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LIFE SCIENCES

Fundamentals and Practice

Part - I

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Preface

Life Sciences have always been a fundamental area of science. The exponential increase in the quantity of scientific information and the rate, at which new discoveries are made, require very elaborate, inter-disciplinary and up-to-date information and their understanding. *Life sciences, Fundamentals and Practice,* is an attempt to provide an extraordinarily large amount of information from enormous and evergrowing field in an easily retrievable form. We have attempted to provide balanced coverage of topics like, biochemistry, cell biology, microbiology, immunology and classical genetics. It is written in clear and concise language to enhance the self motivation and strategic learning skill of students and empowering them with a mechanism to measure and analyze their abilities and the confidence of winning.

Our own experience and comments from students have reinforced our initial purpose of writing this book. Thus in writing the book we have taken account of feedback provided by number of students from various parts of the country who appeared for CSIR-JRF-NET or related examinations like ICMR, GATE, DBT-JRF. Each chapter contains logically organized information to remain current with the discipline and with subtle changes in entrance examinations trends and patterns. Each chapter in this book contains three types of questions: study questions, review the concepts and self tests; which are designed to enable the student to identify the pattern of examinations easily. We have incorporated selected questions from previous year's question papers along with the text to measure the level of understanding and their applications. We hope you will find this study material interesting, relevant and challenging.

Any book of this kind requires meticulous and painstaking efforts by all its contributors. We would like to thank all my colleagues who have helped in various ways and special thanks to Pradeep Verma, Ajay Kumar, Arun Kumar and Murari Kumar Singh for designing, proof-reading and typing the manuscript.

Pranav Kumar

Usha Mina

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Chapter - 01

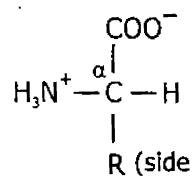
Amino acids and Proteins

1.1 AMINO ACIDS

Proteins are linear, unbranched polymers constructed from 22 standard α-amino acids that are encoded in the DNA of the genome. The fundamental units of protein polymers are α-amino acids.

Amino acids are composed of an amino group (imino group in proline), a carboxyl group, a hydrogen atom, and a distinctive side chain, all bonded to a carbon atom, the α-carbon.

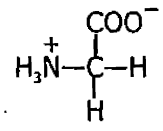
The general structure of an amino acid is



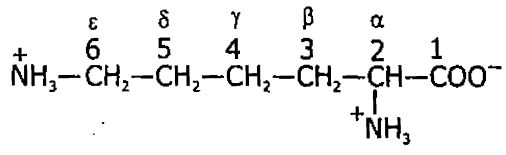
Basic structure of amino acid

This structure is common to all but one of the α-amino acids (proline, a cyclic amino acid, is the exception). The R group or side chain attached to the α carbon is different in each amino acid. In the simplest case, the R group is a hydrogen atom and amino acid is glycine.

Structure of Glycine:



Structure of Lysine:



In α-amino acids both the amino group and the carboxyl group are attached to the same carbon atom. However, many naturally occurring amino acids not found in protein, have structures that differ from the α-amino acids. In these compounds the amino group is attached to a carbon atom other than the α-carbon atom and they are called β, γ, δ, or ε amino acids accordingly.

Amino acids can act as acids and bases

When an amino acid is dissolved in water, it exists in solution as the *dipolar ion* or *zwitterion*. A zwitterion can act as either an acid (proton donor) or a base (proton acceptor). Hence, amino acid is an **amphoteric molecule**. At high concentrations of hydrogen ion (low pH), the carboxyl group accepts a proton and becomes uncharged, so that the overall charge on the molecule is *positive*. Similarly at low concentrations of hydrogen ion (high pH), the amino group loses its proton and becomes uncharged; thus the overall charge on the molecule is *negative*.

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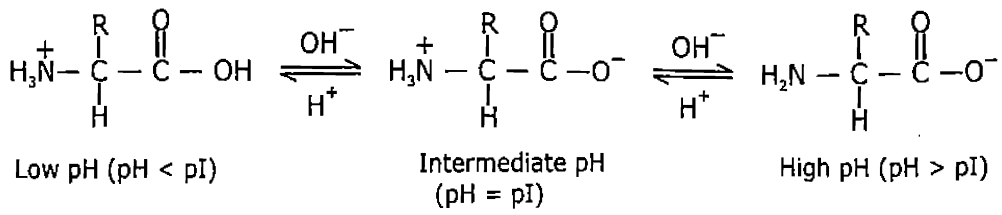
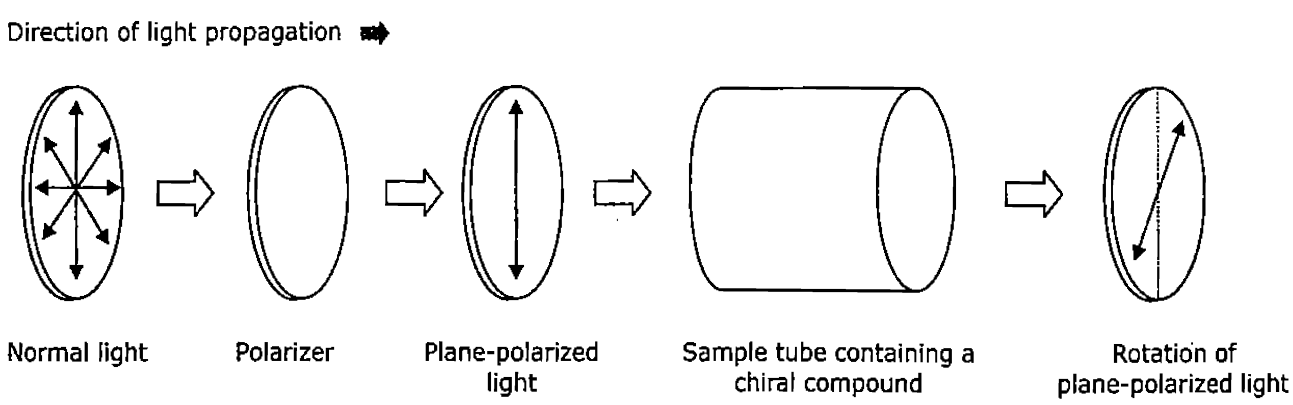


Figure 1.1 : The acid-base behavior of an amino acid in solution. At low pH, the positively charged species predominates. As the pH increases, the electrically neutral zwitterion becomes predominant. At higher pH, the negatively charged species predominates.

Optical Properties

All amino acids except glycine are optically active i.e., they rotate the plane of plane polarized light. Optically active molecules have an asymmetry such that they are not superimposable on their mirror image. This is characteristic of substances that contain chiral centre. A tetrahedral carbon atom with four distinct constituents said to be **chiral**. The one amino acid not exhibiting chirality is glycine since its 'R-group' is a hydrogen atom. Chirality describes the handedness of a molecule that is observable by the ability of a molecule to rotate the plane of polarized light either to the right (**dextrorotatory**) or to the left (**laevorotatory**).



Optical activity is measured by polarimeter. The magnitude of the optical activity is measured as an angle of rotation. A quantitative measure of the optical activity of the molecule is known as its **optical rotation** (α). Optical rotation of an optically active compounds depend on concentration of the compound, light path length, the wavelength of the polarized light and the temperature. Hence experimentally measured α is always converted to and expressed as **specific rotation** $[\alpha]_D^T$. The optical rotation of a solution at a given temperature and wavelength is given by

$$[\alpha]_D^T = \frac{A^\circ}{\ell \times C}$$

where, Å = observed rotation in degrees

$[\alpha]_D^T$ = the specific rotation of compound at fixed temperature and wavelength

C = the concentration of solution in g/ml

l = light path length in decimeter

STUDY QUESTIONS

Q1. A solution of L-leucine (3.0 g/50 ml of 6 N HCl) had an observed rotation of +1.81° in a 20 cm polarimeter tube. Calculate the specific rotation of L-leucine in 6 N HCl.

Ans. $[\alpha]_D^T = \frac{A^\circ}{\ell \times C}$

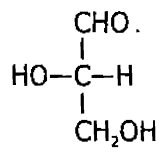
$$[\alpha]_D^T = \frac{+1.81}{2 \times 0.06} \qquad \text{where, } \ell = 20\,\text{cm} = 2\,\text{dm} \text{ and } C = 3\,\text{g}/50\,\text{ml} = 0.06\,\text{g/ml}$$

$$[\alpha] = +15.1^\circ$$

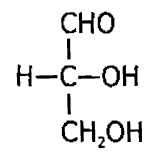
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Absolute configuration

Amino acid with chiral centre, gives two possible isomers, known as *enantiomers*. Enantiomers are mirror image of each other. Thus the two enantiomers have different configurations. For example the glyceraldehyde has two enantiomers describes the exact spatial orientation of all the four specification i.e., *absolute configuration*.

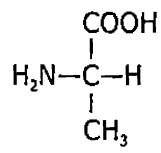


L-Glyceraldehyde

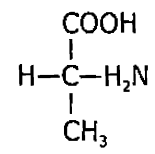


D-Glyceraldehyde

In the above figure, prefixes D- and L- refer to absolute configuration of glyceraldehyde. Similarly absolute configuration of amino acids are specified by the D- and L- system. The designation of D or L to an amino acid refers to its absolute configuration relative to the structure of D- or L-glyceraldehyde, respectively.



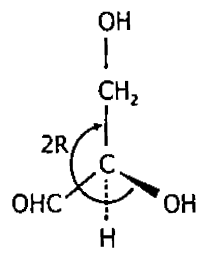
L-Alanine



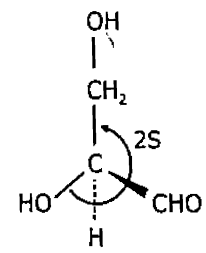
D-Alanine

All amino acids except glycine exist in these two different enantiomeric forms. However, all of the amino acids in proteins exhibit the same absolute steric configuration as *L-glyceraldehyde*. Therefore, they are all L-α-amino acids. D-form of amino acids are not found in proteins, although they exist in nature. D-form of amino acids are found in some peptide antibiotics and peptidoglycan cell wall of bacteria.

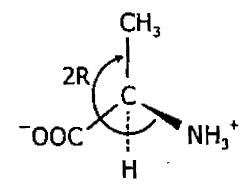
A second absolute configuration notation using the symbols R (from *rectus*, Latin for right) and S (from *sinister*, Latin for left) can also be used. In this approach, the substituents on an asymmetric carbon (a tetrahedral carbon with four different substituents) are prioritized by decreasing atomic number. Configuration is assigned by looking down the bond to the lowest priority substituent and assigning R to the configuration where the remaining substituents are arranged clockwise in decreasing priority. S is then assigned to the molecular form where the substituents are arranged counterclockwise.



D-Glyceraldehyde

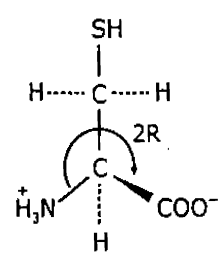


L-Glyceraldehyde

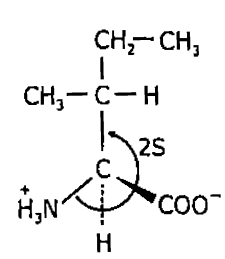


D-Alanine

The absolute configuration of the amino acids at the α-carbon is typically referred to using the D-L notation rather than the more modern R-S designation. In R-S notation change of a single substituent can change assignment. However, all of the amino acids used in proteins (except for glycine which is not optically active) are of L configuration. In R-S notation, cysteine is 2R, whereas the others are 2S.



L-Cysteine



L-Isoleucine

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Standard amino acids

More than 300 amino acids are present in cells but only 22 amino acids present in proteins called *standard amino acids*. Standard L-α-amino acids are specified by simple three letter codons. α-amino acids can be classified on the properties of their R group, in particular, their polarity, or tendency to interact with water at biological pH (near pH 7). The polarity of the R group varies widely, from nonpolar and hydrophobic to highly polar and hydrophilic. The structure of the 20 standard amino acids are shown in figure and some of their properties are listed in table 1.1. Properties of selenocysteine (Sec) and pyrrolysine (Pyl), termed as 21st and 22nd amino acid respectively are mentioned in the next section.

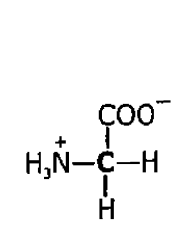
Table 1.1 : Standard amino acids (L-α-amino acids) and their properties

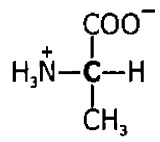
Amino acids with nonpolar side chain				
		Molecular mass	**pI**	**(Occurrence in protein %)**
Glycine	Gly (G)	75 (min)	5.97	
Alanine	Ala (A)	89	6.01	
Valine	Val (V)	117	5.97	
Leucine	Leu (L)	131	5.98	9.1 (max)
Isoleucine	Ile (I)	131	6.02	
Proline	Pro (P)	115	6.48	
Methionine	Met (M)	149	5.74	
Phenylalanine	Phe (F)	165	5.48	
Tryptophan	Trp (W)	204 (max)	5.89	1.4 (min)
Amino acids with uncharged polar side chain				
Serine	Ser (S)	105	5.68	
Threonine	Thr (T)	119	5.87	
Cysteine	Cys (C)	121	5.07	
Asparagine	Asn (N)	132	5.41	
Glutamine	Gln (Q)	146	5.65	
Tyrosine	Tyr (Y)	181	5.66	
Amino acids with charged polar side chain				
Negatively charged R groups				
Aspartate	Asp (D)	133	2.77 (min)	
Glutamate	Glu (E)	147	3.22	
Positively charged R groups				
Lysine	Lys (K)	146	9.74	
Arginine	Arg (R)	174	10.76 (max)	
Histidine	His (H)	155	7.59	

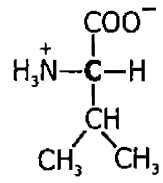
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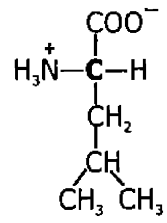
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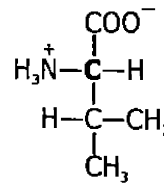
Amino acids with Nonpolar side chain

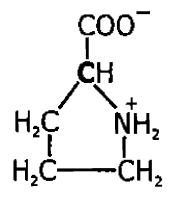

Glycine

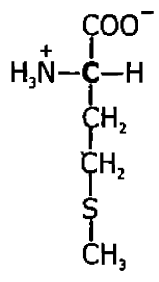

Alanine

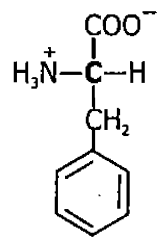

Valine

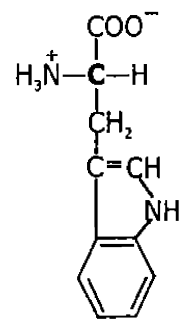

Leucine


Isoleucine

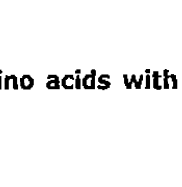

Proline

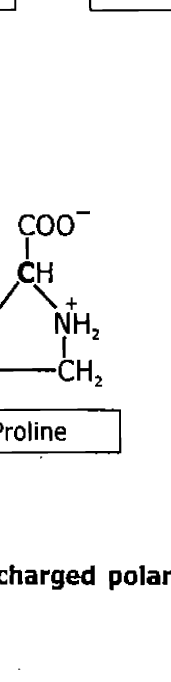

Methionine

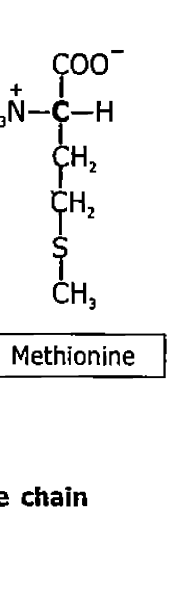

Phenylalanine

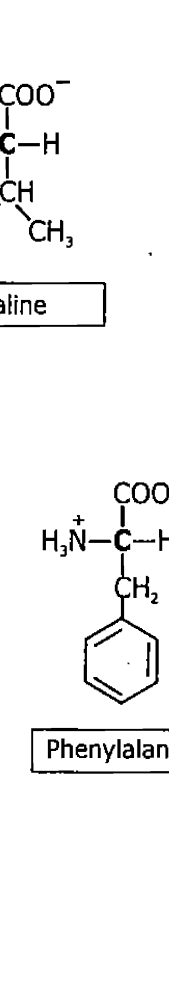

Tryptophan

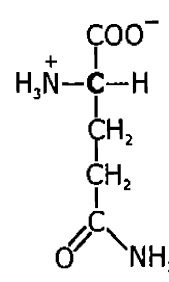
Amino acids with uncharged polar side chain

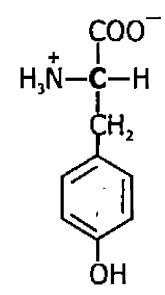

Serine


Threonine


Cysteine

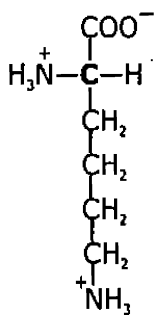

Asparagine

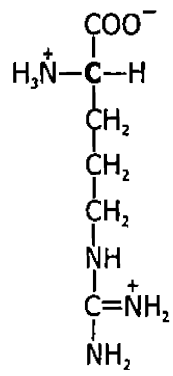

Glutamine

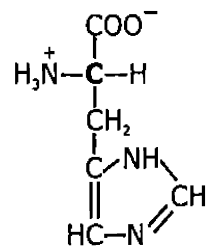

Tyrosine

Amino acids with charged polar side chain

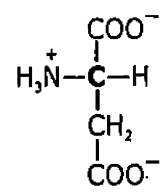
Positively charged R groups

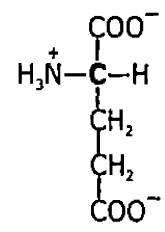

Lysine


Arginine


Histidine

Negatively charged R groups


Aspartate


Glutamate

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Table 1.2 : Classification of amino acids based on chemical structure

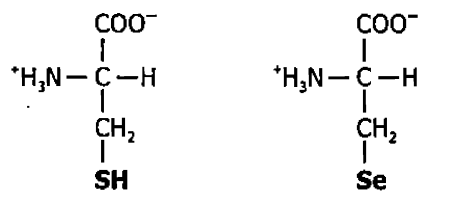
Chemical nature of R group	*Example*
Aliphatic	Gly, Ala, Val, Leu
Aromatic	Phe, Tyr, Trp
Hydroxyl	Ser, Thr
Carboxylic	Asp, Glu
Sulphur containing	Cys, Met
Imino	Pro
Amino	Lys, Arg
Amide	Asn, Gln

Nonstandard amino acids

More than three hundred amino acids have been found in cells. Besides their role in proteins, amino acids and their derivatives have many other biologically important functions. For example, *ornithine* and *citrulline* are key intermediates in the biosynthesis of arginine and in urea cycle. Similarly *azaserine* acts as an antibiotic.

In addition to the 20 standard amino acids, proteins may contain uncommon amino acid residues formed by modification of standard amino acids residues already incorporated into a polypeptide. Examples like 4-Hydroxyproline (derivative of proline), 5-Hydroxylysine (derivative of lysine), Desmosine (derivative of 4-lysine), N-acetylserine, N-formylmethionine, γ-carboxyglutamate (found in the blood clotting protein prothrombin).

It was originally thought that all unconventional amino acids were made by modifying one of the standard amino acids after it was incorporated into protein, a process called post translational modification. But amino acids like *selenocysteine*, *pyrrolysine* are inserted into proteins by the translational machinery. **Selenocysteine** is introduced during protein synthesis rather than created through a postsynthetic modification. It contains selenium rather than sulphur of its structural analog, cysteine. Since selenocysteine is incorporated into polypeptides during translation, it is referred to as 21st amino acid. However, it is specified by triplet codon, UGA (a nonsense codon). Selenocysteine has its own tRNA containing the anticodon UCA and it is formed by modifying a *serine* that has been attached to the selenocysteine tRNA. Enzymes like *glutathione peroxidase* and *formate dehydrogenase* contain selenocysteine in their catalytic center. **Pyrrolysine** is similar to lysine and present in some bacterial proteins. It is coded by UAG codon.



Cysteine Selenocysteine

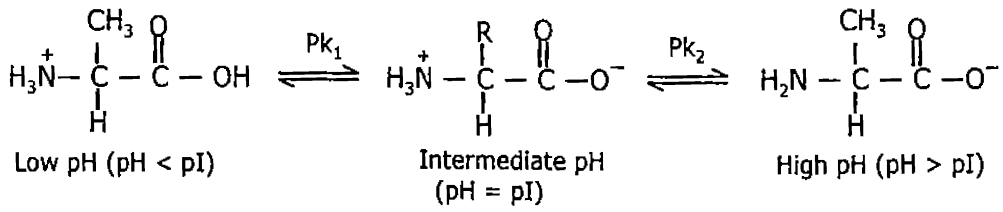
Titration of amino acids

Because amino acids contain ionizable groups, the predominant ionic form of these molecules in solution depends on the pH. Titration of an amino acid illustrates the effect of pH on amino acid structure. Consider alanine, a simple amino acid, which has two titrable groups (α-amino and α-carboxyl group). During titration with a strong base such as NaOH, alanine loses two protons in a stepwise fashion. In a strongly acidic solution, alanine is present mainly in the form in which the carboxyl group is uncharged. Under this condition

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the molecule's net charge is +1, since the ammonium group is protonated. However, increase in the pH results in deprotonation of α-carboxyl group. At this point, alanine has no *net* charge and is electrically neutral. The pH at which this occurs is called the **isoelectric point** (pI). Because there is no net charge at the isoelectric point, amino acids are electrophoretically non-mobile and least soluble at this pH. Further increase in pH i.e., lowering of the H^+ concentration results in the deprotonation of the charged amino group and a uncharged amino group forms. So at high pH, the molecules net charge is -1, since the ammonium group is deprotonated and a net negative charge develops due to the presence of the carboxylate group.



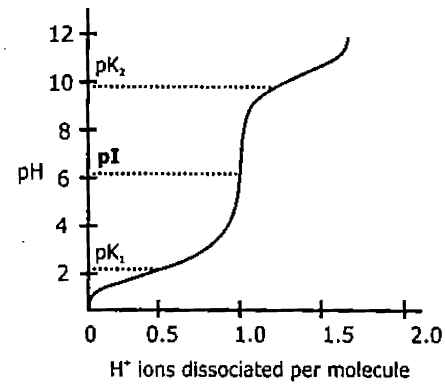


Figure : 1.2
Titration curve of alanine
(monoamino and monocarboxylic acid)

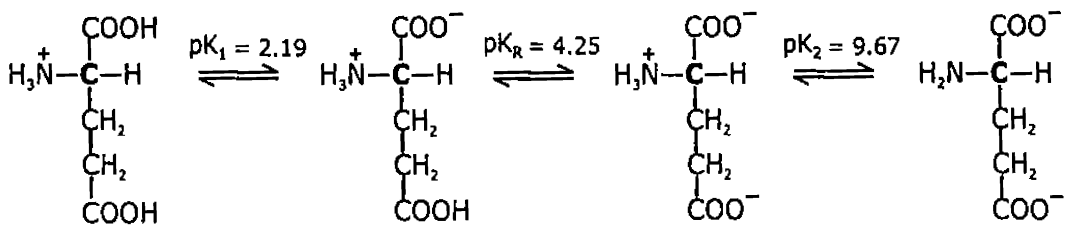
The isoelectric point for alanine may be calculated as follow :

$$pI = \frac{pK_1 + pK_2}{2}$$

The pK_1 and pK_2 values for alanine are 2.34 and 9.7 respectively. The pI value for alanine is therefore

$$pI = \frac{2.34 + 9.7}{2} = 6.0$$

Amino acids with ionizable side chains have more complex titration curves. Glutamic acid, for example, has a carboxyl side chain group. At low pH, glutamic acid has net charge +1. As base is added (pH increases), the α-carboxyl group loses a proton to become a carboxylate group (in a polyprotic acid, the protons are first lost from the group with the lowest pKa). Glutamate now has no net charge. As still more base is added, the second carboxyl group loses a proton, and the molecule has a −1 charge. Adding base further results in loss of proton from ammonium ion. At this point, glutamate has a net charge of −2.



The pI value for Glu is the pH halfway between the pK values for the two carboxyl groups:

$$pI = \frac{2.19 + 4.25}{2} = 3.22$$

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Table 1.3 : pKa values for the ionizing groups of the amino acids

Amino acid	pK_1	pK_2	pK_R
Glycine	2.34	9.6	
Alanine	2.34	9.69	
Valine	2.32	9.62	
Leucine	2.36	9.6	
Isoleucine	2.36	9.6	
Serine	2.21	9.15	
Threonine	2.63	10.43	
Methionine	2.28	9.21	
Phenylalanine	1.83	9.13	
Tryptophan	2.83	9.39	
Asparagine	2.02	8.8	
Glutamine	2.17	9.13	
Proline	1.99	10.6	
Cysteine	1.71	10.78	8.33
Histidine	1.82	9.17	6.04
Aspartic acid	2.09	9.82	3.86
Glutamic acid	2.19	9.67	4.25
Tyrosine	2.2	9.11	10.46
Lysine	2.18	8.95	10.54
Arginine	2.17	9.04	12.48

Absorption of UV-light by aromatic amino acids

Aromatic amino acids such as tryptophan, tyrosine, phenylalanine absorb ultraviolet (UV) light. The aromatic side chains of these amino acids are responsible for UV absorption. Tryptophan and tyrosine absorbs maximum near a wave length of 280 nm. However phenylalanine absorbs maximum at 257.4 nm. Absorbance at 280 nm is used for detection and quantification of purified proteins. The absorbance of each protein depends on the number and positions of its aromatic amino acid residues.

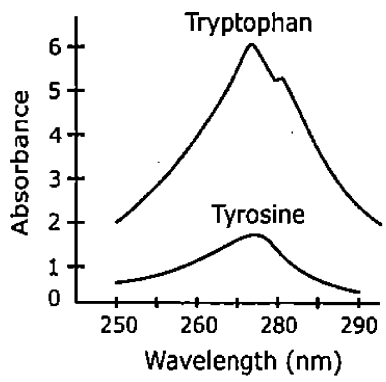


Figure : 1.3
Absorption of UV-light. Maximum light absorption for both tryptophan and tyrosine occurs near a wavelength of 280 nm and absorbance of tryptophan is as much as four times that of tyrosine.

Table 1.4 : Absorbance of the aromatic amino acids at neutral pH

Amino acids	*Absorbance* (λ_{max})
Phenylalanine	257.4 nm
Tyrosine	274.6 nm
Tryptophan	279.8 nm

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STUDY QUESTION

Q2. Determine whether the following statements are true or false. If false, explain why?

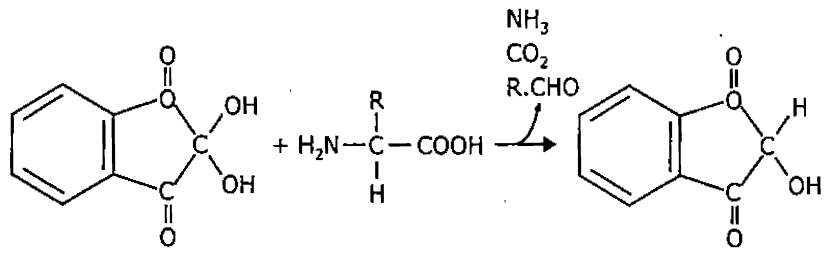
1. All 20 standard amino acids found in proteins have at least one asymmetric carbon atom
2. An equimolar mixture of D- and L-alanine does not rotate the plane of polarized light
3. The observed optical rotation of an amino acid solution is directly proportional to the length of the light path through the polarimeter tube and the concentration of the amino acid solution
4. Alanine obtained from a protein hydrolysate has the same absolute configuration as D-glyceraldehyde

Ans. 1. False; glycine has no asymmetric carbon atom.

2. True.
3. True.
4. False; alanine obtained from a protein hydrolysate has the same absolute configuration as L-glyceraldehyde.

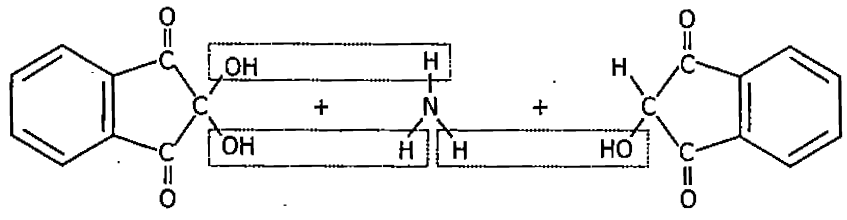
Ninhydrin test

Ninhydrin test is used to detect amino acids, peptides and proteins. Ninhydrin (triketohydrindene hydrate) reacts with α-amino group of the amino acids and proteins. Ninhydrin is a strong oxidizing agent and in its presence, amino acids undergo oxidative deamination liberating ammonia, CO_2, a corresponding aldehyde and reduced form of ninhydrin. Ammonia formed from α-amino group reacts with ninhydrin and its reduced product (hydrindantin) to give a blue substance called diketohydrin (Ruhemann's purple) that absorbs light at 570nm. Proline gives a yellow colour as it lacks an α-amino group. The molar absorption coefficient of the coloured product can be used in the quantitation of individual amino acids but this value varies from one amino acid to another. This is used for both qualitative and quantitative identification of particular amino acids.

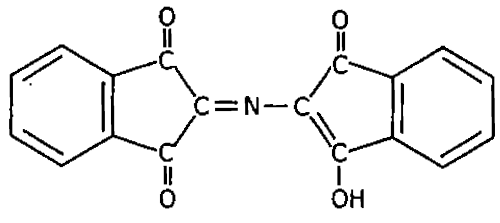


Ninhydrin Reduced ninhydrin

Oxidative decarboxylation of the amino acid and the production of reduced ninhydrin, ammonia and carbon dioxide.



Reduced ninhydrin reacts with more ninhydrin and the liberated ammonia



Blue-coloured complex

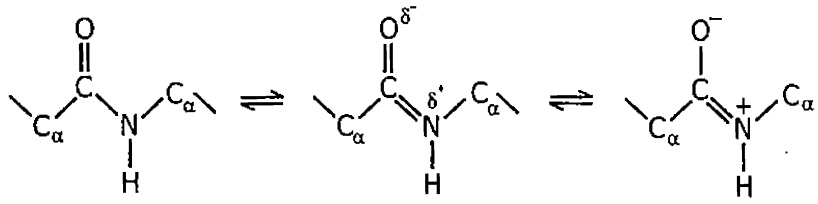
A blue-coloured complex is formed.

1.2 PEPTIDE BOND

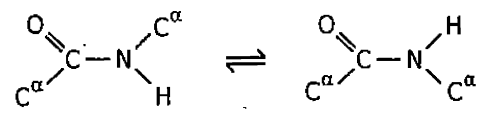
Polypeptides are linear and unbranched polymers composed of amino acids linked together by peptide bonds. Peptide bonds are amide linkages formed when the unshared electron pair of the α-amino nitrogen

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atom of one amino acid attacks the α-carboxyl carbon of another in a nucleophilic acyl substitution reaction. This reaction is a dehydration, that is, a water molecule is removed and the linked amino acids are referred to as amino acid residues. Peptide bond formation is an endergonic process, with $\Delta G \sim +21 kJ/mol$.

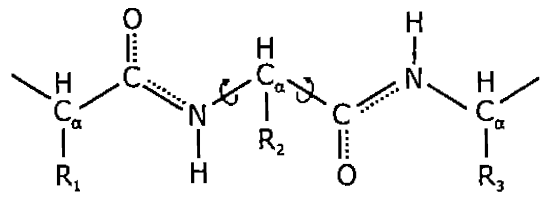


The peptide C—N bond has a partial double bond character that keeps the entire six-atom peptide group in a rigid planar configuration. Consequently, the peptide bond length is only 1.33 Å, shorter than the usual C—N bond length of 1.45 Å. The peptide bond appears to have approximately 40 percent double-bonded character. As a result, rotation of this bond is restricted. The angle of rotation around the peptide bond, ω, usually has the value ω = 180° (*trans*) and occasionally ω = 0° (*cis*). The *trans* form is favoured by a ratio of approximately 1000:1 over the *cis* form because in the *cis* form the C_α atom and the side chains of neighbouring residues are in too close proximity.



trans *cis*

However, rotation is permitted about the N—C_α and the C_α—C bonds. Rotation about bonds are described as *torsion* or *dihedral* or *conformational* angle. By convention, the bond angles resulting from rotations at C_α are labeled ϕ (phi) for the N—C_α bond and ψ (psi) for the C_α—C bond.



In principle, ϕ and ψ can have any value between −180° and + 180° but many values are prohibited by steric interference between atoms in the polypeptide backbone and amino acid side chains. The permitted values for ϕ and ψ were first determined by G. N. Ramachandran. The permitted value of ϕ and ψ are usually indicated on a 2-D map of the ϕ-ψ plane, also known as **Ramachandran plot**.

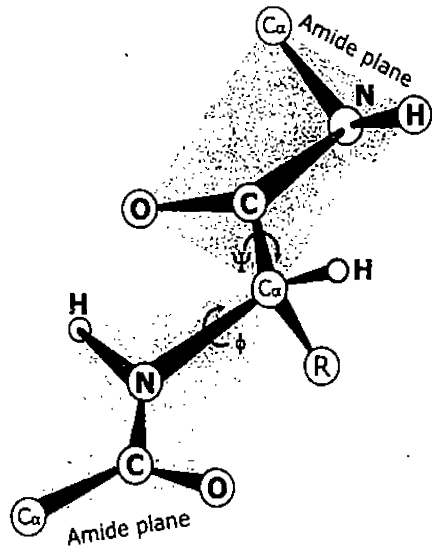


Figure 1.4a : The torsional angle in a peptide unit

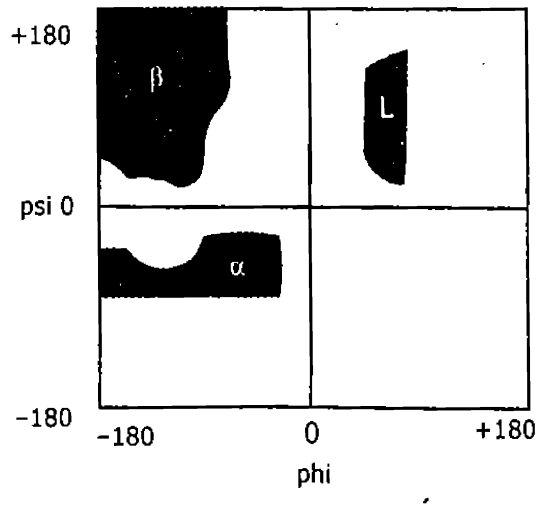


Figure 1.4b : Ramachandran plots showing allowed combinations of the conformational angles phi and psi.

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Since phi (ϕ) and psi (ψ) refer to rotations of two rigid peptide units around the same C_α atom, most combinations produce steric collisions either between atoms in different peptide groups or between a peptide unit and the side chain attached to C_α. These combinations are therefore not allowed. Shaded areas in figure 1.4b show sterically allowed regions. The areas labeled α, β and L correspond approximately to conformational angles found for the usual right-handed α–helices, β-strands, and left handed α-helices, respectively.

Table 1.5 : Parameters for regular secondary structure

	Bond angle (in degree)	
	ϕ	ψ
Antiparallel β-sheet	- 139	+ 135
Parallel β-sheet	- 119	+ 113
Right-handed α-helix (3.6_{13} helix)	- 57	- 47
Right-handed 3_{10}-helix	- 49	- 26

PEPTIDES

When two amino acid molecules are linked, through peptide bond then the product is called a *dipeptide*. Three amino acids can be joined by two peptide bonds to form *tripeptide*, similarly amino acids can be linked to form tetrapeptides, pentapeptides and so forth. Oxytocin, bradykinin, amanitin, enkephalins, glutathione are example of some biologically active peptides. Similarly **aspartame** is a commercially synthesized dipeptide, L-aspartylphenylalanyl methylester, and used as an artificial sweetener. Peptide chains of more than 12 and less than about 20 amino acid residues are usually referred to as **oligopeptide**. When many amino acid residues are joined the product is called a **polypeptide.** Peptides are named by using their amino acid sequences, beginning from their N-terminal residue.

The peptide bonds in proteins form between the α-amino and the α-carboxyl groups. But peptides do occur naturally where the peptide linkage involves a carboxyl or amino group which is attached to a carbon atom other than the α-carbon. For example a dipeptide formed between the γ carboxyl group of glutamic acid and the amino group of alanine is called γ–glutamylalanine.

1.3 PROTEIN STRUCTURE

Protein have four levels of the structural organization. *Primary structure*, the amino acid sequence, is specified by genetic information. As the polypeptide chain folds, it forms certain localized arrangements of adjacent amino acids that constitute *secondary structure*. The overall three-dimensional shape that a polypeptide assumes is called the *tertiary structure*. Proteins that consist of two or more polypeptide chains (or subunits) are said to have a *quaternary structure*.

Primary structure

The primary structure of a polypeptide is its amino acid sequence. The amino acids are connected by peptide bonds. Primary structure of polypeptide determines the higher levels of structural organization.

Secondary structure

The most common types of secondary structure are the **α-helix** and the **β-pleated** sheet. Both α-helix and β-pleated sheet patterns are stabilized by hydrogen bonds between the carbonyl and N–H groups in the polypeptide's backbone.

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α-helix

The α-helix is a rigid, rod like structure that forms when a polypeptide chain twists into a helical conformation. The *screw sense* of α helix can be right-handed (clockwise) or left-handed (counterclockwise). However right-handed helices are energetically more favorable. There are 3.6 amino acid residues per turn of the helix and the pitch (the distance between corresponding points per turn) is 0.54 nm. Each residue is related to the next one by a rise of 1.5 Å (0.15 nm) along the helix axis. A single turn of α-helix involves 13 atoms from O to the H of the H bond. For this reason, the α-helix is referred to as the 3.6_{13} helix. Length of α-helix is usually 10-15 amino acid residues. Hydrogen bonds form between the N—H group of each amino acids and the carbonyl group of the amino acid four residues away.

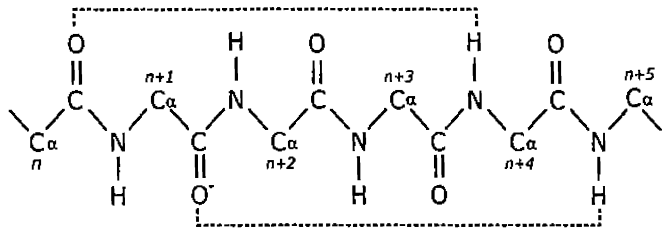


Figure 1.5 : The hydrogen bonding arrangement in the α-helix.

Except for amino acids near the ends of an α-helix, all the main-chain CO and NH groups are hydrogen bonded. Side chain of amino acids extend outward from the helix. All of the H bonds lie parallel to the helix axis and point in the same direction.

Helices can be formed from either D- or L-amino acids, but a given helix must be composed entirely of amino acids of one configuration. α-helix cannot be formed from a mixed copolymer of D- and L-amino acids.

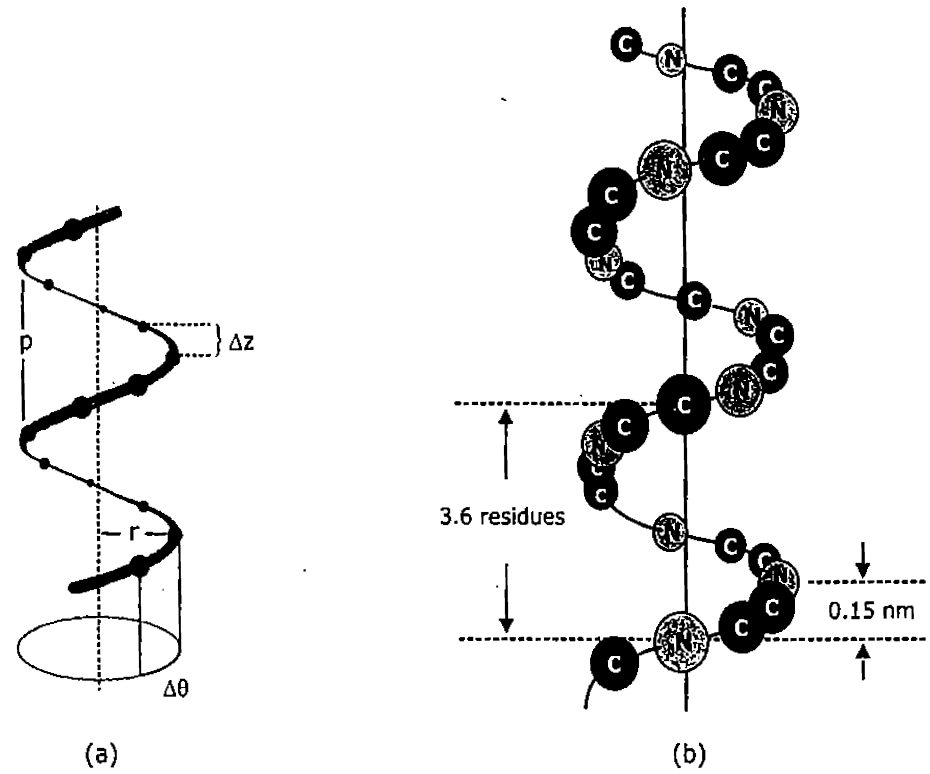


Figure 1.6 (a) : *Describing the geometry of α-helix.* The helix structure is defined by : the *pitch*, P (the distance along the axis between successive turns), the *radius*, r, and the rise per residue, ΔZ. The number of residues per turns is equal to p/ΔZ. The angular difference between successive residues, $\Delta\theta = 360° \times \Delta Z/p$
(b) The right handed α-helix. A complete turn of the helix contains an average of 3.6 aminoacyl residues, and the distance it rises per turn (its pitch) is 0.54 nm. The R groups of each aminoacyl residue in an α-helix face outward (not shown in the figure). In the α-Helix the hydrogen bonds are within a single polypeptide chain and are almost parallel to the helix axis.

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β-pleated sheets

β-pleated sheets form when two or more polypeptide chain segment line up side by side. Each individual segment is referred to as a **β-strand**. Rather than being coiled, each β-strand is fully extended. The distance between adjacent amino acids along a β strand is approximately 3.5 Å, in contrast with a distance of 1.5 Å along an α-helix. β-pleated sheets are stabilized by hydrogen bonds that form between the polypeptide backbone N—H and carbonyl groups of adjacent strand. Adjacent strand can be either *parallel* or *antiparallel*. In parallel β-pleated sheet structures, the polypeptide chains are arranged in the same direction. However in, antiparallel β-pleated sheet chains run in opposite directions. Antiparallel β-sheets are more stable than parallel β-sheets because fully collinear hydrogen bonds form.

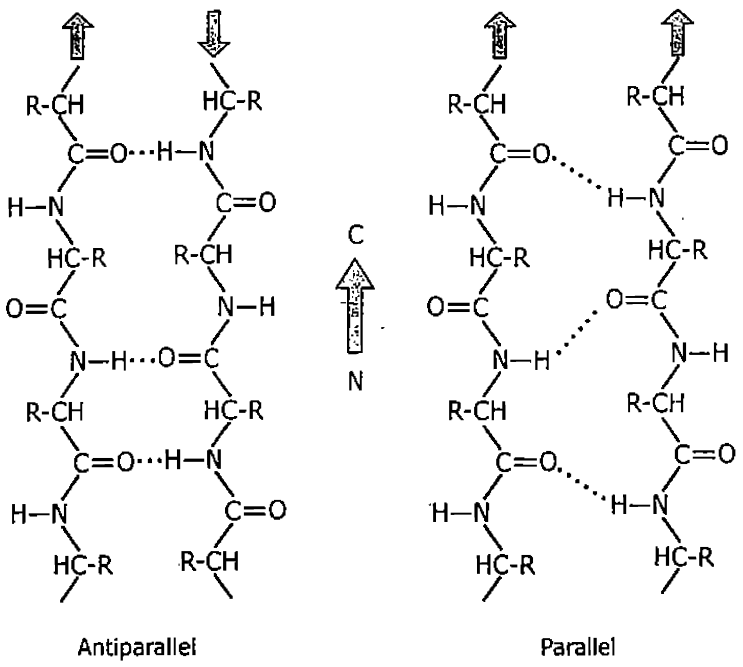


Figure : 1.7
In an antiparallel sheet, adjacent strands run in opposite directions. Hydrogen bonds between NH and CO groups connect each amino acid to a single amino acid on an adjacent strand. In parallel sheet, adjacent strands run in the same direction. Hydrogen bonds connect each amino acid on one strand with two different amino acids on the adjacent strand. For each amino acid, the NH group is hydrogen bonded to the CO group of one amino acid on the adjacent strand, whereas the CO group is hydrogen bonded to the NH group on the amino acid two residues farther along the chain.

Turns

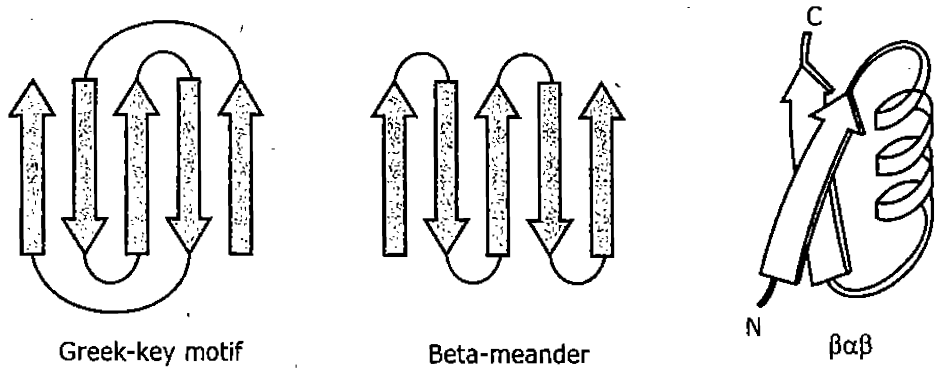
Most proteins have compact, globular shapes, requiring reversals in the direction of their polypeptide chains. Many of these reversals are accomplished by a common structural element called the *turn*. Turns, composed of three or four residues, are classified as a third type of secondary structure. These short, U-shaped secondary structures are stabilized by a hydrogen bond between their end residues. Glycine and proline are commonly present in turns. The lack of a large side chain in glycine and the presence of a built-in bend in proline allow the polypeptide backbone to fold into a tight U shape. Turns allow large proteins to fold into highly compact structures. Turns canbe grouped by their hydrogen bonding. A **β-turn** (the most common form) is characterized by hydrogen bond(s) in which the donor and acceptor residues are separated by *three* residues. Similarly a **γ-turn** and **α-turn** are characterized by hydrogen bond(s) in which the donor and acceptor residues are separated by *two* residues and *four* residues respectively. Turns and loops invariably lie on the surfaces of proteins and thus often participate in interactions between proteins and other molecules.

Supersecondary structures

Many globular proteins contain combinations of α-helix and β-pleated sheet secondary structures. These patterns are called *supersecondary structures* (also called **motifs**). In the **βαβ-motif**, two parallel β-strands are connected by an α-helix segment. In the **β-meander** pattern, two antiparallel β-strands are connected by polar amino acids and glycines to effect an abrupt change in direction of the polypeptide chain called reverse or β-turns. In **αα-motif**, two successive α-helices separated by a loop or nonhelical segment become enmeshed because of compatible side chains. When an antiparallel β-strand folds back on itself in a pattern that resembles a common Greek pottery design, the motif is called the **Greek key**.

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Tertiary structure

The term *tertiary structure* refers to the unique three-dimensional conformations that globular proteins assumes as a consequence of the interactions between the side chains in their primary structure. All of the information needed to fold the protein into its native tertiary structure is contained within the primary structure of the peptide chain itself. The following types of covalent and non-covalent interactions stabilize tertiary structure:

1. Hydrophobic interactions (major form of non-covalent interaction)
2. Electrostatic interactions
3. Hydrogen bonds
4. van der Waal force of interaction
5. Covalent bonds : The most prominent covalent bonds in tertiary structure are the disulfide bridges found in many extracellular proteins. Cysteine has a thiol group (sulfhydryl group) that is unique among the 20 amino acids in that it often forms a **disulfide bond** to another cysteine residue through the oxidation of their thiol groups. This dimeric compound is referred to as the amino acid **cystine.**

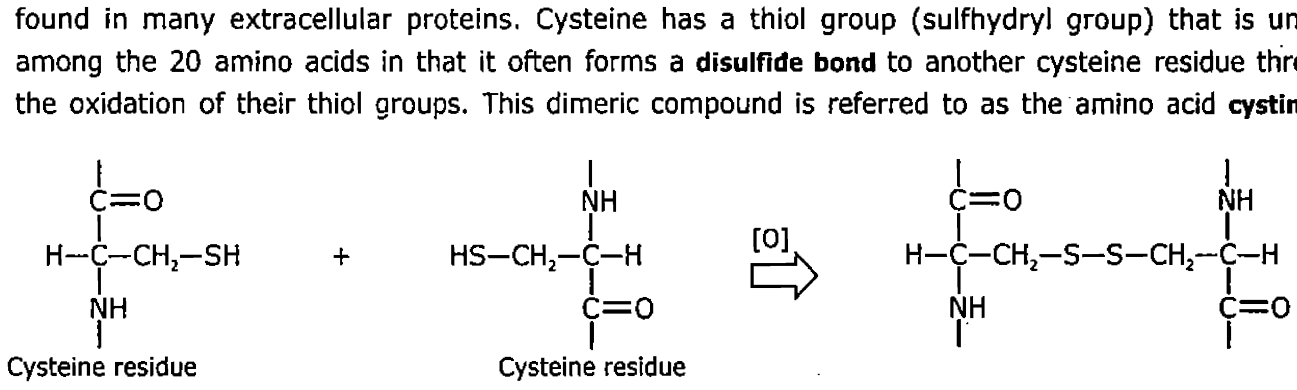


Figure 1.8 : Structure of cystine

STUDY QUESTION

Q3. The peptide bond is a stronger bond than that of esters. What unique feature of the peptide bond make it more stronger than ester bond?

