144
			Jurassic	late	
				middle	
				early	206
			Triassic	late	
				middle	
				early	248

Eon	Era	Sub-Era	Period		Epoch	Age (my)
Phanerozoic	Palaeozoic		Permian		late	
					early	290
			Carboniferous	Pennsylvanian	late	323
				Mississippian	early	354
			Devonian		late	
					middle	
					early	417
			Silurian		late	
					early	443
			Ordovician		late	
					early	490
			Cambrian		late	
					middle	
					early	543
Proterozoic	Precambrian					2500-543
Archaeozoic						3900-2500
Hadean						4600-3900

Standard Geological time scale



Geological Column and Time Scale with major geological events

ERA	PERIOD	EPOCH	CHARACTERISTIC ORGANISM AND SOME MAJOR GEOLOGICAL EVENTS	DURATION (M.Y.)	BEGAN (M.Y.) ago
Cenozoic	Quaternary	Recent		Last 5000 yrs	
		Pleistocene	i. Several advances and retreat of continental ice sheets, accompanied by some elevation of continents and drainage of major synclines. ii. Redistribution of floras reflecting advances and retreats of glaciers. iii. Woolly mammoth and giant bison. iv. **Appearance of modern man**.	2.5	2.5
	Tertiary	Pliocene	i. Elevation of the Andes and general continued uplift of continents bringing about climatic changes in temperate latitudes that allowed spread of grassland caused local restriction or extinction of some species. ii. Mastodons, camels, horses and cats.	4.5	7
		Miocene	i. Marked worldwide continental uplifts and a major orogeny resulting in rise of the Alps. ii. Climatic cooling and restriction of broad leafed evergreens to lower latitudes. iii. Establishment of present day forest association. iv. **Rise and rapid evolution of grazing mammals and apelike creatures**.	19	26

		Oligocene	i. Mild temperate climate and widespread occurrence of now relic taxa (*Metasequoia*, *Cercidiphyllum*) in higher latitudes. ii. Dryer climates in southern-western North America. iii. Saber toothed cats, true cats, dogs, rodents and great rhinoceroses.	12	38
		Eocene	i. Sub tropical climate with heavy rainfall supported distinctive forests in northern and southern latitudes. ii. Grasslands limited. iii. Many genera of angiosperms became extinct with new, more modern types appearing. iv. All modern orders of mammals present. v. **First horses**.	16	54
		Palaeocene	i. Trend from temperate climate to mild sub tropical. ii. Some seasonal variations. iii. Continued inundation of embayments and synclines. iv. Angiosperms having affinities with Magnoliaceae, Lauraceae, Juglandaceae. v. Floras having more in common with Upper Cretaceous than modern ones. vi. **First lemurs**. vii. Some modern groups of birds.	11	65
Mesozoic	Cretaceous	Upper	i. Climate tending to be uniform, temperate because of	76	141

		Lower	extensive inundation of continents. ii. Elevation of rocky mountains. iii. Angiosperms rise to dominance in Upper Cretaceous. iv. **First recognition of angiosperm pollen grains, leaves and flowers in Lower Cretaceous**. v. Monocots and dicots present. vi. Modern groups of insects. vii. **First pouched and placental mammals** viii. **Extinction of giant land and marine reptiles**.		
	Jurassic	Upper Middle Lower	i. Uniform, mild climates from north to south poles. ii. Highly similar plant communities composed of ginkgos, conifers, ferns, cycads, and cycadeoids. iii. Some pteridosperms. iv. Rise of higher insects and birds. v. Dinosaurs abundant.	54	195
	Triassic	Upper Middle Lower	i. Arid to semi arid, savanna type climate. ii. Rise of cycadophytes, Ginkgoales. iii. Diversification of conifers and ferns. iv. Decline of Glossopterids. v. **First mammals, rise of the dinosaurs**.	30	225
Palaeozoic	Permian	Upper	i. Uplift in Appalachian geosynclines.	55	280

			Lower	ii. Cooler, mild, drier climate with extensive glaciations in Southern Hemisphere. iii. Rise of Northern Hemisphere. iv. Voltziales and diversification of Southern Hemisphere glossopterids. v. Extinction of arborescentlycopsids and sphenopsids. vi. Diversification of reptiles.		
	Carboniferous	Pennsylvanian	Upper Middle Lower	i. Uniformly warm, humid climate. ii. Further intrusion of epicontinental seas associated with the formation of the great Carboniferous swamps. iii. Mosses, lycopods, sphenopsids, ferns, seed ferns and *Cordaites*. iv. **Origin of conifers**. v. **Origin of reptiles**, diversification of amphibians, abundance of insects.	45	325
		Mississippian	Upper Lower	i. Warm, equable climate. ii. Continued inundation of low-lying continents and synclines. iii. Primitive ferns, seed ferns, arborescentlycopods and calamites associated with extensive swamps in low lands. iv. Spread of amphibians, sharks and bony fishes. v. **Insects evolved wings**.	20	345
	Devonian		Upper Middle	i. Heavy rainfall and aridity ii. Extensive inundation of continents to form seas in areas	50	395

		Lower	of major synclines. iii. Diversification of vascular plants. iv. All major groups of plant except flowering plants present by end of Devonian. v. **Heterospory and the seed habit in early seed ferns**. vi. Liverworts, fungi. vii. Diversification of fishes. viii. **Origin of amphibians**.		
	Silurian	Upper Lower	i. Mild climate. ii. Low lying continents with some epicontinental flooding. iii. **First vascular plants in mid Silurian (probably among the first land plants).** iv. Scorpions and millipedes, **first air breathing animals**. v. Brachiopods corals and eurypterids.	40	435
	Ordovician	Upper Lower	i. Warm, mild climate. ii. Warm, epicontinental seas with an abundance of green and red algae. iii. **First vertebrates and a great variety of marine invertebrates**. iv. Graptolites, nautiloids, cystoids in maximum abundance.	65	500
	Cambrian	Upper Middle	i. Climate warm and equable. ii. Warm and epicontinental seas supporting an abundance of	70	570

		Lower	cyanophytes, green and red algae. iii. Abundance of marine invertebrates, first trilobites and foraminifera.		
Precambrian into 3 Eons Proterozoic Archaeozoic Hadean			i. Climates were warm enough to support the growth **cyanophytes, red algae and bacteria and possibly green algae.** ii. Questionable invertebrate fossils	4,130	4,700

The evolutionary time scale; eras, periods and epoch; major events in the evolutionary time scale

Era	Period	Epoch	Millions of years from start to present	Evolutionary Events in				Major event
				Biosphere		Lithosphere	Atmosphere	
				Plant	Animal			
Cenozoic	Quaternary (Q)	Recent	0.01		Extinction of large mammal and birds; **evolution of *Homo erectus* to *Homo sapiens***	Continents in modern position	Repeated glaciations and lowering of sea level;	Rise of agriculture and civilizations
		Pleistocene	1.8	Redistribution of floras reflecting advances and retreats of glaciers	Woolly mammoth and giant bison; Appearance of modern man	Some elevation of continents and drainage of major synclines	Several advances and retreat of continental ice sheets, accompanied by	
	Tertiary (T)	Pliocene	5.3	Radiation of angiosperms; spread of grassland caused local restriction or extinction of some species	Radiation of mammals (mastodons, camels, horses and cats), birds, snakes, pollinating insects, teleost fishes	Continents nearing modern positions; elevation of the Andes and general continued uplift of continents	Increasingly cool, dry climate	

Era	Period	Epoch	Millions of years from start to present	Evolutionary Events in				Major event
				Biosphere		Lithosphere	Atmosphere	
				Plant	Animal			
		Miocene	23.0	Restriction of broad leafed evergreens to lower latitudes; establishment of present day forest association	Rise and rapid evolution of grazing mammals and ape like creatures	Marked worldwide continental uplifts and a major orogeny resulting in rise of the Alps	Climatic cooling	
		Oligocene	33.9	Widespread occurrence of now relic taxa (*Metasequoia*, *Cercidiphyllum*) in higher latitudes	Saber toothed cats, true cats, dogs, rodents and great rhinoceroses		Mild temperate climate; dryer climates in southern-western North America	
		Eocene	55.8	Grasslands limited; many genera of angiosperms became extinct with new, more modern types appearing; distinctive forests in northern	All modern orders of mammals present; **First horses**		Sub tropical climate with heavy rainfall	

Era	Period	Epoch	Millions of years from start to present	Evolutionary Events in				Major event
				Biosphere		Lithosphere	Atmosphere	
				Plant	Animal			
				and southern latitudes				
		Palaeocene	65.5	Floras having more in common with Upper Cretaceous than modern ones; Angiosperms having affinities with Magnoliaceae, Lauraceae, Juglandaceae	**First lemurs**; Some modern groups of birds	Continued inundation of embayments and synclines	Trend from temperate climate to mild sub tropical; some seasonal variations	
Mesozoic	Cretaceous (K)		145	Angiosperms rise to dominance in Upper Cretaceous; **first recognition of angiosperm pollen, leaves and flowers in Lower Cretaceous**; increasing diversity of angiosperms-monocots and dicots present	Continued radiation of dinosaurs at the beginning but extinction marine reptiles at end of period; increasing diversity of mammals, birds; modern groups of insects; **first pouched and placental mammals**	Most continents separated; elevation of rocky mountains	Climate tending to be uniform, temperate because of extensive inundation of continents	Mass extinction at end of period, including last ammonoids and dinosaurs

Era	Period	Epoch	Millions of years from start to present	Evolutionary Events in				Major event
				Biosphere		Lithosphere	Atmosphere	
				Plant	Animal			
	Jurassic (J)		200	Gymnosperms dominant- highly similar plant communities composed of ginkgos, conifers, ferns, cycads, and cycadeoids, some pteridosperms	Diverse dinosaurs and other reptiles; **first birds and higher insects**; archaic mammals; ammonoid radiation	Continents separating	Uniform, mild climates from north to south poles	Mesozoic marine revolution
	Triassic (Tr)		251	Gymnosperms become dominant- diversification of conifers and ferns; **Rise of cycadophytes, Ginkgoales;** decline of Glossopterids	Diversification of reptiles, including **first dinosaurs; first mammals**	Continents begin to separate	Arid to semi arid, savanna type climate	Marine diversity increases;
Palaeozoic	Permian (P)		299	Voltziales and diversification of Southern Hemisphere glossopterids; **extinction of**	Increasing advanced fishes; diverse orders of insects; amphibians decline; diversification of	Continents aggregated into Pangaea; uplift in Appalachian geosynclines; rise of Northern Hemisphere	Cooler, mild, drier climate with extensive glaciations	Major mass extinctions, especially of marine life, at end of period

Era	Period	Epoch	Millions of years from start to present	Evolutionary Events in				Major event
				Biosphere		Lithosphere	Atmosphere	
				Plant	Animal			
				arborescentlycopsids and sphenopsids	reptiles, and mammal like forms,		in Southern Hemisphere; low sea level;	
	Carboniferous (C)		359	Extensive forests of early vascular plants, especially mosses, lycopods, sphenopsids, ferns, seed ferns and *Cordaites;* **Origin of conifers**	**First reptiles**, diversification of amphibians, sharks; abundance of insects; early orders of winged insects	Gondwana and small northern continents form; further intrusion of epicontinental seas associated with the formation of the great Carboniferous swamps; continued inundation of low-lying continents and synclines	Uniformly warm, humid, equable climate	
	Devonian (D)		416	**Origin of ferns, seed plants-**heterospory and the seed habit in early seed ferns;diversification of early land vascular plants, liverworts,	Diversification of bony fishes; trilobite diverse; **origin of ammonoids, amphibians, insects**	Extensive inundation of continents to form seas in areas of major synclines	Arid climate	Mass extinction late in period

Era	Period	Epoch	Millions of years from start to present	Evolutionary Events in				Major event
				Biosphere		Lithosphere	Atmosphere	
				Plant	Animal			
				fungi				
	Silurian (S)		444	Earliest terrestrial vascular plants in mid Silurian	Diversification of agnathans; origin of jawed fishes (acanthodians, placoderms, osteichthyes); brachiopods corals and eurypterids; arthropods scorpions and millipedes, first air breathing animals	Low lying continents with some epicontinentalflooding	Mild climate	
	Ordovician (O)		488	Abundance of green and red algae	Diversification of echinoderms, other invertebrate phyla; agnathan vertebrates; graptolites, nautiloids, cystoids in maximum abundance	Warm, epicontinental seas	Warm, mild climate	Mass extinction at end of period
	Cambrian Є		542	Diverse algae-abundance of	Marine animal diversify; first	Warm and epicontinental seas	Climate warm and	

Era	Period	Epoch	Millions of years from start to present	Evolutionary Events in				Major event
				Biosphere		Lithosphere	Atmosphere	
				Plant	Animal			
				cyanophytes, green and red algae	appearance of most animal phyla and many classes within relatively short interval; **earliest agnathan vertebrates**; abundance of marine invertebrates, **first trilobites and foraminifera**		equable	
Proterozoic			2500		Trace fossil of animals (ca. 1000 Mya); multicellular animals from ca. 640 Mya, including possible Cnidaria, AnnelidamArthropoda			Earliest eukaryotes (ca. 1900-1700 Mya); origin of eukaryotic kingdoms
Archaean				Growth of cyanophytes, red algae and bacteria and possibly green algae			Warm climate	Origin of life in remote past; first fossil evidence at ca. 3500 Mya;

Era	Period	Epoch	Millions of years from start to present	Evolutionary Events in				Major event
				Biosphere		Lithosphere	Atmosphere	
				Plant	Animal			
								diversification of prokaryotes (bacteria); photosynthesis generates oxygen, replacing earlier oxygen poor atmosphere; evolution of aerobic respiration

6. Genetic variations: Origin of genetic variation; Mendelian genetics; polygenic traits, linkage and recombination; epistasis, gene - environment interaction; heritability; population genetics; molecular evolution; molecular clocks.

Genetic variation:

Genetic variation can be defined as the genetic makeup of organisms within a population change. Genes are inherited segments of DNA that contain codes for the production of proteins. Genes exists in alternate versions, or alleles that determine distinct traits that can be passed on from parents to offspring.

Among individuals within a population

Genetic variation can be identified at many levels. It is possible to identify genetic variation from observations of phenotypic variation in either quantitative traits (traits that vary continuously and are coded for by many genes (e.g., leg length in dogs)) or discrete traits (traits that fall into discrete categories and are coded for by one or a few genes (e.g., white, pink, red petal color in certain flowers).

Genetic variation can also be identified by examining variation at the level of enzymes using the process of protein electrophoresis. Polymorphic genes have more than one allele at each locus. Half of the genes that code for enzymes in insects and plants may be polymorphic, whereas polymorphisms are less common among vertebrates.

Ultimately, genetic variation is caused by variation in the order of bases in the nucleotides in genes. New technology now allows scientists to directly sequence DNA which has identified even more genetic variation than was previously detected by protein electrophoresis. Examination of DNA has shown genetic variation in both coding regions and in the non-coding intron region of genes.

Genetic variation will result in phenotypic variation if variation in the order of nucleotides in the DNA sequence results in a difference in the order of amino acids in proteins coded by that DNA sequence, and if the resultant differences in amino acid sequence influence the shape, and thus the function of the enzyme.

Between populations

Geographic variation means genetic differences in populations from different locations. This is caused by natural selection or genetic drift.

Origin of genetic variation:

Genetic variation occurs mainly through DNA mutation, gene flow (movement of genes from one population to another), and sexual reproduction. Due to the fact that environments are unstable, populations that are genetically variable will be able to adapt to changing situations better than those that do not contain genetic variation.

DNA Mutation: A mutation is a change in the DNA sequence. These variations in gene sequences can sometimes be advantageous to an organism. Most mutations that result in genetic variation produce traits that confer neither an advantage or disadvantage. Mutations lead to genetic variation by altering genes and alleles in a population. They may impact an individual gene or an entire chromosome. Although mutations change an organism's genotype (genetic makeup), they may not necessarily change an organism's phenotype.

Gene Flow: Also called gene migration, gene flow introduces new genes into a population as organisms migrate into a new environment. New gene combinations are made possible by the availability of new alleles in the gene pool. Gene frequencies may also be altered by emigration of

organisms out of a population. Immigration of new organisms into a population may help organisms better adapt to changing environmental conditions. Migration of organisms out of a population could result in a lack of genetic diversity.

Sexual Reproduction: Sexual reproduction promotes genetic variation by producing different gene combinations. Meiosis is the process by which sex cells or gametes are created. Genetic variation occurs as alleles in gametes are separated and randomly united upon fertilization. The genetic recombination of genes also occurs during crossing over or the swapping of gene segments in homologous chromosomes during meiosis.

Crossing over (genetic recombination) and random segregation during meiosis can result in the production of new alleles or new combinations of alleles. Furthermore, random fertilization also contributes to variation.

Variation and recombination can be facilitated by transposable genetic elements, endogenous retroviruses, LINEs, SINEs, etc.

For a given genome of a multicellular organism, genetic variation may be acquired in somatic cells or inherited through the germline.

Mendelian genetics:

Mendelian inheritance/ genetics is a type of biological inheritance that follows the laws originally proposed by Gregor Mendel in 1865 and 1866 and re-discovered in 1900. These laws were initially controversial. When Mendel's theories were integrated with the Boveri–Sutton chromosome theory of inheritance by Thomas Hunt Morgan in 1915, they became the core of classical genetics. Ronald Fisher combined these ideas with the theory of natural selection in his 1930 book The Genetical Theory of Natural Selection, putting evolution onto a mathematical footing and forming the basis for population genetics within the modern evolutionary synthesis.

Gregor Johann Mendel (1822-1884) is called as Father of Genetics. He was born in 1822 to a family of poor fathers an area that is now part of Czech Republic.

His father had a great love for plants and this influenced Mendel a lot. As he grew older, he became interested in plant hybridization.

Mendel conducted his historic experiments with garden pea (*Pisumsativum*) in the monastery garden for about nine years (1856-1864) and published his results in a less known journal-The Annual proceedings of the Natural History Society of Brunn in 1865.

Unfortunately, his out-standing contributions failed to attract the attention of the scientific world of that time and remained unknown till 1900 when his papers were rediscovered by three different scientists from three different countries of Europe, while working independently on heredity in plants. They were **Hugo Devries** of Netherlands, **Karl Correns** from Germany and **Eric Von Tshermak** of Austria.

Reasons for Mendel's Success:

1. Mendel studied the inheritance of one character at a time unlike his predecessors who considered the organism as a whole.
2. Lie carried out his experiments upto F2 and F3 generations.
3. His knowledge in statistics helped him to maintain accurate records of all the experiments of findings and analysed them carefully.
4 He grew pure lines in separate plots and conducted experiments by crossing two plants from pure strains.
5. The parent plants undergoing crossing belonged to pure lines and had sharply visible contrasting characters.
6. He selected genetically pure plants for his experiments by performing a series of sell crossing tests.
7 All this genetical experiments were conducted with utmost care and meticulous planning.

Mendelian Laws of Heredity:

1. Principle of Unit Characters:

Mendel assumed that the unit of hereditary characters is the factors of determiners which occur in pairs. One of each comes from the mother while the other comes from the father. The unit character or factor is now called as gene.

2. Law of Dominance:

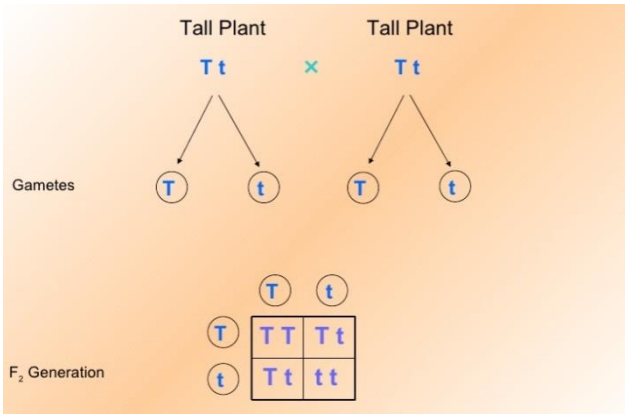
When a pair of contrasting characters (or allelomorphic characters or alleles) are present together, only one of them expresses itself and the other remains suppressed of hidden The character which is expressed (or is visible) is called as dominant and the character which remains hidden is termed as recessive.

3. Law of Segregation or Purity of Gametes:

The allelic factors or genes present together in the hybrids segregate (separate) from one another and are placed in different gametes in the next generation.

4. Law of Independent Assortment:

When two or more pairs of contrasting characters are taken into consideration in a cross, each factor, assort or place itself independently of the other (lining its passages from on; generation to the other.



Monohybrid cross:

In a monohybrid cross the two parents differ through a single character. Mendel took a tall pea plant and crossed with a dwarf plant. Phenotypic ratio (3 tall: 1 dwarf) and

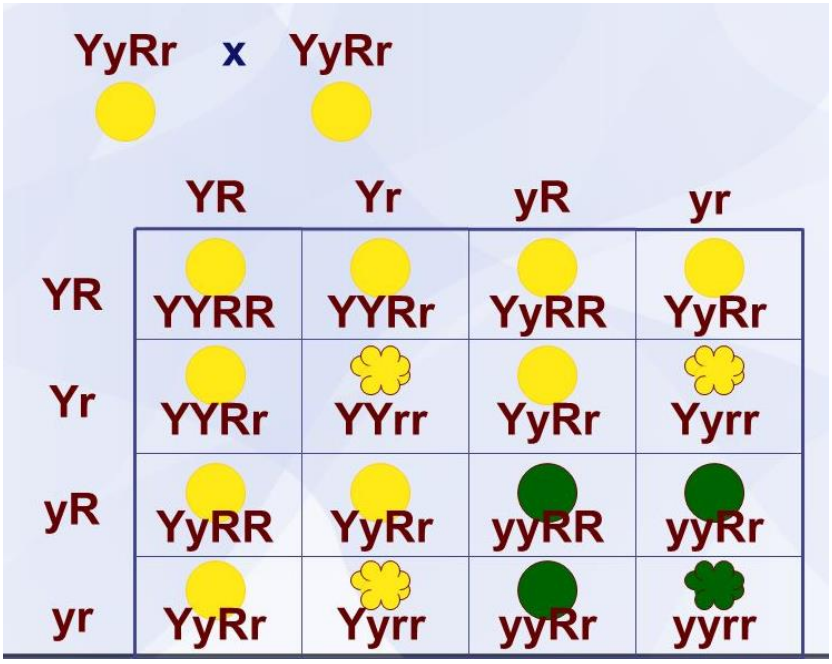
Genotypie ratio (1 pure tall: 2 hybrid tall: 1).

Dihybrid cross:

In a di-hybrid cross the parents differ through two characters. Mendel conducted a cross between a true breeding Round Yellow plants (RRYY) with true breeding Wrinkled Green plant (rryy). Round and Wrinkled are the shapes of seed coat whereas Yellow and Green are the colours of the seed coat.

Phenotypic ratio (9:3:3:1)

Genotypie ratio (1:2:2:4:2:1:2:1:1)



Back Cross & Test Cross:

Crossing of F_1 organism with either of the parents is called back cross. When an organism is crossed with other organism having recessive phenotypic trait (recessive homozygous genotype) is called test cross. This is called test cross because it helps to test the genotype of an organism. In monohybrid cross, tall pea plant of F_2 may be homozygous (TT) or heterozygous (Tt). Test cross results confirm it.

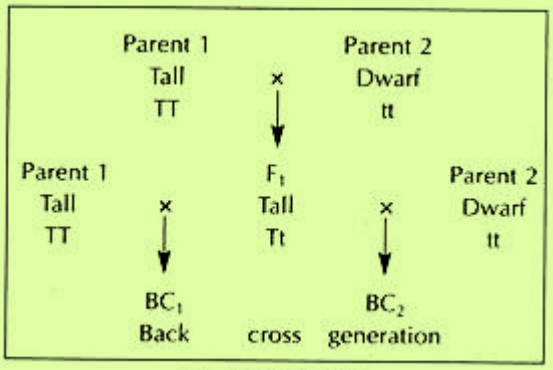


Fig. 6.6: Back cross

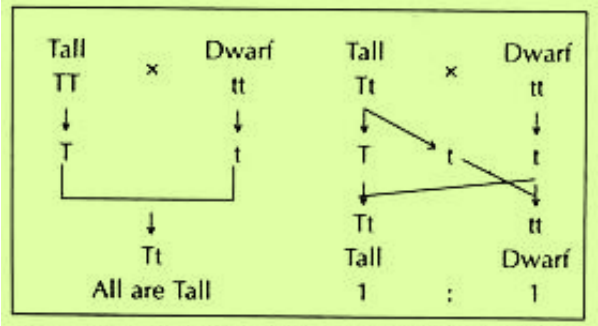


Fig. 6.7: Test cross to confirm the genotype of tall plant.

Polygenic traits:

Character or trait refers to any property of an individual showing heritable variation. It includes morphological, physiological, biochemical and behavioural properties. Some characters are governed by one or few genes. Such traits are referred to as qualitative characters or oligogenic characters.

On the other hand, some characters are controlled by several genes. They are known as quantitative characters or polygenic characters. The mode of inheritance of polygenic characters is termed as polygenic inheritance or quantitative inheritance. Since in polygenic inheritance several genes (factors) are involved, it is also known as multiple factor inheritance.

Features of Polygenic Traits:

The term polygene was introduced by Mather in 1941. This term has found wide usage in quantitative genetics replacing the older term multiple gene.

Main features of polygenic characters are briefly presented below:

1. Each polygenic character is controlled by several independent genes and each gene has cumulative effect.
2. Polygenic characters exhibit continuous variation rather than a discontinuous variation. Hence, they cannot be classified into clear-cut groups.
3. Effect of individual gene is not easily detectable in case of polygenic characters and, therefore, such traits are also known as minor gene characters.
4. The statistical analysis of polygenic variation is based on means, variances and co-variances, whereas the discontinuous variation is analysed with the help of frequencies and ratios. Thus, polygenic characters are studied in quantitative genetics and oligogenic characters in mendelian genetics.
5. Polygenic traits are highly sensitive to environmental changes, whereas oligogenic characters are little influenced by environmental variation.
6. Classification of polygenic characters into different clear-cut groups is not possible because of continuous variation from one extreme to the other. In case of qualitative characters, such grouping is possible because of discrete or discontinuous variation.
7. Generally the expression of polygenic characters is governed by additive gene action, but now cases are known where polygenic characters are governed by dominance and epistatic gene action. In

case of oligogenic characters, the gene action is primarily of non-additive type (dominance and epistasis).

8. In case of polygenic characters, metric measurements like size, weight, duration, strength, etc. are possible, whereas in case of oligogenic characters only the counting of plants with regard to various kinds like colour and shape is possible. Thus, metric measurement is not possible in case of oligogenic characters.

9. Transgressive segregants are only possible from the crosses between two parents with mean values for a polygenic character. Such segregants are not possible in case of qualitative or oligogenic traits.

10. The transmission of polygenic characters is generally low because of high amount of environmental variation. On the other hand, oligogenic characters exhibit high transmission because there is little difference between the genotype and phenotype of such character. Thus, polygenic characters differ from oligogenic ones in several aspects.

TABLE 12.1. Differences between polygenic and oligogenic traits

Polygenic Traits	*Oligogenic Traits*
1. Governed by several genes.	Governed by few genes.
2. Effect of each gene is not detectable.	Effect of each gene is detectable.
3. Usually governed by additive genes.	Governed by non-additive genes.
4. Variation is continuous.	Variation is discontinuous.
5. Separation into different classes is not possible.	Separation into different classes is possible.
6. Highly influenced by environmental factors.	Little influenced by environmental factors.
7. Statistical analysis is based on mean, variances and covariances.	Statistical analysis is based on frequencies or ratios.

In plant breeding both types of characters showing qualitative and quantitative inheritance have equal economic importance.

Similarities between Oligogenic and Polygenic Traits:

East (1916) demonstrated that polygenic characters were perfectly in agreement with Mendelian segregation and later on Fisher (1918) and Wright (1921, 1935) provided a mathematical basis for the genetic interpretation of such characters.

The quantitative characters do not differ in any essential feature from the qualitative characters, as discussed below:

1. Both quantitative and qualitative characters are governed by genes; the former is controlled by polygenes or minor genes and the latter by oligogenes or major genes.

2. Both major as well as minor genes are located on the chromosome in the nucleus.

3. The polygenic traits controlling continuous variation exhibit segregation like major genes controlling discontinuous Mendelian variation.

4. Polygenic characters show variable expression which is due to non-genetic causes i.e., environmental effects. Qualitative characters also exhibit variation in expression but to a lesser degree than polygenic traits.

5. The reciprocal crosses for both types of traits exhibit close agreement in expression of genes.

6. The phenomenon of transgression in polygenes can only be explained by Mendelian principles of inheritance.

7. Polygenes mutate like oligogenes.

8. Dominance and non-allelic interactions are common features of major genes. These features are also observed for polygenes, but are usually complete for major genes and only partial for minor genes.

9. Polygenes exhibit linkage like oligogenes. Many cases of linkage between major genes and polygenes controlling continuous variation have been reported.

Thus, quantitative genetics or biometrical genetics is an extension of Mendelian genetics firmly based on Mendelian principles of heredity.

Analysis of Polygenic Traits:

The method of analysis of quantitative inheritance differs from that of qualitative inheritance in some aspects as given below:

1. It requires various measurements of characters like weight, length, width, height, duration, etc., rather than classification of individuals into groups based on colour or shape.

2. Observations are recorded on several individuals and the mean values are used for genetical studies. Segregation into distinct classes in F2 generation-is not obtained in the inheritance of quantitative characters. The segregants exhibit continuous range of variation from one extreme (low) to other (high) for such traits.

3. The inheritance is studied with the help of mean, variances and covariance's. These estimates can be worked out from data recorded in replicated experiment.

4. Fisher (1918) was the pioneer worker to interpret the quantitative characters in terms of Mendelian genetics. Now several biometrical techniques are available for the genetic analysis of quantitative characters. The science which deals with the genetic interpretations of quantitative characters has got separate entity as quantitative genetics or biometrical genetics.

Assumptions of Polygenic Traits:

Polygenic inheritance is based on several assumptions.

The six important assumptions are given below:

1. Each of the contributing genes involved in the expression of a character produces an equal effect.

2. Each contributing allele has either cumulative or additive effect in the expression of a character.

3. The genes involved in the expression of characters have lack of dominance. They show intermediate expression between two parents.

4. There is no epistasis among genes at different loci.

5. The linkage is in equilibrium, means there is no linkage.

6. The environmental effects are absent or may be ignored. However, last three assumptions are seldom fulfilled.

There are two types of alleles or genes in the polygenic inheritance, viz:

(1) Contributing alleles and

(2) Non-contributing alleles.

Those alleles which contribute to continuous variation are known as contributing alleles and those which do not contribute to continuous variation are referred to as non-contributing alleles. Some scientists refer to these as effective and non-effective alleles, respectively.

Examples of Polygenic Traits:

In plant genetics, examples of polygenic characters include yield per plant, days to flower, days to maturity, seed size, seed oil content, etc. Examples of qualitative characters are colour of stem, flower, pollen, etc. and their shapes.

Polygenic inheritance has been reported for various characters both in plants and animals. The most common examples include kernel colour in wheat, corolla length in tobacco, skin colour in man and ear size in maize.

These are briefly described as follows:

1. Kernel Colour in Wheat:

Nilsson Ehle (1908) studied the inheritance of kernel colour in wheat. He found that seed or kernel colour in wheat is governed by one, two and three gene pairs, because in the crosses between red and white kernel varieties, he observed that the F1 was intermediate between the parental values and in F2 he observed 3:1,15:1 and 63 : 1 ratios of red and white seeds in different crosses.

The last two ratios indicated that there was duplicate gene interaction; however, in depth study of coloured seeds revealed that there were different grades or shades of colour within the red coloured seeds. The red seeds of 15 : 1 ratio could be easily divided into four classes on the basis of shade of colour, viz., dark red, medium dark red, medium red and light red.

These colours were observed in the ratio of 1 : 4 : 6 : 4 : 1. This suggested that the seed colour in wheat is controlled by genes which show lack of dominance and have small cumulative effects.

Here, two types of alleles are involved in the expression of character. Those which contribute to continuous variation and those which do not contribute. The first category of alleles is called effective and second as non-effective. Assume that red seed colour is controlled by two genes R1 and R2 and, white seed colour by r1 and r2.

From the cross between dark red and white seed parents, Nilsson Ehle observed the following results

Parents		Dark red			White
Genotypes		$R_1R_1R_2R_2$		× ↓	$r_1r_1r_2r_2$
F_1				$R_1r_1R_1r_1$	Medium Red
		R_1R_2	R_1r_2	r_1R_2	r_1r_2
F_2	R_1R_2	$R_1R_1R_2R_2$ [DR]	$R_1R_1R_2r_2$ [MDR]	$R_1r_1R_2R_2$ [MDR]	$R_1r_1R_2r_2$ [MR]
	R_1r_2	$R_1R_1R_2r_2$ [MDR]	$R_1R_1r_2r_2$ [MR]	$R_1r_1R_2r_2$ [MR]	$R_1r_1r_2r_2$ [LR]
	r_1R_2	$R_1r_1R_2R_2$ [MDR]	$R_1r_1R_2r_2$ [MR]	$r_1r_1R_2R_2$ [MR]	$r_1r_1R_2r_2$ [LR]
	r_1r_2	$R_1r_1R_2r_2$ [MR]	$R_1r_1r_2r_2$ [LR]	$r_1r_1R_2r_2$ [LR]	$r_1r_1r_2r_2$ [W]

DR = Dark Red, MDR = Medium Dark Red,
MR = Medium Red, LR = Light Red and W = White

Fig. 12.1. Inheritance of kernel colour in wheat.

Summary of Results

Effective alleles for red colour	*No. of individuals*	*Phenotype*
4	1	Dark red
3	4	Medium dark red
2	6	Medium red
1	4	Light red
0	1	White

Where 4 effective alleles were present, the seed colour was dark red, where 3 such alleles were present, the seed colour was medium dark red, with 2 effective alleles, colour was medium red and

with 1 effective allele, seed colour was light red. White seed colour was produced when all the non-effective alleles were present.

2. Corolla Length in Tobacco:

Extreme differences exist in corolla length in Nicotiana longiflora. East (1916) studied the inheritance of corolla length in this species of tobacco. He crossed inbred lines of this species with average corolla length of 40 cm and 93 cm.

The F1 showed intermediate expression for corolla length with 63 cm. In F2, wide variation for corolla length was observed. The results indicated that five or more genes were involved in the expression of corolla length.

3. Skin Colour Inheritance in Man:

The inheritance of skin colour in man was studied by Davenport. The inheritance of Negro x white matings can be explained on the basis of two gene difference. Assume that negro colour is governed by A and B genes and white colour by a and b genes.

A cross between negro and white gives birth to a child with medium skin colour called mullatoes (F1). In F2 generation, four distinct shades of black colour were observed besides one white (Fig. 12.2). Thus, the phenotypic ratio of 1 : 4 : 6 : 4 : 1 was observed. The individuals having 4, 3, 2, 1 and 0 effective alleles had black (negro) dark, medium, light and white colour, respectively.

Transgressive Segregation:

Appearance of transgressive segregants in F2 is an important feature of polygenic inheritance. Segregants which fall outside the limits of both the parents are known as transgressive segregants. Transgressive segregation results due to fixation of dominant and recessive genes in separate individuals.

Such segregation occurs when the parents are intermediate to the extreme values of the segregating population. Plant breeders use this principle to obtain superior combinations in segregating material for polygenic characters.

Parents	AAbb	×	aaBB
		↓	
F_1		AaBb	
F_2	AABB and aabb are transgressive segregants		

Environmental Effect:

Polygenic characters are highly sensitive to environmental changes. In other words, they are more prone to genotype x environmental interactions. The main effect of environment is to mask the small differences among different genotypes resulting in continuous variation in the character.

When the contribution of environment is 50 per cent, the distribution becomes roughly similar to normal curve and with 75 per cent contribution, it tends to reach normal distribution. For polygenic traits, generally the environmental variation ranges from 10 to 50 per cent and even more for some traits like yield. The high environmental variation results in overlapping of various classes resulting in continuous, variation.

Partitioning of Polygenic Variability:

The polygenic variation or variability present in a genetic population is measured in terms of variances.

The polygenic variation is of three types, viz:

(1) Phenotypic,

(2) Genotypic and

(3) Environmental.

These are briefly described below:

1. Phenotypic Variability:

It is the total variability which is observable. It includes both genotypic and environmental variation and hence changes under different environmental conditions. Such variation is measured in terms of phenotypic variance.

2. Genotypic Variability:

It is the inherent or genetic variability which remains unaltered by environmental conditions. This type of variability is more useful to a plant breeder for exploitation in selection or hybridization. Such variation is measured in terms of genotypic variance. The genotypic variance consists of additive, dominance and epistatic components.

3. Environmental Variability:

It refers to non-heritable variation which is entirely due to environmental effects and varies under different environmental conditions. This uncontrolled variation is measured in terms of error mean variance. The variation in true breeding parental lines and their F1 is non-heritable. Fisher was the first to divide in 1918, the genetic variance into additive, dominance and epistatic components.

a. Additive Variance:

It refers to that portion of genetic variance which is produced by the deviations due to average effects of genes at all segregating loci. Thus, it is the component which arises from differences between two homozygotes of a gene, i.e., AA and aa. Additive genes show lack of dominance, i.e., intermediate expression.

The additive genetic variance is associated with homozygosis and, therefore, it is expected to be maximum in self-pollinating crops and minimum in cross-pollinating crops. Additive variance is fixable and, therefore, selection for traits governed by such variance is very effective.

Additive genetic variance is important for the following major reasons:

1. It is required for estimation of heritability in narrow sense and response to selection is directly proportionate to narrow sense heritability.
2. It is a pre-requisite for selection because this is the only variance which responds to selection.
3. Breeding value of an individual is measured directly by the additive gene effects. The general combining ability (gca) effect of a parent is measure of additive gene effects.
4. Additive genetic variance gets depleted proportionate to the improvement made by selection.
5. In natural plant breeding populations, additive variance is the predominant one closely followed by dominance variance.

b. Dominance Variance:

It arises due to the deviation from the additive scheme of gene action resulting from intra-allelic interaction i.e., interaction between alleles of the same gene or same locus. It is due to the deviation of heterozygote (Aa) from the average of two homozygotes (AA and aa).

Such genes show incomplete, complete or over-dominance. The dominance variance is associated with heterozygosis and, therefore, it is expected to be maximum in cross-pollinating crops and minimum in self-pollinating species.

Dominance variance is not fixable and, therefore, selection for traits controlled by such variance is not effective. Heterosis breeding may be rewarding in such situation. Dominance variance differs from additive variance in several ways.

c. Epistatic Variance:

It arises due to the deviation as a consequence of inter-allelic interaction, i.e., interaction between alleles of two or more different genes or loci. The epistatic variance is of three types, viz., (i) additive x additive, (ii) additive x dominance, and (iii) dominance x dominance. They differ from each other in several aspects.

TABLE 12.3. Comparison of three types of epistatic gene action

Additive × Additive	*Additive × Dominance*	*Dominance × Dominance*
1. It refers to interaction between two loci each exhibiting lack of dominance individually.	It refers to interaction between two loci, one exhibiting lack exhibiting lack of dominance and other dominance individually.	It refers to interaction between two loci each exhibiting dominance individually.
2. This type of epistasis is fixable.	This in non-fixable.	This is also non-fixable.
3. This comes under additive type of gene action.	This comes under non-additive type of gene action.	This comes under non-additive type of gene action.
4. Selection is effective for traits governed by this type of epistasis.	Selection is not effective as this is type of epistasis is not fixable.	Selection is not effective as this is not fixable.
5. This is useful for mass selection and progeny selection.	This type of epistasis is useful for exploitation of heterosis.	This type of epistasis is also suitable for exploitation of heterosis.

(i) Additive x Additive:

In this case both the interacting loci exhibit lack of dominance individually. It is denoted as A x A and is fixable.

(ii) Additive x Dominance:

It refers to interaction between two or more loci, one exhibiting lack of dominance and the other dominance individually. It is denoted as A x D and is non-fixable.

(iii) Dominance x Dominance:

In this type of epistasis both the interacting loci exhibit dominance individually. It is represented as D x D and is non-fixable.

The first type of epistasis is fixable and, therefore, selection is effective for traits governed by such variance. The last two types of epistatic variances are unfixable and, therefore, heterosis breeding may be rewarding for traits exhibiting such variance. In natural plant breeding populations, epistatic variance has the lowest magnitude. Epistatic variance differs in many aspects from dominance variance.

Wright (1935) suggested the partitioning of genetic variance into two components, viz., additive and non-additive (dominance and epistatic components), of which only the additive component contributes to genetic advance under selection.

Mather (1949) divided the phenotypic variance into three components, namely, (1) heritable fixable (additive variance), (2) heritable non-fixable (dominance and epistatic components), and (3) non-heritable non-fixable (Environmental fraction).

In fact, the heritable fixable component of phenotypic variance will include the additive x additive fraction of the epistatic variance as well. Further, the total phenotypic variance may be partitioned as (1) fixable (additive and additive x additive components) and (2) non-fixable (dominance, additive x dominance and dominance x dominance types of epistasis and environmental fraction) components.

The above discussion may be summarized as follows:

$VP = VG + VE$; $VG = VA + VD + VI$; and $VI = VAA + VAD + VDD$

Where; VP = phenotypic variance, VG = genotypic variance, VA = additive variance, VD = dominance variance, VI = epistatic variance, VAA = additive x additive variance, VAD = additive x dominance variance, and VDD = dominance x dominance variance.

In homozygous genotypes, the genetic variance is of additive (A) and additive epistatic (AA) types, while in the segregating populations all the three types of genetic variances, viz., additive, dominance and epistasis are observed. In F2, the phenotypic variance has 1/2D (additive) and 1/4H (non-additive) components.

In a random mating populations with no epistasis and zero inbreeding, the covariance between a parent and its offspring is 1/2 VA; the covariance among half-sibs is 1/4 VA; and the covariance among full-sibs is 1/2 VA + 1/4 VD. These relationships change with the level of inbreeding in the population.

Genetic variability for important agronomic traits in almost all the crops is mainly due to the additive genetic variance. The non-additive variance also exists in nearly all crops and for many important traits, but it is generally smaller in magnitude than the additive component.

The variability present in genetic populations can be assessed in four different ways: (1) using simple measures of variability, (2) by variance component analysis, (3) by D2 statistics, and (4) by metro glyph analysis. For details of these procedures refer Singh and Narayanan (1993).

Significance of Polygenes:

Polygenes are of prime importance to plant breeder for evolution of improved cultivars. Polygenes have great evolutionary significance. They provide variation of fine adjustment and are systems of smooth adaptive change and of speciation.

The potential genetic variability is stored in the form of linked polygenic complexes. Such stores bear mixtures of plus and minus alleles. The potential or hidden variability is released, after inter-mating of such genotypes with other genotypes, due to segregation and recombination.

Some characters are controlled by several genes. They are known as quantitative characters or polygenic characters. The mode of inheritance of polygenic characters is termed as polygenic inheritance or quantitative inheritance. Since in polygenic inheritance several genes (factors) are

Linkage and recombination:

Linkage

Linkage is the phenomenon of certain genes staying together during inheritance through generations without any change or separation due to their being present on the same chromosome.

It was Morgan (1910) who clearly proved and defined linkage on the basis of his breeding experiments in fruitfully *Drosophila melanogaster*. In 1911, Morgan and Castle proposed chromosome theory of linkage. It states that

(i) Linked genes occur in the same chromosome.

(ii) They lie in a linear sequence in the chromosome.

(iii) There is a tendency to maintain the parental combination of genes except for occasional crossovers.

(iv) Strength of the linkage between two genes is inversely proportional to the distance between the two, i.e., two linked genes show higher frequency of crossing over if the distance between them is higher and lower frequency if the distance is small.

Linked genes are those genes which occur on the same chromosome while unlinked genes are the ones found on different chromosomes. Linked and unlinked genes can be easily known from breeding experiments. Unlinked genes show independent assortment, a di-hybrid ratio of 9: 3: 3: 1 and the di-hybrid or double test cross ratio of 1: 1: 1: 1 with two parental and two recombinant types.

The linked genes do not show independent assortment but remain together and are inherited en block producing only parental type of progeny. They give a di-hybrid ratio of 3: 1 and a test cross ratio of 1: 1.**Phases:**

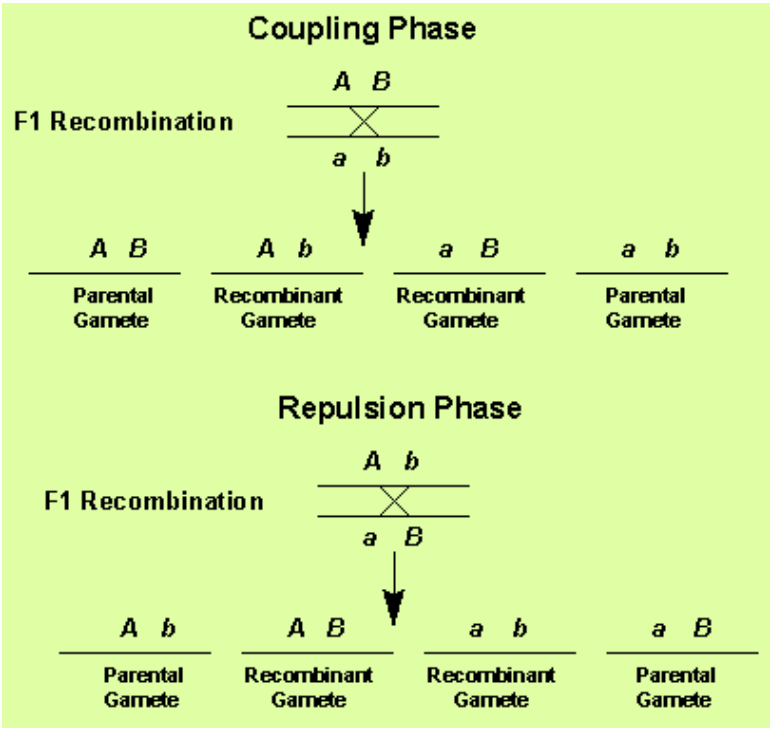
i. Coupling:

The linkage between two or more either dominant (AB) or recessive (ab) alleles is referred to as coupling. A good example of coupling was reported by Hutchinson in maize for the genes governing colour of seed (coloured and colourless) and shape of seed (full and shrunken).
The coloured seed is governed by dominant gene (C) and full seed is also governed by dominant gene (S). He made cross between plants having coloured full seeds (CCSS) and colourless shrunken seeds (ccss). The F1 seeds were coloured full. When the F1 was test crossed with double recessive parent the following results were obtained instead of 1 : 1 : 1 : 1 ratio.

ii. Repulsion:

The linkage of dominant allele with that of the recessive allele (Ab or aB) is known as repulsion. Hutchinson also observed repulsion phase of linkage in maize. He observed this type of linkage when he made cross between plants having coloured shrunken seeds (Cs) with those having colourless full seeds (cS).

In F_1 the seeds were coloured full. By crossing of F_1 with double recessive parent the following results were obtained instead of 1 : 1 : 1 : 1 ratio.



Types of Linkage:

Linkage is of two types, complete and incomplete.

1. Complete Linkage:

The genes located on the same chromosome do not separate and are inherited together over the generations due to the absence of crossing over. Complete linkage allows the combination of parental traits to be inherited as such. It is rare but has been reported in male Drosophila and some other heterogametic organisms.

2. Incomplete Linkage:

Genes present in the same chromosome have a tendency to separate due to crossing over and hence produce recombinant progeny besides the parental type. The number of recombinant individuals is usually less than the number expected in independent assortment. In independent assortment all the four types (two parental types and two recombinant types) are each 25%. In case of linkage, each of the two parental types is more than 25% while each of the recombinant types is less than 25%.

Linkage Groups:

A linkage group is a linearly arranged group of linked genes which are normally inherited together except for crossing over.

Significance of Linkage:

(i) Linkage plays an important role in determining the nature of scope of hybridization and selection programmes.

(ii) Linkage reduces the chance of recombination of genes and thus helps to hold parental characteristics together. It thus helps organism to maintain its parental, racial and other characters. For this reason plant and animal breeders find it difficult to combine various characters.

Recombination:

Genetic recombination (also known as genetic reshuffling) is the production of offspring with combinations of traits that differ from those found in either parent. In eukaryotes, genetic recombination during meiosis can lead to a novel set of genetic information that can be passed on from the parents to the offspring. Most recombination is naturally occurring.

Types of Recombination:

These are classified basically into the following three groups:

(i) General recombination,

General recombination occurs only between the complementary strands of two homologous DNA molecules.

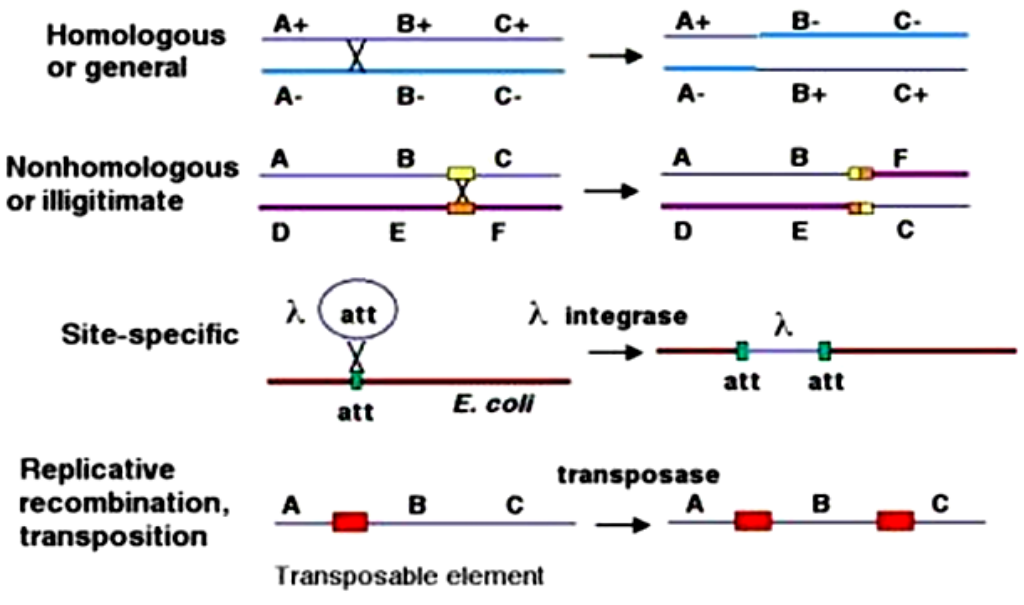
Double helix of two DNA molecules breaks and the two broken ends join to their opposite partners to reunite to form double helix. The site of exchange can occur anywhere in the homologous nucleotide sequence where a strand of one DNA molecule becomes base paired to the second strand to yield heteroduplex just between two double helices

(ii) Non-reciprocail recombination:

The fundamental law of genetics is that the two partners contribute the equal amount of genes to the offsprings. It means that the offsprings inherit the half complete set of genes from the male and half from the female. One diploid cell undergoes meiosis producing four haploid cells; therefore, the number of genes contributed by male gets halved and so the genes of female.