Ans. The amide bond is more highly resonance-stabilized than the ester bond. This stabilization strengthens the peptide bond.

Q4. Most cytosolic proteins lack disulfide bonds, whereas extracellular proteins usually contain them. Why?

Ans. The cytosol is a reducing environment, whereas the extracellular milieu is an oxidizing environment. Disulfide bond forms as a result of oxidation of thiol group and it requires oxidizing environment.

Quaternary structure

Many proteins like hemoglobin, are composed of several polypeptide chains and each polypeptide component is called a *subunit*. Subunits in a multisubunit protein may be identical or quite different. Multisubunit proteins in which some or all subunits are identical are referred to as **oligomers** and identical units are referred to as **protomers**. Polypeptide subunits assemble and are held together by noncovalent interactions such as hydrophobic interactions, electrostatic interactions, and hydrogen bonds, as well as covalent cross-links.

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Determination of approximate molecular weight of proteins

The average molecular weight (a dimensionless quantity defined as the ratio of the particle mass to 1/12th the mass of a ^{12}C-atom and symbolized M) of amino acid residue in protein is nearer to 128. Because a molecule of water (MW = 18) is removed during peptide bond formation, the average molecular weight of amino acid residue in a protein is considered about 110 (128–18). Alternatively, this quantity may be expressed in terms of *molecular mass*. The molecular mass is expressed in units of daltons.

Loss of protein structure

Many physical and chemical agents can disrupt a protein's native conformation. The process of structure disruption is called *denaturation*. Denaturation includes the breaking of non-covalent (ionic interaction, H-bonds, van der Waal force of interaction, hydrophobic interactions) and covalent (disulfide bonds) bonds. Denaturation is not usually considered to include the breaking of peptide bonds. Depending on the degree of denaturation, the molecule may partially or completely lose its biological activity.

Denaturing conditions include the following:

1. Strong acids or bases

   Changes in pH result in protonation or deprotonation of side group of amino acids of protein which alters hydrogen bonding and salt bridge patterns.

2. Organic solvents

   Water-soluble organic solvents such as ethanol interfere with hydrophobic interactions because they interact with nonpolar R groups and form hydrogen bonds with water and polar protein groups. Nonpolar solvents also disrupt hydrophobic interactions.

3. Detergents

   These amphipathic molecules disrupt hydrophobic interactions, causing proteins to unfold into extended polypeptide chains.

4. Reducing agents

   In the presence of reagents such as urea, reducing agents such as β-mercaptoethanol convert disulfide bridges to sulfhydryl groups.

5. Heavy metal ions

   Heavy metals such as mercury (Hg^{2+}) and lead (Pb^{2+}) affect protein structure in several ways. They may disrupt salt bridges by forming ionic bonds with negatively charged groups. Heavy metals also bond with sulfhydryl groups.

6. Temperature change

   As the temperature increases, the rate of molecular vibration increases. Eventually, weak interactions such as hydrogen bonds, van der Waal interaction are disrupted and the protein unfolds.

Anfinsen experiment

The work of the American biochemist Christian Anfinsen on refolding of ribonuclease is particularly interesting. Ribonuclease isolated from bovine pancreas is an enzyme that has a molar mass of 13,700 g. It contains 124 amino acid residues and four disulfide linkages. In the presence of urea, a denaturant, and beta-mercaptoethanol, a reducing agent, ribonuclease is denatured and the disulfide bonds are broken. When the protein is allowed to renature by removing denaturant and the reductant, the protein regains its native conformation, including four correctly paired disulfide bonds. When the reductant is removed while the denaturant is still present, the disulfide bonds again form in protein but most of the disulfide bonds form between incorrect partners. This indicates that weak interactions are required for correct positioning of disulfide bonds and assumption of the native conformation.

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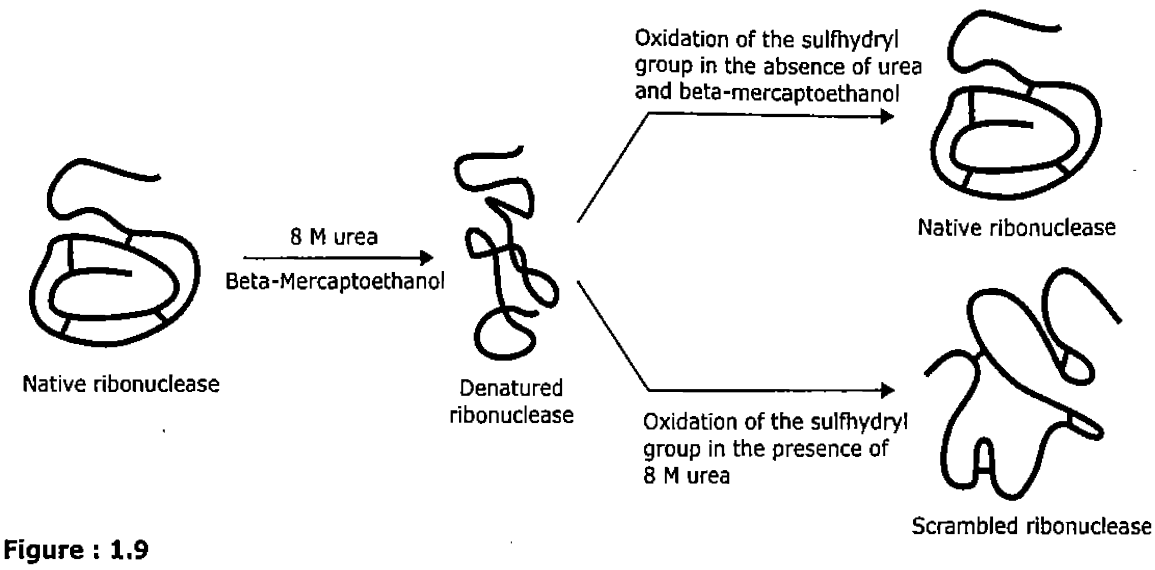


Figure : 1.9
Denaturation and renaturation of ribonuclease. Depending on the conditions for renaturation, we obtain either native ribonuclease or scrambled ribonuclease.

STUDY QUESTION

Q5. The enzyme RNase A requires the formation of four disulphide bonds amongst its eight cysteines. If this is entirely a random phenomenon, the probability that the first correct disulphide bond will be formed is 1/7. Following the same logic, what is the probability of formation of all four *correct* disulphide bonds?

Ans. There are seven possible pairings for the first disulfide bond, five for the second, and three for the third. Therefore, the probability that the correct bonds will form randomly is 1/7 × 1/5 × 1/3 = 1/105.

SOLUBILITIES OF PROTEINS

The solubility of proteins varies considerably in given set of conditions. Solubilities of proteins depend on several factors like pH, ionic strength, nature of solvent, temperature.

Effect of pH : At pI, the protein molecules carries no net charge. At pI, protein has minimum solubility. Hence when the pH of a protein mixture is adjusted to the pI of the protein to be isolated, its precipitation occur due to decrease in solubility (**isoelectric precipitation**).

Effect of ionic strength : The solubility of a protein at low ionic strength generally increases with salt concentration. This process is known as **salting in**. The binding of salt ions to the protein's ionizable groups decreases interaction between oppositely charged groups on the protein molecules. Water molecules then can form solvation spheres around these groups. However, when large amounts of salt are added to a protein in solution, a precipitate forms. This process is referred to as **salting out**. The large number of salt ions can effectively compete with the protein for water molecules, that is, the solvation spheres surrounding the protein's ionized groups are removed. The protein molecules aggregate and then precipitate.

Effect of solvent : Organic solvents such as acetone, ethanol, due to their low dielectric constants lower the solvating power of their aqueous solutions for proteins.

SIMPLE AND CONJUGATED PROTEINS

On the basis of composition, proteins are classified as *simple* or *conjugated*. **Simple proteins**, such as serum albumin, contain only amino acids. In contrast, **conjugated protein** consists of a simple protein combined with a non-protein component. The nonprotein component is called a **prosthetic group**. A conjugated protein without its prosthetic group is called an **apoprotein**. A protein molecule combined with its prosthetic group is referred to as a **holoprotein**. Conjugated proteins are classified according to the nature of their prosthetic groups. For example, glycoproteins contain a carbohydrate component, lipoproteins contain lipid molecules, and metalloproteins contain metal ions. Similarly, *phosphoproteins* contain phosphate groups, and *hemoproteins* possess heme groups.

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Table 1.6 : Example of few conjugated proteins

Class
Glycoproteins
Fibronectin
Proteoglycan
Lipoproteins
Chylomicron
HDL
Metalloproteins
Ferritin (Iron)
Alcohol dehydrogenase (Zinc)
Cytochrome oxidase (Copper and iron)
Nitrogenase (Molybdenum and iron)
Hemoprotein
Hemoglobin (Transport of oxygen in blood)
Myoglobin (Storage of oxygen in muscle)
Cytochrome C (Involvement in electron transport chain)
Cytochrome P450 (Hydroxylation of xenobiotics)
Catalase (Degradation of hydrogen peroxide)

1.4 FIBROUS AND GLOBULAR PROTEINS

Proteins are also classified into two categories - fibrous and globular proteins, on the basis of shape and solubility. **Fibrous proteins** are long, rod-shaped molecules that are insoluble in water and physically tough. Fibrous proteins, such as the keratins found in skin, hair, and nails, have structural and protective functions. **Globular proteins** are compact spherical molecules that are usually water-soluble. In globular proteins the nonpolar residues *Val, Leu, Ile, Met* and *Phe* largely occur in the interior of a protein, out of contact with the aqueous solvent. The charged polar residues *Arg, His, Lys, Asp* and *Glu* are largely located on the surface of protein in the contact with the aqueous solvent. Uncharged polar residues *Ser, Thr, Asn, Gln, Tyr* and *Trp* are usually present on the protein surface but frequently occur in the interior of the molecule.

Table 1.7 : The many functions of proteins and their examples

Function	**Class of protein**	**Example**
Structure	Fibers	Collagen, Keratin, Fibrin
Metabolism	Enzymes	Lysosomes, Proteases, Polymerase, Kinases
Membrane transport	Channels/Carriers	Proton pump, Anion channels
Cell recognition	Cell surface antigens	MHC proteins, ABO blood group
Osmotic regulation	Albumin	Serum albumin
Regulation of gene action	Repressors	*lac* repressor
Regulation of body functions	Hormones	Insulin, Vasopressin, Oxytocin
Transport throughout body	Globins	Hemoglobin, Myoglobin, Cytochromes
Storage	Ion-binding	Ferritin, Casein, Calmodulin
Contraction	Muscle	Actin, Myosin
Defense	Ig, Toxins	Antibodies, Snake venom

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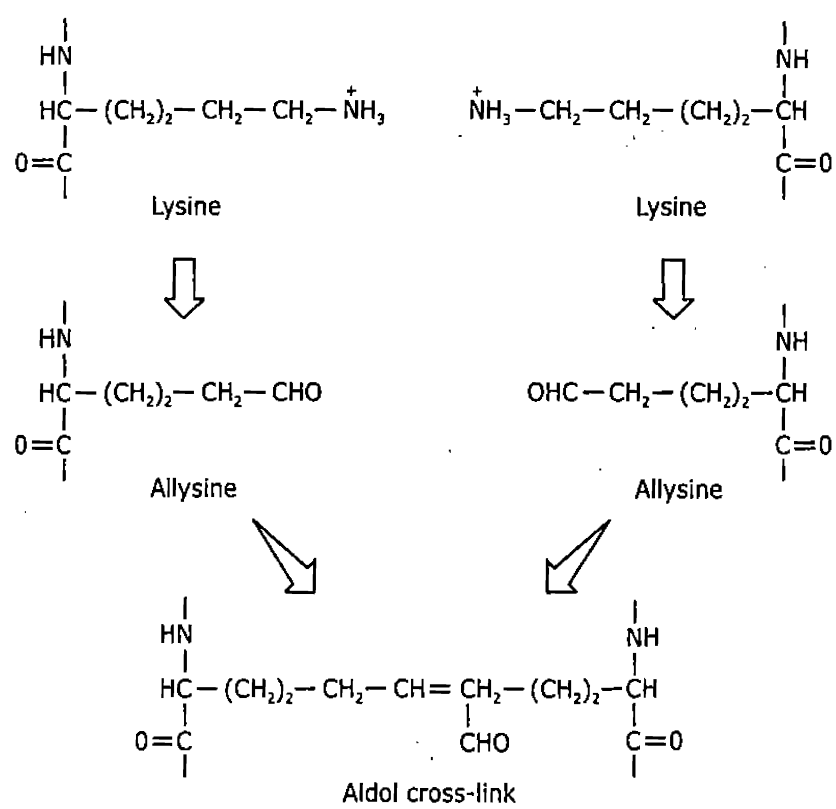
COLLAGEN

Collagen is the major structural protein in the extracellular matrix. It is the most abundant protein in vertebrates. Collagens are a large family of proteins containing at least 19 different members. Fundamental unit of collagen is **tropocollagen** (length 300nm). Tropocollagen consists of 3-coiled polypeptides called α-chains. The α-chains are left-handed polypeptide helices and has 3.3 amino acid residues per turn. Three α-chains wind around one another in a characteristic right-handed triple helix. Vertebrates have about 25 different kinds of α-chains, each coded by different genes and has its own unique amino acid sequence. These different types of α-chains combine in various ways to form at least 19 different types of collagen molecules. Types I, II, and III represent 90% of collagens.

The amino acid sequence in α-chain is generally a repeating tripeptide unit, *Gly–X–Y*, where *X* is often proline and *Y* is often 3- or 4-hydroxyproline or 5-hydroxylysine. Glycine constitutes approximately one-third of the amino acid residues. Proline and hydroxyproline confer rigidity on the collagen molecule. The hydroxylation is carried out during post-translational modifications by two enzymes : *prolyl hydroxylase* and *lysyl hydroxylase*. Ascorbate (vitamin C) acts as cofactors for these enzymes and hence is essential for hydroxylation of proline and lysine residue. Hydroxylation results in formation of interchain H-bonds. It also allows the glycosylation of hydroxylysine residues. Deficiency of ascorbic acid causes **scurvy**, a disease that affects the structure of collagen. It occurs due to impaired synthesis of collagen as a result of deficiencies of prolyl and lysyl hydroxylases.

Cross-linking of collagen

Covalent cross-links are formed both within a tropocollagen molecule and between different molecules. *Intramolecular* cross-links form through the action of *lysyl oxidase*, a copper-dependent enzyme that oxidatively deaminates the ε-amino groups of lysine residues, yielding reactive aldehydes of *allysine* residues. Such aldehydes of two side chains then link covalently in a spontaneous nonenzymatic aldol condensation. Histidine may also be involved in certain cross-links.



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The *intermolecular* cross-linking of tropocollagens involves the formation of a unique **hydroxypyridinum** structure from one lysine and two hydroxylysine residues.

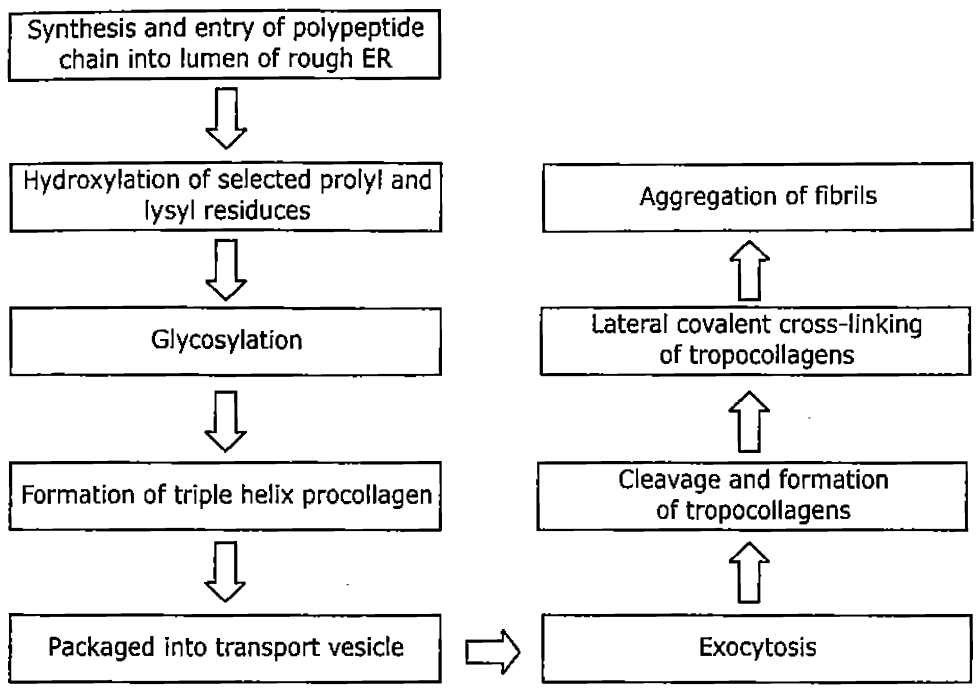


Figure 1.10 : The major events in the biosynthesis of collagen.

ELASTIN

Elastin is a connective tissue protein that is responsible for extensibility and elasticity. It is the second major protein in the extracellular matrix, which is the main component of elastic fibers found in ligaments, large arteries, and lungs. After synthesis and partial hydroxylation of proline residues, a 72 kDa molecule of **tropoelastin** is secreted into the matrix. This protein is rich in nonpolar amino acid. After secretion from the cell, certain lysyl residues of tropoelastin are oxidatively deaminated to aldehydes by **lysyl oxidase**. The condensation of three of these lysine-derived aldehydes with an unmodified lysine results in formation of a tetrafunctional cross-link called **desmosines**. One cross-linked in its mature, extracellular form, elastin is highly insoluble and extremely stable.

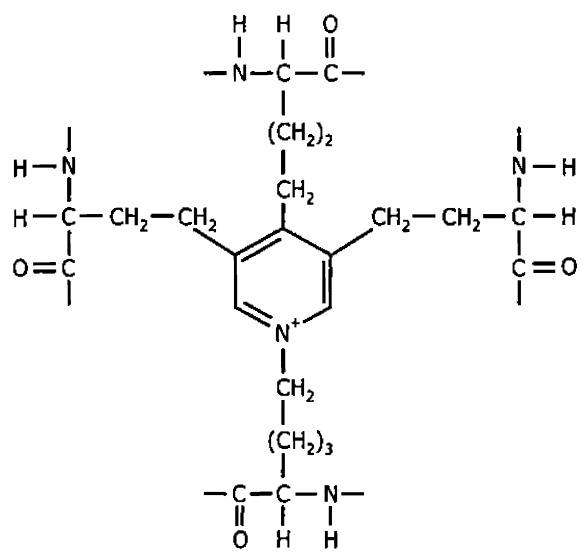


Figure 1.11 : Intramolecular desmosine cross-links in elastin.

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KERATINS

Keratins are intermediate filaments of eukaryotic cells. Keratins form a large family, with about 30 members being distinguished. All individual keratins are heterodimers made up of one member of each class. Two different classes of keratins-α and β, are known. In vertebrates, α-keratins constitute almost the entire dry weight of hair, wool, feathers, nails, claws, quills, scales, horns, hooves, tortoise shell, and much of the outer layer of skin. The α-keratin helix is the right-handed α helix. α-Keratin is rich in the hydrophobic residues Phe, Ile, Val, Met, and Ala.

Proteins	**α-keratin**	**β-keratin**
Characteristics	Tough, insoluble	soft, flexible
Conformation	α-helix	extended chain
Basic unit	protofibril	antiparallel β-pleated sheet
Important amino acids	Cys	Ala, Gly

MYOGLOBIN

Myoglobin, a globular protein, contains a single polypeptide chain of 153 amino acid residues (molecular weight 17,800), and a single heme group. The inside of myoglobin consists almost exclusively of nonpolar residues, whereas the outside contains both polar and nonpolar residues. About 75% of the polypeptide chain is α-helical. There are eight helical segments. These eight helical segments are commonly labeled A-H, starting from the NH_2-terminal end. The interhelical regions are designated as AB, BC, CD, ..., GH, respectively. The iron atom of the heme is directly bonded to a nitrogen atom of a histidine side chain of globin.

Heme

Globin of Mb bind a single *heme* group by forming co-ordinate bond. The heterocyclic ring system of heme is a porphyrin derivative. The porphyrin in heme is known as **protoporphyrin IX**. It is made up of 4-pyrrole ring and 4-pyrroles are linked by methylene bridges to form a tetrapyrrole ring. *Fe atom* is present either in Fe^{2+} or Fe^{3+} oxidation state in the center of the porphyrin ring.

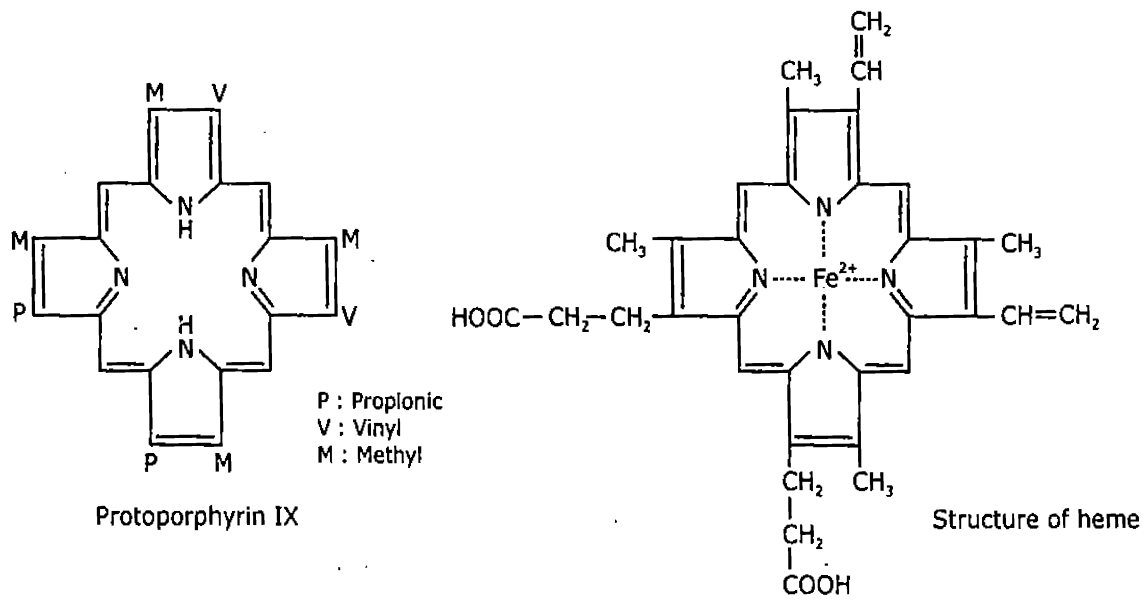


Figure 1.12 : Structure of protoporphyrin IX and heme.

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Only myoglobin in Fe^{2+} state can binds O_2. Iron atom has six co-ordination bond, 4 in plane of flat porphyrin ring and two perpendicular to it. Iron atom of the heme is directly bonded to one of histidine called **proximal histidine** of globin protein. The O_2-binding site is present on the other side of heme plane, at the sixth coordination position. A second histidine residue, termed as **distal histidine** (not bonded to the heme) makes H-bond with oxygen molecule. O_2 binds directly to iron atom of the heme only.

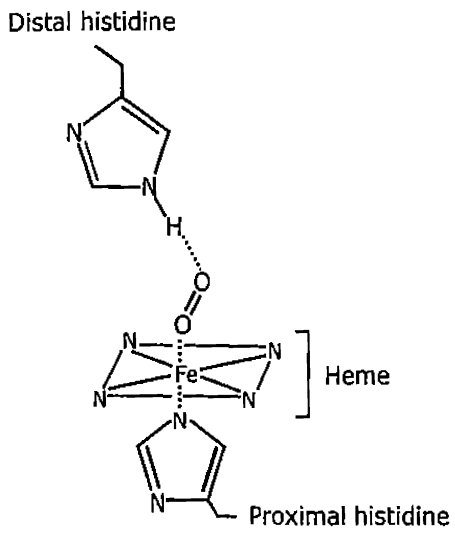


Figure : 1.13
The pyrrole rings and methylene bridge carbons are coplanar, and the iron atom resides in almost the same plane. The fifth and sixth coordination positions of iron atom are directed perpendicular to—and directly above and below—the plane of the heme ring. The fifth coordination position of the iron is linked to a ring nitrogen of the proximal histidine. The distal histidine lies on the side of the heme ring opposite to proximal histidine.

Binding of oxygen to Mb

Myoglobin (Mb) binds with one oxygen molecule. The dissociation of the myoglobin-oxygen complex (MbO_2) is described by

$$MbO_2 \underset{}{\overset{K_d}{\rightleftharpoons}} Mb + O_2$$

K_d is given by

$$K_d = \frac{[Mb][O_2]}{[MbO_2]}$$

The *fractional saturation* (Y) of myoglobin

$$Y = \frac{\text{Number of binding sites occupied}}{\text{Total number of binding sites}} = \frac{[MbO_2]}{[MbO_2]+[Mb]}$$

Because O_2 is gas, expressing its concentration in terms of its partial pressure is more convenient. In terms of the partial pressure of oxygen and the dissociation constant of the myoglobin oxygen complex:

$$Y = \frac{pO_2}{pO_2 + K_d}$$

When all of the Mb molecules in solution are bound to oxygen, Y = 1.0. Myoglobin is saturated with oxygen at this point. When Y = 0.5, myoglobin is half-saturated with oxygen.

Then, $K_d = pO_2 = P_{50}$

$$Y = \frac{pO_2}{pO_2 + P_{50}};$$

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A plot of Y versus pO_2 is *hyperbolic.*

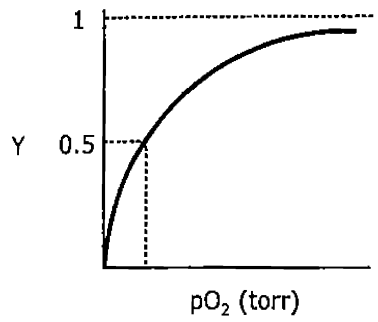


Figure : 1.14
Oxygen binding curve of myoglobin.
A plot of the fractional saturation of myoglobin, Y versus the partial pressure of oxygen pO_2, is hyperbolic.
(1 torr equals the pressure of 1 mm Hg at 0°C and standard gravity).

The ratio of the *fractional saturation* of myoglobin (Y) to free myoglobin (1–Y) is related to the oxygen pressure and P_{50} by following equation.

$$\frac{Y}{1-Y} = \frac{pO_2}{P_{50}}$$

If we take the logarithm of both side

$$\log \frac{Y}{1-Y} = \log pO_2 - \log P_{50} \qquad \ldots(I)$$

A **Hill plot**, which is a graph of log (Y/[1 – Y]) versus log pO_2 for myoglobin gives a straight line whose intercept on the log pO_2 axis equals log P_{50}. The slope of the line, also known as the **Hill coefficient** (n), is 1.0.

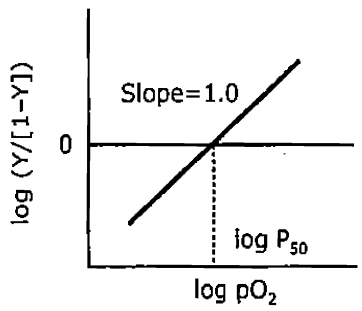


Figure : 1.15
Hill plot for the binding of oxygen to myoglobin. The slope of the line, the Hill coefficient, is 1.0.

HEMOGLOBIN (Hb)

Hemoglobin (Molecular weight of 65,450) is an oligomeric conjugated protein with four peptide chains joined by non-covalent bonds. It contains heme prosthetic group. Heme is an iron-containing porphyrin derivative.

A variety of Hb molecules are produced in humans. All are tetramers consisting of numerous combinations of seven distinct polypeptide chains, each encoded by a separate gene. Hb A_1, which contains two *alpha* and two *beta* chains, represents about 98 percent of all Hb found in adults RBCs. Alpha-chains contain 141 amino acid residues and β-chains contain 146 amino acid residues. Thus, Hb A_1 is designated $\alpha_2\beta_2$. The remaining 2 percent consists of HbA_2, a minor adult Hb. This molecule contains two *alpha* and two *delta* chains and is designated $\alpha_2\delta_2$. The delta chain is very similar to beta chain.

During embryonic and fetal development, a different set of Hb is found. One type of Hb during early embryonic state contains two *zeta* chains, which are very similar to alpha chains, and two *epsilon* chains, which are very similar to beta chains. Whereas during fetal state, Hb contains two *alpha* chains and two *gamma* chains, which are very similar to beta chains. The embryonic and fetal forms have a higher affinity for oxygen. This is necessary in order to obtain oxygen from the mother's blood.

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Table 1.8 : Different Hb expressed during different developmental stages of human

Developmental stages	*Types of globin proteins*	*Type of hemoglobin*
Embryonic (<8 weeks)	$\varepsilon_2\xi_2$	Gower I
Fetal (3–9 months)	$\alpha_2\gamma_2$	HbF
Adult (from birth)	$\alpha_2\beta_2$ (major)	HbA_1
	$\alpha_2\delta_2$ (minor)	HbA_2

Role of hemoglobin

Hemoglobin has four functions:

It facilitates O_2 transport;

It facilitates CO_2 transport;

It has an important role as a buffer and

It transports NO (the ferrous O_2 binding sites in hemoglobin also bind nitric oxide (NO), and an additional NO binding site is present on the β chains).

Sickle-cell hemoglobin (HbS)

HbS forms as a result of a single amino acid substitution in the beta-chain of Hb. Replacement of the glutamate residue at position 6 in the beta-chain by a valine residue is the only chemical difference between Hb A and sickle-cell hemoglobin. This residue present on the outer surface of the molecule, and the change produces a *sticky* hydrophobic spot on the surface that results in abnormal quaternary association of hemoglobin. This makes the deoxyHbS less soluble than deoxyHbA. Insoluble deoxyHbS forms polymers that aggregate into tubular fibers. The formation of insoluble deoxyHbS fibers distorts the red cell into the elongated sickle shape characteristic of the disease, sickle-cell anemia.

Oxygen transport

Oxygen does not dissolve easily in water, so only about 1.5% of inhaled oxygen is dissolved in blood plasma, which is mostly water. About 98.5% of blood oxygen is bound to hemoglobin in red blood cells. The heme portion of hemoglobin contains four atoms of iron, each capable of binding to a molecule of oxygen. Oxygen and hemoglobin bind in an easily reversible reaction to form **oxyhemoglobin**: Reaction between Hb and O_2 is an *oxygenation*, not an oxidation.

$$Hb + O_2 \rightleftharpoons HbO_2$$

It actually reacts with 4 molecules of O_2 to form Hb_4O_8.

$$Hb_4 + O_2 \rightleftharpoons Hb_4O_2$$

$$Hb_4O_2 + O_2 \rightleftharpoons Hb_4O_4$$

$$Hb_4O_4 + O_2 \rightleftharpoons Hb_4O_6$$

$$Hb_4O_6 + O_2 \rightleftharpoons Hb_4O_8$$

Oxygen-hemoglobin dissociation curve

The most important factor that determines how much oxygen binds to hemoglobin is the pO_2; the higher the pO_2, the more oxygen combines with Hb. When reduced hemoglobin (Hb) is completely converted to oxyhemoglobin, the hemoglobin is said to be fully saturated; when hemoglobin consists of a mixture of Hb

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and $Hb-O_2$, it is partially saturated. The percent saturation of hemoglobin expresses the average saturation of hemoglobin with oxygen. For instance, if each hemoglobin molecule has bound two oxygen molecules, then the hemoglobin is 50% saturated because each Hb can bind a maximum of four oxygen. The relation between the percent saturation of hemoglobin is illustrated in the *oxygen-hemoglobin dissociation curve.*

The quaternary structure of hemoglobin also determines its affinity for O_2. In deoxyhemoglobin, the globin units are tightly bound in a *tense* (T) configuration which reduces the affinity of the molecule for O_2. When O_2 is first bound, the bonds holding the globin units are released, producing a *relaxed* (R) configuration which exposes more O_2 binding sites. The net result is a 500-fold increase in O_2 affinity. In the tissues, these reactions are reversed, releasing O_2. The *oxygen-hemoglobin dissociation curve*, the curve relating percentage saturation of the O_2-carrying power of hemoglobin to the pO_2 has a characteristic **sigmoid shape** due to the T-R interconversion. Combination of the first heme in the Hb molecule with O_2 increases the affinity of the second heme for O_2, and oxygenation of the second increases the affinity of the third, etc, so that the affinity of Hb for the fourth O_2 molecule is many times greater than that for the first.

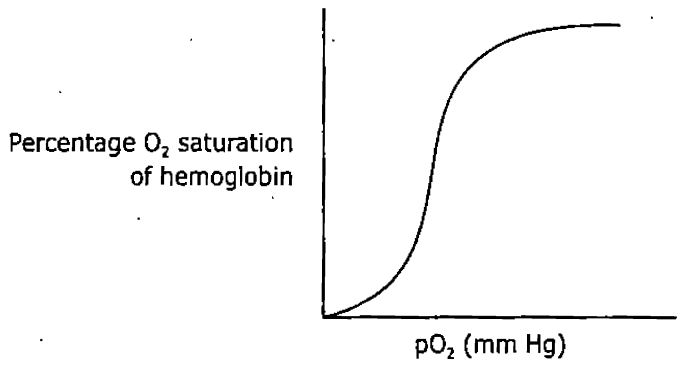


Figure : 1.16
Oxygen-oxygen hemoglobin dissociation curve (pH 7.40, temperature 38°C)

Binding of oxygen to Hb

A single molecule of hemoglobin (Hb), binds up to four oxygen molecules.

The equation for the fractional saturation of hemoglobin would be

$$Y = \frac{(pO_2)^4}{(pO_2)^4 + (P_{50})^4}$$

A plot of Y versus pO_2 for hemoglobin is *S-shaped* or *sigmoidal*. This is in sharp contrast to the hyperbolic plot obtained for myoglobin. The sigmoidal oxygen binding curve of hemoglobin indicates the interactive binding phenomenon termed **cooperativity.**

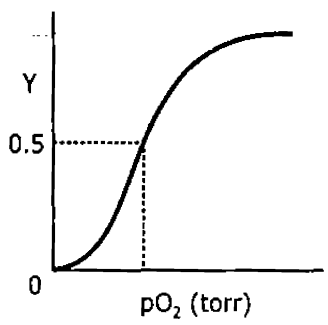


Figure : 1.17
Oxygen binding curve of hemoglobin. A plot of the fractional saturation of hemoglobin, Y versus the partial pressure of oxygen pO_2, is sigmoidal.

So, equation I describes the binding of Mb with O_2 quite well, but it does not hold true for Hb. Instead, it must be modified as follows:

If we take the log of the generalized Hill equation, we obtain

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$$\log \frac{Y}{1-Y} = n \log pO_2 - n \log P_{50}$$

The Hill plot for oxygen binding to hemoglobin (log (Y/[1-Y]) versus pO_2) is not completely linear since binding is cooperative. The Hill coefficient, n, is equal to the slope of the line when pO_2 equals P_{50}.

The Hill coefficient is a measure of cooperativity - the higher n is, the higher the cooperativity. If n =1, there is no cooperativity; If n<1, there is negative cooperativity. The upper limit of n is the number of binding sites, which is 4 for Hb.

Functional differences between myoglobin and hemoglobin

- Hemoglobin is an allosteric protein, whereas myoglobin is not.
- The shape of the oxygen dissociation curve of hemoglobin is sigmoidal, whereas that of myoglobin is rectangular hyperbolic i.e., myoglobin has a higher affinity for oxygen than does hemoglobin.
- The affinity of hemoglobin for oxygen depends on pH, whereas that of myoglobin is independent of pH.
- The binding of oxygen to hemoglobin is cooperative that is, as oxygen is bound to one subunit, the binding to other subunits is facilitated. The degree of cooperativity in the binding reaction is expressed by the **Hill coefficient** (n). For hemoglobin, the value of n is 2.8, whereas for myoglobin it is 1.0.

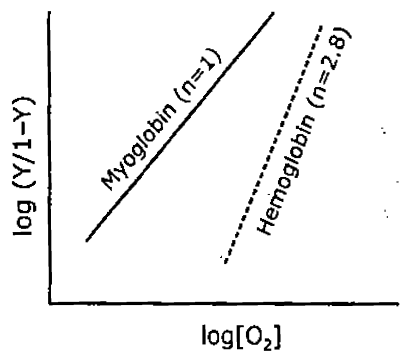


Figure : 1.18
Plot of log (Y/1-Y) against log [O_2] for Mb and Hb. The slope of the line gives Hill's coefficient.

Factors affecting the affinity of hemoglobin for oxygen

Although pO_2 is the most important factor that determines the percent oxygen saturation of hemoglobin, several other factors influence the tightness or affinity with which hemoglobin binds oxygen. In effect, these factors shift the entire curve either to the left (higher affinity) or to the right (lower affinity). Three important conditions affect the oxygen-hemoglobin dissociation curve:

- The pH,
- The temperature, and
- The concentration of 2,3-biphosphoglycerate (BPG).

Effect of pH on the oxygen affinity of Hb

Lowering in pH causes the oxygen affinity of the hemoglobin to decrease and shifts the curve to the right. When the curve is shifted in this direction, a higher pO_2 is required for hemoglobin to bind a given amount of O_2. The effect of pH on the oxygen affinity of Hb is known as the **Bohr effect**. The molecular basis behind the Bohr effect is that the T structure of hemoglobin binds hydrogen more readily than the R structure, so under a condition of low pH (high hydrogen ion concentration) the T structure, which has a decreased oxygen affinity, dominates. The pH of blood falls as its CO_2 content increases, so that when the pCO_2 rises, the curve shifts to the right.

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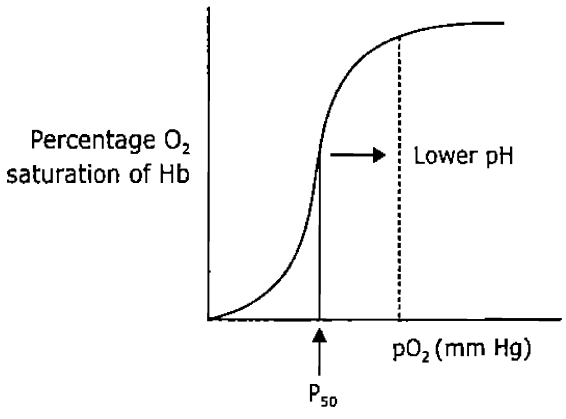


Figure : 1.19
Change in oxygen-hemoglobin dissociation curve to higher P_{50} value with decrease in pH.

Effect of temperature

A rise in temperature shifts the curve to the right. Conversely, a fall in temperature shifts the curve to the left, and a lower PO_2 is required to bind a given amount of O_2.

Effect of BPG

BPG (2, 3-Biphosphoglycerate) is present in RBC of human and other species. It is a highly charged anion that binds to the β-chains of deoxyhemoglobin. One mole of deoxyhemoglobin binds one mol of 2,3-BPG.

In effect, $HbO_2 + 2,3-BPG \rightleftharpoons Hb-2,3-BPG + O_2$

BPG reduces the O_2 affinity of Hb (by factor of 26). BPG exerts its effect on O_2 affinity of Hb by binding to deoxyhemoglobin but not to oxy Hb. An increase in the concentration of 2,3-BPG shifts the reaction to the right, causing more O_2 to be liberated.

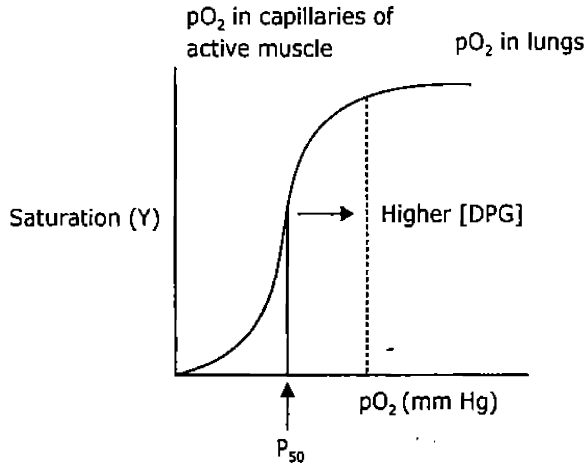


Figure : 1.20
Change in oxygen-hemoglobin dissociation curve to higher P_{50} value with increase in BPG concentration.

Ascent to high altitude triggers a substantial rise in 2,3-BPG concentration in red cells, with a consequent increase in P_{50} and increase in the availability of oxygen to tissues. Similarly the affinity of fetal hemoglobin (hemoglobin F) for O_2, which is greater than that for adult hemoglobin (hemoglobin A), facilitates the movement of O_2 from the mother to the fetus. The cause of this greater affinity is the poor binding of 2,3-BPG by the γ-polypeptide chains that replace β-chains in fetal hemoglobin.

Carbon dioxide transport by hemoglobin

The solubility of CO_2 in blood is about 20 times that of O_2; therefore considerably more CO_2 than O_2 is present in blood plasma. Under normal resting conditions, each 100 mL of deoxygenated blood contains an equivalent of 53 mL of gaseous carbon dioxide, which is transported in the blood in three main forms.

1. *Dissolved carbon dioxide* : The smallest percentage (about 7%) is dissolved in blood plasma.
2. *Carbamino compound* : A somewhat higher percentage, about 23%, combines with the amino groups of amino acids and proteins in blood to from *carbamino compounds*. Because the most prevalent protein in blood is hemoglobin, most of the carbon dioxide transported in this manner is bound to hemoglobin.

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The main carbon dioxide binding sites are the terminal amino acids in the two alpha and two beta globin chains. Hemoglobin that has bound carbon dioxide is termed **carbaminohemoglobin.**

3. *Bicarbonate ions* : The greatest percentage of carbon dioxide (about 70%) is transported in blood plasma as *bicarbonate ions*. As carbon dioxide diffuses into the red blood cells, it reacts with water in the presence of the enzyme *carbonic anhydrase* to form carbonic acids, which dissociates into H^+ and HCO_3^-.

Thus, as blood picks up carbon dioxide, bicarbonate accumulates inside RBCs. Some bicarbonate ions moves out into the blood plasma, down its concentration gradient. In exchange, chloride ions move from plasma into the RBCs. The exchange of negative ions which maintains the electrical balance between blood plasma and RBC cytosol, is known as the **chloride shift.** The amount of carbon dioxide that can be transported in the blood is influenced by the percent saturation of hemoglobin with oxygen. The lower the amount of oxyhemoglobin, the higher the carbon dioxide carrying capacity of the blood, a relationship known as the **Haldane effect.**

Models for the behavior of allosteric proteins/enzymes

Several models have been proposed to explain the behavior of allosteric proteins/enzymes. The *concerted model* proposed by Monod, Wyman and Changeux (MWC) and *Sequential model* suggested by Koshland, Nemethy and Filmer (KNF) have been the most popular. Both these models explain the non-hyperbolic kinetics by assuming that the allosteric behavior is manifested by the interaction between subunits in an oligomer. Both the models proposed two distinct conformational states for the subunits. These two conformation differ with respect to their kinetic and ligand binding behavior.

Concerted model

Monod, Wymann and Changeux in 1965, proposed concerted model to explain the behavior of allosteric proteins and enzymes. This model is based on the following postulates:

1. Allosteric proteins are oligomers composed of even number of identical subunits.
2. Each monomer in an oligomer can exist in two conformations labeled as T (tense) and R (relaxed), which are in equilibrium.
3. The binding affinities of ligand to these two conformations are different.
4. At any given time all the monomers in an oligomeric molecule possess the same conformation.

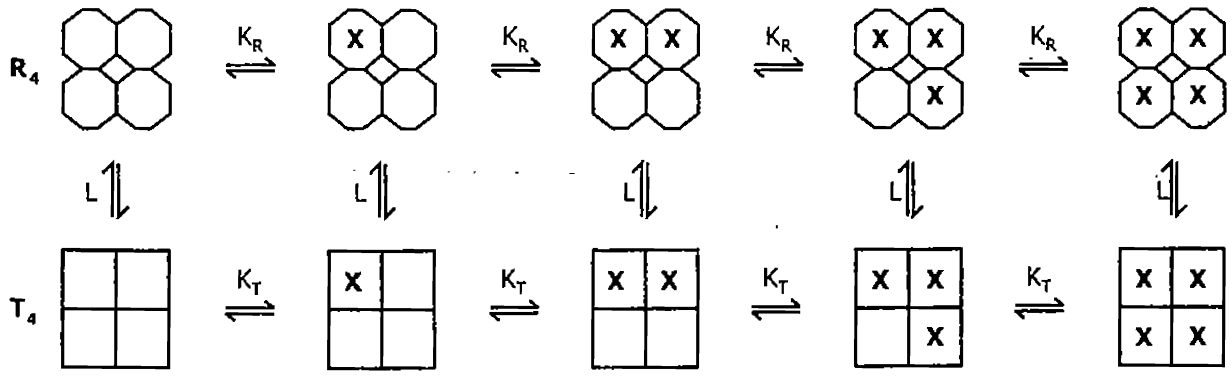


Figure 1.21 A : The concerted model. The squares represent the T state; the octagon represent the R state. In the absence of the ligand, the T state is favored. When ligand are bound to the oligomer (tetramer), the equilibrium gradually shifts to the R state, which has a higher affinity for the ligand.

As shown in the figure 1.21 A, the protein has two conformations, the R conformation, which binds substrate tightly and the T conformation, which binds substrate less tightly. The distinguishing feature of this model is that the conformation of *all* subunits changes simultaneously. All subunits change conformation from the inactive T conformation to the active R conformation at the same time; that is, a concerted change of conformation occurs. The binding of the first molecule of substrate to one subunit facilitates the binding of

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the second substrate molecule to the other subunit. This is exactly what is meant by cooperative binding. In the absence of substrate, the enzyme exists mainly in the T form, in equilibrium with small amounts of the R form. The presence of substrate shifts the equilibrium to produce more of the R form.

Sequential model

The sequential model proposes that as ligand binds to one subunit, there is a change in the conformation of the subunit, which then induces a conformational change in a contiguous subunit. The effect of ligand binding is sequentially transmitted through the interface between subunits producing increased or decreased affinity for the ligand by contiguous protomers. Hence to begin with the monomers in an oligomer exist in the T conformation, binding of ligand to the protomer induces a conformational change from T to R and thus the conformation of the protomers in a oligomer changes sequentially to R. As for example when an oxygen molecule binds to a vacant site on one of the four subunits of Hb, the interaction causes the site to change its conformation, which in turn affects the binding constants of the three sites that are still vacant. For this reason, this model is called the *sequential model*. Unlike the *concerted model,* the sequential model can have tetramers that consist of both R and T state subunits.

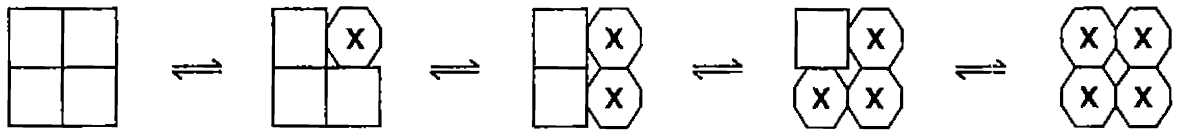


Figure 1.21 B : The sequential model. The squares represent the T state; octagon represent the R state. The binding of a ligand to a subunit changes the conformation of that subunit. This transition increases the affinity of the remaining subunits for the ligand.

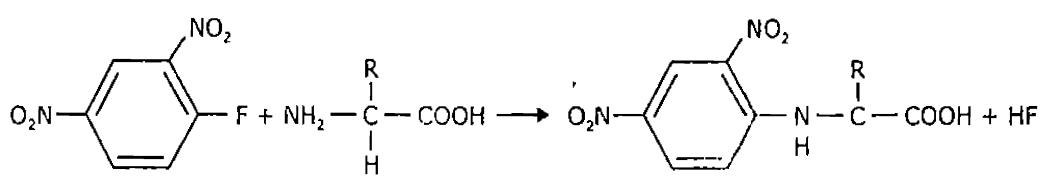
1.5 PROTEIN SEQUENCING

Determination of amino acids compositions

Peptide bonds of proteins are hydrolyzed by either strong acid or strong base. The peptide canbe hydrolyzed into its constituent amino acids by heating it in 6 M HCl at 110°C for 24 hours. Mixture of amino acids in hydrolysates can be separated by ion exchange chromatography or by reversed phase HPLC. The identity of the amino acid is revealed by its elution volume and quantified by reaction with *ninhydrin.*

N-terminal analysis

Reagent *1-Fluoro-2,4-dinitrobenzene* (FDNB) and *Dansyl chloride* are used for determination of N-terminal amino acid residue. FDNB reacts in alkaline solution (pH 9.5) with the free amino group of the N-terminal amino acid residue of a peptide to form a characteristic yellow dinitrophenyl (DNP) derivative. It can be released from the peptide by either acid or enzymic hydrolysis of the peptide bond and subsequently identified. Sanger first used this reaction to determine the primary structure of the polypeptide hormone insulin. This reagent is often referred to as Sanger's reagent.