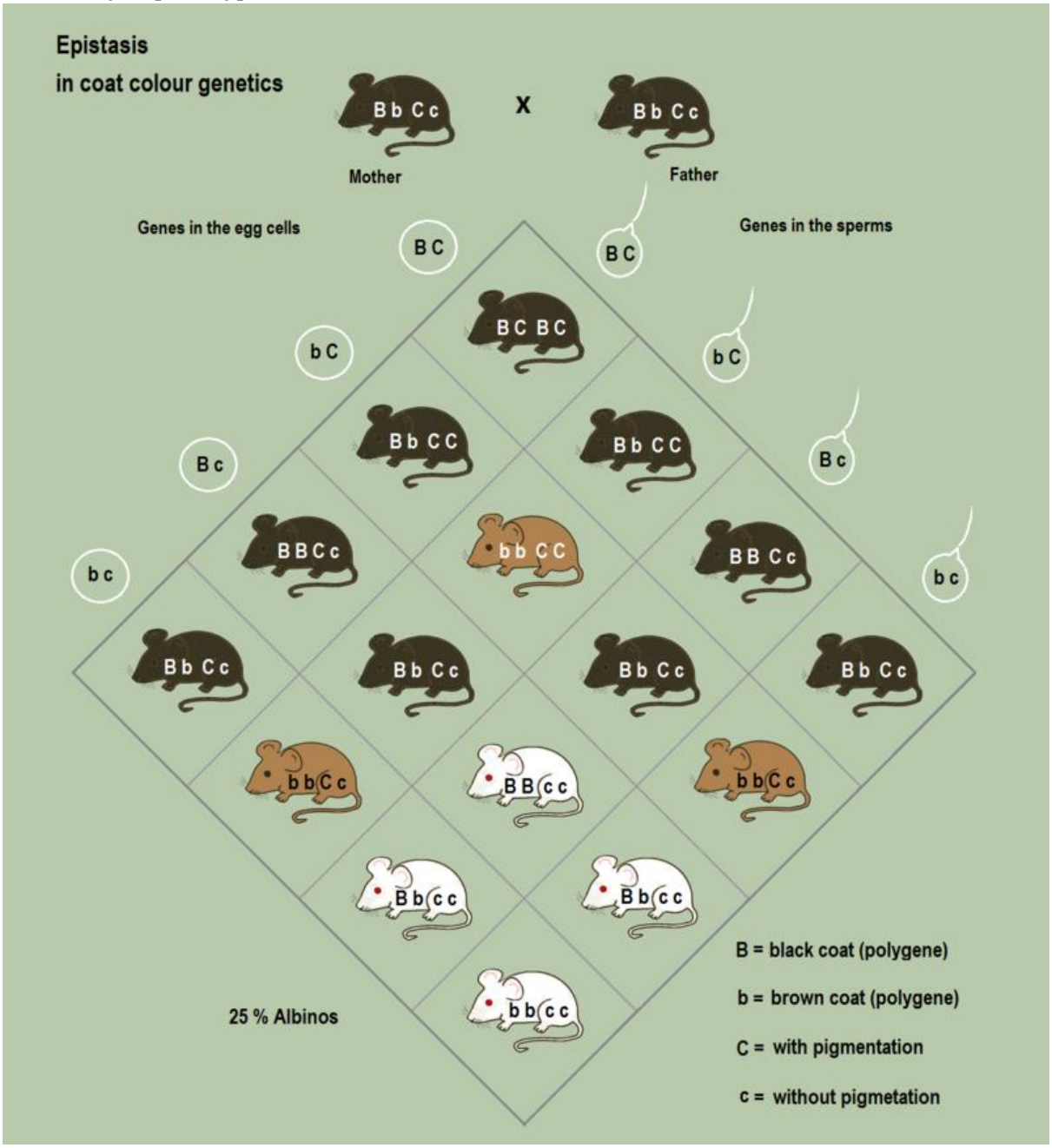
(iii) Site specific recombination:

Site specific recombination alters the relative position of nucleotide sequences in chromosome. The base pairing reaction depends on protein mediated recognition of the two DNA sequences that will combine. Very long homologous sequence is not required.



Epistasis:

Epistasis is the phenomenon where the effect of one gene (locus) is dependent on the presence of one or more 'modifier genes', i.e. the genetic background. Originally the term meant that the phenotypic effect of one gene is masked by a different gene (locus). Thus, epistatic mutations have different effects in combination than individually. It was originally a concept from genetics but is now used in biochemistry, computational biology and evolutionary biology. It arises due to interactions, either between genes, or within them, leading to non-linear effects. Epistasis has a large influence on the shape of evolutionary landscapes, which leads to profound consequences for evolution and evolvability of phenotypic traits.



Example of epistasis in coat colour genetics: If no pigments can be produced the other coat colour genes have no effect on the phenotype, no matter if they are dominant or if the individual is homozygous. Here the genotype "c c" for no pigmentation is epistatic over the other genes.

Classification:

In general, epistasis is used to denote the departure from 'independence' of the effects of different genetic loci. Confusion often arises due to the varied interpretation of 'independence' among different branches of biology. The classifications below attempt to cover the various terms and how they relate to one another.

Additivity

Two mutations are considered to be purely additive if the effect of the double mutation is the sum of the effects of the single mutations. This occurs when genes do not interact with each other, for example by acting through different metabolic pathways. Simple, additive traits were studied early on in the history of genetics, however they are relatively rare, with most genes exhibiting at least some level of epistatic interaction.

Magnitude epistasis

When the double mutation has a fitter phenotype than expected from the effects of the two single mutations, it is referred to as **positive epistasis**. Positive epistasis between beneficial mutations generates greater improvements in function than expected. Positive epistasis between deleterious mutations protects against the negative effects to cause a less severe fitness drop.

Conversely, when two mutations together lead to a less fit phenotype than expected from their effects when alone, it is called **negative epistasis**. Negative epistasis between beneficial mutations causes smaller than expected fitness improvements, whereas negative epistasis between deleterious mutations causes greater-than-additive fitness drops.

Independently, when the effect on fitness of two mutations is more radical than expected from their effects when alone, it is referred to as **synergistic epistasis**. The opposite situation, when the fitness difference of the double mutant from the wild type is smaller than expected from the effects of the two single mutations, it is called **antagonistic epistasis**. Therefore, for deleterious mutations, negative epistasis is also synergistic, while positive epistasis is antagonistic; conversely, for advantageous mutations, positive epistasis is synergistic, while negative epistasis is antagonistic.

The term **genetic enhancement** is sometimes used when a double (deleterious) mutant has a more severe phenotype than the additive effects of the single mutants. Strong positive epistasis is sometimes referred to by creationists as irreducible complexity (although most examples are misidentified).

Haploid organisms

In a haploid organism with genotypes (at two loci) *ab*, *Ab*, *aB* or *AB*, we can think of different forms of epistasis as affecting the magnitude of a phenotype upon mutation individually (Ab and aB) or in combination (AB).

Interaction type	**ab**	**Ab**	**aB**	**AB**	
No epistasis (additive)	0	1	1	2	$AB = Ab + aB + ab$
Positive (synergistic) epistasis	0	1	1	3	$AB > Ab + aB + ab$
Negative (antagonistic) epistasis	0	1	1	1	$AB < Ab + aB + ab$
Sign epistasis	0	1	-1	2	*AB* has opposite sign to *Ab* **or** *aB*
Reciprocal sign epistasis	0	-1	-1	2	*AB* has opposite sign to Ab **and** *aB*

Diploid organisms

Epistasis in diploid organisms is further complicated by the presence of two copies of each gene. Epistasis can occur between loci, but additionally, interactions can occur between the two copies of

each locus in heterozygotes. For a two locus, two allele system, there are eight independent types of gene interaction.

Additive A locus

	aa	**aA**	**AA**
bb	1	0	–1
bB	1	0	–1
BB	1	0	–1

Additive B locus

	aa	**aA**	**AA**
bb	1	1	1
bB	0	0	0
BB	–1	–1	–1

Dominance A locus

	aa	**aA**	**AA**
bb	–1	1	–1
bB	–1	1	–1
BB	–1	1	–1

Dominance B locus

	aa	**aA**	**AA**
bb	–1	–1	–1
bB	1	1	1
BB	–1	–1	–1

Additive by Additive Epistasis

	aa	**aA**	**AA**
bb	1	0	–1
bB	0	0	0
BB	–1	0	1

Additive by Dominance Epistasis

	aa	**aA**	**AA**
bb	1	0	–1
bB	–1	0	1
BB	1	0	–1

Dominance by Additive Epistasis

	aa	**aA**	**AA**
bb	1	–1	1
bB	0	0	0
BB	–1	1	–1

Dominance by Dominance Epistasis

	aa	**aA**	**AA**
bb	–1	1	–1
bB	1	–1	1
BB	–1	1	–1

Genetic and Molecular cause:

Additivity

This can be the case when multiple genes act in parallel to achieve the same effect. For example, when an organism is in need of phosphorus, multiple enzymes that break down different phosphorylated components from the environment may act additively to increase the amount of phosphorus available to the organism. However, there inevitably comes a point where phosphorus is no longer the limiting factor for growth and reproduction and so further improvements in phosphorus metabolism have smaller or no effect (negative epistasis).

Epistasis between genes

Epistasis within the genomes of organisms occurs due to interactions between the genes within the genome. This interaction may be direct if the genes encode proteins that, for example, are separate components of a multi-component protein (such as the ribosome), inhibit each other's activity, or if the protein encoded by one gene modifies the other (such as by phosphorylation). Alternatively the interaction may be indirect, where the genes encode components of a metabolic pathway or network, developmental pathway, signalling pathway or transcription factor network. For example, the gene encoding the enzyme that synthesizes penicillin is of no use to a fungus without the enzymes that synthesize the necessary precursors in the metabolic pathway.

Epistasis within genes

Just as mutations in two separate genes can be non-additive if those genes interact, mutations in two codons within a gene can be non-additive. In genetics this is sometimes called intragenic complementation when one deleterious mutation can be compensated for by a second mutation within that gene. This occurs when the amino acids within a protein interact. Due to the complexity of protein folding and activity, additive mutations are rare.

Heterozygotic epistasis

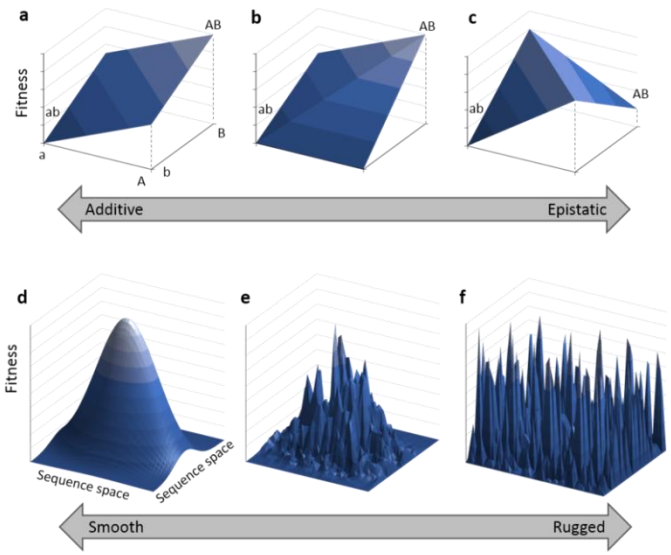
Diploid organisms contain two copies of each gene. If these are different (heterozygous / heteroallelic), the two different copies of the allele may interact with each other to cause epistasis. This is sometimes called **allelic complementation**, or **interallelic complementation**. It may be caused by several mechanisms, for example transvection, where an enhancer from one allele acts in trans to activate transcription from the promoter of the second allele. Alternately, trans-splicing of two non-functional RNA molecules may produce a single, functional RNA. Similarly, at the protein level,

proteins that function as dimers may form a heterodimer composed of one protein from each alternate gene and may display different properties to the homodimer of one or both variants.

Evolutionary consequences:

Fitness landscapes and evolvability

In evolutionary genetics, the sign of epistasis is usually more significant than the magnitude of epistasis. This is because magnitude epistasis (positive and negative) simply affects how beneficial mutations are together, however sign epistasis affects whether mutation combinations are beneficial or deleterious.



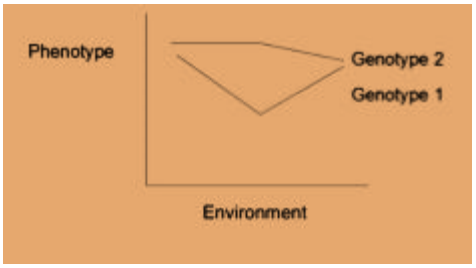
A fitness landscape is a representation of the fitness where all genotypes are arranged in 2D space and the fitness of each genotype is represented by height on a surface. It is frequently used as a visual metaphor for understanding evolution as the process of moving uphill from one genotype to the next, nearby, fitter genotype.

Evolution of sex:

Negative epistasis and sex are thought to be intimately correlated. Experimentally, this idea has been tested in using digital simulations of asexual and sexual populations. Over time, sexual populations move towards more negative epistasis, or the lowering of fitness by two interacting alleles. It is thought that negative epistasis allows individuals carrying the interacting deleterious mutations to be removed from the populations efficiently. This removes those alleles from the population, resulting in an overall more fit population. This hypothesis was proposed by Alexey Kondrashov, and is sometimes known as the deterministic mutation hypothesis and has also been tested using artificial gene networks.

Gene–environment interaction:

Gene–environment interaction (or genotype–environment interaction or G×E) is when two different genotypes respond to environmental variation in different ways. A norm of reaction is a graph that shows the relationship between genes and environmental factors when phenotypic differences are continuous. They can help illustrate GxE interactions. When the norm of reaction is not parallel, as shown in the figure below, there is a gene by environment interaction. This indicates that each genotype responds to environmental variation in a different way. Environmental variation can be physical, chemical, biological, behavior patterns or life events.



In genetic epidemiology, gene-environment interactions are useful for understanding some diseases. Sometimes, sensitivity to environmental risk factors for a disease are inherited rather than the disease itself being inherited.

e.g.- A classic example of gene–environment interaction was performed on *Drosophila* by Gupta and Lewontin in 1981. In their experiment they demonstrated that the mean bristle number on Drosophila could vary with changing temperatures.

A sorghum bi-parental population was repeatedly grown in seven diverse geographic locations across years. A group of genotypes requires similar growing degree-day (GDD) to flower across all environments, while another group of genotypes need less GDD in certain environments, but higher GDD in different environments to flower. The complex flowering time patterns is attributed to the interaction of major flowering time genes (Ma_1, Ma_6, FT, ELF3) and an explicit environmental factor, photothermal time (PTT) capturing the interaction between temperature and photoperiod.

Methods of Analysis:

Adoption studies

Adoption studies have been used to investigate how similar individuals that have been adopted are to their biological parents with whom they did not share the same environment with. For example, an adoption study showed that Swedish men with disadvantaged adoptive environments and a genetic predisposition were more likely to abuse alcohol.

Twin studies

Using monozygotic twins, the effects of different environments on identical genotypes could be observed. Later studies leverage biometrical modelling techniques to include the comparisons of dizygotic twins to ultimately determine the different levels of gene expression in different environments.

Family studies

Family-based research focuses on the comparison of low-risk controls to high risk children to determine the environmental effect on subjects with different levels of genetic risk. For example, a Danish study on high-risk children with schizophrenic mothers depicted that children without a stable caregiver were associated with an increased risk of schizophrenia.

Molecular Analyses

Interaction with single genes

The often used method to detect gene-environment interactions is by studying the effect a single gene variation (candidate gene) has with respect to a particular environment. Single nucleotide polymorphisms (SNP's) are compared with single binary exposure factors to determine any effects.

Candidate studies such as these require strong biological hypotheses which are currently difficult to select given the little understanding of biological mechanisms that lead to higher risk.

These studies are also often difficult to replicate commonly due to small sample sizes which typically results in disputed results.

Interaction with multiple genes

Since the same environmental factor could interact with multiple genes, a polygenic approach can be taken to analyzeGxE interactions. A polygenic score is generated using the alleles associated with a trait and their respective weights based on effect and examined in combination with environmental exposure.

Genome-wide association studies and genome wide interaction studies

A genome wide interaction scan (GEWIS) approach examines the interaction between the environment and a large number of independent SNP's. An effective approach to this all-encompassing study occurs in two-steps where the genome is first filtered using gene-level tests and pathway based gene set analyses. The second step uses the SNP's with G-E association and tests for interaction.

Gene × Environment × Environment Interactions

New studies have also revealed the interactive effect of multiple environment factors. For example, a child with a poor quality environment would be more sensitive to a poor environment as an adult which ultimately led to higher psychological distress scores. This depicts a three way interaction Gene

x Environment x Environment. The same study suggests taking a life course approach to determining genetic sensitivity to environmental influences within the scope of mental illnesses.

Medical significance

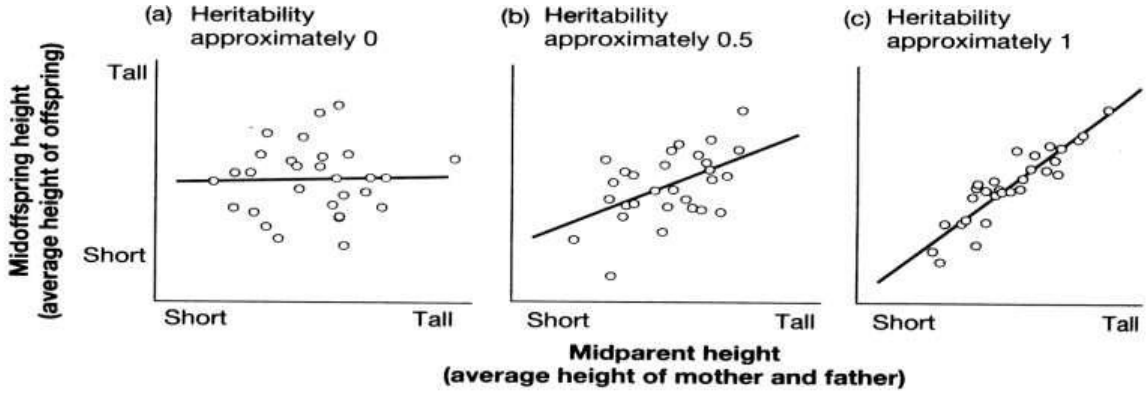
Doctors are interested in knowing whether disease can be prevented by reducing exposure to environmental risks. Some people carry genetic factors that confer susceptibility or resistance to a certain disorder in a particular environment. The interaction between the genetic factors and environmental stimulus is what results in the disease phenotype. There may be significant public health benefits in using gene by environment interactions to prevent or cure disease.

Heritability

eritability is a statistic used in the fields of breeding and genetics that estimates the degree of variation in a phenotypic trait in a population that is due to genetic variation between individuals in that population. Heritability is estimated by comparing individual phenotypic variation among related individuals in a population. Heritability is an important concept in quantitative genetics, particularly in selective breeding and behavior genetics.

Heritability, amount of phenotypic (observable) variation in a population that is attributable to individual genetic differences. Heritability, in a general sense, is the ratio of variation due to differences between genotypes to the total phenotypic variation for a character or trait in a population. The concept typically is applied in behaviour genetics and quantitative genetics, where heritability estimates are calculated by using either correlation and regression methods or analysis of variance (ANOVA) methods.

- Heritability is the ratio of genotypic variance to the phenotypic variance
- Heritability denotes the proportion of phenotypic variance that is due to genotype i.e., heritable.
- It is generally expressed in percent (%)
- It is a good index of transmission of characters from parents to their offspring



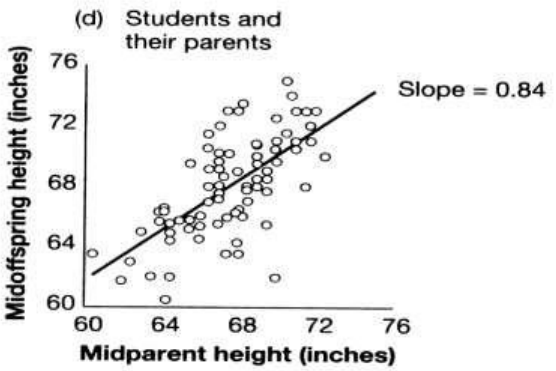


Fig: heritability in between parents & off springs height

Heritability is expressed as

$H^2 = Vg/Vp$,

Where, *H* is the heritability estimate, *Vg* the variation in genotype, and *Vp* the variation in phenotype. Heritability estimates range in value from 0 to 1. If $H = 1$, then all variation in a population is due to differences or variation between genotypes (i.e., there is no environmentally caused variation). If $H = 0$, there is no genetic variation; in this case all variation in the population comes from differences in the environments experienced by individuals.

Depending upon the components of variance used as numerator in the calculation ,there are 2 definitions of Heritability

1. Broad sense heritability

2. Narrow sense heritability

Broad sense heritability:

Broad sense heritability includes all components of genetic variance. Often it is informative to calculate the proportion of the total phenotypic variance that is due to genetic differences among individuals in a population. This proportion is called the broad-sense heritability, symbolized H^2. In terms of Fisher's variance components,

According to Falconer, broad sense heritability is the ratio of genotypic variance to total or phenotypic variance

It is calculated with the help of following formula

$$\text{Heritability } (h^2) = V_g / V_p \times 100 = V_g / V_g + V_e \times 100$$

The total phenotypic variance may be decomposed:

V_p = total phenotypic variance

V_g = total genetic variance

V_e = environmental variance

- Broad heritability (h2) separates genotypic from environmentally induced variance: h2 = Vg / Vp
- It can be estimated from both parental as well as segregating populations
- It express the extent to which the phenotype is determined by the genotype , so called degree of genetic determination
- It is most useful in clonal or highly selfing species in which genotypes are passed from parents to offspring more or less intact
- It is useful in selection of superior lines from homozygous lines

The symbol for the broad-sense heritability, H_2 , is written with the exponent 2 to remind us that this statistic is calculated from variances, which are squared quantities. Because of the way it is calculated, the broad-sense heritability must lie between 0 and 1. If it is close to 0, little of the observed variability in the population is attributable to genetic differences among individuals. If it is close to 1, most of the observed variability is attributable to genetic differences. The broad-sense heritability therefore summarizes the relative contributions of genetic and environmental factors to the observed variability in a population. However, it is important to note that this statistic is population-specific. For a given trait, different populations may have different values of the broad-sense heritability. Thus, the broad-sense heritability of one population cannot automatically be assumed to represent the broad-sense heritability of another population. In the F^2 wheat population, H^2= 11.98/14.26 = 0.84. This result tells us that in this population 84 percent of the observed variability in wheat maturation time is due to genetic differences among individuals. However, it does not tell us what these differences are. The genetic variance upon which the broad-sense heritability depends

includes all the factors that cause genotypes to have different phenotypes: the effects of individual alleles, the dominance relationships between alleles, and the epistatic interactions among different genes. In Chapter 4 we saw how these factors influence phenotypes. In the next two sections, we will see that by breaking out these components of genetic variability and by focusing on the component that involves the effects of individual alleles, we can predict the phenotypes of offspring from the phenotypes of their parents.

Narrow sense heritability includes only the additive genetic variance and it is this form of heritability that usually of interest.

The ability to make predictions in quantitative genetics depends on the amount of genetic variation that is due to the effects of individual alleles. Genetic variation that is due to the effects of dominance and epistasis has little predictive power.

- It is the ratio of additive or fixable genetic variance to the total or phenotypic variance
- Also known as degree of genetic resemblance
- It plays an important role in the selection process in plant breeding
- For estimation of narrow sense heritability , crosses have to be made in a definite fashion
- It is estimated from additive genetic variance
- It is useful for plant breeding in selection of elite types from segregating populations

It is calculated with the help of following formula

Where VA or D = additive genetic variance

VP

To see how dominance limits the ability to make predictions, consider the ABO blood types in humans. This trait is determined strictly by the genotype; environmental variation has essentially no effect on the phenotype.

However, because of dominance, two individuals with the same phenotype can have different genotypes. For example, a person with type A blood could be either I A I A or I A i. If two people with type A blood produce a child, we cannot predict precisely what phenotype the child will have. It could be either type A or type O, depending on the genotypes of the parents; however, we know that it will not have type B or type AB blood. Thus, although we can make some kind of prediction about the child's phenotype, dominance prevents us from making a precise prediction. Our ability to make predictions about an offspring's phenotype is improved in situations where the genotypes are not confused by dominance. Consider, for example, the inheritance of flower color in the snapdragon, *Antirrhinummajus*. Flowers in this plant are white, red, or pink, depending on the genotype. As with the ABO blood types, variation in flower color has essentially no environmental component; all the variance is the result of genetic differences. However, for the flower color trait, the genotype of an individual is not obscured by the complete dominance of one allele over the other. A plant with two w alleles has white flowers, a plant with one w allele and one W allele has pink flowers, and a plant with two W alleles has red flowers. In this system, the phenotype depends simply on the number of W alleles present; each W allele intensifies the color by a fixed amount. Thus, we can say that the color-determining alleles contribute to the phenotype in a strictly additive fashion. This kind of allele action improves our ability to make predictions in crosses between different plants. A mating between two red plants produces only red offspring; a mating between two white plants produces only white offspring; and a mating between red and white plants produces only pink offspring. The only uncertainty is in a cross involving heterozygotes, and in this case the uncertainty is due to Mendelian segregation, not to dominance.

Quantitative geneticists distinguish between genetic variance that is due to alleles that act additively (such as those in the flower color example just discussed) and genetic variance that is due to dominance. These different variance components are symbolized as:

V_a = additive genetic variance

V_d = dominance variance

In addition, geneticists define a third variance component that measures variation due to epistatic interactions between alleles of different genes:

V_i = epistatic variance

Epistatic interactions, like dominance, are of little help in predicting phenotypes. Altogether, these three variance components constitute the total genetic variance:

$V_g = V_a + V_d + V_i$

If we recall that $V_T = V_g + V_e$, we can express the total phenotypic variance as the sum of four components:

$V_T = Va + V_d + V_i + V_e$

Of these four variance components, only the additive genetic variance, V_a, is useful in predicting the phenotypes of offspring from the phenotypes of their parents. This variance, as a fraction of the total phenotypic variance, is called the narrow-sense heritability, symbolized h^2. Thus,

$h_2 = V_a/V_T$

Like the broad-sense heritability, h^2 lies between 0 and 1. The closer it is to one, the greater is the proportion of the total phenotypic variance that is additive genetic variance, and the greater is our ability to predict an offspring's phenotype. Human stature is highly heritable, but litter size in pigs is not. Thus, if we knew the parental phenotypes, we would be better able to predict the height of a human's offspring than the litter size of a pig's offspring.

It is also referred to as **resemblance between relatives**.

Because individual components of variance are not directly measurable, it is necessary to use comparative measurements of phenotype to determine the contribution of individual variance components.

For example:

- By measuring a specific trait such as height in individual organisms from several populations, one could determine the range of height measurements for that species.
- Individuals from different populations could then be grown in a common garden and measured at the same point of maturity as the original organisms. The common garden would eliminate the environmental variance experienced between the different populations.
- Therefore, the difference between the phenotype variance of the wild populations and that of the common garden would give an estimate of the total genetic variance.

Factor affecting heritability:

- **Type of genetic material:** the magnitude of heritability is largely governed by the amount of genetic variance present in a population for the character under study
- **Sample size:** Large sample is necessary for accurate estimates
- **Sampling methods**: 2 sampling methods , Random and Biased. The random sampling methods provide true estimates of genetic variance and hence of heritability
- **Layout or conduct of experiment:** Increasing the plot size and no. of replications we can reduce experimental error and get reliable estimates
- **Method of calculation :** heritability is estimated by several methods
- **Effect of linkage :** high frequency of coupling phase (AB/ab) causes upward bias in estimates of additive and dominance variances

- **Excess of repulsion phase linkage** (Ab/aB) leads to upward bias in dominance variance and downward bias in additive variances

Genetic advance:

Improvement in the mean genotypic value of selected plants over the parental population is known as genetic advance. It is the measure of genetic gain under selection. The success of genetic advance under selection depends upon three factors (Allard , 1960).

- Genetic variability : greater the amount of genetic variability in base populations higher the genetic advance
- Heritability : the G.A. is high with characters having high heritability
- Selection intensity : the proportion of individuals selected for the study is called selection intensity . high selection intensity gives better results

Selection differential:

It is the difference between the mean phenotypic value of selected population and mean phenotype of original population

This is the measure of the selection intensity and denoted by K.

$K = Xs – Xo$

where Xs = mean of phenotypic value of selected plants

Xo = mean of phenotypic value of parental population

Genetic gain:

The difference between the mean phenotypic value of the progeny of selected plants and the original parental population is known as genetic gain

It is denoted by R

$R = Xp – Xo$

where , Xp = mean phenotypic value of progeny of selected plants

Xo = mean of phenotypic value of base population

Merits of heritability:

- It is useful in predicting the effectiveness of selection.
- It is also helpful for deciding breeding methods to be followed, for effective selection.
- It gives an idea about the response of various characters to selection pressure.
- It is useful in predicting the performance under different degree of intensity of selection.
- It helps for construction of selection index.
- Estimates of heritability serve as a useful guide to the breeder, to appreciate the proportion of variation, which is due to genotypic or additive effects

The Limitations of Heritability

• Heritability does not indicate the degree to which a characteristic is genetically determined.

• Pure breed no polydactilly rabbits: still polydactilly can happen

• An individual does not have heritability.

• Narrow-sense heritability of 0.6 in population does not indicate that an individual's characteristic is 60% additive

• There is no universal heritability for a characteristic.

• Two populations will have different heritability due to environment

• Even when heritability is high, environmental factors may influence a characteristic.

• Human height

• Heritability indicates nothing about the nature of population differences in a characteristic.

Population genetics

Population genetics is a subfield of genetics that deals with genetic differences within and between populations, and is a part of evolutionary biology. Studies in this branch of biology examine such phenomena as adaptation, speciation, and population structure.

Population genetics was a vital ingredient in the emergence of the modern evolutionary synthesis. Its primary founders were Sewall Wright, J. B. S. Haldane and Ronald Fisher, who also laid the foundations for the related discipline of quantitative genetics. Traditionally a highly mathematical discipline, modern population genetics encompasses theoretical, lab, and field work. Population genetic models are used both for statistical inference from DNA sequence data and for proof/disproof of concept.

Populations

A population is a group of organisms of the same species that are found in the same area and can interbreed. A population is the smallest unit that can evolve—in other words, an individual can't evolve.

Alleles

An allele is a version of a gene, a heritable unit that controls a particular feature of an organism.

For instance, Mendel studied a gene that controls flower color in pea plants. This gene comes in a white allele, w, and a purple allele, W. Each pea plant has two gene copies, which may be the same or different alleles. When the alleles are different, one—the dominant allele, W—may hide the other—the recessive allele, w. A plant's set of alleles, called its genotype, determines its phenotype, or observable features, in this case flower color.

Population genetics is the branch of genetics that deals with frequencies of alleles and genotypes in breeding populations. Population genetics examines allelic variation among individuals, the transmission of allelic variants from parents to offspring generation after generation, and the temporal changes that occur in the genetic makeup of a population because of systematic and random evolutionary forces.

Allele frequency

Allele frequency refers to how frequently a particular allele appears in a population. For instance, if all the alleles in a population of pea plants were purple alleles, W, the allele frequency of W would be 100%, or 1.0. However, if half the alleles were W and half were w, each allele would have an allele frequency of 50%, or 0.5.

Estimation of allelic frequencies:

Frequency of the M-N Blood Types in a Sample of 6129 Individuals

Blood Type	Genotype	Number of Individuals
M	$L^M L^M$	1787
MN	$L^M L^N$	3039
N	$L^N L^N$	1303

Because an entire population is usually too large to study, we resort to analyzing a representative sample of individuals from it. The blood types are determined by two alleles of a gene on chromosome 4: *LM*, which produces the M blood type, and *LN*, which produces the N blood type. People who are *LMLN* heterozygotes have the MN blood type. To estimate the frequencies of the *LM* and *LN* alleles, we simply calculate the incidence of each allele among all the alleles sampled:

1. Because each individual in the sample carries two alleles of the blood-type locus, the total number of alleles in the sample is two times the sample size: 2 × 6129 = 12,258.

2. The frequency of the *LM* allele is two times the number of *LMLM* homozygotes plus the number of *LMLN* heterozygotes, all divided by the total number of alleles

sampled: [(2 × 1787) + 3039]/12,258 = 0.5395.
3. The frequency of the *LN* allele is two times the number of *LNLN* homozygotes plus the number of *LMLN* heterozygotes, all divided by the total number of alleles sampled: [(2 × 1303) + 3039]/12,258 = 0.4605.
Thus, letting *p* represent the frequency of the *LM* allele and letting *q* represent the frequency of the *LN* allele, we estimate that in the population from which the sample was taken, $p = 0.5395$ and $q = 0.4605$. Furthermore, because *LM* and *LN* represent 100 percent of the alleles of this particular gene, $p + q = 1$.

The Hardy–Weinberg principle:
In the first decade of the twentieth century, these questions were posed independently by G. H. Hardy, a British mathematician, and by Wilhelm Weinberg, a German physician. In 1908 Hardy and Weinberg each published papers describing a mathematical relationship between allele frequencies and genotype frequencies. This relationship, now called the Hardy–Weinberg principle, allows us to predict a population's genotype frequencies from its allele frequencies.
The Law states that gene frequencies in a population remain constant from generation to generation if no evolutionary processes like migration, mutation, selection and drift are operating.
Thus if matings are random, and no other factors disturb the reproductive abilities of any genotype, the equilibrium genotypic frequencies are given by the square of the allelic frequencies.
Let's suppose that in a population a particular gene is segregating two alleles, *A* and *a*, and that the frequency of *A* is *p* and that of *a* is *q*. Thus, on the assumption of random mating, the predicted frequencies of the three genotypes in the population are:

Genotype	Frequency
AA	p^2
Aa	$2pq$
aa	q^2

These predicted frequencies can be obtained by expanding the binomial expression $(p + q)^2 = p^2 + 2pq + q^2$. Population geneticists refer to them as the Hardy–Weinberg genotype frequencies.
Hardy–Weinberg equilibrium:
Random mating and no differential survival or reproduction among the members of the population, the Hardy–Weinberg genotype frequencies—and, of course, the underlying allele frequencies—persist generation after generation. This condition is referred to as the Hardy–Weinberg equilibrium.

Factor affecting gene frequencies:
Some of the major factors which affect the genetic equilibrium and induce the variability in population are as follows:
(A) Mutations (B) Recombinations during Sexual Reproduction (C) Genetic Drift (D) Gene Migration (Gene Flow) (E) Natural Selection.
(A) Mutations:
These are characterized by:
(i) These are sudden, large and inheritable changes in the genetic material.
(ii) Mutations are random (indiscriminate) and occur in all directions.
(iii) Most mutations are harmful or neutral. It is estimated that only one out of 1,000 mutations is useful.

(iv) Rate of mutation is very low, i.e., one per million or one per several million genie loci. But rate of mutation is sufficient to produce considerable genetic variability.
(v) Certain mutations are pre-adaptive and appear even without exposure to a specific environment. These express and become advantageous only when after exposure to new environment which only selects the pre-adaptive mutations that occurred earlier.
(viii) Significance of mutations:
(a) Mutations create and maintain variations within a population.
(b) These also introduce new genes and alleles in a gene pool.
(c) Accumulation of mutations over a number of generations may lead to speciation.

(B) Recombinations during Sexual Reproduction:

Recombination involves reshuffling of genes of chromosomes. Chances of recombination are more in those organisms which undergo sexual reproduction which involves gametogenesis followed by fertilization.
Sexual reproduction involves recombinations during three stages:
(i) Crossing over
(ii) By independent assortment of chromosomes
(iii) By random fertilization
Significance:
Due to recombination's, though only reshuffling of already existing characters takes place and no new genes are produced but it leads to redistribution of different traits to different individuals of a population. Different combinations bring diversity in genotype and phenotype of different organisms. So recombination is an agent of evolution.

(C) Genetic Drift:

It is the random change in the frequency of alleles occurring by chance fluctuations. It is characterized by:
(i) It is a binomial sampling error of the gene pool, i.e., that alleles which form the gene pool of the next generation are a sample of the alleles of present population.
(ii) Genetic drift always influences frequencies of alleles and is inversely proportional to the size of population. So genetic drift is most important in very small populations in which there are increased chances of inbreeding which increases the frequency of individuals homozygous for recessive alleles, many of which maybe deleterious.
(iii) Genetic drift occurs when a small group separates from a larger population and may not have all the alleles or may differ from the parental population in the frequencies of certain genes. This explains for the difference between island populations and mainland population.
(iv) In a small population, a chance event (e.g. snow storm) may increase the frequency of a character having little adaptive value.
(v) Genetic drift can also operate through founder effect. In this, genetic drift can cause dramatic changes in the allele frequencies in a population derived from small groups of colonisers, called founders, to a new habitat.
These founders do not have all of the alleles found in their source population. These founders become quickly different from the parental population and may form a new species, e.g. evolution of Darwin finches on Galapagos Islands which were probably derived from a few initial founders.

(vi) Population bottleneck:

It is reduction in allele frequencies caused by drastic reduction in population size called population crash e.g. decrease in cheetah population in Africa due to over-hunting. As the given gene pool is limited, population bottleneck often prevents the species to reestablish its former richness so new population has a much restricted gene pool than the larger parent population.

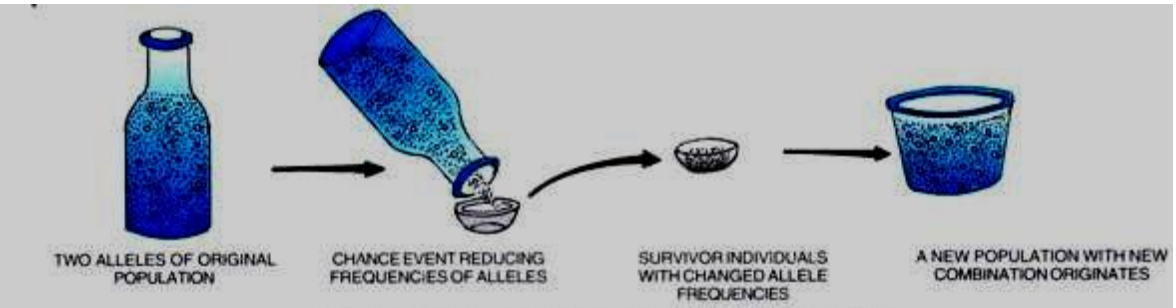


Fig. 7.47. Bottleneck effect on the frequencies of two alleles.

(D) Gene Migration (Gene Flow):

Most populations are only partially isolated from other populations of same species. Usually some migration-emigration (moving out of some individuals out of a population) or immigration (entry of some members of a population into another population of same species) occurs between the populations.

Immigration results in the addition of new alleles into the existing gene pool and changes the allele frequencies. Degree of changes in allele frequencies depends upon the differences between the genotypes of immigrants and native population.

If there is no much genetic differences, then entry of a small number of migrants will not change the allele frequencies much. However, if the populations are genetically quite different, a small amount of immigration can result in large changes in allele frequencies.

If the migrating individuals interbreed with the members of local population, called hybridization, these may bring many new alleles into the local gene pool of the host population. This is called gene migration. If the inter specific hybrids are fertile, then these may initiate a new trend in evolution which lead to formation of new species.

(E) Natural Selection:

The process by which comparatively better adapted individuals out of a heterogeneous population are favored by the Nature over the less adapted individuals is called natural selection.

Types of Natural selection:

The three different types of natural selections observed are:

1. Stabilizing or balancing selection:

It leads to the elimination of organisms having overspecialized characters and maintains homogenous population which is genetically constant. It favors the average or normal phenotypes, while eliminates the individuals with extreme expressions. In this, more individuals acquire mean character value.

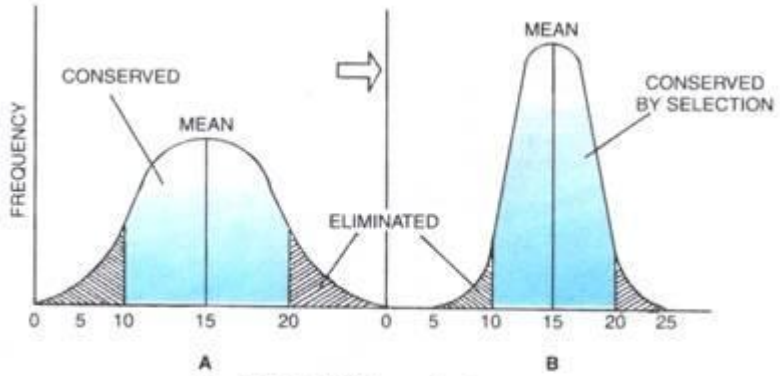


Fig. 7.49. Stabilizing selection.

It reduces variation but does not change the mean value. It results very slow rate of evolution. If we draw a graphical curve of population, it is bell-shaped. The bell-shaped curve narrows due to elimination of extreme variants.

Example:

Sickle-cell anaemia in human beings (Explained in Neo-Darwinism).

2. Directional or Progressive selection:

In this selection, the population changes towards one particular direction along with change in environment. As environment is undergoing a continuous change, the organisms having acquired new characters survive and others are eliminated gradually.

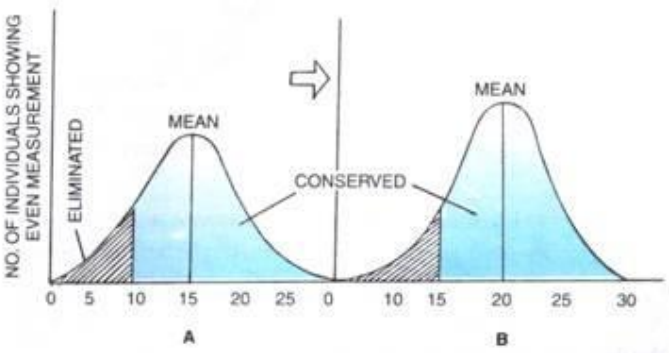


Fig. 7.50. Directional selection.

In this, individuals at one extreme (less adapted) are eliminated while individuals at other extreme (more adapted) are favored. This produces more and more adapted individuals in the population when such a selection operates for many generations. In this type of selection, more individuals acquire value other than mean character value.

Examples:

Industrials melanism (Explained in Neo-Darwinism):

In this, number of the light coloured moths {*Biston betularia*) decreased gradually while that of the melanic moths (*B. carbonaria*) increased showing directional selection.

3. Disruptive selection:

It is a type of natural selection which favors extreme expressions of certain traits to increase variance in a population. It breaks a homogeneous population into many adaptive forms. It results in balanced polymorphism.

In this type of selection, more individuals acquire peripheral character value at both ends of the distribution curve. This kind of selection is rare and eliminates most of the members with mean expression so producing two peaks in the distribution of a trait.

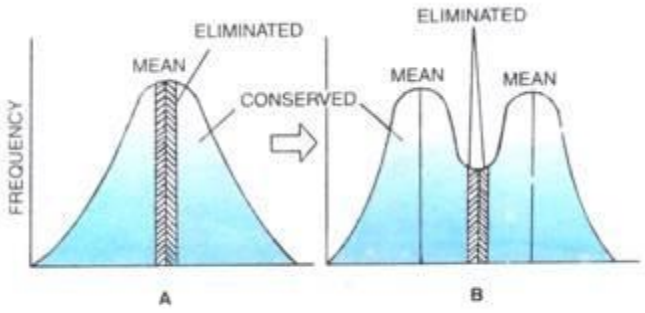


Fig. 7.51. Disruptive selection.

Example:

In sea, the three types of snails i.e., white colored; brown colored and black colored are present. The white colored snails are invisible when covered by barnacles. The black colored snails are invisible when rock is bare. But brown colored snails are eaten by predators in both the conditions. So these are eliminated gradually.

Molecular evolution:

Molecular evolution is the process of change in the sequence composition of cellular molecules such as DNA, RNA, and proteins across generations. The field of molecular evolution uses principles of evolutionary biology and population genetics to explain patterns in these changes. Major topics in molecular evolution concern the rates and impacts of single nucleotide changes, neutral evolution vs. natural selection, origins of new genes, the genetic nature of complex traits, the genetic basis of speciation, evolution of development, and ways that evolutionary forces influence genomic and phenotypic changes.

Forces in molecular evolution

Mutation

Mutations are permanent, transmissible changes to the genetic material (DNA or RNA) of a cell or virus. Mutations result from errors in DNA replication during cell division and by exposure to radiation, chemicals, and other environmental stressors, or viruses and transposable elements. Most mutations that occur are single nucleotide polymorphisms which modify single bases of the DNA sequence, resulting in point mutations. Other types of mutations modify larger segments of DNA and can cause duplications, insertions, deletions, inversions, and translocations.

Most organisms display a strong bias in the types of mutations that occur with strong influence in GC-content. Transitions (A ↔ G or C ↔ T) are more common than transversions (purine (adenine or guanine)) ↔ pyrimidine (cytosine or thymine, or in RNA, uracil) and are less likely to alter amino acid sequences of proteins.

Recombination

Recombination is a process that results in genetic exchange between chromosomes or chromosomal regions. Recombination counteracts physical linkage between adjacent genes, thereby reducing genetic hitchhiking. The resulting independent inheritance of genes results in more efficient selection, meaning that regions with higher recombination will harbor fewer detrimental mutations, more selectively favored variants, and fewer errors in replication and repair. Recombination can also generate particular types of mutations if chromosomes are misaligned.

Gene conversion

Gene conversion is a type of recombination that is the product of DNA repair where nucleotide damage is corrected using an homologous genomic region as a template. Damaged bases are first excised, the damaged strand is then aligned with an undamaged homolog, and DNA synthesis repairs the excised region using the undamaged strand as a guide. Gene conversion is often responsible for homogenizing sequences of duplicate genes over long time periods, reducing nucleotide divergence.

Genetic drift

Genetic drift is the change of allele frequencies from one generation to the next due to stochastic effects of random sampling in finite populations. Some existing variants have no effect on fitness and may increase or decrease in frequency simply due to chance. "Nearly neutral" variants whose selection coefficient is close to a threshold value of 1 / the effective population size will also be affected by chance as well as by selection and mutation. Many genomic features have been ascribed to accumulation of nearly neutral detrimental mutations as a result of small effective population sizes. With a smaller effective population size, a larger variety of mutations will behave as if they are neutral due to inefficiency of selection.

Selection

Selection occurs when organisms with greater fitness, i.e. greater ability to survive or reproduce, are favored in subsequent generations, thereby increasing the instance of underlying genetic variants in a population. Selection can be the product of natural selection, artificial selection, or sexual selection. Natural selection is any selective process that occurs due to the fitness of an organism to its environment. In contrast sexual selection is a product of mate choice and can favor the spread of genetic variants which act counter to natural selection but increase desirability to the opposite sex or increase mating success. Artificial selection, also known as selective breeding, is imposed by an outside entity, typically humans, in order to increase the frequency of desired traits.

Applications:

Molecular evolution analysis has clarified:

•the evolutionary relationships between humans and other primates;

•the origins of AIDS;

•the origin of modern humans and population migration;
•speciation events;
•genetic material exchange between species.
•origin of some deseases (cancer, etc...)

Protein evolution:

This chart compares the sequence identity of different lipase proteins throughout the human body. It demonstrates how proteins evolve, keeping some regions conserved while others change dramatically. Evolution of proteins is studied by comparing the sequences and structures of proteins from many organisms representing distinct evolutionary clades. If the sequences/structures of two proteins are similar indicating that the proteins diverged from a common origin, these proteins are called as homologous proteins. More specifically, homologous proteins that exist in two distinct species are called as orthologs. Whereas, homologous proteins encoded by the genome of a single species are called paralogs.

The phylogenetic relationships of proteins are examined by multiple sequence comparisons. Phylogenetic trees of proteins can be established by the comparison of sequence identities among protoeins. Such phylogenetic trees have established that the sequence similarities among proteins reflect closely the evolutionary relationships among organisms.

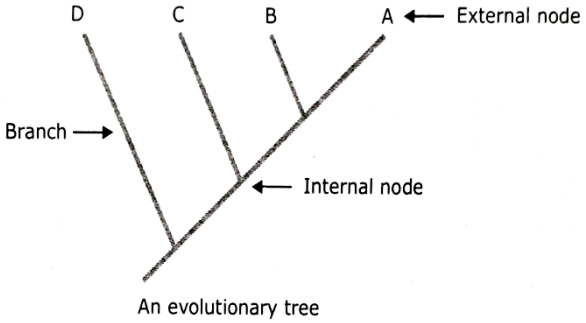
Protein evolution describes the changes over time in protein shape, function, and composition. Through quantitative analysis and experimentation, scientists have strived to understand the rate and causes of protein evolution. Using the amino acid sequences of hemoglobin and cytochrome c from multiple species, scientists were able to derive estimations of protein evolution rates. What they found was that the rates were not the same among proteins. Each protein has its own rate, and that rate is constant across phylogenies (i.e., hemoglobin does not evolve at the same rate as cytochrome c, but hemoglobins from humans, mice, etc. do have comparable rates of evolution).

Molecular phylogeny

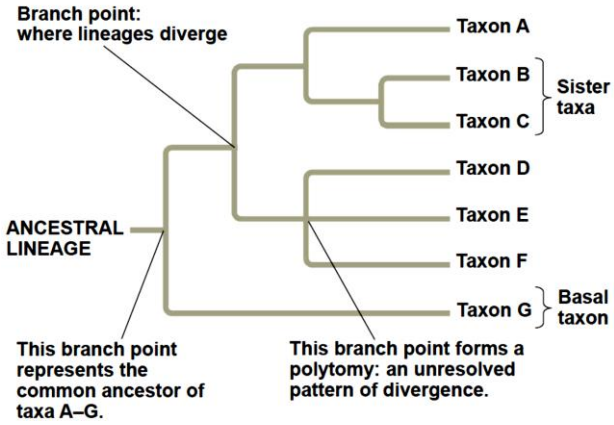
Phyogenetics is the science of estimating and analyzing evolutionary relatedness among various group of organisms. Molecular phylogenetics is the use of the structure of molecules to gain information on an organism's evolutionary relationships. Nucleic acids (DNA and RNA) and proteins are 'information molecules' in that they retain information of an organism's evolutionary history. The approach is to compare nucleic acid or protein sequences from different organisms using computer programs and estimate the evolutionary relationships based on the degree of homology between the sequences. Nucleic acids and proteins are linear molecules made of smaller units called nucleotides and amino acids, respectively. The nucleotide differences within a gene or amino acid differences within a protein reflect the evolutionary distance between two organisms. In other words, closely related organisms will exhibit fewer sequence differences than distantly related organisms.

Phylogenetic tree

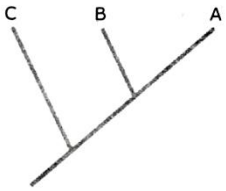
In phylogenetic studies, the most convenient way of visually presenting evolutionary relationships among a group of organisms is through illustrations called phylogenetic trees. Phylogenetic tree is represented by lines and nodes. Nodes can be internal or external (terminal). The different sequences of DNA/proteins which are compared are located at external nodes but are connected via branches to interior nodes which represent ancestral forms for two or more sequences. The terminal nodes at the tips of trees represent operational taxonomic units (OTUs). Branch defines the relationsnip between the taxa in terms of descent and ancestry. The lengths of the branches indicate the degree or diffrence between the sequences represented by the nodes. The branch lengths are proportional to the predicted evolutionary time between organisms or sequences. The branching pattern or the tree is termed as topology.



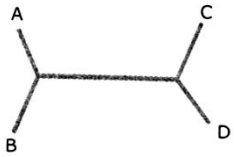
An evolutionary tree



Phylogenetic tree may be rooted or unrooted. A rooted tree infers the existence of a common ancestor and indicates the direction on the evolutionary process. A rooted tree in which every node has two descendants is called a binary tree. An unrooted tree does not infer a common ancestor and shows only the evolutionary relationships between the organisms.



Rooted tree

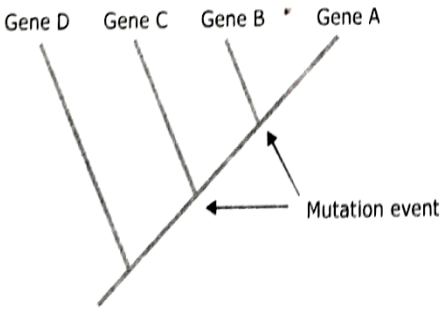


Unrooted tree

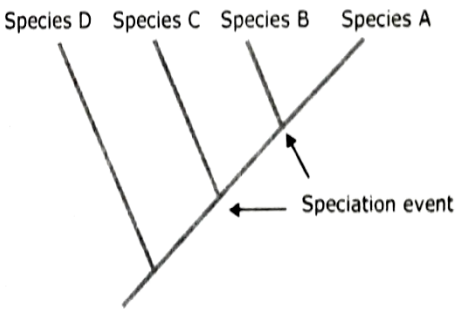
Gene trees versus Species trees

A gene tree is a model of how a gene evolves through duplication, loss, and nucleotide substitution. As a gene at a locus in the genome replicates and its copies are passed on to more than one offspring, branching points are generated in the gene tree. A species tree depicts the pattern of branching or species lineages via the process of speciation. When reproductive communities are split by speciation, the gene copies within these communities likewise are split into separate bundles of descent.

An internal node in a gene tree indicates the divergence of an ancestral gene into two genes with different DNA sequences, usually resulting from a mutation of one sort or another. An internal node in a species tree represents what is called a speclation event, whereby the population of the ancestral species splits into two groups that are no longer able to interbreed. These two events, mutation and speciation, do not always occur at the same time.

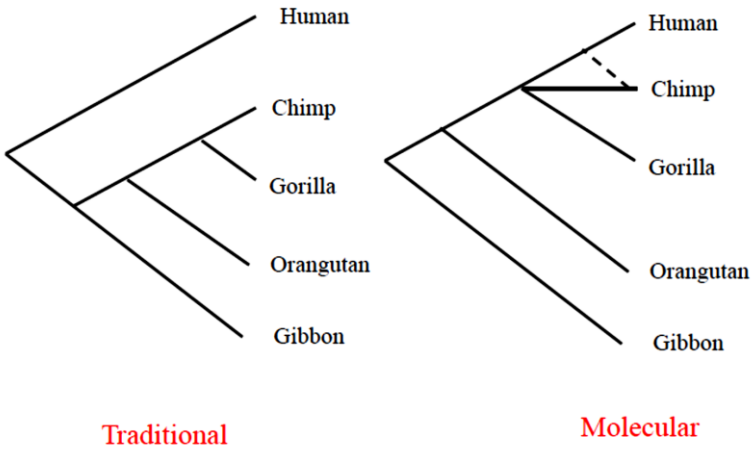


An evolutionary gene tree



An evolutionary species tree

Phylogenetic analysis using DNA sequence



Traditional Molecular

Construction of phylogenetic tree

Phylogenetic studies construct the tree like pattern that describes the evolutionary relationship between the organisms being studied. To construct a molecular phylogenetic tree it is necessary to compare nucleic acid sequence (or protein sequence). A sequence of DNA yields more phylogenetic information than protein, the nucleotide sequences a pair of homologous genes having a higher information content than the amino acid sequences of the corresponding proteins, because mutations that result in synonymous changes alter the DNA sequence but do not affect the amino acia sequences.

Many different ways of constructing phylogenetic trees from aligned sequences have been developed, and numerous programs are available that perform the operations automatically. Although some procedures actually construct the alignment and the tree simultaneously, in most cases the alignment is made first, after which the tree is calculated from it. Constructing a phylogenetic tree from molecular data is a complex process. There are four methods for tree reconstruction:

1. Distance matrix method
2. Maximum parsimony method
3. Maximum likeihood method.
4. Bayesian method

However, regardless of different methods being applied, the ultimate goal is the same, which is to obtain a best-estimated tree.

Distance based methods:

Distance-matrix methods of phylogenetic analysis explicitly rely on a measure of "genetic distance" between the sequences being classified and therefore they require an MSA(multiple sequnce alignment) as an input.

Type:

1. UPGMA
2. NJ(Neighbor Joining) etc.

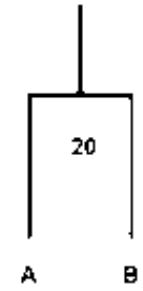
UPGMAstands for Unweighted pair group method with arithmetic mean, originally developed for numeric taxonomy in 1958 by Sokal and Michener. This method uses sequential clustering algorithm.

This method follows a clustering procedure:

- Assume that initially each species is a cluster on its own.
- Join closest 2 clusters and recalculate distance of the joint pair by taking the average.
- Repeat this process until all species are connected in a single cluster.

CONSTRUCTION OF PHYLOGENETIC TREE

A	0				
B	20	0			
C	60	50	0		
D	100	90	40	0	
E	90	80	50	30	0



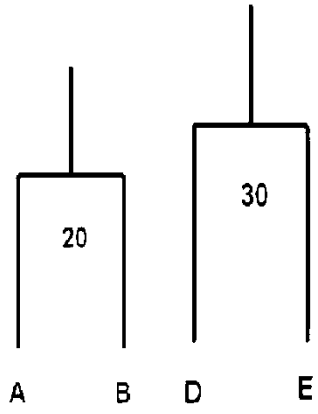
New average distance between AB and C is:

C to AB = (60 + 50) / 2 = 55

Distance between D to AB is:

D to AB = (100 + 90) / 2 = 95

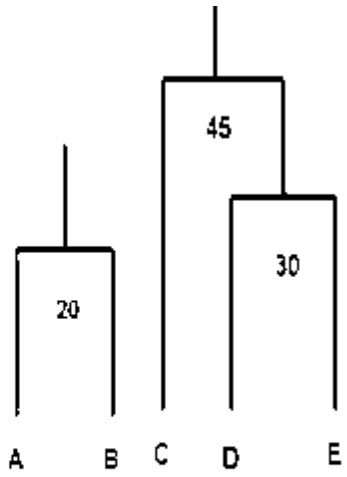
AB	0			
C	55	0		
D	95	40	0	
E	85	50	30	0



New average distance between AB and DE is:

AB to DE = (95 + 85) / 2 = 90

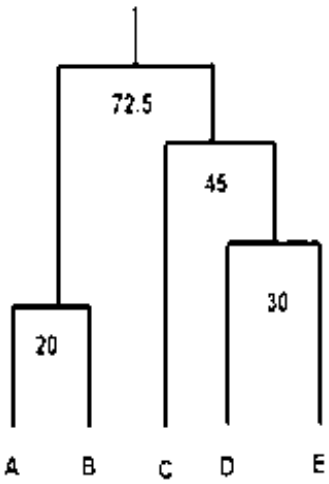
AB	0		
C	55	0	
DE	90	45	0



New Average distance between CDE and AB is:

CDE to AB = (90 + 55) / 2 = 72.5

AB	0	
CDE	72.5	0



There are only two clusters. so this completes the calculation!

Molecular clock

Molecular clock is a technique in molecular evolution to relate the time that the two species diverged to the number of molecular differences measured between the species DNA sequences or proteins. It is sometimes called a gene clock or evolutionary clock. The concept of molecular clock is based on hypothesis that DNA and protein sequences evolve at a rate that is relatively constant over time and among different organism. This constancy is used to estimate the length of time that various organisms have been diverging from one another by measuring the degree of difference between two sequences. The molecular clock hypothesis was originally proposed by researchers Emile Zuckerkandl and Linus Pauling on the basis of empirical observations, but it soon received theoretical backing when biologist Motoo Kimura developed the neutral theory of molecular evolution in 1968.

Neutral theory of molecular evolution:

The neutral theory of molecular evolution (also, simply the neutral theory of evolution) is an influential theory that was introduced by Motoo Kimura. Kimura suggested that a large fraction of new mutations do not have an effect on evolutionary fitness, so natural selection would neither favor nor disfavor them. Eventually, each of these neutral mutations would either spread throughout a population and become fixed in all of its members, or they would be lost entirely in a stochastic process called genetic drift. Kimura then showed that the rate at which neutral mutations become fixed in a population (known as the substitution rate) is equivalent to the rate of appearance or new mutations in each number of the population (the mutation rate). Provided that the mutation rate is consistent across species, the substitution rate would remain constant throughout the tree of life.

The neutral theory is the foundation or the molecular clock tecnnique, which evolutionary molecular biologists use to measure how much time has passed since species diverged from a common ancestor. A strict molecular clock based on Kimura's neutral theory is too simplistic, because rate of molecular evolution can vary significantly among organisms. Thus, researchers have undertaken efforts to retain some aspects of the original clock hypothesis while relaxing the assumption of a strictly constant rate. Such efforts have led to the development of so-called relaxed molecular clocks, which allow the molecular rate to vary among lineages, albeit in a limited manne

Calibrating the molecular clock

When using either a strict- or relaxed- clock method of genetic analysis, the most important consideration is how to calibrate the molecular clock. Assume, for example, that researches have two DNA sequences that have content difference of 5%. From this information alone, it is not possible to tell whether these sequences have diverged from each other at a rate of 1% per 1 million years over a period of 5 million years, or whether they have diverged at a five told higher rate over a period of just 1 million years. This is equivalent to trying to determine the average speed of a car merely by looking at its odometer. To deduce the average speed, one would also need to know the length of time for which the car has been travelling.

Thus, to calibrate the molecular clock, one must know the absolute age of some evolutionary divergence event, such as the split between mammals and birds. An estimate of the timing of this event can be gained by examining the fossil record, or by correlating this particular instance of evolutionary divergence with some geological event of known antiquty (Such as the formation of a mountain range that split the geographic range of a species in two, thus initiating a process of speciation). Once the evolutionary rate is calculated using a calibration, this calibration can then be applied to other organisms to estimate the timing of evolutionary events. For example, fossils suggest that the most recent common ancestor of humans and orangutans lived 13 million years ago. To calibrate the human molecular clock we can compare human and orangutans DNA sequence to determine the amount of nucleotide substitution (x) that has occurred, and then divided this figure by 13, followed by 2 to obtain a rate of substitution per million years. Hence, number of substitutions per lineage per million years = $x/2 \times 13$.

7. Let's sum up

- Evolution is change in the heritable characteristics of biological populations over successive generations. Evolutionary processes give rise to biodiversity at every level of biological organisation, including the levels of species, individual organisms, and molecules.
- In the mid-19th century, Charles Darwin formulated the scientific theory of evolution by natural selection, published in his book On the Origin of Species (1859). Evolution by natural selection is a process first demonstrated by the observation that often, more offspring are produced than can possibly survive.
- Spontaneous generation was a concept proposed by Aristotle around mid-300 B.C. which is the hypothesis that living things arise from non-living material
- Primordial soup is a hypothetical condition of the Earth's atmosphere before the emergence of life. It is a chemical environment in which the first biological molecules were formed under natural forces. According to the theory, simple organic compounds were created from non-living inorganic molecules through physical and chemical reactions on the Earth's surface.
- Early living cells were RNA life forms, self- replicating RNA covered by lipoprotein vesicles were the pre-prokaryotes, with time the proteins replaced the catalytic function of RNA, and DNA replaced the coding function of RNA, the progenitors of modern prokaryotes with DNA-RNA-protein functioning types evolved.
- The eukaryotic cell seems to have evolved from a symbiotic community of prokaryotic cells. DNA-bearing organelles like the mitochondria and the chloroplasts are descended from ancient symbiotic oxygen-breathing proteobacteria and cyanobacteria, respectively, which were endosymbiosed by an ancestral archaean prokaryote.
- The geologic time scale is a system of chronological dating that relates geological strata (stratigraphy) to time. It is used by geologists, paleontologists, and other Earth scientists to describe the timing and relationships of events that have occurred during Earth's history. Geologic time is divided into Eon, Era, Peroids and Epoch.
- Genetic variation is brought about, fundamentally, by mutation, which is a permanent change in the chemical structure of chromosomes. Genetic recombination also produces changes within alleles.
- Mendel assumed that the unit of hereditary characters is the factors of determiners which occur in pairs. One of each comes from the mother while the other comes from the father. The unit character or factor is now called as gene..
- Crossing of F_1 organism with either of the parents is called back cross. When an organism is crossed with other organism having recessive phenotypic is called test cross.
- Some characters are controlled by several genes. They are known as quantitative characters or polygenic characters. The mode of inheritance of polygenic characters is termed as polygenic inheritance or quantitative inheritance. Since in polygenic inheritance several genes (factors) are involved, it is also known as multiple factor inheritance.
- Linked genes Linked genes are those genes which occur on the same chromosome while unlinked genes are the ones found on different chromosomes. Genetic recombination is the production of offspring with combinations of traits that differ from those found in either parent.
- Epistasis is the phenomenon where the effect of one gene (locus) is dependent on the presence of one or more 'modifier genes', i.e. the genetic background. Originally the term meant that the phenotypic effect of one gene is masked by a different gene (locus).
- Gene–environment interaction is when two different genotypes respond to environmental variation in different ways. A norm of reaction is a graph that shows the relationship between genes and environmental factors when phenotypic differences are continuous.
- Heritability is a statistic used in the fields of breeding and genetics that estimates the degree of variation in a phenotypic trait in a population that is due to genetic variation between

individuals in that population. Heritability is estimated by comparing individual phenotypic variation among related individuals in a population.

- Population genetics is a subfield of genetics that deals with genetic differences within and between populations, and is a part of evolutionary biology. Hardy and Weinberg each published papers describing a mathematical relationship between allele frequencies and genotype frequencies. This relationship, now called the Hardy–Weinberg principle**,** allows us to predict a population's genotype frequencies from its allele frequencies.
- Molecular evolution is the process of change in the sequence composition of cellular molecules such as DNA, RNA, and proteins across generations. The field of molecular evolution uses principles of evolutionary biology and population genetics to explain patterns in these changes.
- The molecular clock is figurative term for a technique that uses the mutation rate of biomolecules to deduce the time in prehistory when two or more life forms diverged. The biomolecular data used for such calculations are usually nucleotide sequences for DNA or amino acid sequences for proteins.

8. Suggested readings

1. Strickberger Monroe W , Brian K. Hall, BenediktHallgrimssonStrickberger's Evolution (5th ed.) 2014 Jones & Bartlett Learning.
2. Futuyma., D. Evolution. 2015. (3rd Ed.) Sinauer Associates
3. Rastogi V. B. (2014) Organic Evolution Medtec; 1st Edition edition
4. Kumar Pranav & Mina Usha (2011) Life Sciences: Fundamentals and Practice (part II), Pathfinder Academy Private Limited
5. http://www.biologydiscussion.com/
6. https://en.wikipedia.org/wiki/

9. Assignment

1. What is Coacervate?
2. Differentiate between ortholog and paralog.
3. What do you mean by bottle-neck effect?
4. Write down the full form of UGMA.
5. What is prebiotic soup? Describe Urey-Miller experiment of chemical evolution.
6. Explain major events in the geological time scale with major plant groups.
7. Define geological time scale.
8. How are heredity and natural selection involved in the process of evolution?
9. In a population, the genotype frequencies are AA: 0.64, Aa: 0.20 and aa: 0.16. What are the frequencies of A and a allele?
10. What is gene pool?
11. What causes variation to occur?
12. Why modern theory of evolution is called synthetic theory?
13. Briefly describe Oparine and Handle hypothesis of origin of life.
14. Write a short note on RNA world hypothesis.
15. In which time flowering angiosperms are evolved?
16. What is molecular clock?
17. Define serial endosymbiotic theory.
18. According to endosymbiotic theory which organism is the supposed ancestor of mitochondria?
19. Write a explanatory note on endosymbiotic theory of eukaryotic cell evolution.
20. When the organisms with aerobic metabolism originated?

21. Which geological period is known as “Age of ferns”?
22. What is mean by “Survival of the Fittest”?
23. How does the first cell evolved?
24. Why test cross is important?
25. Mention the features of polygenic traits.
26. Distinguish between additive and dominanace.
27. What is heterozygotic epistasis?
28. How do you construct a phylogenetic tree using UPGMA distance matrix.
29. What is binary tree?
30. Why sequence of DNA yields more phylogenetic information than protein or amino acid sequence?

All the materials are self writing and collected from ebook, journals and websites.

BOTANY

POST GRADUATE DEGREE PROGRAMME
(CBCS CURRICULUM)

SEMESTER: IV

PAPER: BOSCT 4.2

Immunology

Directorate of Open and Distance Learning
UNIVERSITY OF KALYANI
Kalyani, Nadia
West Bengal

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Open & Distance Learning, University of Kalyani
[redacted]
Phone : (033) 2502 2212, 2502 2213
Website : www.klyuniv.ac.in

POST GRADUATE DEGREE PROGRAMME
(CBCS)
IN
BOTANY

SEMESTER - IV

Course: BOSCT4.2

(IMMUNOLOGY)

Self-Learning Material

DIRECTORATE OF OPEN AND DISTANCE LEARNING
UNIVERSITY OF KALYANI
KALYANI - 741 235,
WEST BENGAL,INDIA

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May, 2020

Directorate of Open and Distance Learning, University of Kalyani

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Director's Message

Satisfying the varied needs of distance learners, overcoming the obstacle of distance and reaching the unreached students are the threefold functions catered by Open and Distance Learning (ODL) systems. The onus lies on writers, editors, production professionals and other personnel involved in the process to overcome the challenges inherent to curriculum design and production of relevant Self Learning Materials (SLMS). At the University of Kalyani a dedicated team under the able guidance of the Hon'ble Vice-Chancellor has invested its best efforts, professionally and in keeping with the demands of Post Graduate CBCS Programmes in Distance Mode to devise a self-sufficient curriculum for each course offered by the Directorate of Open and Distance Learning (DODL), University of Kalyani.

Development of printed SLMS for students admitted to the DODL within a limited time to cater to the academic requirements of the Course as per standards set by Distance Education Bureau of the University Grants Commission, New Delhi, India under Open and Distance Mode UGC Regulations, 2017 had been our endeavour. We are happy to have achieved our goal.

Utmost care and precision have been ensured in the development of the SLMS, making them useful to the learners, besides avoiding errors as far as practicable. Further suggestions from the stakeholders in this would be welcome.

During the production-process of the SLMS, the team continuously received positive stimulations and feedback from Professor (Dr.) Sankar Kumar Ghosh, Hon'ble Vice- Chancellor, University of Kalyani, who kindly accorded directions, encouragements and suggestions, offered constructive criticism to develop it within proper requirements. We gracefully, acknowledge his inspiration and guidance.

Sincere gratitude is due to the respective chairpersons as well as each and every member of PGBOS (DODL), University of Kalyani. Heartfelt thanks are also due to the Course Writers-faculty members at the DODL, subject-experts serving at University Post Graduate departments and also to the authors and academicians whose academic contributions have enriched the SLMS. We humbly acknowledge their valuable academic contributions. I would especially like to convey gratitude to all other University dignitaries and personnel involved either at the conceptual or operational level of the DODL of University of Kalyani.

Their persistent and co-ordinated efforts have resulted in the compilation of comprehensive, learner-friendly, flexible texts that meet the curriculum requirements of the Post Graduate Programme through Distance Mode.

All the Self Learning Materials are self-writing and collected from e-book, journals and websites.

Prof Manas Mohan Adhikary
Director
Directorate of Open and DistanceLearning
University of Kalyani

COURSE –BOSCT4.2

IMMUNOLOGY
(Full Marks – 40)

TEE points:40 **Classes/Semister:40**

Course	Group	Details Contents Structure		Study hour
BOSCT4.2	Immunology	**Unit1.Introduction; Cells and organs of Immune Syestem**	Overview of the immune system, cells and organs of Immune system-Hematopoietic stem cells,stromal cells,hematopoietic growth factors ,lymphoid organs(primary and secondary) and cells, mononuclear cells,granulocytic cells,mast cells,dendritic cells-characteristics and function	1
		Unit2. Types of Immunity and Antigens	Types of immunity: (i) Innate immunity and adaptive immunity, Major Histocompatibility Complex (MHC) and their role in antigen presentation, cytokine. Antigens: Chemical nature, antigenicity, immunogenicity, hapten, epitops, mitogens (definition, properties, examples); adjuvant (definition, examples, function).	1
		Unit3. Immunoglobulins and Complement	Immunoglobulins: Types, monoclonal antibody (definition and characteristics). Complement: Components, function, mode of action.	1

		Unit4. Antigen and Antibody interactions and Hypersensitivity	Antigen – Antibody interactions: Agglutination, precipitation, immunodiffusion, immuunoelectrophoresis. Hypersensitivity: Definition, types, examples	1
		Unit5. Vaccines	Vaccines: Active and passive immunization (definition, characteristics, functions, examples).	1
		Unit6. Diagnostic immunology	Diagnostic immunology: ELISA, RIA, Immunofluorescence, Flow cytometry, Fluorescence activated cell sorting (FACS).	1

Content

COURSE –BOSCT4.2
IMMUNOLOGY

Soft Core Theory Paper Credits:2

Content Structure

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1. Introduction
2. Course Objective
3. **Introduction; Cells and organs of Immune Syestem:** Overview of the immune system, cells and organs of Immune system-Hematopoietic stem cells,stromal cells,hematopoietic growth factors ,lymphoid organs(primary and secondary) and cells, mononuclear cells,granulocytic cells,mast cells,dendritic cells-characteristics and function
4. **Types of Immunity and Antigens:** Types of immunity: (i) Innate immunity and adaptive immunity, Major Histocompatibility Complex (MHC) and their role in antigen presentation, cytokine. Antigens: Chemical nature, antigenicity, immunogenicity, hapten, epitops, mitogens (definition, properties, examples); adjuvant (definition, examples, function).
5. **Immunoglobulins and Complement:** Immunoglobulins: Types, monoclonal antibody (definition and characteristics). Complement: Components, function, mode of action.
6. **Antigen and Antibody interactions and Hypersensitivity:** Antigen – Antibody interactions: Agglutination, precipitation, immunodiffusion, immuunoelectrophoresis. Hypersensitivity: Definition, types, examples
7. **Vaccines:** Vaccines: Active and passive immunization (definition, characteristics, functions, examples).
8. **Diagnostic immunology:** Diagnostic immunology: ELISA, RIA, Immunofluorescence, Flow cytometry, Fluorescence activated cell sorting (FACS)
9. Suggest reading
10. Assignment

1. Introduction

Immunology is the study of the immune system and is a very important branch of the medical and biological sciences. The immune system protects us from infection through various lines of defence. If the immune system is not functioning as it should, it can result in disease, such as autoimmunity, allergy and cancer. It is also now becoming clear that immune responses contribute to the development of many common disorders not traditionally viewed as immunologic, including metabolic, cardiovascular, and neurodegenerative conditions such as Alzheimer's.

By the mid 20th century, coincident with the molecular revolution, immunology grew rapidly including discoveries about tumor regulation, allergies, anaphylaxis, and the structure and synthesis of immunoglobulins. In general, the **immune system** of higher organisms can be broken down into two primary response systems that work together to create immunity. The two primary response systems are innate and adaptive immune responses, with the latter further divided into cell-mediated and antibody-mediated responses. The **cell-mediated response** is produced when a subset of sensitized white blood cells or **lymphocytes** directly attack material (e.g., usually a cell or a virus) that has been determined to be foreign to the body. The **antibody-mediated response** involves the transformation of a subset of lymphocytes into cells that produce and secrete specific antibodies against the foreign material.

2. Course Objective

A student who has completed the course should have solid knowledge of:

1.Explain the ideas of Immunology.what are immunologic assays important diagnostics.

2. Illustrate the types of immunology and the role of antigens

3. To know about Hypersensitivity,definition and examples

4.To understand what are the main organs of immune system and the function of each organ in the immune system.

✓ *Part- 1:Introduction: overview of the immune system:*

The immune system is a host defence system comprising many biological structures and processes within an organism that protects against disease. To function properly, an immune system must detect a wide variety of agents, known as pathogens, from viruses to parasitic worms, and distinguish them from the organism's own healthy tissue.

In many species, there are two major subsystems of the immune system: the innate immune system and the adaptive immune system. Both subsystems use humoral immunity and cell-mediated immunity to perform their functions. In humans, the blood–brain barrier, blood–cerebrospinal fluid barrier, and similar fluid–brain barriers separate the peripheral immune system from the neuroimmune system, which protects the brain.

Pathogens can rapidly evolve and adapt, and thereby avoid detection and neutralization by the immune system; however, multiple defense mechanisms have also evolved to recognize and neutralize pathogens. Even simple unicellular organisms such as bacteria possess a rudimentary immune system in the form of enzymes that protect against bacteriophage infections. Other basic immune mechanisms evolved in ancient eukaryotes and remain in their modern descendants, such as plants and invertebrates. These mechanisms include phagocytosis, antimicrobial peptides called defensins, and the complement system. Jawed vertebrates, including humans, have even more sophisticated defence mechanisms, including the ability to adapt over time to recognize specific pathogens more efficiently. Adaptive (or acquired) immunity creates immunological memory after an initial response to a specific pathogen, leading to an enhanced response to subsequent encounters with that same pathogen. This process of acquired immunity is the basis of vaccination.

✓ ***Part- 2: Cells and organs of Immune system:***

i. **Hematopoietic stem cells:** All functionally specialized, mature blood cells (red blood cells, granulocytes, macrophages, dendritic cells, and lymphocytes) arise from a single cell type, the hematopoietic stem cell (HSC). The process by which HSCs differentiate into mature blood cells is called hematopoiesis. Two primary lymphoid organs are responsible for the development of stem cells into mature immune cells: the bone marrow, where HSCs reside and give rise to all cell types; and the thymus, where T cells complete their maturation.HSCs are rare—fewerthan one HSC is present per 5 $x10^4$ cells in the bone marrow—and their numbers are strictly controlled by a balance of cell division, death, and differentiation. Under conditions where the immune system is not being challenged by a pathogen (steady state or homeostatic conditions), most HSCs are quiescent. A small number divide, generating daughter cells. Some daughter cells retain the stem-cell characteristics of the mother cell—that is, they remain self-renewing and able to give rise to all blood cell types. Other daughter cells differentiate into progenitor cells that lose their self-renewal capacity and become progressively more committed tparticular blood cell lineage. As an organism ages, the number of HSCs decreases, demonstrating that there are limits to an HSC's self-renewal potential. When there is an increased demand for hematopoiesis (e.g., during an infection or after chemotherapy), HSCs display an enormous proliferative capacity. This can be demonstrated inmice whose hematopoietic systems have been completely destroyed by a lethal dose of x-rays (950 rads). Such irradiated mice die within 10 days unless they are infused with normal bone marrow cells from a genetically identical mouse.

Although a normal mouse has 3 108 bone marrow cells, infusion of only 104 to 105 bone marrow cells from a donor is sufficient to completely restore the hematopoietic system, which demonstrates the enormous capacity of HSCs for self-renewal. Our ability to identify and purify this tiny subpopulation has improved considerably, and investigators can now theoretically rescue irradiated animals with just a few purified stem cells, which give rise to progenitors that proliferate rapidly and populate the blood system relatively quickly.

Hematopoiesis Is the Process by Which Hematopoietic Stem Cells Develop into Mature Blood Cells. An HSC that is induced to differentiate (undergo hematopoiesis) loses its self-

renewal capacity and makes one of two broad lineage commitment choices (see Figure 2-1). It can become a common myeloid-erythroid progenitor (CMP), which gives rise to all red blood cells (the erythroid lineage), granulocytes, monocytes, and macrophages (the myeloid lineage), or it can become a common lymphoid progenitor (CLP), which gives rise to B lymphocytes, T lymphocytes, and NK cells. Myeloid cells and NK cells are members of the innate immune system, and are the first cells to respond to infection or other insults. Lymphocytes are members of the adaptive immune response and generate a refined antigen specific immune response that also gives rise to immune memory. As HSCs progress along their chosen lineages, they lose the capacity to contribute to other cellular lineages. Interestingly, both myeloid and lymphoid lineages give rise to dendritic cells, antigen-presenting cells with diverse features and functions that play an important role in initiating adaptive immune responses. The concentration and frequency of immune cells in blood are listedin Table 2-1.

TABLE 2-1 Concentration and frequency of cells in human blood

Cell type	Cells/mm^3	Total leukocytes (%)
Red blood cells	5.0×10^6	
Platelets	2.5×10^5	
Leukocytes	7.3×10^3	
Neutrophil	$3.7–5.1 \times 10^3$	50–70
Lymphocyte	$1.5–3.0 \times 10^3$	20–40
Monocyte	$1–4.4 \times 10^2$	1–6
Eosinophil	$1–2.2 \times 10^2$	1–3
Basophil	$<1.3 \times 10^2$	<1

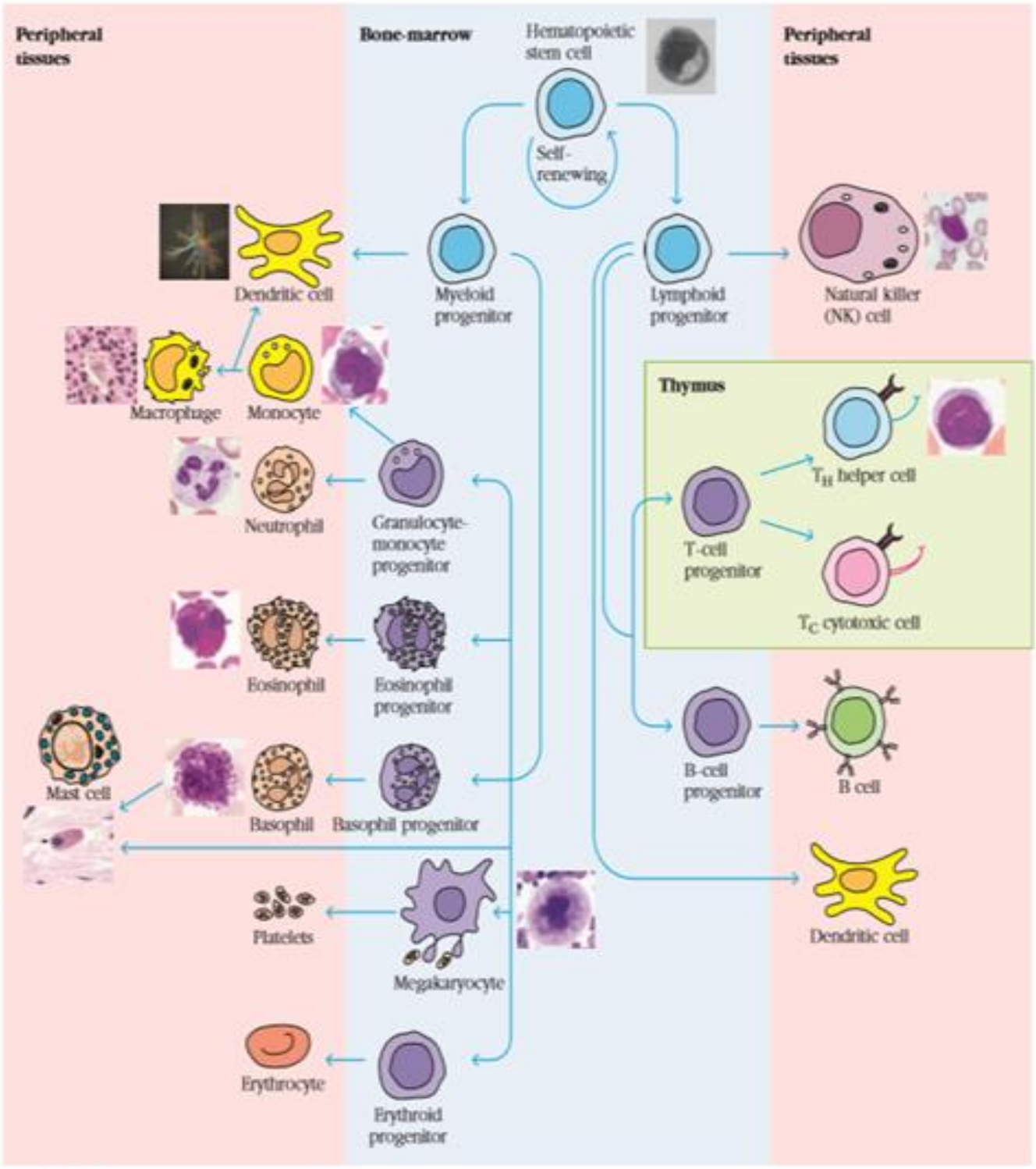
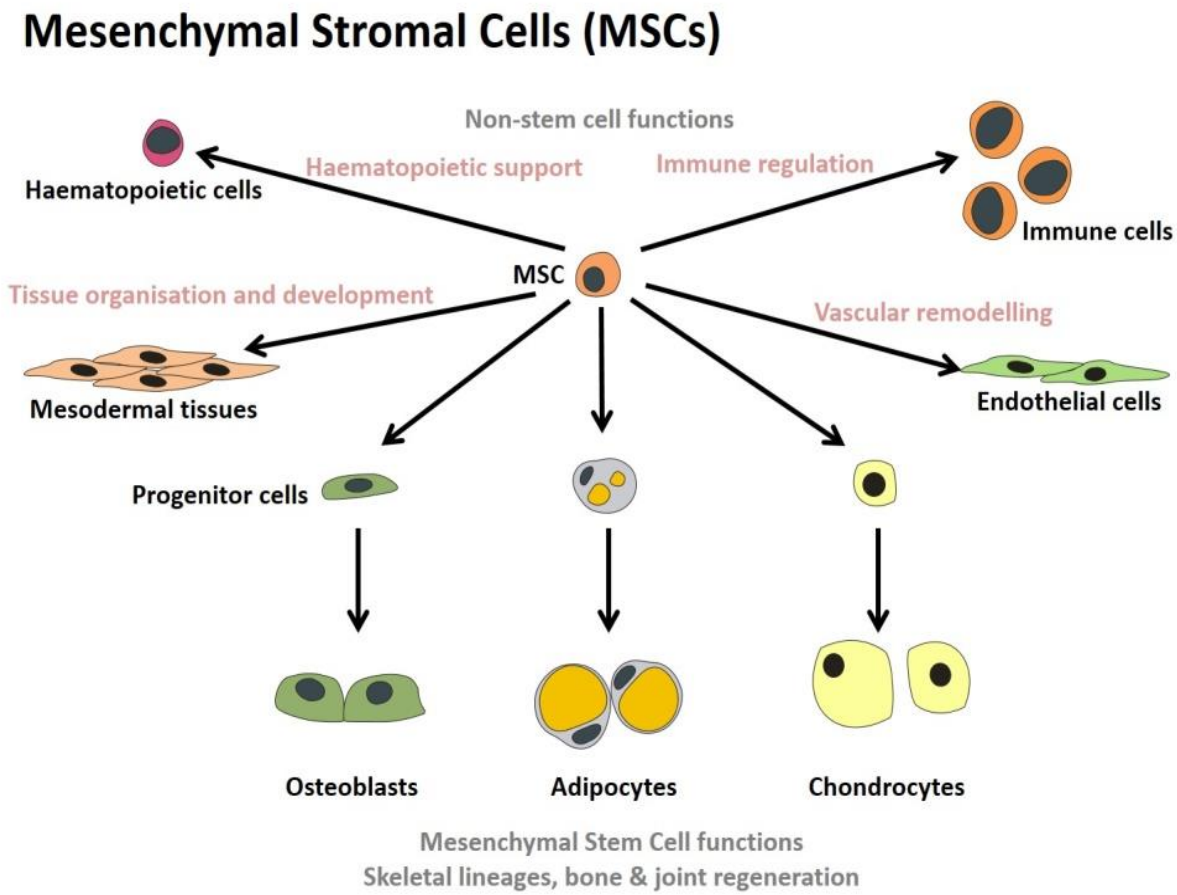


FIGURE 2-1 Hematopoiesis. Self-renewing hematopoietic stem cells give rise to lymphoid and myeloid progenitors. Most immune cells mature in the bone marrow and then travel to peripheral organs via the blood. Some, including mast cells and macrophages, undergo further maturation outside the bone marrow. T cells develop to maturity in the thymus.

ii. **Stromal cell:**

Stromal cells are connective tissue cells of any organ, for example in the uterine mucosa (endometrium), prostate, bone marrow, lymph node and the ovary. They are cells that support the function of the parenchymal cells of that organ. The most common stromal cells include fibroblasts and pericytes. The term stromal comes from Latin stromat-, "bed covering", and Ancient Greek strôma, "bed". The interaction between stromal cells and tumor cells is known

to play a major role in cancer growth and progression. In addition, by regulating local



cytokine networks (e.g. M-CSF, LIF), bone marrow stromal cells have been described to be involved in human haematopoiesis and inflammatory processes. Stromal cells (in the dermis layer) adjacent to the epidermis (the top layer of the skin) release growth factors that promote cell division. This keeps the epidermis regenerating from the bottom while the top layer of cells on the epidermis are constantly being "sloughed" off the body. Additionally, stromal cells play a role in inflammation responses, and controlling the amount of cells accumulating at an inflamed region of tissue. Certain types of skin cancers (basal cell carcinomas) cannot spread throughout the body because the cancer cells require nearby stromal cells to continue their division. The loss of these stromal growth factors when the cancer moves throughout the body prevents the cancer from invading other organs.Stroma is made up of the non-malignant cells, but can provide an extracellular matrix on which tumor cells can grow. Stromal cells may also limit T-cell proliferation via nitric oxide production, hindering immune capability.

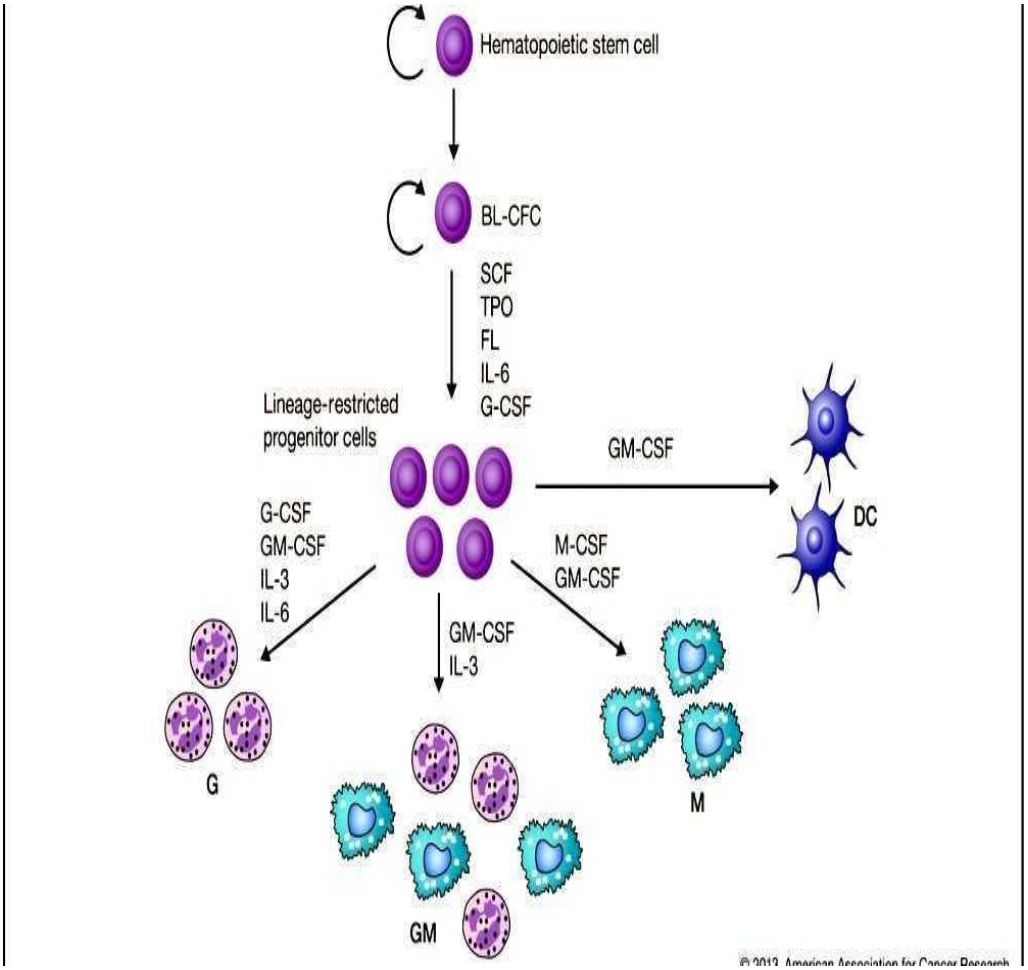
iii. **Hematopoietic growth factors:**

Hematopoietic growth factors are a family of cytokines that interact with specificreceptors on hematopoietic cells. Thesemolecules regulate the functional activation of the specific cells with which theyinteract and are required for the survival,proliferation, and differentiation of hematopoietic progenitors. The development of recombinant DNA technologyhas made it possible to synthesize and purify pharmacologic doses of a variety ofhematopoietic growth factors. A numberof growth factors have been studied inclinical trials since the mid-1980s, including granulocyte colony-stimulatingfactor (G-CSF), granulocyte-macrophagecolony-stimulating factor (GM-CSF),macrophage colony-stimulating factor(M-CSF), erythropoietin (EPO), interleukin-3 (IL-3), and, more recently, themolecule putatively thought to representthrombopoietin.

G-CSF:G-CSF acts primarily on the neutrophil component of the blood. Its action occurs by a variety of mechanisms, including stimulation of granulocyte colonies, differentiation of progenitor cells toward neutrophil lineage, and stimulation of neutrophil maturation. Overall, it increases the number of neutrophils capable of fighting bacteria. G-CSF is available in various formulations throughout the world. In the United States, it is available as filgrastim. G-CSF can be administered subcutaneously or intravenously. Depending on the specific use, the dose ranges from 5 to 10 µg/kg per day. It should not be administered 24 hours prior to or 24 hours after chemotherapy. G-CSF is usually continued until the absolute neutrophil count has been greater than 500 cells/mm3 (500,000 cells/mL) for at least 3 days. G-CSF is clinically indicated for use in:

- Chemotherapy-induced neutropenia
- Collection of stem cells for transplantation
- Bone marrow or peripheral stem cell transplantation
- Congenital neutropenia

The major side effect of G-CSF is bone pain (due to the expansion of the cell population within the marrow). Transient minor side effects that have been reported with relative frequency include fever, hyperuricemia, and skin rash. Rarely, severe reactions such as anaphylaxis, capillary leak syndrome, and diffuse alveolar haemorrhage have been reported, although a causal relationship between these reactions and filgrastim administration is yet to be determined.

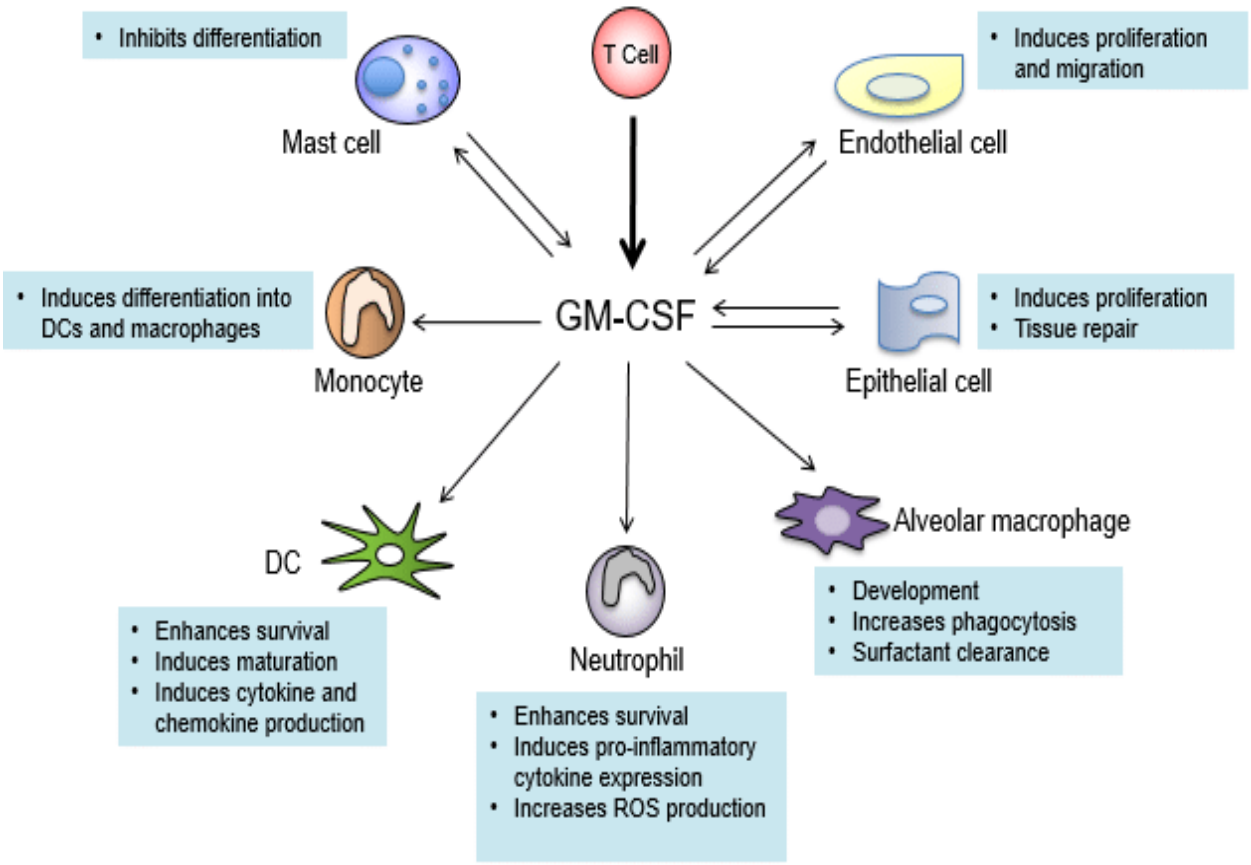


GM-CSF:

GM-CSF exerts its effect via stimulation of colonies containing neutrophils, eosinophils, and monocytes (Fig 1). In the clinical setting, the effects of GM-CSF include increasing the number of neutrophils, eosinophils, and monocytes and improving the function of mature neutrophils, eosinophils, and monocytes. GM-CSF is available as sargramostim in the United States. The dose of GM-CSF varies from 250 to 500 µg/m2, depending on the specific use.5 It is approved for use in:

- Chemotherapy-induced neutropenia
- Bone marrow or peripheral stem cell transplantation
- Collection of stem cells for transplantation

GM-CSF has many side effects similar to GCSF, although the frequency of these may be higher due to its effect on inflammatory cytokines. These side effects include skin rash, diarrhoea, fever, malaise, chills, and headaches.

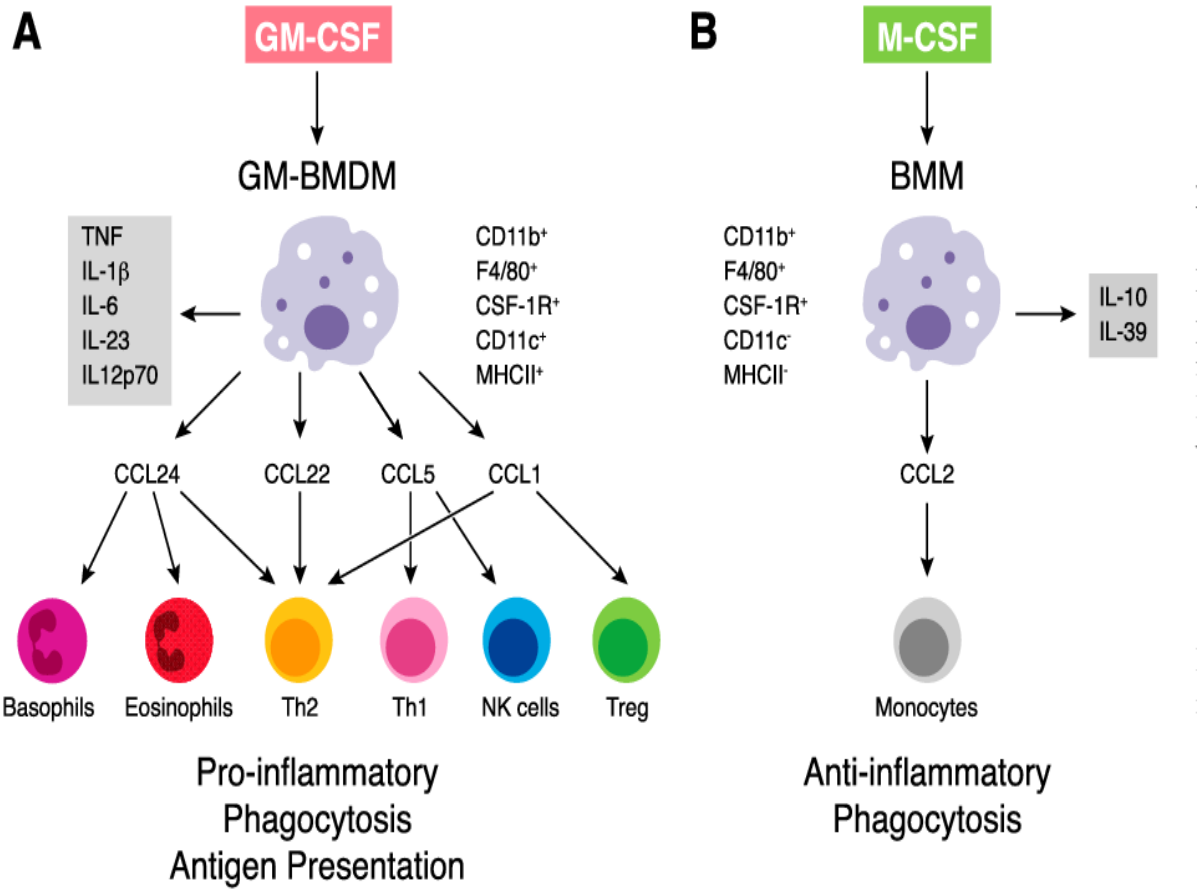


M-CSF: Macrophage colony-stimulating factor (M-CSF), is a secreted cytokine which causes hematopoietic stem cells to differentiate into macrophages or other related cell types. Eukaryotic cells also produce M-CSF in order to combat intercellular viral infection. It is one of the three experimentally described colony-stimulating factors. M-CSF binds to the colony stimulating factor 1 receptor. It may also be involved in development of the placenta.