FDNB Yellow-coloured derivative

Similarly, **Dansyl chloride** reacts with free amino group of the N-terminal amino acid residue of a peptide in alkaline solution to form strongly fluorescent derivatives of free amino acids and N-terminal amino acid residue of peptides.

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Cleavage of disulfide bonds

If protein is made up of two or more polypeptide chains and held together by noncovalent bonds then denaturing agents, such as urea or guanidine hydrochloride, are used to dissociate the chains from one another. But polypeptide chains linked by disulfide bonds can be separated by two common methods. These methods used to break disulfide bonds and also prevent their reformation.

Oxidation of disulfide bonds with performic acid produces two cysteic acid residues. Because these cysteic acid side chains are ionized SO_3^- groups, electrostatic repulsion prevents S-S recombination. Second method involves reduction by β-mercaptoethanol or dithiothreitol to form cysteine residues. This reaction is followed by further modification of the reactive -SH groups to prevent reformation of the disulfide bond. Acetylation by iodoacetate serves this purpose which modify the -SH group.

Edman degradation

Edman degradation method for determining the sequence of peptides and proteins from their N-terminus was developed by Pehr Edman. This chemical method uses a three-stage reaction cycle for sequential removal of amino acid residues from the *N-terminus* of a polypeptide chain. The three stages of the Edman degradation method are

1. Coupling 2. Cyclization 3. Conversion

1. *Coupling* involves a nucleophilic attack of the N-terminal α-amino group on the thiocyanate carbon of phenylisothiocyanate (PITC), to form the phenylthiocarbamyl-peptide derivative (PTC-peptide).
2. *Cyclization* constitutes the formation of the anilinothiazolinone derivative (ATZ-amino acid) in anhydrous trifluoroacetic acid, thereby liberating the N-terminal amino acid residue in a cyclic form, and leaving the remaining peptide truncated by one residue.
3. *Conversion* involves the rearrangement of the liberated ATZ derivative to the corresponding phenylthiohydantoin derivative (PTH-amino acid) by treatment with aqueous acid.

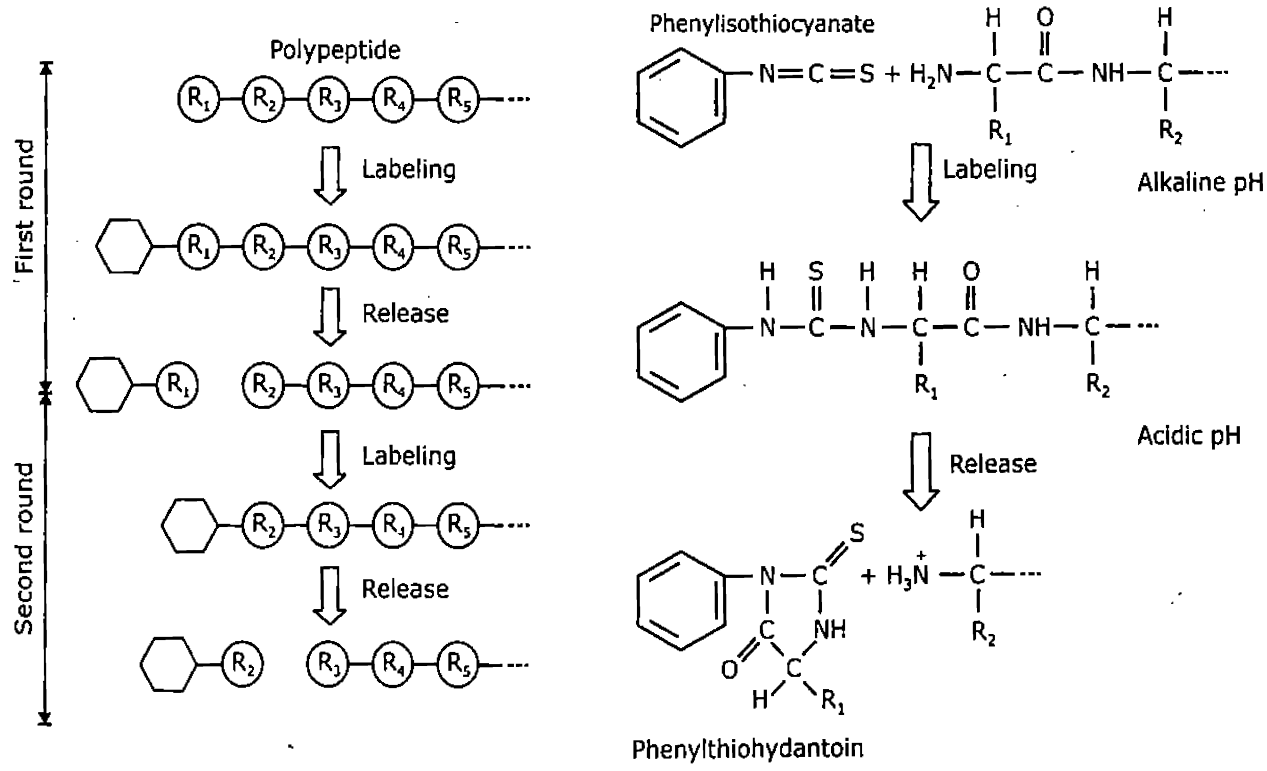


Figure 1.22 : The Edman degradation.

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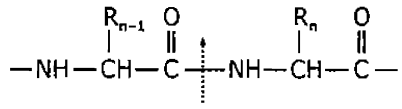
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SPECIFIC CLEAVAGE OF PEPTIDES/POLYPEPTIDES

Sequencing techniques currently in use cannot determine the amino acid sequence of proteins with more than about 50 amino acid residues. Thus it is necessary to cleave large proteins into smaller peptides and to determine the amino acid sequences of the peptides individually.

Proteolytic enzymes are most commonly used, especially those that cleave proteins at specific locations. The specificities are determined by the side chains of the amino acid on either side of the peptide bond that is hydrolyzed in the polypeptide chain. Various other chemicals also cleave polypeptide chains at specific locations. The most widely used is *cyanogen bromide* (CNBr), which cleaves peptide bond at Met residues. Similarly hydroxylamine cleaves the polypeptide chain at Asn-Gly sequences.

Table 1.9 : Specificities of proteolytic enzymes.



Agents	Site of Cleavage
Trypsin	Carboxyl side of Lys or Arg, $R_n \neq$ Pro
Chymotrypsin	Carboxyl side of aromatic amino acid residues, $R_n \neq$ Pro
Pepsin	Amino side of aromatic amino acids like Tyr, Phe and Trp, $R_{n-1} \neq$ Pro
Elastase	Carboxyl side of Ala, Gly and Ser, $R_n \neq$ Pro
Carboxypeptidase A	A bulky, nonpolar carboxy-terminal residue, $R_n \neq$ Pro, Lys, Pro; $R_n \neq$ Pro
Aminopeptidase	Any amino-terminal residue

STUDY QUESTION

Q6. The following reagents are often used in protein chemistry

CNBr, Performic acid, Phenyl isothiocyanate, Urea, Dabsyl chloride, Chymotrypsin, Mercaptoethanol, 6 N HCl, Trypsin, Ninhydrin

Which one is the best suited for accomplishing each of the following tasks?

a. Determination of the amino acid sequence of a small peptide.
b. Identification of the amino-terminal residue of a peptide (of which you have less than 0.1 μg).
c. Reversible denaturation of a protein devoid of disulfide bonds. Which additional reagent would you need if disulfide bonds were present?
d. Hydrolysis of peptide bonds on the carboxyl side of aromatic residues.
e. Cleavage of peptide bonds on the carboxyl side of methionines.
f. Hydrolysis of peptide bonds on the carboxyl side of lysine and arginine residues.

Ans. a. Phenyl isothiocyanate;
b. dansyl chloride or dabsyl chloride;
c. urea; β-mercaptoethanol to reduce disulfides;
d. chymotrypsin;
e. CNBr;
f. trypsin

Q7. A peptide was broken into two smaller peptides by cyanogen bromide (CNBr), and into two different peptides by trypsin (Trp). Their sequences were as follows: CNBr 1, Gly-Thr-Lys-Ala-Glu; CNBr 2, Ser-Met; Trp 1, Ser-Met-Gly,Thr-Lys; Trp 2, Ala-Glu. Determine the sequence of the parent peptide.

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Ans. By arranging the sequences in an overlapping set, as follows, it is possible to determine the parent sequence:

CNBr 2 : Ser – Met
Trp 1 : Ser – Met – Gly – Thr – Lys –
CNBr 1 : Gly – Thr – Lys – Ala – Glu
Trp 2 : Ala – Glu

Ser – Met – Gly – Thr – Lys – Ala – Glu

Protein assays

To determine the amount of protein in an unknown sample is termed *protein assays*. The simplest and most direct assay method for proteins in solution is to measure the absorbance at 280 nm (UV range). Amino acids containing aromatic side chains (i.e., tyrosine, tryptophan and phenylalanine) exhibit strong UV-light absorption. Consequently, proteins absorb UV-light in proportion to their aromatic amino acid content and total concentration. Several colorimetric, reagent-based protein assay techniques have also been developed. Protein is added to the reagent, producing a color change in proportion to the amount added. Proteins concentration is determined by reference to a standard curve consisting of known concentrations of a purified reference protein. There are several colorimetric, reagent-based methods for determining the protein concentration. Some most commonly used methods are:

Biuret method : Biuret method is based on the direct complex formation between the peptide bonds of the protein and Cu^{2+} ion. This method is not highly sensitive since the complex does not have a high extinction coefficient.

Folin method : The Folin assay (also called *Lowry* method) is dependent on the presence of aromatic amino acids in the protein. First, a cupric/peptide bond complex is formed and then this is enhanced by a phosphomolybodate complex with the aromatic amino acids.

Bradford method : Bradford method is based on a blue dye (Coomassie Brilliant Blue) that binds to free amino groups in the side chains of amino acids, especially Lys. This assay is as sensitive as the Folin assay.

Estimation of protein concentration by measuring the absorbance at 280 nm

The relationship between protein concentration and absorbance (A_{280}) is linear. Hence protein concentration can be estimated by measuring absorbance at 280nm. As the absorbance at 280nm (A_{280}) is due to the aromatic rings, the sensitivity of the assay is dependent on the number of aromatic rings containing amino acids. Peptide bonds absorb strongly at 190nm. Thus protein concentration can be measured at this wavelength because of the large number of peptide bonds in a protein.

For determination of protein concentration in unknown sample, the following formula can be used:

$$\text{Concentration (mg/ml)} = \frac{A_{280}}{\text{Path length (in cm)}}$$

Since nucleic acids also absorb at 280 nm, unknown sample containing nucleic acids (RNA and DNA) produce erroneously high protein estimates. Thus the absorbance of the sample at 260 nm (A_{260}) is measured and the protein concentration of the sample is corrected using the following formula.

Protein concentration (mg/ml) = $1.55\ A_{280} - 0.76\ A_{260}$

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Purification of proteins

The basic aim of purification of protein is to isolate the particular protein of interest from the mixture of proteins. Different features of proteins such as solubilities, size, charge, adsorption properties are utilized during purification process. The main separation methods used in purifying proteins based on the characteristic properties are as follows:

Properties		*Method*
Size or mass	:	Centrifugation, Gel filtration, Dialysis and ultrafiltration, Gel electrophoresis
Charge	:	Ion-exchange chromatography, Electrophoresis, Isoelectric focusing
Solubility	:	Change in pH, Change in ionic strength, Decrease in dielectric constant
Specific binding	:	Affinity chromatography

1.6 AMINO ACID SYNTHESIS

Living organisms differ in their capacity to synthesize the amino acids required for protein synthesis. While plants and many microorganisms can produce all 20 standard amino acids from readily available precursors, other organisms must obtain some preformed amino acids from their diet. Mammals and other animals can only synthesize a subset of these standard amino acids; known as the 12 *non-essential* amino acids. The remaining amino acids (8 *essential* amino acids) must be obtained from the diet.

Non essential amino acids

Alanine, Arginine#, Asparagine, Aspartate, Cysteine, Glutamate, Glutamine, Glycine, Histidine#, Proline, Serine, Tyrosine (# Amino acids that are essential for infants).

Essential amino acids

Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine

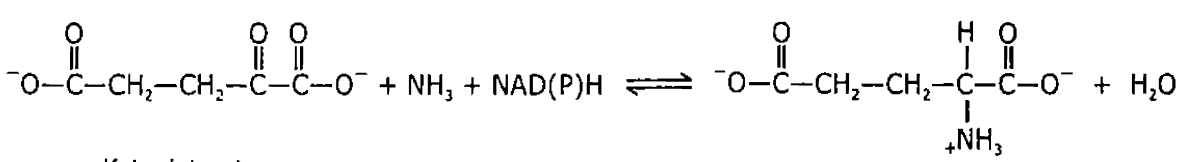
Nitrogen is a key component of amino acids. Nitrogen in the form of ammonia is the source of nitrogen for all the amino acids. In living system, reduced nitrogen (NH_4^+) is incorporated first into amino acids then into a variety of other biomolecules, including nucleotides. Two amino acids glutamate and glutamine provide the critical entry point. Glutamate is the source of amino group for most other amino acids through transamination reactions.

There are two principal means by which ammonium ions are incorporated into amino acids and eventually other metabolites

1. Reductive amination of α-keto acids.
2. Formation of the amides of aspartic and glutamic acids.

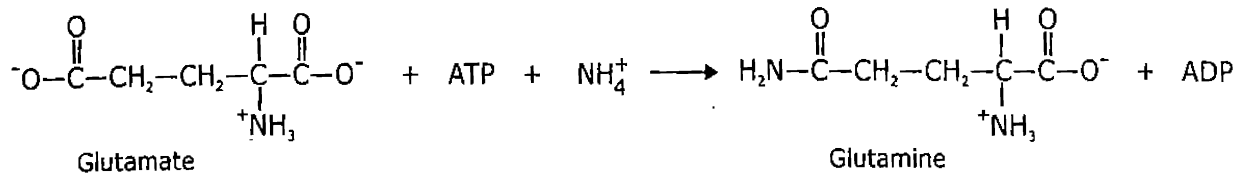
Reductive amination

Glutamate dehydrogenase catalyzes the reductive amination of α-ketoglutarate and forms glutamate. *This pathway present in all organisms*. In eukaryotes, this is a minor pathway and occurs in the mitochondria. Glutamate dehydrogenase is an unusual enzyme in that it does not discriminate between NADH and NADPH.

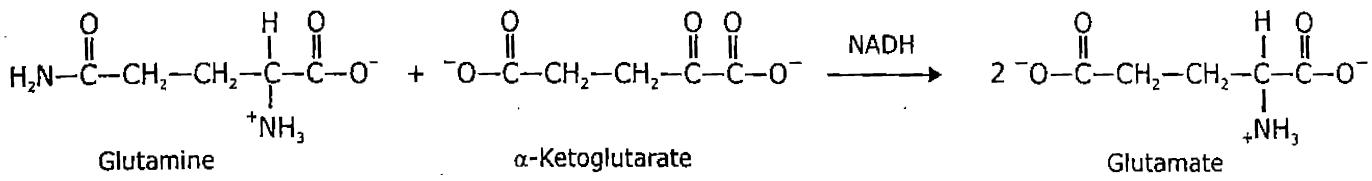


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Ammonium ions are also incorporated into cell metabolites by the formation of glutamine, the amide of glutamate. This amination reaction is catalyzed by *glutamine synthetase* (present in all organisms).



In plants, after NH_4^+ is incorporated into glutamine by glutamine synthetase, the amide amino group is transferred to the 2-keto group of α-ketoglutarate by *glutamate synthase* (also known as glutamine:2-oxo-glutarate aminotransferase or GOGAT). Glutamate synthase transfer amide group of glutamine to α-ketoglutaric acid and forms 2 molecules of glutamate (amination of α-Ketoglutarate).

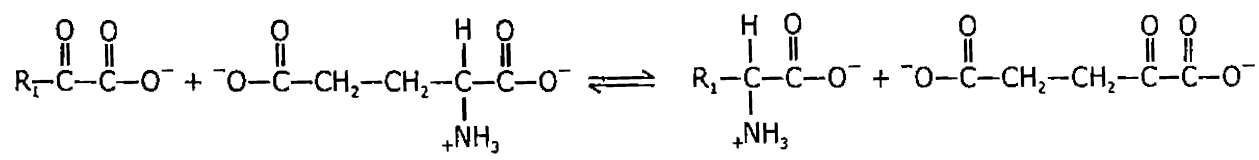


Transamination

Many amino acid biosynthetic pathways involve transamination reactions. The α-amino group from one amino acid is transferred to an α-keto acid to produce a new amino acid. This reaction is catalyzed by enzyme called *aminotransferases* (formerly called transaminases). Eukaryotic cells possess a large variety of aminotransferase. Found within both the cytoplasm and mitochondria, these enzymes possess two types of specificities :

1. The type of α-amino acid that donates the α-amino group
2. The α-keto acid that accepts the α-amino group

Although the aminotransferase vary widely in the type of amino acids they bind, most of them use glutamate as the amino group donor. The amino group from glutamate can be transferred to other α-ketoacids by transamination reactions. Transamination reactions participate in the synthesis of most amino acids.



Carbon sources

The amino acids differ from other classes of biomolecules in that each member of this class is synthesized by a unique pathway. Despite the tremendous diversity of amino acid synthetic pathways, they have one common feature. The carbon skeletons of each amino acid are derived from commonly available metabolic intermediates. All amino acids are derived from intermediates of *glycolysis*, the *citric acid cycle* or the *pentose phosphate pathway*. The 20 amino acids can be divided into six biosynthetic families on the basis of the primary carbon sources of each amino acid. The primary carbon sources are glycerate-3-phosphate, pyruvate, α-ketoglutarate, oxaloacetate, ribose 5-phosphate, phosphoenol pyruvate and erythrose 4-phosphate.

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Overview of amino acids biosynthesis

There are six families of amino acids - glutamate, serine, aspartate, pyruvate, the aromatics, and histidine. The amino acids in each family are ultimately derived from one precursor molecule.

Glutamate family : The glutamate family includes — glutamate, glutamine, proline, and arginine and derive their carbon skeletons from α-ketoglutarate.

Serine family : The members of the serine family — serine, glycine, and cysteine—derive their carbon skeletons from glycerate-3-phosphate.

Aspartate family : The aspartate family includes aspartate, asparagine, lysine, methionine, and threonine and derive their carbon skeletons from oxaloacetate.

Pyruvate family : The pyruvate family consists of alanine, valine, leucine, and isoleucine and derive their carbon skeletons from pyruvate.

Aromatic family : The members of the aromatic family — phenylalanine, tyrosine, and tryptophan — derive their carbon skeletons from phosphoenol pyruvate and erythrose 4-phosphate.

Histidine : The member histidine family–histidine–derive their carbon skeletons from ribose 5-phosphate.

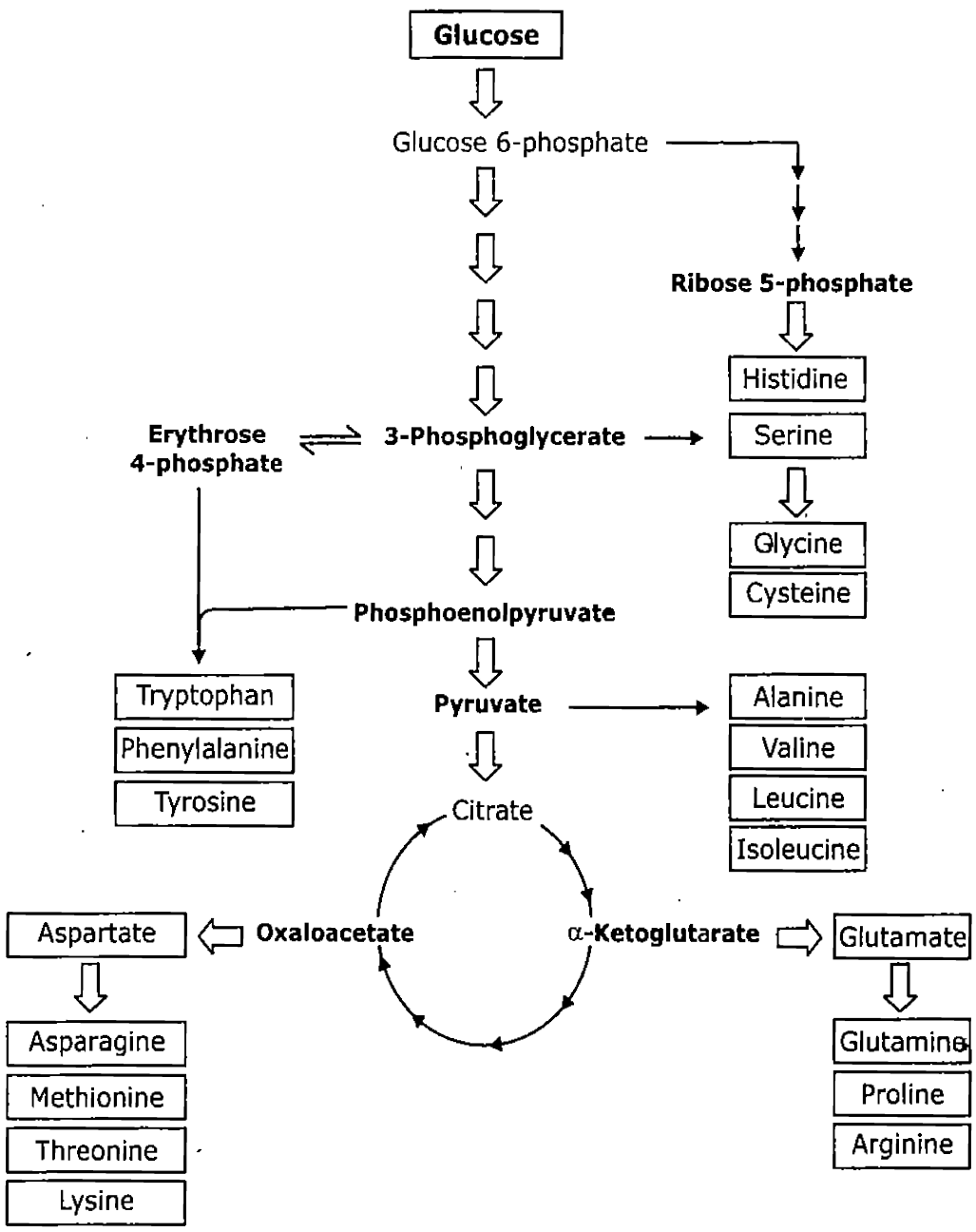


Figure 1.23 : Overview of amino acid biosynthesis. The carbon skeleton precursors are derived from three sources—glycolysis, TCA cycle and pentose phosphate pathway.

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1.7 NITROGEN FIXATION

Certain prokaryotic organisms convert nitrogen gas into ammonia. The biosynthetic process of reduction of atmospheric nitrogen to ammonia is called *nitrogen fixation*. All eukaryotic organisms are unable to fix nitrogen. Nitrogen fixing prokaryotes are termed *diazotroph*. Nitrogen fixation is a reductive process and the reduction of N_2 to NH_3 is a six-electron process.

$$\boxed{N_2} \xrightleftharpoons{6e^- + 6H^+} 2\boxed{NH_3}$$

The biological reaction also generates at least 1 mol of H_2 in addition to 2 mol of NH_3 for each mole of $N \equiv N$. Hence, an input of two additional electrons is required.

$$\boxed{N_2} \xrightleftharpoons{8e^- + 8H^+} 2\boxed{NH_3} + \boxed{H_2}$$

Thus the actual reduction of N_2 occurs in three discrete steps, each involving an electron pair:

$$N \equiv N \xrightarrow[2H^+ + 2e^-]{} NH = NH \xrightarrow[2H^+ + 2e^-]{} H_2N - NH_2 \xrightarrow[2H^+ + 2e^-]{} 2NH_2 + H_2$$

The biological process of nitrogen fixation is catalyzed by an enzyme complex called **nitrogenase complex**. There are three different forms of nitrogenase that differ in their requirement for molybdenum, vanadium, or iron as a critical metallic component of the cofactor associated with the catalytic site. Most of the nitrogenases that have been studied extensively contain a Mo cofactor. Nitrogenase consists of two proteins: a *dinitrogenase reductase,* which provides electrons with high reducing power, and *dinitrogenase,* which uses these electrons to reduce N_2 to NH_3. The transfer of electrons from the dinitrogenase reductase to the dinitrogenase component is coupled to the hydrolysis of ATP by the reductase.

The *dinitrogenase reductase* (also called the *Fe protein*) is a dimer of identical 30-kd subunits bridged by a 4Fe-4S cluster. Dinitrogenase is a tetramer with two copies of two different subunits. It contains both Fe and Mo. Because *molybdenum* is present in this cluster, the dinitrogenase component is also called the *molybdenum-iron protein (MoFe protein)*. The FeMo cofactor is the site of nitrogen fixation. The genes involved collectively in synthesis of nitrogenase and the catalytic process of N_2 fixation are called *nif* genes. Accessory genes are called *fix* genes, and they are also necessary for the function and regulation of nitrogenase in microaerobic and aerobic nitrogen-fixing bacteria.

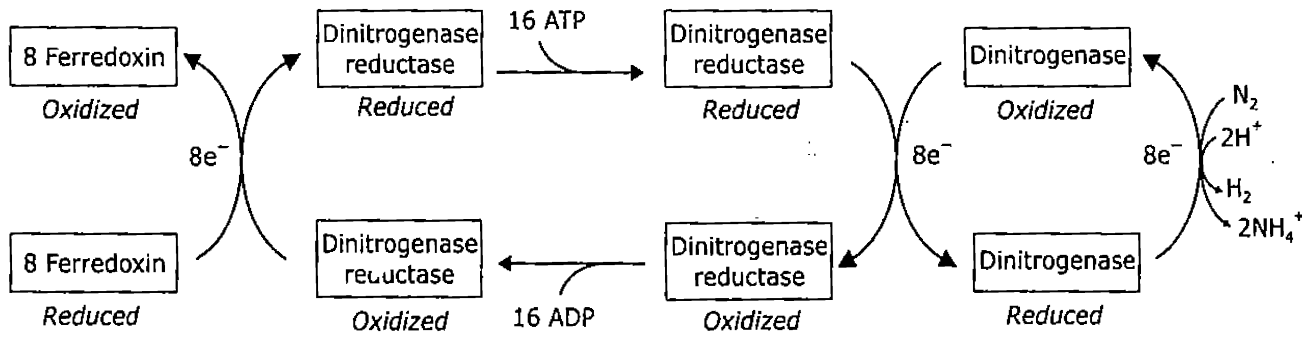


Figure 1.24 : Nitrogen fixation by nitrogenase complex

In most nitrogen-fixing microorganisms, *reduced ferredoxin,* acts as donor for electrons. Ferredoxin is a small (14 to 24 kDa) protein containing an Fe-S group. At least 16 molecules of ATP are required for each molecule of N_2 reduced; two for each electron transferred.

The nitrogenase complex is very sensitive to inactivation by O_2. Since nitrogenase is inactivated by O_2, the fixation of N_2 must occur under conditions which are anaerobic at least locally. For anaerobes there is no problem. Facultative organisms such as purple photosynthetic bacteria fix N_2 only when anaerobic. Other

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organisms have protective mechanisms. In *Azotobacter*, an obligate aerobe, the O_2 concentration inside the cell is held down by partial uncoupling of a highly active respiratory chain. This wastes carbohydrate, but if growth is limited by absence of nitrogen compounds then this is justifiable. In cyanobacteria O_2 is actually generated by photosynthesis. Fixation of N_2 occurs in special cells known as *heterocysts* which do *not* photosynthesize but are devoted solely to N_2 fixation. Leguminous plants maintain a very low concentration of free O_2 in their root nodules by binding O_2 to *leghemoglobin*, a homolog of hemoglobin.

Nitrogen fixing organisms

Nitrogen fixing prokaryotes can be divided into those that carry out biological nitrogen fixation in a symbiotic or commensalistic relationship with a eukaryote and those that fix N_2 in a free living state. Free-living, nitrogen-fixing bacteria are widespread. They include photosynthetic as well as nonphotosynthetic genera.

Examples of free living nitrogen fixing organisms

Archaebacteria	
Methanogens	*Methanococcus volate*
Eubacteria	
Heterotrophs	
Anaerobes	*Clostridium pasteurianum*
Facultative anaerobes	*Klebsiella pneumoniae,* Purple and Green sulphur bacteria
Aerobes	*Azospirillum*
Autotrophs	
Chemotrophic bacteria	*Thiobacillus ferrooxidans*
Photosynthetic bacteria	*Rhodospirillum rubrum*
Cyanobacteria	
Unicelluler	*Gloeothece spp.*
Filamentous	*Oscillatoria spp., Anabaena, Nostoc spp.*

Symbiotic nitrogen fixing bacteria

The most common form of symbiotic association results in the formation of enlarged, multicellular structures, called *nodules*, on the root (or occasionally the stem) of the host plant. In case of Leguminous plants (e.g., peas, beans, clover, alfalfa, soybean), the symbiont is a bacterium of one of three genera: *Rhizobium, Bradyrhizobium* and *Azorhizobium*. Only one nonleguminous genus, *Parasponia* is known to form root nodules with a rhizobia symbiont. Although most leguminous plants form nodules on their roots, a few leguminous plants bear nodules on their stems. One common example of stem nodulated leguminous plant is *Sesbania.*

Nodules are also found in certain nonleguminous plants such as *Alnus*, *Myrica, Casuarina*. However, the symbiont in these nonleguminous nodules is a filamentous bacterium (*Frankia*) of the group actinomycetes. Both *Rhizobium* and *Frankia* live freely in the soil, but fix dinitrogen only when in symbiotic association with an appropriate host plant. Other symbiotic associations occur but are less important. *Anabaena azollae*, a nitrogen fixing cyanobacterium, lives in pores on the fronds of a water fern called *Azolla*. This symbiotic partnership is used to enrich rice paddies with organic nitrogen.

Rhizobium - legume symbiosis

Rhizobium is gram-negative motile rod-shaped proteobacteria that can grow free-living in soil or can infect leguminous plants and establish a symbiotic existence. Infection of the roots of a legume with species of *Rhizobium* genera leads to the formation of *root nodules.*

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Chemotactic movement of free-living soil bacteria towards roots is mediated by chemicals such as flavionoids, homoserine, secreted by roots. A specific adhesion protein called *rhicadhesin* is present on the surfaces of *Rhizobium* species. Rhicadhesin, a calcium binding protein, play role in plant-bacterium attachment. Root exudates also induces the *nodulation* genes responsible for nodule formation. These genes synthesize *nodulation factors* or **nod factors**. Nod factors are *lipo-chitooligosaccharide*, the derivatives of chitin. Nod factors induce root hair curling and trigger plant cell division.

The process of nodule development begins with the infection of a root hair by a *Rhizobium* bacterium. The first recognizable reaction of the plant is the curling of the root hair. The cell wall is then broken down and the bacterium is able to enter. An *infection thread* (a cellulosic tube) develops that penetrates the inner cortex of the root. The infected cortex cells increase in size and divide to from a sphere surrounded by uninfected cells. Division these root cells ultimately gives rise to the *nodule*. In the infected cells the rhizobia divide and enlarge to form *bacteroids*, which are separated from the plant cytoplasm by the peribacteroid membrane. Bacteroids can neither divide nor revert back to the non differentiated state. Reduction of nitrogen to ammonia occurs in bacteroid. Nitrogenase enzyme catalyzes this reaction is very sensitive to oxygen. In root nodules the O_2 level is regulated by *leghemoglobin*. Leghemoglobin is the most abundant (up to 20%) protein in root nodules. The globin portion is about 145 amino acid residue monomeric oxygen binding protein. The globin protein is encoded by the host plant, whereas the heme group is primarily synthesized by the bacterium and transported into the host cytoplasm. Leghemoglobin is localized in the cytoplasm of the infected plant cells and not inside the peribacteroid membrane.

Stages in root nodule formation

The stages in the infection and development of root nodules are:

1. *Recognition* of the plant by bacterium and *attachment* of the bacterium to root hairs. Attachment of *Rhizobium* to roots involves interaction between specific bacterial proteins and their receptors present on the cells of host plant that affect the pattern of attachment and *nod* gene expression.

   Example of host plant and bacterial species that recognize and initiate nodule formation

Pea	*Rhizobium leguminosarum*
Bean	*Rhizobium leguminosarum*
Clover	*Rhizobium leguminosarum*
Alfalfa	*Sinorhizobium meliloti*
Soybean	*Bradyrhizobium elkanii*

2. Excretion of *Nod* factors by the bacterium. Nod factor promotes root hair curling and plant cortical cell division.
3. Bacterial invasion of the root hair;
4. Formation of *infection thread* (cellulosic tube) and invasion into the cortical cells;
5. Formation of modified bacterial cells, **bacteroids**, within the plant cells;
6. Plant cells division and formation of the mature *root nodule*.

Assimilation of ammonium

Because NH_3, the primary product of N_2 fixation, is toxic to cells in high concentrations, N_2-fixing bacteria possess highly efficient mechanisms of NH_3 assimilation that prevent its accumulation. NH_4^+ first assimilated into the amide group of amino acid *glutamine*. Assimilation of NH_4^+ into glutamine is accomplished by the **glutamate synthase cycle**, a pathway involving the sequential action of two enzymes: *glutamine synthetase* and *glutamate synthase*. Both glutamine synthetase and glutamate synthase are nodulin proteins that are expressed at high levels in the host cytoplasm of infected cells, outside the peribacteroid membrane.

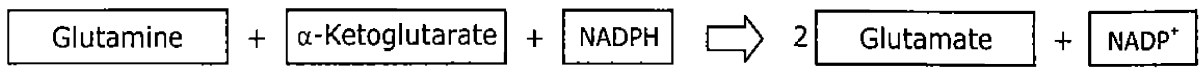
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In the first reaction of the glutamate synthase cycle, catalyzed by *glutamine synthetase* (present in all organisms), the addition of an NH_4^+ group to glutamate forms the corresponding amide, glutamine:



Glutamine is then converted back to glutamate by the transfer of the amide group to a molecule of α-ketoglutarate.



It is catalyzed by *glutamate synthase* and requires reducing potential in the form of NADPH. This enzyme is not present in animals.

There is a possible alternative, albeit minor, pathway for nitrogen assimilation, involving the direct *reductive amination* of α-ketoglutarate by the enzyme **glutamate dehydrogenase**, an enzyme present in all organisms.



1.8 AMINO ACID CATABOLISM

The catabolism of the amino acids usually begins by removing the amino group. Amino groups can then be disposed of in urea synthesis. The carbon skeletons produced from the standard amino acids are then degraded to TCA intermediates or their precursors so that they can be metabolized to CO_2 and H_2O or used in gluconeogenesis. So, catabolic pathway of amino acids involves three common stages:

A. Removal of alpha-amino group from amino acids (*amino acid deamination*) and conversion of amino group to ammonia.

B. Incorporation of ammonia into urea.

C. Conversion of amino acid's carbon skeletons to common metabolic intermediate.

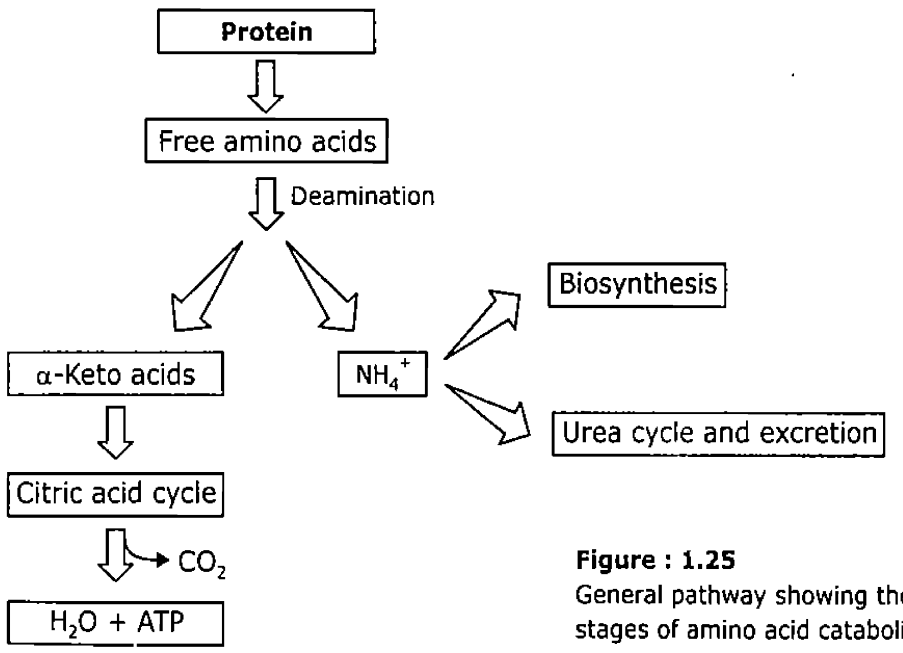


Figure : 1.25
General pathway showing the stages of amino acid catabolism

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Amino acid deamination

The removal of the α-amino group from amino acids involve two types of biochemical reactions: *transamination* and *oxidative deamination*.

Transamination

The dominant reactions involved in removing amino acid nitrogen are known as *transaminations*. This class of reactions funnels nitrogen from all free amino acids into a small number of compounds; then, either they are oxidatively deaminated, producing ammonia, or their amine groups are converted to urea by the urea cycle. Transaminations involve moving an α-amino group from a donor α-amino acid to the keto carbon of an acceptor α-keto acid. As a result of transfer, α-keto derivatives of amino acid and corresponding amino acid forms.

All amino acids except lysine, threonine and proline participate in transamination during catabolism. Transamination is readily reversible. This reaction is catalyzed by enzyme called **aminotransferases** (also called transaminase). Each aminotransferase is specific for one or at most a few amino group donors. Aminotransferases are named after the specific amino group donor, because the acceptor of the amino group is almost always α-ketoglutarate.

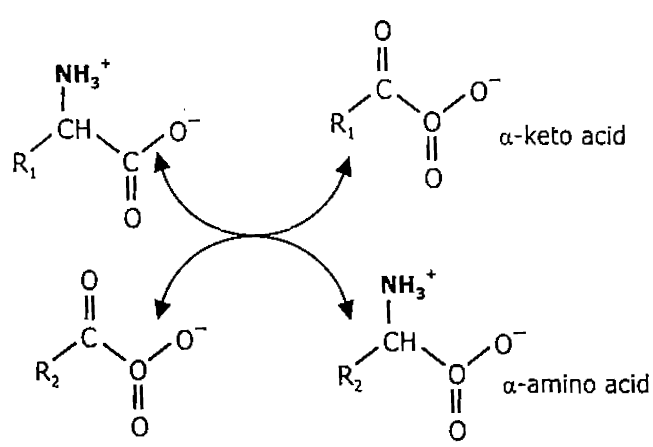


Figure : 1.26
Transamination involves the transfer of amino group to an α-keto acid to yield the α-keto acid of the original amino acid and a new amino acid.

Aminotransferases require participation of an aldehyde-containing coenzyme, pyridoxal-5-phosphate, a derivative of pyridoxine (vitamin B_6). Pyridoxal-5-phosphate is covalently attached to the enzyme via a schiff base linkage formed by the condensation of its aldehyde group with the ε-amino group of *Lys* residue. Aminotransferases act by transferring the amino group of an amino acid to the pyridoxal part of the coenzyme to generate pyridoxamine phosphate. The pyridoxamine form of the coenzyme then reacts with an α-keto acid to form an amino acid and regenerates the original aldehyde form of the coenzyme.

The most common compounds involved as a donor/acceptor pair in transamination reactions are glutamate and α-ketoglutarate, which participate in reactions with many different aminotransferases.

Amino acid + α-Ketoglutarate ⇌ α-Keto acid + Glutamate

All the amino nitrogen from amino acid that undergo transamination can be concentrated in glutamate. This is important because L-glutamate is the only amino acid that undergoes oxidative deamination at an appreciable rate.

Glucose-alanine cycle

In skeletal muscle, excess amino groups are generally transferred to pyruvate to form alanine, which finally enters into the liver where it undergoes transamination to yield pyruvate for use in gluconeogenesis. The resulting glucose is returned to the muscles, where it is glycolytically degraded to pyruvate.

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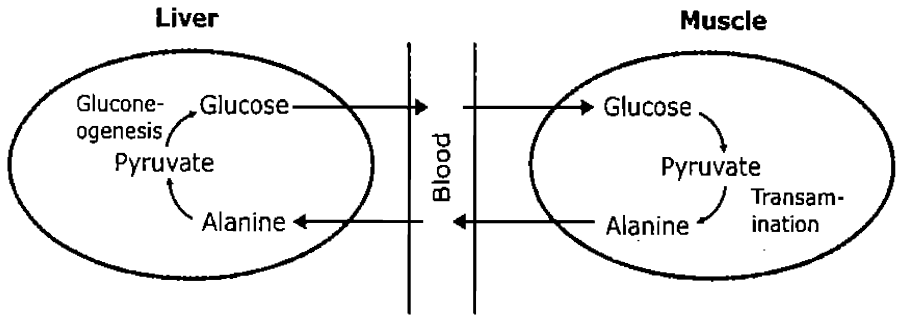
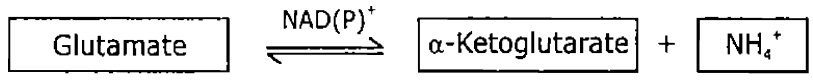


Figure 1.27 : Glucose-Alanine cycle.

Oxidative deamination

Transamination does not result in any net deamination. During *oxidative deamination,* an amino acid is converted into the corresponding keto acid by the removal of the amine functional group as ammonia and the amine functional group is replaced by the ketone group. The ammonia eventually goes into the urea cycle.

Deamination occurs mainly through the oxidative deamination of glutamate by **glutamate dehydrogenase.** The reaction requires an oxidizing agent NAD^+ or $NADP^+$. Glutamate dehydrogenase is allosterically inhibited by GTP and NADH and activated by ADP and NAD^+.



UREA CYCLE

Living organisms excrete the excess nitrogen resulting from the metabolic breakdown of amino acids in one of three ways. Many aquatic animals simply excrete *ammonia*. Where water is less plentiful, however, processes have evolved that convert ammonia to less toxic waste products that therefore require less water for excretion. One such product is *urea* and other is *uric acid.* Accordingly, living organisms are classified as being either **ammonotelic** (ammonia excreting), **ureotelic** (urea excreting) or **uricotelic** (uric acid excreting).

Urea is formed from ammonia, CO_2 and aspartate in a cyclic pathway referred to as the **urea cycle.** Because the urea cycle was discovered by Krebs and Henseleit, it is often referred to as **Krebs-Henseleit cycle.**

Urea synthesis, which occurs in the hepatocytes (liver cells), consists of five sequential enzymatic reactions. The first two reactions occur in the mitochondria and the remaining three reactions take place in the cytosol. Urea cycle begins with the formation of carbamoyl phosphate in the mitochondria. The substrates for this reaction, catalyzed by **carbamoyl phosphate synthetase I** (CPSI), are NH_4^+ and HCO_3^-. Because two molecules of ATP are required in carbamoyl phosphate synthesis, this reaction is essentially irreversible (one is used to activate HCO_3^- and the second molecule is used to phosphorylate carbamate). Carbamoyl phosphate subsequently reacts with ornithine to form citrulline. Citrulline passes into the cytosol.

Next three steps that occur in cytosol involves

- Formation of argininosuccinate by ATP dependent reaction of citrulline with aspartate (aspartate provides second nitrogen that is ultimately incorporated into urea).
- Formation of arginine from argininosuccinate. This reaction releases fumarate, which enters the citric acid cycle.
- Formation of urea and regeneration of ornithine.

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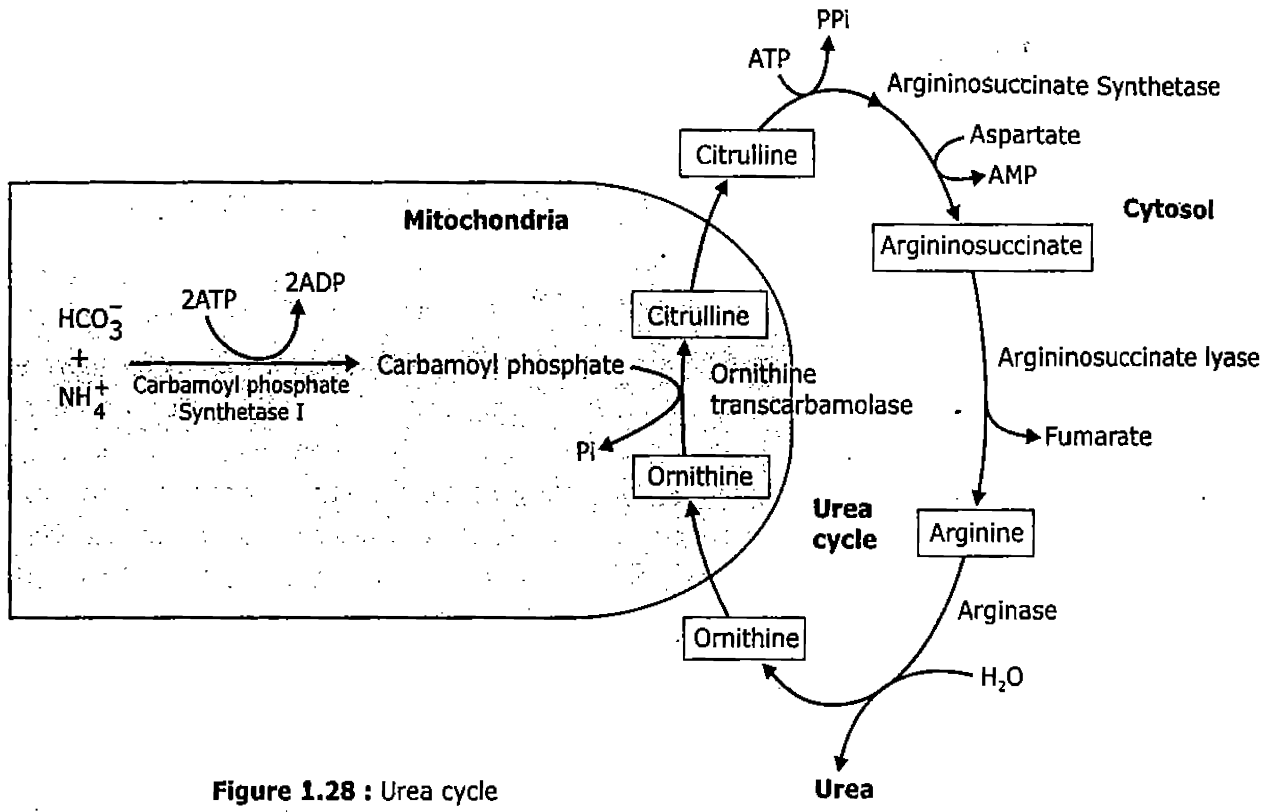


Figure 1.28 : Urea cycle

Net reaction of urea cycle;

$CO_2 + NH_4^+ + Aspartate + 3ATP + 2H_2O \Rightarrow Urea + Fumarate + 2ADP + AMP$

i.e., four high energy phosphates are consumed in the synthesis of one molecule of urea.

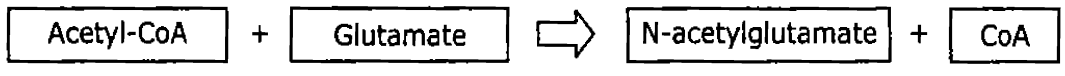
Hyperammonemia

Hyperammonemia is a condition in which the concentration of NH_4^+ is excessive (i.e., greater than 60 μM) in blood. Elevated concentrations of ammonia are serious; the consequences of ammonia intoxication include lethargy, tremors, slurred speech, blurred vision, protein-induced vomiting, coma, and death.

Regulation of the urea cycle

The urea cycle operates only to eliminate excess nitrogen. On high-protein diets the carbon skeletons of the amino acids are oxidized for energy or stored as fat and glycogen, but the amino nitrogen must be excreted. To facilitate this process, enzymes of the urea cycle are controlled at the gene level. With long-term changes in the quantity of dietary protein, changes of 20-fold or greater in the concentration of cycle enzymes are observed. When dietary proteins increase significantly, enzyme concentrations rise. On return to a balanced diet, enzyme levels decline. Under conditions of starvation, enzyme levels rise as proteins are degraded and amino acid carbon skeletons are used to provide energy, thus increasing the quantity of nitrogen that must be excreted.

Short-term regulation of the cycle occurs principally at CPS-I, which is relatively inactive in the absence of its allosteric activator **N-acetylglutamate.** This metabolite is synthesized from glutamate and acetyl CoA by N-acetylglutamate synthase.



Increased urea synthesis is required when amino acid breakdown rates increase, generating excess nitrogen that must be excreted. Increases in these breakdown rates are signaled by an increase in glutamate concentration through transamination reactions. This situation, in turn, causes an increase in N-acetyl-glutamate synthesis, stimulating carbomoyl phosphate synthetase and thus the entire urea cycle.

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CATABOLISM OF AMINO ACID'S CARBON SKELETONS

The carbon skeletons produced from the standard amino acids are then degraded to form seven metabolic products: acetyl-CoA, acetoacetyl-CoA, pyruvate, α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate. Depending on the animal's metabolic requirements, these molecules are used to synthesize fatty acids or glucose or to generate energy. Amino acids canbe classified into three categories based on their catabolic pathway:

1. Glucogenic 2. Ketogenic 3. Glucogenic and ketogenic

Glucogenic amino acids are those that give rise to a net production of pyruvate or TCA cycle intermediates, such as α-ketoglutarate or oxaloacetate, all of which are precursors to glucose via gluconeogenesis. All amino acids except lysine and leucine are at least partly glucogenic. Lysine and leucine are the only amino acids that are solely ketogenic, giving rise only to acetyl–CoA or acetoacetyl–CoA, neither of which can bring about net glucose production. A small group of amino acids comprised of isoleucine, phenylalanine, threonine, tryptophan, and tyrosine give rise to both glucose and fatty acid precursors and are thus characterized as being glucogenic and ketogenic.