M-CSF is a hematopoietic growth factor that is involved in the proliferation, differentiation, and survival of monocytes, macrophages, and bone marrow progenitor cells. M-CSF affects macrophages and monocytes in several ways, including stimulating increased phagocytic and chemotactic activity, and increased tumour cell cytotoxicity. The role of M-CSF is not only restricted to the monocyte/macrophage cell lineage. By interacting with its membrane receptor (CSF1R or M-CSF-R encoded by the c-fms proto-oncogene), M-CSF also modulates the proliferation of earlier hematopoietic progenitors and influence numerous physiological

processes involved in immunology, metabolism, fertility and pregnancy. M-CSF released by osteoblasts (as a result of endocrine stimulation by parathyroid hormone) exerts paracrine effects on osteoclasts. M-CSF binds to receptors on osteoclasts inducing differentiation, and ultimately leading to increased plasma calcium levels—through the resorption (breakdown) of bone. Additionally, high levels of CSF-1 expression are observed in the endometrial epithelium of the pregnant uterus as well as high levels of its receptor CSF1R in the placental trophoblast. Studies have shown that activation of trophoblastic CSF1R by local high levels of CSF-1 is essential for normal embryonic implantation and placental development. More recently, it was discovered that CSF-1 and its receptor CSF1R are implicated in the mammary gland during normal development and neoplastic growth.

Locally produced M-CSF in the vessel wall contributes to the development and progression of atherosclerosis. M-CSF has been described to play a role in renal pathology including acute kidney injury and chronic kidney failure. The chronic activation of monocytes can lead to multiple metabolic, hematologic and immunologic abnormalities in patients with chronic kidney failure. In the context of acute kidney injury, M-CSF has been implicated in promoting repair following injury, but also been described in anopposing role, driving proliferation of a pro-inflammatory macrophage phenotype.



Erythropoietin (EPO): Erythropoietin stimulates stem cells toward the production of RBCs. Its clinical result is usually not seen before 7 days after initiating therapy and may take as long as 14 days. It can be administered intravenously, intramuscularly, or subcutaneously. Prior to initiating therapy, one should ascertain that the patient has adequate iron stores to sustain the increased haemoglobin production. Erythropoietin is indicated for treating patients with anaemia from a variety of causes, including:

- ■chronic renal failure (with or without dialysis)
- ■ AIDS
- ■ Chemotherapy
- ■ Neonatal anaemia due to prematurity
- ■ Rheumatoid arthritis

It is also indicated to prevent or reduce the need for allogeneic blood transfusions in surgical patients. The dose of erythropoietin ranges from 50-150U/kg 3 times weekly for most patients, although in chemotherapy patients and those undergoing elective surgery, the dose can be increased to as high as 300 U/kg. Studies have demonstrated that erythropoietin can be effective at 40,000 U once a week. This dose schedule has received approval from the Food and Drug Administration. Once the haematocrit has reached the 30% to 36% (0.30-0.36) range, the erythropoietin should be reduced to a maintenance dose. The side effects of erythropoietin are minimal with the predominantly reported adverse events being bone pain, headaches, hypertension, and, rarely,thrombocytosis with venous fistula occlusion.

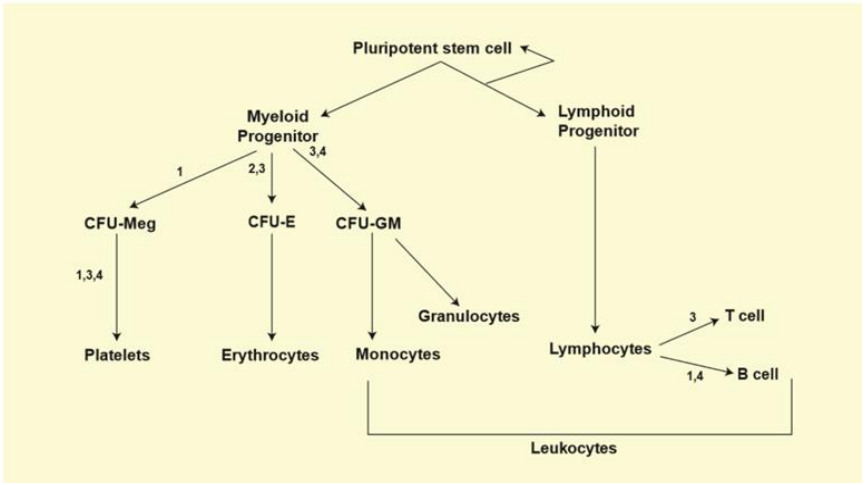


Fig 1. Differentiation of the pluripotent stem cell with the major sites of action of the various growth factors. CFU, colony-forming units; CSF, colony-stimulating factor; E, erythrocyte; G, granulucyte; GM, granuloctye-macrophage; IL, interleukin; Meg, megakaryocyte; 1, IL-11; 2, erythropoietin; 3, GM-CSF; 4, G-CSF.

Hematopoietic Growth Factors Currently Approved for Clinical Use

Growth Factor	Synonyms	Indications
Granulocyte colony-stimulating factor	G-CSF, rG-CSF, rhG-CSF, filgrastim	Chemotherapy-induced neutropenia; stem cell collection for transplantation; bone marrow and/or peripheral stem cell transplant; congenital neutropenia, idiopathic neutropenia, cyclic neutropenia
Granulocye-macrophage colony-stimulating factor	GM-CSF, sargramostim, rGM-CSF, rhGM-CSF	Chemotherapy-induced neutropenia; stem cell collection for transplantation; bone marrow and/or peripheral stem cell transplant
Erythropoietin	Epoetin alfa, epoetin beta	Anemia associated with chronic renal failure, chemotherapy, AIDS, prematurity, rheumatoid arthritis, and prior to elective surgery to reduce need for allogeneic blood transfusions
Oprelvekin interleukin-11	rhIL-11, rIL-11, recombinant human	Prevention of chemotherapy-induced severe thrombocytopenia

CSF indicates colony-stimulating factor; G, granuloctye; GM, granuloctye-macrophage; IL, interleukin.

iv. **Lymphoid organs:**

Lymphoid organs, such as the spleen, thymus, and bone marrow, are innervated. This fact indicates that the CNS is in permanent contact with the IS. In addition, neurotransmitters and neuropeptides regulate inflammation and immunity.The mediators involved may stimulate or inhibit immune and inflammatory processes. Substance P, neurokinin A and B (collectively known as tachykinins), calcitonin gene-related peptide (CGRP), and vasoactive intestinal peptide (VIP) are proinflammatory mediators capable of eliciting an inflammatory response. These mediators also enhance various immune responses. In contrast, somatostatin and galanin are anti-inflammatory and immunosuppressive mediators.

➢ Primary Lymphoid Organs—Where Immune Cells Develop:

The ability of any stem cell to self-renew and differentiate depends on the structural organization and cellular function of specialized anatomic microenvironments known as stem cell niches. These sequestered regions are typically populated by a supportive network of stromal cells. Stem cell niche stromal cells express soluble and membrane-bound proteins that regulate cell survival, proliferation, differentiation, and trafficking. The organs that have microenvironments that support the differentiation of hematopoietic stem cells actually change over the course of embryonic development. However, by mid to late gestation, HSCs take up residence in the bonemarrow, which remains the primary site of haematopoiesis throughout adult life. The bone marrow supports the maturation of all erythroid and myeloid cells and, in humans and mice, the maturation of B lymphocytes (as described in Chapter 10). HSCs are also found in blood and may naturally recirculate between the bone marrow and other tissues. This observation has simplified the process used to transplant blood cell progenitors from donors into patients who are deficient (e.g., patients who have undergone chemotherapy). Whereas once it was always necessary to aspirate bone marrow from the donor—a painful process that requires anesthesia—it is now sometimes possible to use enriched hematopoietic precursors from donor blood, which is much more easily obtained. Unlike B lymphocytes, T lymphocytes do not complete their maturation in the bone marrow. T lymphocyte precursors need to leave the bone marrow and travel to the unique microenvironments providedby the other primary lymphoid organ, the thymus, in order to develop into functional cells.

- ***The Bone Marrow Provides Niches for Hematopoietic Stem Cells to Self-Renew and Differentiate into Myeloid Cells and B Lymphocytes:***

The bone marrow is a primary lymphoid organ that supports self-renewal and differentiation of hematopoietic stem cells (HSCs) into mature blood cells. Although all bones contain marrow, the long bones (femur, humerus), hip bones (ileum), and sternum tend to be the most active sites of haematopoiesis. The bone marrow is not only responsible for the development and replenishment of blood cells, but it is also responsible for maintaining the pool of HSCs throughout the life of an adult vertebrate. The adult bone marrow (Figure 2-5), the paradigmatic adult stem cell niche, contains several cell types that coordinate HSC development, including (1) osteoblasts, versatile cells that both generate bone and control the differentiation of HSCs, (2) endothelial cells that line the blood vessels and also regulate HSC differentiation, (3) reticular cells that send processes connecting cells to bone and blood vessels, and, unexpectedly, (4) sympathetic neurons, which can control the release of

hematopoietic cells from the bone marrow. A microscopic cross-section reveals that the bone marrow is tightly packed with stromal cells and hematopoietic cells at every stage of differentiation. With age, however, fat cells gradually replace 50% or more of the bone marrow compartment, and the efficiency of haematopoiesis decreases. The choices that an HSC makes depend largely on the environmental cues it receives. The bone marrow is packed with hematopoietic cells at all stages of development, but it is likely that the precursors of each myeloid and lymphoid subtype mature in distinct environmental micro-niches within the bone marrow. Our understanding of the microenvironments within the bone marrow that support specific stages of haematopoiesis is still developing. Evidence suggests, however, that the endosteal niche (the area directly surrounding the bone and in contact with bone-producing osteoblasts) and the vascular niche (the area directly surrounding the blood vessels and in contact with endothelial cells) play different roles (see Figure 2-5c). The endosteal niche appears to be occupied by quiescent HSCs in close association with osteoblasts that regulate stem cell proliferation. The vascular niche appears to be occupied by HSCs that have been mobilized to leave the endosteal niche to either differentiate or circulate. In addition, the more differentiated a cell is, the farther it appears to migrate from its supportive osteoblasts and the closer it moves to the more central regions of the bone. For example, the most immature B lymphocytes are found closest to the endosteum and osteoblasts, while the more mature B cells have moved into the more central sinuses of the bone marrow that are richly served by blood vessels. Finally, it is important to recognize that the bone marrow is not only a site for lymphoid and myeloid development but is also a site to which fully mature myeloid and lymphoid cells can return. Mature antibody-secreting B cells (plasma cells) may even take up long-term residence in the bone marrow. Whole bone marrow transplants, therefore, do not simply include stem cells but also include mature, functional cells that can both help and hurt the transplant effort.

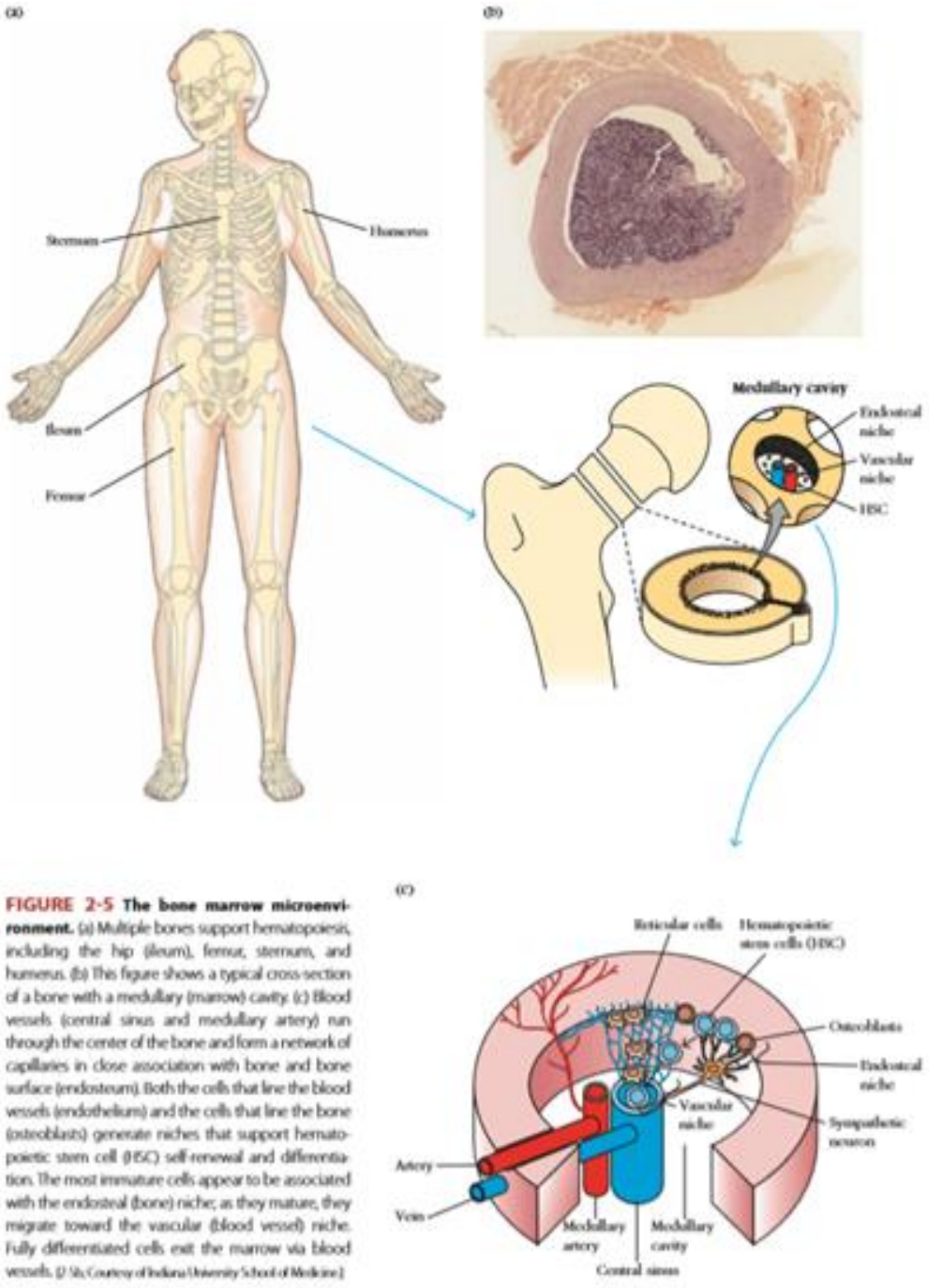


FIGURE 2-5 The bone marrow microenvironment. (a) Multiple bones support hematopoiesis, including the hip (ileum), femur, sternum, and humerus. (b) This figure shows a typical cross-section of a bone with a medullary (marrow) cavity. (c) Blood vessels (central sinus and medullary artery) run through the center of the bone and form a network of capillaries in close association with bone and bone surface (endosteum). Both the cells that line the blood vessels (endothelium) and the cells that line the bone (osteoblasts) generate niches that support hematopoietic stem cell (HSC) self-renewal and differentiation. The most immature cells appear to be associated with the endosteal (bone) niche; as they mature, they migrate toward the vascular (blood vessel) niche. Fully differentiated cells exit the marrow via blood vessels. (*b:* Courtesy of Indiana University School of Medicine.)

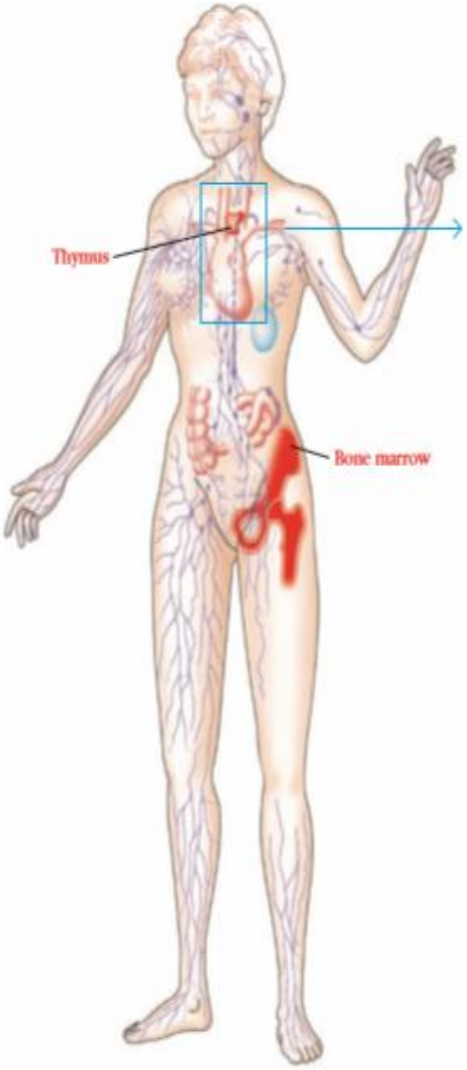
- ***The Thymus Is a Primary Lymphoid Organ Where T Cells Mature:***

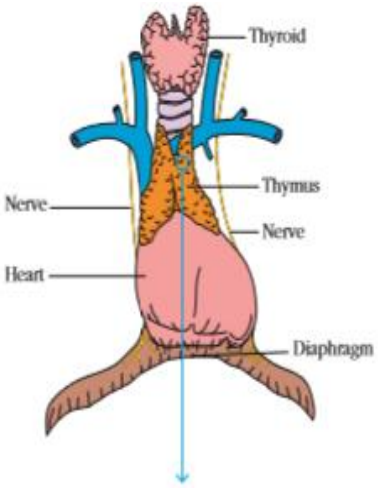
T-cell development is not complete until the cells undergo selection in the thymus (Figure 2-6). The importance of the thymus in T-cell development was not recognized until the early 1960s, when J.F.A.P. Miller, an Australian biologist, worked against the power of popular assumptions to advance his idea that the thymus was something other than a graveyard for cells. It was an underappreciated organ, very large in prepubescent animals, that was thought by some to be detrimental to an organism, and by others to be an evolutionary dead-end. The cells that populated it—small, thin-rimmed,featureless cells called thymocytes—looked dull and inactive. However, Miller proved that the thymus was the all-important site for the maturation of T lymphocytes. T-cell precursors, which still retain the ability to give rise to multiple hematopoietic cell types, travel via the blood from the bone marrow to the thymus. Immature T cells, known as thymocytes (thymus cells) because of their site of maturation, pass through defined developmental stages in specific thymic microenvironments as they mature into functional T cells. The thymus is a specialized environment where immature T cells generate unique antigen receptors (T cell receptors, or TCRs) and are then selected on the basis of their reactivity to self MHC-peptide complexes expressed on the surface of thymic stromal cells. Those thymocytes whose T-cell receptors bind self MHC-peptide complexes with too high affinity are induced to die (negative selection), and those thymocytes that bind self MHC-peptides with an intermediate affinity undergo positive selection, resulting in their survival, maturation, and migration to the thymic medulla. Most thymocytes do not navigate the journey through the thymus successfully; in fact, it is estimated that 95% of thymocytes die in transit. The majority of cells die because they have too low an affinity for the self-antigen MHC combinations that they encounter on the surface of thymic epithelial cells and fail to undergo positive selection. These developmental events take place in several distinct thymic microenvironments (see Figure 2-6). T-cell precursors enter the thymus in blood vessels at the corticomedullary junction between the thymic cortex, the outer portion of the organ, and the thymic medulla, the inner portion of the organ. At this stage thymocytes express neither CD4 nor CD8, markers associated with mature T cells.

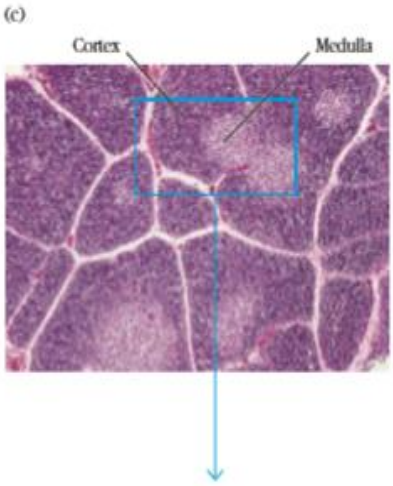
They are therefore called double negative (DN) cells. DN cells first travel to the region under the thymic capsule, a region referred to as the subcapsular cortex, where they proliferate and begin to generate their T-cell receptors. Thymocytes that successfully express TCRs begin to

express both CD4 and CD8, becoming double positive (DP) cells, and populate the cortex, the site where most (85% or more) immature T cells are found. The cortex features a distinct set of stromal cells, cortical thymic epithelial cells (cTECs), whose long processes are perused by thymocytes testing the ability of their T-cell receptors to bind MHC-peptide complexes (Video 2-1). Thymocytes that survive selection move to the thymic medulla, where positively selected thymocytes encounter specialized stromal cells, medullary thymic epithelial cells (mTECs). Not only do mTECs support the final steps of thymocyte maturation, but they also have a unique ability to express proteins that are otherwise found exclusively in other organs. This allows them to negatively select a group of potentially very damaging, auto reactive T cells that could not be deleted in the cortex.

Mature thymocytes, which express only CD4 or CD8 and are referred to as single positive (SP), leave the thymus as they entered: via the blood vessels of the corticomedullary junction. Maturation is finalized in the periphery, where these new T cells (recent thymic emigrants) explore antigens presented in secondary lymphoid tissue, including spleen and lymph nodes.







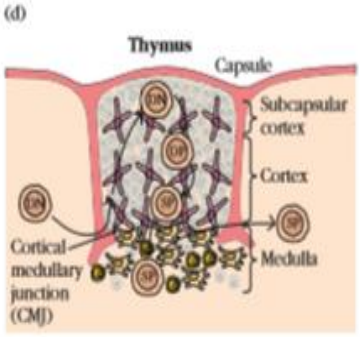


FIGURE 2-6 The structure of the thymus. The thymus is found just above the heart (a, b) and is largest prior to puberty, when it begins to shrink. Panel (c) depicts a stained thymus tissue section and (d) a cartoon of the microenvironments: the cortex, which is densely populated with DP immature thymocytes (blue) and the medulla, which is sparsely populated with SP mature thymocytes. These major regions are separated by the corticomedullary junction (CMJ), where cells enter from and exit to the bloodstream. The area between the cortex and the thymic capsule, the subcapsular cortex, is a site of much proliferation of the youngest (DN) thymocytes. The route taken by a typical thymocyte during its development from the DN to DP to SP stages is shown. Thymocytes are positively selected in the cortex. Autoreactive thymocytes are negatively selected in the medulla; some may also be negatively selected in the cortex. [2-6c: Dr. Gladden Willis/Getty Images.]

➢ Secondary Lymphoid Organs—Where the Immune Response Is Initiated:

As just described, lymphocytes and myeloid cells develop to maturity in the primary lymphoid system: T lymphocytes in the thymus, and B cells, monocytes, dendritic cells, and granulocytes in the bone marrow. However, they encounter antigen and initiate an immune response in the microenvironments of secondary lymphoid organs (SLOs).

• *Secondary Lymphoid Organs Are Distributed Throughout the Body and Share Some Anatomical Features:*

Lymph nodes and the spleen are the most highly organized of the secondary lymphoid organs and are compartmentalized from the rest of the body by a fibrous capsule. A somewhat less organized system of secondary lymphoid tissue, collectively referred to as mucosa-associated lymphoid tissue (MALT), is found associated with the linings of multiple organ systems, including the gastrointestinal (GI) and respiratory tracts. MALT includes tonsils, Peyer's patches (in the small intestine), and the appendix, as well as numerous lymphoid follicles within the lamina propria of the intestines and in the mucous membranes lining the upper airways, bronchi, and genitourinary tract (Figure 2-7). Although secondary lymphoid organs vary in their location and degree of organization, they share key features. All SLOs include anatomically distinct regions of T-cell and B-cell activity, and all develop lymphoid follicles, which are highly organized microenvironments that are responsible for the development and selection of B cells that produce high-affinity antibodies.

• *Lymphoid Organs Are Connected to Each Other and to Infected Tissue by Two Different Circulatory Systems: Blood and Lymphatics:*

The immune cells are the most mobile cells in a body and use two different systems to traffic through tissues: the blood system and the lymphatic system. The blood has access to virtually every organ and tissue and is lined by endothelial cells that are very responsive to inflammatory signals. Hematopoietic cells can transit through the blood system—away from the heart via active pumping networks (arteries) and back to the heart via passive valve-based

systems (veins) within minutes. Most lymphocytes enter secondary lymphoid organs via specialized blood vessels, and leave via the lymphatic system.

The lymphatic system is a network of thin walled vessels that play a major role in immune cell trafficking, including the travel of antigen and antigen-presenting cells to secondary lymphoid organs and the exit of lymphocytes from lymph nodes.

Lymph vessels are filled with a protein-rich fluid (lymph) derived from the fluid component of blood (plasma) that seeps through the thin walls of capillaries into the surrounding tissue. In an adult, depending on size and activity, seepage can add up to 2.9 litres or more during a 24-hour period. This fluid, called interstitial fluid, permeates all tissues and bathes all cells. If this fluid were not returned to the circulation, the tissue would swell; causing edema that would eventually become life threatening. We are not afflicted with such catastrophic edema because much of the fluid is returned to the blood through the walls of venules. The remainder of the interstitial fluid enters the delicate network of primary lymphatic vessels. The walls of the primary vessels consist of a single layer of loosely apposed endothelial cells. The porous architecture of the primary vessels allows fluids and even cells to enter the lymphatic network. Within these vessels, the fluid, now called lymph, flows into a series of progressively larger collecting vessels called lymphatic vessels (see Figures 2-7b and 2-7c).

All cells and fluid circulating in the lymph are ultimately returned to the blood system. The largest lymphatic vessel, the thoracic duct,empties into the left subclavian vein. It collects lymph from all of the body except the right arm and right side of the head. Lymph from these areas is collected into the right lymphatic duct, which drains into the right subclavian vein (see Figure 2-7a).

By returning fluid lost from the blood, the lymphatic system ensures steady-state levels of fluid within the circulatory system. The heart does not pump the lymph through the lymphatic system; instead, the slow, low-pressure flow of lymph is achieved by the movements of the surrounding muscles. Therefore, activity enhances lymph circulation. Importantly, a series of one-way valves along the lymphatic vessels ensures that lymph flows in only one direction.

When a foreign antigen gains entrance to the tissues, it is picked up by the lymphatic system (which drains all the tissues of the body) and is carried to various organized lymphoid tissues such as lymph nodes, which trap the foreign antigen. Antigen-presenting cells that engulf and process the antigen also can gain access to lymph. In fact, as lymph passes from the tissues to lymphatic vessels, it becomes progressively enriched in specific leukocytes, including lymphocytes, dendritic cells, and macrophages. Thus, the lymphatic system also serves as a means of transporting white blood cells and antigen from the connective tissues to organized lymphoid tissues, where the lymphocytes can interact with the trapped antigen and undergo activation. Most secondary lymphoid tissues are situated along the vessels of the lymphatic system. The spleen is an exception and is served only by blood vessels. All immune cells that traffic through tissues, blood, and lymph nodes are guided by small molecules known as chemokines. These proteins are secreted by stromal cells, antigen presenting cells, lymphocytes, and granulocytes, and form gradients that act as attractants and guides for other immune cells, which express an equally diverse set of receptors for these chemokines. The interaction between specific chemokines and cells expressing specific chemokine receptors allows for a highly refined organization of immune cell movements.

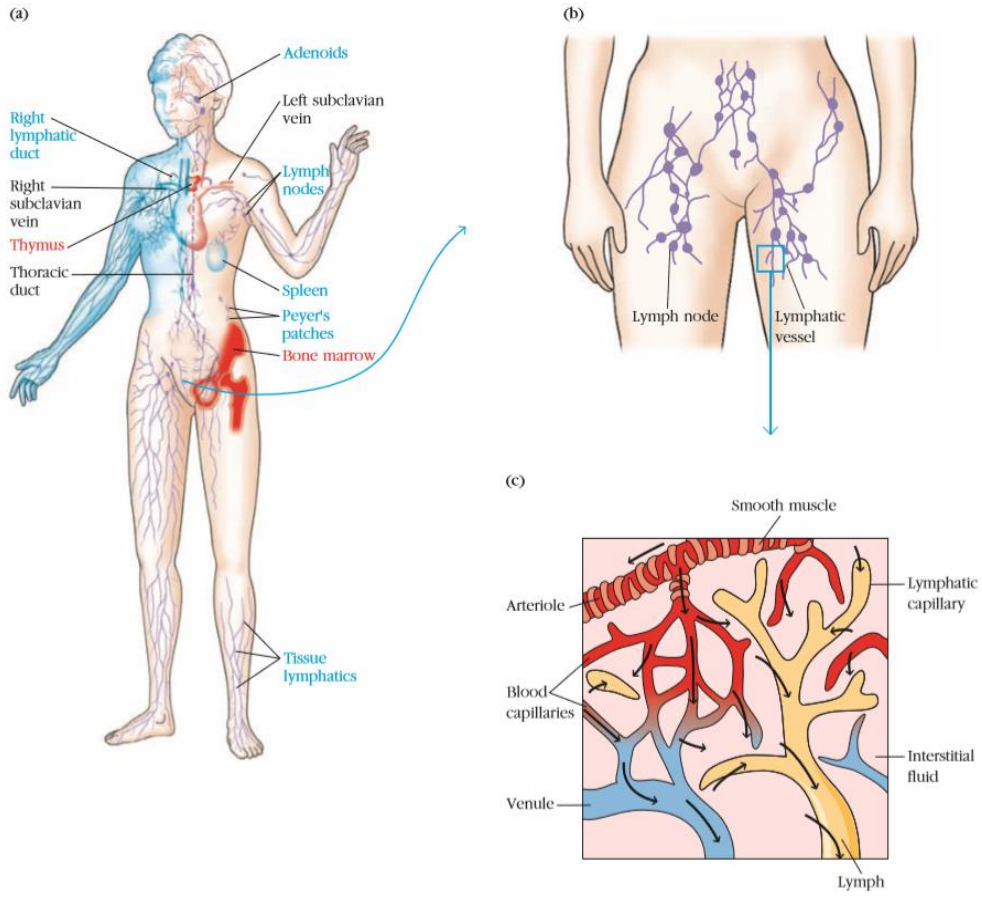


FIGURE 2-7 The human lymphoid system. The primary organs (bone marrow and thymus) are shown in red; secondary organs and tissues, in blue. These structurally and functionally diverse lymphoid organs and tissues are interconnected by the blood vessels (not shown) and lymphatic vessels (purple). Most of the body's lymphatics eventually drain into the thoracic duct, which empties into the left subclavian vein. However, the vessels draining the right arm and right side of the head (shaded blue) converge to form the right lymphatic duct, which empties into the right subclavian vein. The inset (b) shows the lymphatic vessels in more detail, and (c) shows the relationship between blood and lymphatic capillaries in tissue. The lymphatic capillaries pick up interstitial fluid, particulate and soluble proteins, as well as immune cells from the tissue surrounding the blood capillaries (see arrows). *[Part (a): Adapted from H. Lodish et al., 1995,* Molecular Cell Biology, *3rd ed., Scientific American Books, New York.]*

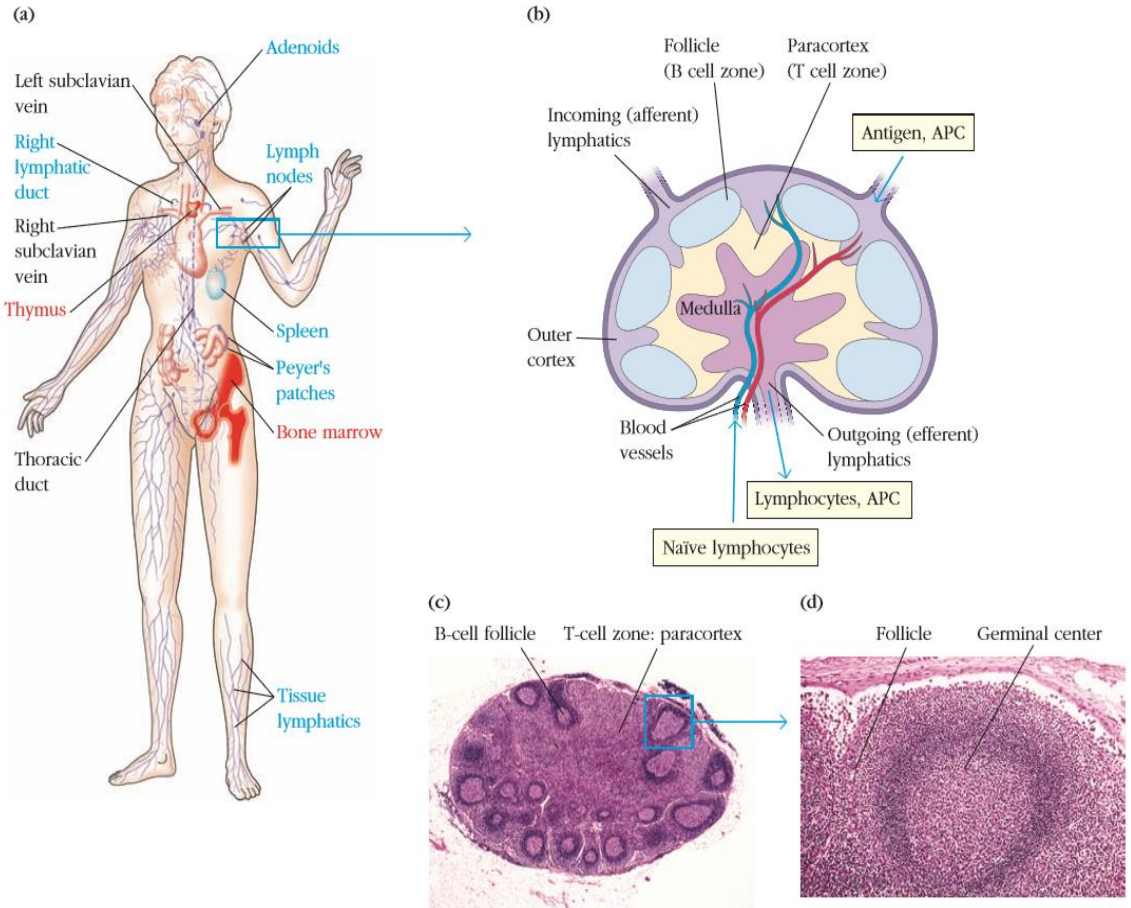
- ***The Lymph Node Is a Highly Specialized Secondary Lymphoid Organ:***

.Lymph nodes (Figure 2-8) are the most specialized SLOs. Unlike the spleen, which also regulates red blood cell flow and fate, lymph nodes are fully committed to regulating an immune response. They are encapsulated, bean-shaped structures that include networks of stromal cells packed with lymphocytes, macrophages, and dendritic cells. Connected to both blood vessels and lymphatic vessels, lymph nodes are the first organized lymphoid structure to encounter antigens that enter the tissue spaces. The lymph node provides ideal

microenvironments for encounters between antigen and lymphocytes and productive, organized cellular and humoral immune responses.

Structurally, a lymph node can be divided into three roughly concentric regions: the cortex, the paracortex, and the medulla, each of which supports a distinct microenvironment (see Figure 2-8). The outermost layer, the cortex, contains lymphocytes (mostly B cells), macrophages, and follicular dendritic cells arranged in follicles. Beneath the cortex is the paracortex, which is populated largely by T lymphocytes and also contains dendritic cells that migrated from tissues to the node. The medulla is the innermost layer, and the site where lymphocytes exit (egress) the lymph node through the outgoing (efferent) lymphatics. It is more sparsely populated with lymphoid lineage cells, which include plasma cells that are actively secreting antibody molecules.

Antigen travels from infected tissue to the cortex of the lymph node via the incoming (afferent) lymphatic vessels, which pierce the capsule of a lymph node at numerous sites and empty lymph into the sub capsular sinus (see Figure 2-8b). It enters either in particulate form or is processed and presented as peptides on the surface of migrating antigen presenting cells. Particulate antigen can be trapped by resident antigen-presenting cells in the subcapsular sinus or cortex, and it can be passed to other antigen-presenting cells, including B lymphocytes. Alternatively, particulate antigen can be processed and presented as peptide-MHC complexes on cell surfaces of resident dendritic cells that are already in the T-cell-rich



paracortex

Mononuclear Cells:

Mononuclear cells refer to blood cells that have a single, round nucleus, such as lymphocytes and monocytes. When isolated from circulating blood, they are called peripheral blood mononuclear cells (PBMC), but other sources exist, such as the umbilical cord, spleen, and bone marrow. The established method for separating mononuclear cells from blood is through density gradient centrifugation using the polysaccharide, Ficoll. Upon spinning, the cells collect in a layer called the buffy coat, which comprise about 1% of the total sample volume. From this fraction, more specific cell types can be further isolated by purification methods that target specific cell surface proteins: CD4 for T helper cells, CD8 for cytotoxic T cells, CD19 for B cells, CD14/CD16 for monocytes, among others. Mononuclear cells have been essential in the research areas of disease, therapeutics, vaccines, immunology, diagnostics and sequencing.

➢ Granulocytic cells:

Granulocytes are at the front lines of attack during an immune response and are considered part of the innate immune system. Granulocytes are white blood cells (leukocytes) that are classified as neutrophils, basophils, mast cells, or eosinophils on the basis of differences in cellular morphology and the staining of their characteristic cytoplasmic granules (Figure 2-2). All granulocytes have multi-lobed nuclei that make them visually distinctive and easily distinguishable from lymphocytes, whose nuclei are round. The cytoplasm of all granulocytes is replete with granules that are released in response to contact with pathogens. These granules contain a variety of proteins with distinct functions: Some damage pathogens directly; some regulate trafficking and activity of other white blood cells, including lymphocytes; and some contribute to the remodelling of tissues at the site of infection. See Table 2-2 for a partial list of granule proteins and their functions.

Neutrophils: Constitutethe majority (50% to 70%) of circulating leukocytes (see Figure 2-2a) and are much more numerous than eosinophils (1%–3%), basophils (1%), or mast cells (1%). After differentiation in the bone marrow, neutrophils are released into the peripheral blood and circulate for 7 to 10 hours before migrating into the tissues, where they have a life

span of only a few days. In response to many types of infections, the number of circulating neutrophils increases significantly and more are recruited to tissues, partially in response to cues the bone marrow receives to produce and release more myeloid cells. The resulting transient increase in the number of circulating neutrophils, called leukocytosis, is used medically as an indication of infection. Neutrophils are recruited to the site of infection in response to inflammatory molecules (e.g.,chemokines) generated by innate cells (including other neutrophils) that have engaged a pathogen. Once in tissues, neutrophils phagocytose (engulf) bacteria very effectively and also secrete a range of proteins that have antimicrobial effects and tissue remodelling potential. Neutrophils are the dominant first responders to infection and the main cellular components of pus, where they accumulate at the end of their short lives. Although once considered a simple and "disposable" effector cell, the neutrophil has recently inspired renewed interest from investigations indicating that it may also regulate the adaptive immune response.

Basophilsare nonphagocytic granulocytes (see Figure 2-2b) that contain large granules filled with basophilic proteins (i.e., they stain blue in standard H&E staining protocols). Basophils are relatively rare in the circulation, but can be very potent. In response to binding of circulating antibodies, basophils release the contents of their granules. Histamine, one of the best known proteins in basophilic granules, increases blood vessel permeability and smooth muscle activity. Basophils (and eosinophils, below) are critical to our response to parasites, particularly helminths (worms), but in areas where worm infection is less prevalent, histamines are best appreciated as the cause of allergy symptoms. Like neutrophils, basophils may also secrete cytokines that modulate the adaptive immune response.

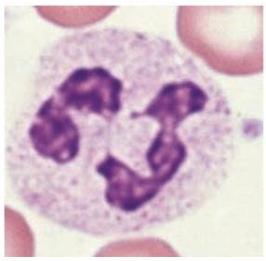
TABLE 2-2 Examples of proteins contained in neutrophil, eosinophil, and basophil granules

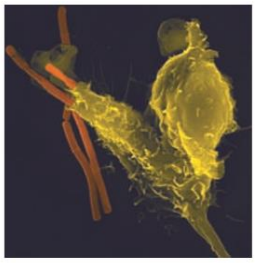
Cell type	Molecule in granule	Examples	Function
Neutrophil	Proteases	*Elastase, Collagenase*	Tissue remodeling
	Antimicrobial proteins	*Defensins, lysozyme*	Direct harm to pathogens
	Protease inhibitors	*α1-anti-trypsin*	Regulation of proteases
	Histamine		Vasodilation, inflammation
Eosinophil	Cationic proteins	*EPO*	Induces formation of ROS
	Ribonucleases	*MBP*	Vasodilation, basophil degranulation
	Cytokines	*ECP, EDN*	Antiviral activity
	Chemokines	*IL-4, IL-10, IL-13, TNFα*	Modulation of adaptive immune responses
		RANTES, MIP-1α	Attract leukocytes
Basophil/Mast Cell	Cytokines	*IL-4, IL-13*	Modulation of adaptive immune response
	Lipid mediators	*Leukotrienes*	Regulation of inflammation
	Histamine		Vasodilation, smooth muscle activation

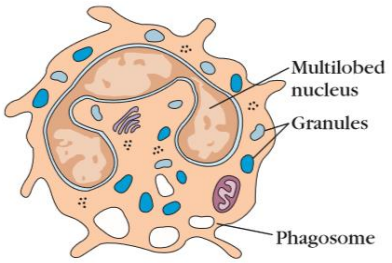
Eosinophils, like neutrophils, are motile phagocytic cells (see Figure 2-2d) that can migrate from the blood into the tissue spaces. Their phagocytic role is significantly less important than that of neutrophils, and it is thought that they play their most important role in the defense against multicellular parasitic organisms, including worms. They can be found clustering around invading worms, whose membranes are damaged by the activity of proteins released from eosinophilic granules. Like neutrophils and basophils, eosinophils may also secrete cytokines that regulate B and T lymphocytes, thereby influencing the adaptive immune response. In areas where parasites are less of a health problem, eosinophils are better appreciated as contributors to asthma and allergy symptoms.

- **Mast cells:**(see Figure 2-2c) are released from the bone marrow into the blood as undifferentiated cells; they mature only after they leave the blood. Mast cells can be found in a wide variety of tissues, including the skin, connective tissues of various organs, and mucosal epithelial tissue of the respiratory, genitourinary, and digestive tracts. Like circulating basophils, these cells have large numbers of cytoplasmic granules that contain histamine and other pharmacologically active substances. Mast cells also play an important role in the development of allergies.

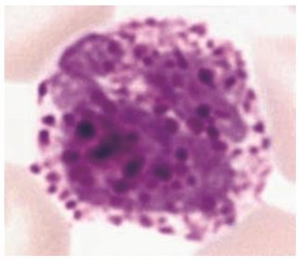
(a) Neutrophil

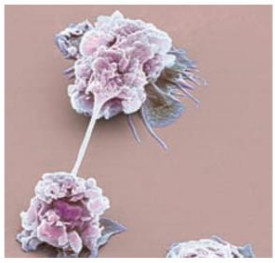


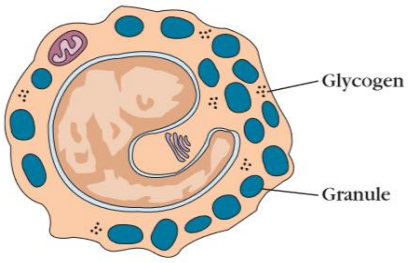




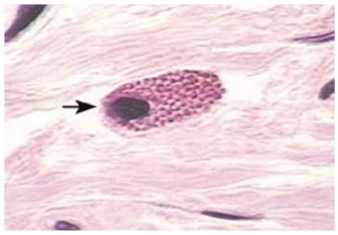
(b) Basophil

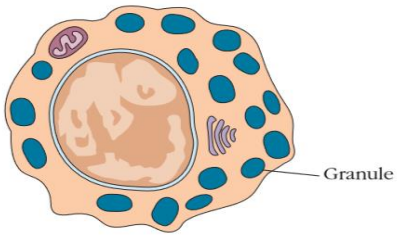




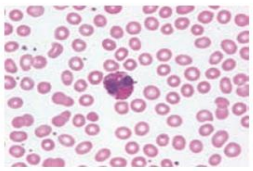


(c) Mast cell





(d) Eosinophil



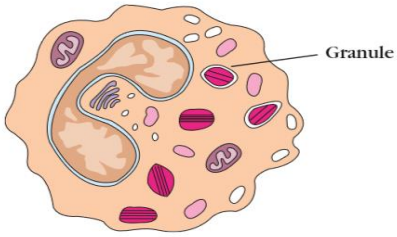


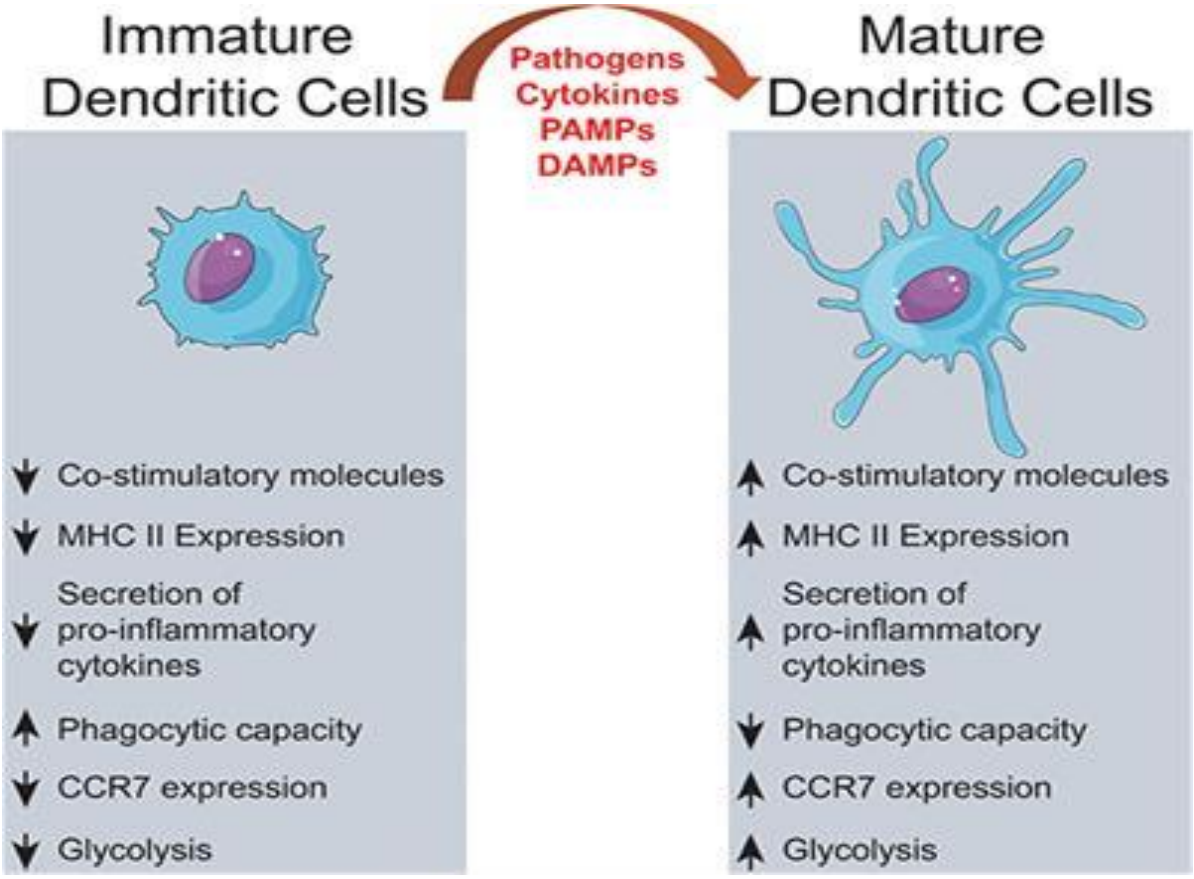
FIGURE 2-2 Examples of granulocytes. (a, b, c, d) Hematoxylin and eosin (H&E) stains of indicated cells in blood smears. (a, middle) Neutrophil engulfing bacteria visualized by scanning electron microscopy (SEM) and colorized digitally. (b, middle) SEM of activated granulocytes (colorized). Each image is accompanied by a cartoon depicting the typical morphology of the indicated granulocyte. Note differences in the shape of the nucleus and in the number, color, and shape of the cytoplasmic granules. [2-2a, left: Science Source/Getty Images; 2-2a, right: Creative Commons, http://es.wikipedia.org/wiki/Archivo:Neutrophil_with_anthrax_copy.jpg; 2-2b, left: Dr. Gladden Willis/Visuals Unlimited, Inc.; 2-2b, right: Steve Gschmeissner/Photo Researchers; 2-2c, left: Courtesy Gwen V. Childs, Ph.D., University of Arkansas for Medical Sciences; 2-2d, left; Pathpedia.com.]

➢ Dendritic Cell:

The discovery of the dendritic cell (DC) by Ralph Steinman in the mid-1970s resulted in awarding of the Nobel Prize in 2011. Dendritic cells are critical for the initiation of the immune response and acquired their name because they are covered with long membranous extensions that resemble the dendrites of nerve cells and extend and retract dynamically,increasing the surface area available for browsing lymphocytes. They are more diverse a population of cells than once was thought, and seem to arise from both the myeloid and lymphoid lineages of hematopoietic cells. The functional distinctions among these diverse cells are still being clarified and are likely critically important in tailoring immune responses to distinct pathogens and targeting responding cells to distinct tissues.

Dendritic cells perform the distinct functions of antigen capture in one location and antigen presentation in another. Outside lymph nodes, immature forms of these cells monitor the body for signs of invasion by pathogens and capture intruding or foreign antigens. They process these antigens, thenmigrate to lymph nodes, where they present the antigen to naïve T cells, initiating the adaptive immune response.

When acting as sentinels in the periphery, immature dendritic cells take on their cargo of antigen in three ways. They engulf it by phagocytosis, internalize it by receptor-mediated endocytosis, or imbibe it by pinocytosis. Indeed, immature dendritic cells pinocytose fluid volumes of 1000 to 1500 m3 per hour, a volume that rivals that of the cell itself. Through a process of maturation, they shift from an antigen-capturing phenotype to one that is specialized for presentation of antigen to T cells. In making the transition, some attributes are lost and others are gained. Lost is the capacity for phagocytosis and large-scale pinocytosis. However, the ability to present antigen increases significantly, as does the expression of costimulatory molecules that are essential for the activation of naïve T cells. After activation, dendritic cells abandon residency in peripheral tissues, enter the blood or lymphatic circulation, and migrate to regions of the lymphoid organs, where T cells reside, and present antigen.



It is important to note that, although they share a name, follicular dendritic cells do not arise in bone marrow and have completely different functions from those described for the dendritic cells discussed above. Follicular dendritic cells do not function as antigen-presenting cells for TH-cell activation. These dendritic cells were named for their exclusive location in organized structures of the lymph node called lymph follicles, which are rich in B cells.

✓ Part- 3:*Types of immunity:*

➢ **Innate immunity:**Innate immunity can be seen to comprise four types of defensive barriers: anatomic, physiologic, phagocytic, and inflammatory(Table 1-2).