The α-amino acids can be grouped into following classes according to their end products.

Amino acids forming acetyl-CoA

Ten standard α-amino acids yield acetyl-CoA. This group is further divided according to whether or not pyruvate is an intermediate in acetyl-CoA formation. The amino acids whose degradation involves pyruvate are alanine, serine, glycine, cysteine, and threonine. The other five amino acids converted to acetyl-CoA by pathways not involving pyruvate are lysine, tryptophan, tyrosine, phenylalanine, and leucine.

Amino acids forming α-Ketoglutarate

Five amino acids (arginine, proline, histidine, glutamate, and glutamine) are degraded to α-ketoglutarate.

Amino acids forming succinyl-CoA

Succinyl-CoA is formed from the carbon skeletons of methionine, isoleucine, and valine.

Amino acids forming oxaloacetate

Both aspartate and asparagine are degraded to form oxaloacetate.

Some human genetic disorders affecting amino acid catabolism

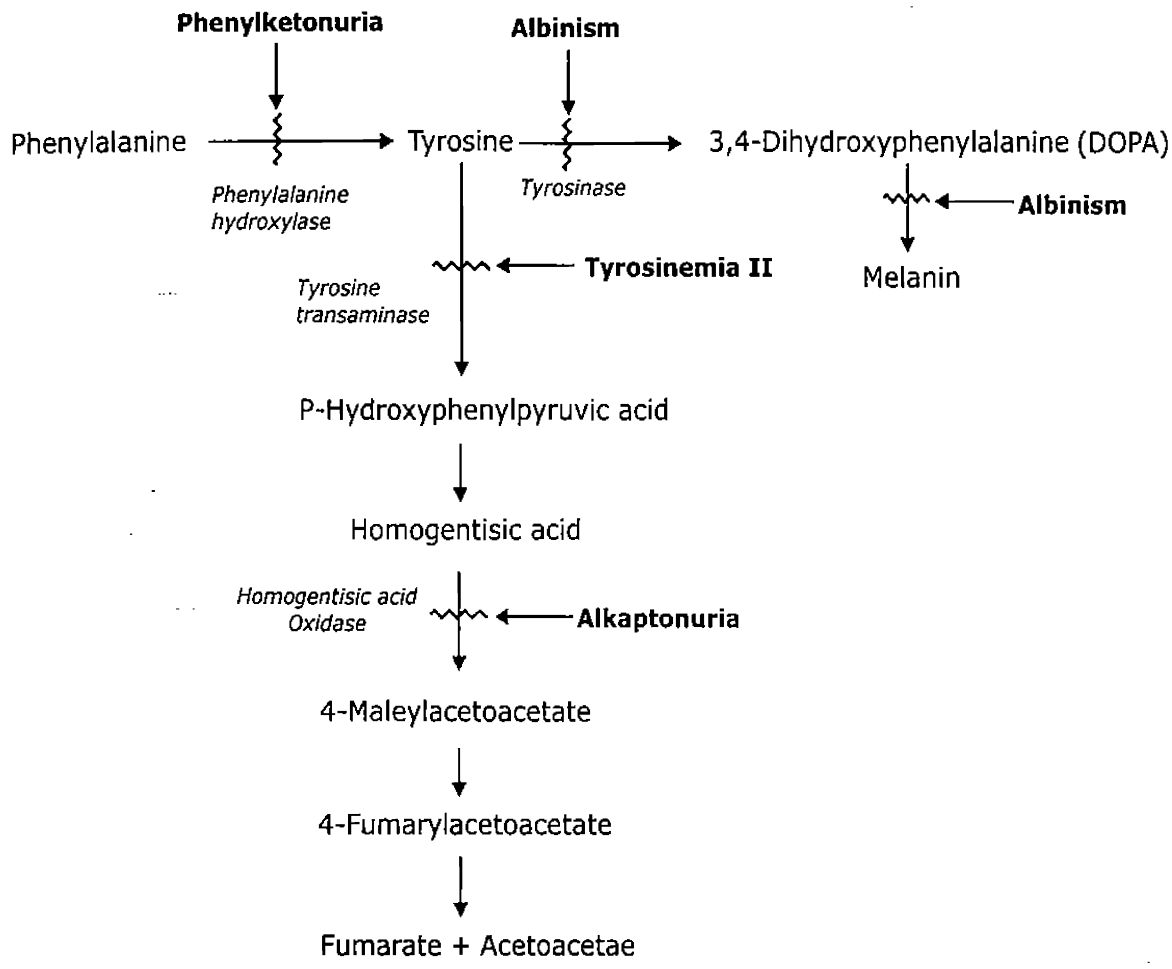
More than 50 metabolic disorders, most are rare, have been described as a result of defects in amino acid metabolism especially in catabolic pathways. Some of the most commonly encountered diseases of amino acid catabolism given in the table 1.10 :

Table 1.10 : Name of some genetic disorder and associated enzyme symptoms

Medical condition	*Defective process*	*Defective enzyme*	*Symptoms and effects*
Albinism	Melanin synthesis from tyrosine	Tyrosine 3-mono-oxygenase (tyrosinase)	Lack of pigmentation; white hair, pink skin
Alkaptonuria	Tyrosine degradation	Homogentisate 1,2-dioxygenase	Dark pigment in urine
Homocystinuria	Methionine degradation	Cystathionine beta-synthase	Faulty bone development; mental retardation
Maple syrup urine disease	Isoleucine, leucine, and valine degradation	Branched-chain alpha-keto acid dehydrogenase complex	Vomiting; convulsions; mental retardation; early death
Phenylketonuria	Conversion of phenyl-alanine to tyrosine	Phenylalanine hydroxylase	Neonatal vomiting; mental retardation

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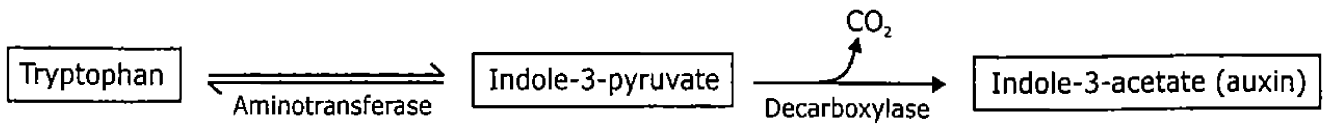
1.9 MOLECULES DERIVED FROM AMINO ACIDS

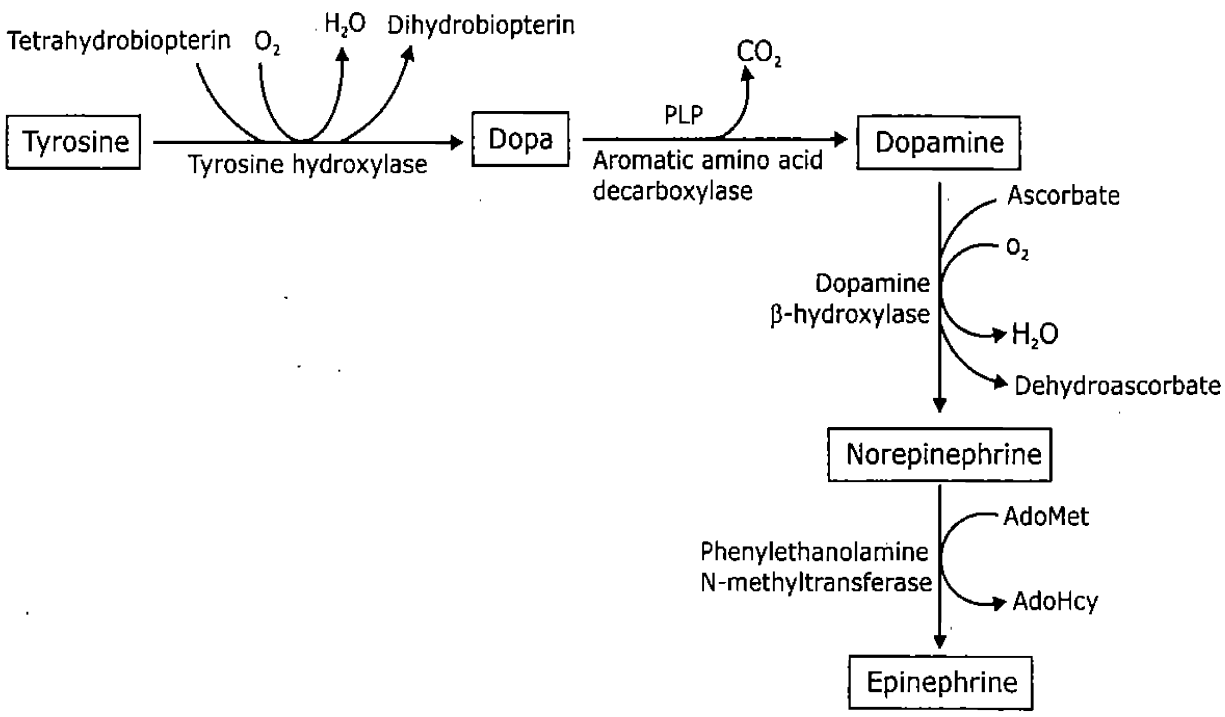
Many important biomolecules including harmones, coenzymes, porphyrins, antibiotics, pigments, neurotransmitters are derived from amino acids.

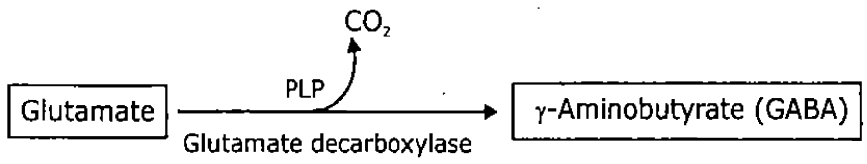
Biomolecules	Precursor amino acid/s
Auxin (Indole 3-acetic acid)[1]	Tryptophan
Catecholamines (Dopamine, norepinephrine and epinephrine)[2]	Tyrosine
GABA (γ-amino butyrate)[3]	Glutamate
Serotonin[4]	Tryptophan
Histamine[5]	Histidine
Polyamines (like spermine and spermidine)	Methionine and Ornithine
Nitric oxide (Biological messenger)[6]	Arginine
Creatine (Energy buffer)	Glycine and Arginine
Lignin	Phenylalanine and Tyrosine
Porphyrin[7]	Glycine/Glutamate
Glutathione (Reducing agent)	Glycine, Glutamate and Cysteine

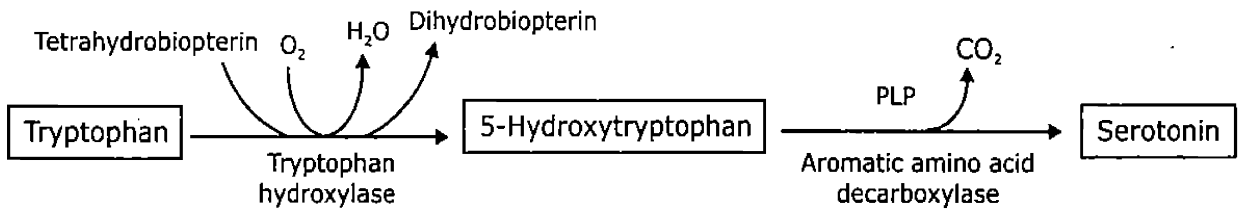
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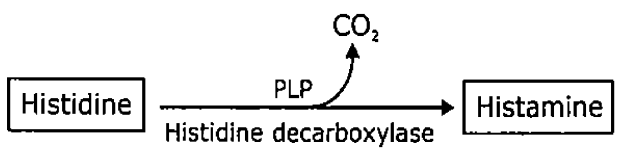
Amino acids and Proteins

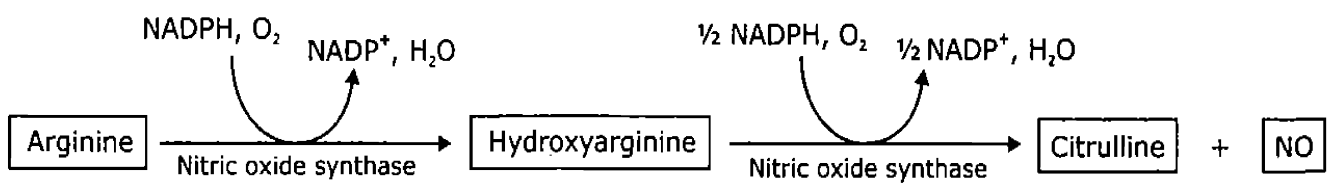
1. 

2. 

3. 

4. 

5. 

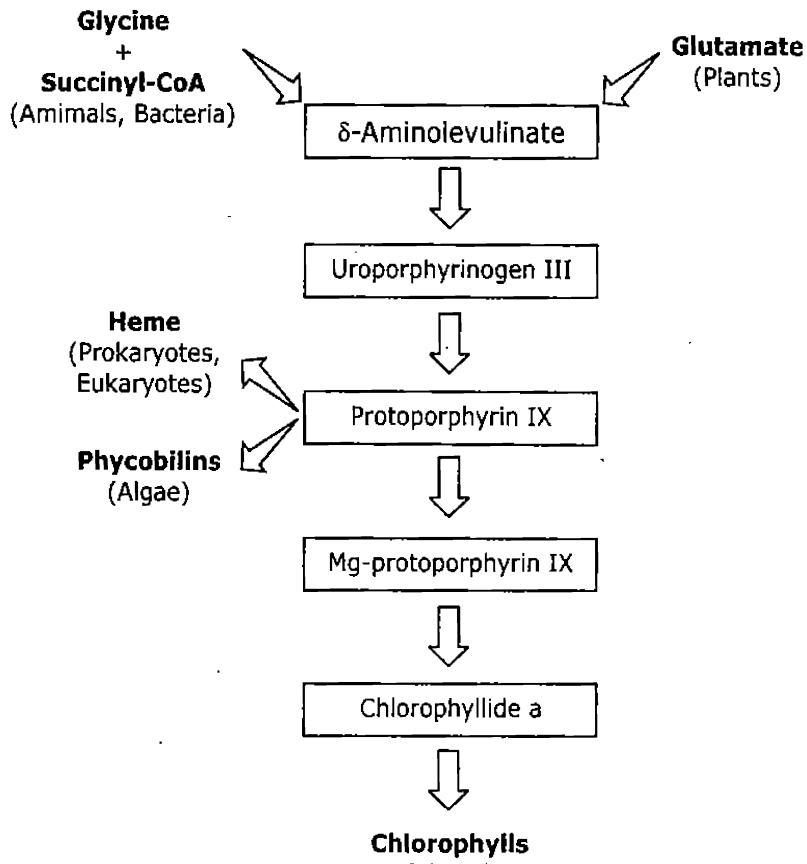
6. 

The nitrogen of the NO is derived from the guanidino group of arginine.

7. Porphyrin (tetrapyrrole is the generic term for compounds containing four linked pyrrole rings) include heme, chlorophylls, phycobilins, and cobalamins. All are synthesized from **δ-aminolevulinic acid** (ALA), which is formed differently in plants from the way it is formed in bacterial and animal cells (*as shown in figure below*).

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(Plants)

In animals, all of the nitrogen of heme is derived from glycine and all of the carbons is derived from succinate and glycine. Hence, this synthesis is often called the **succinate-glycine pathway**.

Porphyrin biosynthesis involves three distinct process

a. Synthesis of a substituted pyrrole compound, **porphobilinogen** from ALA.

b. Condensation of four porphobilinogen molecules to yield a partly reduced precursor called a **porphyrinogen**.

c. Modification of the side chains, dehydrogenation of the ring system, and introduction of iron, to give the porphyrin product, heme.

STUDY QUESTION

Q8. Describe how the glucose-alanine cycle acts to transport ammonia to the liver.

Ans. In the muscle, pyruvate undergoes a transamination reaction and is converted to alanine. Alanine is then transferred to the liver, where it is reconverted to pyruvate. The amino group is transferred to α-ketoglutarate thus forming glutamate, which is subsequently oxidatively deaminated to form α-ketoglutarate and ammonia.

Q9. Urea formation is energetically expensive, requiring the expenditure of four moles of ATP per mole of urea formed. However, NADH is produced when fumarate is reconverted to aspartate. How many ATP molecules are produced by the mitochondrial oxidation of the NADH? What is the net ATP requirement for urea synthesis?

Ans. The NADH formed during the conversion of fumarate to aspartate results in the synthesis of approximately 2.5 ATP. Therefore, the net ATP requirement for urea synthesis is approximately 1.5 moles of ATP per mole of urea

Q10. The synthesis of δ-aminolevulinate takes place in the mitochondrial matrix, whereas the formation of porphobilinogen takes place in the cytosol. Propose a reason for the mitochondrial location of the first step in heme synthesis.

Ans. Succinyl-CoA is formed in the mitochondrial matrix.

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BOX 1.1 BIOLOGICAL INTERACTION

Bonds in biological systems fall under two categories- covalent and non-covalent bonds or interactions. Covalent bonds are very strong whereas non-covalent bonds are weak bonds. The main types of weak bonds are hydrogen bonds, ionic bonds, van der Waals forces, and hydrophobic interactions.

Covalent bond

In covalent bond, the nuclei of two atoms are held close together because electrons in the outermost shell are shared by both atoms. Covalent bonds are very stable and the large energy changes are associated with the making and breaking of these bonds.

Noncovalent bonds

Noncovalent bonds (i.e., hydrogen bonds, electrostatic interactions, van der Waals forces, and hydrophobic interactions) play important roles in determining the structure and function of biomolecules. Non-covalent interactions are weak interactions. The energy required to break non-covalent interactions is only 1-5 kcal/mol, much less than the bond energies of covalent bonds.

Hydrogen bonds

Bonds between hydrogen and oxygen or nitrogen are sufficiently polar so that the hydrogen nucleus is weakly attracted to the lone pair electrons of an oxygen or nitrogen of a neighbouring molecule. The resulting intermolecular 'bond' acts as a bridge between water molecules. Although considerably weaker than ionic and covalent bonds, hydrogen bonds are stronger than most noncovalent bonds.

Electrostatic interactions

Electrostatic interactions occurs between oppositely charged atoms or groups. Like other noncovalent bonds, they are of great significance in determining the shape and function of biomolecules. For example, the attraction between the $-COO^-$ and $-NH_3^+$ groups is a factor in determining the three-dimensional structure of proteins. An important aspect of all electrostatic interactions in aqueous solution is the hydration of ions. Because water molecules are polar, they are attracted to charged ions. Shells of water molecules, referred to as **salvation spheres,** cluster around both positive and negative ions. As ions become hydrated, the attractive force between them is reduced, and the charged species dissolves in the water. Electrostatic interactions between charged groups or polar groups on biomolecules are most significant in water depleted regions since water competes with great success with electrostatic interactions between other groups. The *dielectric constant* of a solvent is a measure of its capacity to reduce the attractive force between ions. Water, sometimes called the *universal solvent* because of the large variety of ionic and polar substances it can dissolve, due to its large dielectric constant.

Van der Waals forces

Van der Waals forces are relatively weak, transient electrostatic interactions. They occur between permanent or induced dipoles. They may be attractive or repulsive, depending on the distance between the atoms or groups involved. The attraction between molecules is greatest at a distance called the *van der Waals radius.* If molecules approach each other more closely, a repulsive force develops. The magnitude of van der Waals forces depend on how easily an atom is polarized. Electronegative atoms with unshared pairs of electrons are easily polarized. There are three types of van der Waals forces:

1. **Dipole-dipole interactions**

   These forces, which occur between molecules containing electronegative atoms cause molecules to orient themselves so that the positive end of one molecule is directed toward the negative end of another.

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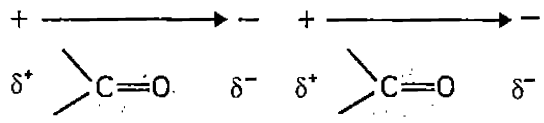
2. Dipole–induced dipole interactions

A permanent dipole induces a transient dipole in a nearby molecule by distorting its electron distribution.

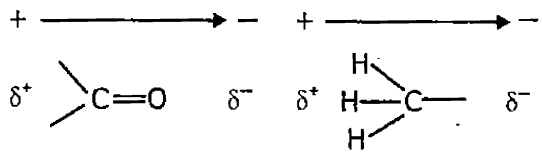
3. Induced dipole–induced dipole interactions

The motion of electrons in nearby nonpolar molecules results in transient charge imbalances in adjacent molecules. A transient dipole in one molecule polarizes the electrons in neighboring molecule. This attractive interaction, often called **London dispersion forces**, is extremely weak.

1. Dipole –dipole interactions



2. Dipole –induced dipole interactions



3. Induced dipole –induced dipole interaction

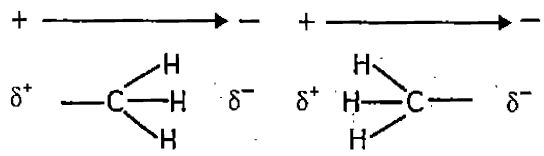


Table 1.11 : Comparisons of covalent and non-covalent chemical bonds

Bond Type	**Length (nm)**	**Strength (kcal/mole)**	
		In Vacuum	*In Water*
Covalent	0.15	90	90
Noncovalent			
Ionic	0.25	80	3
Hydrogen	0.30	4	1
van der Waals	0.35	0.1	0.1

Hydrophobic interaction

When mixed with water, small amounts of nonpolar substances coalesce into droplets. This process results from *hydrophobic interactions.* Hydrophobic (*water-hating*) molecules, such as the hydrocarbons, are virtually insoluble in water. Their association into droplets (or, in larger amounts, into a separate layer) results from the solvent properties of water, not from the relatively weak attraction between the associating nonpolar molecules. When nonpolar molecules enter an aqueous environment hydrogen-bonded water molecules attempt to form a cage like structure around them. Sufficient energy is not available in the surroundings to form this cage-like structure. And nonpolar molecules are expelled. The droplets that form result from the most energetically favorable configuration of the surrounding water molecules. Hydrophobic interactions have a profound effect on living cells. For example they are primarily responsible for the structure of membranes and the stability of proteins.

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REVIEW THE CONCEPT

Q1. a. What is the principle behind Ramachandran Plot?
b. Write the Ramachandran plot for a dipeptide alanyl-alanine. Indicate the positions of occurrence of the various secondary structures.
c. How does the Ramachandran plot change when glycine replaces alanine? **[CSIR-UGC]**

Q2. a. Define hydrogen bonding giving one example. What are the factors influencing the strength of hydrogen bonds.
b. Describe the tertiary and quaternary structures of proteins giving one example each. **[CSIR-UGC]**

SELF TEST

Q1. Which one of the following amino acids has the largest number of rotatable bonds in the side chain
a. tryptophan
b. leucine
c. lysine
d. cysteine **[IISc]**

Q2. The amino acid with pK_a closest to physiological pH is
a. serine
b. histidine
c. threonine
d. proline **[IISc]**

Q3. Histidine is often found at the active-site of enzymes because
a. it has a cyclic group
b. it can form hydrogen bonds
c. it has a pKa of 6.8
d. it is an imino acid

Q4. Which of the following is *not* true about alanine
a. at pH 1, the overall charge is +1
b. at pH 1, it will move towards cathode
c. an equimolar mixture of D and L alanine does not rotate the plane polarized light
d. contains branched side chain

Q5. Lysine is an amino acid with three ionizable groups. These are the α-COOH, 2 α-amino and ε-amino groups with pKa values of 2.2, 9.2 and 10.8, respectively. The isoelectric point (pI) for lysine is
a. 5.7
b. 6.5
c. 9.2
d. 10.0

Q6. One of the characteristics of a peptide bond is that
a. there is no restriction in rotation around peptide bond
b. there is a restricted rotation around peptide bond
c. its bond energy is approximately 5 Kcal/mole
d. it is longer than 0.2 nm **[CSIR-UGC]**

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Q7. Among the various interactions that help in the sustenance of the tertiary structure of proteins, the weakest one is
a. hydrogen bonding
b. van der Waals interactions
c. S—S bonding
d. hydrophobic interactions

Q8. The bonds in protein structure that are not broken on denaturation
a. hydrogen bonds
b. peptide bonds
c. ionic bonds
d. disulfide bonds

Q9. In an alpha-helix hydrogen bonds are
a. within a single chain
b. between chains that run side by side
c. between polar amino acids and water
d. only between amino acids of opposite charge

Q10. The Bohr effect in hemoglobin refers to the
a. effect of pH on hemoglobin and myoglobin
b. higher pH found in actively metabolizing tissues
c. increased affinity for O_2 at lower pH
d. reduced affinity for O_2 at lower pH **[CSIR-UGC]**

Q11. How does hemoglobin carry carbon dioxide generated in tissues back to the lungs?
a. by coordination with heme
b. by forming N-terminal carbamate
c. by forming C-terminal carbamate
d. by linking to the epsilon-amino group of lysine **[GATE]**

Q12. Highly cooperative binding of a ligand to multiple binding sites on a macromolecule is best demonstrated by
a. Adair equation
b. Hill plot
c. Lineweaver-Burk plot
d. Arrhenius plot **[JNU]**

Q13. Which of the following would produce a Hill plot with negative cooperativity
a. a protein has multiple subunits each with a single binding site. Binding a ligand to one site decreases affinity of other sites for ligand
b. a protein is a single polypeptide chain with two ligand binding sites each with different ligand affinities
c. a protein has a single binding site. But during purification the protein preparation is heterogeneous, hence there are different protein molecules with different ligand affinities
d. a protein has multiple binding sites for different ligands. Binding of one type of ligand at a higher affinity site promotes binding of the other ligand at a lower affinity site **[CSIR-UGC]**

Q14. Hemoglobin and myoglobin both have all of the following characteristics *except*
a. consist of subunits designed to provide hydrogen bonds and nonpolar interaction with other subunits
b. highly alpha helical
c. bind one molecules of heme per globin chain
d. bind heme in a hydrophobic pocket

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Q15. The amino acids are said to be ketogenic when the carbon skeleton is finally degraded to

a. succinyl-CoA
b. fumarate
c. acetyl-CoA
d. pyruvate

Q16. All of the following are correct about ornithine *except*

a. it may be formed from or converted to glutamic semialdehyde
b. it can be converted to proline
c. it plays a major role in the urea cycle
d. it is chemically spermidine

Q17. Hemoglobin and myoglobin both have all of the following characteristics *except*

a. consist of subunits designed to provide hydrogen bonds and nonpolar interaction with other subunits
b. highly alpha helical
c. bind one molecules of heme per globin chain
d. bind heme in a hydrophobic pocket

Q18. DNA polymerase contains a lysine residue that is important for binding to DNA. Mutations were found that converted this lysine to either glutamate, glycine, valine, or arginine. Which mutations would be predicted to be the most and least harmful to the ability of the enzyme to bind DNA

	Most	*Least*
a.	Glycine	Arginine
b.	Arginine	Glycine
c.	Glutamate	Valine
d.	Glutamate	Arginine

Q19. Amino transferases

a. usually require alpha-ketoglutarate or glutamine as one of the reacting pair
b. catalyze reactions that result in a net use or production of amino acids
c. catalyze irreversible reactions
d. require pyridoxal phosphate as an essential cofactor for the reaction

Q20. Match the entries in Column A against those in Column B

Column A	*Column B*
P. The amino acid with maximum number of codons	1. Cys
Q. The characteristic amino acid in lysozyme active site	2. Trp
R. The amino acid precursor in heme biosynthesis	3. Ser
S. The amino acid contributing the most to protein absorbance at 280 nm	4. Gly
	5. Glu

a. P-2, Q-3, R-1, S-4
b. P-3, Q-5, R-4, S-2
c. P-5, Q-3, R-2, S-1
d. P-4, Q-1, R-5, S-3

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Chapter - 02

Nucleic Acids

2.1 NUCLEOTIDES AND NUCLEIC ACID

Nucleic acid was first discovered by Friedrich Miescher from the nuclei of the pus cells (Leukocytes) from discarded surgical bandages and called it **nuclein**. Nuclein was later shown to be a mixture of a basic protein and a phosphorus-containing organic acid, now called **nucleic acid**. There are two types of nucleic acid: ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

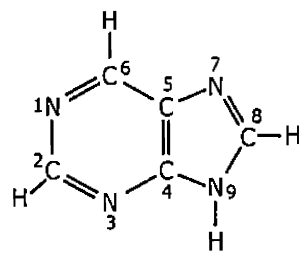
NUCLEOTIDES

The monomeric units of nucleic acids are called *nucleotides*. Nucleic acids therefore are also called *polynucleotides*. Nucleotides are phosphate esters of nucleosides and made up of three components:

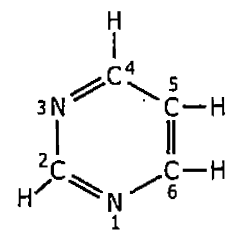
1. A base that has a nitrogen atom
2. A five carbon sugar
3. An ion of phosphoric acid

Nitrogenous bases

Nitrogenous bases are heterocyclic, planar and relatively water insoluble aromatic molecules. There are two general types of nitrogenous bases in both DNA and RNA, pyrimidines and purines.



Purine



Pyrimidine

Purines

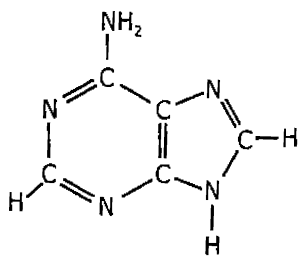
Two different nitrogenous bases with a purine ring (composed of carbon and nitrogen) are found in DNA. The two common purine bases found in DNA and RNA are *adenine* (6-aminopurine) and *guanine* (6-oxy-2-aminopurine). Adenine has an amino group ($-NH_2$) on the C6 position of the ring (carbon at position 6 of the ring). Guanine has an amino group at the C2 position and a carbonyl group at the C6 position.

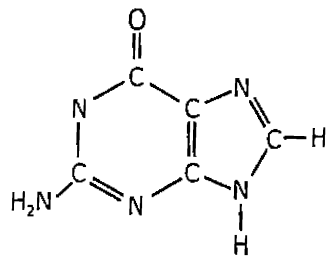
Pyrimidines

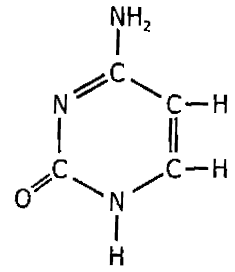
The two major pyrimidine bases found in DNA are *thymine* (5-methyl-2,4-dioxypyrimidine) and *cytosine* (2-oxy-4-aminopyrimidine) and in RNA they are *uracil* (2,4-dioxypyrimidine) and *cytosine*. Thymine contains a methyl group at the C5 position with carbonyl groups at the C4 and C2 positions. Cytosine contains a hydrogen atom at the C5 position and an amino group at C4. Uracil is similar to thymine but lacks the methyl group at the C5 position. Uracil is not usually found in DNA. It is a component of ribonucleic acid (RNA).

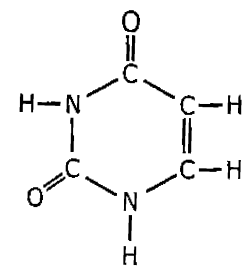
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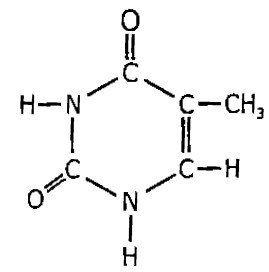
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Adenine


Guanine


Cytosine


Uracil


Thymine

Sugars

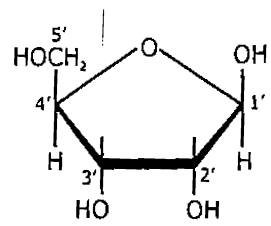
Naturally occurring nucleic acids have two types of pentose sugars: Ribose and Deoxyribose sugar. All known sugars in nucleic acids have the D-stereoisomeric configuration.

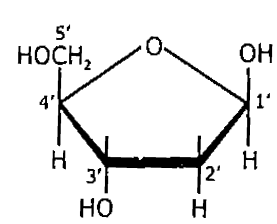
Ribose sugar is found in RNA

β-D-Ribose is a 5-carbon sugar with a hydroxyl group (—OH) on each carbon (the carbon atoms of the ribose/deoxyribose present in nucleoside/nucleotides are designated with a prime (′) mark to distinguish them from the backbone numbering in the bases).

Deoxyribose sugar is found in DNA

Hydroxyl (-OH) group present at the 2′ position of ribose sugar is replaced by a hydrogen (-H).


β-D-ribose


β-D-2-Deoxyribose

Sugar pucker

In the envelope form, the four carbons of the pentose sugar form a plane at the corners of the body of the envelope. The oxygen is at the position representing the top of the envelope flap. The oxygen can be bent out of the plane of the envelope. Twisting the C2′ and C3′ carbons relative to the other atoms results in various twist forms of the sugar ring. To form the C2′ endo form of the ribose sugar, C2′ twists up from the plane of the four carbons. To form the C3′ endo, C3′ twists up of the plane of the four carbons.

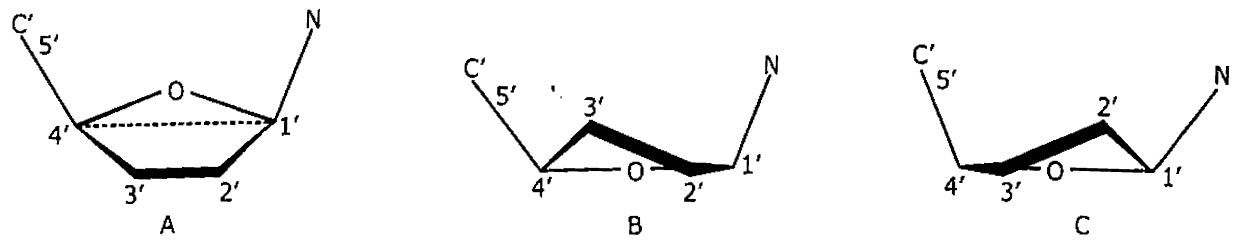
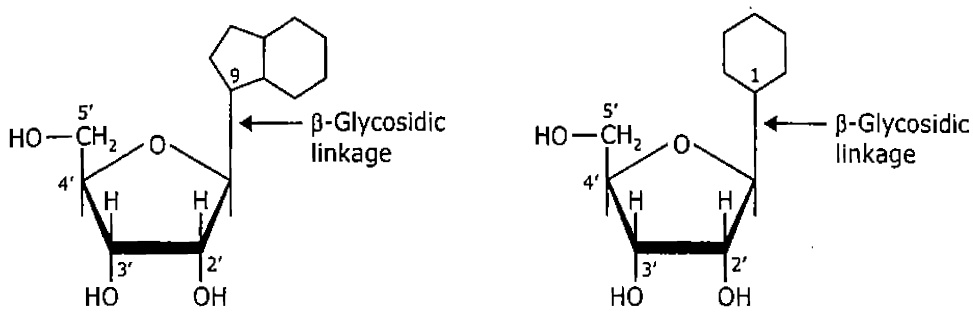

A B C

Figure 2.1 : Sugar puckers A. A representation of an envelope conformation of a ribose sugar. B. C3′ endo conformation of the ribose sugar C. C2′ endo conformation of the ribose sugar.

Nucleoside

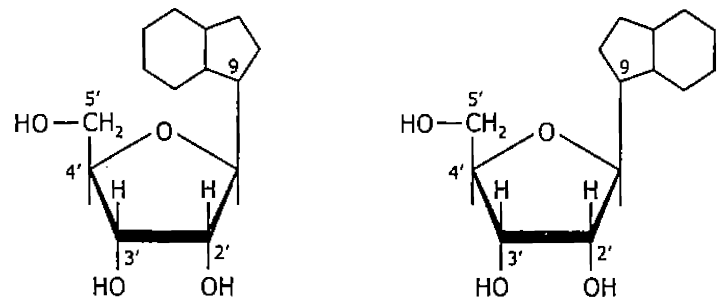
Sugar and nitrogenous base joins to form **nucleoside**. The bond between the sugar and the base is called the **glycosidic bond**. This bond is said to be in the β (up) configuration with respect to the ribose sugar.

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Nucleoside

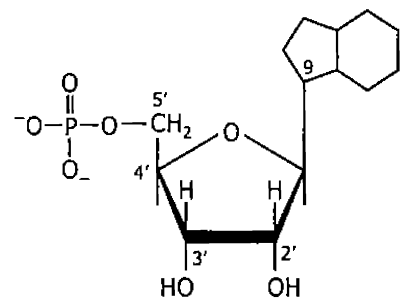
The base is free to rotate around the glycosidic bond. Due to rotation of the glycosidic bond two different conformations are possible. The two standard conformation of the base around the glycosidic bond are *syn* and *anti*. Pyrimidines tend to adopt the *anti* conformation whereas purines are able to assume both forms (*syn* as well as *anti*).



Syn *Anti*

Nucleotides

The nucleotides are phosphoric acid esters of nucleosides, with phosphate at position C-5′. The nucleotide can have one, two, or three phosphate groups designated α, β, and γ for the first, second, and third, respectively.



Nucleotide

Nucleotides are found primarily as the monomeric units comprising the major nucleic acids of the cell, RNA and DNA. However, they also are required for numerous other important functions within the cell. These functions include

- Formation of energy currency like ATP, GTP.
- Acts as a precursor for several important coenzymes such as NAD^+, $NADP^+$, FAD and coenzyme A.
- Serving as precursor for secondary messengers like cyclic-AMP (cAMP), a cyclic derivative of AMP formed from ATP.
- Serving as activated intermediates in numerous biosynthetic reactions. These activated intermediates include S-adenosylmethionine (S-AdoMet) involved in methyl transfer reactions.

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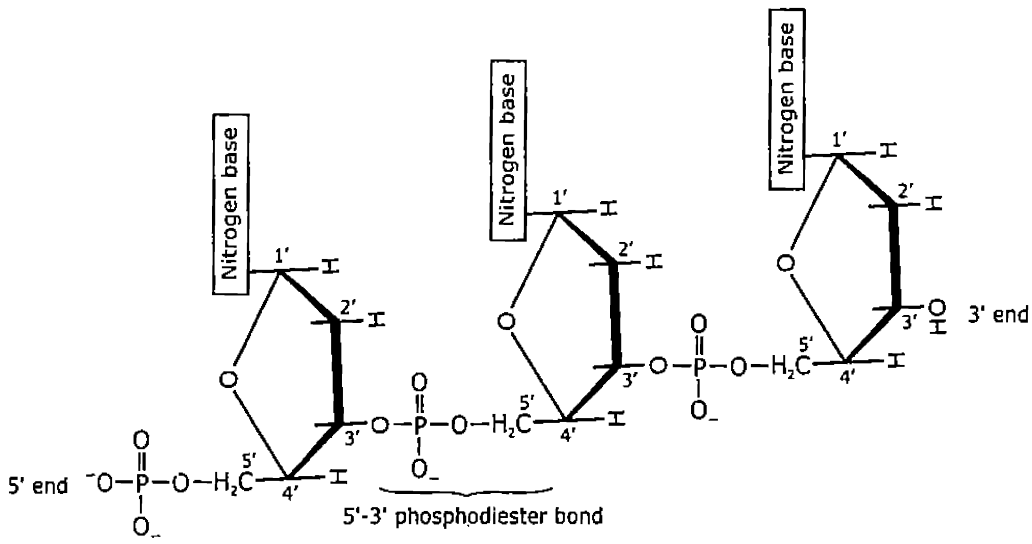
Table 2.1 : Naming nucleosides and nucleotides

	BASES			
	PURINES		PYRIMIDINES	
	Adenine (A)	**Guanine (G)**	**Cytosine (C)**	**Uracil (U)/Thymine (T)**
Nucleosides–in RNA	Adenosine	Guanosine	Cytidine	Uridine
in DNA	Deoxyadenosine	Deoxyguanosine	Deoxycytidine	Deoxythymidine
Nucleotides– in RNA	Adenylate	Guanylate	Cytidylate	Uridylate
in DNA	Deoxyadenylate	Deoxyguanylate	Deoxycytidylate	Deoxythymidylate
Nucleoside monophosphate	AMP	GMP	CMP	UMP/TMP
Nucleoside diphosphate	ADP	GDP	CDP	UDP/TDP
Nucleoside triphosphate	ATP	GTP	CTP	UTP/TTP

Polynucleotides

Polynucleotides are formed by the condensation of two or more nucleotides. The condensation most commonly occurs between the alcohol of a 5'-phosphate of one nucleotide and the 3'-hydroxyl of a second, with the elimination of H_2O, forming a *phosphodiester bond.* All nucleotides in a polynucleotide chain have the same relative orientation. The formation of phosphodiester bonds in DNA and RNA exhibits directionality. The primary structure of DNA and RNA (the linear arrangement of the nucleotides) proceeds in the 5' → 3' direction. The common representation of the primary structure of DNA or RNA molecules is to write the nucleotide sequences from left to right synonymous with the 5' → 3' direction as shown below.

5'-pGpApTpC-3'



The order in which the bases occur in a polynucleotide describes the *primary structure*. The convention is to list them left to right from the 5' end to the 3' end. The backbone consists of the phosphate-sugar chain that runs the length of the polynucleotide. The *secondary structure* is the three-dimensional structure of the polynucleotide strand or strands. For DNA the most important secondary structure consists of a double helix (two strands) held together by hydrogen bonding between the bases.

CHARGAFF'S RULES

In the early 1950s, Chargaff found that in DNA sample, the number of moles of A equaled the number of moles of T, and the number of moles of G equaled the number of moles of C. Mathematically, Chargaff's rules can be expressed as A + T + G + C = 1, A = T, and G = C where A, T, G, and C are given in mole fractions; that is, A = moles of A/total moles of nucleotides.

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Hence, in dsDNA

1. The amount of adenine residues is proportional to the amount of thymine residues in the DNA. Also, the amount of guanine residues is proportional to the amount of cytosine residues.
2. The sum of the purines (A+G) equals the sum of the pyrimidines (C+T).
3. The percentage of C+G does not necessarily equal the percentage of A+T.

STUDY QUESTIONS

Q1. Sea urchin DNA, which is double stranded, was shown to contain 17.5 percent of its bases in the form of cytosine (C). What percentage of the other three bases are present in this DNA?

Ans. The amount of C is equal to G, so guanine is also present as 17.5 percent. The remaining bases, A and T, are present in equal amounts, and together they represent the rest of the bases (100–35 = 65). Therefore, A = T = 65/2 = 32.5 percent.

2.2 STRUCTURE OF DOUBLE STRANDED DNA

Watson and Crick first described the structure of the DNA double helix in 1953 using X-ray diffraction data of DNA fibers obtained by R. Franklin and M. Wilkins. *Watson, Crick,* and *Wilkins* were awarded the 1962 Nobel Prize for Medicine for discovering the molecular structure of DNA. R. Franklin died of cancer before the Nobel prize was awarded. Nobel prizes are not awarded posthumously.

Features of B-form of DNA (Watson-Crick model)

Watson-Crick model have following major features:

- Two long polynucleotide strands coiled around a central axis
- Strands are wrapped plectonemically in a *right handed helix*.
- Strands are antiparallel i.e., one strand is oriented in the 5′→3′ direction and the other in the 3′→5′ direction.
- Strands interact by hydrogen bonds between *complementary* base pairs

  G forms *three* hydrogen bonds with C.

  A forms *two* hydrogen bonds with T.

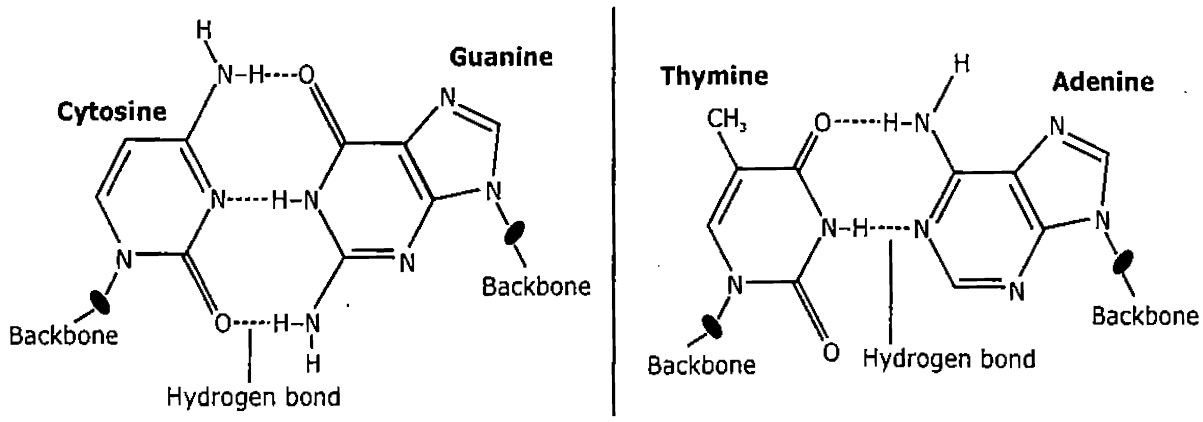


Figure 2.2 : Specific pairing between adenine and thymine and between guanine and cytosine via hydrogen bonds : These are the typical base pairs found in double-stranded DNA.

- Angle of interaction between base pairs result in *major* and *minor* grooves.
- Helix diameter is 20Å.

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- Helix rise per base pair is 3.32Å.
- Helix pitch (distance along the axis per 360 degree turn) is 33.2Å.
- 10.4 base pairs per helical turn.
- Base pairs are in the inside of the molecule stacked close to each other.

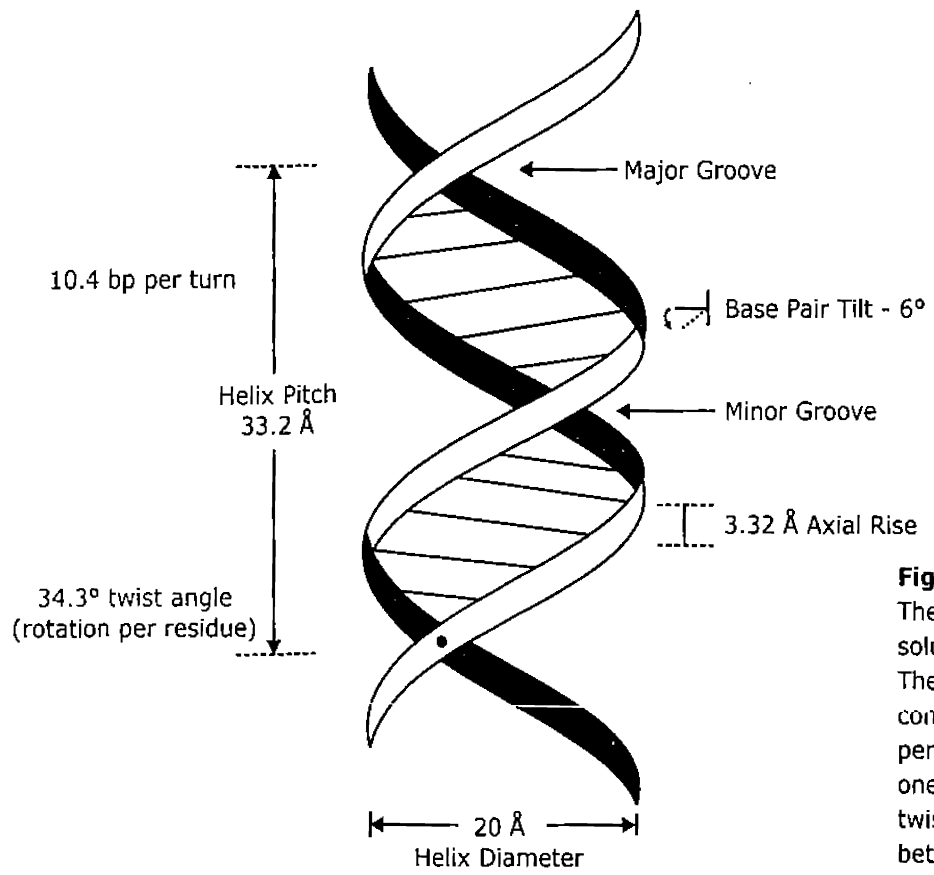


Figure : 2.3
The DNA double helix in solution: structural parameters. The Watson-Crick double helix is composed of about 10.4 base pairs per helical turn. Since 360° constitutes one helical turn, there would be a 34.3° twist angle or rotation per residue between adjacent base pairs.

Tilt, Roll and Propeller twist

Base pair tilt refers to the angle of the planar bases with respect to the helical axis. The tilt angle is measured by considering the angle made by a line drawn through the two hydrogen bonded bases relative to a line drawn perpendicular to the helix axis. The tilt angle opens in the direction of the phosphate backbone.

Base pair roll refers to the angle of deflection of a base pair with respect to the helix axis along a line drawn between two adjacent base pairs relative to a line drawn perpendicular to the helix axis. Roll angle occurs at 90° to the direction of hydrogen bonding. It can open toward either the major or the minor groove.

Propeller twist refers to the angle between the planes of two paired bases. A base pair is rarely a perfect flat plane with each aromatic base in the same plane. Rather, each base has a slightly different roll angle with respect to the other base. This makes the two bases look like an airplane propeller.



Tilt Roll Propeller Twist

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Z-DNA

Left-handed Z-DNA has been mostly found in alternating purine-pyrimidine sequences $(CG)_n$ and $(TG)_n$. Z-DNA is thinner (18 Å) than B-DNA (20 Å), the bases are shifted to the periphery of the helix, and there is only one deep, narrow groove equivalent to the minor groove in B-DNA. In contrast to B-DNA where a repeating unit is 1 base pair, in Z-DNA the repeating unit is 2 bp. The backbone follows a zigzag path as opposed to a smooth path in B-DNA. The sugar and glycosidic bond conformations alternate; C2′ endo in *anti* dC or dT and C3′ endo in *syn* dG or dA. Electrostatic interactions play a crucial role in Z-DNA formation. Therefore, Z-DNA is stabilized by high salt concentrations or polyvalent cations that shield interphosphate repulsion better than monovalent cations.

Z-DNA can form in regions of alternating purine-pyrimidine sequence; $(GC)_n$ sequences form Z-DNA most easily. $(GT)_n$ sequences also form Z-DNA but they require a greater stabilization energy. $(AT)_n$ sequences generally does not form Z-DNA since it easily forms cruciforms.

Table 2.2 : Comparisons of different forms of DNA

Geometry attribute	**A-form**	**B-form**	**Z-form**
Helix sense	right-handed	right-handed	left-handed
Repeating unit	1 bp	1 bp	2 bp
Rotation/bp	33.6°	34.3°	60°/2
Mean bp/turn	10.7	10.4	12
Base pair tilt	20°	−6°	7°
Rise/bp along axis	2.3Å	3.32Å	3.8Å
Pitch/turn of helix	24.6Å	33.2Å	45.6Å
Mean propeller twist	+18°	+16°	0°
Glycosidic bond	anti	anti	anti for A, C ,T, syn for G
Sugar pucker	C3'-endo	C2'-endo	A, C, T:C2'-endo, G:C3'-endo
Diameter	23Å	20Å	18Å
Major groove	Narrow and deep	Wide and deep	Flat
Minor groove	Wide and shallow	Narrow and deep	Narrow and deep

Triplex DNA structure

In certain circumstances (e.g., low pH), a DNA sequence containing a long segment consisting of a polypurine strand, hydrogen bonded to a polypyrimidine strand and form a *triple helix*. The triple helix will be written as (*dT*).(dA).(dT) with the third strand in italics. Triple-stranded DNA is formed by laying a third strand into the major groove of DNA. Third strand makes hydrogen bond to another surface of the duplex. The third strand pairs in a Hoogsteen base-pairing scheme. The central strand of the triplex must be purine rich. Thus, triple-stranded DNA requires a homopurine: homopyrimidine region of DNA. If the third strand is purine rich, it forms *reverse Hoogsteen* hydrogen bonds in an antiparallel orientation with the purine strand of the Watson-Crick helix. If the third strand is pyrimidine rich, it forms *Hoogsteen bonds* in a parallel orientation with the Watson-Crick-paired purine strand.

Triple helix can be intermolecular or intramolecular. In the *intermolecular Pu*.Pu.Py triple helix, the polypurine third strand is organized antiparallel with respect to the purine strand of the original Watson-Crick duplex. In the intermolecular *Py*.Pu.Py triplex, the polypyrimidine third strand is organized parallel with respect to the purine strand and the phosphate backbone is positioned.

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An *intramolecular triplex* (also referred to as **H-DNA**) could form within a single homopurine.homopyrimidine duplex DNA region in supercoiled DNA. As in intermolecular triplexes, when the third strand is the pyrimidine strand, it forms Hoogsteen pairs in a parallel fashion with the central purine strand. When the third strand is the purine strand, it forms reverse Hoogsteen pairs in an antiparallel fashion with the central purine strand.

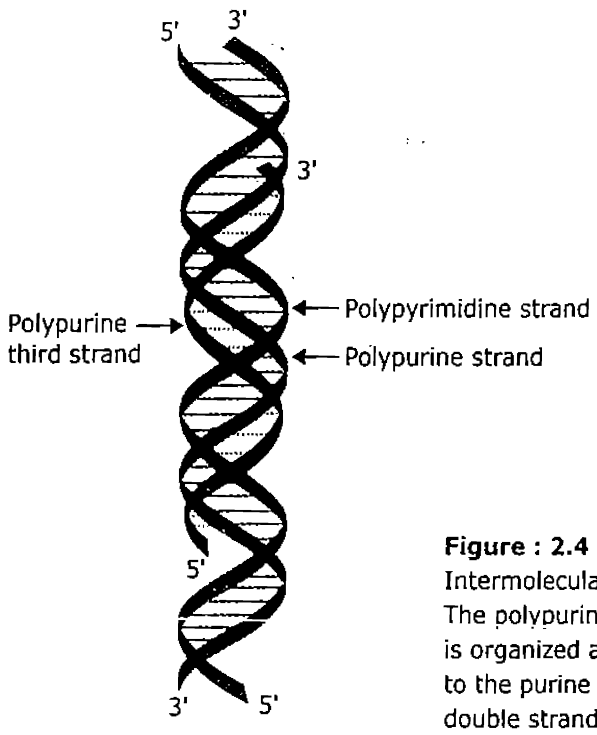


Figure : 2.4
Intermolecular *Pu*.Pu.Py triple helix.
The polypurine third strand (black coloured) is organized antiparallel with respect to the purine strand of the original double strand DNA.

Quadruplex structure

Nucleic acid sequences which are rich in guanine are capable of forming four-stranded structures called *G-quadruplexes* (also called G-quartat). These consist of a square arrangement of guanines (a tetrad), stabilized by Hoogsteen hydrogen bonding. The formation and stability of G-quadruplexes is monovalent cation-dependent. A monovalent cation present in the center of the tetrads. G-quadruplexes can be formed of DNA or RNA. They can be formed from one, two or four separate strands of DNA or RNA. Depending on the direction of the strands or parts of a strand that form the tetrads, structures may be described as parallel or antiparallel. All parallel quadruplexes have all guanine glycosidic angles in an *anti* conformation. Anti-parallel quadruplexes have both *syn* and *anti* conformations.

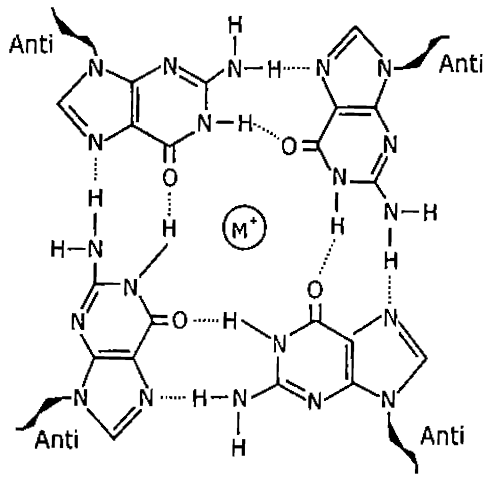


Figure : 2.5
Four-stranded structures can arise from square arrangement of guanines.

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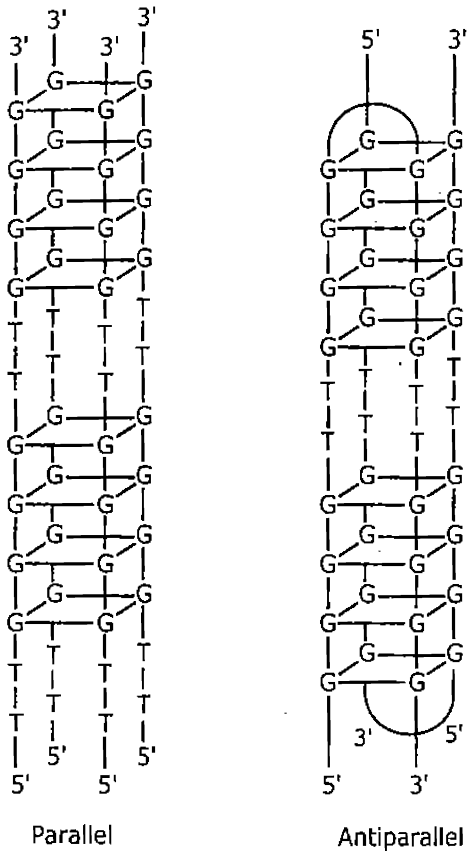


Figure : 2.6
G-Quadruplex DNA.
Quadruplex structures
may be parallel or antiparallel.

Stability of the double helical structure of DNA

First : Internal and external hydrogen bonds stabilize the double helix. The two strands of DNA are held together by H-bonds that form between the complementary purines and pyrimidines, two in an A:T pair and three in a G:C pair, while polar atoms in the sugar-phosphate backbone form external H bonds with surrounding water molecules.

Second : The negatively charged phosphate groups are all situated on the exterior surface of the helix in such a way that they have minimal effect on one another and are free to interact electrostatically with cations in solution such as Mg^{2+}.

Third : The core of the helix consists of the base pairs, which, in addition to being H-bonded, stack together through hydrophobic interactions and van der waals forces that contribute significantly to the overall stability.

2.3 THERMAL DENATURATION AND HYPERCHROMIC SHIFT

When duplex DNA molecules are subjected to conditions of pH, temperature, or ionic strength that disrupt hydrogen bonds, the strands are no longer held together. That is, the double helix is **denatured** and the strands separate as individual random coils. If temperature is the denaturing agent, the double helix is said to **melt**. This dissociation increases the relative absorbance of the DNA solution at 260-nm (as much as 40% as the bases unstack). This absorbance increase, or **hyperchromic shift**, is due to the fact that the aromatic bases in DNA interact via their π-electron clouds when stacked together in the double helix. Because the ultraviolet absorbance of the bases is a consequence of π-electron transitions, and because the potential for these transitions is diminished when the bases stack, the bases in duplex DNA absorb less 260-nm radiation than expected for their numbers. The rise in absorbance coincides with strand separation, and the midpoint of the absorbance increase is termed the melting temperature, T_m. Temperature at which the change in A_{260} is half maximal is called T_m.

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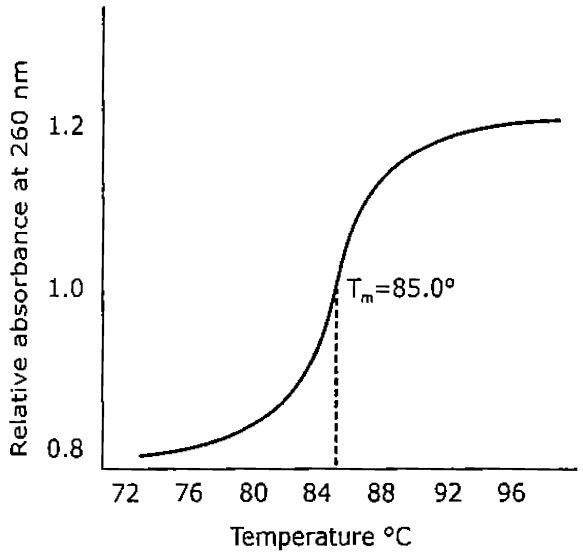


Figure : 2.7
Thermal denaturation of DNA. The DNA absorbs more ultraviolet radiation at 260 nm as the double helix is denatured. The transition is quite abrupt, and the temperature of the midpoint, Tm, is proportional to the GC content of the DNA. Although the denatured DNA can be renatured by a slow cooling, the process does not follow a similar curve. Renaturation becomes progressively more complete at temperatures well below the Tm, and then only after a considerable incubation time.

Factors affecting Tm

GC-content

Double-stranded DNA with a high GC-content has a higher Tm than DNA with a lower GC-content. The higher the G + C content of a DNA, the higher its melting temperature because G:C pairs are held by three H-bonds whereas A:T pairs have only two.

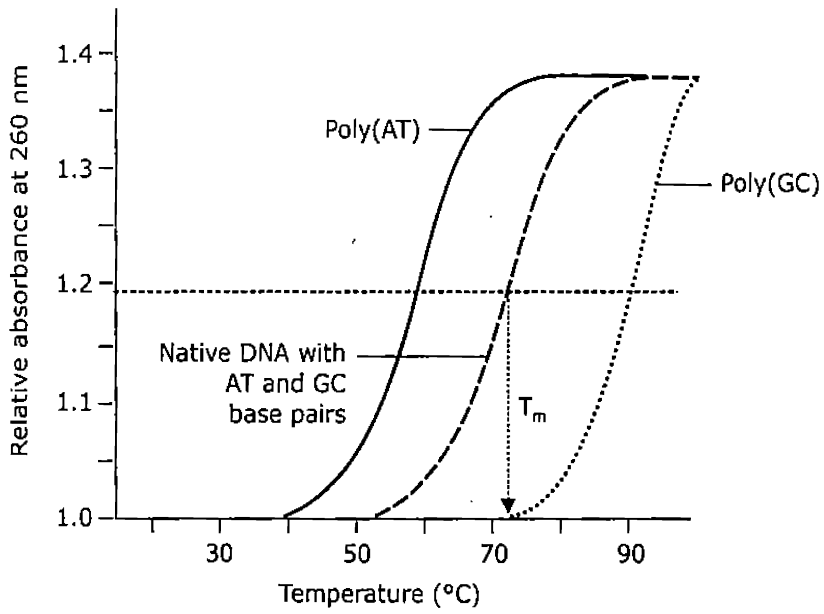


Figure : 2.8
Melting curve for denaturation of DNA. The temperature at which DNA is half unwound is the melting temperature, T_m, of the DNA.

Ionic strength

Tm is also dependent on the ionic strength of the solution; the lower the ionic strength, the lower the melting temperature. DNA is a polyanionic molecule. The salt shields the negative charges on each phosphate. When the charges are not shielded, the electrostatic repulsion makes it energetically more favorable to separate the strands.

pH

Change in the pH also affects the Tm. At pH values greater than 10, extensive deprotonation of the bases occurs, destroying their hydrogen bonding potential and denaturing the DNA duplex. Similarly extensive protonation of the bases below pH 3 also disrupts base pairing.

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2.4 QUANTIFICATION OF NUCLEIC ACIDS

Quantification of nucleic acids by Ultraviolet Spectroscopy

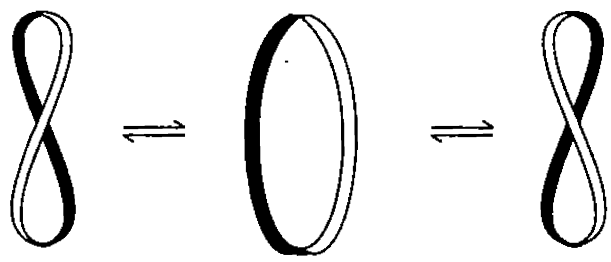
Nucleic acids show a strong absorbance in the region of 240 to 275 nm. It originates from the $\pi \rightarrow \pi^*$ transitions of the pyrimidine and purine ring systems of the nucleobases. The bases can be protonated, and therefore the spectra of DNA and RNA are sensitive to pH. At neutral pH, the absorption maxima range from 253 nm (for guanosine) to 271 nm (for cytidine), and therefore polymeric DNA and RNA show a broad and strong absorbance near 260 nm. At this wavelength absorption is proportional to nucleic acid concentration. This relationship is so well characterized that UV absorption is used to accurately determine the concentration of nucleic acids in solution. The relationship between DNA concentration and absorption is linear.

The absorbance at 260 nm is used to determine the concentration of nucleic acids. Amounts of nucleic acid are often given as A_{260} units. For double-stranded DNA, one A_{260} unit is equivalent to 50 µg DNA (i.e. a solution of DNA with a concentration of 50 µg/mL will have an absorbance at 260nm equal to 1.0); for single-stranded DNA, it is equivalent to 33 µg DNA; for single-stranded RNA, it is equivalent to 40 µg RNA.

2.5 SUPERCOIL FORMS OF DNA

Supercoiling represents a twisting of the DNA double helix upon itself. Supercoiling can arise in any helical or coiled structure in which the two ends are constrained in some way. In a double helical molecule of DNA, the two polynucleotide backbone chains have rotational freedom about one another as long as the ends are not fixed. If any part of a linear molecule of DNA is locally twisted, then that twist can be redistributed throughout the molecule and it can reassume its most stable (Watson-Crick) conformation. However, if the ends of the molecule are fixed then any rotational freedom that the two backbones might have about one another is removed and as a result any local twisting or untwisting will cause a stress on the structure. It occurs in circular DNAs and in DNAs that are topologically constrained by being complexed with protein.

If a circular DNA molecule in a *relaxed* conformation is broken across both strands and one or more additional right-handed helical turns are inserted before rejoining the ends, the molecule would twist on itself to form a *positive supercoil*. If, on the other hand, the helix is unwound (given left-handed turns) before rejoining, the result is a *negative supercoil*.



Negative supercoil Relaxed conformation Positive supercoil

In order to understand the origin of supercoiling; imagine a linear DNA 5200 base pairs in length. If the DNA were in the B-form one would expect the two strands of the helix to be wrapped around each other 500 times (5200 bp/10.4 bp/turn). Imagine a linear DNA in which the two ends become connected to form an open circle. This is referred to as a *relaxed* circular DNA. On the other hand, if the linear DNA were unwound 10%, say 50 turns, before its ends were joined, then the DNA molecule would be under stress. When the molecule is free in solution it will coil about itself in space as the two strands simultaneously twist about each other in order to return to equilibrium value of 10.4 base pairs per turn.

DNA that is underwound is referred to as *negatively* supercoiled. The helices wind about each other in a right handed path in space. DNA that is overwound will also relax and assume a supercoiled conformation

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but this is referred to as a *positively* supercoiled DNA helix. Positively coiled DNA has its DNA helices wound around each other in a left-handed path in space.

Linking number

Supercoiling in circular DNA molecules is described mathematically by the number of times the two phosphodiester backbones wrap around one another in a given distance. This quantity is the **Linking number** (L_k). So linking number is defined as the number of times one strand crosses over the other in space in closed circular DNA. Because the entire molecule will be a closed circle, the linking number will always be an integer. Hence a B-DNA molecule of 208 bp in length, will have a linking number of 20.

Features related to linking number

1. The linking number is only defined for covalently closed DNA and its value is fixed as long as the molecule remains covalently closed.
2. The linking number does not change whether the covalently closed circle is forced to lie in a plane in a stressed conformation or whether it is allowed to supercoil about itself freely in space.
3. The linking number L_k of a circular DNA can only be changed by breaking a phosphodiester bond in one of the two strands, allowing the intact strand to pass through the broken strand and then rejoining the broken strand.
4. L_k is always an integer since two strands must always be wound about each other an integral number of times upon closure.

Twisting number and **Writhing number**

Linking number is made up of two components- **Twisting number** (represent total number of helical turns in DNA) and **Writhing number** (represents the supercoiling of the helix in space).

Linking number can be mathematically expressed in following form $\mathbf{L_k = Tw + Wr}$

In the relaxed DNA, the linking number equals the twist number ($L_k = Tw$) and $Wr = 0$. The linking of relaxed DNA, L_{k0}, is defined as $L_{k0} = N/10.4$; Where N is the number of base pairs in the DNA molecule and 10.4 refers to the helical repeat. The level of supercoiling may be quantified in terms of the change in linking number (ΔL_k) from that of the relaxed closed circular molecule (ΔL_{k0}).

Negatively supercoiled DNA

DNA is said to be negatively supercoiled when it has a deficiency in the linking number compared with relaxed DNA, or $L_k < L_{k0}$. Negatively supercoiled DNA is underwound because it has fewer helical turns than the molecule would contain as a linear or relaxed molecule. This underwinding in the number of helical turns results in more base pairs per helical turn.

Positively supercoiled DNA

Most DNA isolated from natural sources is negatively supercoiled. However, DNA can exist in a positively supercoiled form when it has a greater linking number than relaxed DNA, or $L_k > L_{k0}$. Positively supercoiled DNA is overwound in terms of the number of helical turns. This overwinding creates a situation in which there are fewer base pairs per helical turn. The tension in the winding of the helix is relieved by the positive supercoiling of the DNA forming a left-handed supercoil. Few archaebacterium living at high temperature and low pH, has been shown to contain positive supercoils.

Super helix density of DNA

Superhelical density, σ, is a quantitative measure of the intensity of supercoiling. Mathematically

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it can be expressed as

$\sigma = \Delta Lk/Lk_0$

ΔLk = change in linking number = $Lk - Lk_0$

Lk_0 = linking number of DNA in the relaxed state

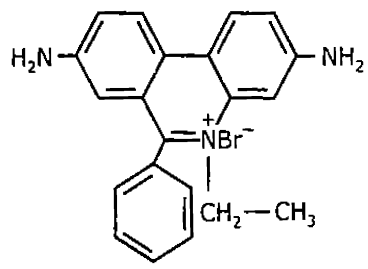
Circular DNA from bacteria and eukaryotes are usually negatively supercoiled having value of σ about –0.06 i.e., degree of underwinding is about 6%. The negative sign indicates that the change in linking number is due to underwinding of the DNA. The supercoiling induced by underwinding is therefore defined as negative supercoiling.

In vivo change in linking number

Linking number *in vivo* is regulated by enzymes called *topoisomerases* that transiently break and reseal the DNA double helix. Type I topoisomerases break only one strand of the DNA, allowing one strand to rotate around the other. Type II topoisomerases break and reseal both DNA strands. Correspondingly, the linking number changes in increments of 1 and 2 for type I and II topoisomerases, respectively. In bacterial cells the level of supercoiling is carefully maintained by topoisomerase I, that relaxes supercoils, and topoisomerase II (gyrase), that introduces negative supercoils.

Effect of EtBr on supercoiling

In B-form DNA, with 10.4 base pairs per turn, two adjacent base pairs are rotated with respect to each other by 36° (360° per complete turn). One molecule of ethidium bromide (EtBr) creates about 27° of unwinding, affecting the hydrodynamic properties of circular DNA.



Ethidium bromide (EtBr)

EtBr is a fluorescent dye generally used to stain DNA molecule for their visualization. EtBr binding decreases the number of turns needed to create an unconstrained duplex; it decreases Tw, the twist. Since Lk doesn't change unless the molecule is broken, the writhe, Wr component is affected. The number of turns devoted to writhe increases. In other words, EtBr increases the positive superhelicity of the molecule. Thus, an underwound molecule tends to become relaxed, because it loses negative superhelicity, whereas a relaxed closed duplex becomes overwound, and an overwound molecule becomes more so.

2.6 DNA : A GENETIC MATERIAL

DNA contains the genetic instructions specifying the biological development of all cellular forms of life, and many viruses. DNA acts as genetic material and used to store the genetic information of an organic life form. DNA encodes the sequence of the amino acid residues in proteins using the genetic code, a triplet code of nucleotides. For all currently known living organisms, the genetic material is almost exclusively DNA. Some viruses use Ribonucleic acid as their genetic material.

The first genetic material is generally believed to have been RNA, initially manifested by self-replicating RNA molecules floating on bodies of water. This hypothetical period in the evolution of cellular life is known as the *RNA world*. This hypothesis is based on RNA's ability to act both as genetic material and as a catalyst, known as ribozyme or a ribosome. However, once proteins, which can form enzymes, came into

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existence, the more stable molecule DNA became the dominant genetic material, a situation continued today. Not only does DNA's double-stranded nature allow for correction of mutations but RNA is inherently unstable. Modern cells use RNA mainly for the building of proteins from DNA instructions, in the form of messenger RNA, ribosomal RNA, and transfer RNA.

GRIFFITH'S EXPERIMENT

In 1928, Griffith experimented with virulence in *Pneumococcus*. During the 1920s Frederick Griffith studied the difference between a disease-causing strain of the pneumonia causing bacteria (*S. peumoniae*) and a strain that did not cause pneumonia. The pneumonia-causing strain (the S strain) was surrounded by a capsule. The other strain (the R strain) did not have a capsule and also did not cause pneumonia.

Serotype	*Morphology*	*Capsule*	*Virulence*
IIR	Rough	Absent	Avirulent
IIIS	Smooth	Present	Virulent

Griffith injected the different strains of bacteria into mice. The S strain killed the mice; the R strain did not. He further noted that if heat killed S strain was injected into a mouse, it did not cause pneumonia. When he combined heat-killed S with live R and injected the mixture into a mouse (remember neither alone will kill the mouse) that the mouse developed pneumonia and died. Bacteria recovered from the mouse had a capsule and killed other mice when injected into them.

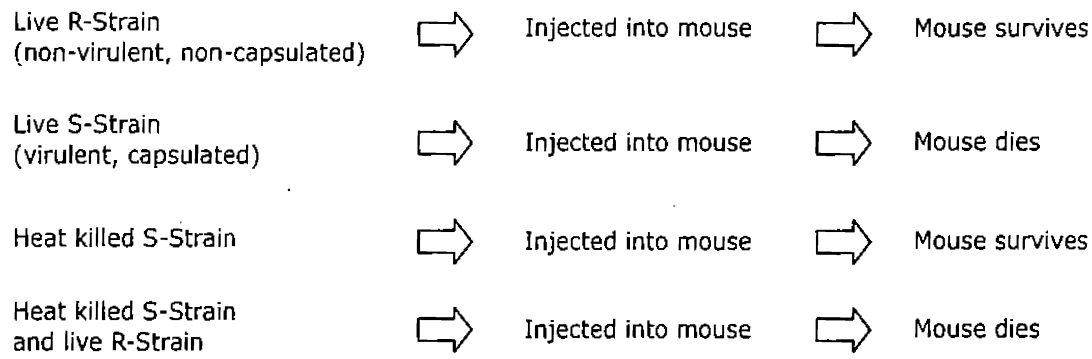


Figure 2.9 : Griffith's demonstration of transformation in pneumococcus.

Frederick Griffith (1928) was able to induce a nonpathogenic strain of the bacterium *Streptococcus pneumoniae* to become pathogenic. He determined that nonvirulent strains (rough-strain) could be transformed (genetically changed) to virulent (smooth) strains if the remains of dead virulent bacteria were made available to the living nonvirulent bacteria. Griffith called the genetic information which could be passed from one bacteria to another the **transforming principle**.

In 1944, Oswald Avery, Colín MacLeod, and Maclyn McCarty revisited Griffith's experiment and concluded that the transforming material was pure DNA not protein, lipid or carbohydrate.

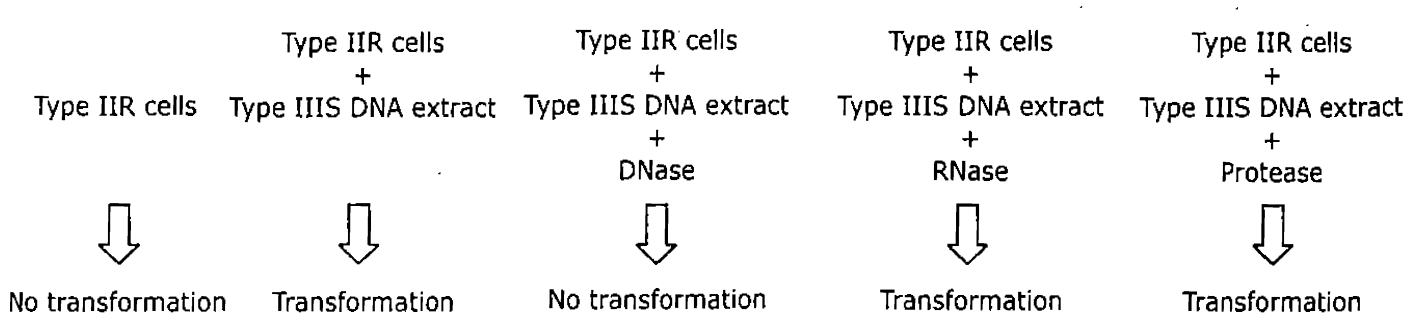


Figure 2.10 : Avery, MacLeod, and McCarty had shown that only DNA extracts from S cells caused transformation of R cells to S cells. To demonstrate that contaminating molecules in the DNA extract were not responsible for transformation, the DNA extract from S cells was treated with RNase, DNase, and protease and then mixed with R cells.

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HERSHEY-CHASE EXPERIMENT

The Hershey-Chase experiment was conducted in 1952 by Alfred Hershey and Martha Chase that identified *DNA to be the genetic material of phages*. A phage is a small virus that infects bacteria. It consists of a protein coat that encloses the genetic material. When a phage infects a bacterium, it inserts its genetic material into the bacterium, while its coat remains outside.

In a first experiment, T2 phages with radioactive ^{32}P-labeled DNA infected bacteria. In a second experiment, T2 phages with radioactive ^{35}S-labeled protein infected bacteria. In both experiments, bacteria were separated from the phage coats by blending followed by centrifugation. In the first experiment, most radioactivity was found in the infected bacteria, while in the second experiment most radioactivity was found in the phage coat. These experiments demonstrated that DNA is the genetic material of phage and that protein does not transmit genetic information.

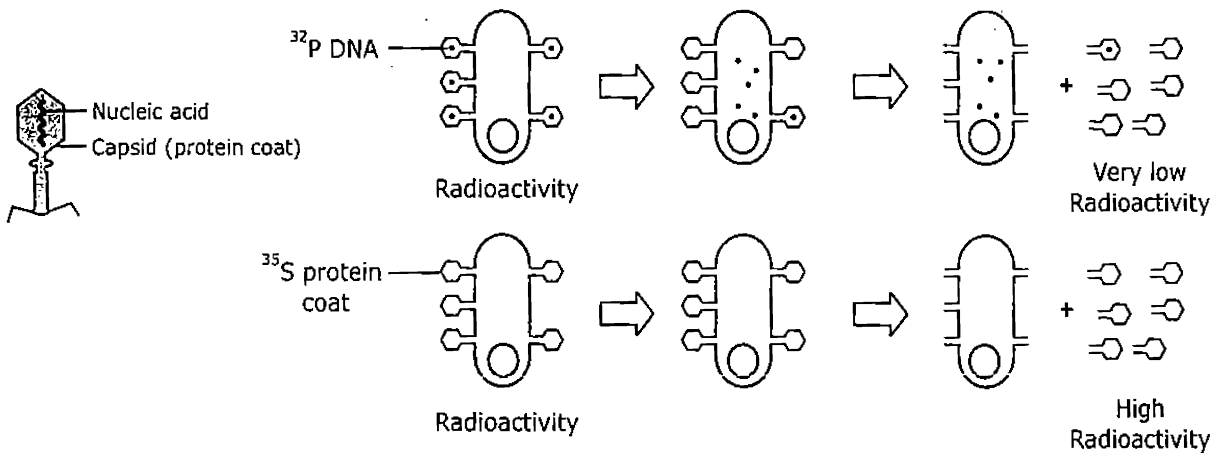


Figure 2.11 : The Hershey-Chase experiment

Hershey shared the 1969 Nobel Prize in Physiology or Medicine for his *discoveries concerning the genetic structure of viruses*.

2.7 STRUCTURE AND TYPES OF RNA

DNA contains all the information needed to maintain a cell's processes, but these precious blueprints never leave the protected nucleus. How, then, is all this data transmitted to the body of the cell itself where it may be put to use? The answer: by way of RNA.

RNA molecules play essential roles in the transfer of genetic information during protein synthesis and in the control of gene expression. The diverse functions of RNA molecules in living organisms also include the enzymatic activity of ribozymes and the storage of genetic information in RNA viruses and viroids. So, ribonucleic acid may be genetic or non genetic, catalytic or non-catalytic and coding (mRNA) or noncoding (like tRNA, rRNA).

Thermodynamic stability of RNA structure

Primary structure of RNA refers to the sequence of nucleotides. Secondary structure in RNA is dominated by Watson-Crick base pairing. This fundamental interaction between nucleobases leads to the formation of double-helical structures of varying length. In RNA, double-helical tracts are generally short. RNA double helices adopt the A-form structure, which differs significantly from the canonical B-form adopted by DNA double helices. RNA secondary structure is generally more stable than its tertiary structure. Thus, formation of the secondary structure dominates the process of RNA folding. RNA tertiary structure forms through relatively weak interactions between preformed secondary structure elements.

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TYPES OF RNA

Within a given cell, RNA molecules are found in multiple copies and in multiple forms. Major RNA classes are rRNA, tRNA, snRNA, SnoRNA, miRNA, XIST, scRNA, siRNA, tmRNA and telomerase RNA. Features of few major forms of RNA present in prokaryotic and eukaryotic cells are given below.

tRNA

tRNA are small, well-characterized RNA molecules with a key role in **protein biosynthesis.** Transfer RNA also known as adaptor RNA. The concept of an adaptor to provide the interface between nucleic acid language and protein language was introduced by Crick in 1955. tRNAs also participate in nonprotein synthetic capacities. They prime reverse transcription in retrovirus life cycles.

Structural properties

tRNA are single RNA chains of 75-93 nucleotides, present in the cytosol and organelles of all living cells. Holley and his co-workers determined the first tRNA sequence in 1965. Dictated by their primary sequence, tRNA fold into cloverleaf-like secondary structures with well-defined stems and loops that make up the acceptor arm, D arm and loop, anticodon arm and loop, and the T arm and loop. Regardless of the length of the tRNA, the numbering of conserved nucleotides remain constant.

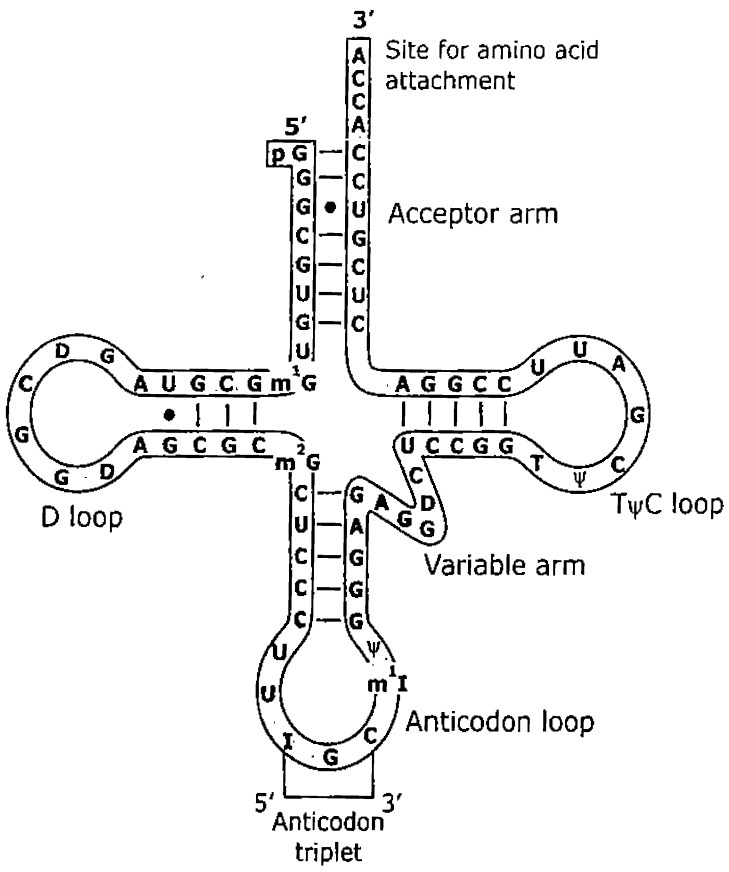


Figure : 2.12
Nucleotide sequence of yeast $tRNA^{Ala}$.
This structure was deduced in 1965 by Robert W. Holley and his colleagues; it is shown in the cloverleaf conformation in which intrastrand base pairing is maximal. The following symbols are used for the modified nucleotides :
ψ, pseudouridine; I, inosine; T, ribothymidine; D-5, 6-dihydrouridine.

The acceptor stem always has 7 base pairs and 4 single-stranded nucleotides, including an absolutely conserved CCA sequence. The D stem and loop are of variable length, whereas the anticodon stem has 5 nucleotides and the anticodon loop has 7 nucleotides. The variable region usually has 4-5 nucleotides but can contain up to 24 nucleotides. Finally, the T stem always has 5 base pairs and the T loop has 7 nucleotides. All tRNAs share the same secondary structure. tRNA are classified according to the length of their variable region, which can be 4 or 5 nucleotides (class I tRNA) or 10-24 nucleotides (class II tRNA). A new class II tRNA, discovered more recently in many organisms, serves as the adaptor for **selenocysteine**, the 21st amino acid. tRNA are thought to fold into the L-shaped 3D structures necessary to fulfill their functions. All tRNAs also share the same L-shaped three-dimensional structure. In each cell or organelle, there are 20 families of tRNA, one per

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each amino acid. Isoacceptor tRNAs belong to the same family and are charged with the same amino acid during protein synthesis.

rRNA

Ribosomal RNA is a component of the ribosomes, the protein synthesis factories in the cell. Eukaryotic ribosomes contain four different rRNA molecules: 18s, 5.8s, 28s, and 5s rRNA (16s rRNA, 5s rRNA and 23s rRNA in *E. coli*). Three of the rRNA molecules are synthesized in the nucleolus, and one is synthesized elsewhere. rRNA molecules are extremely abundant. They make up at least 80% of the RNA molecules found in a typical eukaryotic cell.

Synthesis of the three nucleolar rRNA molecules is unusual because they are made on one primary transcript that is chopped up into three mature rRNA molecules. These rRNA molecules and the 5s rRNA combine with the ribosomal proteins in the nucleolus to form pre 40s and pre 60s ribosomal subunits. These pre-subunits are exported to the nucleus where they mature and assume their role in protein synthesis.

The rRNA molecules have several roles in protein synthesis. First, the 28s rRNA has a catalytic role, it forms part of the *peptidyl transferase activity* of the 60s subunit. Second, 18s rRNA has a recognition role, involved in correct positioning of the mRNA and the peptidyl tRNA.

snRNA

snRNA (small nuclear RNA) is a class of small RNA molecules that are found within the nucleus of eukaryotic cells. They are involved in a variety of important processes such as RNA splicing (removal of **introns** from hnRNA) and maintaining the telomeres. They are always associated with specific proteins, and the complexes are referred to as *small nuclear ribonucleoproteins* (snRNP) or sometimes as snurps.

snoRNA

snoRNA (small nucleolar RNA) is a class of small RNA molecules that are involved in chemical modifications of ribosomal RNAs (rRNAs) and other forms of RNA, for example by *methylation*. snoRNAs are a component in the small nucleolar ribonucleoprotein (snoRNP), which contains snoRNA and proteins. The snoRNA guides the snoRNP complex to the modification site of the target RNA gene via sequences in the snoRNA that hybridize to the target site. The proteins then catalyze modification of the RNA gene.

gRNAs

gRNAs (guide RNA) are RNA genes that function in RNA editing. Thus far, RNA editing has been found only in the mitochondria of *kinetoplastids*, in which mRNAs are edited by inserting or deleting stretches of *uridylates* (Us). The gRNA forms part of the **editosome** and contains sequences that hybridize to matching sequences in the mRNA, to guide the mRNA modifications.

miRNA

A **miRNA** (micro-RNA) is a form of single-stranded RNA which is typically 20-25 nucleotides long, and is thought to regulate the expression of other genes. The DNA sequence that codes for an miRNA gene is longer than the miRNA. This DNA sequence includes the miRNA sequence and an approximate reverse complement. When this DNA sequence is transcribed into a single-stranded RNA molecule, the miRNA sequence and its reverse-complement base pair to form a double stranded RNA hairpin loop; this forms a primary miRNA structure (pri-miRNA). Drosha, a nuclear enzyme, cleaves the base of the hairpin to form pre-miRNA. The pre-miRNA molecule is then actively transported out of the nucleus into the cytoplasm by Exportin protein. The **Dicer enzyme** then cuts 20-25 nucleotides from the base of the hairpin to release the mature miRNA.

The function of miRNAs appears to be in gene regulation. For that purpose, a miRNA is complementary to a part of one or more messenger RNAs (mRNAs), usually at a site in the 3' UTR. The annealing of the miRNA to

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the mRNA inhibits protein translation. In some cases, the formation of the double-stranded RNA through the binding of the miRNA triggers the degradation of the mRNA transcript through a process similar to **RNA interference** (RNAi), though in other cases it is believed that the miRNA complex blocks the protein translation machinery or otherwise prevents protein translation without causing the mRNA to be degraded.

tmRNA

tmRNA has a complex structure with tRNA-like and mRNA-like regions. It has currently only been found in bacteria, but is ubiquitous in all bacteria. tmRNA recognizes ribosomes that have trouble translating or reading an mRNA and stall, leaving an unfinished protein that may be detrimental to the cell. tmRNA acts like a tRNA first, and then an mRNA that encodes a peptide tag. The ribosome translates this mRNA region of tmRNA and attaches the encoded peptide tag to the C-terminus of the unfinished protein. This attached tag targets the protein for destruction or proteolysis.

Alkali-catalyzed cleavage of RNA

Under alkaline conditions, RNA is hydrolyzed rapidly and generates a mixture of 2′- and 3′ nucleoside monophosphate. In the presence of a hydroxide ion, the 2′-hydroxyl group of the ribose is converted to a 2′-alkoxide ion. The 2′-alkoxide attacks the 3′-phosphodiester group, breaking the 5′-3′ phosphodiester bond and forming a cyclic 2′,3′-nucleoside monophosphate. Another hydroxide ion attacks the cyclic 2′,3′-nucleoside monophosphate, yielding a mixture of 2′-and 3′-nucleoside monophosphates. DNA is stable in basic solution because DNA lacks a 2′-hydroxyl group to carry out intramolecule catalysis.

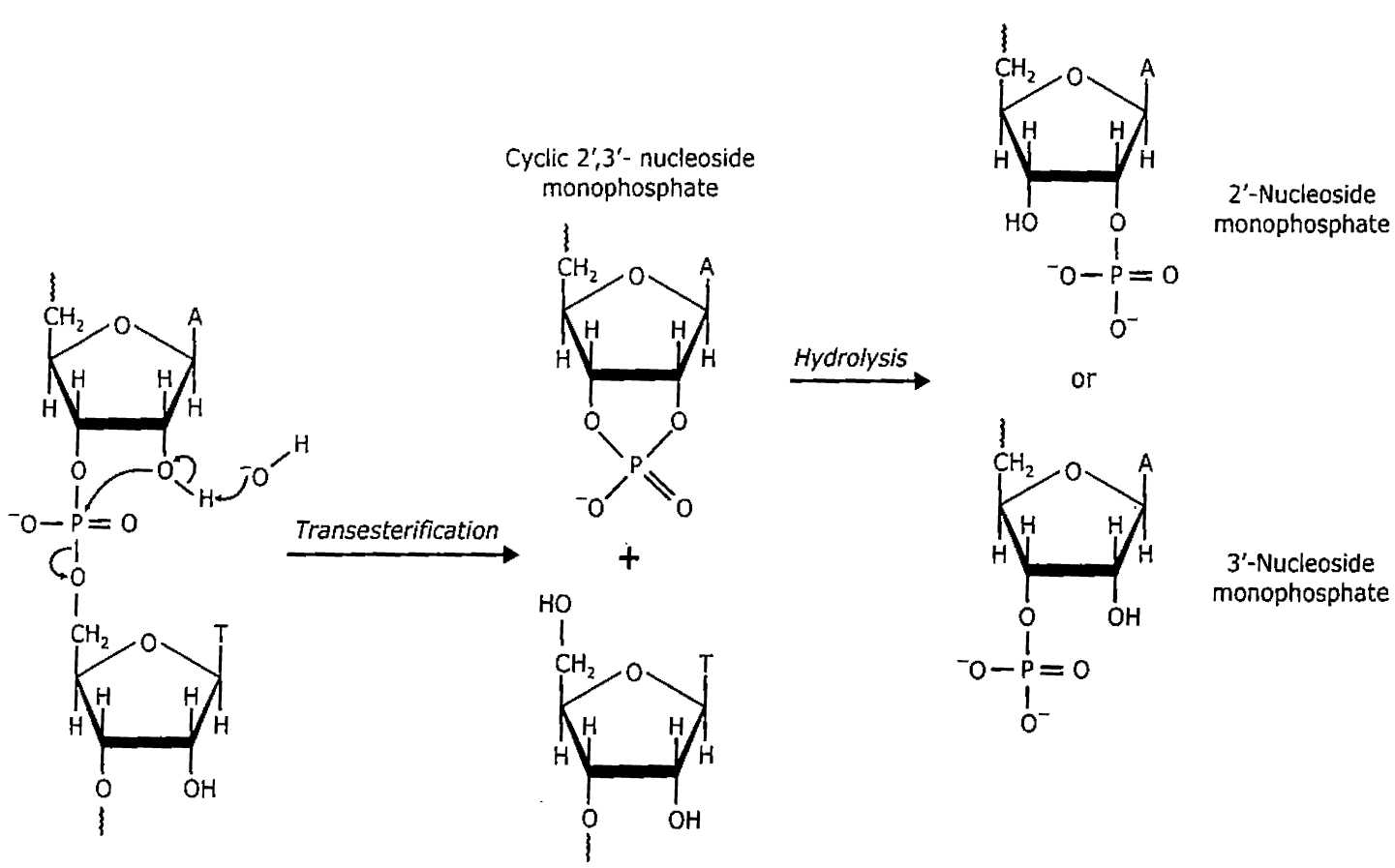


Figure 2.13 : Alkali-catalyzed cleavage of RNA

RNA world hypothesis

The RNA world hypothesis proposes that RNA was actually the first life-form on earth, later developing a cell membrane around it and becoming the first prokaryotic cell (the phrase *RNA World* was first used by *Walter Gilbert* in 1986). This hypothesis is supported by the RNA's ability to store, transmit, and duplicate genetic

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information, just like DNA does. RNA can also act as a ribozyme (an enzyme made of ribonucleic acid). Because it can reproduce on its own, performing the tasks of both DNA and proteins (enzymes), RNA is believed to have once been capable of independent life.

RNA as genetic material

Some viruses contain an RNA as genetic material. One of the first experiments that established RNA as the genetic material in RNA viruses was the *reconstitution experiment* of H.Fraenkel-Conrat and B.Singer. They took two different strains of TMV, separated the RNAs from their protein coats, and reconstituted *hybrid* viruses by mixing the proteins of one strain with the RNA of the second strain, and vice versa. When the *hybrid* virus was spread on tobacco leaves, the lesions that developed corresponded to the TMV from which the RNA had been obtained. It was concluded that RNA serves as the genetic material in TMV.

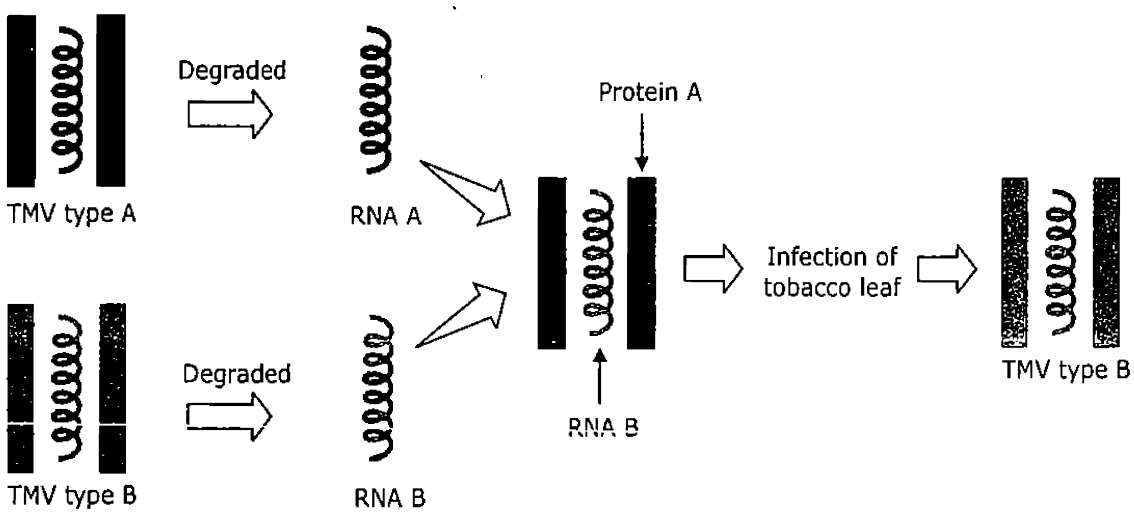


Figure 2.14 : Proof that the genetic material of tobacco mosaic virus (TMV) is RNA, not protein.

2.8 NUCLEOTIDE SYNTHESIS

The pathways for the biosynthesis of nucleotides fall into two classes: *de novo pathways* and *salvage pathways*.

In *de novo* pathways, the nucleotide bases are assembled from simpler compounds. The framework for a *pyrimidine* base is assembled first and then attached to ribose. In contrast, the framework for a *purine* base is synthesized piece by piece directly onto a ribose-based structure.

In *salvage pathways*, preformed bases are recovered and reconnected to a ribose unit.

Salvage pathway	***de novo* pathway**
Activated ribose (PRPP) + Base	PRPP + Amino acids + ATP + CO_2 + ...
⇩	⇩
Nucleotide	Nucleotide

Both *de novo* and *salvage pathways* lead to the synthesis of *ribo*nucleotides. All deoxyribonucleotides are synthesized from the corresponding ribonucleotides. The deoxyribose sugar is generated by the reduction of ribose within a fully formed nucleotide. Furthermore, the methyl group that distinguishes the thymine of DNA from the uracil of RNA is added at the last step in the pathway.

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de novo Synthesis

Pyrimidine ribonucleotide

In *de novo* synthesis of pyrimidines, the ring is synthesized first and then it is attached to ribose to form a *pyrimidine nucleotide*. In *de novo* synthesis, the pyrimidine ring is assembled from *bicarbonate, aspartate*, and *glutamine*.