TABLE 1-2 Summary of nonspecific host defenses

Type	Mechanism
Anatomic barriers	
Skin	Mechanical barrier retards entry of microbes. Acidic environment (pH 3–5) retards growth of microbes.
Mucous membranes	Normal flora compete with microbes for attachment sites and nutrients. Mucus entraps foreign microorganisms. Cilia propel microorganisms out of body.
Physiologic barriers	
Temperature	Normal body temperature inhibits growth of some pathogens. Fever response inhibits growth of some pathogens.
Low pH	Acidity of stomach contents kills most ingested microorganisms.
Chemical mediators	Lysozyme cleaves bacterial cell wall. Interferon induces antiviral state in uninfected cells. Complement lyses microorganisms or facilitates phagocytosis. Toll-like receptors recognize microbial molecules, signal cell to secrete immunostimulatory cytokines. Collectins disrupt cell wall of pathogen.
Phagocytic/endocytic barriers	Various cells internalize (endocytose) and break down foreign macromolecules. Specialized cells (blood monocytes, neutrophils, tissue macrophages) internalize (phagocytose), kill, and digest whole microorganisms.
Inflammatory barriers	Tissue damage and infection induce leakage of vascular fluid, containing serum proteins with antibacterial activity, and influx of phagocytic cells into the affected area.

- ***The Skin and the Mucosal Surfaces Provide Protective Barriers Against Infection:***

Physical and anatomic barriers that tend to prevent the entry of pathogens are an organism's first line of defense against infection. The skin and the surface of mucous membranes are included in this category because they are effective barriers to the entry of most microorganisms. The skin consists of two distinct layers: a thinner outer layer—the **epidermis** and a thicker layer—the **dermis**. The epidermis contains several layers of tightly packed epithelial cells.

The outer epidermal layer consists of dead cells and is filled with a waterproofing protein called keratin. The dermis, which is composed of connective tissue, contains blood vessels, hair follicles, sebaceous glands, and sweat glands. The sebaceous glands are associated with the hair follicles and produce an oily secretion called **sebum**. Sebum consists of lactic acid and fatty acids, which maintain the pH of the skin between 3 and 5; this pH inhibits the growth of most microorganisms. A few bacteria that metabolize sebum live as commensals on the skin and sometimes cause a severe form of acne. One acne drug, isotretinoin (Accutane), is a vitamin A derivative that prevents the formation of sebum.

Breaks in the skin resulting from scratches, wounds, or abrasion are obvious routes of infection. The skin may also be penetrated by biting insects (e.g.,mosquitoes,mites,ticks, fleas, and sand flies); if these harbor pathogenic organisms, they can introduce the pathogen into the body as they feed. The protozoan that causes malaria,for example,is deposited in humans by mosquitoes when they take a blood meal.Similarly,bubonic plague is spread by the bite of fleas,and Lyme disease is spread by the bite of ticks.

The conjunctivae and the alimentary, respiratory, and urogenital tracts are lined by mucous membranes,not by the dry,protective skin that covers the exterior ofthe body.These membranes consist of an outer epithelial layer and an underlying layer of connective tissue. Although many pathogens enter the body by binding to and penetrating mucous membranes,a number of nonspecific defense mechanisms tend to prevent this entry.For example,saliva,tears,and mucous secretions act to wash away potential invaders and also contain antibacterial or antiviral substances.The viscous fluid called mucus,which is secreted by epithelial cells of mucous membranes, entraps foreign microorganisms. In the lower respiratory tract, the mucous membrane is covered by cilia, hairlike protrusions of the epithelial-cell membranes. The synchronous movement of cilia propels mucus-entrapped

microorganisms from these tracts. In addition, non-pathogenic organisms tend to colonize the epithelial cells of mucosal surfaces. These normal flora generally outcompete pathogens for attachment sites on the epithelial cell surface and for necessary nutrients.

- ***Physiologic Barriers to Infection Include General Conditions and Specific Molecules:***

The physiologic barriers that contribute to innate immunity include temperature, pH, and various soluble and cell associated molecules. Many species are not susceptible to certain diseases simply because their normal body temperature inhibits growth of the pathogens. Chickens, for example, have innate immunity to anthrax because their high body temperature inhibits the growth of the bacteria. Gastric acidity is an innate physiologic barrier to infection because very few ingested microorganisms can survive the low pH of the stomach contents. One reason new borns are susceptible to some diseases that do not afflict adults is that their stomach contents are less acid than those of adults.

A variety of soluble factors contribute to innate immunity, among them the soluble proteins lysozyme, interferon, and complement. **Lysozyme**, a hydrolytic enzyme found in mucous secretions and in tears, is able to cleave the peptidoglycan layer of the bacterial cell wall. **Interferon** comprises a group of proteins produced by virus-infected cells. Among the many functions of the interferon is the ability to bind to nearby cells and induce a generalized antiviral state. **Complement**, is a group of serum proteins that circulate in an inactive state. A variety of specific and nonspecific immunologic mechanisms can convert the inactive forms of complement proteins into an active state with the ability to damage the membranes of pathogenic organisms, either destroying the pathogens or facilitating their clearance. Complement may function as an effector system that is triggered by binding of antibodies to certain cell surfaces, or it may be activated by reactions between complement molecules and certain components of microbial cell walls. Reactions between complement molecules or fragments of complement molecules and cellular receptors trigger activation of cells of the innate or adaptive immune systems. Recent studies on **collectins**indicate that these surfactant proteins may kill certain bacteria directly by disrupting their lipid membranes or, alternatively, by aggregating the bacteria to enhance their susceptibility to phagocytosis.

Many of the molecules involved in innate immunity have the property of pattern recognition, the ability to recognize a given class of molecules. Because there are certain types of molecules that are unique to microbes and never found in multicellular organisms, the ability

to immediately recognize and combat invaders displaying such molecules is a strong feature of innate immunity. Molecules with pattern recognition ability may be soluble, like lysozyme and the complement components described above, or they may be cell-associated receptors. Among the class of receptors designated the toll-like receptors (TLRs), TLR2 recognizes the lipopolysaccharide (LPS) found on Gram-negative bacteria. It has long been recognized that systemic exposure of mammals to relatively small quantities of purified LPS leads to an acute inflammatory response (see below). The mechanism for this response is via a TLR on macrophages that recognizes LPS and elicits a variety of molecules in the inflammatory response upon exposure. When the TLR is exposed to the LPS upon local invasion by a Gram-negative bacterium, the contained response results in elimination of the bacterial challenge.

- ***Cells That Ingest and Destroy Pathogens Make Up a Phagocytic Barrier to Infection:***

Another important innate defense mechanism is the ingestion of extracellular particulate material by phagocytosis. Phagocytosis is one type of endocytosis, the general term for the uptake by a cell of material from its environment. In phagocytosis, a cell's plasma membrane expands around the particulate material, which may include whole pathogenic microorganisms, to form large vesicles called phagosomes (Figure 1-3). Most phagocytosis is conducted by specialized cells, such as blood monocytes, neutrophils, and tissue macrophages. Most cell types are capable of other forms of endocytosis, such as receptor-mediated endocytosis, in which extracellular molecules are internalized after binding by specific cellular receptors, and pinocytosis, the process by which cells take up fluid from the surrounding medium along with any molecules contained in it.

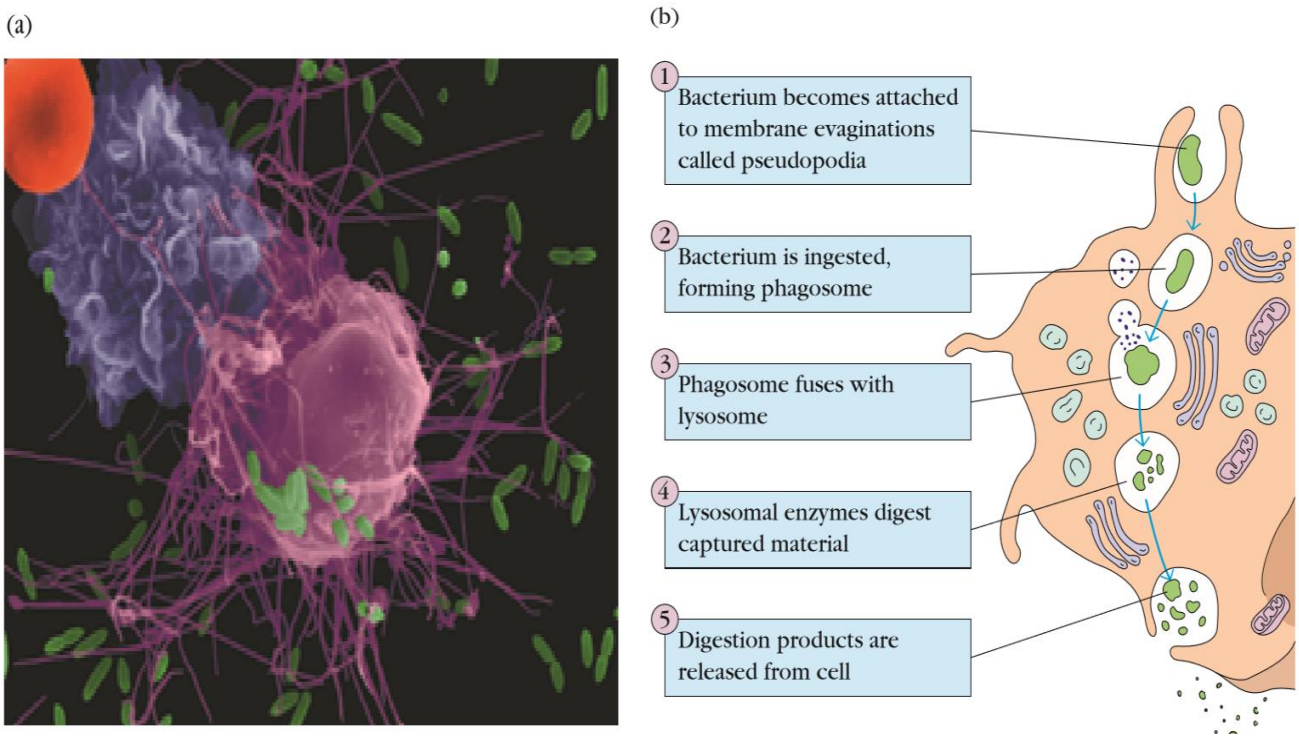


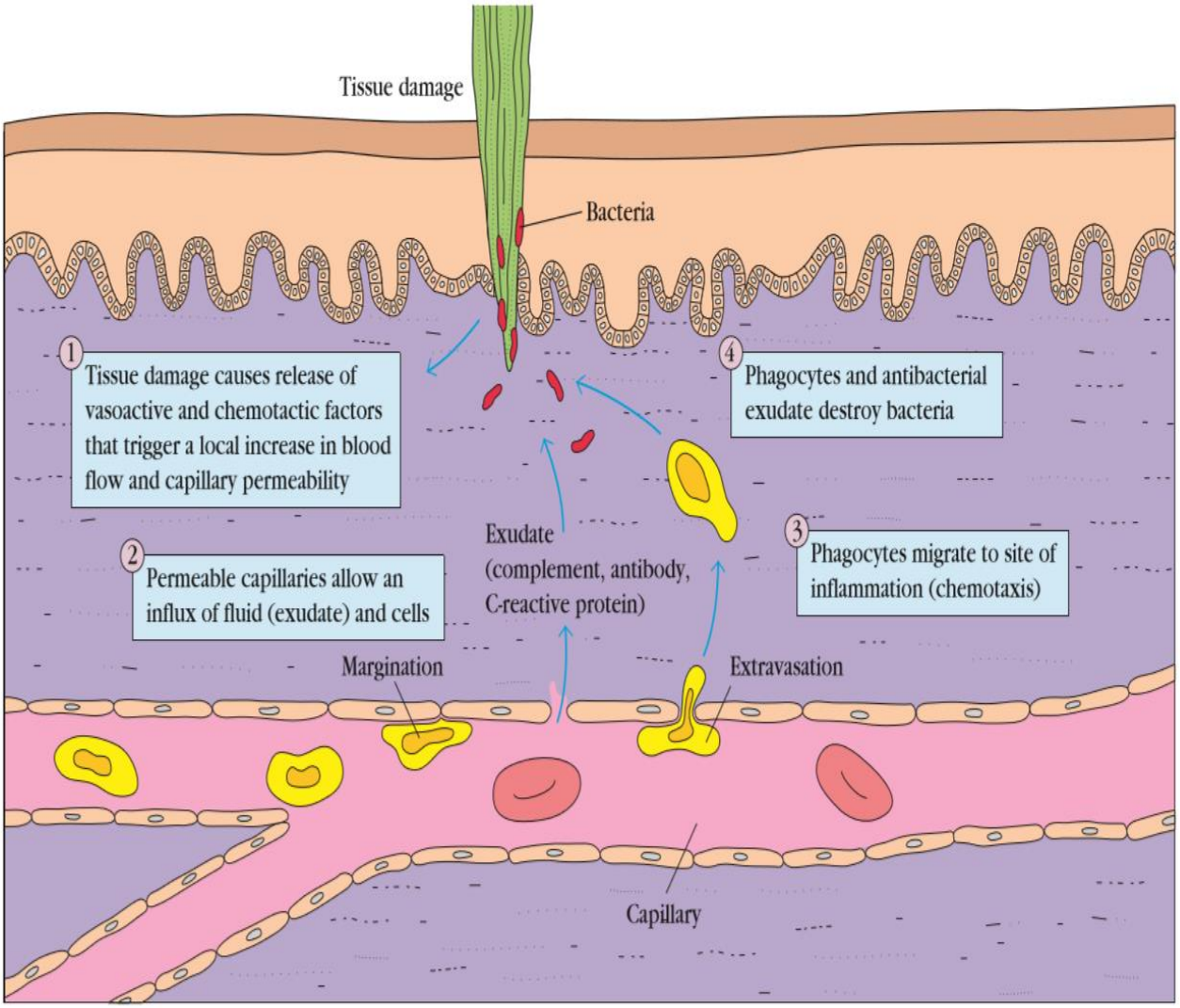
FIGURE 1-3 (a) Electronmicrograph of macrophage (pink) attacking *Escherichia coli* (green). The bacteria are phagocytized as described in part b and breakdown products secreted. The monocyte (purple) has been recruited to the vicinity of the encounter by soluble factors secreted by the macrophage. The red sphere is an erythrocyte. (b) Schematic diagram of the steps in phagocytosis of a bacterium. [*Part a, Dennis Kunkel Microscopy, Inc./Dennis Kunkel.*]

- ***Inflammation Represents a Complex Sequence of Events That Stimulates Immune Responses:***

Tissue damage caused by a wound or by an invading pathogenic microorganism induces a complex sequence of events collectively known as the inflammatory response. As described above, a molecular component of a microbe, such as LPS, may trigger an inflammatory response via interaction with cell surface receptors. The end result of inflammation may be the marshalling of a specific immune response to the invasion or clearance of the invader by components of the innate immune system. Many of the classic features of the inflammatory response were described as early as 1600 BC, in Egyptian papyrus writings. In the first century AD, the Roman physician Celsus described the "four cardinal signs of inflammation" as rubor (redness), tumor (swelling), calor (heat), and dolor (pain). In the second century AD, another physician, Galen, added a fifth sign: functiolaesa (loss of function). The cardinal

signs of inflammation reflect the three major events of an inflammatory response (Figure 1-4):

1. Vasodilation—an increase in the diameter of blood vessels—of nearby capillaries occurs as the vessels that carry blood away from the affected area constrict, resulting in engorgement of the capillary network.The engorged capillaries are responsible for tissue redness (erythema) and an increase in tissue temperature.
2. *An increase in capillary permeability* facilitates an influx of fluid and cells from the engorged capillaries into the tissue.The fluid that accumulates (exudate) has much higher protein content than fluid normally released from the vasculature.Accumulation of exudate contributes to tissue swelling (**edema**).
3. *Influx of phagocytes* from the capillaries into the tissues is facilitated by the increased permeability of the capillaries.The emigration of phagocytes is a multistep process that includes adherence of the cells to the endothelial wall of the blood vessels (margination), followed by their emigration between the capillaryendothelial cells into the tissue (diapedesis orextravasation),and,finally,their migration through the tissue to the site of the invasion (chemotaxis).As phagocytic cells accumulate at the site and begin to phagocytose bacteria, theyrelease lytic enzymes,which can damage nearby healthy cells.The accumulation of dead cells,digested material,and fluidforms a substance called pus.


Tissue damage
Bacteria
1
Tissue damage causes release of vasoactive and chemotactic factors that trigger a local increase in blood flow and capillary permeability
2
Permeable capillaries allow an influx of fluid (exudate) and cells
Exudate
(complement, antibody, C-reactive protein)
3
Phagocytes migrate to site of inflammation (chemotaxis)
4
Phagocytes and antibacterial exudate destroy bacteria
Margination
Extravasation
Capillary

The events in the inflammatory response are initiated by a complex series of events involving a variety of chemical mediators whose interactions are only partly understood. Some of these mediators are derived from invading microorganisms, some are released from damaged cells in response to tissue injury, some are generated by several plasma enzyme systems, and some are products of various white blood cells participating in the inflammatory response.

One of the principal mediators of the inflammatory response is histamine, a chemical released by a variety of cells in response to tissue injury. Histamine binds to receptors on nearby capillaries and venules, causing vasodilation and increased permeability. Another important group of inflammatory mediators, small peptides called kinins, are normally present in blood plasma in an inactive form. Tissue injury activates these peptides, which then cause vasodilation and increased permeability of capillaries. A particular kinin, called bradykinin, also stimulates pain receptors in the skin. This effect probably serves a protective role, because pain normally causes an individual to protect the injured area.

Vasodilation and the increase in capillary permeability in an injured tissue also enable enzymes of the blood-clotting system to enter the tissue. These enzymes activate an enzyme cascade that results in the deposition of insoluble strands of fibrin, which is the main component of a blood clot. The fibrin strands wall off the injured area from the rest of the

FIGURE 1-4 Major events in the inflammatory response. A bacterial infection causes tissue damage with release of various vasoactive and chemotactic factors. These factors induce increased blood flow to the area, increased capillary permeability, and an influx of white blood cells, including phagocytes and lymphocytes, from the blood into the tissues. The serum proteins contained in the exudate have antibacterial properties, and the phagocytes begin to engulf the bacteria, as illustrated in Figure 1-3.

body and serve to prevent the spread of infection.

Once the inflammatory response has subsided and most of the debris has been cleared away by phagocytic cells, tissue repair and regeneration of new tissue begins. Capillaries grow into the fibrin of a blood clot. New connective tissue cells, called fibroblasts, replace the fibrin as the clot dissolves. As fibroblasts and capillaries accumulate, scar tissue forms.

➢ Adaptive immunity:

Adaptive immunity is capable of recognizing and selectively eliminating specific foreign microorganisms and molecules (i.e., foreign antigens). Unlike innate immune responses, adaptive immune responses are not the same in all members of a species but are reactions to specific antigenic challenges. Adaptive immunity displays four characteristic attributes:

- **Antigenic specificity**
- **Diversity**
- **Immunologic memory**
- **Self/nonself recognition**

The antigenic specificity of the immune system permits it to distinguish subtle differences among antigens. Antibodies can distinguish between two protein molecules that differ in only a single amino acid. The immune system is capable of generating tremendous diversity in its recognition molecules, allowing it to recognize billions of unique structures on foreign antigens. Once the immune system has recognized and responded to an antigen, it exhibits immunologic memory; that is, a second encounter with the same antigen induces a heightened state of immune reactivity. Because of this attribute, the immune system can confer life-long immunity to many infectious agents after an initial encounter. Finally, the immune system normally responds only to foreign antigens, indicating that it is capable of self/nonself recognition. The ability of the immune system to distinguish self from nonself and respond only to nonself molecules is essential, for, as described below, the outcome of an inappropriate response to self-molecules can be fatal.

Adaptive immunity is not independent of innate immunity. The phagocytic cells crucial to nonspecific immune responses are intimately involved in activating the specific immune response. Conversely, various soluble factors produced by a specific immune response have been shown to augment the activity of these phagocytic cells. As an inflammatory response develops, for example, soluble mediators are produced that attract cells of the immune system. The immune response will, in turn, serve to regulate the intensity of the inflammatory

response. Through the carefully regulated interplay of adaptive and innate immunity, the two systems work together to eliminate a foreign invader.

- ***The Adaptive Immune System Requires Cooperation Between Lymphocytes and Antigen-Presenting Cells:***

An effective immune response involves two major groups of cells: T lymphocytes and antigen-presenting cells. Lymphocytes are one of many types of white blood cells produced in the bone marrow by the process of hematopoiesis. Lymphocytes leave the bone marrow, circulate in the blood and lymphatic systems, and reside in various lymphoid organs. Because they produce and display antigen binding cell-surface receptors, lymphocytes mediate the defining immunologic attributes of specificity, diversity, memory, and self/nonself recognition. The two major populations of lymphocytes—**B lymphocytes (B cells)** and **T lymphocytes (T cells)**.

B lymphocytes:

B lymphocytes mature within the bone marrow; when they leave it,each expresses a unique antigen-binding receptor on its membrane (Figure 1-5a). This antigen-binding or B-cell receptor is a membrane-bound antibody molecule. Antibodies are glycoproteins that consist of two identical heavy polypeptide chains and two identicallight polypeptide chains.Each heavy chain is joined with a light chain by disulfide bonds,and additional disulfide bonds hold the two pairs together.The amino-terminal ends of the pairs of heavy and light chains form a cleft within which antigen binds.When a naive B cell (one that has not previously encountered antigen) first encounters the antigen that matches its membranebound antibody, the binding of the antigen to the antibody causes the cell to divide rapidly;its progeny differentiate into memory B cells and effector B cells called plasma cells. Memory B cells have a longer life span than naive cells, and they express the same membrane-bound antibody as their parent B cell. Plasma cells produce the antibody in a form that can be secreted and have little or no membrane-bound antibody.Although plasma cells live for only a few days,they secrete enormous amounts of antibody during this time. It has been estimated that a single plasma cell can secrete more than 2000 molecules of antibody per second. Secreted antibodies are the major effector molecules of humoral immunity.

T-lymphocytes:T lymphocytes also arise in the bone marrow.Unlike B cells, which mature within the bone marrow,T cells migrate to the thymus gland to mature. During its

maturation within the thymus,the T cell comes to express a unique antigen-binding molecule,called the T-cell receptor, on its membrane.Unlike membrane-bound antibodies on B cells,which can recognize antigen alone, T-cell receptors can recognize only antigen that is bound to cell-membrane proteins called **major histocompatibility complex (MHC)** molecules. MHC molecules that function in this recognition event,which is termed "antigen presentation,"are polymorphic (genetically diverse) glycoproteins found on cell membranes. There are two major types of MHC molecules: Class I MHC molecules,which are expressed by nearly all nucleated cells of vertebrate species, consist of a heavy chain linked to a small invariant protein called 2-microglobulin. Class II MHC molecules,which consist of an alpha and a beta glycoprotein chain,are expressed only by antigen-presenting cells.

When a naive T cell encounters antigen combined with a MHC molecule on a cell, the T cell proliferates and differentiates into memory T cells and various effector T cells.

There are two well-defined subpopulations of T cells: T helper (TH) and T-cytotoxic (TC) cells. Although a third type of T cell, called a T suppressor (TS) cell, has been postulated, recent evidence suggests that it may not be distinct from TH and TC sub populations. T helper and T cytotoxic cells can be distinguished from one another by the presence of either

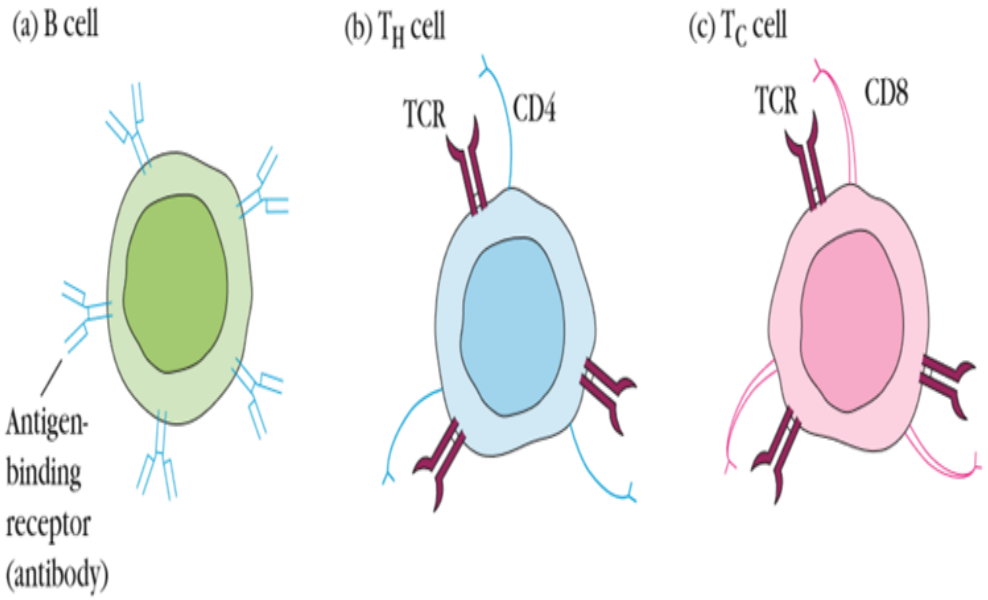


FIGURE 1-5 Distinctive membrane molecules on lymphocytes. (a) B cells have about 10^5 molecules of membrane-bound antibody per cell. All the antibody molecules on a given B cell have the same antigenic specificity and can interact directly with antigen. (b) T cells bearing CD4 ($CD4^+$ cells) recognize only antigen bound to class II MHC molecules. (c) T cells bearing CD8 ($CD8^+$ cells) recognize only antigen associated with class I MHC molecules. In general, $CD4^+$ cells act as helper cells and $CD8^+$ cells act as cytotoxic cells. Both types of T cells express about 10^5 identical molecules of the antigen-binding T-cell receptor (TCR) per cell, all with the same antigenic specificity.

CD4or CD8membrane glycoproteins on their surfaces (Figure 1-5b, c). T cells displaying CD4 generally function asTH cells, whereas those displaying CD8 generally function as T_C cell.

- ***ANTIGEN-PRESENTING CELLS:***

Activation of both the humoral and cell-mediated branches of the immune system requires cytokines produced by TH cells. It is essential that activation of TH cells themselves be carefully regulated, because an inappropriate T-cell response to self-components can have fatal autoimmune consequences. To ensure carefully regulated activation of TH cells, they can recognize only antigen that is displayed together with class MHC II molecules on the surface of antigen-presenting cells (APCs). These specialized cells, which include macrophages, B lymphocytes, and dendritic cells, are distinguished by two properties:

1. They express class II MHC molecules on their membranes,
2. They are able to deliver a co-stimulatory signal that is necessary for TH-cell activation.

Antigen-presenting cells first internalize antigen, either by phagocytosis or by endocytosis, and then display a part of that antigen on their membrane bound to a class II MHC molecule. The TH cell recognizes and interacts with the antigen–class II MHC molecule complex on the membrane of the antigen-presenting cell (Figure 1-6). An additional

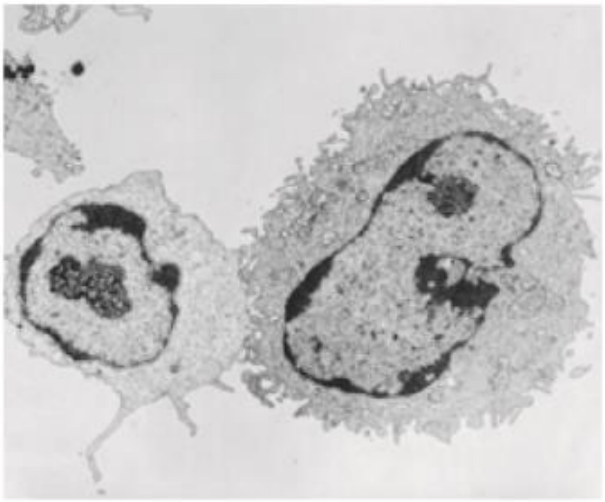


FIGURE 1-6 Electron micrograph of an antigen-presenting macrophage (*right*) associating with a T lymphocyte. [*From A. S. Rosenthal et al., 1982, in* Phagocytosis—Past and Future, *Academic Press, p. 239.*]

costimulatory signal is then produced by the antigen-presenting cell, leading to activation of the TH cell.

- ***Antigen Is Recognized Differently by B and T Lymphocytes:***

Antigens, which are generally very large and complex, are not recognized in their entirety by lymphocytes. Instead, both B and T lymphocytes recognize discrete sites on the antigen called antigenic determinants, or epitopes. Epitopes are the immunologically active regions on a complex antigen, the regions that actually bind to B-cell or T-cell receptors.

Although B cells can recognize an epitope alone, T cells can recognize an epitope only when it is associated with an MHC molecule on the surface of a self-cell (either an antigen-presenting cell or an altered self-cell).Each branch of the immune system is therefore uniquely suited to recognize antigen in a different milieu. The humoral branch (B cells) recognizes an enormous variety of epitopes: those displayed on the surfaces of bacteria or viral particles, as well as those displayed on soluble proteins, glycoproteins, polysaccharides, or lipopolysaccharides that have been released from invading pathogens. The cell-mediated branch (T cells) recognizes protein epitopes displayed together with MHC molecules on self-cells, including altered self-cells such as virus-infected self-cells and cancerous cells.

Thus, four related but distinct cell-membrane molecules are responsible for antigen recognition by the immune system:

- **Membrane-bound antibodies on B cells**
- **T-cell receptors**
- **Class I MHC molecules**

- **Class II MHC molecules**

Each of these molecules plays a unique role in antigen recognition, ensuring that the immune system can recognize and respond to the different types of antigen that it encounters.

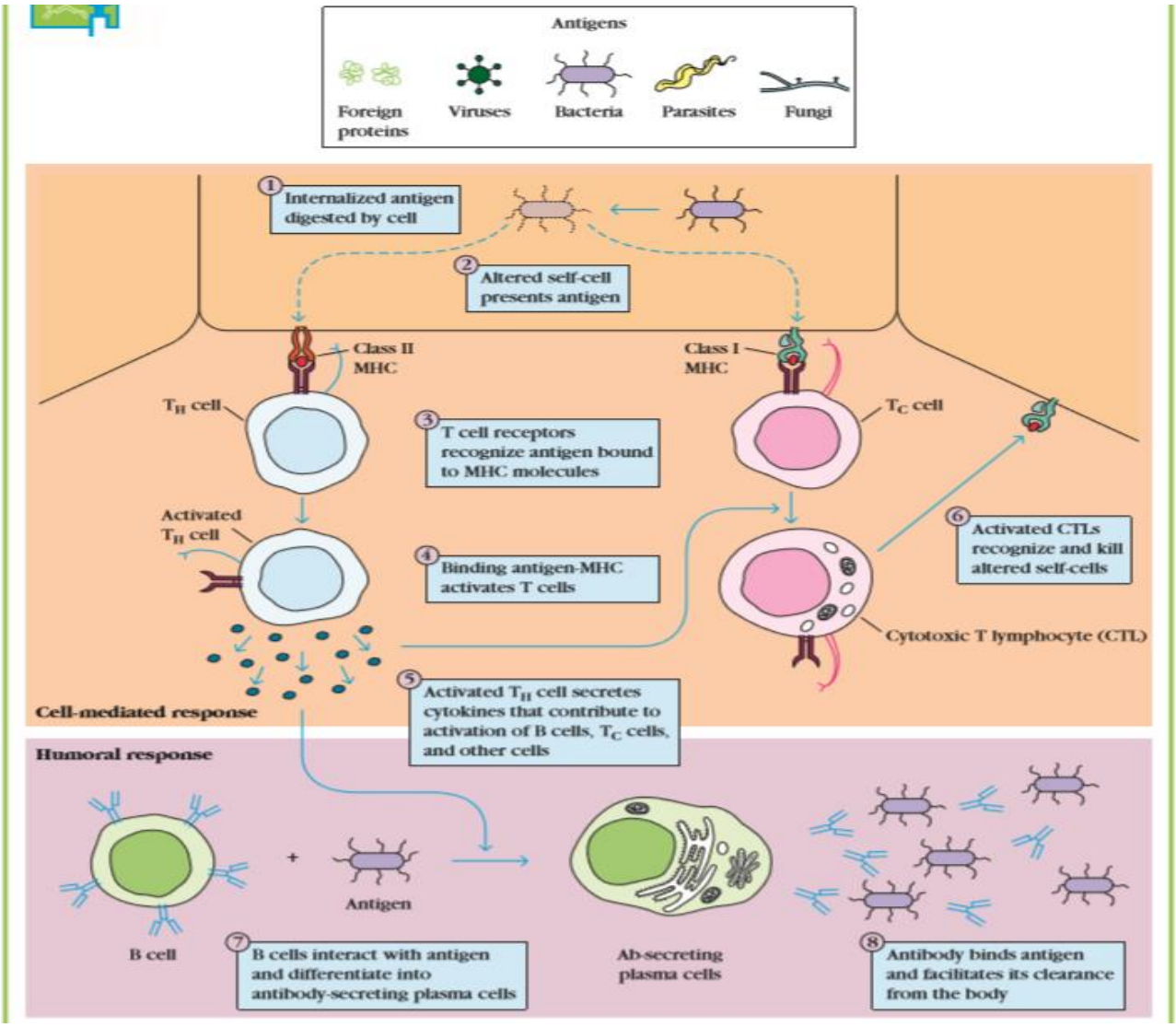


FIGURE 1-7 Overview of the humoral and cell-mediated branches of the immune system. In the humoral response, B cells interact with antigen and then differentiate into antibody-secreting plasma cells. The secreted antibody binds to the antigen and facilitates its clearance from the body. In the cell-mediated response, various subpopulations of T cells recognize antigen presented on self-cells. T_H cells respond to antigen by producing cytokines. T_C cells respond to antigen by developing into cytotoxic T lymphocytes (CTLs), which mediate killing of altered self-cells (e.g., virus-infected cells).

- ***The Major Histocompatibility Molecules Bind Antigenic Peptides:***

The major histocompatibility complex (MHC) is a large genetic complex with multiple loci.The MHC loci encode two major classes of membrane-bound glycoproteins:class Iand class II MHC molecules. As noted above, TH cells generally recognize antigen combined with class II molecules,whereas TC cells generally recognize antigen combined with class I molecules (Figure 1-8).

MHC molecules function as antigen-recognition molecules, but they do not possess the fine specificity for antigen characteristic of antibodies and T-cell receptors.Rather,each MHC molecule can bind to a spectrum ofantigenic peptides derived from the intracellular degradation of antigen molecules. In both class I and class II MHC molecules the distal regions (farthest from the membrane) of different alleles display wide variation in their amino acid sequences. These variable regions form a cleft within which the antigenic peptide sits and is presented to T lymphocytes (see Figure 1-8). Different allelic forms of the genes encoding class I and classII molecules confer different structures on the antigen-binding cleft with different specificity.Thus the ability to present an antigen to T lymphocytes is influenced by the particular set of alleles that an individual inherits.

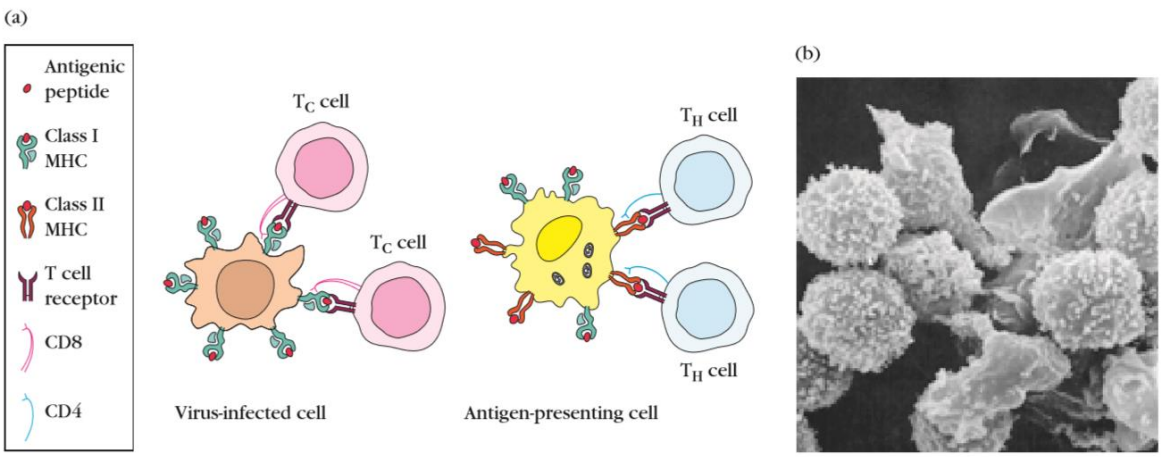


FIGURE 1-8 The role of MHC molecules in antigen recognition by T cells. (a) Class I MHC molecules are expressed on nearly all nucleated cells. Class II MHC molecules are expressed only on antigen-presenting cells. T cells that recognize only antigenic peptides displayed with a class II MHC molecule generally function as T helper (T_H) cells. T cells that recognize only antigenic peptides displayed with a class I MHC molecule generally function as T cytotoxic (T_C) cells. (b) This scanning electron micrograph reveals numerous T lymphocytes interacting with a single macrophage. The macrophage presents processed antigen combined with class II MHC molecules to the T cells. [*Photograph from W. E. Paul (ed.), 1991,* Immunology: Recognition and Response, *W. H. Freeman and Company, New York; micrograph courtesy of M. H. Nielsen and O. Werdelin.*]

- ***Complex Antigens Are Degraded (Processed) and Displayed (Presented) with MHC Molecules on the Cell Surface:***

Exogenous antigen is produced outside of the host cell and enters the cell by endocytosis or phagocytosis. Antigen presenting cells (macrophages, dendritic cells, and B cells) degrade ingested exogenous antigen into peptide fragments within the endocytic processing pathway. Experiments suggest that class II MHC molecules are expressed within the endocytic processing pathway and that peptides produced by degradation of antigen in this pathway bind to the cleft within the class II MHC molecules. The MHC molecules bearing the peptide are then exported to the cell surface. Since expression of class II MHC molecules is limited to antigen-presenting cells, presentation of exogenous peptide– class II MHC complexes is limited to these cells. T cells displaying CD4 recognize antigen combined with class II MHC molecules and thus are said to be class II MHC restricted. These cells generally function as T helper cells.

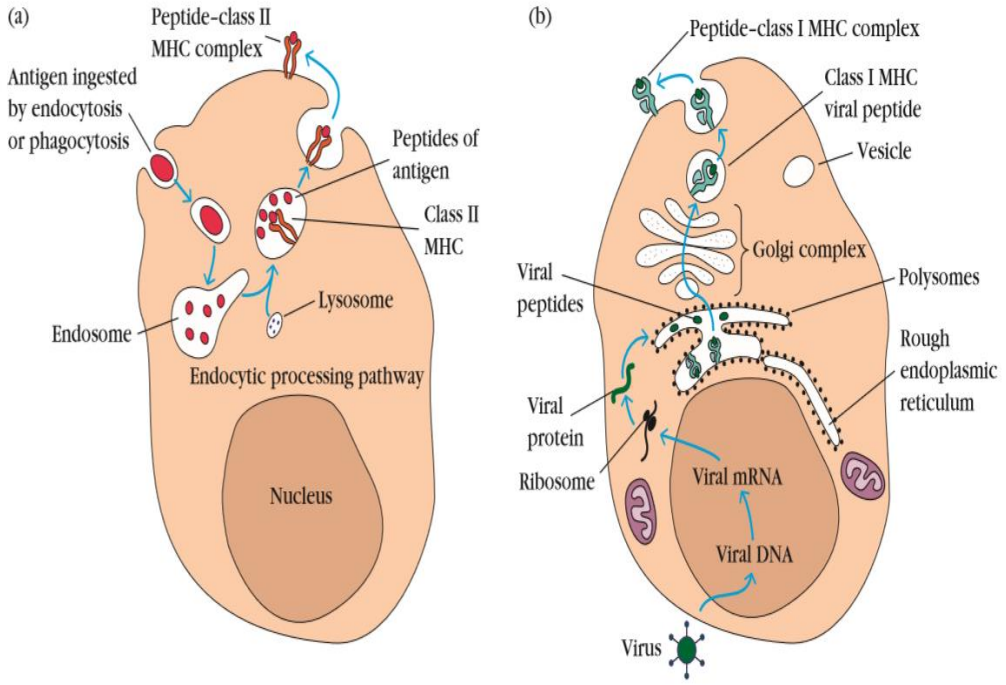


FIGURE 1-9 Processing and presentation of exogenous and endogenous antigens. (a) Exogenous antigen is ingested by endocytosis or phagocytosis and then enters the endocytic processing pathway. Here, within an acidic environment, the antigen is degraded into small peptides, which then are presented with class II MHC molecules on the membrane of the antigen-presenting cell. (b) Endogenous antigen, which is produced within the cell itself (e.g., in a virus-infected cell), is degraded within the cytoplasm into peptides, which move into the endoplasmic reticulum, where they bind to class I MHC molecules. The peptide–class I MHC complexes then move through the Golgi complex to the cell surface.

Endogenous antigen is produced within the host cell itself. Two common examples are viral proteins synthesized within virus-infected host cells and unique proteins synthesized by cancerous cells. Endogenous antigens are degraded into peptide fragments that bind to class I MHC molecules within the endoplasmic reticulum. The peptide– class I MHC complex is then transported to the cell membrane. Since all nucleated cells express class I MHC molecules, all cells producing endogenous antigen use this route to process the antigen. T cells displaying CD8 recognize antigen associated with class I MHC molecules and thus are said to be class I MHC restricted. These cytotoxic T cells attack and kill cells displaying the antigen–MHC class I complexes for which their receptors are specific.

- ***Cytokines:***

After a TH cell recognizes and interacts with an antigen–MHC class II molecule complex, the cell is activated—it becomes an effector cell that secretes various growth factors known collectively as cytokines. The secreted cytokines play an important role in activating B cells, TC cells, macrophages, and various other cells that participate in the immune response. Differences in the pattern of cytokines produced by activated TH cells result in different

types of immune response.Under the influence of TH-derived cytokines, a TC cell that recognizes an antigen– MHC class I molecule complex proliferates and differentiates into an effector cell called a cytotoxic T lymphocyte (CTL). In contrast to the TC cell, the CTL generally does not secrete many cytokines and instead exhibits cell-killing or cytotoxic activity. The CTL has a vital function in monitoring the cells of the body and eliminating any that display antigen, such as virus-infected cells, tumor cells, and cells of a foreign tissue graft. Cells that display foreign antigen complexed with a class I MHC molecules are called altered self-cells; these are targets of CTLs.

✓ Part- 4:

Antigens: Substances that can be recognised by the immunoglobulin receptor of B cells, or by the T-cell receptor when complexed with MHC, are called Antigens.

The molecular properties of antigens and the way in which these properties ultimately contribute to immune activation are central to our understanding of the immune system.

1. *Immunogenicity Versus Antigenicity:*

Immunogenicity and antigenicity are related but distinct immunologic properties that sometimes are confused. Immunogenicityis the ability to induce a humoral and/or cell mediated immune response:

B cells + antigen -> effector B cells + memory B cells

||

(plasma cells)

T cells + antigen ->effector T cells + memory T cells

||

(e.g.,CTLs, THs).

Although a substance that induces a specific immune response is usually called an antigen, it is more appropriately called an immunogen.

Antigenicity is the ability to combine specifically with the final products of the above responses (i.e., antibodies and/or cell-surface receptors). Although all molecules that have the property of immunogenicity also have the property of antigenicity, the reverse is not true. Some small molecules, called haptens, are antigenic but incapable, by themselves, of inducing a specific immune response. In other words, they lack immunogenicity.

2. *Factors That Influence Immunogenicity:*

To protect against infectious disease, the immune system must be able to recognize bacteria, bacterial products, fungi, parasites, and viruses as immunogens. In fact, the immune system actually recognizes particular macromolecules of an infectious agent, generally either proteins or polysaccharides. Proteins are the most potent immunogens, with polysaccharides ranking second. In contrast, lipids and nucleic acids of an infectious agent generally do not serve as immunogens unless they are complexed with proteins or polysaccharides. Immunologists tend to use proteins or polysaccharides as immunogens in most experimental studies of humoral immunity (Table 3-1). For cell-mediated immunity, only

TABLE 3-1 Molecular weight of some common experimental antigens used in immunology

Antigen	Approximate molecular mass (Da)
Bovine gamma globulin (BGG)	150,000
Bovine serum albumin (BSA)	69,000
Flagellin (monomer)	40,000
Hen egg-white lysozyme (HEL)	15,000
Keyhole limpet hemocyanin (KLH)	>2,000,000
Ovalbumin (OVA)	44,000
Sperm whale myoglobin (SWM)	17,000
Tetanus toxoid (TT)	150,000

proteins and some lipids and glycolipids serve as immunogens. These molecules are not recognized directly. Proteins must first be processed into small peptides and then presented together with MHC molecules on the membrane of a cell before they can be recognized as immunogens. Recent work shows that those lipids and glycolipids that can elicit cellmediated immunity must also be combined with MHC-like membrane molecules called CD1.

Immunogenicity is not an intrinsic property of an antigen but rather depends on a number of properties of the particular biological system that the antigen encounters. The next two sections describe the properties that most immunogens share and the contribution that the biological system makes to the expression of immunogenicity.

3. *Haptens and the Study of Antigenicity:*

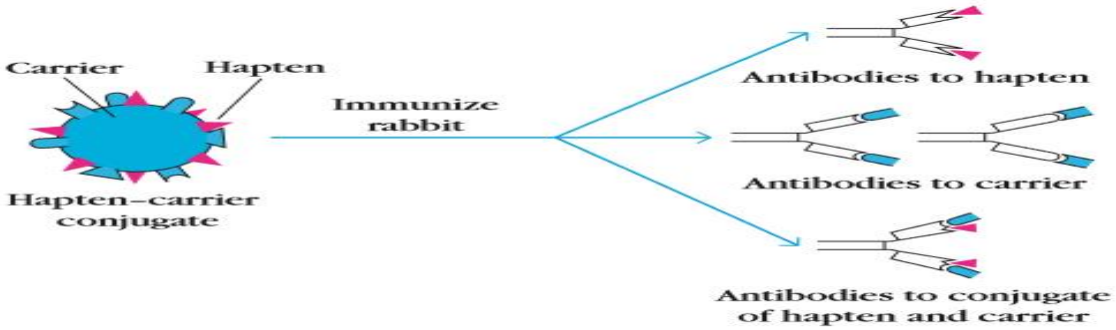
The pioneering work of Karl Landsteiner in the 1920s and 1930s created a simple, chemically defined system for studying the binding of an individual antibody to a unique epitope on a complex protein antigen. Landsteiner employed various haptens, small organic molecules that are antigenic but not immunogenic. Chemical coupling of a hapten to a large protein, called a carrier, yields an immunogenic hapten-carrier conjugate. Animals immunized with such a conjugate produce antibodies specific for:

(1) The hapten determinant,

(2) Unaltered epitopes on the carrier protein, and

(3) New epitopes formed by combined parts of both the hapten and carrier

By itself, a hapten cannot function as an immunogenic epitope. But when multiple molecules of a single hapten are coupled to a carrier protein (or nonimmunogenichomopolymer), the hapten becomes accessible to the immune system and can function as an immunogen.



Injection with:	Antibodies formed:
Hapten (DNP)	None
Protein carrier (BSA)	Anti–BSA
Hapten–carrier conjugate (DNP-BSA)	Anti–DNP (major) Anti–BSA (minor) Anti–DNP/BSA (minor)

FIGURE 3-10 A hapten-carrier conjugate contains multiple copies of the hapten—a small nonimmunogenic organic compound such as dinitrophenol (DNP)—chemically linked to a large protein carrier such as bovine serum albumin (BSA). Immunization with DNP alone elicits no anti-DNP antibodies, but immunization with DNP-BSA elicits three types of antibodies. Of these, anti-DNP antibody is predominant, indicating that in this case the hapten is the immunodominant epitope in a hapten-carrier conjugate, as it often is in such conjugates.

4. *Epitops:*

Immune cells do not interact with, or recognize, an entire immunogen molecule; instead, lymphocytes recognize discrete sites on the macromolecule called epitopes, or antigenic determinants. Epitopes are the immunologically active regions of an immunogen that bind to antigen-specific membrane receptors on lymphocytes or to secreted antibodies. Studies with small antigens have revealed that B and T cells recognize different epitopes on the same antigenic molecule. For example, when mice were immunized with glucagon, a small human hormone of 29 amino acids,antibody was elicited to epitopes in the aminoterminal portion, whereas the T cells responded only to epitopes in the carboxyl-terminal portion. Lymphocytes may interact with a complex antigen on several levels of antigen structure.An epitope on a protein antigen may involve elements of the primary,secondary,tertiary, and even quaternary structure ofthe protein (see Figure 3-1). In polysaccharides, branched chains are commonly present, and multiple branches may contribute to the conformation of epitopes.

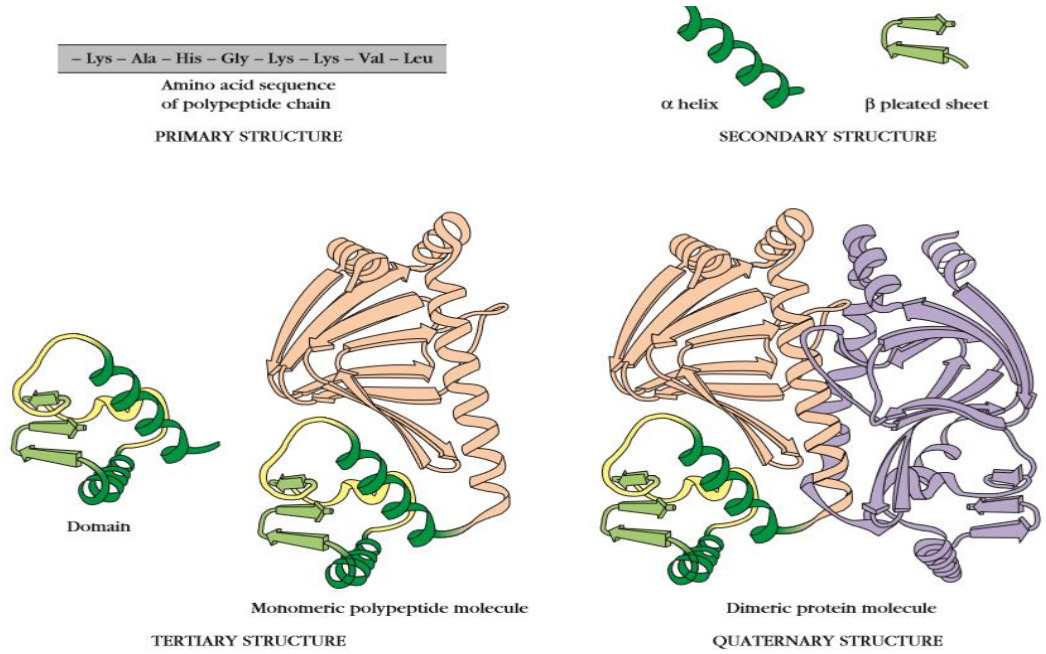


FIGURE 3-1 The four levels of protein organizational structure. The linear arrangement of amino acids constitutes the primary structure. Folding of parts of a polypeptide chain into regular structures (e.g., α helices and β pleated sheets) generates the secondary structure. Tertiary structure refers to the folding of regions between secondary features to give the overall shape of the molecule or parts of it (domains) with specific functional properties. Quaternary structure results from the association of two or more polypeptide chains into a single polymeric protein molecule.

The recognition of antigens by T cells and B cells is fundamentally different (Table 3-4).B cells recognize soluble antigen when it binds to their membrane-bound antibody. Because B cells bind antigen that is free in solution, the epitopes they recognize tend to be highly

TABLE 3-4 Comparison of antigen recognition by T cells and B cells

Characteristic	B cells	T cells
Interaction with antigen	Involves binary complex of membrane Ig and Ag	Involves ternary complex of T-cell receptor, Ag, and MHC molecule
Binding of soluble antigen	Yes	No
Involvement of MHC molecules	None required	Required to display processed antigen
Chemical nature of antigens	Protein, polysaccharide, lipid	Mostly proteins, but some lipids and glycolipids presented on MHC-like molecules
Epitope properties	Accessible, hydrophilic, mobile peptides containing sequential or nonsequential amino acids	Internal linear peptides produced by processing of antigen and bound to MHC molecules

accessible sites on the exposed surface of the immunogen. As noted previously, most T cells recognize only peptides combined with MHC molecules on the surface of antigen-presenting cells and altered self-cells; T-cell epitopes, as a rule, cannot be considered apart from their associated MHC molecules.

5. *ADJUVANTS:*

Adjuvants (from Latin adjuvare, to help) are substances that, when mixed with an antigen and injected with it, enhance the immunogenicity of that antigen. Adjuvants are often used to boost the immune response when an antigen has low immunogenicity or when only small amounts of an antigen are available. For example, the antibody response of mice to immunization with BSA can be increased fivefold or more if the BSA is administered with an adjuvant. Precisely how adjuvants augment the immune response is not entirely known, but they appear to exert one or more of the following effects (Table 3-3):

- Antigen persistence is prolonged.

- Co-stimulatory signals are enhanced.
- Local inflammation is increased.
- The nonspecific proliferation of lymphocytes is stimulated.

Aluminum potassium sulfate (alum) prolongs the persistence of antigen. When an antigen is mixed withalum, the salt precipitates the antigen. Injection of this alum precipitate results in a slower release of antigen from the injection site, so that the effective time of exposure to the antigen increases from a few days without adjuvant to several weeks with the adjuvant. The alum precipitate also increases the size of the antigen, thus increasing the likelihood of phagocytosis.

Water-in-oil adjuvants also prolong the persistence of antigen. A preparation known as Freund's incomplete adjuvant contains antigen in aqueous solution, mineral oil, and an

TABLE 3-3 Postulated mode of action of some commonly used adjuvants

	POSTULATED MODE OF ACTION			
Adjuvant	Prolongs antigen persistence	Enhances co-stimulatory signal	Induces granuloma formation	Stimulates lymphocytes nonspecifically
Freund's incomplete adjuvant	+	+	+	–
Freund's complete adjuvant	+	++	++	–
Aluminum potassium sulfate (alum)	+	?	+	–
Mycobacterium tuberculosis	–	?	+	–
Bordetella pertussis	–	?	–	+
Bacterial lipopolysaccharide (LPS)	–	+	–	+
Synthetic polynucleotides (poly IC/poly AU)	–	?	–	+

emulsifying agent such as mannidemonooleate, which disperses the oil into small droplets surrounding the antigen; the antigen is then released very slowly from the site of injection. This preparation is based on Freund's complete adjuvant, the first deliberately formulated highly effective adjuvant, developed by Jules Freund many years ago and containing heat-killed Mycobacteria as an additional ingredient. Muramyl dipeptide, a component of the mycobacterial cell wall, activates macrophages, making Freund's complete adjuvant far more potent than the incomplete form. Activated macrophages are more phagocytic than

unactivated macrophages and express higher levels of class II MHC molecules and the membrane molecules of the B7 family. The increased expression of class II MHC increases the ability of the antigen-presenting cell to present antigen to TH cells. B7 molecules on the antigen presenting cell bind to CD28, a cell-surface protein on TH cells, triggering co-stimulation, an enhancement of the T-cell immune response. Thus, antigen presentation and the requisite co-stimulatory signal usually are increased in the presence of adjuvant.

Alum and Freund's adjuvants also stimulate a local, chronic inflammatory response that attracts both phagocytes and lymphocytes. This infiltration of cells at the site of the adjuvant injection often results in formation of a dense, macrophage-rich mass of cells called a granuloma. Because the macrophages in a granuloma are activated, this mechanism also enhances the activation of TH cells.

Other adjuvants (e.g., synthetic polyribonucleotides and bacterial lipopolysaccharides) stimulate the nonspecific proliferation of lymphocytes and thus increase the likelihood of antigen-induced clonal selection of lymphocytes.

✓ Part- 5:

1. *Immunoglobulins:*

Immunoglobulins, also known as antibodies, are glycoprotein molecules produced by plasma cells (white blood cells). They act as a critical part of the immune response by specifically recognizing and binding to particular antigens, such as bacteria or viruses, and aiding in their destruction. The antibody immune response is highly complex and exceedingly specific. The various immunoglobulin classes and subclasses (isotypes) differ in their biological features, structure, target specificity and distribution. Hence, the assessment of the immunoglobulin isotype can provide useful insight into complex humoral immune response. Assessment and knowledge of immunoglobulin structure and classes is also important for selection and preparation of antibodies as tools for immunoassays and other detection applications.

The first evidence that antibodies were contained in particular serum protein fractions came from a classic experiment by A.Tiselius and E.A.Kabat,in 1939.They immunized rabbits with the protein ovalbumin (the albumin of egg whites) and then divided the immunized rabbits' serum into two aliquots.Electrophoresis of one serum aliquot revealed four peaks corresponding to albumin and the alpha (α),beta (β), and gamma (γ) globulins. The other serum aliquot was reacted with ovalbumin, and the precipitate that formed was removed;the remaining serum proteins,which did not react with the antigen, were then electrophoresed. A comparison of the electrophoretic profiles of these two serum aliquots revealed that there was a significant drop in the γ-globulinpeak in the aliquot that had been reacted with antigen (Figure 4-1).Thus, the γ-globulin fractionwas identified as containing serum antibodies, which were called immunoglobulins,to distinguish them from any other proteins that might be contained in the γ-globulin fraction.

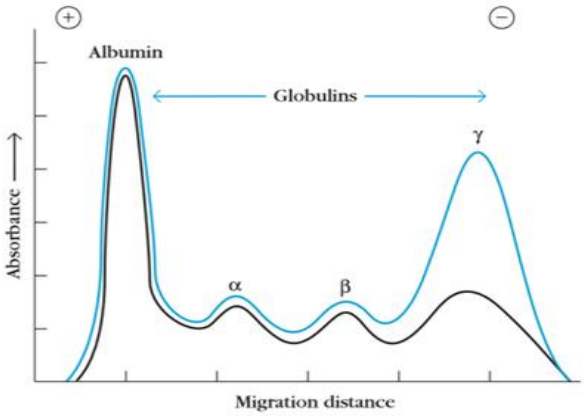


FIGURE 4-1 Experimental demonstration that most antibodies are in the γ-globulin fraction of serum proteins. After rabbits were immunized with ovalbumin (OVA), their antisera were pooled and electrophoresed, which separated the serum proteins according to their electric charge and mass. The blue line shows the electrophoretic pattern of untreated antiserum. The black line shows the pattern of antiserum that was incubated with OVA to remove anti-OVA antibody and then electrophoresed. *[Adapted from A. Tiselius and E. A. Kabat, 1939,* J. Exp. Med. ***69:****119, with copyright permission of the Rockefeller University Press.]*

2. *ImmunoglobulinsAre Heterodimers:*

Antibody molecules have a common structure of four peptide chains (Figure 4-2). This structure consists of two identical light (L) chains, polypeptides of about 25,000 molecular weight, and two identical heavy (H) chains, larger polypeptides of molecular weight 50,000 or more. Like the antibody molecules they constitute, H and L chains are also called immunoglobulins. Each light chain is bound to a heavy chain by a disulfide bond,and by such noncovalent interactions as salt linkages,hydrogen bonds,and hydrophobic bonds,to form a heterodimer (H-L).Similar noncovalent interactions and disulfide bridges link the two identical heavy and light (H-L) chain combinations to each other to form the basic four-chain $(H\text{-}L)_2$ antibody structure, a dimer of dimers. The first 110 or so amino acids of the amino-terminal region of a light or heavy chain vary greatly among antibodies of different specificity. These segments of highly variable sequence are called V regions: V_L in light chains and V_H in heavy. The regions of relatively constant sequence beyond the variable regions have been dubbed C regions, C_L on the light chain and C_H on the heavy chain.

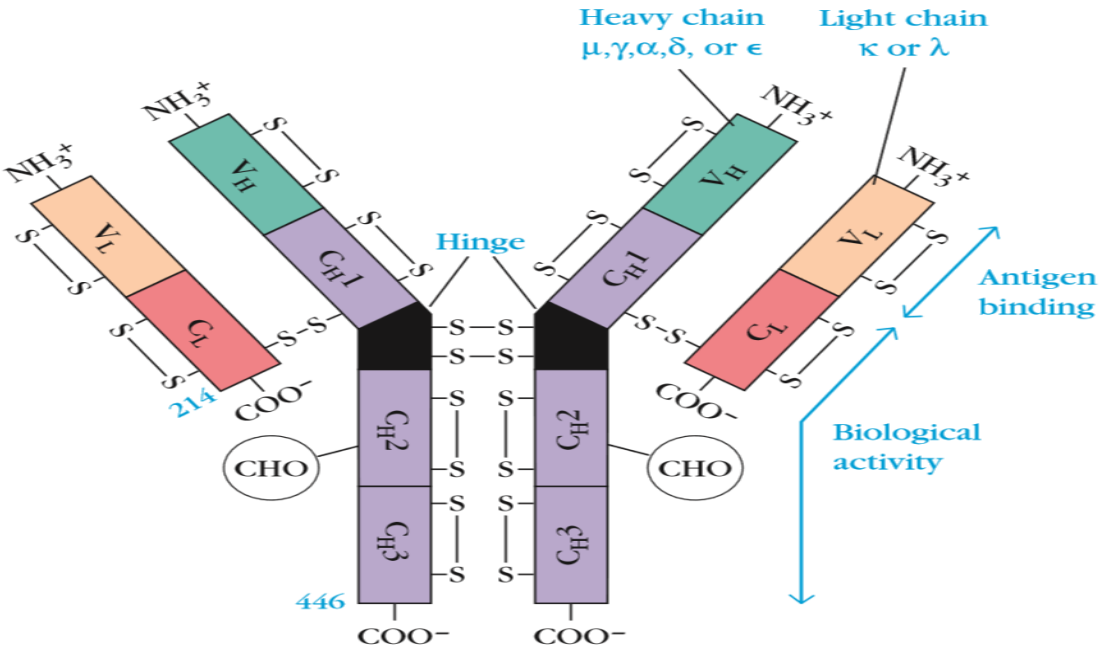


FIGURE 4-2 Schematic diagram of structure of immunoglobulins derived from amino acid sequencing studies. Each heavy and light chain in an immunoglobulin molecule contains an amino-terminal variable (V) region (aqua and tan, respectively) that consists of 100–110 amino acids and differs from one antibody to the next. The remainder of each chain in the molecule—the constant (C) regions (purple and red)—exhibits limited variation that defines the two light-chain subtypes and the five heavy-chain subclasses. Some heavy chains (γ, δ, and α) also contain a proline-rich hinge region (black). The amino-terminal portions, corresponding to the V regions, bind to antigen; effector functions are mediated by the other domains. The μ and ϵ heavy chains, which lack a hinge region, contain an additional domain in the middle of the molecule.

3. ***Light-Chain Sequencing Revealed That Immunoglobulins Have Constant and Variable Regions:***

When the amino acid sequences of several Bence-Jones proteins (light chains) from different individuals were compared, a striking pattern emerged. The amino-terminal half of the chain, consisting of 100–110 amino acids, was found to vary among different Bence-Jones proteins. This region was called the variable (V) region. The carboxyl-terminal half of the molecule, called the constant (C) region, had two basic amino acid sequences. This led to the recognition that there were two light chain types, kappa (κ) and lambda (λ). In humans, 60% of the light chains are kappa and 40% are lambda, whereas in mice, 95% of the light chains are kappa and only 5% are lambda. A single antibody molecule contains only one light chain type, either or, never both.

4. ***Heavy-Chain Sequencing Revealed Five Basic Varieties of Heavy Chains:***

For heavy-chain sequencing studies, myeloma proteins were reduced with mercaptoethanol and alkylated, and the heavy chains were separated by gel filtration in a denaturing solvent. When the amino acid sequences of several myeloma protein heavy chains were compared,a pattern similar to that of the light chains emerged. The amino-terminal part of the chain, consisting of 100–110 amino acids, showed great sequence variation among myeloma heavy chains and was therefore called the variable (V) region.

TABLE 4-1 Chain composition of the five immunoglobulin classes in humans

Class	Heavy chain	Subclasses	Light chain	Molecular formula
IgG	γ	γ1, γ2, γ3, γ4	κ or λ	$\gamma_2\kappa_2$ $\gamma_2\lambda_2$
IgM	μ	None	κ or λ	$(\mu_2\kappa_2)_n$ $(\mu_2\lambda_2)_n$ $n = 1$ or 5
IgA	α	α1, α2	κ or λ	$(\alpha_2\kappa_2)_n$ $(\alpha_2\lambda_2)_n$ $n = 1, 2, 3$, or 4
IgE	ϵ	None	κ or λ	$\epsilon_2\kappa_2$ $\epsilon_2\lambda_2$
IgD	δ	None	κ or λ	$\delta_2\kappa_2$ $\delta_2\lambda_2$

The remaining part of the protein revealed five basic sequence patterns, corresponding to five different heavy-chain constant (C) regions (μ, δ, γ, ε, and α).Each of these five

different heavy chains is called an isotype. The length of the constant regions is approximately 330 amino acids for δ, γ, and α and 440 amino acids for μ and ε.The heavy chains of a given antibody molecule determine the class of that antibody: IgM(μ), IgG(γ), IgA(α), IgD(δ), or IgE(ε). Each class can have either κ or λ light chains.

A single antibody molecule has two identical heavy chains and two identical light chains,H_2L_2,or a multiple $(H_2L_2)_n$ of this basic four-chain structure (Table 4-1).

Minor differences in the amino acid sequences of the α and γheavy chains led to further classification of the heavy chains into subisotypes that determine the subclass of antibody molecules they constitute. In humans, there are two subisotypes of αheavy chains- α1 and α2 (and thus IgG3, and IgG4). In mice, thereare four subisotypes, γ1, γ2a, γ2b,and γ3,and the corresponding subclasses.

Careful analysis ofthe amino acid sequences ofimmunoglobulin heavy and light chains showed that both chains contain several homologous units of about 110 amino acid residues. Within each unit, termed a domain, an intrachaindisulfide bond forms a loop of about 60 amino acids.

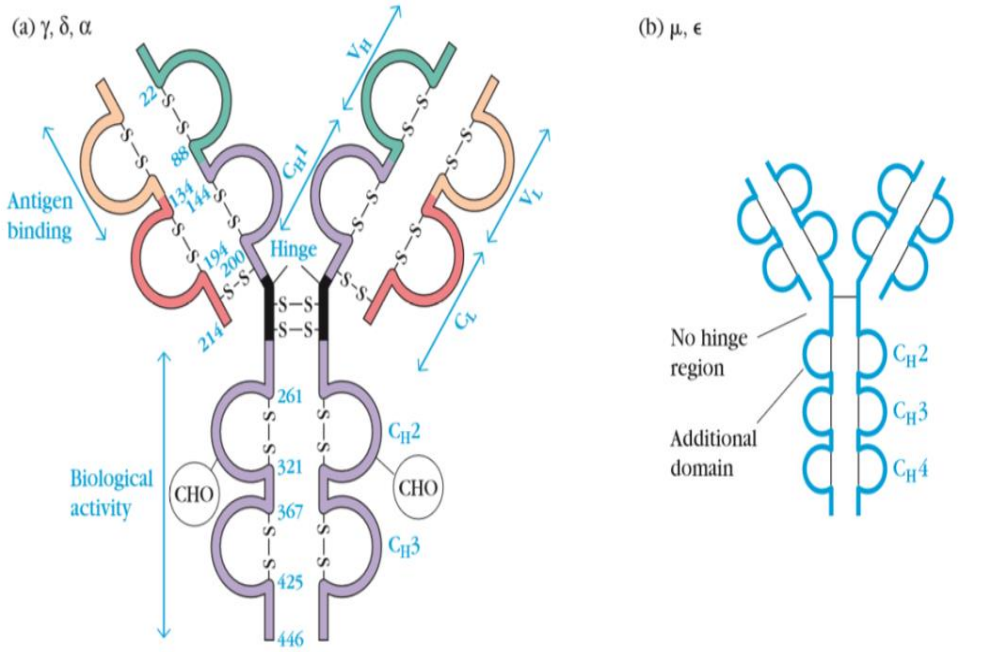


FIGURE 4-6 (a) Heavy and light chains are folded into domains, each containing about 110 amino acid residues and an intrachain disulfide bond that forms a loop of 60 amino acids. The amino-terminal domains, corresponding to the V regions, bind to antigen; effector functions are mediated by the other domains. (b) The μ and ε heavy chains contain an additional domain that replaces the hinge region.

Light chains contain one variable domain (V_L), and one constant domain (C_L);heavy chains contain one variable domain (V_H),and either three or four constant domains two subclasses,IgA1 and IgA2)—and four subisotypes of γ heavy chains: γ1, γ2, γ3, and γ4 (therefore four subclasses, IgG1, IgG2, (C_H1, C_H2, C_H3, and C_H4),depending on the antibody class (Fig 4-6).

5. *Antibody Classes and Biological Activities:*

i. Immunoglobulin G (IgG):

IgG, the most abundant class in serum, constitutes about 80% of the total serum immunoglobulin. The IgG molecule consists of two γ heavy chains and two κ or two λ light chains (see Figure 4-13a).There are four human IgG subclasses,distinguished by differences in γ-chain sequence and numbered according to their decreasing average serum concentrations: IgG1, IgG2, IgG3,and IgG4 (Table 4-2).

TABLE 4-2 Properties and biological activities* of classes and subclasses of human serum immunoglobulins

Property/Activity	IgG1	IgG2	IgG3	IgG4	IgA1	IgA2	IgM‡	IgE	IgD
Molecular weight†	150,000	150,000	150,000	150,000	150,000–600,000	150,000–600,000	900,000	190,000	150,000
Heavy-chain component	γ1	γ2	γ3	γ4	α1	α2	μ	ϵ	δ
Normal serum level (mg/ml)	9	3	1	0.5	3.0	0.5	1.5	0.0003	0.03
In vivo serum half life (days)	23	23	8	23	6	6	5	2.5	3
Activates classical complement pathway	+	+/−	++	−	−	−	+++	−	−
Crosses placenta	+	+/−	+	+	−	−	−	−	−
Present on membrane of mature B cells	−	−	−	−	−	−	+	−	+
Binds to Fc receptors of phagocytes	++	+/−	++	+	−	−	?	−	−
Mucosal transport	−	−	−	−	++	++	+	−	−
Induces mast-cell degranulation	−	−	−	−	−	−	−	+	−

*Activity levels indicated as follows: ++ = high; + = moderate; +/− = minimal; − = none; ? = questionable.

†IgG, IgE, and IgD always exist as monomers; IgA can exist as a monomer, dimer, trimer, or tetramer. Membrane-bound IgM is a monomer, but secreted IgM in serum is a pentamer.

‡IgM is the first isotype produced by the neonate and during a primary immune response.

The amino acid sequences that distinguish the four IgG subclasses are encoded by different germ-line C_H genes, whose DNA sequences are 90%–95% homologous. The structural characteristics that distinguish these subclasses from one another are the size of the hinge region and the number and position of the interchaindisulfide bonds between the heavy chains (Figure 4-14).

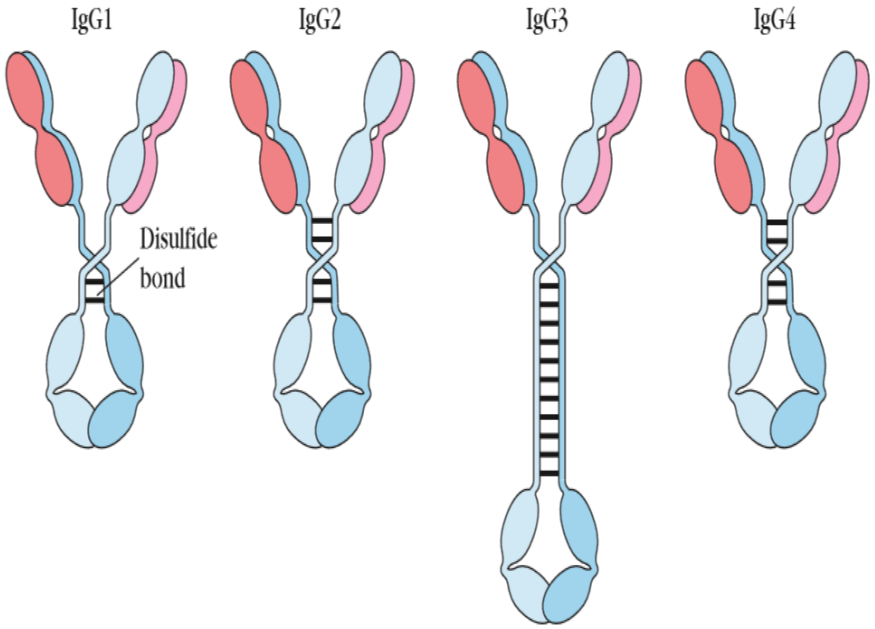


FIGURE 4-14 General structure of the four subclasses of human IgG, which differ in the number and arrangement of the interchain disulfide bonds (thick black lines) linking the heavy chains. A notable feature of human IgG3 is its 11 interchain disulfide bonds.

The subtleamino acid differences between subclasses of IgG affect the biological activity of the molecule:

- IgG1,IgG3,and IgG4 readily cross the placenta and play an important role in protecting the developing fetus.
- IgG3 is the most effective complement activator, followed by IgG1; IgG2 is less efficient,and IgG4 is not able to activate complement at all.
- IgG1 and IgG3 bind with high affinity to Fc receptors on phagocytic cells and thus mediate opsonisation.IgG4 has an intermediate affinity for Fc receptors,and IgG2 has an extremely low affinity.

ii. Immunoglobulin M (IgM):

IgM accounts for 5%–10% of the total serum immunoglobulin, with an average serum concentration of 1.5 mg/ml. Monomeric IgM, with a molecular weight of 180,000, is expressed as membrane-bound antibody on B cells.IgM is secreted by plasma cells as a pentamer in which five monomer units are held together by disulfide bonds that link their carboxyl-terminal heavy chain domains (Cμ4/Cμ4) and their Cμ3/Cμ3 domains (see Figure 4-13e). The five monomer subunits are arranged with their Fc regions in the center of the pentamer and the ten antigen-binding sites on the periphery of the molecule. Each pentamer contains anadditional Fc-linked polypeptide called the J (joining) chain, which is disulfide-bonded to the carboxyl-terminal cysteine residue of two of the ten μ chains. The J chain appears to be required for polymerization of the monomers to form pentamericIgM;it is added just before secretion of the pentamer.

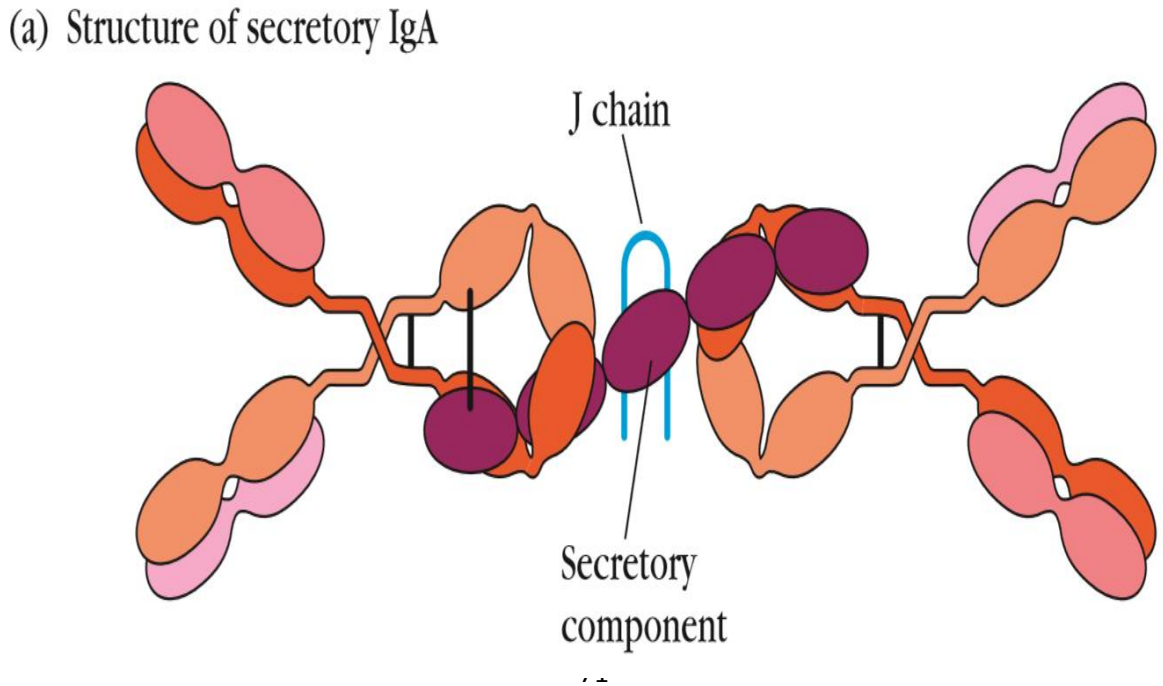
IgM is the first immunoglobulin class produced in a primary response to an antigen, and it is also the first immunoglobulin to be synthesized by the neonate. Because of its pentameric structure with 10 antigen-binding sites,serum IgM has a higher valency than the other isotypes. An IgM molecule can bind 10 small hapten molecules; however, because of steric hindrance,only 5 or fewer molecules of larger antigens can be bound simultaneously.Because ofits high valency,pentamericIgM is more efficient than other isotypes in binding antigens with many repeating epitopes such as viral particles and red blood cells (RBCs). It takes 100 to 1000 times more molecules of IgG than of IgM to achieve the same level of agglutination.A similar phenomenon occurs with viral particles:less IgM than IgG is requiredto neutralize viral infectivity. IgM is also more efficient than IgG at activating complement. Complement activation requires two Fc regions in close proximity,and the pentameric structure ofa single molecule ofIgMfulfills this requirement.

Because of its large size, IgM does not diffuse well and therefore is found in very low concentrations in the intercellular tissue fluids. The presence of the J chain allows IgM to bind to receptors on secretory cells,which transport it across epithelial linings to enter the external secretions that bathe mucosal surfaces. Although IgA is the major isotype found in these secretions,IgM plays an important accessory role as a secretory immunoglobulin.

iii. ImmunoglobulinA (IgA):

Although IgA constitutes only 10%–15% of the total immunoglobulin in serum, it is the predominant immunoglobulin class in external secretions such as breast milk, saliva, tears, and mucus of the bronchial, genitourinary,and digestive tracts.In serum,IgA exists primarily as a monomer, but polymeric forms (dimers, trimers, and some tetramers) are sometimes seen, all containing a J-chainpolypeptide (see Figure 4-13d). The IgA of external secretions,called secretory IgA, consists of a dimer or tetramer,a J-chain polypeptide, and a polypeptide chain called secretory component (Figure 4-15a).The J-chain polypeptide in IgA is identical to that found in pentamericIgM and serves a similar function in facilitating the polymerization of both serum IgA and secretory IgA.The secretory component is a 70,000-MW polypeptide produced by epithelial cells of mucous membranes. It consists of five immunoglobulin-like domains that bind to the Fc region domains of the IgA dimer. This interaction is stabilized by a disulfide bond between the fifth domain of the secretory component and one of the chains of the dimeric IgA.

The daily production of secretory IgA is greater than that of any other immunoglobulin class. IgA-secreting plasma cells are concentrated along mucous membrane surfaces.Breast milk contains secretory IgA and many other molecules that help protect the newborn against infection during the first month of life. Because the immune system of infants is not fully functional,breast-feeding plays an important role in maintaining the health of newborns.



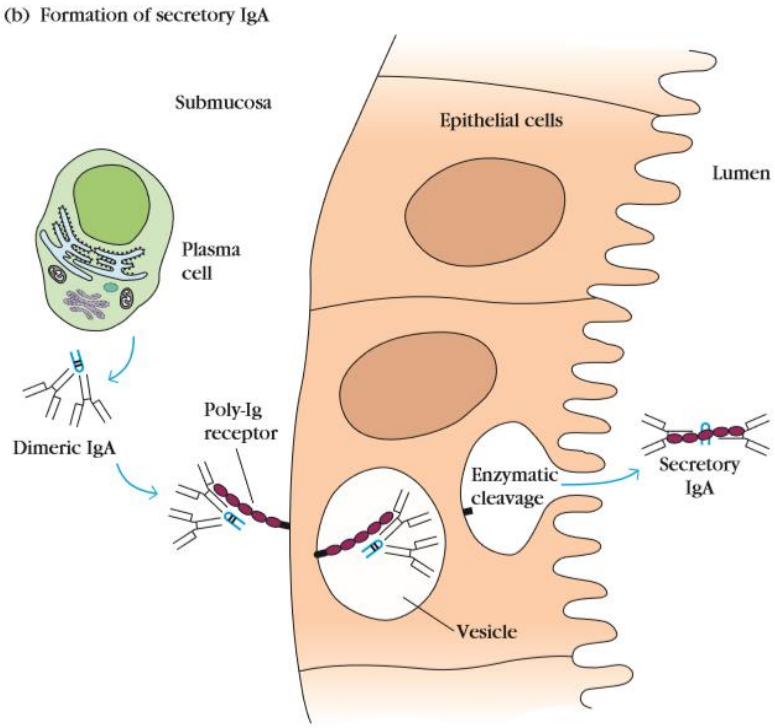


FIGURE 4-15 Structure and formation of secretory IgA. (a) Secretory IgA consists of at least two IgA molecules, which are covalently linked to each other through a J chain and are also covalently linked with the secretory component. The secretory component contains five Ig-like domains and is linked to dimeric IgA by a disulfide bond between its fifth domain and one of the IgA heavy chains. (b) Secretory IgA is formed during transport through mucous membrane epithelial cells. Dimeric IgA binds to a poly-Ig receptor on the basolateral membrane of an epithelial cell and is internalized by receptor-mediated endocytosis. After transport of the receptor-IgA complex to the luminal surface, the poly-Ig receptor is enzymatically cleaved, releasing the secretory component bound to the dimeric IgA.

Secretory IgA serves an important effector function at mucous membrane surfaces, which are the main entry sites for most pathogenic organisms. Because it is polymeric, secretory IgA can cross-link large antigens with multiple epitopes. Binding of secretory IgA to bacterial and viral surface antigens prevents attachment of the pathogens to the mucosal cells, thus inhibiting viral infection and bacterial colonization. Complexes of secretory IgA and antigen are easily entrapped in mucus and then eliminated by the ciliated epithelial cells of the respiratory tract or by peristalsis of the gut. Secretory IgA has been shown to provide an important line of defense against bacteria such as *Salmonella, Vibrio cholerae,*and *Neisseria gonorrhoeaeand* viruses such as polio, influenza,andreovirus.

iv. ImmunoglobulinE (IgE):

The potent biological activity of IgE allowed it to be identified in serum despite its extremely low average serum concentration (0.3 µg/ml).IgE antibodies mediate the immediate hypersensitivity reactions that are responsible for the symptoms of hay fever,asthma,hives,and anaphylactic shock.The presence of a serum component responsible

for allergic reactions was first demonstrated in 1921 by K. Prausnitz and H. Kustner, who injected serum from an allergic person intra-dermally into a nonallergic individual. When the appropriate antigen was later injected at the same site, a wheal and flare reaction (analogous to hives) developed there. This reaction, called the P-K reaction (named for its originators,Prausnitz and Kustner),was the basis for the first biological assay for IgE activity.

IgE binds to Fc receptors on the membranes of blood basophils and tissue mast cells.

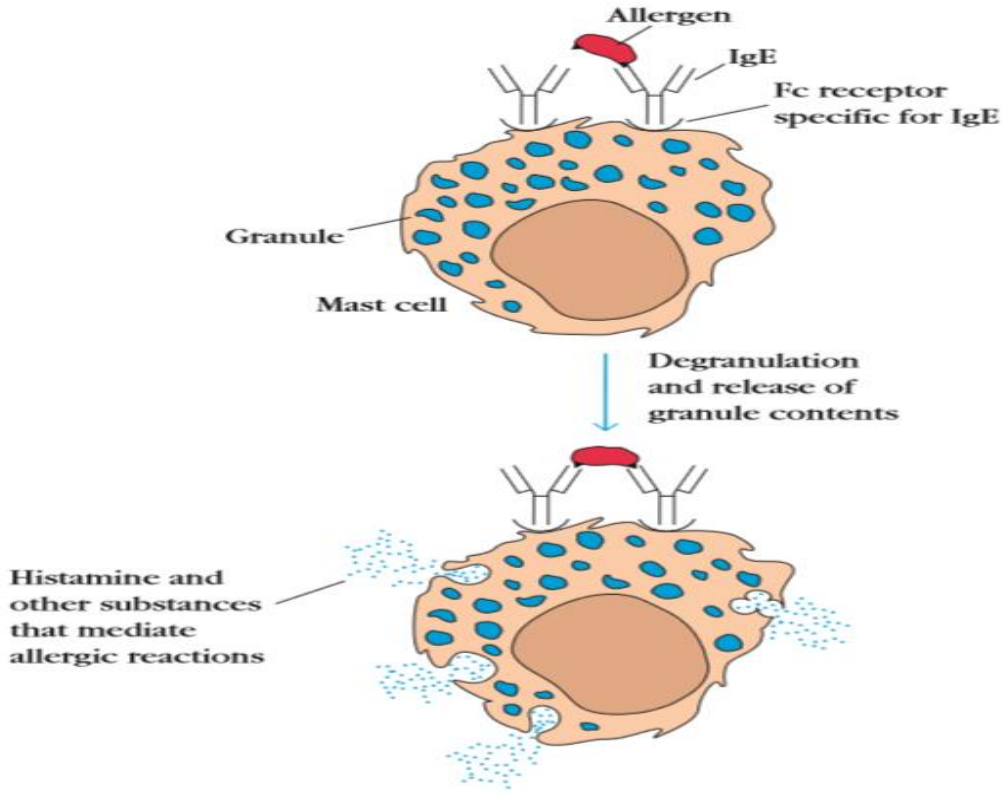


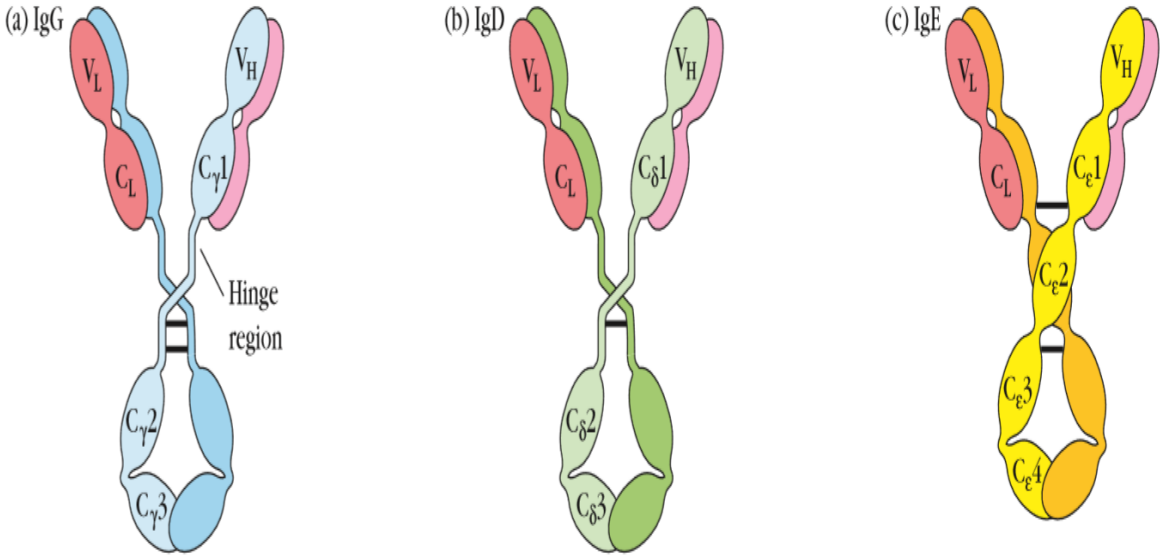
FIGURE 4-16 Allergen cross-linkage of receptor-bound IgE on mast cells induces degranulation, causing release of substances (blue dots) that mediate allergic manifestations.

Cross-linkage of receptorboundIgE molecules by antigen (allergen) induces basophils and mast cells to translocate their granules to the plasma membrane and release their contents to the extracellular environment, a process known as degranulation. As a result, a variety of pharmacologically active mediators are released and give rise to allergic manifestations (Figure 4-16).

Localized mast-cell degranulation induced by IgE also may release mediators that facilitate a buildup of various cells necessary for antiparasiticdefense.

v. Immunoglobulin D (IgD):

IgD was first discovered when a patient developed a multiple myeloma whose myeloma protein failed to react with antiisotype antisera against the then-known isotypes: IgA, IgM, and IgG. When rabbits were immunized with this myeloma protein,the resulting antisera were used to identify the same class of antibody at low levels in normal human serum. The new class,called IgD,has a serum concentration of 30 g/ml and constitutes about 0.2% of the total immunoglobulin in serum. IgD, together with IgM, is the major membraneboundimmunoglobulin expressed by mature B cells,and its role in the physiology of B cells is under investigation.No biological effector function has been identified for IgD.



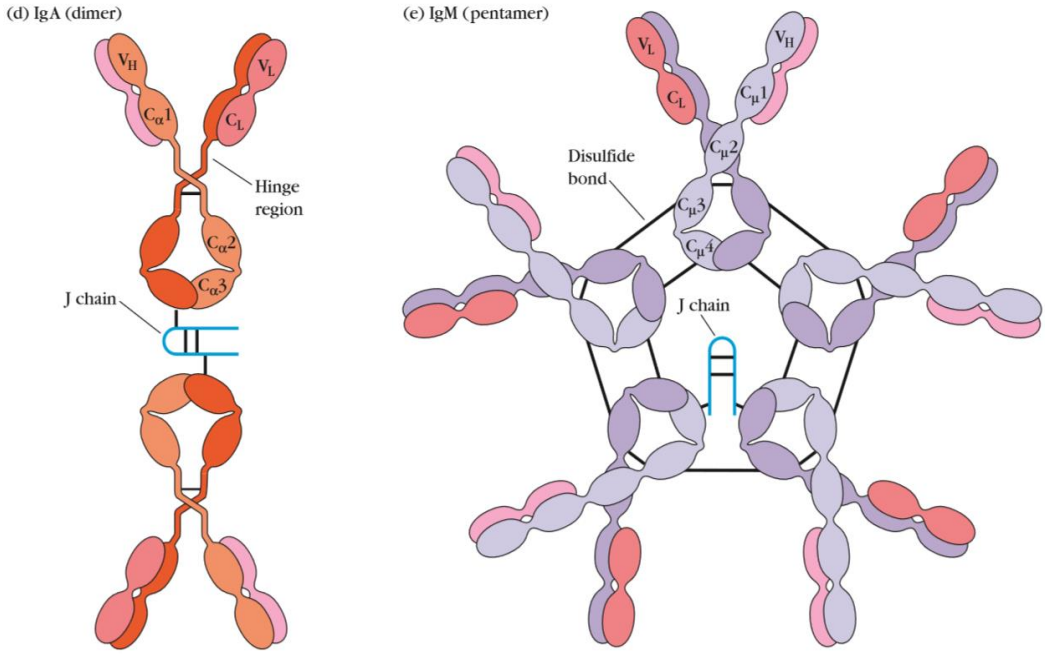


FIGURE 4-13 General structures of the five major classes of secreted antibody. Light chains are shown in shades of pink, disulfide bonds are indicated by thick black lines. Note that the IgG, IgA, and IgD heavy chains (blue, orange, and green, respectively) contain four domains and a hinge region, whereas the IgM and IgE heavy chains (purple and yellow, respectively) contain five domains but no hinge region. The polymeric forms of IgM and IgA contain a polypeptide, called the J chain, that is linked by two disulfide bonds to the Fc region in two different monomers. Serum IgM is always a pentamer; most serum IgA exists as a monomer, although dimers, trimers, and even tetramers are sometimes present. Not shown in these figures are intrachain disulfide bonds and disulfide bonds linking light and heavy chains (see Figure 4-2).

6. ***Monoclonal Antibody:***

Most antigens offer multiple epitopes and therefore induce proliferation and differentiation of a variety of B-cell clones,each derived from a B cell that recognizes a particular epitope.The resulting serum antibodies are heterogeneous, comprising a mixture of antibodies, each specific for one epitope (Figure4-21).Such a polyclonal antibodyresponse facilitates the localization,phagocytosis,and complement-mediated lysis of antigen; it thus has clear advantages for the organism in vivo. Unfortunately, the antibody heterogeneity that increases immune protection in vivo often reduces the efficacy of an antiserum for various in vitro

uses. For most research, diagnostic, and therapeutic purposes, monoclonal antibodies, derived from a single clone and thus specific for a single epitope,are preferable.

Direct biochemical purification of a monoclonal antibody from a polyclonal antibody preparation is not feasible. In 1975, Georges Köhler and Cesar Milstein devised a method for preparing monoclonal antibody, which quickly became one of immunology's key technologies. By fusing a normal activated,antibody-producing B cell with a myeloma cell (a cancerous plasma cell),they were able to generate a hybrid cell, called a hybridoma, that possessed the immortalgrowth properties of the myeloma cell and secreted the antibody produced by the B cell.The resulting clones of hybridoma cells, which secrete large quantities of monoclonal antibody, can be cultured indefinitely. The development of techniques for producing monoclonal antibodiesgave immunologists a powerful and versatile research tool. The significance of the work by Köhler and Milstein was acknowledged when each was awarded a Nobel Prize.

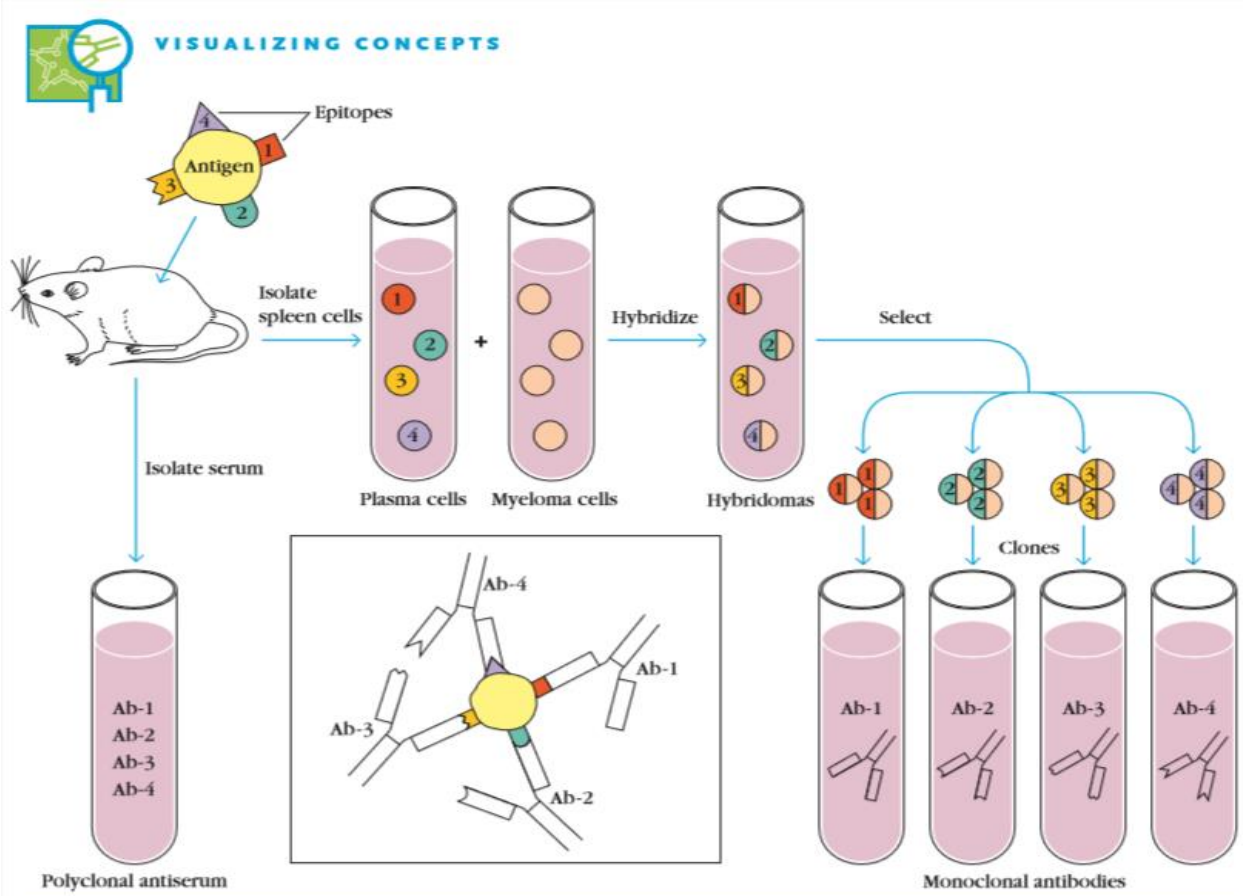


FIGURE 4-21 The conventional polyclonal antiserum produced in response to a complex antigen contains a mixture of monoclonal antibodies, each specific for one of the four epitopes shown on the antigen (inset). In contrast, a monoclonal antibody, which is derived from a single plasma cell, is specific for one epitope on a complex antigen. The outline of the basic method for obtaining a monoclonal antibody is illustrated here.

- ***Monoclonal Antibodies Have Important Clinical Uses:***

Monoclonal antibodies are proving to be very useful as diagnostic, imaging, and therapeutic reagents in clinical medicine.Initially,monoclonal antibodies were used primarily as in vitro diagnostic reagents. Among the many monoclonal antibody diagnostic reagents now available are products for detecting pregnancy, diagnosing numerous pathogenic microorganisms, measuring the blood levels of various drugs, matching histocompatibility antigens, and detecting antigens shed by certain tumors.

Radiolabeled monoclonal antibodies can also be used in vivo for detecting or locating tumor antigens,permitting earlier diagnosis of some primary or metastatic tumors in patients. For example, monoclonal antibody to breast-cancer cells is labeled with iodine-131 and

introduced into the blood to detect the spread of a tumor to regional lymph nodes.This monoclonal imaging technique can reveal breast-cancer metastases that would be undetected by other, less sensitive scanning techniques.

Immunotoxins composed of tumor-specific monoclonal antibodies coupled to lethal toxins are potentially valuable therapeutic reagents.The toxins used in preparing immunotoxins include ricin, Shigella toxin, and diphtheria toxin, all of which inhibit protein synthesis.

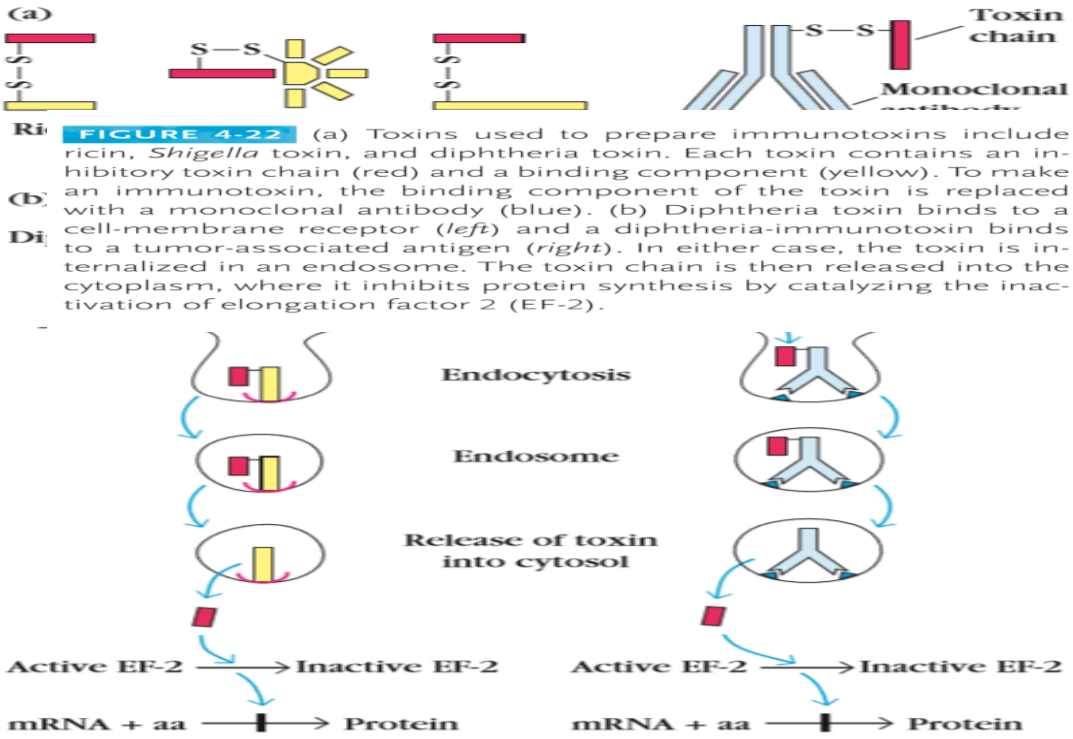


FIGURE 4-22 (a) Toxins used to prepare immunotoxins include ricin, *Shigella* toxin, and diphtheria toxin. Each toxin contains an inhibitory toxin chain (red) and a binding component (yellow). To make an immunotoxin, the binding component of the toxin is replaced with a monoclonal antibody (blue). (b) Diphtheria toxin binds to a cell-membrane receptor (*left*) and a diphtheria-immunotoxin binds to a tumor-associated antigen (*right*). In either case, the toxin is internalized in an endosome. The toxin chain is then released into the cytoplasm, where it inhibits protein synthesis by catalyzing the inactivation of elongation factor 2 (EF-2).

These toxins are so potent that a single molecule has been shown to kill a cell. Each of these toxins consists of two types of functionally distinct polypeptide components, an inhibitory (toxin) chain and one or more binding chains,which interact with receptors on cell surfaces; without the binding polypeptide(s) the toxin cannot get into cells and therefore is harmless.An immunotoxin is prepared by replacing the binding polypeptide(s) with a monoclonal antibody that is specific for a particular tumor cell (Figure 4-22a). In theory, the

attached monoclonal antibody will deliver the toxin chain specifically to tumor cells, where it will cause death by inhibiting protein synthesis (Figure 4-22b). The initial clinical responses to such immunotoxins in patients with leukemia, lymphoma, and some other types of cancer have shown promise,and research to develop and demonstrate their safety and effectiveness is underway.