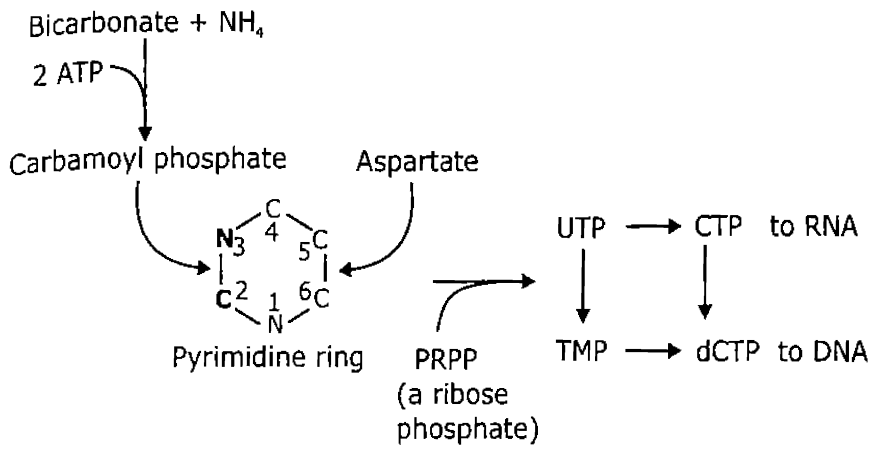


Figure : 2.15
de novo pathway for pyrimidine nucleotide synthesis. The C-2 and N-3 atoms in the pyrimidine ring come from carbamoyl phosphate, whereas the other atoms of the ring come from aspartate.

Pyrimidine rings are assembled from carbamoyl phosphate and aspartate. Carbamoyl phosphate derived from bicarbonate and ammonia. The first step in *de novo* pyrimidine biosynthesis is the synthesis of *carbamoyl phosphate* from bicarbonate and ammonia in a multistep process, requiring the cleavage of two molecules of ATP. This reaction is catalyzed by *cytosolic carbamoyl phosphate synthetase II (CPS).*

Carbamoyl phosphate reacts with aspartate to form carbamoylaspartate in a reaction catalyzed by *aspartate transcarbamoylase*. Carbamoylaspartate then cyclizes to form dihydroorotate which is then oxidized by NAD^+ to form orotate.

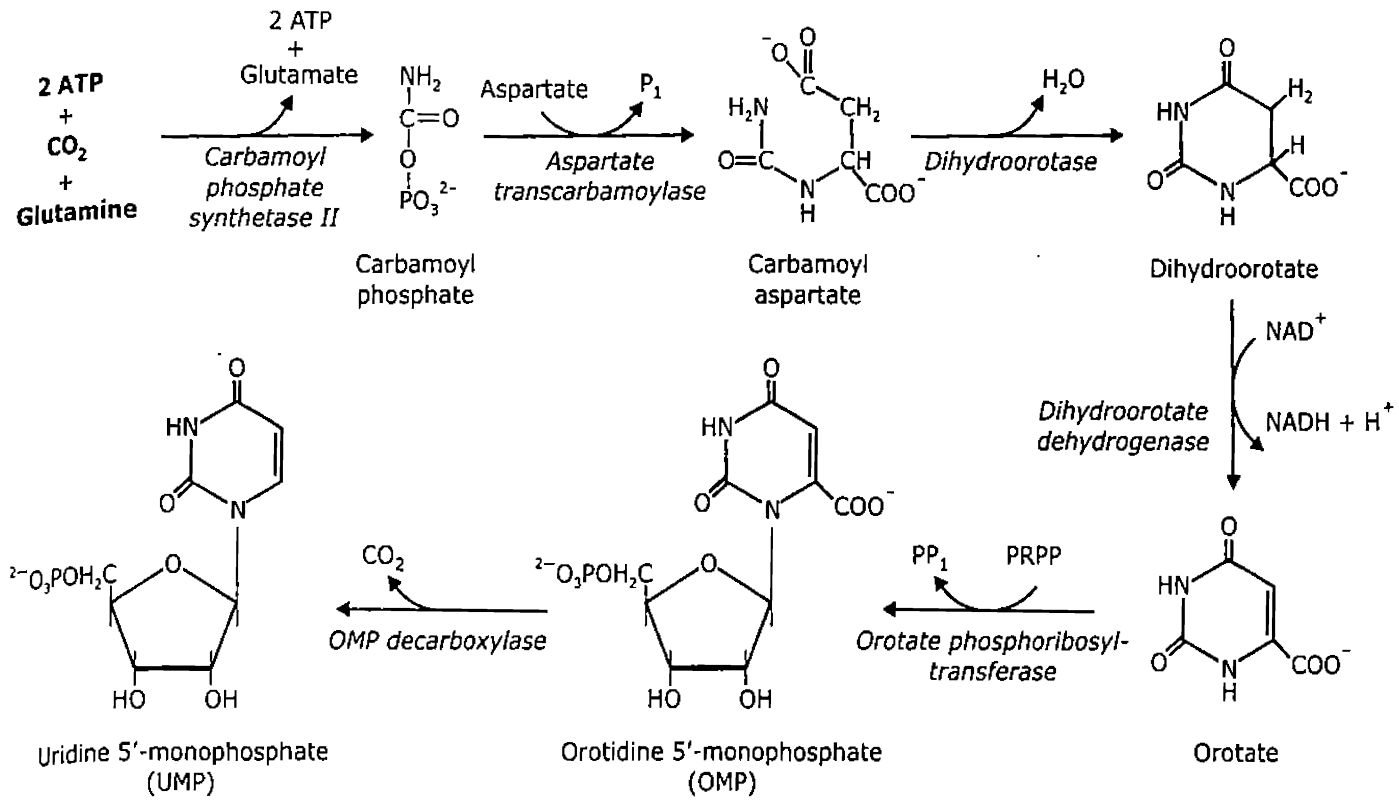


Figure 2.16 : Pyrimidine synthesis.

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Orotate couples to ribose, in the form of *5-phosphoribosyl-1-pyrophosphate (PRPP),* a form of ribose activated to accept nucleotide bases. (PRPP is synthesized from ribose-5-phosphate, formed by the pentose phosphate pathway, by the addition of pyrophosphate from ATP). Orotate reacts with PRPP to form *orotidylate,* a pyrimidine nucleotide. This reaction is driven by the hydrolysis of pyrophosphate. The enzyme that catalyzes this addition, *pyrimidine phosphoribosyltransferase,* is homologous to a number of other phosphoribosyl-transferases that add different groups to PRPP to form the other nucleotides. Orotidylate is then decarboxylated to form *uridylate (UMP),* a major pyrimidine nucleotide that is a precursor to RNA. This reaction is catalyzed by *orotidylate decarboxylase.*

Synthesis of pyrimidine ribonucleotide, cytidine

It is synthesized from the uracil base of UMP, but UMP is converted into UTP before the synthesis can take place. Nucleoside monophosphates are converted into nucleoside triphosphates in stages. First, nucleoside monophosphates are converted into diphosphates then triphosphates. After uridine triphosphate has been formed, it can be transformed into *cytidine triphosphate* by the replacement of a carbonyl group by an amino group.

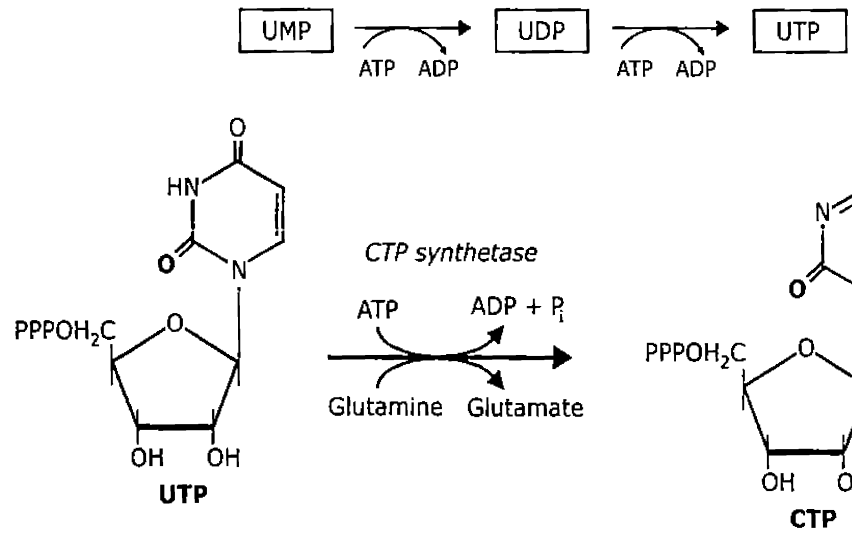
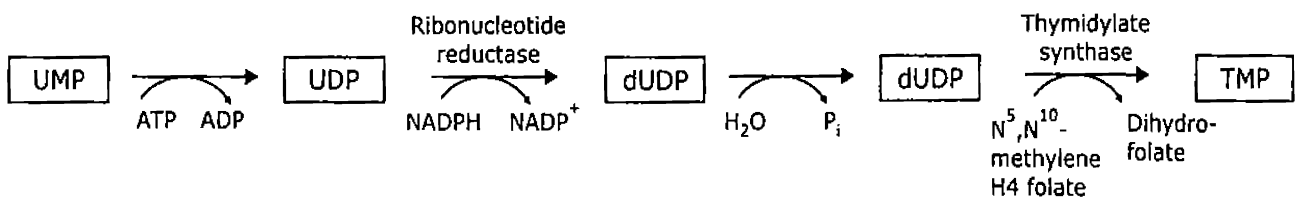


Figure 2.17 : Synthesis of CTP from UMP.

Deoxyribonucleotides are synthesized by the reduction of ribonucleotides

Deoxyribonucleotides, the precursors of DNA, are formed in *E. coli* by the reduction of ribonucleoside diphosphates. These conversions are catalyzed by *ribonucleotide reductase*. Electrons are transferred from NADPH to sulfhydryl groups at the active sites of this enzyme by thioredoxin or glutaredoxin. *TMP is formed by methylation of dUMP*. The donor of a methylene group and a hydride in this reaction is N^5,N^{10}-methylenetetrahydrofolate, which is converted into dihydrofolate. Tetrahydrofolate is regenerated by the reduction of dihydrofolate by NADPH in the presence of enzyme *dihydrofolate reductase*.

Chemotherapeutic agents like **methotrexate** (amethopterin) and **aminopterin** inhibit the activity of *dihydrofolate reductase*. These folate analog act as a competitive inhibitor and used as anticancer drugs.



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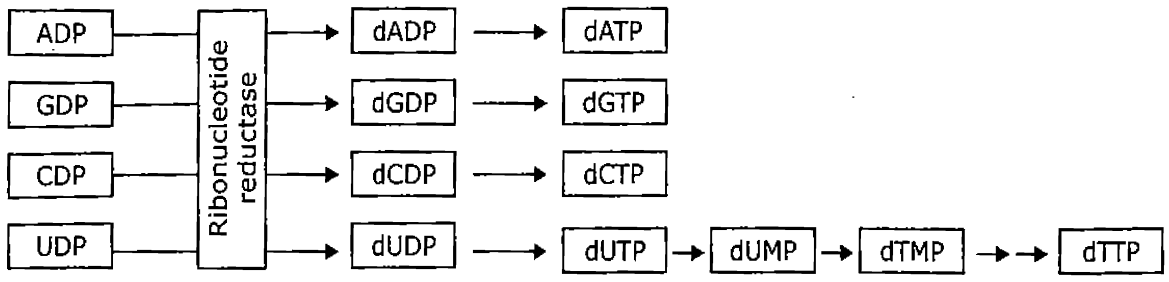


Figure 2.18 : Overview of deoxyribonucleoside triphosphate biosynthesis.

Purine ribonucleotide

The purine ring is assembled from a variety of precursors: glutamine, glycine, aspartate, N^{10}-formyltetrahydrofolate, and CO_2.

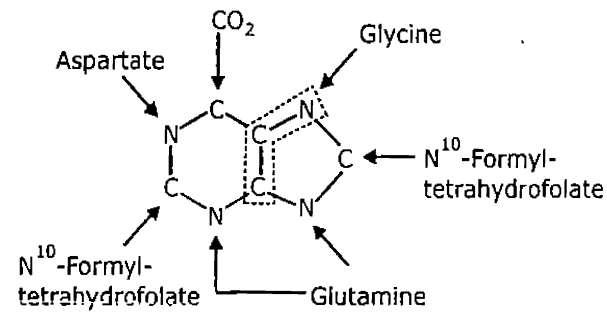
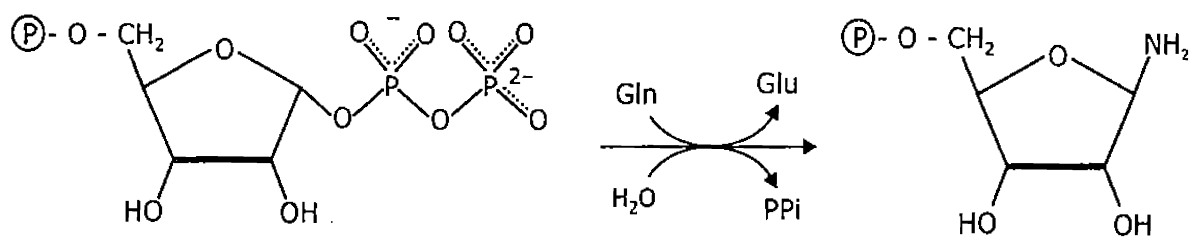


Figure : 2.19
Sources of the individual atoms in the puring ring.

de novo synthesis begins with simple starting materials such as amino acids and bicarbonate. Unlike the case for pyrimidines, the purine bases are assembled already attached to the ribose ring.

De novo purine biosynthesis, like pyrimidine biosynthesis, requires PRPP, but for purines, PRPP provides the foundation on which the bases are constructed step by step. The initial committed step is the displacement of pyrophosphate by ammonia, rather than by a preassembled base, to produce *5-phosphoribosyl-1-amine.*



PRPP *PRPP amidotransferase* 5-Phosphoribosyl-1-amine

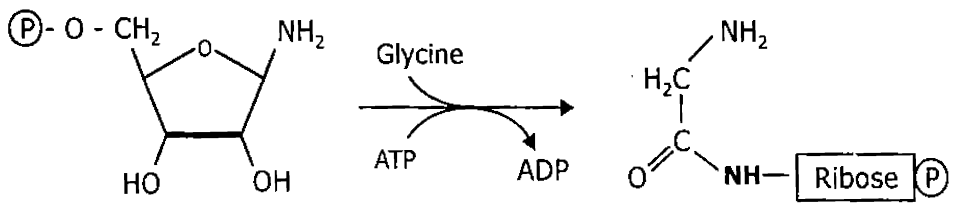
Glutamine phosphoribosyl amidotransferase catalyzes this reaction. To prevent wasteful hydrolysis of either substrate, the amidotransferase assumes the active configuration only on binding of both PRPP and glutamine.

The purine ring is assembled on ribose phosphate, in contrast with the *de novo* synthesis of pyrimidine nucleotides. The addition of glycine, followed by formylation, amination, and ring closure, yields 5-aminoimidazole ribonucleotide. This intermediate contains the completed five-membered ring of the purine skeleton. The addition of CO_2, the nitrogen atom of aspartate, and a formyl group, followed by ring closure, yields inosinate (IMP), a purine ribonucleotide. AMP and GMP are formed from IMP.

de novo purine biosynthesis proceeds as follows:

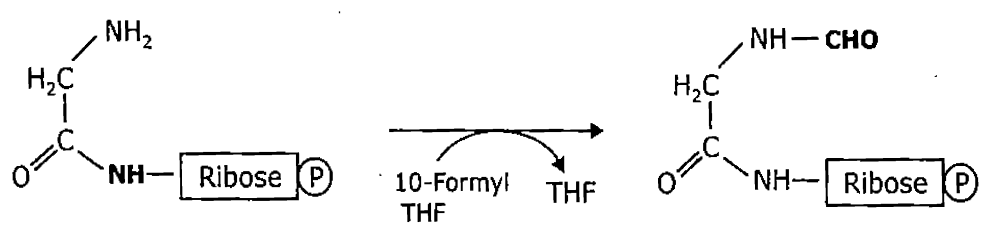
1. The carboxylate group of a glycine residue is activated by phosphorylation and then coupled to the amino group of phosphoribosylamine. A new amide bond is formed while the amino group of glycine is free to act as a nucleophile in the next step.

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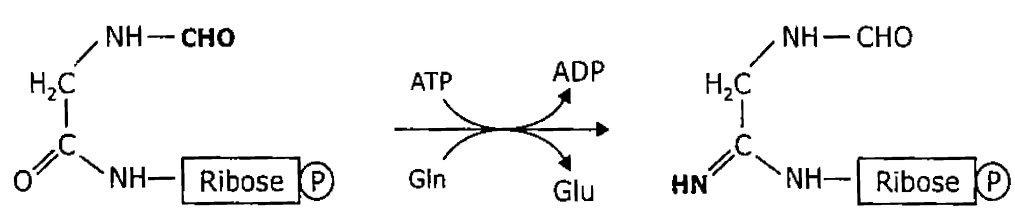
5-Phosphoribosyl-1-amine — Glycinamide ribonucleotide

2. Formate is activated and then added to this amino group to form formylglycinamide ribonucleotide. In some organisms, two distinct enzymes can catalyze this step. One enzyme transfers the formyl group from N^{10}-formyltetrahydrofolate. The other enzyme activates formate as formyl phosphate, which is added directly to the glycine amino group.



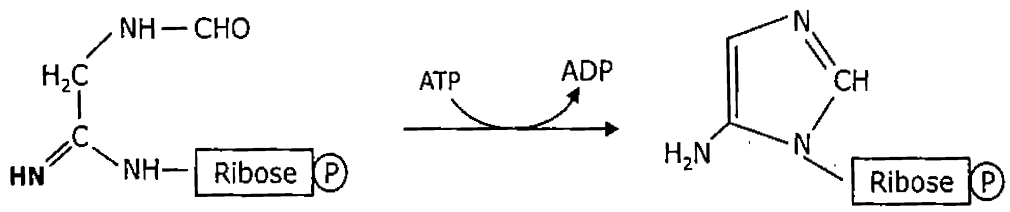
Glycinamide ribonucleotide — Formylglycinamide ribonucleotide

3. The inner amide group is activated and then converted into an amidine by the addition of ammonia derived from glutamine.



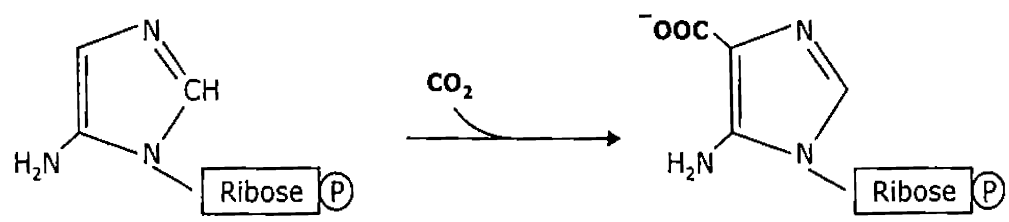
Formylglycinamide ribonucleotide — Formylglycinamidine ribonucleotide

4. Formylglycinamidine ribonucleotide, cyclizes to form the five-membered imidazole ring found in purines. Although this cyclization is likely to be favorable thermodynamically, a molecule of ATP is consumed to ensure irreversibility.



Formylglycinamidine ribonucleotide — 5-Aminoimidazole ribonucleotide

5. Bicarbonate is activated by phosphorylation and then attacked by the exocyclic amino group. The product of the reaction rearranges to transfer the carboxylate group to the imidazole ring. Interestingly, mammals do not require ATP for this step; bicarbonate apparently attaches directly to the exocyclic amino group and is then transferred to the imidazole ring.

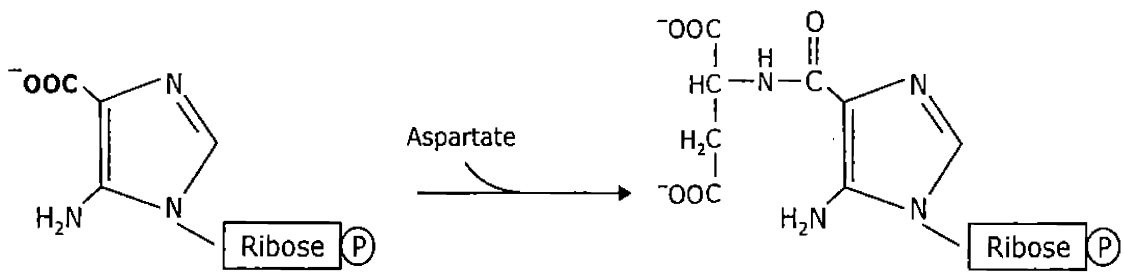


5-Aminoimidazole ribonucleotide — 4-Carboxy-5-aminoimidazole ribonucleotide

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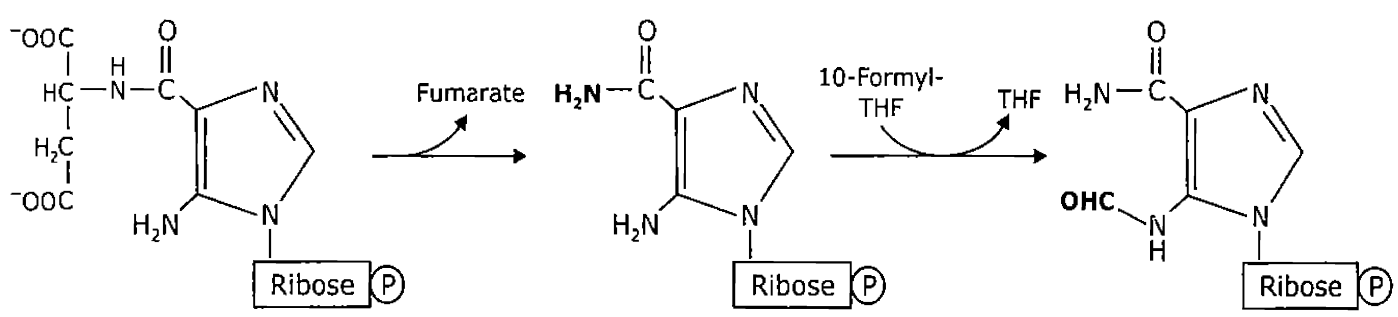
6. The imidazole carboxylate group is phosphorylated again and the phosphate group is displaced by the amino group of aspartate. Thus, a six-step process links glycine, formate, ammonia, bicarbonate, and aspartate to form an intermediate that contains all but two of the atoms necessary for the formation of the purine ring.



4-Carboxy-5-aminoimidazole ribonucleotide

N-Succinylo-5-aminoimidazole-4-carboxamide ribonucleotide

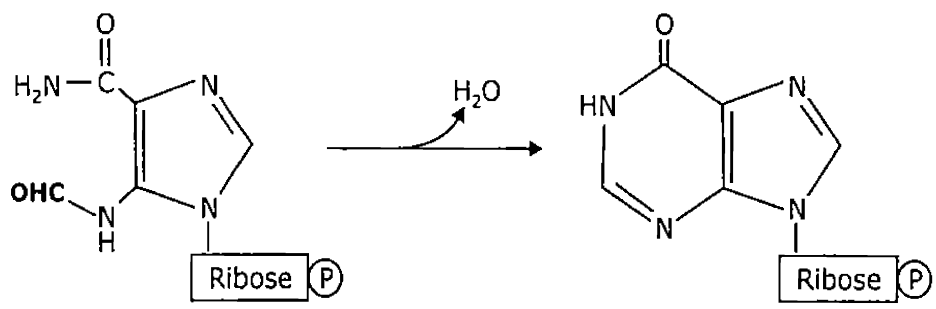
Three more steps complete the ring construction. Fumarate, an intermediate in the citric acid cycle, is eliminated, leaving the nitrogen atom from aspartate joined to the imidazole ring. The use of aspartate as an amino-group donor and the concomitant release of fumarate are reminiscent of the conversion of citrulline into arginine in the urea cycle and these steps are catalyzed by homologous enzymes in the two pathways. A formyl group from N^{10}-formyltetrahydrofolate is added to this nitrogen atom to form a final intermediate that cyclizes with the loss of water to form inosinate.



N-Succinylo-5-aminoimidazole-4-carboxamide ribonucleotide

5-aminoimidazole-4-carboxamide ribonucleotide

N-Formylaminoimidazole-4-carboxamide ribonucleotide



N-Formylaminoimidazole-4-carboxamide ribonucleotide

IMP

AMP and GMP are formed from IMP

The conversion of IMP to either AMP or GMP utilizes a two-step, energy-requiring pathway. Note that the synthesis of AMP requires GTP as an energy source, whereas the synthesis of GMP requires ATP.

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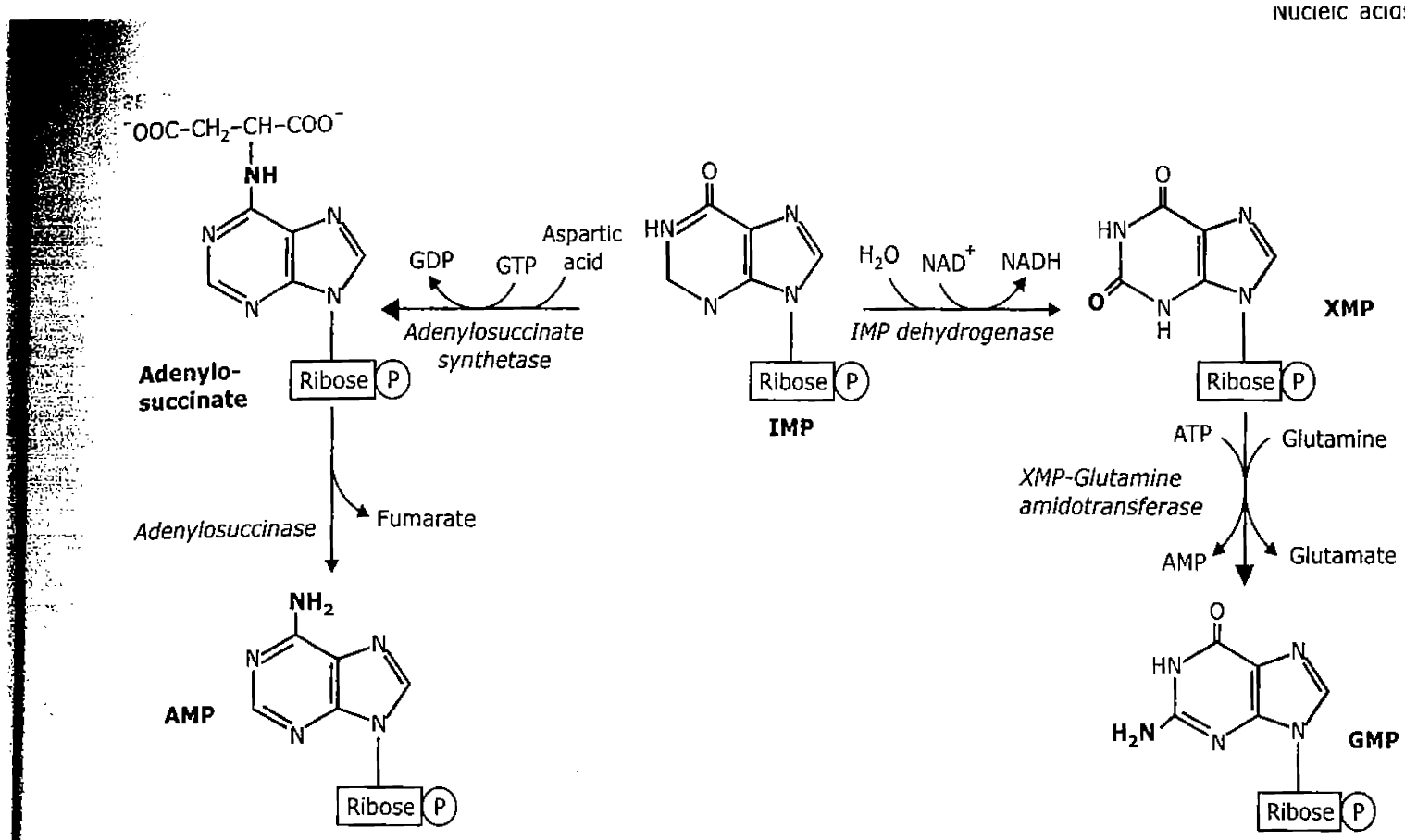


Figure 2.20 : Conversion of IMP to AMP and GMP showing feedback inhibition.

Conversion of nucleoside monophosphates to nucleoside diphosphates and triphosphates

Nucleoside diphosphates (NDP) are synthesized from the corresponding nucleoside monophosphates (NMP) by base-specific nucleoside monophosphate kinases. (Note: These kinases do not discriminate between ribose or deoxyribose in the substrate). ATP is generally the source of the transferred phosphate because it is present in higher concentrations than the other nucleoside triphosphates.

For example, adenylate kinase: AMP + ATP $\rightleftharpoons$ 2 ADP

For example, guanylate kinase: GMP + ATP $\rightleftharpoons$ GDP + ADP

Nucleoside diphosphates and triphosphates are interconverted by nucleoside diphosphate kinase—an enzyme that, unlike the monophosphate kinases, has broad specificity.

For example, GDP + ATP $\rightleftharpoons$ GTP + ADP

For example, CDP + ATP $\rightleftharpoons$ CTP + ADP

SALVAGE PATHWAYS FOR PURINES

Purines that result from the normal turnover of cellular nucleic acids of that are obtained from the diet and not degraded can be reconverted into nucleoside triphosphates and used by the body. This is referred to as the *salvage pathway* for purines. This pathway involves two enzymes : *adenine phosphoribosyl transferase (APRT)* and *hypoxanthine-guanine phosphoribosyl transferase (HGPRT)*. Both enzymes utilize PRPP as the source of the ribose 5-phosphate group.

Adenine phosphoribosyltransferase catalyzes the formation of adenylate

Adenine + PRPP $\rightarrow$ Adenylate + PP_i

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whereas *hypoxanthine-guanine phosphoribosyltransferase* (*HGPRT*) catalyzes the formation of guanylate as well as *inosinate* (inosine monophosphate, IMP), a precursor of guanylate and adenylate.

Guanine + PRPP → Guanylate + PP_i

Hypoxanthine + PRPP → Inosinate + PP_i

Similar salvage pathways exist for pyrimidines. Pyrimidine phosphoribosyltransferase will reconnect uracil, but not cytosine, to PRPP.

2.9 NUCLEOTIDE DEGRADATION

Degradation of purine nucleotides

Purine nucleotides are sequentially degraded by the removal or alteration of portions of the nucleotide. The end product of purine catabolism in humans is uric acid. Mammals other than primates oxidize uric acid further to allantoin, which, in some animals other than mammals, may be further degraded to urea or even ammonia. *In human beings, uric acid is the final product of purine degradation and is excreted in the urine.*

Formation of uric acid

A summary of the steps in the production of uric acid :

1. An amino group is removed from AMP to produce IMP, or from adenosine to produce inosine.
2. IMP and GMP are converted into their nucleoside forms-inosine and guanosine.
3. Inosine and guanosine converted into their respective purine bases, hypoxanthine and guanine.
4. Guanine is deaminated to form xanthine.
5. Hypoxanthine is oxidized by xanthine oxidase to xanthine, which is further oxidized to uric acid, the final product of human purine degradation. Uric acid is excreted in the urine.

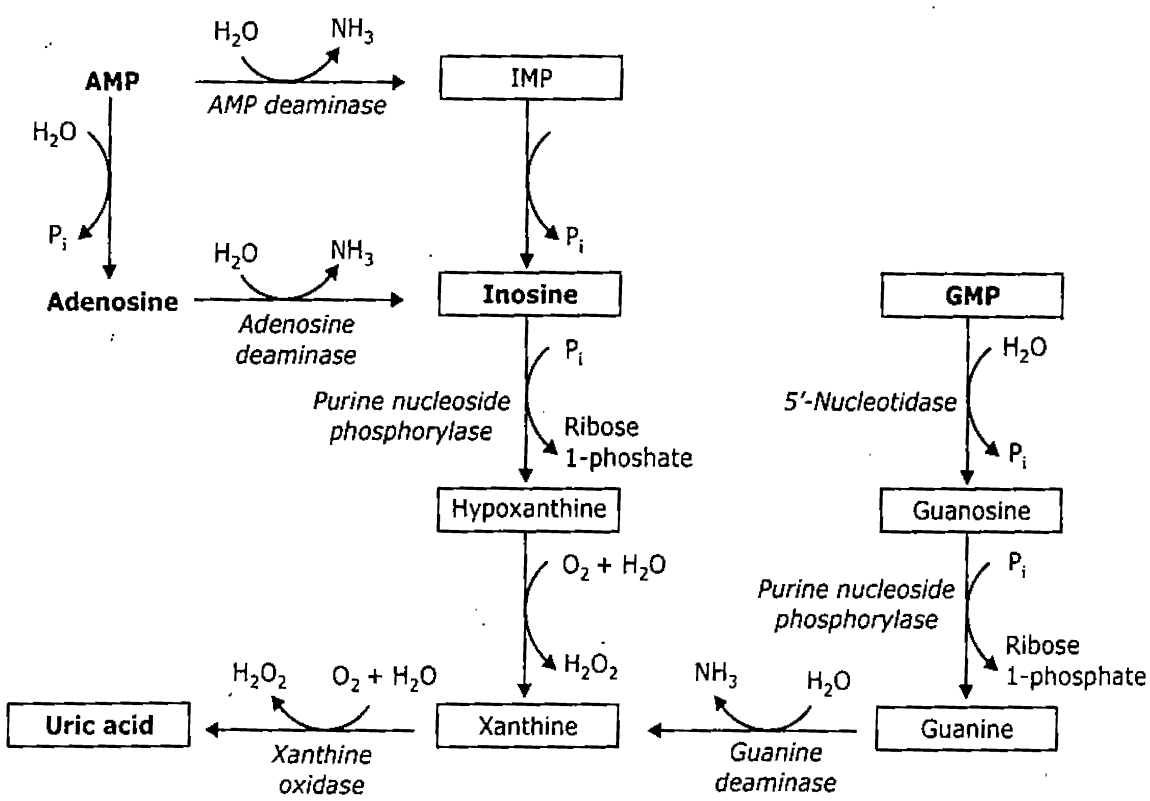


Figure 2.21 : Degradation of purine nucleotides to uric acid.

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Adenosine deaminase deficiency

Adenosine deaminase (ADA) deficiency leads to severe combined immunodeficiency (SCID). In SCID T and B lymphocytes do not develop properly. Lack of ADA leads to a increase in the cellular concentration of dATP. dATP acts as a strong inhibitor of *ribonucleotide reductase*. High levels of dATP produce a general deficiency of other dNTPs in T lymphocytes. The basis of B-lyphocytes toxicity is less clear.

Gout : The major biochemical feature of gout is an elevated level of serum urate. It causes inflammation of the joints by precipitation as sodium urate crystals. The treatment of gout includes the use of the drug **allopurinol**. This drug has a structure similar to xanthine and acts by inhibiting the enzyme *xanthine oxidase*. *Xanthine oxidase,* a molybdenum- and iron-containing flavoprotein, oxidizes hypoxanthine to *xanthine* and then to *uric acid.* Hence inhibition of the activity of xanthine oxidase inhibits uric acid synthesis.

Lesch-Nyhan Syndrome : This genetic disorder is caused by a deficiency of the salvage enzyme hypoxanthine-guanine phosphoribosyltransferase. Children with the deficiency display compulsive self-destructive behavior. They also show signs of mental retardation. Because there is less salvaging of bases, more uric acid is produced, causing gout.

Degradation of pyrimidine nucleotides

Unlike the purine rings, which are not cleaved in human cells, the pyrimidine ring can be opened and degraded to highly water soluble structures, such as β–aminoisobutyrate, β–alanine.

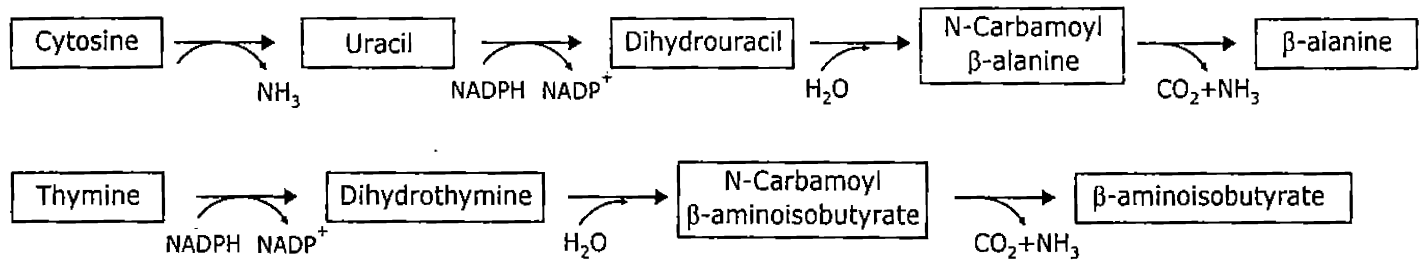


Figure 2.22 : Catabolism of pyrimidine.

2.10 ELECTROPHORESIS OF NUCLEIC ACIDS

Gel electrophoresis is the process by which scientists can sort pieces of DNA of different size. An agarose or polyacrylamide gel is loaded with the DNA fragments and current is passed through the gel. Since DNA is *negatively* charged, it will migrate towards the positive pole. The DNA will not migrate at the same rate, however. Larger pieces of DNA collide with the gel matrix more often and are slowed down, while smaller pieces of DNA move through more quickly. Electrophoretic mobility of DNA in gel not only depends on the size but also on shape.

DNA molecules are invisible to the naked eye, but can be seen in gels by staining them with a solution of planar aromatic cations such as **ethidium bromide**, **acridine organe** or **proflavin.** Ethidium bromide is a molecule that becomes intercalated between the stacked bases of the DNA molecule. Soaking a DNA containing gel in ethidium bromide will result in concentration of the chemical within the DNA. Illumination of the soaked gel with light in the ultraviolet range (260-300 nm) results in fluorescence of ethidium bromide, and the DNA shows up on the gel as a band of fluorescence (orange).

The sizes of the DNAs that can be separated by conventional gel electrophoresis are limited to ~100,000 bp. Very large DNA fragments are unable to penetrate the pores in agarose gel and thus cannot readily be resolved. However, larger DNA fragments can be resolved from one another if the electric field is applied in pulses that are oriented orthogonally to each other. This technique is known as *pulsed-field gel electrophoresis* (developed by Cantor and Smith).

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Electrophoretic separation of DNA topoisomers

Electrophoresis separates DNA molecules, not only according to their molecular weight, but also according to their shape and topological properties. DNA topoisomers have same length but different linking numbers. Even though topoisomers have the same molecular weight, they can be separated from each other by gel electrophoresis. The basis of this separation is that the greater the writhe, the more compact the shape of a covalently closed circular DNA (cccDNA). The more compact the DNA, the more easily it is able to migrate through the gel. Thus a relaxed cccDNA migrates more slowly than a highly supercoiled form of same circular DNA.

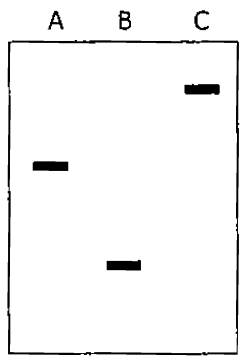


Figure : 2.23
Electrophoretic separation of DNA topoisomers. Band on *lane C* represent relaxed circular DNA, *lane B* highly supercoiled and *lane A* less supercoiled cccDNA.

STUDY QUESTIONS

Q2. What is the approximate molecular weight of duplex DNA required to code for glyceraldehyde phosphate dehydrogenase (MW 40,000)?

Ans. The average molecular weight of an amino acid residue in a protein is 110. Thus, a protein whose molecular weight is 40,000 contains 40,000/110 = 364 amino acids and requires a minimum DNA duplex of 3 × 364 = 1090, nucleotide pairs. Since each nucleotide pair has a molecular weight of about 650, the molecular weight of this gene would be about 1090 × 650 = 708,000. On the average, the molecular weight of coding DNA is about 18 times that of the corresponding protein.

Q3. A sample of purified DNA obtained from tobacco leaf contains 20.0 mole percent of cytosine. Assuming that only the four principal bases are present; calculate the approximate mole percent of purine residues in this DNA.

Ans. Adenine and guanine are the purine residues in DNA, Recall that the number of cytosine residues in all DNA is equal to the number of guanine residues. Then, C = G = 20.0%

The number of thymine residues is equal to the number of adenine residues, and the sum of purine and pyrimidine residues equal 100 percent. Therefore,

$$\begin{aligned}(A + G) + (T + C) &= 100.0\% \\ (A + 20.0) + (T + 20.0) &= 100.0\% \\ (A + 20.0) + (A + 20.0) &= 100.0\% \\ 2A &= 100.0 - 40.0\% \\ A &= 30.0\%\end{aligned}$$

Hence, the mole percent of purines (adenine plus guanine) present in the DNA sample is 50 percent.

Nucleic acid conversion factors

Average MW of a DNA basepair = 650 Da

1 pmol of 1000 bp DNA = 0.66 microgram

1 A_{260} unit = ~50 microgram/ml of double strand DNA

1 A_{260} unit = ~40 microgram/ml of single strand RNA

1 A_{260} unit = ~33 microgram/ml of single strand DNA

1000 bp DNA open reading frame = 333 amino acids = 37,000 Da protein

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SELF TEST

Q1. What will be the molecular weight of poly (A) chain consisting of 100 residues, where the molecular weight of AMP is 300?

a. 30000
b. 29700
c. 28218
d. 29982

Q2. What is the number of hydrogen bonds in a double helical B-DNA structure of 100 base pairs with 20 adenines and 10 thymines in one of the two strands?

a. 200
b. 230
c. 270
d. 300

Q3. Which of the following favours Z-DNA conformation?

a. 2′-OH steric hindrance
b. anti-G and C2′-endo sugar
c. syn-G and C3′-endo sugar
d. G4-DNA quartet **[JNU]**

Q4. In DNA structure *propeller twist* means

a. two bases orient antiparallel to each other
b. two bases flip with respect to each other
c. two flat bases counter rotate relative to each other
d. two bases stack with respect to each other with 3.4 Å space **[JNU]**

Q5. Triple stranded DNA

a. is a characteristic of SV 40 genome
b. involves Hoogsteen base pair
c. involves only intermolecular interactions
d. is characterized by in the presence of a string of alternating purine-pyrimidine bases **[JNU]**

Q6. Which of the following feature is *not* true about Z-DNA?

a. left handed helical structure
b. dinucleotide repeating unit
c. 10.4 bp per helical turn
d. anti and syn conformation of glycosidic bond both

Q7. In which of the following respect A form of DNA differs from B-form of DNA?

a. helix handedness
b. repeating unit
c. conformation of glycosidic bond
d. base pair per helical turn

Q8. Purified duplex DNA molecules cannot exist in which one of the following forms

a. linear
b. circular and supercoiled
c. linear and supercoiled
d. circular and relaxed **[GATE]**

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Nucleic acids

Q9. An unwound DNA and a supercoiled DNA with the same linking number are
a. topologically and geometrically identical
b. topologically identical but geometrically different
c. topologically and geometrically different
d. topologically different but geometrically identical **[GATE]**

Q10. Ribosomal RNA modification such as methylation or pseudouridylation are guided by which of the following RNA in eukaryotic cells
a. mi-RNA
b. si-RNA
c. sno-RNA
d. sh-RNA

Q11. Which of the following is *not* true about RNA?
a. RNA is chemically more reactive than DNA
b. 2′-OH prevents RNA to adopt B-conformation
c. RNA was discovered by Hoppe-Seyler
d. Feulgen reaction is performed by both DNA and RNA

Q12. Which of the following is *not* true? When RNA undergo for alkali-catalyzed cleavage
a. transesterification reaction occurs
b. cyclic 2′-3′-nucleoside monophosphate forms
c. cyclic 5′-3′-nucleoside monophosphate forms
d. 2′-OH is converted into 2′-alkoxide ion **[IISc]**

Q13. According to the RNA-world theory
a. RNA molecules were the first organic molecules formed on earth
b. life evolved on another planet called the 'RNA World'
c. all RNA molecules in cells are 'ribozymes'
d. primitive RNA molecules evolved before protein and DNA

Q14. Which of the following statements about ribozymes is *false*?
a. ribozymes are capable of self-replication, but cannot catalyze other types of reactions
b. Manfred Eigen found that RNA's could replicate themselves in solution without the aid of proteins
c. in *Tetrahymena thermophila*, an intron was found that carries out its own excision and splicing
d. a tRNA-processing enzyme containing RNA was found in which the RNA portion provided the catalysis

Q15. Which of the following contributes nitrogen atoms to both purine and pyrimidine rings?
a. aspartate
b. carbamoyl phosphate
c. carbon dioxide
d. glutamine

Q16. The atoms of the pyrimidine ring are derived from
a. glutamine
b. carbamoyl phosphate and aspartate
c. glutamine and N^{10} formyl tetrahydrofolate
d. aspartate and N^{10} formyl tetrahydrofolate

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Q17. Which of the following answers completes the sentence correctly? The first product of purine nucleotide biosynthesis that contains a complete purine ring (hypoxanthine) is

a. AMP
b. IMP
c. GMP
d. xynthylate

Q18. Folate derivatives are required for the synthesis of which deoxynucleotides?

a. adenylate and guanylate
b. cytidylate and thymidylate
c. adenylate, guanylate and thymidylate
d. adenylate, guanylate and cytidylate

Q19. Which of the following is *not* a characteristic of DNA?

a. it is less susceptible to alkaline hydrolysis than RNA
b. it has a hydroxyl group attached to the 3' carbon at the 3' end
c. it is stabilized by hydrogen bonding, van der Waals forces and covalent interaction
d. it can exist in four or more than four different helical conformations

Q20. The molecular weight of a bacterial DNA molecule is 2.64×10^9. The average molecular weight of a nucleotide pair is 660. Assume that the average protein is made up of a chain of 400 amino acid residues. What is the maximum number of proteins that can be coded by the bacterial DNA molecule?

a. 20000
b. 3333
c. 6667
d. none

[GATE]

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Chapter - 03

Biophysical techniques

3.1 CHROMATOGRAPHY

Chromatography (*color* and *to write*) is a physical method for separation of compounds. Tswet, Russian botanist (referred as *father of Chromatography*) is credited for the development of chromatography. He first devised a method to separate plant pigments using a tube filled with $CaCO_3$.

Chromatography is based on the fact that sample distributes or partitions itself to different extents in two different, immiscible phases, which is described by *partition* or *distribution coefficient*, K_d. If we consider two immiscible phases A and B,

$$K_d = \frac{\text{Concentration of sample in phase A}}{\text{Concentration of sample in phase B}}$$

The two immiscible phase could be a solid and a liquid, or a gas and a liquid, or a liquid and another liquid. One of the two phases is a **stationary phase** (a solid, or a liquid supported on a solid) and does not move and the other is a **mobile phase** and move with respect to first. Mobile phase may be a liquid (liquid chromatography) or a gas (gas chromatography). All chromatographic methods involve passing a mobile phase through a immobile phase. Based on stationary phase there are two subgroups of gas chromatography and liquid chromatography, namely; gas-solid chromatography and gas-liquid chromatography, together with liquid-solid chromatography and liquid-liquid chromatography.

Planar and column chromatography

In **planar chromatography**, the stationary phase is present as or on a plane. The plane can be a paper, serving as such or impregnated by a substrate as the stationary bed (paper chromatography, PC) or a layer of solid particles spread on a support e.g., a glass plate (thin layer chromatography, TLC). Planar chromatography is also termed *open-bed chromatography*. In thin layer chromatography, the stationary phase is a thin layer of silica gel or alumina on a glass, metal or plastic plate. Most commonly silica gel is used as a stationary phase. In silica gel, the silicon atoms are joined via oxygen atoms in a giant covalent structure. The other commonly used stationary phase is alumina - aluminium oxide.

In **column chromatography**, the stationary bed is within a tube. The particles of the solid stationary phase or support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open-tubular column).

Partition and adsorption chromatography

Chromatographic techniques can be classified into two main categories - *partition* and *adsorption* chromatography - depending on how solute molecules interact with the stationary phase. In *partition chromatography*, the distribution of solutes between the two liquid phases is based primarily on solubility differences. Adsorption chromatography relies on specific interactions between the solutes and the molecules of stationary phase. These interactions may be ionic, hydrogen bonding, or hydrophobic interactions.

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Partition chromatography

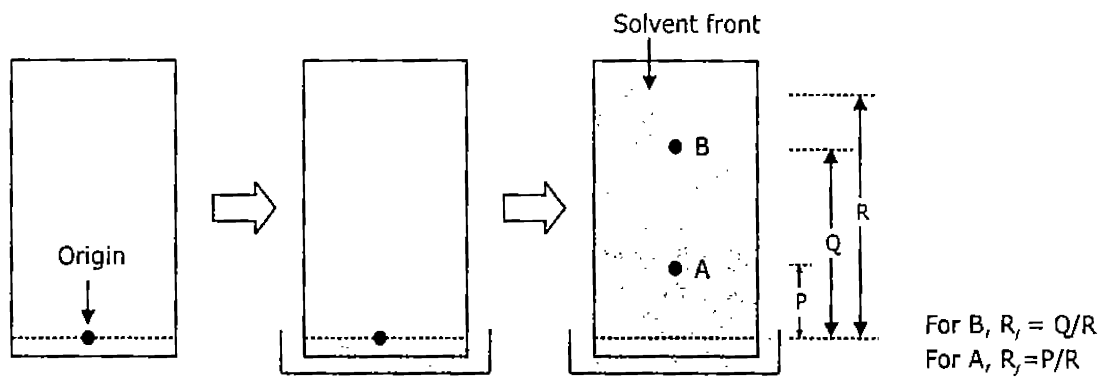
Martin and Synge in 1944 developed the methodology of partition chromatography and got Noble Prize. In this chromatographic technique, separation is based on solute partitioning between stationary liquid phase and mobile liquid phase. Substances which are more soluble in mobile phase will pass rapidly through the system while those which favour the stationary phase will be retarded.