✓ Part- 6:

1. Complement: Components, function, mode of action:

The complement system, also known as complement cascade, is a part of the immune system that enhances (complements) the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism, promote inflammation, and attack the pathogen's cell membrane. It is part of the innate immune systemwhich is not adaptable and does not change during an individual's lifetime. The complement system can, however, be recruited and brought into action by antibodies generated by the adaptive immune system.

- ***Components:***

The complement system consists of a number ofconstitutive serum small proteins that are synthesized by the liver, and circulate in the blood as inactive precursors. When stimulated by one of several triggers, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. The end result of this complement activation or complement fixation cascade is stimulation of phagocytes to clear foreign and damaged material, inflammation to attract additional phagocytes, and activation of the cell-killing membrane attack complex. Over 30 proteins and protein fragments make up the complement system, including serum proteins, and cell membrane receptors. They account for about 10% of the globulin fraction of blood serum.The proteins and glycoproteins composing the complement system are synthesized largely by liver hepatocytes, some by blood monocytes, tissue macrophages and epithe-lial cells of the gastro-intestinal and genito-urinary tracts.

i. The proteins that form the complement system are labelled numerically with the prefix C (e.g., C1 –C9).

ii. Some complement components are designated by letter symbols (e.g., factor B, D, P) or by trivial names (e.g., homologous factor).

iii. There are at least 19 of these components; they are all serum proteins and together they make up about 10% globulin fraction of serum.

iv. The molecular weights of the complement components vary between 24 kDa for factor D and 460 kDa for C19.

v. Serum concentration in humans varies between 20 μg/ml of C2 and 1300 μg/ml of C3.

vi. Complement components are synthesized at various sites like C2, C3, C4, C5; B, D, P and I are from macrophages, C3, C6, C8 and B from liver (Table 7.1 and7.2).

Table 7.1: Complement components

Name	MW (kDa)	Serum concentration (μg/ml)
Classical pathway		
C1q	460	80
C1r	83	50
C1s	83	50
C4	200	600
C2	102	20
C3	185	1300
Alternative pathway		
D	24	1
B	90	210
Terminal components		
C5	204	70
C6	120	65
C7	120	55
C8	160	55
C9	70	60
Control proteins		
C1-INH	105	200
C4-bp	550	250
H	150	480
I	88	35
P	4 × 56	20
Vitronectin	83	500

Table 7.2: The components of the complement system and their functions

Functionally distinct classes of complement protein	
Function	**Protein**
Binding to antigen; antibody complexes	C1q
Activating enzymes	C1r C1s C2b Bb D
Membrane-binding proteins and opsonins	C4b C3b
Peptide mediators of inflammation	C5a C3a C4a
Membrane attack proteins	C5b C6 C7 C8 C9

- ***Mode of action:***

Mainly two biochemical pathways activate the complement system:

1. The classical complement pathway:

The classical pathway is triggered by activation of the C1-complex. The C1-complex is composed of 1 molecule of C1q, 2 molecules of C1r and 2 molecules of C1s, or $C1qr^2s^2$. This occurs when C1q binds to IgM or IgGcomplexed with antigens. A single pentamericIgM can initiate the pathway, while several, ideally six, IgGs are needed. This also occurs when C1q binds directly to the surface of the pathogen. Such binding leads to conformational changes in the C1q molecule, which leads to the activation of two C1r molecules. C1r is a serine protease. They then cleave C1s (another serine protease). The $C1r^2s^2$ component now splits C4 and then C2, producing C4a, C4b, C2a, and C2b (historically, the larger fragment of C2 was called C2a but is now referred to as C2b). C4b and C2a bind to form the classical pathway C3-convertase (C4b2a complex), which promotes cleavage of C3 into C3a and C3b. C3b later joins with C4b2a to make C5 convertase (C4b2a3b complex).

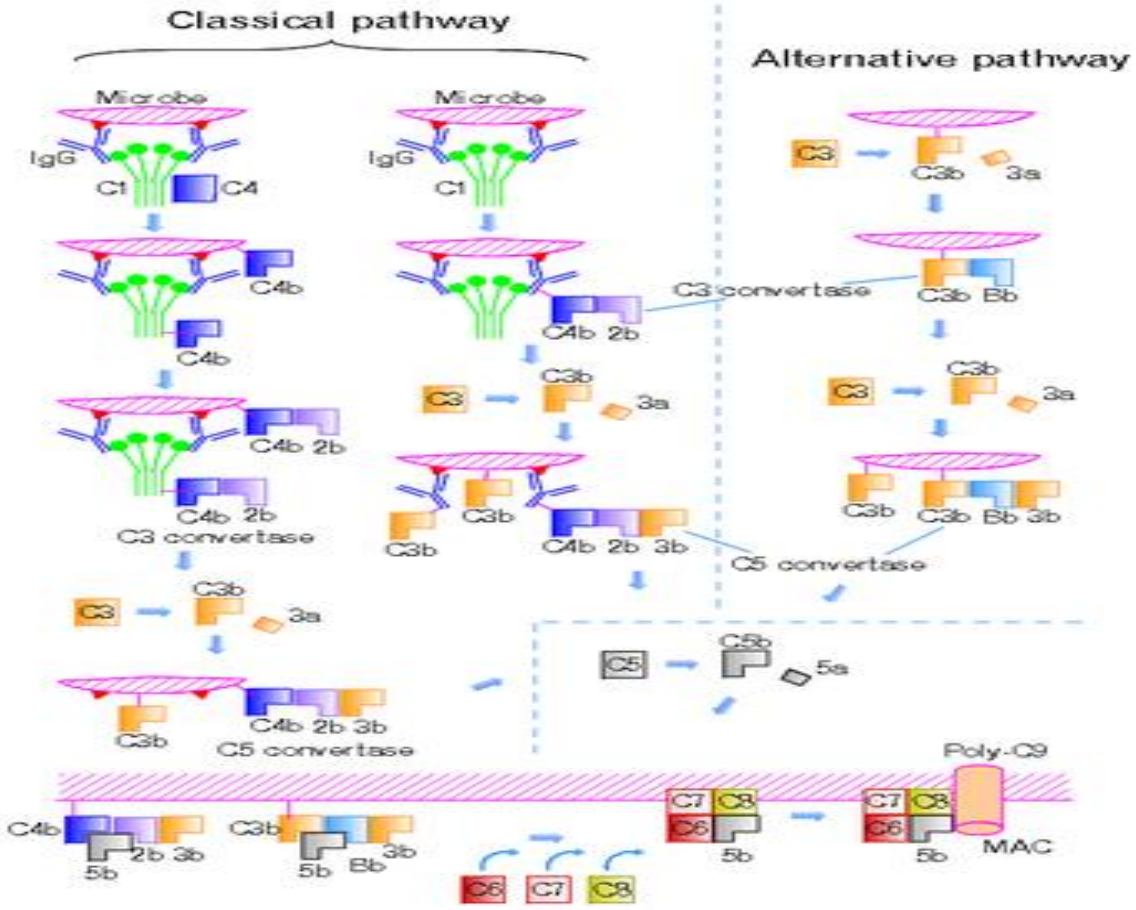
2. The alternative complement pathway:

The alternative pathway is continuously activated at a low level, analogous to a car engine at idle, as a result of spontaneous C3 hydrolysis due to the breakdown of the internal thioester bond (C3 is mildly unstable in aqueous environment). The alternative pathway does not rely on pathogen-binding antibodies like the other pathways. C3b that is generated from C3 by a C3 convertase enzyme complex in the fluid phase is rapidly inactivated by factor H and factor I, as is the C3b-like C3 that is the product of spontaneous cleavage of the internal thioester. In contrast, when the internal thioester of C3 reacts with a hydroxyl or amino group of a molecule on the surface of a cell or pathogen, the C3b that is now covalently bound to the surface is protected from factor H-mediated inactivation. The surface-bound C3b may now bind factor B to form C3bB. This complex in the presence of factor D will be cleaved into Ba and Bb. Bb will remain associated with C3b to form C3bBb, which is the alternative pathway C3 convertase.

The C3bBb complex is stabilized by binding oligomers of factor P (properdin). The stabilized C3 convertase, C3bBbP, then acts enzymatically to cleave much more C3, some of which becomes covalently attached to the same surface as C3b. This newly bound C3b recruits more B, D and P activity and greatly amplifies the complement activation. When complement is activated on a cell surface, the activation is limited by endogenous complement regulatory proteins, which include CD35, CD46, CD55 and CD59, depending on the cell. Pathogens, in

general, don't have complement regulatory proteins (there are many exceptions, which reflect adaptation of microbial pathogens to vertebrate immune defenses). Thus, the alternative complement pathway is able to distinguish self from non-self on the basis of the surface expression of complement regulatory proteins. Host cells don't accumulate cell surface C3b (and the proteolytic fragment of C3b called iC3b) because this is prevented by the complement regulatory proteins, while foreign cells, pathogens and abnormal surfaces may be heavily decorated with C3b and iC3b. Accordingly, the alternative complement pathway is one element of innate immunity.

Once the alternative C3 convertase enzyme is formed on a pathogen or cell surface, it may bind covalently another C3b, to form C3bBbC3bP, the C5 convertase. This enzyme then cleaves C5 to C5a, a potent anaphylatoxin, and C5b. The C5b then recruits and assembles C6, C7, C8 and multiple C9 molecules to assemble the membrane attack complex. This creates a hole or pore in the membrane that can kill or damage the pathogen or cell.



- ***Function:***

1. **Opsonization and phagocytosis:**

   C3b, bound to immune complex or coated on the surface of pathogen, activate phagocytic cells. These proteins bind to specific receptors on the phagocytic cells to get engulfed.

2. **Cell lysis:**

   Membrane attack complex formed by C5b6789 components ruptures the microbial cell surface which kills the cell.

3. **Chemotaxis:**

   Complement fragments attract neutrophils and macrophages to the area where the antigen is present. These cell surfaces have receptors for complements, like C5a, C3a, thus, run towards the site of inflammation, i.e. chemotaxis.

4. **Activation of mast cells and basophils and enhancement of inflammation:**

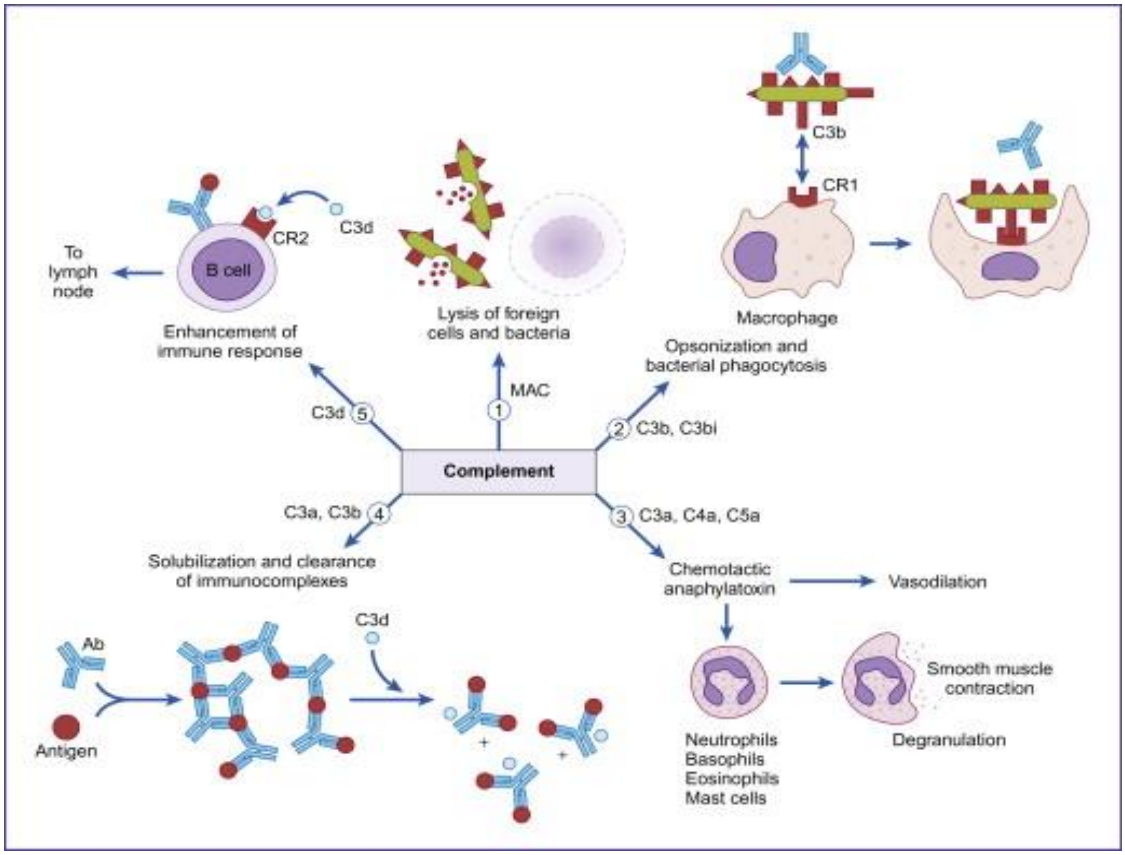
   The proteolytic complement fragments, C5a, C4a, and C3a induce acute inflammation by activating mast cells and neutrophils. All three peptides bind to mast cells and induce degranulation, with the release of vasoactive mediators such as histamine. These peptides are also called anaphylatoxins because the mast cell reactions they trigger are characteristic of anaphylaxis. Binding to specific complement receptors on cells of the immune system, they trigger specific cell functions, inflammation, and secretion of immunoregulatory molecules.

5. **Production of antibodies:**

   B cells have receptor for C3b. When C3b binds to B-cell, it secretes more antibodies. Thus C3b is also an antibody producing amplifiers which converts it into an effective defense mechanism to destroy invading microorganism.

6. **Immune clearance:**

   The complement system removes immune complexes from the circulation and deposits them in the spleen and liver. Thus it acts as anti-inflammatory function. Complement proteins promote the solubilization of these complexes and their clearance by phagocytes.



✓ <u>Part- 7:</u>*Antigen – Antibody interactions: Agglutination, precipitation, immune diffusion, immuunoelectrophoresis.*

i. Strength of Antigen-Antibody Interactions:

The noncovalent interactions that form the basis of antigen-antibody (Ag-Ab) binding include hydrogen bonds, ionic bonds,hydrophobic interactions,and van der Waals interactions (Figure 6-1). Because these interactions are individually weak (compared with a covalent bond), a large number of such interactions are required to form a strong Ag-Ab interaction. Furthermore, each of these noncovalent interactions operates over a very short distance, generally about $1x10^{-7}$ mm (1 angstrom, Å); consequently, a strong Ag-Ab interaction depends on a very close fit between the antigen and antibody. Such fits require a high degree of complementarity between antigen and antibody, a requirement that underlies the exquisite specificity that characterizes antigen-antibody interactions.

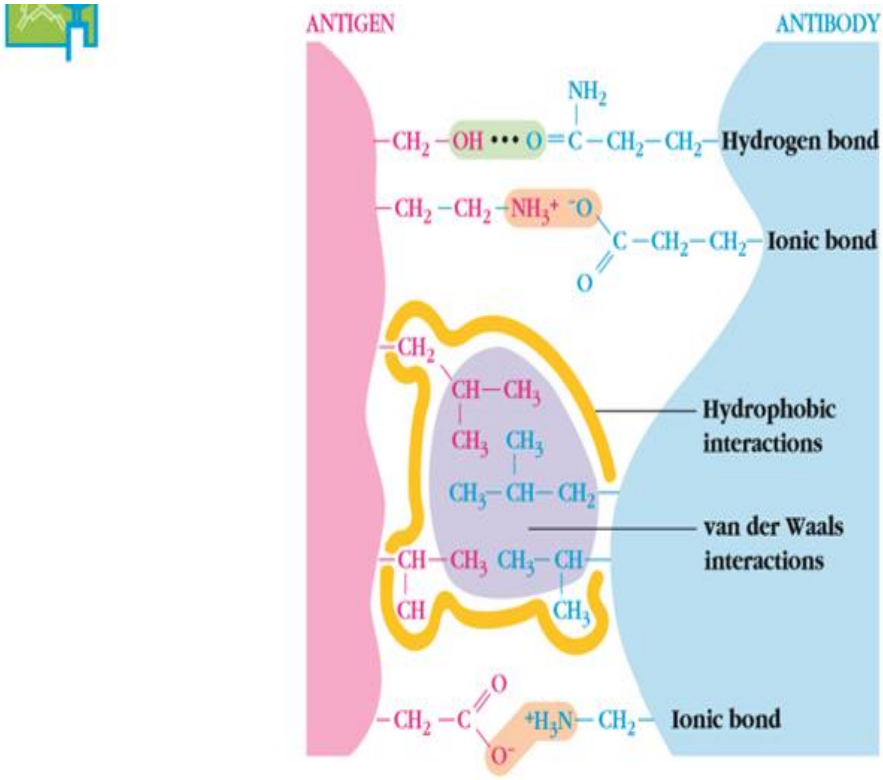


FIGURE 6-1 The interaction between an antibody and an antigen depends on four types of noncovalent forces: (1) hydrogen bonds, in which a hydrogen atom is shared between two electronegative atoms; (2) ionic bonds between oppositely charged residues; (3) hydrophobic interactions, in which water forces hydrophobic groups together; and (4) van der Waals interactions between the outer electron clouds of two or more atoms. In an aqueous environment, noncovalent interactions are extremely weak and depend upon close complementarity of the shapes of antibody and antigen.

ii. Precipitation Reactions:

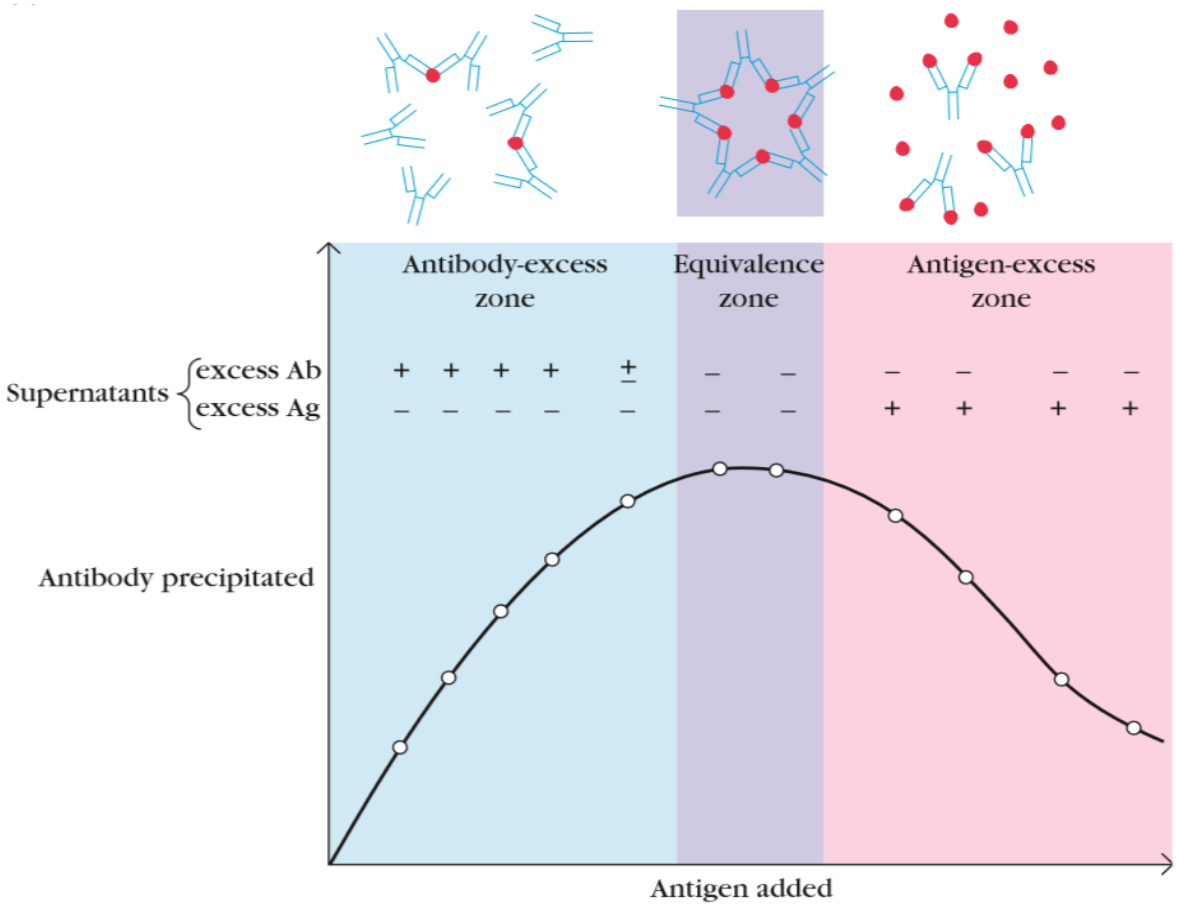
Antibody and soluble antigen interacting in aqueous solution form a lattice that eventually develops into a visible precipitate. Antibodies that aggregate soluble antigens are called precipitins. Although formation of the soluble Ag-Ab complex occurs within minutes, formation of the visible precipitate occurs more slowly and often takes a day or two to reach completion.

Formation of an Ag-Ab lattice depends on the valency of both the antibody and antigen:

- The antibody must be bivalent; a precipitate will not form with monovalent Fab fragments.
- The antigen must be either bivalent or polyvalent; that is, it must have at least two copies of the same epitope,or have different epitopes that react with different antibodies present in polyclonal antisera.

- ***Precipitation Reactions in Fluids Yield a Precipitin Curve:***

A quantitative precipitation reaction can be performed by placing a constant amount of antibody in a series of tubes and adding increasing amounts of antigen to the tubes. At one time this method was used to measure the amount of antigen or antibody present in a sample of interest. After the precipitate forms, each tube is centrifuged to pellet the precipitate, the supernatant is poured off, and the amount of precipitate is measured. Plotting the amount of precipitate against increasing antigen concentrations yields a precipitin curve. As Figure shows, excess of either antibody or antigen interferes with maximal precipitation, which occurs in the so-called equivalence zone, within which the ratio of antibody to antigen is optimal. As a large multimolecular lattice is formed at equivalence, the complex increases in size and precipitates out of solution. As shown in Figure under conditions of antibody excess or antigen excess, extensive lattices do not form and precipitation is inhibited. Although the quantitative precipitation reaction is seldom used experimentally today, the principles of antigen excess, antibody excess, and equivalence apply to many Ag-Ab reactions.



- ***Precipitation Reactions in Gels Yield Visible Precipitin Lines:***

Immune precipitates can form not only in solution but also in an agar matrix.When antigen and antibody diffuse toward one another in agar,or when antibody is incorporated into the agar and antigen diffuses into the antibody-containing matrix, a visible line of precipitation will form.As in a precipitation reaction in fluid, visible precipitation occurs in the region of equivalence,whereas no visible precipitate forms in regions of antibody or antigen excess. Two types of immune diffusion reactionscan be used to determine relative concentrations ofantibodies or antigens,to compare antigens,or to determine the relative purity of an antigen preparation. They are radial immune diffusion (the Mancini method) and double immunodiffusion(the Ouchterlony method);both are carried out in a semisolid medium such as agar.In radial immune diffusion, an antigen sample is placed in a well and allowed to diffuse intoagar containing a suitable dilution of an antiserum.

As the antigen diffuses into the agar, the region of equivalence is established and a ring of precipitation, a precipitin ring, forms around the well (Figure 6-5,upper panel).

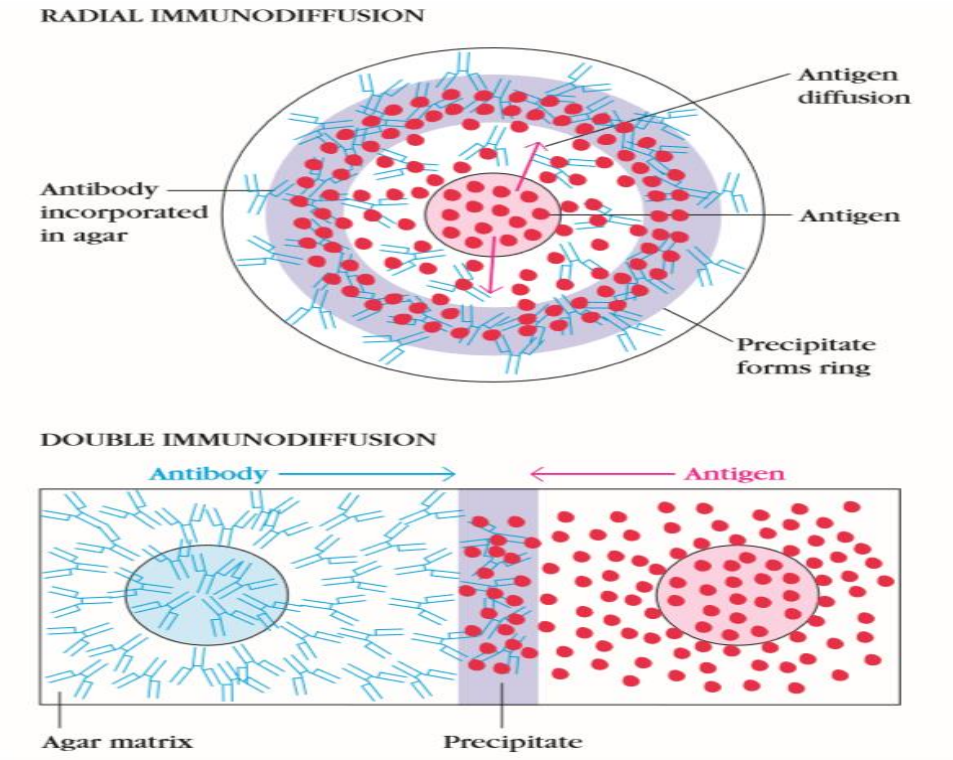


FIGURE 6-5 Diagrammatic representation of radial immunodiffusion (Mancini method) and double immunodiffusion (Ouchterlony method) in a gel. In both cases, large insoluble complexes form in the agar in the zone of equivalence, visible as lines of precipitation (purple regions). Only the antigen (red) diffuses in radial immunodiffusion, whereas both the antibody (blue) and antigen (red) diffuse in double immunodiffusion.

The area of the precipitin ring is proportional to the concentration of antigen.By comparing the area ofthe precipitin ring with a standard curve (obtained by measuring the precipitin areas ofknown concentrations of the antigen),the concentration of the antigen sample can be determined. In the Ouchterlony method, both antigen and antibody diffuse radially from wells toward each other,thereby establishing a concentration gradient.As equivalence is reached, a visible line of precipitation, a precipitin line,forms (Figure 6-5,lower panel).

- ***Immunoelectrophoresis Combines Electrophoresis and Double Immunodiffusion:***

In immunoelectrophoresis,the antigen mixture is first electrophoresed to separate its components by charge. Troughs are then cut into the agar gel parallel to the direction ofthe electric field, and antiserum is added to the troughs. Antibody and antigen then diffuse toward each other and produce lines of precipitation where they meet in appropriate proportions (Figure 6-6a). Immunoelectrophoresis is used in clinical laboratories to detect the presence or absence of proteins in the serum. A sample of serum is electrophoresed, and the individual serum components are identified with antisera specific for a given protein or immunoglobulin class (Figure 6-6b).This technique is useful in determining whether a patient produces abnormally low amounts of one or more isotypes, characteristic of certain immunodeficiency diseases. It can also show whether a patient overproduces some serum protein, such as albumin, immunoglobulin, or transferrin. The immunoelectrophoretic pattern of serum from patients with multiple myeloma, for example, shows a heavy distorted arc caused by the large amount of myeloma protein, which is monoclonal Ig and therefore uniformly charged (Figure 6-6b). Because immune electrophoresis is a strictly *qualitative* technique that only detects relatively high antibody concentrations (greater than several hundred g/ml), it utility is limited to the detectionof quantitative abnormalities only when the departure from normal is striking, as in immunodeficiency states and immune proliferative disorders.

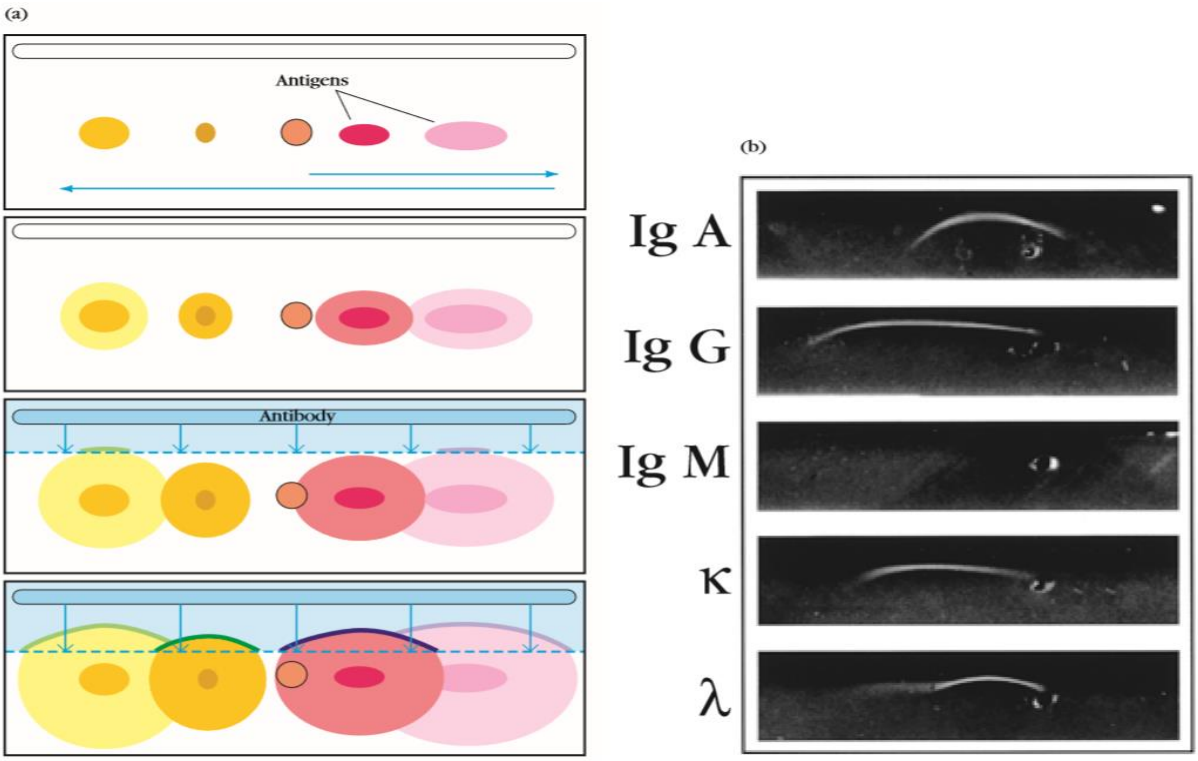


FIGURE 6-6 Immunoelectrophoresis of an antigen mixture. (a) An antigen preparation (orange) is first electrophoresed, which separates the component antigens on the basis of charge. Antiserum (blue) is then added to troughs on one or both sides of the separated antigens and allowed to diffuse; in time, lines of precipitation (colored arcs) form where specific antibody and antigen interact. (b) Immunoelectrophoretic patterns of human serum from a patient with myeloma. The patient produces a large amount of a monoclonal IgG (λ-light-chain-bearing) antibody. A sample of serum from the patient was placed in the well of the slide and electrophoresed. Then antiserum specific for the indicated antibody class or light chain type was placed in the top trough of each slide. At the concentrations of patient's serum used, only anti-IgG and anti-λ antibodies produced lines of precipitation. *[Part(b), Robert A. Kyle and Terry A. Katzman, Manual of Clinical Immunology, 1997, N. Rose, ed., ASM Press, Washington, D.C., p. 164.]*

iii. Agglutination Reactions:

The interaction between antibody and a particulate antigen results in visible clumping called agglutination. Antibodies that produce such reactions are called agglutinins. Agglutination reactions are similar in principle to precipitation reactions; they depend on the crosslinking of polyvalent antigens. Just as an excess of antibody inhibits precipitation reactions, such excess can also inhibit agglutination reactions; this inhibition is called the prozone effect. Because prozone effects can be encountered in many types of immunoassays, understanding the basis of this phenomenon is of general importance.

- ***Hemagglutination Is Used in Blood Typing:***

Agglutination reactions (Figure 6-7) are routinely performed to type red blood cells (RBCs). In typing for the ABO antigens, RBCs are mixed on a slide with antisera to the A or B blood-group antigens. If the antigen is present on the cells, they agglutinate, forming a visible clump on the slide. Determination of which antigens are present on donor and recipient RBCs is the basis for matching blood types for transfusions.

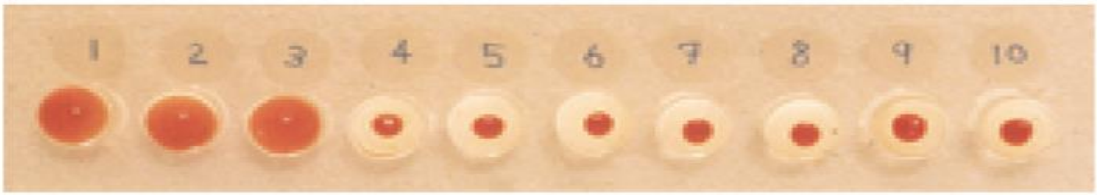


FIGURE 6-7 Demonstration of hemagglutination using antibodies against sheep red blood cells (SRBCs). The control tube (10) contains only SRBCs, which settle into a solid "button." The experimental tubes 1–9 contain a constant number of SRBCs plus serial two-fold dilutions of anti-SRBC serum. The spread pattern in the experimental series indicates positive hemagglutination through tube 3. *[Louisiana State University Medical Center/MIP. Courtesy of Harriet C. W. Thompson.]*

- ***Bacterial Agglutination Is Used To Diagnose Infection:***

A bacterial infection often elicits the production of serum antibodies specific for surface antigens on the bacterial cells. The presence of such antibodies can be detected by bacterial agglutination reactions. Serum from a patient thought to be infected with a given bacterium is serially diluted in an array of tubes to which the bacteria is added. The last tube showing visible agglutination will reflect the serum antibody titer of the patient. The agglutinin titer is defined as the reciprocal of the greatest serum dilution that elicits a positive agglutination reaction. For example, if serial twofold dilutions of serum are prepared and if the dilution of 1/640 shows agglutination but the dilution of 1/1280 does not, then the agglutination titer of the patient's serum is 640. In some cases serum can be diluted up to 1/50,000 and still show agglutination of bacteria. The agglutinin titer of an antiserum can be used to diagnose a bacterial infection. Patients with typhoid fever, for example, show a significant rise in the agglutination titer to *Salmonella typhi.* Agglutination reactions also provide a way to type bacteria. For instance, different species of the bacterium *Salmonella* can be distinguished by agglutination reactions with a panel of typing antisera.

- ***In Agglutination Inhibition, Absence of Agglutination Is Diagnostic of Antigen:***

A modification of the agglutination reaction, called agglutination inhibition, provides a highly sensitive assay for small quantities of an antigen .For example, one of the early types of home pregnancy test kits included latex particles coated with human chorionic gonadotropin (HCG) and antibody to HCG (Figure 6-8). The addition of urine from a pregnant woman, which contained HCG, inhibited agglutination of the latex particles when the anti-HCG antibody was added; thus the absence of agglutination indicated pregnancy.

Agglutination inhibition assays can also be used to determine whether an individual is using certain types of illegal drugs, such as cocaine or heroin. A urine or blood sample is first incubated with antibody specific for the suspected drug. Then red blood cells (or other particles) coated with the drug are added. If the red blood cells are not agglutinated by the antibody, it indicates the sample contained an antigen recognized by the antibody, suggesting that the individual was using the illicit drug. One problem with these tests isthat some legal drugs have chemical structures similar to those of illicit drugs, and these legal drugs may cross-react with the antibody, giving a false-positive reaction. For this reason a positive reaction must be confirmed by a nonimmunologic method.

Agglutination inhibition assays are widely used in clinical laboratories to determine whether an individual has been exposed to certain types of viruses that cause agglutination of red blood cells. If an individual's serum contains specific antiviral antibodies, then the antibodies will bind to the virus and interfere with hemagglutination by the virus. This technique is commonly used in premarital testing to determine the immune status of women with respect to rubella virus. The reciprocal of the last serum dilution to show inhibition of rubella hemagglutination is the titer of the serum. A titer greater than 10 (1:10 dilution) indicates that a woman is immune to rubella, whereas a titer of less than 10 is indicative of a lack of immunity and the need for immunization with the rubella vaccine.

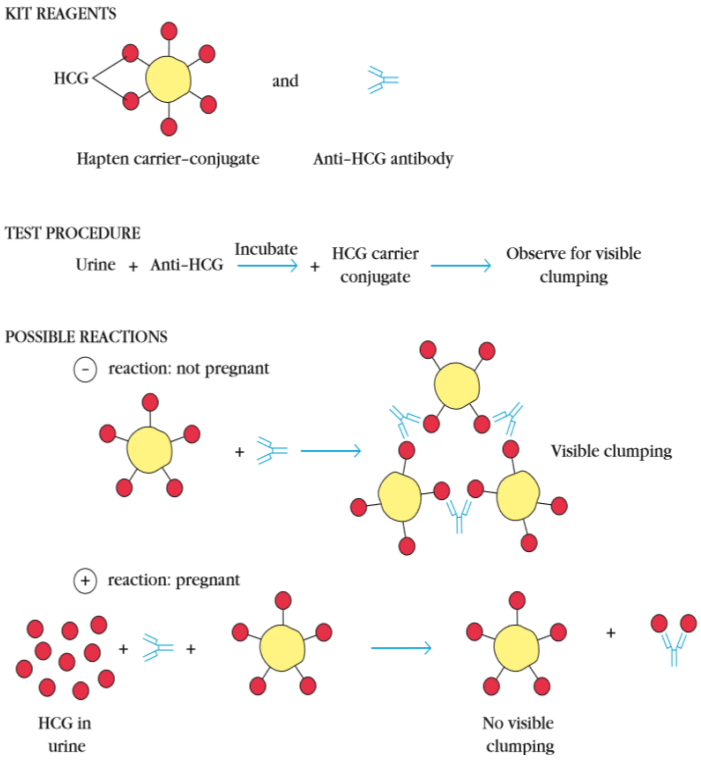


FIGURE 6-8 The original home pregnancy test kit employed hapten inhibition to determine the presence or absence of human chorionic gonadotropin (HCG). The original test kits used the presence or absence of visible clumping to determine whether HCG was present. If a woman was not pregnant, her urine would not contain HCG; in this case, the anti-HCG antibodies and HCG-carrier conjugate in the kit would react, producing visible clumping. If a woman was pregnant, the HCG in her urine would bind to the anti-HCG antibodies, thus inhibiting the subsequent binding of the antibody to the HCG-carrier conjugate. Because of this inhibition, no visible clumping occurred if a woman was pregnant. The kits currently on the market use ELISA-based assays (see Figure 6-10).

✓ Part- 8:Hypersensitivity: Definition, types, examples

Hypersensitivity (also called hypersensitivity reaction or intolerance) refers to undesirable reactions produced by the normal immune system, including allergies and autoimmunity. They are usually referred to as an over-reaction of the immune system and these reactions may be damaging, uncomfortable, or occasionally fatal. Hypersensitivity reactions require a pre-sensitized (immune) state of the host. The Gell and Coombs classification of hypersensitivity is the most widely used, and distinguishes four types of immune response which result in bystander tissue damage.Anaphylactic reactions within the humoral branch initiated by antibody or antigen-antibody complexes as immediate hypersensitivity, because

the symptoms are manifest within minutes or hours after a sensitized recipient encounters antigen. Delayed-type hypersensitivity (DTH) is so named in recognition of the delay of symptoms until days after exposure.

- ***Types:***

  The response of the host to the presence of foreign substances can trigger four types of hypersensitivity reactions:

  i. ***IgE-Mediated (Type I) Hypersensitivity(Anaphylactic Reaction):***

A type I hypersensitive reaction is induced by certain types of antigens referred to as allergens, and has all the hallmarks of a normal humoral response.That is, an allergen induces a humoral antibody response by the same mechanismsfor other soluble antigens, resulting in the generation of antibody-secreting plasma cells and memory cells. What distinguishes a type I hypersensitive response from a normal humoral response is that the plasmacells secrete IgE.This class of antibody binds with high affinity to Fc receptors on the surface of tissue mast cells and blood basophils. Mast cells and basophils coated by IgE are said to be sensitized. A later exposure to the same allergen cross-links the membrane-bound IgE on sensitized mast cells and basophils, causing degranulation of these cells (Figure 16-2).The pharmacologically active mediators released from the granules act on the surrounding tissues. The principal effects—vasodilation and smooth-muscle contraction—may be either systemic or localized, depending on the extent of mediator release.

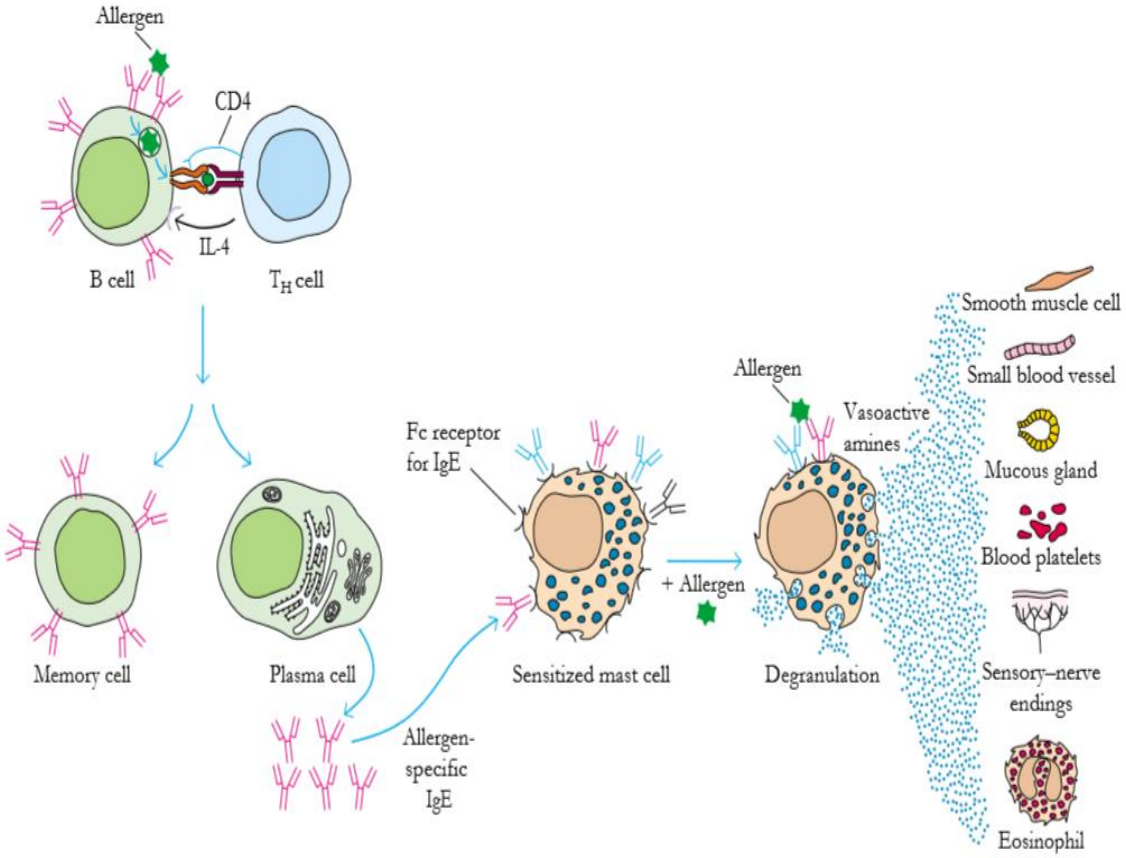


FIGURE 16-2 General mechanism underlying a type I hypersensitive reaction. Exposure to an allergen activates B cells to form IgE-secreting plasma cells. The secreted IgE molecules bind to IgE-specific Fc receptors on mast cells and blood basophils. (Many molecules of IgE with various specificities can bind to the IgE-Fc receptor.) Second exposure to the allergen leads to crosslinking of the bound IgE, triggering the release of pharmacologically active mediators, vasoactive amines, from mast cells and basophils. The mediators cause smooth-muscle contraction, increased vascular permeability, and vasodilation.

These allergic reactions are systemic or localized, as in allergic dermatitis (e.g., hives, wheal and erythema reactions). The reaction is the result of an antigen cross-linking with membrane-bound IgE antibody of a mast cell or basophil. Histamine, serotonin, bradykinin,and lipid mediators (e.g., platelet activating factor, prostaglandins, and leukotrienes) are released during the anaphylactic reaction. These released substances have the potential to cause tissue damage.

TABLE 16-1 Common allergens associated with type I hypersensitivity

Proteins	**Foods**
Foreign serum	Nuts
Vaccines	Seafood
	Eggs
Plant pollens	Peas, beans
Rye grass	Milk
Ragweed	
Timothy grass	**Insect products**
Birch trees	Bee venom
	Wasp venom
Drugs	Ant venom
Penicillin	Cockroach calyx
Sulfonamides	Dust mites
Local anesthetics	
Salicylates	**Mold spores**
	Animal hair and dander

TABLE 16-4 Mechanism of action of some drugs used to treat type I hypersensitivity

Drug	Action
Antihistamines	Block H_1 and H_2 receptors on target cells
Cromolyn sodium	Blocks Ca^{2+} influx into mast cells
Theophylline	Prolongs high cAMP levels in mast cells by inhibiting phosphodiesterase, which cleaves cAMP to 5′-AMP*
Epinephrine (adrenalin)	Stimulates cAMP production by binding to β-adrenergic receptors on mast cells*
Cortisone	Reduces histamine levels by blocking conversion of histidine to histamine and stimulates mast-cell production of cAMP*

*Although cAMP rises transiently during mast-cell activation, degranulation is prevented if cAMP levels remain high.

ii. *Antibody-Mediated Cytotoxic (Type II) Hypersensitivity:*

Type II hypersensitive reactions involve antibody-mediated destruction of cells. Antibody can activate the complement system, creating pores in the membrane of a foreign cell (see

Figure 13-5), or it can mediate cell destruction by antibodydependent cell-mediated cytotoxicity (ADCC). In this process,cytotoxic cells with Fc receptors bind to the Fc region of antibodies on target cells and promote killing of the cells. Antibody bound to a foreign cell also can serve as an opsonin, enabling phagocytic cells with Fc or C3b receptors to bind and phagocytose the antibody-coated cell.

In a cytotoxic reaction, the antibody reacts directly with the antigen that is bound to the cell membrane to induce cell lysis through complement activation. These antigens may be intrinsic or "self" as in autoimmune reactions or extrinsic or "non-self." Cytotoxic reactions are mediated by IgG and IgM. Examples of cytotoxic reaction are the Rh incompatibility of a newborn, blood transfusion reactions, and autoimmune diseases like Pemphigus Vulgaris, Bullous Pemphigoid, autoimmune hemolyticanemia and Goodpasture's syndrome to name a few.

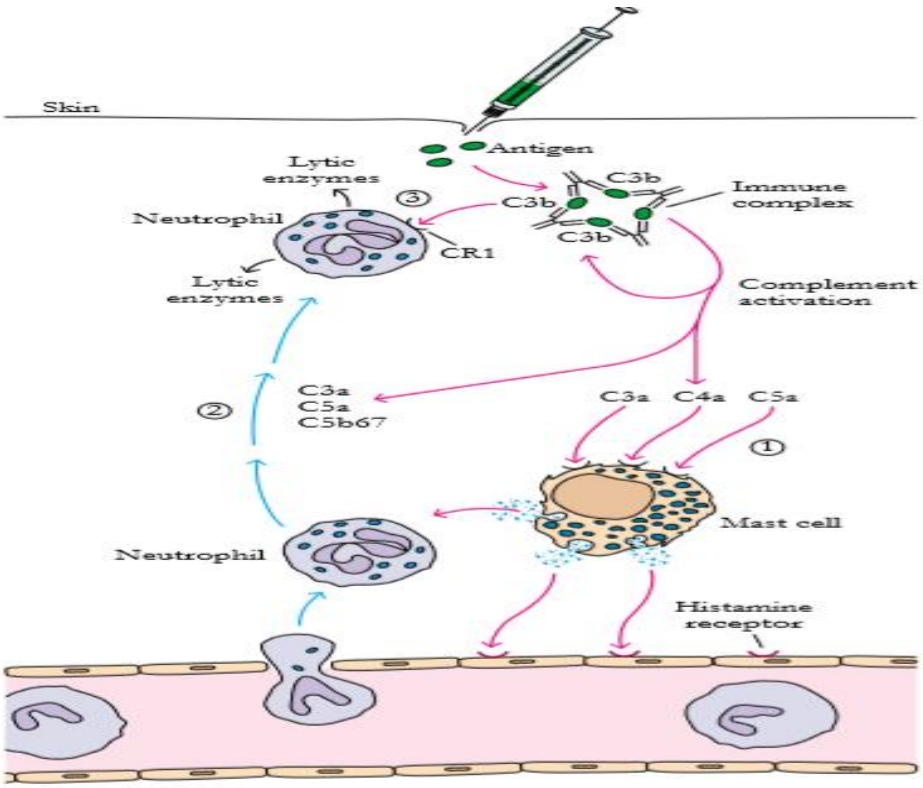


FIGURE 16-15 Development of a localized Arthus reaction (type III hypersensitive reaction). Complement activation initiated by immune complexes (classical pathway) produces complement intermediates that (1) mediate mast-cell degranulation, (2) chemotactically attract neutrophils, and (3) stimulate release of lytic enzymes from neutrophils trying to phagocytose C_3b-coated immune complexes.

iii. ***Immune Complex–Mediated (Type III) Hypersensitivity:***

Type III hypersensitive reactions develop when immune complexes activate the complement system's array of immune effector molecules. The C3a, C4a, and C5a complement split products are anaphylatoxins that cause localized mast-cell degranulation and consequent increase in local vascular permeability. C3a, C5a, and C5b67 are also chemotactic factors for neutrophils, which can accumulate in large numbers at the site of immune-complex deposition.Larger immune complexes are deposited on the basement membrane of bloodvessel walls or kidney glomeruli, whereas smaller complexes may pass through the basement membrane and be deposited in the sub-epithelium.The type of lesion that results depends on the site of deposition of the complexes.

Much of the tissue damage in type III reactions stems from release of lytic enzymes by neutrophils as they attempt to phagocytose immune complexes. The C3b complement component acts as an opsonin, coating immune complexes. A neutrophil binds to a C3b-coated immune complex by means of the type I complement receptor, which is specific for C3b. Because the complex is deposited on the basementmembrane surface, phagocytosis is impeded, so that lytic enzymes are released during the unsuccessful attempts of the neutrophil to ingest the adhering immune complex. Further activation of the membrane-attack mechanism of the complement system can also contribute to the destruction of tissue. In addition, the activation of complement can induce aggregation of platelets, and the resulting release of clotting factors can lead to formation of microthrombi.

IgG and IgM bind antigen, forming antigen-antibody (immune) complexes. These activate complement, which results in PMN chemotaxis and activation. PMNs then release tissue damaging enzymes. Tissue damage present in autoimmune diseases (e.g., systemic lupus erythematosus), and chronic infectious diseases (e.g., leprosy) can be attributed, in part, to immune complex reactions.

iv. *Type IV or Delayed-Type Hypersensitivity (DTH):*

When some subpopulations of activated TH cells encounter certain types of antigens,they secrete cytokines that induce a localized inflammatory reaction called delayed-type hypersensitivity (DTH). The reaction is characterized by large influxes of nonspecific inflammatory cells,in particular,macrophages. This type of reaction was first described in 1890 byRobert Koch, who observed that individuals infected with Mycobacterium

tuberculosis developed a localized inflammatory response when injected intradermally with a filtrate derived from a mycobacterial culture.He called this localized skin reaction a "tuberculin reaction." Later, as it became apparent that a variety of other antigens could induce this response (Table 16-6),its name was changed to delayed-type or type IV hypersensitivity in reference to the delayed onset of the reaction and to the tissue damage (hypersensitivity) that is often associated with it.

TABLE 16-6 Intracellular pathogens and contact antigens that induce delayed-type (type IV) hypersensitivity

Intracellular bacteria	Intracellular viruses
Mycobacterium tuberculosis	Herpes simplex virus
Mycobacterium leprae	Variola (smallpox)
Listeria monocytogenes	Measles virus
Brucella abortus	
Intracellular fungi	Contact antigens
Pneumocystis carinii	Picrylchloride
Candida albicans	Hair dyes
Histoplasma capsulatum	Nickel salts
Cryptococcus neoformans	Poison ivy
	Poison oak
Intracellular parasites	
Leishmania sp.	

The term hypersensitivity is somewhat misleading, for it suggests that a DTH response is always detrimental.Although in some cases a DTH response does cause extensive tissue damage and is in itself pathologic, in many cases tissue damage is limited, and the response plays an important role in defense against intracellular pathogens and contact antigens. The hallmarks of a type IV reaction are the delay in time required for the reaction to develop and the recruitment of macrophages as opposed to neutrophils, as found in a type III reaction. Macrophages are the major component of the infiltrate that surrounds the site of inflammation.

Cell-mediated reactions are initiated by T-lymphocytes and mediated by effector T-cells and macrophages. This response involves the interaction of antigens with the surface of lymphocytes. Sensitized lymphocytes can produce cytokines, which are biologically active substances that affect the functions of other cells. This type of reaction takes 48-72 hours, or longer, after contact with the antigen to fully develop. Many chronic infectious diseases, including tuberculosis and fungal infections, exhibit delayed hypersensitivity.

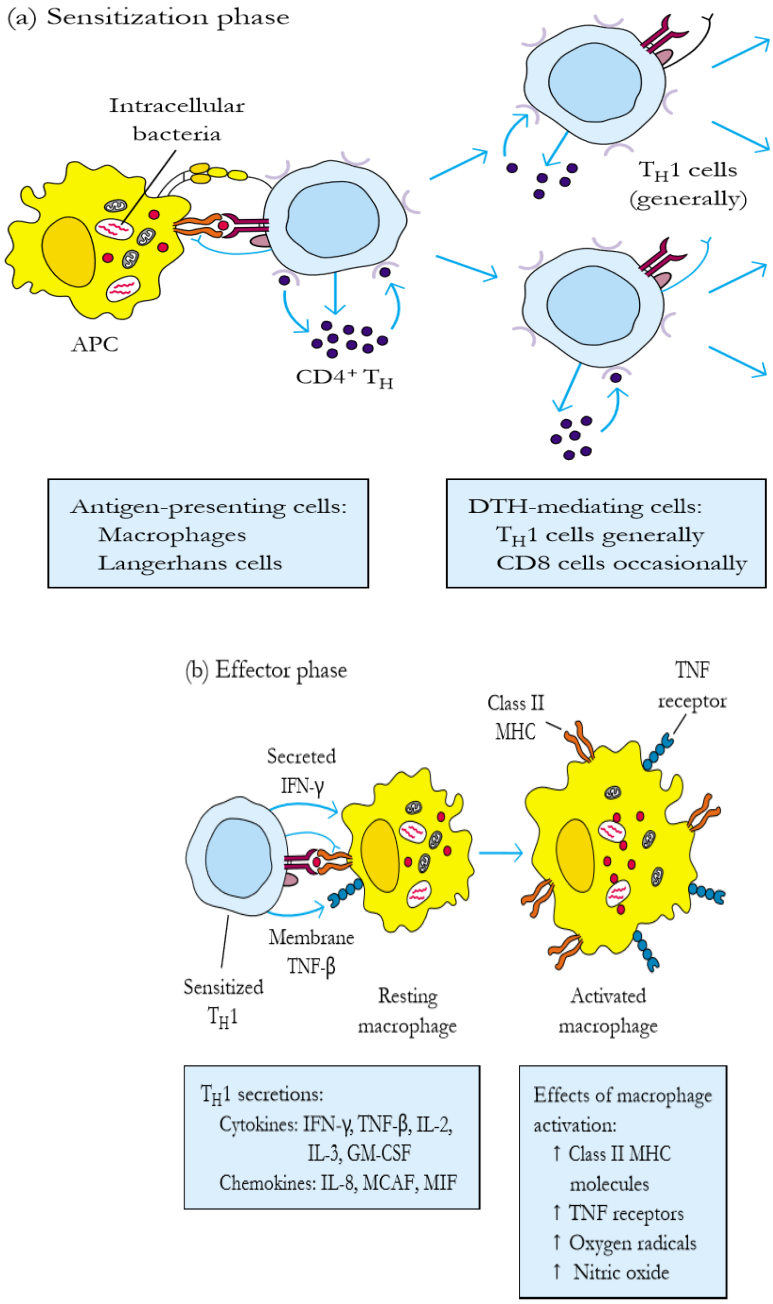


FIGURE 16-17 Overview of the DTH response. (a) In the sensitization phase after initial contact with antigen (e.g., peptides derived from intracellular bacteria), T_H cells proliferate and differentiate into T_H1 cells. Cytokines secreted by these T cells are indicated by the dark blue balls. (b) In the effector phase after subsequent exposure of sensitized T_H1 cells to antigen, the T_H1 cells secrete a variety of cytokines and chemokines. These factors attract and activate macrophages and other nonspecific inflammatory cells. Activated macrophages are more effective in presenting antigen, thus perpetuating the DTH response, and function as the primary effector cells in this reaction.

✓ **<u>Part- 9:</u>Vaccines: Active and passive immunization (definition, characteristics, functions, examples).**

1. Active and passive immunization:

Immunity to infectious microorganisms can be achieved by active or passive immunization. In each case, immunity can be acquired either by natural processes (usually by transfer from mother to fetus or by previous infection by the organism) or by artificial means such as injection of antibodies or vaccines (Table 18-1). The agents used for inducing passive immunity include antibodies from humans or animals, whereas active immunization is achieved by inoculation with microbial pathogens that induce immunity but do not cause disease or with antigenic components from the pathogens. This section describes current usage of passive and active immunization techniques.

TABLE 18–1 Acquisition of passive and active immunity

Type	Acquired through
Passive immunity	Natural maternal antibody
	Immune globulin*
	Humanized monoclonal antibody
	Antitoxin†
Active immunity	Natural infection
	Vaccines‡
	Attenuated organisms
	Inactivated organisms
	Purified microbial macromolecules
	Cloned microbial antigens
	Expressed as recombinant protein
	As cloned DNA alone or in virus vectors
	Multivalent complexes
	Toxoid§

*An antibody-containing solution derived from human blood, obtained by cold ethanol fractionation of large pools of plasma; available in intramuscular and intravenous preparations.

†An antibody derived from the serum of animals that have been stimulated with specific antigens.

‡A suspension of attenuated live or killed microorganisms, or antigenic portions of them, presented to a potential host to induce immunity and prevent disease.

§A bacterial toxin that has been modified to be nontoxic but retains the capacity to stimulate the formation of antitoxin.

- ***Passive immunizationInvolves Transfer of Preformed Antibodies:***

Jenner and Pasteur are recognized as the pioneers of vaccination, or induction of active immunity, but similar recognition is due to Emil von Behring and HidesaburoKitasato for their contributions to passive immunity. These investigators were the first to show that immunity elicited in one animal can be transferred to another by injecting it with serum from the first.

Passive immunization, in which preformed antibodies are transferred to a recipient, occurs naturally by transfer of maternal antibodies across the placenta to the developing fetus. Maternal antibodies to diphtheria, tetanus, streptococci, rubeola, rubella, mumps, and poliovirus all afford passively acquired protection to the developing fetus. Maternal antibodies present in colostrum and milk also provide passive immunity to the infant. Passive immunization can also be achieved by injecting a recipient with preformed antibodies. In the past, before vaccines and antibiotics became available,passive immunization provided a major defense against various infectious diseases. Despite the risks incurred by injecting animal sera,usually horse serum,this was the only effective therapy for otherwise fatal diseases. Currently, there are several conditions that warrant the use of passive immunization. These include:

- Deficiency in synthesis of antibody as a result of congenital or acquired B-cell defects, alone or together with other immunodeficiencies.
- Exposure or likely exposure to a disease that will cause complications (e.g., a child with leukemia exposed to varicella or measles), or when time does not permit adequate protection by active immunization.
- Infection by pathogens whose effects may be ameliorated by antibody. For example, if individuals who have not received up-to-date active immunization against tetanus suffer a puncture wound, they are given an injection of horse antiserum to tetanus toxin. The preformed horse antibody neutralizes any tetanus toxin produced by *Clostridium tetani* in the wound.

Passive immunization is routinely administered to individuals exposed to botulism, tetanus, diphtheria, hepatitis, measles,and rabies (Table 18-2).Passively administered antiserum is also used to provide protection from poisonous snake and insect bites.Passive immunization can provide immediate protection to travelers or health-care workers who will soon be exposed to an infectious organism and lack active immunity to

it. Because passive immunization does not activate the immune system, it generates no memory response and the protection provided is transient.

For certain diseases such as the acute respiratory failure in children caused by respiratory syncytial virus (RSV), passiveimmunization is the best preventative currently available. A monoclonal antibody or a combination of two monoclonal antibodies may be administered to children at risk for RSV disease.

TABLE 18-2 Common agents used for passive immunization

Disease	Agent
Black widow spider bite	Horse antivenin
Botulism	Horse antitoxin
Diphtheria	Horse antitoxin
Hepatitis A and B	Pooled human immune gamma globulin
Measles	Pooled human immune gamma globulin
Rabies	Pooled human immune gamma globulin
Respiratory disease	Monoclonal anti-RSV*
Snake bite	Horse antivenin
Tetanus	Pooled human immune gamma globulin or horse antitoxin

- ***Active Immunization Elicits Long-Term Protection:***

Whereas the aim of passive immunization is transient protection or alleviation of an existing condition,the goal of active immunization is to elicit protective immunity and immunologic memory. When active immunization is successful,a subsequent exposure to the pathogenic agent elicits a heightened immune response that successfully eliminates the pathogen or prevents disease mediated by its products. Active immunization can be achieved by natural

infection with a microorganism,or it can be acquired artificially by administration of a vaccine (see Table 18-1). In active immunization, as the name implies, the immune system plays an active role—proliferation of antigen-reactive T and B cells results in the formation of memory cells. Active immunization with various types of vaccines has played an importantrole in the reduction of deaths from infectious diseases,especially among children.

- Vaccination of children is begun at about 2 months of age. The recommended program of childhood immunizations in this country, updated in 2002 by the American Academy of Pediatrics. The program includes the following vaccines:
- Hepatitis B vaccine
- Diphtheria-pertussis (acellular)- tetanus (DPaT) combined vaccine
- Inactivated (Salk) polio vaccine (IPV); the oral (Sabin) vaccine is no longer recommended for use in the United States
- Measles-mumps-rubella (MMR) combined vaccine
- Haemophilusinfluenzae (Hib) vaccine
- Varicella zoster (Var) vaccine for chickenpox
- Pneumococcal conjugate vaccine (PCV); a new addition to the list.

In addition, hepatitis A vaccine at 18 months and influenza vaccines after 6 months are recommended for infants in high-risk populations.

✓ Part- 10:Diagnostic immunology: ELISA, RIA, Immunofluorescence, Flow cytometry, Fluorescence activated cell sorting (FACS).

1. ELISA:

Enzyme-linked immunosorbent assay, commonly known as ELISA (or EIA), is similar in principle to RIA but depends on an enzyme rather than a radioactive label. An enzyme conjugated with an antibody reacts with a colorless substrate to generate a colored reaction product. Such a substrate is called a chromogenic substrate. A number of enzymes have been employed for ELISA, including alkaline phosphatase, horseradish peroxidase, and β-galactosidase. These assays approach the sensitivity of RIAs and have the advantage of being safer and less costly.

- **INDIRECT ELISA:**

Antibody can be detected or quantitatively determined with an indirect ELISA (Figure 6-10a).Serum or some other sample containing primary antibody (Ab1) is added to an antigen-coated microtiter well and allowed to react with the antigen attached to the well. After any free Ab_1 is washed away, the presence of antibody bound to the antigen is detected by adding an enzyme-conjugated secondary anti-isotype antibody (Ab_2), which binds to the primary antibody. Any free Ab_2 then is washed away,and a substrate for the enzyme is added.The amount of colored reaction product that forms is measured by specialized spectrophotometric plate readers,which can measure the absorbance of all of the wells of a 96-well plate in seconds.

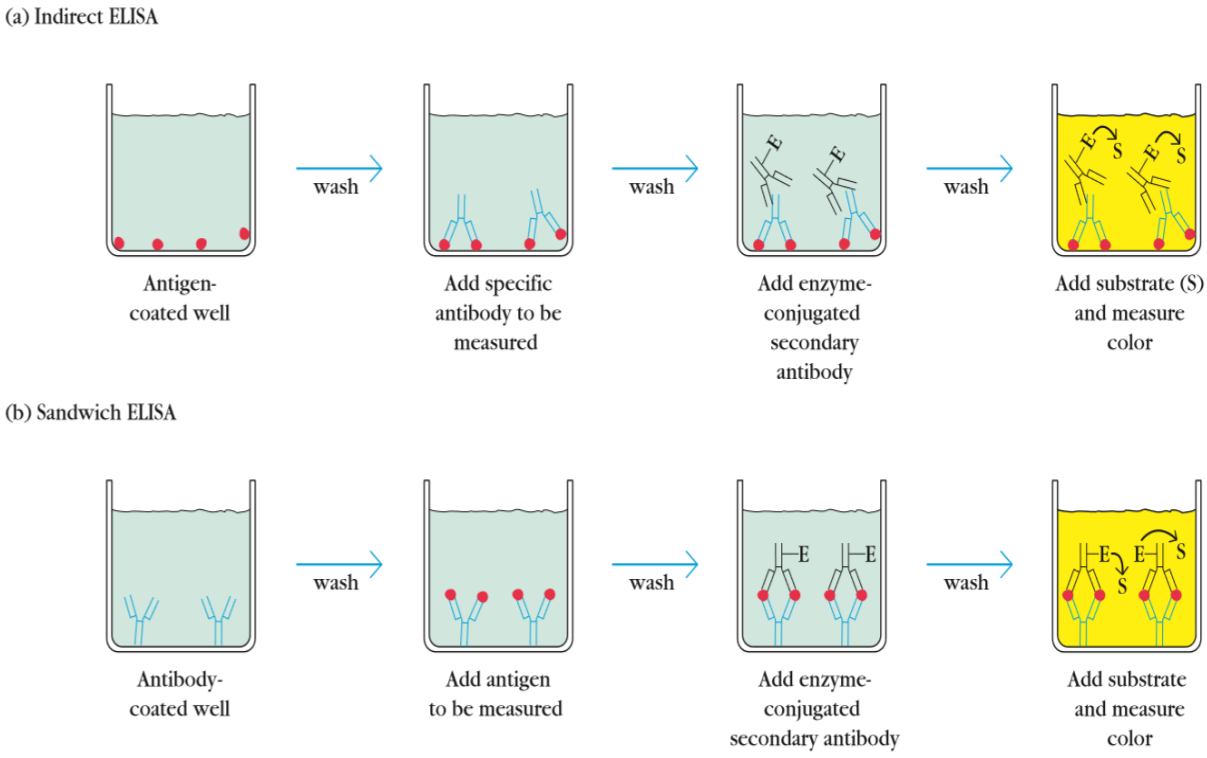
Indirect ELISA is the method of choice to detect the presence of serum antibodies against human immunodeficiency virus (HIV), the causative agent of AIDS. In this assay, recombinant envelope and core proteins of HIV are adsorbedas solid-phase antigens to microtiter wells. Individuals infected with HIV will produce serum antibodies to epitopes on these viral proteins. Generally, serum antibodies to HIV can be detected by indirect ELISA within 6 weeks of infection.

- **SANDWICHELISA:**

Antigen can be detected or measured by a sandwich ELISA (Figure 6-10b). In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well. A sample containing antigen is added and allowed to react with the immobilized antibody.After the well is washed,a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen.After any free second antibody is removed by washing, substrate is added, and the colored reaction product is measured.

- **COMPETITIVEELISA:**

Another variation for measuring amounts of antigen is competitive ELISA (Figure 6-10c). In this technique, antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to an antigencoated microtiter well.The more antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well.Addition of an enzyme-conjugated secondary antibody (Ab_2) specific for the isotype of the primary antibody can be used to determine the amount of primary antibody bound to the well as in an indirect ELISA. In thecompetitive assay, however, the higher the concentration of antigen in the original sample,the lower the absorbance.



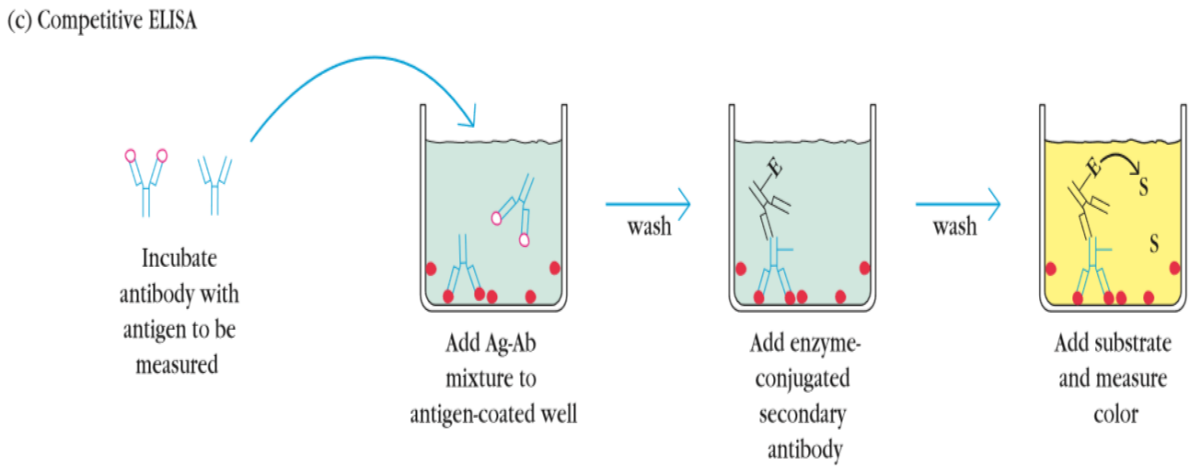


FIGURE 6-10 Variations in the enzyme-linked immunosorbent assay (ELISA) technique allow determination of antibody or antigen. Each assay can be used qualitatively, or quantitatively by comparison with standard curves prepared with known concentrations of antibody or antigen. Antibody can be determined with an indirect ELISA (a), whereas antigen can be determined with a sandwich ELISA (b) or competitive ELISA (c). In the competitive ELISA, which is an inhibition-type assay, the concentration of antigen is inversely proportional to the color produced.

2. Radioimmunoassay (RIA):

One of the most sensitive techniques for detecting antigen or antibody is radioimmunoassay (RIA). The technique was first developed in 1960 by two endocrinologists,S.A.Berson and Rosalyn Yalow, to determine levels of insulin–anti-insulin complexes in diabetics. Although their technique encountered some skepticism, it soon proved its value for measuring hormones,serum proteins,drugs,and vitamins at concentrations of 0.001 micrograms per milliliter or less. In 1977,some years after Berson's death,the significance of the technique was acknowledged by the award ofa Nobel Prize to Yalow.

The principle of RIA involves competitive binding of radiolabeled antigen and unlabeled antigen to a high-affinity antibody. The labeled antigen is mixed with antibody at a concentration that saturates the antigen-binding sites of the antibody. Then test samples of unlabeled antigen of unknown concentration are added in progressively larger amounts. The antibody does not distinguish labeled from unlabeled antigen, so the two kinds of antigen compete for available binding sites on the antibody.As the concentration of unlabeled antigen increases, more labeled antigen will be displaced from the binding sites.The decrease in the

amount of radiolabeled antigen bound to specific antibody in the presence of the test sample is measured in order to determine the amount of antigen present in the test sample.

The antigen is generally labeled with a gamma-emitting isotope such as ^{125}I,but beta-emitting isotopes such as tritium (^{3}H) are also routinely used as labels. The radiolabeled antigen is part of the assay mixture; the test sample may be a complex mixture, such as serum or other body fluids,that contains the unlabeled antigen.The first step insetting up an RIA is to determine the amount of antibody needed to bind 50%–70% of a fixed quantity of radioactive antigen (Ag∗) in the assay mixture. This ratio of antibody to Ag∗ is chosen to ensure that the number of epitopes presented by the labeled antigen always exceeds the total number of antibody binding sites.Consequently,unlabeled antigen added to the sample mixture will compete with radiolabeled antigen for the limited supply of antibody. Even a small amount of unlabeled antigen added to the assay mixture of labeled antigen and antibody will cause a decrease in the amount of radioactive antigen bound,and this decrease will be proportional to the amount of unlabeled antigen added.To determine the amount of labeled antigen bound, the Ag-Ab complex is precipitated to separate it from free antigen (antigen not bound to Ab), and the radioactivity in the precipitate is measured.A standard curve can be generated using unlabeled antigen samples of known concentration (in place of the test sample), and from this plot the amount of antigen in the test mixture may be precisely determined.

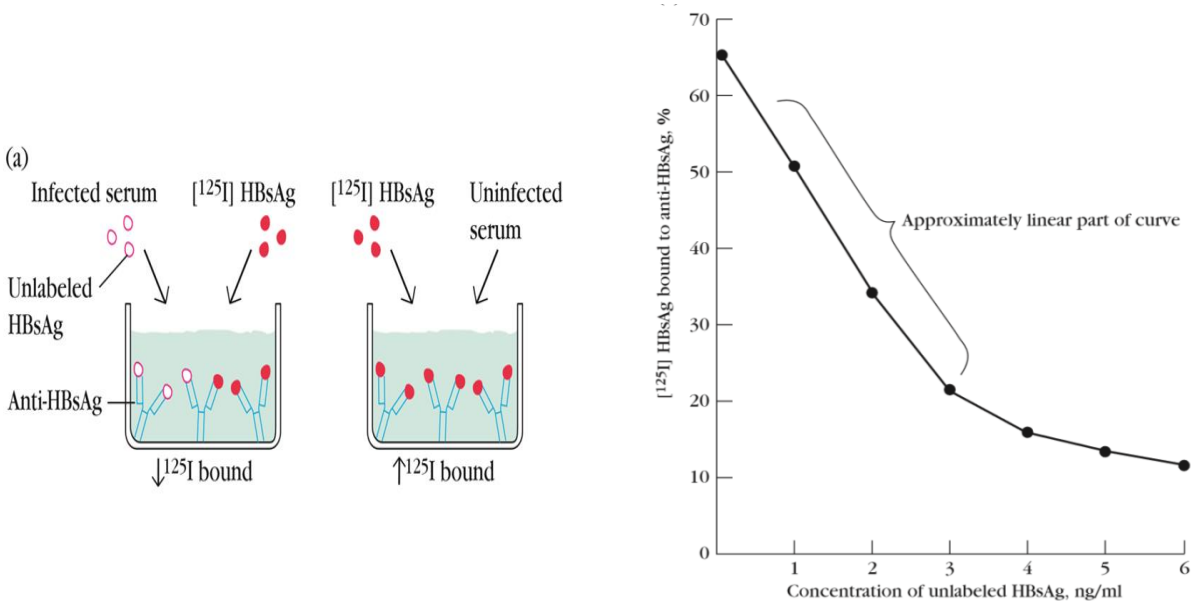


FIGURE 6-9 A solid-phase radioimmunoassay (RIA) to detect hepatitis B virus in blood samples. (a) Microtiter wells are coated with a constant amount of antibody specific for HBsAg, the surface antigen on hepatitis B virions. A serum sample and [^{125}I]HBsAg are then added. After incubation, the supernatant is removed and the radioactivity of the antigen-antibody complexes is measured. If the sample is infected, the amount of label bound will be less than in controls with uninfected serum. (b) A standard curve is obtained by adding increasing concentrations of unlabeled HBsAg to a fixed quantity of [^{125}I]HBsAg and specific antibody. From the plot of the percentage of labeled antigen bound versus the concentration of unlabeled antigen, the concentration of HBsAg in unknown serum samples can be determined by using the linear part of the curve.

3. Immunofluorescence:

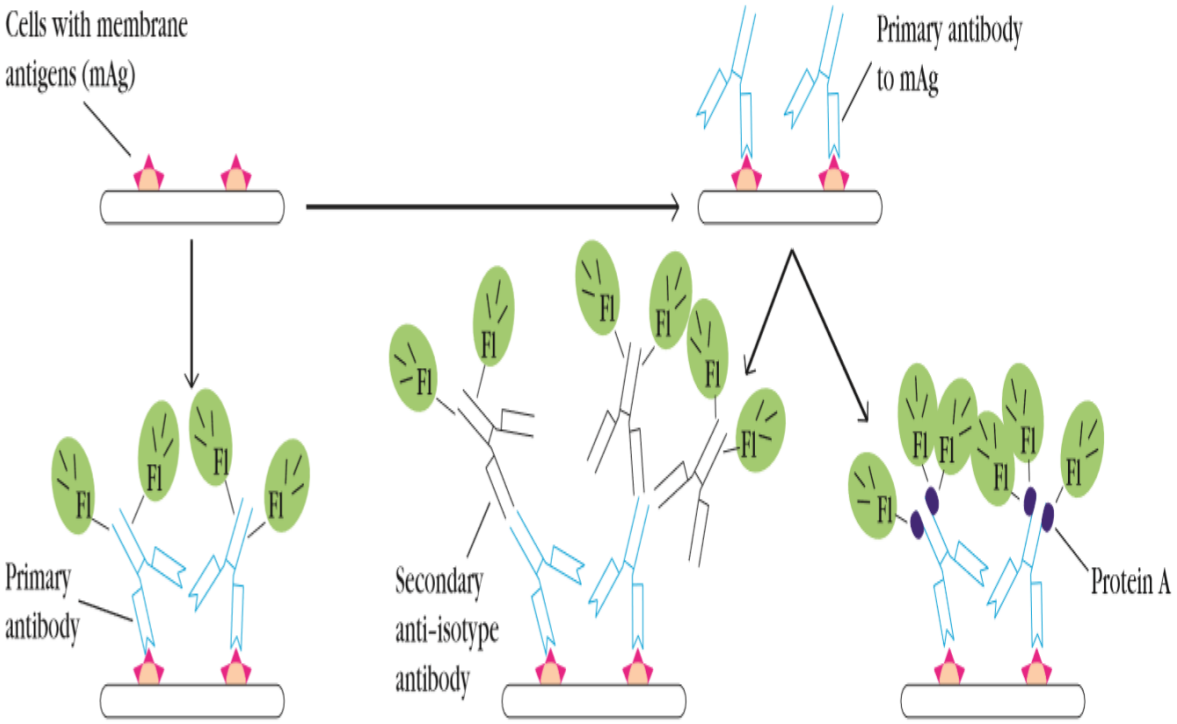
In 1944, Albert Coons showed that antibodies could be labeled with molecules that have the property of fluorescence. Fluorescent molecules absorb light of one wavelength(excitation) and emit light ofanother wavelength (emission). If antibody molecules are tagged with a fluorescent dye, or fluorochrome,immune complexes containing these fluorescently labeled antibodies (FA) can be detected by colored light emission when excited by light of the appropriate wavelength.Antibody molecules bound to antigens in cells or tissue sections can similarly be visualized.The emitted light can be viewed with a fluorescence microscope,which is equipped with a UV light source. In this technique, known as immunofluorescence, fluorescent compounds such as fluorescein and rhodamine are in common use, but other highly fluorescent substances are also routinely used,such as phycoerythrin, an intensely colored and highly fluorescent pigment obtained from algae. These molecules can be conjugated to the Fc region of an antibody molecule without affecting the specificity of the antibody. Each of the fluorochromes below absorbs light at one wavelength and emits light at a longer wavelength:

- Fluorescein, an organic dye that is the most widely used label for immunofluorescence procedures,absorbs blue light (490 nm) and emits an intense yellow-green fluorescence (517 nm).
- Rhodamine, another organic dye,absorbs in the yellow-green range (515 nm) and emits a deep redfluorescence (546 nm).Because it emits fluorescence at a longer wavelength than fluorescein, it can be used in two-color immunofluorescence assays. An antibody specific to one determinant is labeled with fluorescein, and an antibody recognizing a different antigen is labeled with rhodamine. The location of the fluorescein-tagged antibody will be visible by its yellow-green color,easy to distinguish from the red color emitted where the rhodamine-tagged antibody has bound.By conjugating fluorescein to one antibody and rhodamine to another antibody,one can,for example, visualize simultaneously two different cell-membrane antigens on the same cell.
- Phycoerythrin is an efficient absorber of light (~30-fold greater than fluorescein) and a brilliant emitter of red fluorescence,stimulating its wide use as a label for immunofluorescence.

Fluorescent-antibody staining of cell membrane molecules or tissue sections can be direct or indirect (Figure 6-14). In direct staining, the specific antibody (the primary antibody) is directly conjugated with fluorescein; in indirect staining, the primary antibody is unlabeled and is detected with an additional fluorochrome-labeled reagent. A number of reagents have been developed for indirect staining.The most common is a fluorochrome-labeled secondary antibody raised in one species against antibodies of another species, such as fluorescein-labeled goat anti-mouse immunoglobulin.

Indirect immunofluorescence staining has two advantages over direct staining. First, the primary antibody does not need to be conjugated with a fluorochrome.Because the supply of primary antibody is often a limiting factor,indirect methods avoid the loss of antibody that usually occurs during the conjugation reaction. Second, indirect methods increase the sensitivity of staining because multiple molecules of the fluorochrome reagent bind to each primary antibody molecule,increasing the amount of light emitted at the location of each primary antibody molecule.

Immunofluorescence has been applied to identify a number of subpopulations of lymphocytes, notably the $CD4^+$ and $CD8^+$ T-cell subpopulations.The technique is also suitable for identifying bacterial species, detecting Ag-Ab complexes in autoimmune disease, detecting complement components in tissues, and localizing hormones and other cellular products stained in situ.Indeed,a major application of the fluorescent-antibody technique is the localization of antigens in tissue sections or in subcellular compartments. Because it can be used to map the actual location of target antigens,fluorescence microscopy is a powerful tool for relating the molecular architecture of tissues and organs to their overall gross anatomy.



(d)

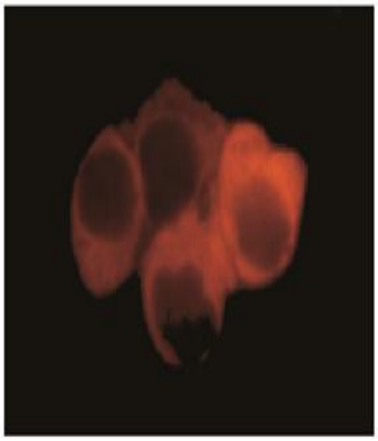


FIGURE 6-14 Direct and indirect immunofluorescence staining of membrane antigen (mAg). Cells are affixed to a microscope slide. In the direct method (a), cells are stained with anti-mAg antibody that is labeled with a fluorochrome (Fl). In the indirect methods (b and c), cells are first incubated with unlabeled anti-mAg antibody and then stained with a fluorochrome-labeled secondary reagent that binds to the primary antibody. Cells are viewed under a fluorescence microscope to see if they have been stained. (d) In this micrograph, antibody molecules bearing μ heavy chains are detected by indirect staining of cells with rhodamine-conjugated second antibody. *[Part (d), H. A. Schreuder et al., 1997, Nature* ***386:****196, courtesy H. Schreuder, Hoechst Marion Roussel.]*

4. Flow cytometry:

The fluorescent antibody techniques described are extremely valuable qualitative tools, but they do not give quantitative data. This shortcoming was remedied by development of the flow cytometer, which was designed to automate the analysis and separation of cells stained with fluorescent antibody. The flow cytometer uses a laser beam and light detector to count single intact cells in suspension (Figure 6-15).Every time a cell passes the laser beam,light is deflected from the detector, and this interruption of the laser signal is recorded. Those cells having a fluorescently tagged antibody bound to their cell surface antigens are excited by the laser and emit light that is recorded by a second detector system located at a right angle to the laser beam. The simplest form of the instrument counts each cell as it passes the laser beam and records the level of fluorescence the cell emits; an attached computer generates plots of the number of cells as the ordinate and their fluorescence intensity as the abscissa. More sophisticated versions of the instrument are capable of sorting populations of cells into different containers according to their fluorescence profile. Use of the instrument to determine which and how many members of a cell population bind fluorescently labeled antibodies is called analysis;use of the instrument to place cells having different patterns of reactivity into different containers is called cell sorting.

The flow cytometer has multiple applications to clinical and research problems.A common clinical use is to determine the kind and number of white blood cells in blood samples. By treating appropriately processed blood samples with a fluorescently labeled antibody and performing flow cytometric analysis, one can obtain the following information:

- How many cells express the target antigen as an absolute number and also as a percentage of cells passing the beam.For example,if one uses a fluorescent antibody specific for an antigen present on all T cells,it would be possible to determine the percentage of T cells in the total white blood cell population.Then,using the cell-sorting capabilities of the flow cytometer,it would be possible to isolate the T-cell fraction of the leukocyte population.
- The distribution of cells in a sample population according to antigen densities as determined by fluorescence intensity.It is thus possible to obtain a measure of the distribution of antigen density within the population of cells that possess the antigen.This is a powerful feature of the instrument,since the same type of cell may

express different levels of antigen depending upon its developmental or physiological state.

- The size of cells.This information is derived from analysis of the light-scattering properties of members of the cell population under examination.

Flow cytometry also makes it possible to analyze cell populations that have been labeled with two or even three different fluorescent antibodies. For example, if a blood sample is reacted with a fluorescein-tagged antibody specific for T cells,and also with a phycoerythrin-tagged antibody specific for B cells, the percentages of B and T cells may be determined simultaneously with a single analysis.Numerous variations of such "two-color"analyses are routinely carried out, and "three-color"experiments are common.Aided by appropriate software, highly sophisticated versions of the flow cytometer can even perform "five-color"analyses.

Flow cytometry now occupies a key position in immunology and cell biology,and it has become an indispensable clinical tool as well. In many medical centers, the flow cytometer is one of the essential tools for the detection and classification of leukemias (see the Clinical Focus). The choice of treatment for leukemia depends heavily on the cell types involved,making precise identification of the neoplastic cells an essential part of clinical practice. Likewise, the rapid measurement of T-cell subpopulations, an important prognostic indicator in AIDS, is routinely done by flowcytometric analysis. In this procedure, labeled monoclonal antibodies against the major T-cell subtypes bearing the CD4 and CD8 antigens are used to determine their ratios in the patient's blood. When the number of CD4 T-cells falls below a certain level, the patient is at high risk for opportunistic infections.

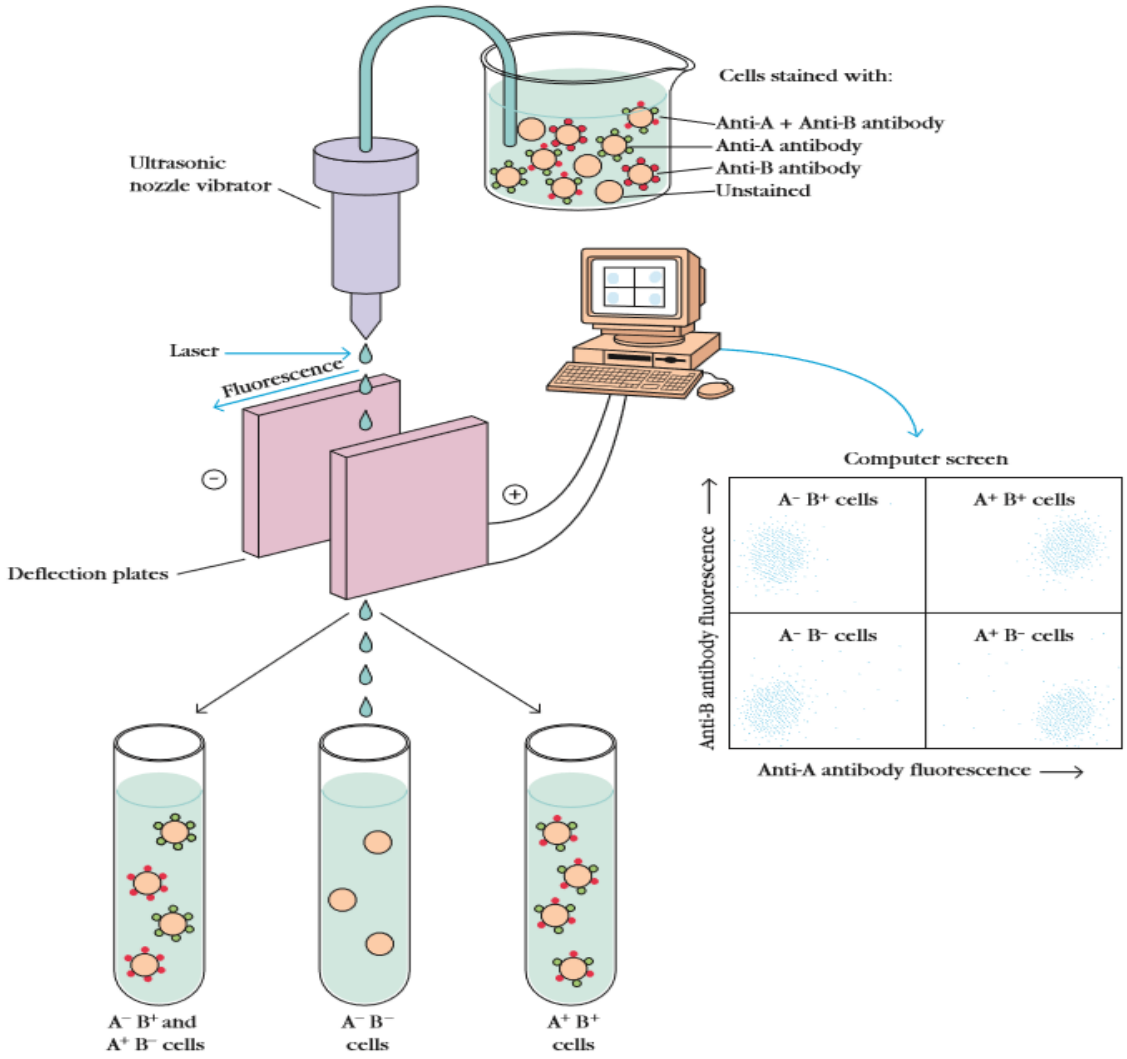


FIGURE 6-15 Separation of fluorochrome-labeled cells with the flow cytometer. In the example shown, a mixed cell population is stained with two antibodies, one specific for surface antigen A and the other specific for surface antigen B. The anti-A antibodies are labeled with fluorescein (green) and the anti-B antibodies with rhodamine (red). The stained cells are loaded into the sample chamber of the cytometer. The cells are expelled, one at a time, from a small vibrating nozzle that generates microdroplets, each containing no more than a single cell. As it leaves the nozzle, each droplet receives a small electrical charge, and the computer that controls the flow cytometer can detect exactly when a drop generated by the nozzle passes through the beam of laser light that excites the fluorochrome. The intensity of the fluorescence emitted by each droplet that contains a cell is monitored by a detector and displayed on a computer screen. Because the computer tracks the position of each droplet, it is possible to determine when a particular droplet will arrive between the deflection plates. By applying a momentary charge to the deflection plates when a droplet is passing between them, it is possible to deflect the path of a particular droplet into one or another collecting vessel. This allows the sorting of a population of cells into subpopulations having different profiles of surface markers.

In the computer display, each dot represents a cell. Cells that fall into the lower left-hand panel have background levels of fluorescence and are judged not to have reacted with either antibody anti-A or anti-B. Those that appear in the upper left panel reacted with anti-B but not anti-A, and those in the lower right panel reacted with anti-A but not anti-B. The upper right panel contains cells that react with both anti-A and anti-B. In the example shown here, the A^-B^-—and the A^+B^+—subpopulations have each been sorted into a separate tube. Staining with anti-A and anti-B fluorescent antibodies allows four subpopulations to be distinguished: A^-B^-, A^+B^+, A^-B^+, and A^+B^-.

5. Fluorescence activated cell sorting (FACS):

Some cell types differ sufficiently in density that they can be separated on the basis of this physical property. White blood cells (leukocytes) and red blood cells (erythrocytes), for instance, have very different densities because erythrocytes have no nucleus; thus these cells can be separated by equilibrium density-gradient centrifugation. Most cell types cannot be differentiated so easily, so other techniques, such as flow cytometry, must be used to separate them.

To separate one type of cell from a complex mixture, it is necessary to have some way to mark and then sort out the desired cells. As we will see below, it is possible to mark cells by expressing a fluorescent protein in them, but if only a few cells in the population express the protein, how can we sort them from the nonfluorescent ones? The cells can be analyzed in a flow cytometer. This machine flows cells past a laser beam that measures the light that they scatter and the fluorescence that they emit; thus it can quantify the cells expressing the fluorescent protein in a mixture. A fluorescence-activated cell sorter (FACS), which is based on flow cytometry, can both analyze the cells and select the few fluorescent cells from thousands of others and sort them into a separate culture dish (Figure 4-2). To achieve this, the cells are mixed with a buffer and forced through a vibrating nozzle to generate tiny droplets. The concentration of cells is adjusted so that most of the droplets do not contain cells, and the ones that do contain only one. Just before the nozzle, the stream of cells passes through a laser beam so that the presence and size of a cell can be recorded from the scattered light using one detector, and the amount of fluorescent light emitted can be quantified using a second, fluorescent light detector. If a cell is present in a droplet, the droplet is given a negative electric charge as it emerges from the nozzle. The stream of droplets then passes through two plates that generate an electric field proportional to the fluorescence detected from the cell in the droplet. This field generates a force that moves charged droplets out of the stream of uncharged droplets and into a collection tube. Since the amount of force applied is proportional to the fluorescence emitted by the cell in the droplet, cells with different levels of fluorescence can be collected. Having been sorted from other cells, the selected cells can be grown in culture.

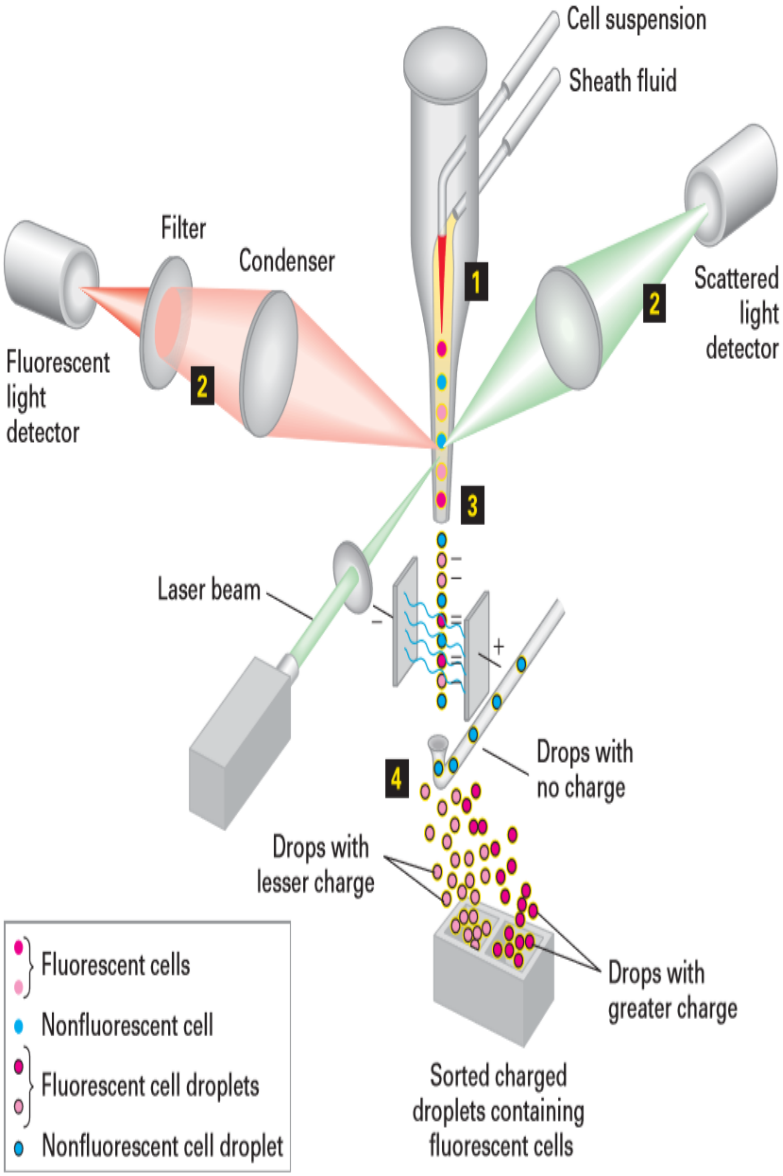
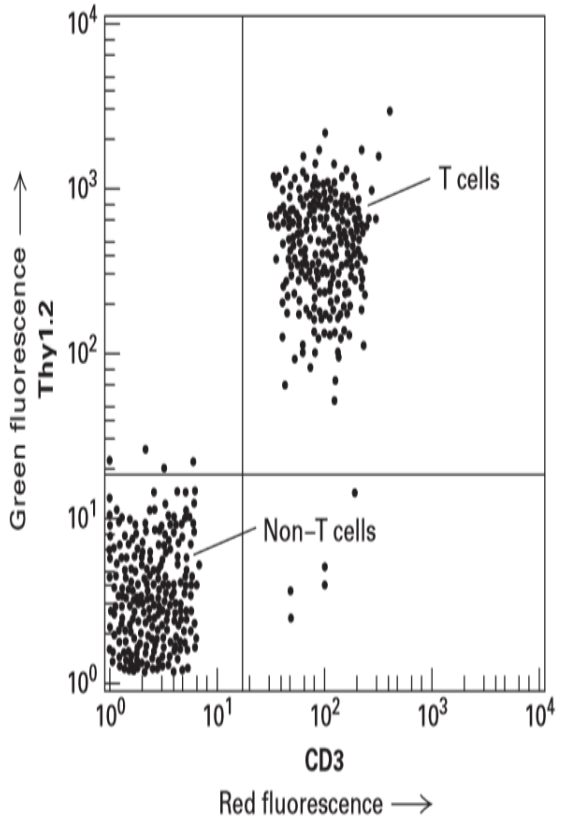


FIGURE 4-2 A fluorescence-activated cell sorter (FACS) separates cells having different levels of fluorescence. Step 1: A concentrated suspension of labeled cells is mixed with a buffer (the sheath fluid) so that the cells pass single file through a laser light beam. Step 2: Both the fluorescent light emitted and the light scattered by each cell are measured; from measurements of the scattered light, the size and shape of the cell can be determined. Step 3: The suspension is then forced through a nozzle, which forms tiny droplets containing at most a single cell. At the time of formation at the nozzle tip, each droplet containing a cell is given a negative electric charge proportional to the fluorescence of that cell determined from the earlier measurement. Step 4: Droplets now pass through an electric field, so that those with no charge are discarded, whereas those with different electric charges are separated and collected. Because it takes only milliseconds to sort each droplet, as many as 10 million cells per hour can pass through the machine.

The FACS procedure is commonly used to purify the different types of white blood cells, each of which bears on its surface one or more distinctive proteins and so will bind monoclonal antibodies specific for its proteins. If a cell mixture is incubated with a fluorescent dye linked to the antibody to a specific cell-surface protein, only the desired cells will be fluorescent. Only the T cells of the immune system, for instance, have both CD3 and Thy1.2 proteins on their surfaces. The presence of these surface proteins allows T cells to be separated easily from other types of blood cells or spleen cells (Figure 4-3).



EXPERIMENTAL FIGURE 4-3 T cells bound to fluorescence-tagged antibodies to two cell-surface proteins are separated from other white blood cells by FACS. Spleen cells from a mouse were treated with a red fluorescent monoclonal antibody specific for the CD3 cell-surface protein and with a green fluorescent monoclonal antibody specific for a second cell-surface protein, Thy1.2. As the cells were passed through a FACS, the intensity of the green and red fluorescence emitted by each cell was recorded. Each dot represents a single cell. This plot of green fluorescence (vertical axis) versus red fluorescence (horizontal axis) for thousands of spleen cells shows that about half of them—the T cells—express both CD3 and Thy1.2 proteins on their surfaces (upper-right quadrant). The remaining cells, which exhibit low fluorescence (lower-left quadrant), express only background levels of these proteins and are other types of white blood cells. Note the logarithmic scale on both axes. [Data from Chengcheng Zhang, Whitehead Institute.]

Other uses of flow cytometry include the measurement of a cell's DNA and RNA content and the determination of its general shape and size. The FACS can make simultaneous measurements of the size of a cell (from the amount of scattered light) and the amount of DNA that it contains (from the amount of fluorescence emitted from a DNA-binding dye). Measurements of the DNA content of individual cellsare used to follow replication of DNA as the cells progress through the cell cycle.

9.Suggested reading

1. Kuby Immunology(7th edition), Janeway's Immunobiology(2007)
2. Abdul Abbas, Andrew H. Lichtman, Shiv Pillai – Cellular and Molecular Immunology (9th edition)
3. Christopher J. Woolverton, joanne Willey,and Linda Sherwood. -Prescott's Microbiology
4. Berdell R. Funke, Gerard J. Tortora,and Christine L. Case. –Microbiology: An Introduction
5. http://www.wikipedia.org/

10.Assignments

1. What are the signs of a weak immune syetem? What are the three major functions of the immune syetem?
2. What is mast cell and what does it do?? How many mast cells are in the body?
3. Write down the interactions between mast cels and dendritic cells.
4. How does blood cell develop? What are the functions of bone marrow?
5. What are the circulatory syetem found in human? How does lymph fluids forms? How does the lymph fluid return to blood? Classify the primary lymphoid organs and describe it.
6. What is immunity and its types? What is cytokines?
7. What is an example of hypersensitivity? What are the signs and symptoms of hypersensitivity?
8. What does vaccine mean? What are the purpose of vaccine? What are the common vaccines?

All the materials are self-written and collected from eBooks, journals and websites.