It is of two types

1. *Normal phase partition chromatography* : Stationary phase is polar (usually water) and mobile phase is relatively non-polar organic solvent. During elution least polar is eluted first and most polar last.
2. *Reverse phase partition chromatography* : Stationary phase is non-polar and mobile phase is relatively polar. In this case polar elute first and non-polar elute last.

Paper chromatography is a type of partition chromatography. In paper chromatography, end of the paper is dipped into a solvent mixture consisting of aqueous and organic components. The solvent soaks into the paper by capillary action because of the fibrous nature of the paper. The aqueous component of the solvent binds to the cellulose of the paper and thereby forms a stationary phase with it. The organic component of the solvent continues migrating, thus forming the mobile phase. The rates of migration of the various substances being separated are governed by their relative solubilities in the polar stationary phase and the nonpolar mobile phase. During separation process, a given solute is distributed between the mobile and stationary phases according to its *partition coefficient*. The nonpolar molecules move faster than polar ones. The migration rate of a substance during paper chromatography is usually expressed as the dimensionless term R_f, which is the ratio of distance traveled by substance and solvent front.

$$R_f = \frac{\text{Distance traveled by substance}}{\text{Distance traveled by solvent front}}$$



HPLC

High performance liquid chromatography (HPLC) is a column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures. There are two variants of HPLC depending on the relative polarity of the solvent and the stationary phase.

In normal phase HPLC, the column is filled with tiny silica particles, and the solvent is non-polar. Polar compounds in the mixture being passed through the column will stick longer to the polar silica than non-polar compounds will. The non-polar ones will therefore pass more quickly through the column.

In reversed phase HPLC, the column filled with the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol. In this case, there will be a strong attraction between the polar solvent and polar molecules present in the mixture being passed through the column. Polar molecules in the mixture will therefore spend most of their time moving with the solvent. Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. This means

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that it is the polar molecules that will travel through the column more quickly. Reversed phase HPLC is the most commonly used form of HPLC.

Size exclusion chromatography

Size exclusion chromatography or molecular sieve chromatography separates molecules on the basis of size and shape. A column matrix filled with porous beads, made up of an insoluble and hydrated polymer such as polyacrylamide (Sephacryl or BioGel P) or dextran (Sephadex) or agarose (Sepharose) acts as stationary phase. Size exclusion chromatography includes: *gel permeation chromatography* and *gel filtration chromatography*. Gel permeation chromatography uses organic mobile solvent while gel filtration chromatography uses aqueous mobile phase to separate and characterize molecules.

The basis of size exclusion chromatography is very simple. If a solution contains molecules of various sizes is passed through the column, molecules smaller than the pores can enter the pores in the beads whereas larger molecules cannot. So larger molecules move faster and elute first. Since smaller molecules can enter the pores present in the beads therefore have longer path and longer transit time than larger molecules that cannot enter the pores.

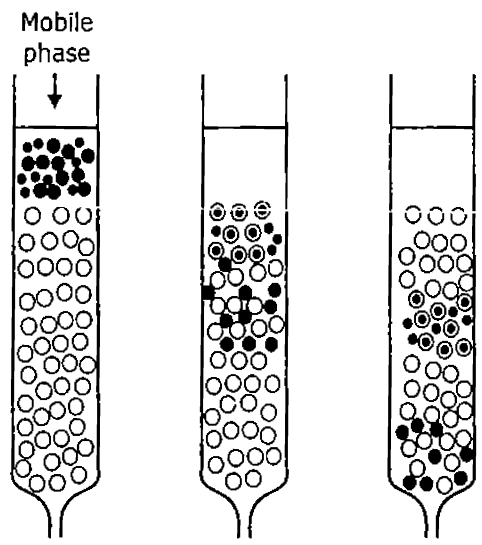


Figure : 3.1
Separation of different size molecules by exclusion chromatography. Large molecules are eluted first.

The molecular mass of the smallest molecule unable to penetrate the pores of a given gel is said to be the gel's **exclusion limit**. For a given sample, the distribution coefficient K_d is dependent upon its size. If the molecule is completely excluded by the sieve then $K_d=0$, whereas if it enters into the gel and has accessibility to inner solvent then $K_d=1$. For all other intermediate sizes the K_d value will lie in the range 0 to 1.

In gel filtration, a sample of protein is applied to a column, which is then washed with an appropriate buffer. The first proteins to emerge from the column are those that are too large to enter the pores of the sieve. Other proteins are eluted in decreasing order of their molecular size.

Size measurements by size exclusion chromatography

In order to obtain size information about a solute from a size exclusion chromatography experiment, the column must first be characterized in terms of the volumes accessible to analytes. The total volume (V_T) of a size exclusion chromatography column is divisible into three parts:

1. The volume external to the packing material i.e., *void volume*, V_0;
2. The volume contained within the porous beads that is accessible to small molecules i.e., *internal volume*, V_i;
3. The volume occupied by the packing material itself i.e, *bed volume*, V_g.

   Therefore, $V_T = V_0 + V_i + V_g$

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The values of V_0 and V_t are determined experimentally by measuring the elution volumes of, respectively, a large solute that is totally excluded from the interior of the resin and a small solute that has access to all pores of the resin. The elution volume of a given solute, V_e, is the volume of solvent required to elute the solute from the column after it has first contacted the gel. The elution volume, V_e, of a solute that is partially included in the pores of the gel filtration resin can be related to the void and internal volumes of a column by the following equation:

$$V_e = V_0 + \sigma V_i \quad \text{where } \sigma \text{ is the partition coefficient of the solute.}$$

It is the partition coefficient, σ, that, when compared with the values measured for proteins of known size, provides information about the molecular size of an unknown macromolecule. If a series of proteins of known size are subjected to size exclusion chromatography, a linear relationship between partition coefficient and size is observed.

Ion exchange chromatography

Ion exchange chromatography is applicable for separation of charged molecules. It is based on the reversible exchange of ions in solution with ions electrostatically bound to support media (ion exchanger).

Ion exchanger

Ion exchangers are made up of two parts-an insoluble matrix and chemically bonded charged groups within and on the surface of the matrix. An ion exchanger is classified as *cationic* or *anionic* depending on whether it exchanges cations or anions.

Cation exchanger (also called acidic ion exchanger) : It is used for cation separation.

Anion exchanger (also called basic ion exchanger) : It is used for anion separation.

Each type of exchanger is also classified as strong or weak according to the ionizing strength of the functional group. An exchanger with a quaternary amino group is, therefore, a strongly basic anion exchanger, whereas primary or secondary aromatic or aliphatic amino groups would lead to a weakly basic anion exchanger. A strongly acidic cation exchanger contains the sulfonic acid group.

Table 3.1 : Commonly used ion exchangers

Name	*Type*	*Ionizable group*	
DEAE-cellulose	Weakly basic	Diethylaminoethyl	Anion exchanger
CM-cellulose	Weakly acidic	Carboxymethyl	Cation exchanger
SP-Sepharose	Strongly acidic	Methyl sulfonate	Cation exchanger
CM Bio-Gel A	Weakly acidic	Carboxylmethyl	Cation exchanger

A molecular species in a given sample which has opposite charge to that of the charged group of the resin column binds to the column. Separation of charged molecules occur because different molecules have different degree of interaction with the ion-exchanger due to difference in their charges, charge density and distribution of charge on their surfaces. If a protein has a net positive charge at pH 7, it will usually bind to ion exchanger (cation exchanger) containing carboxylate groups, whereas a negatively charged protein will not. The bound molecules can be eluted by altering the pH of the eluting buffer or by increasing the salt concentration of the eluting buffer. A positively charged protein bound to cation exchanger can be eluted by increasing the salt concentration in the eluting buffer because cations present in the buffer compete with positively charged groups on the protein for binding to the ion exchanger. Proteins that have a low density of net positive charge will tend to emerge first, followed by those having a higher charge density.

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Affinity chromatography

Affinity chromatography is a technique enabling purification of a biomolecule with respect to biological function or individual chemical structure. The substance to be purified is specifically and reversibly adsorbed to a ligand (binding substance), immobilized by a covalent bond to a chromatographic bed material (matrix). Samples are applied under favourable conditions for their specific binding to the ligand. Substances of interest are consequently bound to the ligand while unbound substances are washed away. Recovery of molecules of interest can be achieved by changing experimental conditions to favour desorption.

3.2 ELECTROPHORESIS

Electrophoresis (*Electro* refers to the energy of electricity and *Phoresis*, from the Greek verb *phoros*, means to carry across) is a technique for separating, or resolving, charged molecules (such as amino acids, peptides, proteins, nucleotides, and nucleic acids) in a mixture under the influence of an applied electric field. Charged molecules in an electric field move or migrate, at a speed determined by their charge : mass ratio. According to the laws of electrostatics an ion with charge 'Q' in an electric field of strength 'E' will experience a electric force, $F_{electrical}$

$$F_{electrical} = QE$$

The resulting migration of the charged molecule through the solution is opposed by a frictional force

$$F_{frictional} = Vf$$

Where V is the rate of migration of ion and f is its **frictional coefficient.**

Frictional coefficient depends on the size, shape and viscosity of the solution. In constant electric field, the force on ion balance each other;

$$QE = Vf$$

So that each ion moves with a constant characteristic velocity. An ion's **electrophoretic mobility,** μ is defined as

$$\mu = V/E = Q/f$$

So according to equation, if two molecules have the same mass and shape, the one with the greater net charge will move faster toward an electrode.

Electrophoresis is of two types - *moving boundary electrophoresis* and *zone electrophoresis.* In zone electrophoresis sample is constrained to move in a solid support such as filter paper (paper electrophoresis) or a gel (gel electrophoresis).

GEL

There are two basic types of materials used to make gels: *agarose* and *polyacrylamide*. Agarose is a natural colloid extracted from sea weed. Agarose gels have very large pore size and are used primarily to separate very large molecules with a molecular mass greater than 200 kDa. Agarose is a linear polysaccharide made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose. Agarose is usually used at concentrations between 1% and 3%.

A polyacrylamide gel consists of chains of acrylamide monomers (CH_2 = CH–CO–NH_2) cross-linked with *N, N*-methylenebisacrylamide units (CH_2 = CH–CO–NH–CH_2–NH–CO–CH–CH_2), the latter commonly called *bis*. The pore size of the gel is determined by both the total concentration of monomers (acryiamide + bis) and the ratio of acrylamide to bis. Polymerization of the acrylamide : bis solution is initiated by ammonium persulfate and catalyzed by TEMED (*N, N, N′ N′*-tetramethylethylenediamine).

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SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic separation of proteins is most commonly performed in **polyacrylamide gels**. When a mixture of proteins is applied to a gel and an electric current applied, smaller proteins migrate faster than larger proteins through the gel. The rate of movement is influenced by the gel's pore size and the strength of the electric field. The pores in a highly cross-linked polyacrylamide gel are quite small. Such a gel could resolve small proteins and peptides, but large proteins would not be able to move through it.

Proteins are exposed to the negatively charged ionic detergent **sodium dodecylsulfate** (SDS) before and during gel electrophoresis. SDS denatures proteins, causing multimeric proteins to dissociate into their subunits, and all polypeptide chains are forced into extended conformations with similar charge:mass ratio. SDS treatment thus eliminates the effect of differences in shape, so that chain length, which reflects mass, is the sole determinant of the migration rate of proteins in SDS-PAGE. The molecular weight of a protein can be estimated by comparing the distance it migrates through a gel with the distances that proteins of known molecular weight migrate.

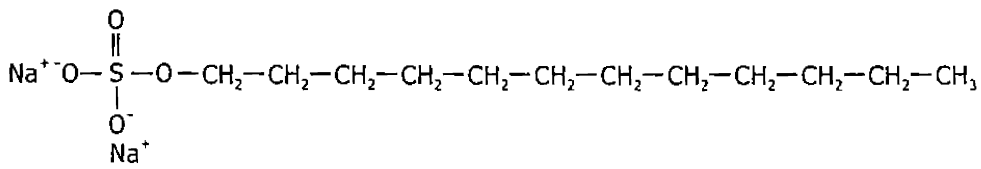


Figure 3.2 : Structure of sodium dodecylsulfate (SDS)

SDS-PAGE is rapid, sensitive, and capable of a high degree of resolution. Bands resulting from electrophoretic separation can be located by a variety of techniques. Proteins are often visualized by staining. **Coomassie brilliant blue** is the most widely used dye. **Fluorescamine**, a nonfluorescent molecule is also used as an alternative type of protein stain.

Discontinuous electrophoresis

In *continuous gel system*, separating media consists of a single gel with an uniform pH throughout. In *discontinous gel system*, polyacrylamide gel is divided into three regions called the *sample gel*, *stacking gel*, and *separating gel*. These gels can have different concentrations of the same support media, or may be completely different agents. The sample gel contains the mixture of proteins to be separated and is prepared using low concentration of acrylamide so that pore sizes are large and do not influence the rates of migration of different size proteins. The stacking gel is similar to the sample gel. The proteins in the sample gel will concentrate into a small zone in the stacking gel before entering the separating gel.

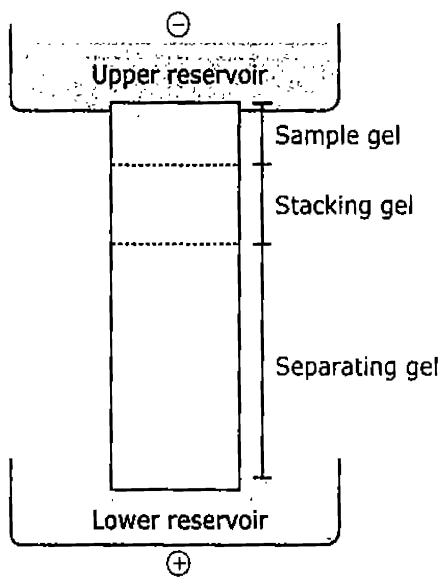


Figure : 3.3
Essential components of discontinuous electrophoresis.

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The sample gel, stacking gel, and reservoirs have the same pH. The separating gel differs from the other two regions due to greater concentrations of acrylamide; this results in smaller pore sizes and provides the sieving effect. The sample is usually dissolved in glycine-chloride buffer, pH 8 to 9, before loading on the gel. Glycine exists primarily in two forms at this pH, a zwitterion and an anion. When the voltage is turned on, buffer ions (glycinate and chloride) and protein sample move into the stacking gel, which has a pH of 6.9. Upon entry into the upper gel, the concentration of glycine zwitterion increases and hence no electrophoretic mobility. Since most proteins samples are still anionic at pH 6.9, they replace glycinate as mobile ions. The ion having a greater charge will move faster and is thus the *leading* ion, while the ion with the lesser charge will be the *trailing* ion. Therefore, the relative ion mobilities in the stacking gel are chloride> protein sample> glycinate. The sample will tend to accumulate and form a thin, concentrated band sandwiched between the chloride and glycinate as they move through the upper gel. Now, when the ionic front reaches the lower gel with pH 8 to 9 buffer, the glycinate concentration increases and anionic glycine and chloride carry most of the current. The protein sample molecules, now in a narrow band, encounter both an increase in pH and a decrease in pore size. The increase in pH would, of course, tend to increase electrophoretic mobility, but the smaller pores decrease mobility. The relative rate of movement of anions in the separating gel is chloride> glycinate > protein sample.

The separation of sample components in the resolving gel occurs as described in an earlier section on gel electrophoresis. Each component has a unique charge/mass ratio and a discrete size and shape, which directly influence its mobility.

Two-dimensional gel electrophoresis

Electrophoresis of all cellular proteins through an SDS gel can separate proteins having relatively large difference in molecular weights (e.g., a 41-kDa protein from a 42-kDa protein). To separate proteins of similar mass, another physical characteristic must be exploited.

In two-dimensional electrophoresis, proteins are separated in two sequential steps: first by their charge and then by their mass. In the first step, a cell extract is fully denatured by high concentrations (8 M) of urea and then layered on a glass tube filled with polyacrylamide that is saturated with a solution of **ampholytes**, a mixture of polyanionic and polycationic molecules. When placed in an electric field, the ampholytes will separate and form a continuous gradient based on their net charge. The most highly polyanionic ampholytes will collect at one end of the tube, and the most polycationic ampholytes will collect at the other end. This gradient of ampholytes establishes a pH gradient. Charged proteins will migrate through the gradient until they reach their pI, or isoelectric point, the pH at which the net charge of the protein is zero. This technique, called **isoelectric focusing** (IEF), can resolve proteins that differ by only one charge unit.

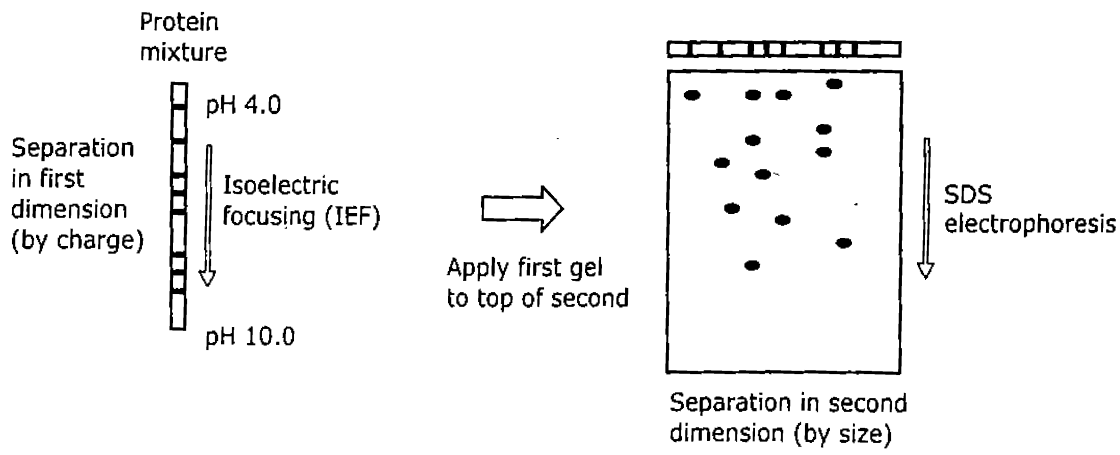


Figure 3.4 : Two-dimensional gel electrophoresis

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Proteins that have been separated on an IEF gel can then be separated in a second dimension based on their molecular weights. To accomplish this, the IEF gel is extruded from the tube and placed lengthwise on a second polyacrylamide gel, this time formed as a slab saturated with SDS. When an electric field is imposed, the proteins will migrate from the IEF gel into the SDS slab gel and then separate according to their mass. The sequential resolution of proteins by their charge and mass can achieve excellent separation of cellular proteins.

Native PAGE

SDS-PAGE is not used if a particular protein (e.g. an enzyme) has to be separated on the basis of its biological activity, as the protein is denatured by the SDS-PAGE. In native gels, non-denaturing condition are used. SDS is not used and the proteins are not denatured prior to loading. Since all the proteins in the sample being analyzed carry their native charge at the pH of the gel, proteins separate according to their different electrophoretic mobilities.

Immunoblotting

Separation of a mixture of proteins by electrophoretic techniques usually results in a complex pattern of protein bands or zones. Specific proteins can often be identified using an **immunoblotting** technique (also known as Western blotting). This technique requires an antibody against the test protein. After the initial separation by electrophoretic technique in a gel, the proteins are transferred (or blotted) from the gel to a membrane, usually nitrocellulose. The next step involves treating the membrane with a suitable antibody and allowing the reaction to take place. Excess antibodies are then washed from the membrane and the bound antibody which remains is detected using a second antibody against the first.

STUDY QUESTIONS

Q1. Amino acids are frequently separated by either paper electrophoresis or ion exchange chromatography.

a. What is the basis for these separation techniques?

b. Do both techniques use the acid-base properties of the amino acids to effect a separation? How?

c. How are the locations of the amino acids on the chromatogram detected?

d. Can these techniques be used to separate peptides? Explain.

Ans. a. Separation of amino acids is based on variations in electric charge and hydrophobicity, which lead to different migration rates in an electric field (paper electrophoresis) and through a cation-exchange resin (ion-exchange chromatography).

b. Yes. The average net charge on an amino acid is a function of the pH of the solution.

c. The amino acids are made visible on the chromatogram by treatment with ninhydrin, which reacts with the amino group on the N terminus to give a purple spot.

d. Yes. Peptides, like amino acids, exhibit a pH-dependent average charge variation.

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3.3 SPECTROSCOPY

Spectroscopy is the study of the interaction between electromagnetic radiation and matter. The matter can be atoms, molecules, atomic or molecular ions, or solids. It is the use of the absorption, emission, or scattering of electromagnetic radiation by matter to qualitatively or quantitatively study the matter or to study physical processes. A study of the wavelength absorbed or emitted by an atom or a molecule will give information about its identity and this technique is known as *qualitative spectroscopy*. Measurement of the total amount of radiation will give information about the number of absorbing or emitting atoms or molecules and is called *quantitative spectroscopy*.

Electromagnetic radiation

Electromagnetic radiation is a form of energy and has both electrical and magnetic characteristics. A representation of electromagnetic radiation with electric field (E) and the magnetic field (M) at right angle to the direction of the wave is depicted in the figure.

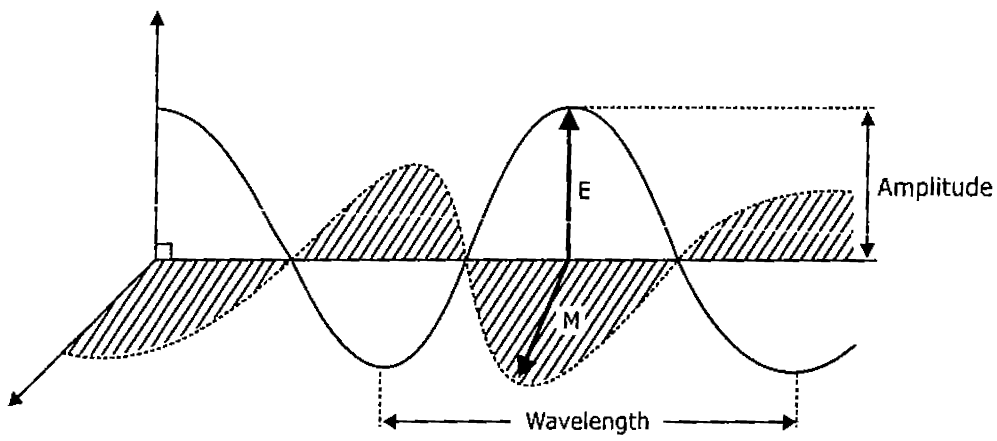


Figure 3.5 : Electromagnetic radiation. A representation of electromagnetic radiation with the electric field (E) and the magnetic field (M) at right angles to the direction of the wave movement. Both fields oscillate at the same frequency.

Electromagnetic spectrum ranges from very short wavelengths (such as gamma ray) to very long wavelengths (radio waves). The visible region of the spectrum extends approximately over the wavelength range 400-700 nm, the shorter wavelengths being the blue end of the spectrum and the longer wavelengths the red and wavelength between 400 and 200 nm make up the near ultraviolet region of the spectrum and wavelengths above 700 nm to approximately 2000 nm (2 μm) the infrared region. The energy associated with a given segment of the spectrum is proportional to its frequency.

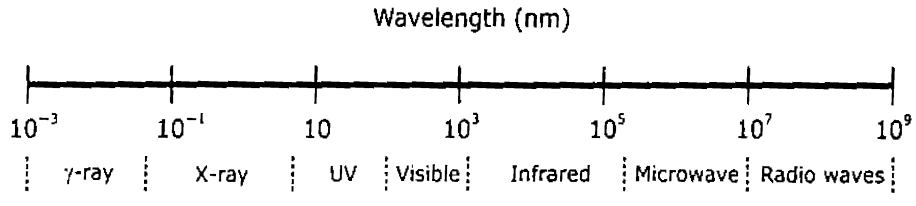


Figure 3.6 : Range of electromagnetic radiation.

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of spectroscopy

When radiation meets matter, the radiation is either scattered, emitted, or absorbed. This gives rise to three principal branches of spectroscopy:

Absorption spectroscopy

Absorption spectroscopy studies radiation *absorbed* at various frequencies (wavelengths). Common example of absorption spectroscopy are ultraviolet/visible (UV/Vis) spectroscopy (to detect molecular content) and infrared (IR) spectroscopy (to determine molecular information, including structural information).

Emission spectroscopy

Emission spectroscopy observes light *emitted* by atoms excited by radiation-matter interactions. The substance first absorbs energy and then radiates this energy as light.

Scattering spectroscopy

Scattering spectroscopy measures certain physical properties by measuring the amount of light that a substance scatters at certain wavelengths. One of the most useful applications of light scattering spectroscopy is Raman spectroscopy.

Principles of absorption spectroscopy

When electromagnetic radiation passes through a material, a portion of the electromagnetic radiation may be absorbed. If that occurs, the remaining radiation, when it is passed through a prism, yields a spectrum with gap in it, called an *absorption spectrum*. The absorption spectrum is characteristic for a particular element or compound, and does not change with varying concentration. At a given wavelength the measured absorbance has been shown to be proportional to the molar concentration of the absorbing species and the thickness of the sample the light passes through. This is known as the *Beer-Lambert law*.

Beer-Lambert law

When radiation fall on homogeneous medium, a portion of incident light is reflected, a portion is absorbed remainder is transmitted. The two laws governing the absorption of radiation are known as Lambert's law and Beer's law. In the combined form they are referred to as *Beer-Lambert law*.

Lambert's law : It states that when monochromatic light passes through a transparent medium, intensity of transmitted light decreases exponentially as the thickness of absorbing material increases.

Beer's law : It states that the intensity of transmitted monochromatic light decreases exponentially as the concentration of the absorbing substance increases.

Mathematical expression of Beer-Lambert law:

The relationship between concentration, length of the light path, and the light absorbed by a particular substance are expressed mathematically as shown below.

$$A = \log \frac{I_0}{I} = \varepsilon c l$$

A = Absorbance

I_0 = Intensity of incident light

I = Intensity of light transmitted through the sample

ε = Extinction coefficient or absorption coefficient for a particular absorbing compound

c = Concentration of absorbing material in the sample

l = Pathlength (cm)

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If the concentration is expressed in molarity, ε is termed *molar absorption coefficient.* If the concentration is expressed in g/liter, ε becomes the *specific absorption coefficient.* The ratio I/I_0 is called the *transmittance* (T); it measures the amount of light transmitted after passing through the medium. The smaller the transmittance, the greater the absorption of light. The absorbance is a dimensionless quantity. Theoretically, A can have any positive value; in practice, for UV and visible spectrometers, 'A' normally varies between zero and one. The Beer-Lambert law forms the quantitative basis for all types of absorption spectroscopy.

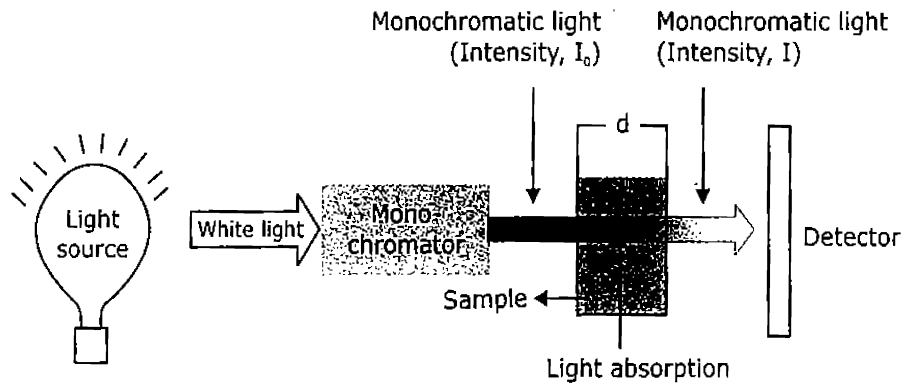


Figure 3.7 : A spectrophotometer. Light from a lamp passes through a monochromator for wavelength selection. Sample is contained in cuvette in a cuvette holder. Light passes through a cuvette and detected by detector.

The spectroscopic technique used to assess the concentration or amount of a given species is termed **spectrometry**. An instrument used to measure the absorbance by measuring the amount of light of a given wave length that is transmitted by a sample is termed **spectrophotometer**. A spectrophotometer consists of a light source, a monochromator, sample holder (cuvette) and a light detector.

STUDY QUESTION

Q2. On passing monochromatic light through a 0.04 molar solution in a cell of 1 cm path length, the intensity of the transmitted light was reduced to 50%. Calculate the molar extinction coefficient of the solute in the solution (given log 2 = 0.3010).

Ans. $A = \log\frac{I_0}{I} = \varepsilon c \ell = 7.5 \text{ liter mol}^{-1} \text{ cm}^{-1}$

UV/VIS absorption spectroscopy

UV/VIS absorption spectroscopy is based on the transitions of electrons from one atomic orbital or molecular orbital to another in that substance due to the absorption of electromagnetic radiation. Atoms and molecules can absorb electromagnetic radiation, but only at certain wavelengths. Various kinds of electronic excitation may occur in organic molecules. As a rule, energetically favored electron promotion will be from the *highest occupied molecular orbital* to the *lowest unoccupied molecular orbital*, and the resulting species is called an *excited state*. When sample molecules are exposed to light having an energy that matches a possible electronic transition within the molecule, some of the light energy will be absorbed as the electron is promoted to a higher energy orbital. An spectrometer records the wavelengths at which absorption occurs, together with the degree of absorption at each wavelength.

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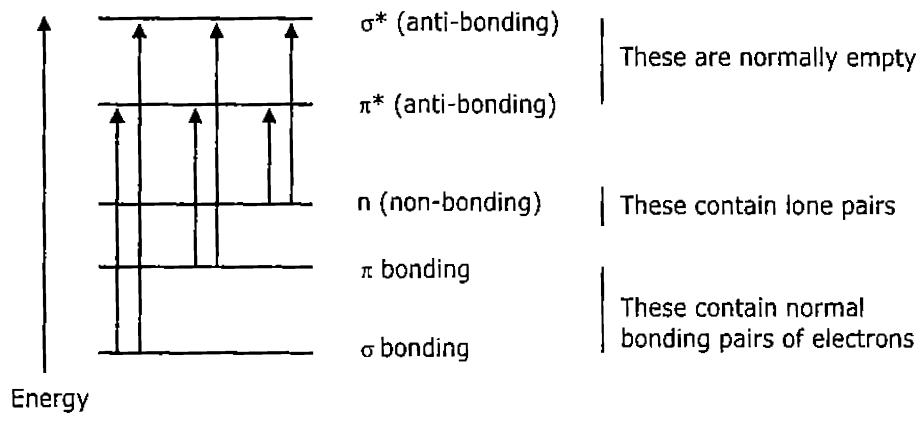


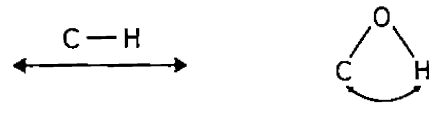
Figure 3.8 : The possible electron jumps that light might cause are. In each possible case, an electron is excited from a full orbital into an empty anti-bonding orbital. Each jump takes energy from the light, and a big jump obviously needs more energy than a small one. Each wavelength of light has a particular energy associated with it. If that particular amount of energy is just right for making one of these energy jumps, then that wavelength will be absorbed - its energy will have been used in promoting an electron.

Note : Very energetic radiations (UV to X-ray region of the spectrum) may cause an electron to be ejected from the molecule (ionization). Radiations in the infrared region of the spectrum have much less energy than radiations in the visible or UV regions of the electromagnetic spectrum. They can cause *vibrations* in molecules. Microwave radiation is even less energetic than infrared radiation. It cannot excite electrons in molecules, nor can it excite vibrations; it can only cause molecules to *rotate*.

IR spectroscopy

Each frequency of light has a certain energy. If a particular frequency is being absorbed as it passes through the compound being investigated, it must mean that its energy is being transferred to the compound. As with other types of energy absorption, molecules are excited to a higher energy state when they absorb *infrared* radiation. The term *infrared* covers the range of the electromagnetic spectrum between 1 micrometer to 100 micrometer. A molecule absorbs only selected frequencies of infrared radiation. The absorption of infrared radiation corresponds to energy changes on the order of 8 to 40 KJ/mole.

IR spectroscopy is one type of *vibrational spectroscopy*. IR radiation does not have enough energy to induce electronic transitions as seen with UV. Energies in infra-red radiation correspond to the energies involved in bond vibrations. At temperatures above absolute zero, all the atoms in molecules are in continuous vibration with respect to each other. Vibrational motion fall into the two main catagories of *stretching* and *bending*.



Stretching Bending

When the frequency of a specific vibration is equal to the frequency of the IR radiation directed on the molecule, the molecule absorbs the radiation. But not all bonds in a molecule are capable of absorbing infrared energy, even if the frequency of the radiation exactly matches that of the bond motion. Only those bonds that have a *dipole moment* are capable of absorbing infrared radiation. This means that for homonuclear diatomic molecules such as H_2, N_2 and O_2 no infrared absorption is observed, as these molecules have zero dipole moment and stretching of the bonds will not produce one. For heteronuclear diatomic molecules such as CO and HCl, which do possess a permanent dipole moment, infrared activity occurs.

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However all molecules having permanent dipole moment are not infrared active. In order to infrared active, a vibration must cause a change in the dipole moment of the molecule. In the case of the *asymmetric stretch* a dipole moment will be periodically produced and destroyed resulting in a changing dipole moment and therefore infrared active. The symmetric stretch will not be infrared active because stretching of this bond leads to no change in dipole moment. For example, in the case of CO_2 the symmetrical stretch is IR inactive because this vibration produces no change in the dipole moment of the molecule. In general, the larger the dipole change, the stronger the intensity of the band in an IR spectrum.

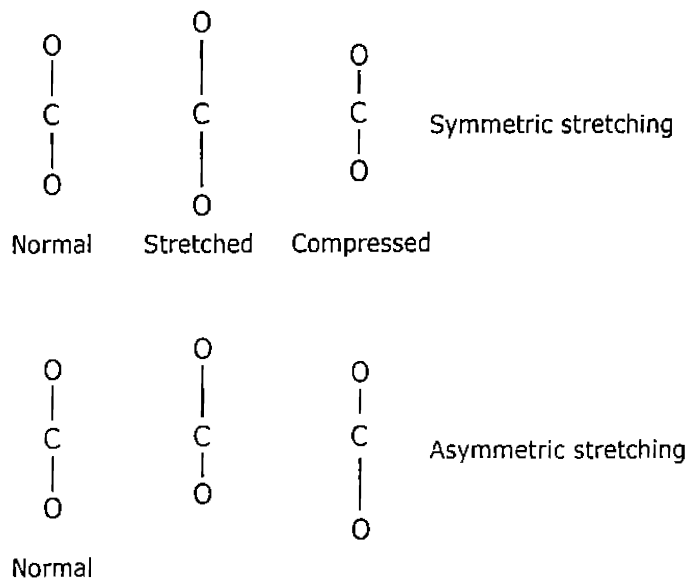


Figure 3.9 : Symmetric and asymmetric stretching vibration of the carbon dioxide molecule.

The energy involved in vibration depends on things like the length of the bond and the mass of the atoms at either end. This means that each different bond will vibrate in a different way, involving different amounts of energy. The amount of energy it needs to do this will vary from bond to bond, and so each different bond will absorb a different frequency (and hence energy) of infrared radiation.

Some general trends:

1. Stretching frequencies are higher than corresponding bending frequencies. It is easier to bend a bond than to stretch or compress it.
2. Bonds to hydrogen have higher stretching frequencies than those to heavier atoms.
3. Triple bonds have higher stretching frequencies than corresponding double bonds, which in turn have higher frequencies than single bonds (except for bonds to hydrogen).

Uses of the infrared spectrum

Since every type of bond has a different natural frequency of vibration, and since two of the same type of bond in two different compounds are in two slightly different environments, no two molecules of different structure have exactly the same infrared absorption pattern, or infrared spectrum. Thus, the infrared spectrum can be used for molecules just like a fingerprint. By comparing the infrared spectra of two substances one can establish whether they are identical or non-identical.

A second use of the infrared spectrum is to determine structural information about a molecule. The absorption of each type of bond (N-H, C-H, O-H, C-X, C=O, C-O, C-C and other) are found only in certain small portions of the infrared region. Thus, a small range of absorption can be defined for each type of bond.

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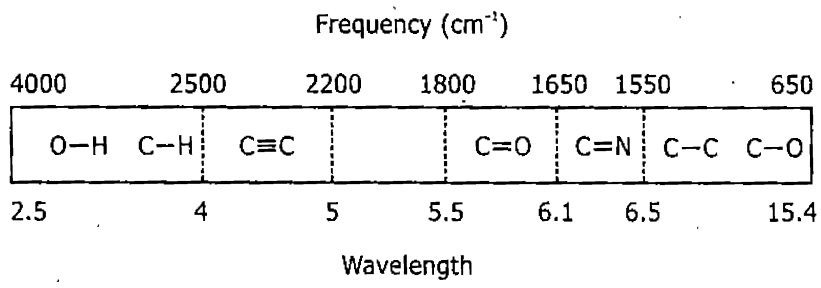


Figure 3.10 : The approximate regions where various common types of bonds absorb (stretching vibrations only).

IR spectroscopy is used to determine the functional groups in the sample. Different functional groups absorb characteristic frequencies of IR radiation.

Fluorescence spectroscopy

Fluorescence is an emission phenomenon. Fluorescence emission of light is observed when, after excitation by the absorption of a photon, an electron returns from the first excited state back to the ground state. In the excited state, some energy is always lost by nonradiative processes (such as transitions between vibrational states). Therefore, the energy of the emitted light is always less than that of the absorbed light, and the fluorescence of a chromophore thus occurs always at greater wavelengths than its absorption. Fluorescence emission is much more sensitive to changes in the environment of the chromophore than light absorption. Changes in protein conformation, very often lead to large changes in the wavelength and intensity of fluorescence emission.

Optical Rotatory Dispersion (ORD) **and Circular Dichroism** (CD)

Most of biological compounds are optically active and thus able to rotate the plane polarized light. The technique of ORD measures the ability of optically active compounds to rotate plain polarized light as a function of the wavelength (i.e., wavelength dependence of optical rotation). The instrument that measures ORD curves is called a *spectropolarimeter*, which differ from a polarimeter in that the latter employs only one wavelength.

The physical basis of CD is same as ORD. In case of CD, circularly polarized light is used. Circularly polarized light is obtained by superimposing two plane polarized light of same wavelengths and amplitudes which are polarized in two perpendicular planes but there is a phase difference of 90° between them. A phase difference of 90° means that when one wave is at its peak then the other one is just crossing the zero line. Similar to plane polarized light, circularly plane polarized light may be right or left handed. If a right and a left circularly polarized light, both of equal amplitude, are superimposed, the result is plane polarized light.

Dichroism is a word derived from Greek which means *two-colors*, because the sample under analysis has one color if illuminated with the right polarized light and a different color if illuminated with the left one. The color, in fact, depends on light absorption. Circular Dichroism (CD) is observed when optically active matter absorbs left and right circularly polarized light slightly different. In fact, circular dichroism is the absorption difference between left and right circularly polarized light at a given wavelength.

Absorption is quantitated by the *molar extinction coefficient,* ε. Optically active samples have distinct molar extinction coefficients for left (ε_L) and right (ε_R) circularly polarized light. The difference in absorbance, A, of the two components, is a measure of **Circular Dichroism** (CD).

Thus, $CD = A_L - A_R$

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The difference between ε_L and ε_R may be expressed as $\Delta\varepsilon$. From Beer-Lambert law the difference in the absorbance of left and right circularly polarized light ΔA, can be given by,

$$\Delta A = \Delta\varepsilon c l, \qquad c \text{ is the concentration and } l, \text{ is the path length.}$$

Because differential absorption of the left and right circularly polarized light means that the amplitudes of the transmitted waves will differ, the result is elliptically polarized light. The ellipticity is proportional to the difference in absorbance of the two components, $A_L - A_R$. Thus, CD is equivalent to ellipticity. The relationships between CD and ellipticity (θ).

The relationships between CD and ellipticity (θ) is given by

$$\theta = 2.303\,(A_L - A_R)\frac{180}{4\pi} = 33\,(A_L - A_R) = 33\,\Delta A \text{ degree}$$

Applications

1. Determination of protein's secondary structure. CD spectra can be readily used to estimate the fraction of a molecule that is in the alpha-helix, the beta-sheet, or random coil conformation. Each of the three basic secondary structures of a polypeptide chain shows a characteristic CD spectrum.

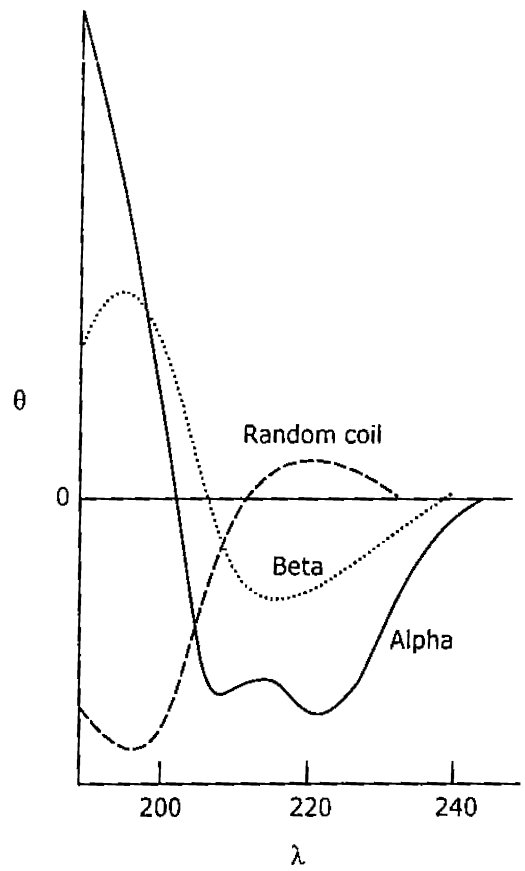


Figure : 3.11
Circular dichroism spectra for poly-L-lysine in the alpha helical, beta and random coil conformations.

2. Determination of optical purity.
3. Analysis of the tertiary structure of proteins and conformational changes.
4. Comparison of the secondary and tertiary structure of wild type and mutant proteins.
5. Nucleic acid structure and changes upon binding or melting.

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Nuclear magnetic resonance (NMR)

All electrons and some nuclei possess a property conveniently called *spin*. All electrons have a spin of 1/2. Protons and neutrons also have a spin of 1/2. Hence if a particular nucleus is composed of *P* protons and *n* neutrons, its total spin will be a vector combination of *p+n* spins each of magnitude 1/2. Each nuclear isotope, being composed of a different number of protons and neutrons, will have its own total spin value. The spin of the H-nucleus (^1H) is 1/2 since it consists of one proton only. The spin of a nucleus is usually expressed by a symbol *I*, called the *spin quantum number.* The nuclear spin (*I*) can have various values such as 0, 1/2, 1, 3/2. Due to spin nuclei of certain isotopes of some elements (^1H, ^{13}C, ^{19}F) behave like minute magnets. These atomic nuclei have different energy levels when placed in a magnetic field. Nuclei that interact with magnetic field are said to possess nuclear spin. With the help of nuclear spin value, the number of energy levels can be determined by using formula $2I + 1$. The ^1H, and ^{13}C nuclei possess nuclear spin and have two different energy levels. When a magnetic field is applied to these nuclei, they can either align themselves with it, which would be the lowest energy state, or they can align themselves against the field, which is higher in energy.

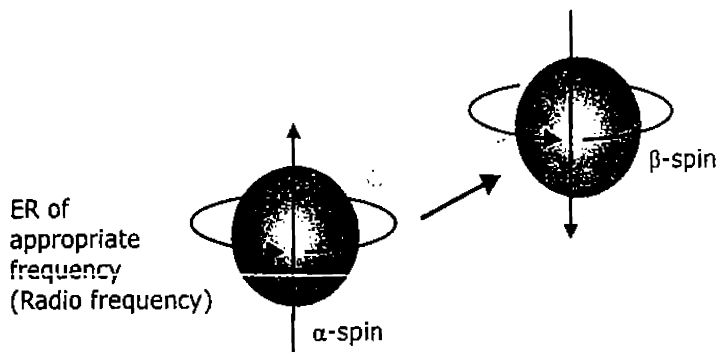
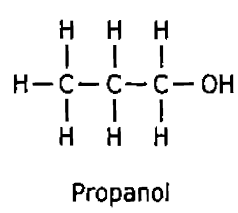


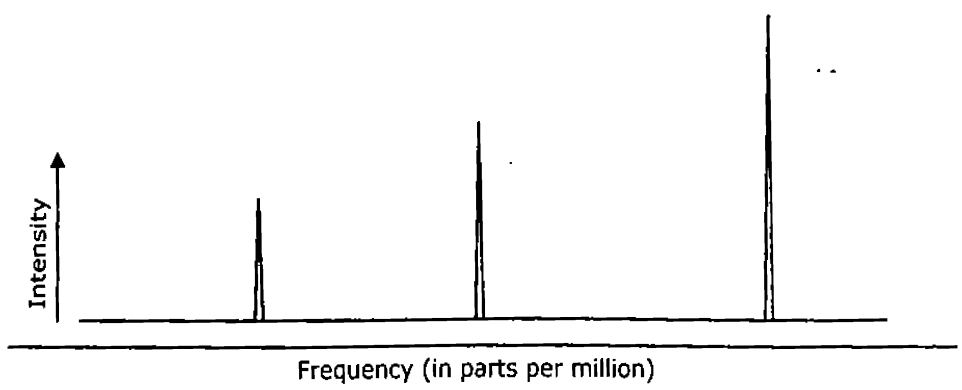
Figure : 3.12
A rotating nucleus can take up either of two orientation in an applied electromagnetic field. The energy separation between the α and β spin states is determined by measuring the frequency of electromagnetic radiation needed to induce $\alpha \rightarrow \beta$ spin.

But since the energy difference between the two states is so small, we don't need to do much work. In fact, the amount of energy needed to flip the nucleus can be provided by electromagnetic radiation of radio-wave frequency. Radio waves flip the nucleus from the lower energy state to the higher state. The nucleus now wants to return to the lower energy state and, when it does so, the energy comes out again and this is what we detect.

In the spectrum of carbon NMR of *propanol* given below 3 peaks form. Each peak represents a different kind of carbon atom: each one absorbs energy at a different frequency. But why should carbon atoms be different? Two factors that affect the energy difference - the magnetic field strength and what sort of nucleus is being studied. So we might expect all carbon-13 nuclei to resonate at one particular frequency. But they don't.



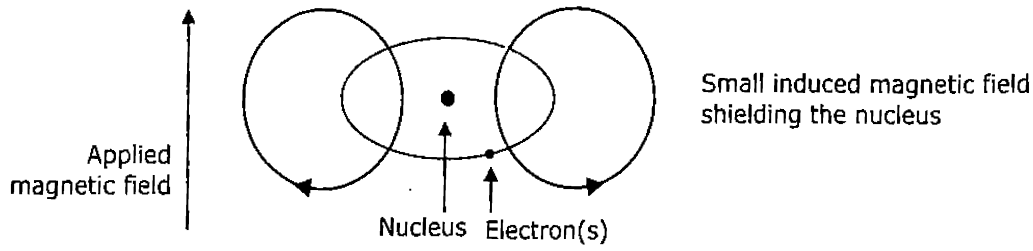
Propanol



The variation in frequency for different carbon atoms must mean that there are different types of carbon atom and their nuclei experience a magnetic field that is not quite the same as the magnetic field that we

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apply. Each nucleus is surrounded by electrons, and in a magnetic field these will set up a tiny electric current. This current will set up its own magnetic field, which will oppose the magnetic field that we apply. The electrons are said to **shield** the nucleus from the external magnetic field. If the electron distribution varies from ^{13}C atom to ^{13}C atom, so does the local magnetic field, and so does the resonating frequency of the ^{13}C nuclei.

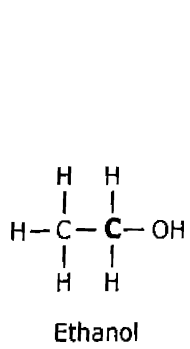


Thus, the changes in the distribution of electrons around a nucleus affect:

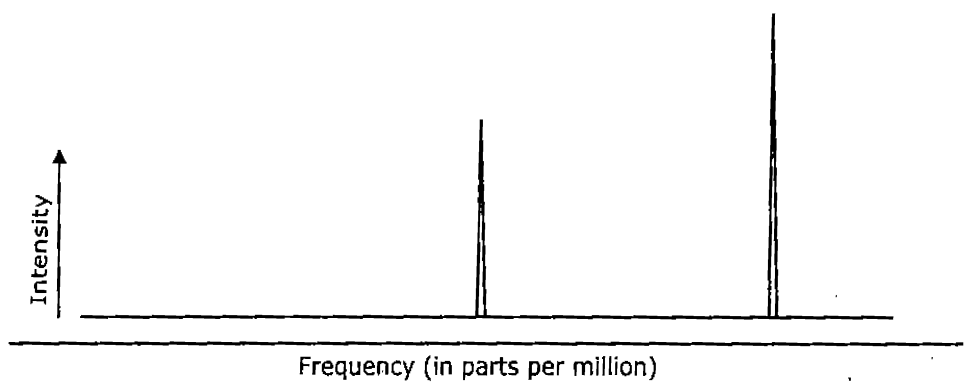
- The local magnetic field that the nucleus experiences
- The frequency at which the nucleus resonates
- The chemistry of the molecule at that atom

This variation in frequency is known as the **chemical shift**.

As an example, consider ethanol. The *bold* carbon attached to the OH group will have relatively fewer electrons around it compared to the *unbold* carbon since the oxygen atom is more electronegative and draws electrons towards it, away from the carbon atom.



Ethanol



The magnetic field that this bold carbon nucleus feels will therefore be slightly greater than the felt by the unbold carbon with more electrons since the bold carbon is less shielded from the applied externai magnetic field – in other words it is **deshielded**. Since the carbon attached to the oxygen feels a stronger magnetic field, there will be a greater energy difference between the two alignments of the nucleus. The greater the energy difference, the higher the resonance frequency. So for ethanol we would expect the bold carbon with the OH group attached to resonate at a higher frequency than the unbold carbon, the indeed this is exactly what the ^{13}C NMR spectrum shows.

MASS SPECTROSCOPY

A mass spectroscopy determines the molecular weight by measuring the **mass-to-charge** ratio for ions in the gas phase. In this process the ions are generated in the ionization source by inducing either the loss or the gain of a charge (e.g., electron ejection, protonation, or deprotonation). Once the ions are formed in the gas phase, they can be electrostatically directed into a mass analyzer, separated according to mass, and finally detected. Thus, a mass spectrometer has three basic components of : the *ionization source*, the *mass analyzer*, and the *detector*.

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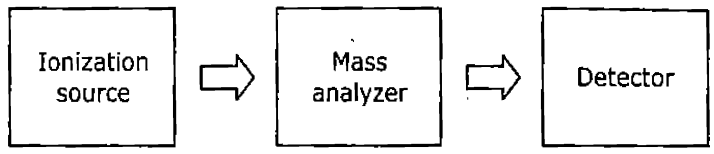


Figure 3.13 : Components of a mass spectrometer.

The operation of the mass spectrometer involves the following steps:

1. Production of the sample in an ionized form in the vapour phase;
2. Acceleration of the ions in an electric field, each ion emerging with a velocity proportional to its charge/mass ratio;
4. Passage of the ions into a field-free region;
3. Detection of the times of arrival of the ions, the *time-of-flight* indicating the mass-to-charge ratio of the ions.

Ionization process

A sample molecule studied by mass spectrometer must be converted to charged particles by ionization process before they can be analyzed and detected. Several methods are used for converting the sample to ions. These methods include:

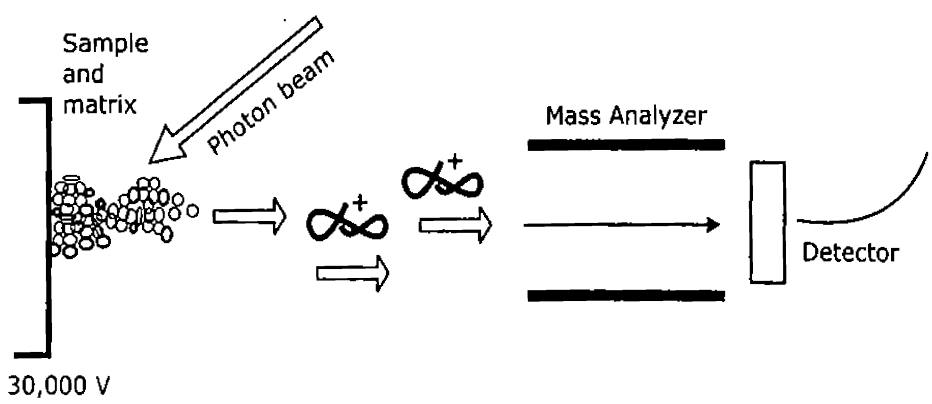
Electron ionization : In electron ionization, a beam of high energy electrons strikes the molecules. The electron- molecule collision strips an electron from the molecule, creating a cation.

Chemical ionization : In chemical ionization, the sample molecules are combined with a *ionized* reagent gas. When the sample molecules collide with the ionized reagent gas, some of the sample molecules are ionized by various mechanisms like proton transfer, electron transfer and adduct formation.

Desorption ionization : In desorption ionization, the sample to be analyzed is dissolved in a matrix and placed in the path of high energy beam of ions or high intensity protons. In case of high intensity photon, it is termed as MALDI (*matrix-assisted laser desorption/ionization*). In MALDI, analytes are placed in a light-absorbing solid matrix. Solid matrix strongly absorbs the laser radiation and acts as a receptacle for energy deposition. With a short pulse of laser light, the analytes are ionized and then desorbed from the matrix into the vacuum system.

Electrospray ionization : In *ESI,* a solution containing the sample molecules is dispersed into highly charged droplets by passage through a needle under the influence of a high-voltage electric field. The droplets evaporate, and the ions enter the mass analyzer for m/z measurement.

All these are fundamentally different ionization techniques but they achieve essentially the same end result. The generation of gas phase ions *via* non destructive vaporization and ionization.



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Mass analysis

Once the sample has been ionized, the beam of ions is accelerated by an electric field and then passes into the mass analyzer, the region of the mass spectrometer where the ions are separated according to their mass to charge ratio. Just like ionization methods there are several types of mass analyzer.

The most common type of mass analyzer is TOF (time of flight) mass analyzer. The TOF mass analyzer measure ion flight time. It is based on the simple idea that velocities of two ions with the same kinetic energy will vary depending on the mass of ion – the lighter ion will have higher velocity.

Mass spectrometry can be used to measure the mass of biomolecules well over 200,000 Da, to provide sequence information of unknown peptides and proteins, to detect noncovalent complexes.

3.4 CENTRIFUGATION

Centrifugation is a process used to separate or concentrate materials suspended in a liquid medium. It is a method to separate molecules based on their sedimentation rate under centrifugal field. It is also used to measure physical properties (e.g. molecular weight, density, and shape).
Centrifugation used for separation of one type of material from others is termed as *preparative centrifugation* whereas if it is used for measurement of physical properties (e.g., molecular weight, density, shape) of macromolecules then termed as *analytical centrifugation*.

Sedimentation in the ultracentrifuge

Particles suspended in a solution are pulled downward by Earth's gravitational force. In a solution, particles whose mass or density is higher than that of the solvent sink (*sediment*), and particles that are lighter than it *float* to the top. The greater the difference in mass or density, the faster they move. This movement is partially offset by the buoyancy of the particle. Because Earth's gravitational field is weak, a solution containing structure like cell organelles or macromolecules is usually homogeneous, due to the random thermal motion of the molecules. However sedimentation of cell organelles or macromolecules can be enhanced by applying centrifugal forces. A ultracentrifuge increases the sedimentation by subjecting particles to centrifugal forces as great as 1,000,000 times the force of gravity *g*, which can sediment particles as small as 10 kDa.

Consider a solution under the influence of a strong gravitational field, such as a solution being spun in a centrifuge tube. The centrifugal force acting on a solute particle of mass m is $m\omega^2 r$, where ω is the angular velocity in radians per second, r is the distance from the center of rotation to the particle, and $\omega^2 r$ is the centrifugal acceleration.

In addition to centrifugal force, we must also consider the particle's buoyancy due to the displacement of the solvent molecules by the particle. This buoyancy reduces the force on the particle by $\omega^2 r$ times the mass of the displaced solvent. Thus, the net force acting on the particle is given by

$$\text{Net force} = \text{centrifugal force} - \text{buoyancy force}$$

$$= \omega^2 rm - \omega^2 rv\rho$$

Where v and ρ are the volume of the particle and the density of the solution, respectively.

At the same time, the motion of particle through the solution is opposed by the frictional force. The frictional force is equal to the product of the frictional coefficient, f (in units $Nm^{-1}s$), and the sedimentation velocity, v. It acts in the opposite direction to the net force.

$$F_{friction} = vf$$

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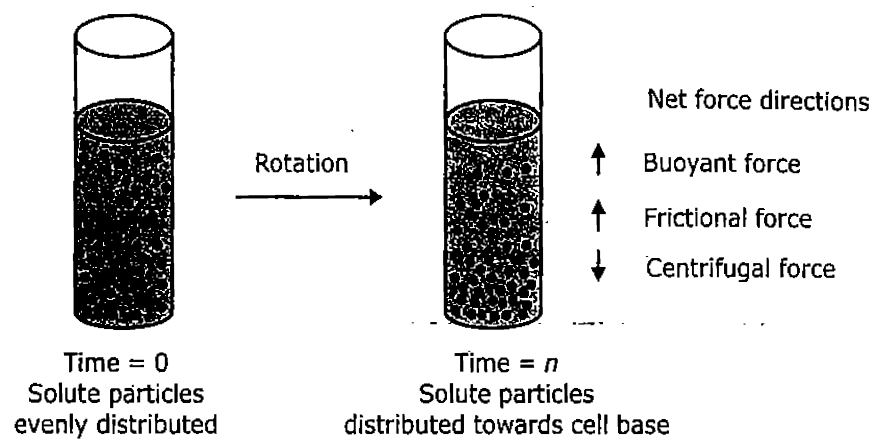


Figure 3.14 : A schematic diagram of a centrifugation experiment.

At steady state, then, the *frictional force* is equal to the net force and the molecule moves with velocity v toward the bottom of the cell

$$fv = \omega^2 rm - \omega^2 rv\rho$$

Because measuring the volume of the particle is difficult, for convenience we use a term called *partial specific volume*, $\bar{v}$.

$$fv = \omega^2 rm - \omega^2 rm\bar{v}\rho$$

$$fv = \omega^2 rm(1 - \bar{v}\rho)$$

The mass of 1 mol of particles, M is; M = mN, where N = Avogadro's number

$$fv = \frac{M\omega^2 r(1 - \bar{v}\rho)}{N}$$

Now, the *sedimentation coefficient*, S,

$$S = \frac{v}{\omega^2 r} = \frac{M}{N}\frac{(1 - \bar{v}\rho)}{f}$$

The sedimentation coefficient, a quantity that is analogous to the electrophoretic mobility in that it is a velocity per unit force, is usually expressed in units of 10^{-13} sec, which are known as *Svedbergs* [s].

Important conclusions drawn from equation:

- The sedimentation velocity of a particle is proportional to its mass.
- A dense particle moves more rapidly than a less dense one because the opposing buoyant force is smaller for a dense particle.
- Shape, too, is important because it affects the viscous drag. The frictional coefficient of a compact particle is smaller than that of an extended particle of the same mass. A parachutist with a defective unopened parachute falls much more quickly than one with a functioning opened parachute.
- The sedimentation velocity depends also on the density of the solution (ρ). Particle sink when $\bar{v}\rho < 1$, float when $\bar{v}\rho > 1$.

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Differential centrifugation

Differential centrifugation separates particles based on differences in sedimentation rate, which reflect differences in size/mass and densities. Differential centrifugation is used for the separation of sub-cellular components.

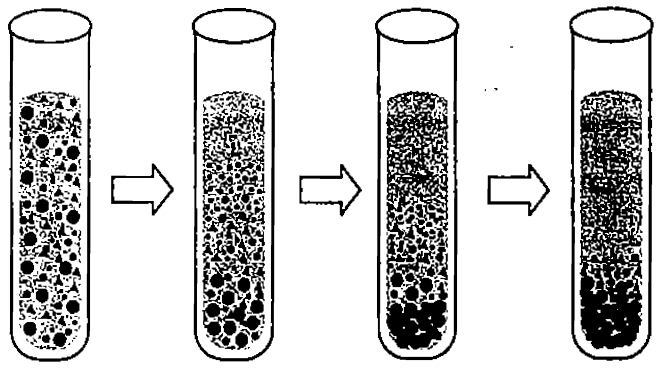


Figure : 3.15
Differential centrifugation separates particles based on differences in sedimentation rate, which reflect differences in size and/or density. Particles that are large or dense sediment rapidly, those that are intermediate in size or density sediment less rapidly, and the smallest or least dense particles sediment very slowly. Eventually, all of the particles reach the bottom of the tube.

In the process of separation of sub-cellular components the preparation of broken cells is poured into a centrifuge tube and is initially centrifuged at low centrifugal force long enough to completely sediment the largest and heaviest sub-cellular component. The supernatant obtained is carefully decanted and is again centrifuged at a higher centrifugal force for sedimenting the next heavier entity in the extract. This process is continued and at each ensuring step the centrifugal force as well as the centrifugal time is increased to successively sediment the lighter components and particles. In this method, the separation occurs due to the differential sedimentation rates of the sub-cellular organelles because of the differences in their sizes and densities.

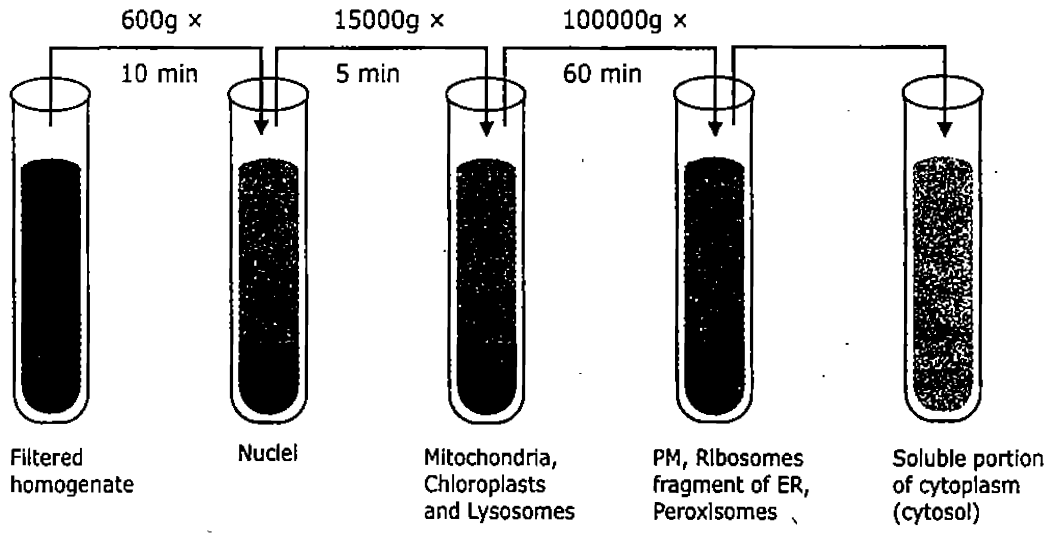


Figure 3.16 : Differential centrifugation. The different sedimentaton rate of various cellular components make their separation possible through differential centrifugation. A force of 600g is generated by rotating at 500 rpm, where g denotes the force of gravity.

Density gradient centrifugation

In differential centrifugation, the particles about to be separated were uniformly distributed throughout the solution prior to centrifugation. Density gradient centrifugation is a variation of differential centrifugation in which the sample is centrifuged in a medium that gradually increases in density from top to bottom. The gradient consists of an increasing concentration of solute (and therefore density) from the top of the tube to

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...ottom. It is of two types : *zonal centrifugation*, in which the sample is centrifuged in a preformed ...nt and *isopycnic centrifugation*, in which a self generating gradient forms during centrifugation.

...zonal centrifugation

...zonal centrifugation is used to separate particles on the basis of differences in their sedimentation ... Separation of particles occurs according to their mass or size. Although the rate of sedimentation is ...ighly influenced by the mass of particles but even slight variation in shape also affect the rate of ...mentation. Materials used for the preparation of density gradients are sucrose, glycerol, ficoll etc. A ...ncentrated sucrose solution is commonly used to form density gradient. In this centrifugation process ...mples are centrifuged just long enough to separate the molecules of interest into discrete zones. If a ...mple is centrifuged much longer than necessary, all the components of sample will end up in a pellet at ...e bottom of the tube.

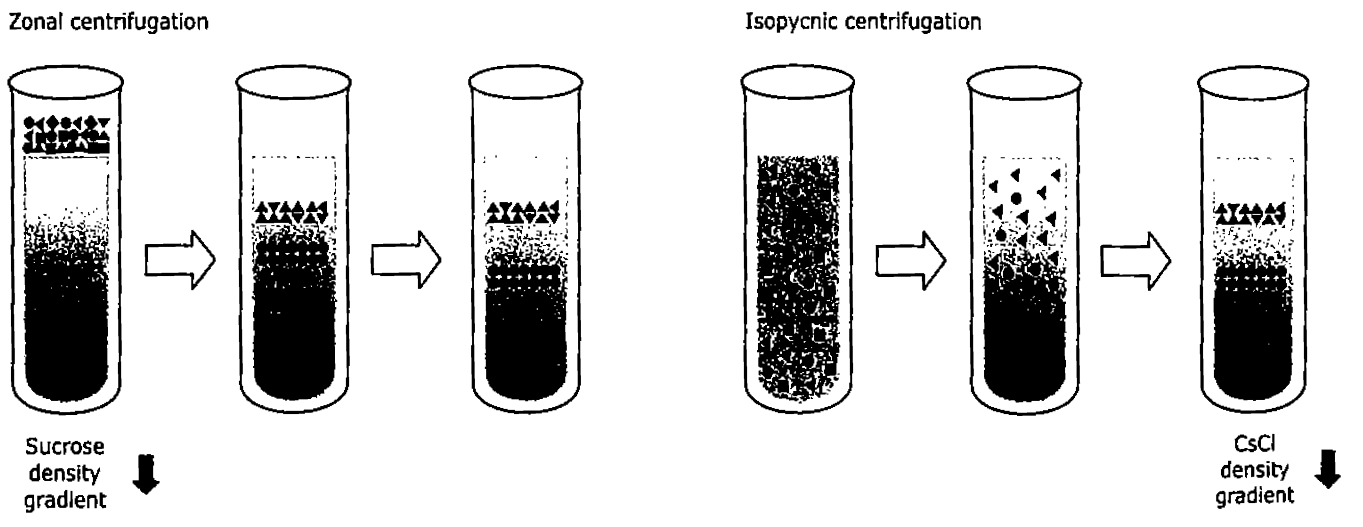
In addition to the preparation and purification of macromolecules and cellular components, rate zonal centrifugation can be used to determine the sedimentation coefficients and molecular weights of biological macromolecules. If a purified molecule such as a protein is spun in a centrifugal field, the molecule will eventually sediment toward the bottom at a constant velocity. At this point, the molecular weight (M) can be calculated as $M = f \times v/\omega^2 r$.

Where f is the *frictional coefficient* of the solvent system (which has been calculated from other measurements) and $v/\omega^2 r$ is the rate of sedimentation per unit applied centrifugal field. The latter value is given the symbol S, or *sedimentation coefficient*. A sedimentation coefficient of 1×10^{-13} is defined as one *Svedberg unit* (S); this unit is named for The Svedberg, a pioneer in the field of centrifugation.

Isopycnic centrifugation or **equilibrium density gradient centrifugation**

In contrast to rate zonal centrifugation, isopycnic centrifugation separates particles solely on the basis of buoyant density and independent of shape and size of particles. It is also independent of time of centrifugation. Thus technique is used to separate particles of similar size but different density. Sedimentation of particles in density gradient occurs until the buoyant density of particle is equal to the density of the gradient.

The zonal and equilibrium centrifugation methods are often confused because both utilize gradients through which the sedimenting molecules move (sucrose and CsCl, respectively). However, the sucrose plays no direct role in the separation of the sedimenting molecules while the CsCl gradient performs the separation. Another distinction is that when the centrifugation time of a zonal experiment is too long all of the sedimenting molecules will be found on the bottom of the centrifuge tube. In contrast, lengthening the centrifugation time of a CsCl gradient experiment will have no effect because the distribution of the molecules is at equilibrium.



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3.5 MICROSCOPY

Microscopy is a technique for making very small things visible to the unaided eye. An instrument used to make the small things visible to the naked (unaided) eye is called *microscope*. There are two fundamentally different types of microscope: the *light microscope* and the *electron microscope*.

Light microscope

Light microscope uses visible light as a source of illumination. Light microscopes are able to magnify to a maximum of approximately 1500x and has limit of resolution of about 0.2 μm. Both living and dead specimens are viewed with a light microscope.

The simplest form of light microscope consists of a single lens, a *magnifying glass*. Microscope made up of more than one glass lens in combination is termed *compound microscope*. Compound microscope includes *condenser* lens, the *objective* lens and the *eyepiece* lens. Condenser lens focus the light from the light source at the specimen. The objective lens is responsible for producing the magnified image. Objective lens are available in many different varieties (4x, 10x, 20x, 40x, 60x, 100x). Lenses can either be dry or immersion lenses. The eyepiece (also referred to as the *ocular*) works in combination with the objective lens to further magnify the image. A compound microscope with a single eyepiece is said to be *monocular*; one with two eyepieces is said to be *binocular*. Eyepieces usually magnify by 10x, since an eyepiece of higher magnification merely enlarges the image, with no improvement in resolution.

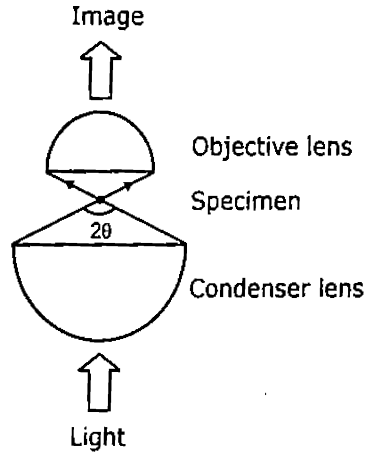
Magnifying power and Resolving power

The *magnifying power* of a compound microscope is defined as the ratio of the size of the final image as seen through the microscope to the size of the object as seen with a naked eye. The magnifying power of a compound microscope is the product of the magnification of the objective and the magnifying power of the eyepiece. The power of a microscope is described with a number followed by the letter "x". For example, if through a microscope you can see something 25 times larger than actual size, its magnification power is 25x.

Resolving power is the ability of magnifying instrument to distinguish two objects that are close together. The resolving power of light microscope depends upon the wavelength (l) and the numerical aperture (NA) of the objective lens. The *limit of resolution*, defined as the minimum distance between two points that allows for their discrimination as two separate points is:

$$\text{Limit of resolution} = \frac{0.61\lambda}{NA}$$

The numerical aperture is, NA = n × sin θ. Here, n is the refractive index of the medium and sin θ is the sine of the semiangle of aperture. *The limit of resolution is inversely related to the resolving power*; i.e., the higher the resolving power, the smaller the limit of resolution.



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Since sin θ cannot exceed 1, and the refractive index of most optical material does not exceed 1.6, the maximal NA of lenses, using oil immersion, is about 1.4. The resolution power of unaided human eye is 100 micrometer.

Microscope type

Brightfield microscopy

The original, and most commonly used, form of microscopy, in which the specimen is viewed by transmitted light from a condenser lens. In a bright field microscope, light is aimed toward a condenser, through the specimen, through an objective lens, and to the eye through a second magnifying lens, the ocular or eyepiece. Objects are visible in the light path because natural pigmentation or stains absorb light differentially, or because they are thick enough to absorb a significant amount of light despite being colorless.

Darkfield microscopy

A dark-field microscope is a type of microscope in which objects are illuminated at a very low angle from the side so that the background appears dark and the objects show up against this dark background. Dark field microscopy is a technique for improving the contrast of unstained, transparent specimens. Darkfield illumination uses a carefully aligned light source to minimize the quantity of directly-transmitted light and collecting only the light scattered by the sample. To view a specimen in dark field, an opaque disc is placed underneath the condenser lens, that blocks light from entering the objective directly; light reflected by specimen enters the objective, and the specimen appears light against a black background.

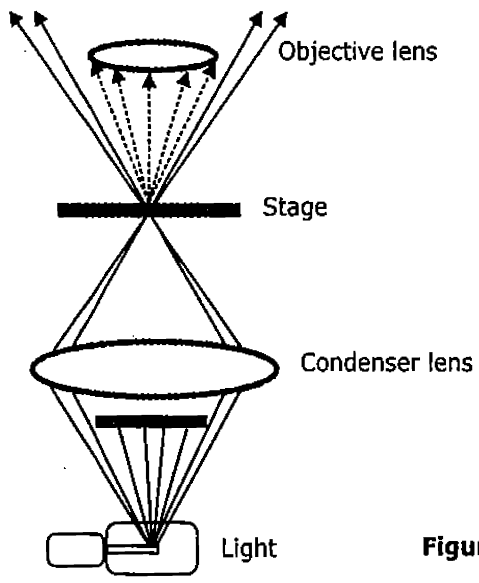


Figure 3.17 : Darkfield microscopy

Phase-contrast microscopy

When light passes through a living cell, the phase of the light wave is changed according to the cell's refractive index: light passing through a relatively thick or dense part of the cell, such as the nucleus, is retarded; its phase, consequently, is shifted relative to light that has passed through an adjacent thinner region of the cytoplasm. The phase-contrast microscope exploit the interference effects produced when these two sets of waves recombine, thereby creating an image of the cell's structure. The specimen appears as different degrees of brightness and contrast. It is used for the study of living cells, which are, in general, transparent to light.

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Fluorescence microscopy

A chemical is said to be fluorescent if it absorbs light at one wavelength and emits light (fluoresces) at a specific and longer wavelength. Most fluorescent dyes, or *flurochromes,* emit visible light, but some emit infrared light. In fluorescence microscopes, only fluorescent light emitted by the sample is used to form an image; light of the exciting wavelength induces the fluorescence but is then not allowed to pass the filters placed between the objective lens and the eye. Fluorescence microscopy is most often used to detect specific proteins or other molecules in cells and tissues. Two fluorescent dyes that are commonly used are *fluorescein,* which emits an intense green fluorescence when excited with blue light, and *rhodamine,* which emits a deep red fluorescence when excited with green-yellow light.

Confocal microscopy

Confocal is defined as *having the same focus*. What this means in the microscope is that the final image has the same focus as or the focus corresponds to the point of focus in the object. The object and its image are *confocal*. The microscope is able to filter out the out-of-focus light from above and below the point of focus in the object. Normally when an object is imaged in the fluorescence microscope, the signal produced is from the full thickness of the specimen which does not allow most of it to be in focus to the observer. The confocal microscope eliminates this out-of-focus information by means of a confocal *pinhole* situated in front of the image plane which acts as a spatial filter and allows only the in-focus portion of the light to be imaged. Light from above and below the plane of focus of the object is eliminated from the final image. The confocal microscope uses a laser beam to illuminate a specimen, usually one that has been fluorescently stained. A diagram of the confocal principle is shown below.

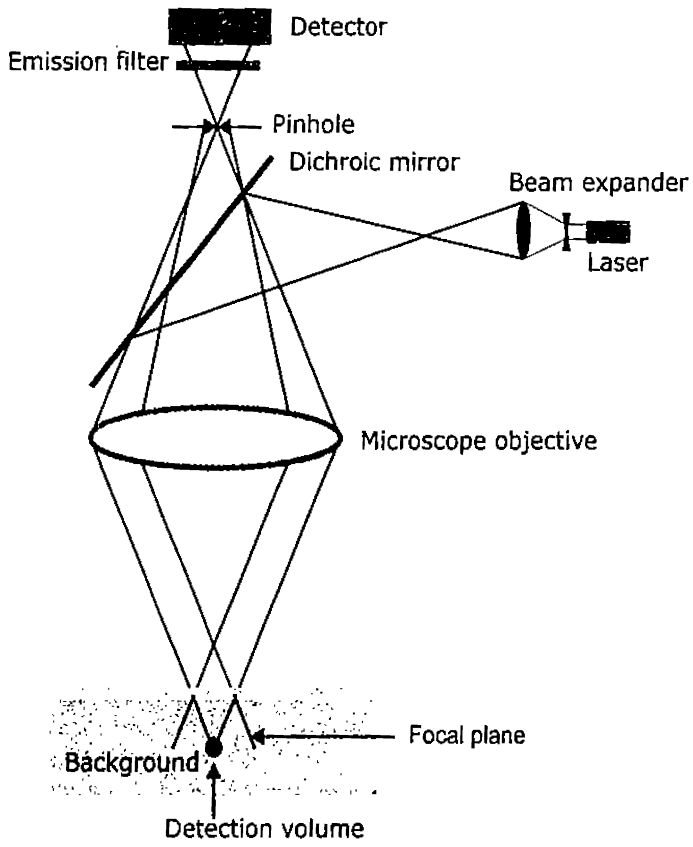


Figure : 3.18
Ray path in a confocal microscope.
A microscope objective is used to focus a laser beam onto the specimen, where it excites flourescence. The flourescent radiation is collected by the objective and efficiently directed onto the detector via a dichroic beam splitter. The interesting wavelength range of the flourescence spectrum is selected by an emission filter, which also acts as a barrier blocking the excitation laser line. The pin hole is arranged infront of the detector, on a plane conjugate to the focal plane of the objective. Light coming from planes above or below the focal plane is out of focus when it hits the pin hole, so most of it cannot pass the pin hole and therefore does not contribute to forming the image.

Electron microscope

The fundamental principles of electron microscopy are similar to those of light microscopy except one major difference of using electromagnetic lenses, rather than optical lenses to focus a high-velocity electron beam instead of visible light. Due to the short wavelength of electrons, the resolving power of electron microscope is very high. Electron microscopy is not used to study live cells.

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Table 3.2 : Differences between light and electron microscopes

Feature	Light Microscope	Electron Microscope
Highest practical magnification	About 1,000-1,500	Over 100,000
Best resolution	0.2 μm	0.5 nm
Radiation source	Visible light	Electron beam
Medium of travel	Air	High vacuum
Type of lens	Glass	Electromagnet
Source of contrast	Differential light absorption	Scattering of electrons
Specimen mount	Glass slide	Metal grid (usually copper)

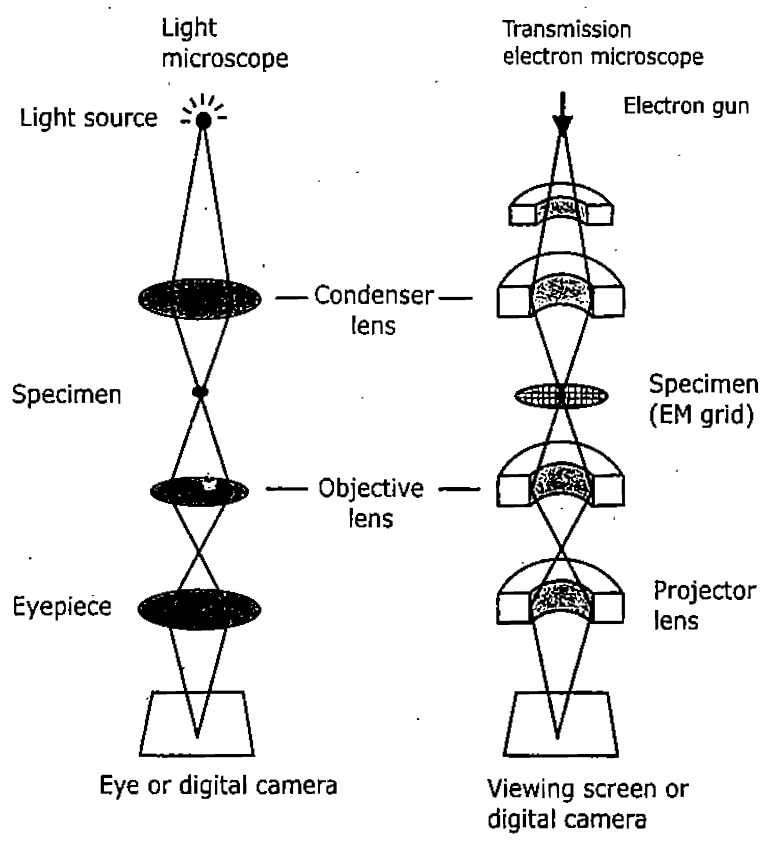


Figure 3.19 : Light and electron microscopy.

Transmission electron microscope

In the *transmission electron microscope* (TEM), the beam of electrons are emitted from a filament and accelerated in an electric field. A condenser lens focuses the electron beam onto the specimen. The beam passes through the specimen so that some of the electrons are absorbed and some scattered. The remaining electrons are focused onto a fluorescent screen or special photographic plates via the use of magnetic lenses. Because electrons are absorbed by atoms in air, the entire tube between the electron source and the detector is maintained under an ultrahigh vacuum.

Scanning electron microscope

The scanning electron microscope views the surfaces of specimens. The sample is fixed, dried, and coated with a thin layer of a heavy metal, such as gold or a mixture of gold and palladium. An intense electron beam inside the microscope scans rapidly over the sample. Molecules in the specimen are excited and release

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secondary electrons that are focused onto a scintillation detector; the resulting signal is displayed on a cathode-ray tube. The resolving power of scanning electron microscopes, which is limited by the thickness of the metal coating, is only about 10 nm, much less than that of transmission electron microscope. The image produced appear three-dimensional (transmission electron microscope produces two-dimensional image).

Scanning tunneling electron microscopy

The scanning tunneling electron microscopy (STM) utilizes a tiny probe that does not emit an electron beam, but instead possesses a tip made of a conducting material such as platinum-iridium. It can move in three dimensions over a surface. The *x* and *y* dimensions scan the surface, while the z dimension governs the distance of the tip above the surface. When the tip and sample are connected with a voltage source, a small tunneling current flows between the tip and specimen surface (the specimen must be an electrical conductor). If the tip is close enough to the surface and the surface is electrically conductive, electrons will begin to leak or *tunnel* across the gap between the probe and the sample. This current can be measured, and the magnitude depends on the distance between the tip and the surface. The tunneling is highly dependent on the distance, so that even small irregularities in the size will affect the rate of electron tunneling. The instrument is designed to move the tip in the z direction to maintain a constant current flow. The movement is therefore a function of the tunneling current and is presented on a video screen.

Atomic-Force microscopy

Atomic-force microscopy (AFM)is similar to scanning-tunneling microscopy in that it can image surfaces at atomic-scale resolution. The difference between AFM and STM is that AFM does not require that the sample to be an electrically conducting material. Like STM it uses an atomically-sharp tip that is brought very close to the surface. The tip will feel a chemical attraction or repulsion and will move up or down on its supporting cantilever. The key to the sensitivity of AFM is in monitoring the movement of the tip. A common means of monitoring the tip movement is to use a laser beam that is reflected or diffracted by the tip or cantilever. Up or down movement of the tip is then detected by changes in the laser beam position. As in STM, moving the tip across the surface produces a topographic map of the surface with atomic resolution.

Sample preparation : Fixation and staining

Fixation

The first step in sample preparation, has the aim of preserving tissue in its original state. Specimens for light and electron microscopy are commonly fixed with a solution containing chemicals that crosslink most proteins and nucleic acids. Fixatives kill the cells while preserve their structural appearance.

The most widely employed fixatives are acids and aldehydes such as acetic acid, picric acid, formaldehyde, and glutaraldehyde. Glutaraldehyde is the most commonly used primary fixative. It penetrates rapidly and stabilizes proteins by forming cross links, but does not fix lipids. Other common fixative is formaldehyde which cross-links amino groups on adjacent molecules. Osmium tetroxide is used as a secondary fixative, reacting with lipids and acting as a stain. Following each fixation step, excess fixative must be washed out of the tissue.

Staining

Most biological materials show little contrast with their surroundings unless they are stained. In the case of light microscopy, contrast can be enhanced by using coloured stains which selectively absorb certain wavelengths. Specimens for light microscopy is stained to visualize the structural features. Many chemical stains bind to specific molecules present in specimens. For example, hematoxylin binds to basic amino acids (lysine and arginine) of different proteins, whereas ecsin binds to acidic molecules (such as DNA and side chains of aspartate and glutamate).

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...ative staining

...electrons in the electron microscope are absorbed very little by biological material and contrast is ...tained mainly by electron scattering. To heighten the contrast between specimen and the background ...ectron-dense stains are used. These are usually compounds of heavy metals of high atomic number that ...erve to scatter the electrons from regions covered with the stain. If specimens are coated with stain (positive staining), fine detail may be obscured. Negative staining overcomes this problem by staining the background and leaving the specimens relatively untouched. The negative stain is moulded round the specimen.

3.6 FLOW CYTOMETRY

Flow cytometry is a technique for counting, examining, and sorting microscopic objects suspended in a fluid. A flow cytometer, also known as *fluorescence activated cell sorter (FACS)*, identifies different cells by measuring the light that they scatter and the fluorescence that they emit as they flow through a laser beam; thus it can sort out cells of a particular type from a mixture. The flow cytometer uses a laser beam and light detector to count single intact cells in suspension. 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 detector.

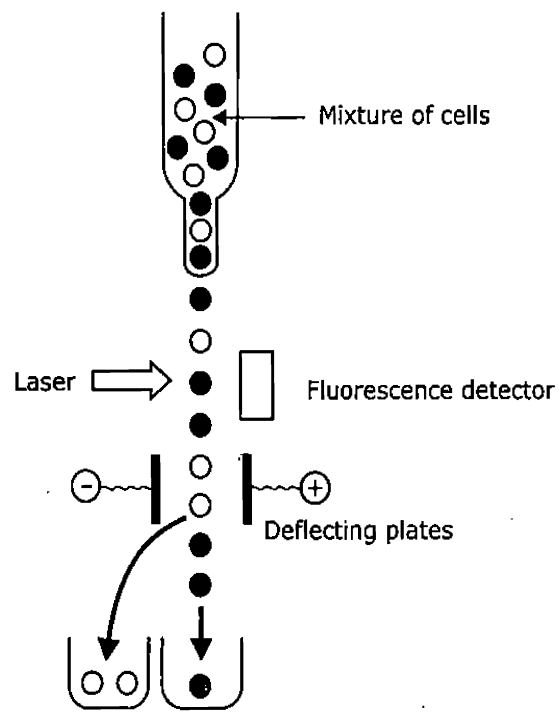


Figure : 3.20
Fluorescence-activated cell sorter (FACS) seperates cells that are labeled differentially with a fluorescent reagent. A mixture of fluorescently stained cells is passed through a small aperture so that each drop that emerges contains just one cell. The fluorescence detector identifies the signal from drops containing the correct cell and applies an electric charge to these drops. when the drops reach the electric plates, the charged ones are deflected into a separate beaker. All other drops fall straight through the deflecting plates and are collected in the waste beaker.

The flow cytometer has multiple applications. The FACS is commonly used to separate different types of cells (cell sorting), each of which bears on its surface one or more distinctive proteins and will thus bind monoclonal antibodies specific for that protein. 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. It is also used to measure density of particular antigen.

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3.7 DIALYSIS

Dialysis is a separation technique that facilitates the removal of small, unwanted compounds from macromolecules in solution by passive diffusion through a *dialysis membrane*. A dialysis membrane is a semi-permeable membrane such as a cellulose membrane with pores. Molecules larger than the pores cannot pass through the membrane but small molecules can do so freely. Dialysis works by diffusion, a process that results from the thermal, random movement of molecules in solution and leads to the net movement from areas of higher to lower concentration (until an equilibrium is reached).

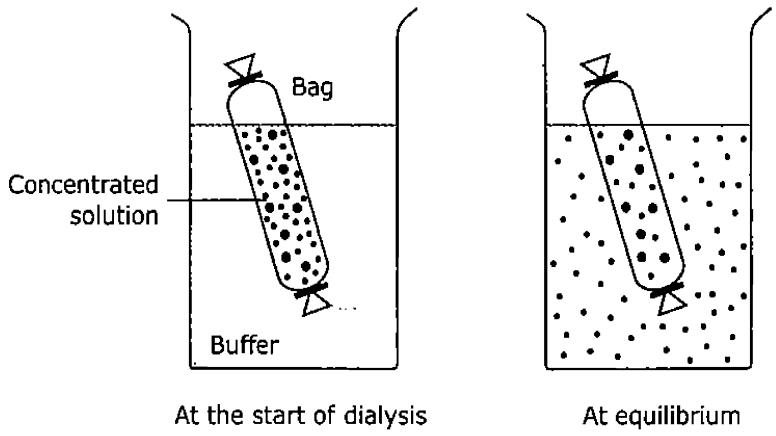


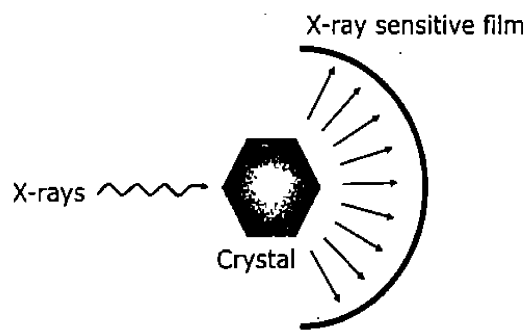
Figure : 3.21
During dialysis only small molecules diffuse through the membrane. At equilibrium, the concentration of small molecules is the same inside and outside the membrane. Macromolecules remain inside the bag.

In dialysis, a sample and a buffer solution (called the *dialysate*, usually 200-500 times the volume of the sample) are placed on opposite sides of the membrane. Sample molecules that are larger than the membrane-pores are retained on the sample side of the membrane, but small molecules and buffer salts pass freely through the membrane, reducing the concentration of those molecules in the sample. Changing the dialysate buffer removes the small molecules that are no longer in the sample and allows more contaminants to diffuse into the dialysate. In this way, the concentration of small contaminants within the sample can be decreased to acceptable or negligible levels.

3.8 X-RAY CRYSTALLOGRAPHY

X-ray crystallography is a method of determining the arrangement of atoms within a crystal. Crystal structure is a unique arrangement of atoms or molecules in space. X-ray crystallography technique is based on **X-ray diffraction**, a non destructive technique. When a beam of X-ray strikes a crystal, the beam may be differacted. From the angles and intensities of these differacted beams, a 3-dimentional structure of the density of electrons within the crystal can be derived.

When X-rays interact with a single particle, it scatters the incident beam uniformly in all directions. However, when X-rays interact with a solid material the scattered beams can add together in a few directions and reinforce each other to yield diffraction. The regularity of the material is responsible for the diffraction of the beams.



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Why are X-rays used in crystallography? The wavelengths of X-rays (very short wave length between 0.01 and 10nm) are of the same order of magnitude as the distances between atoms or ions in a molecule or crystal. When X-rays are scattered from a crystalline solid they can constructively interfere, producing a diffracted beam. Constructive interference occurs when the waves are moving in phase with each other.

The electrons that surround the atoms are the entities which physically interact with the incoming X-ray to diffract them, not the atomic nuclei. Diffraction pattern is recorded on photographic plate and then analyzed to reveal the nature of that lattice. For X-ray crystallography, molecule must be crystallized.

The relationship describing the angle at which a beam of X-rays of a particular wavelength diffracts from a crystalline surface was discovered by Sir William H. Bragg and Sir W. Lawrence Bragg and is known as *Bragg's Law*

$$n\lambda = 2 d \sin \theta$$

where, n is an integer

λ is the wave length of X-ray

d is the spacing between the planes

θ is the angle between the incident ray and the scattering planes.

3.9 DNA SEQUENCING

The term *DNA sequencing* encompasses biochemical methods for determining the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a DNA oligonucleotide.

The methodologies for DNA sequencing

- The *chain termination method* (by Sanger), in which the sequence of a single-stranded DNA molecule is determined by enzymatic synthesis of complementary polynucleotide chains, these chains terminating at specific nucleotide positions.
- The *chemical degradation method* (by Maxam and Gilbert), in which the sequence of a double-stranded DNA molecule is determined by treatment with chemicals that cut the molecule at specific nucleotide positions.

Chain termination method

Chain termination method relies on the use of dideoxyribonucleoside triphosphates, derivatives of the normal deoxyribonucleoside triphosphates that lack the 3′ hydroxyl group.

Purified DNA is synthesized *in vitro* in a mixture that contains single-stranded molecules of the DNA to be sequenced, the enzyme DNA polymerase, a short primer DNA to enable the polymerase to start DNA synthesis, and the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP: A, C, G, and T). If a dideoxyribonucleotide analog of one of these nucleotides is also present in the nucleotide mixture, it can become incorporated into a growing DNA chain. Because this chain now lacks a 3′ OH group, the addition of the next nucleotide is blocked, and the DNA chain terminates at that point.

To determine the complete sequence of a DNA fragment, the double-stranded DNA is first separated into its single strands and one of the strands is used as the template for sequencing. Four different chain-terminating dideoxyribonucleoside triphosphates (ddATP, ddCTP, ddGTP, ddTTP,) are used in four separate DNA synthesis

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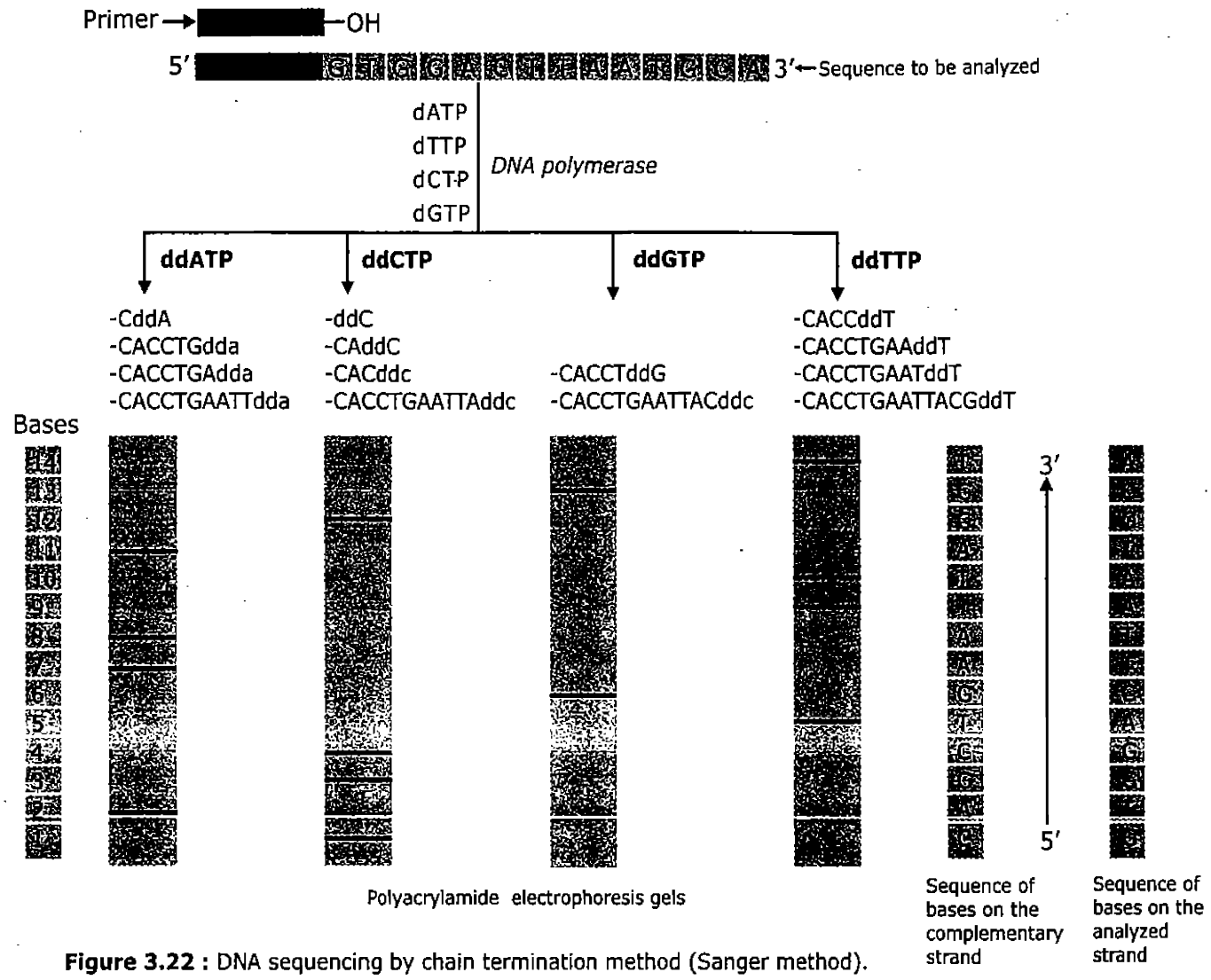


Figure 3.22 : DNA sequencing by chain termination method (Sanger method).

reactions on copies of the same single-stranded DNA template. Each reaction produces a set of DNA copies that terminate at different points in the sequence. The products of these four reactions are separated by electrophoresis in four parallel lanes of a polyacrylamide gel. The newly synthesized fragments are detected by a label (either radioactive or fluorescent) that has been incorporated either into the primer or into one of the deoxyribonucleoside triphosphates used to extend the DNA chain. In each lane, the bands represent fragments that have terminated at a given nucleotide but at different positions in the DNA. By reading off the bands in order, starting at the bottom of the gel and working across all lanes, the DNA sequence of the newly synthesized strand can be determined.

Automated sequencing

The standard chain termination sequencing methodology employs radioactive labels, and the banding pattern in the polyacrylamide gel is visualized by autoradiography.

Fluorescent primers are the basis of automated sequencing. The fluorolabel is attached to the ddNTPs, with a different fluorolabel used for each one. Chains terminated with A are therefore labeled with one fluorophore, chains terminated with C are labeled with a second fluorophore, and so on. Now it is possible to carry out the four sequencing reactions - for A, C, G and T - in a single tube and to load all four families of molecules into just one lane of the polyacrylamide gel, because the fluorescent detector can discriminate between the different labels and hence determine if each band represents an A, C, G or T. The sequence can be read directly as the bands pass in front of the detector and either printed out in a form readable by eye or sent straight to a computer for storage.

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Chemical degradation method

The starting material is double-stranded DNA, which is first labeled by attaching a radioactive phosphorus group to the 5′ end of each strand. The strands are then separated by electrophoresis under denaturing conditions, and analysed separately. DNA labelled at one end is divided into four aliquots and each is treated with chemicals that act on specific bases by methylation or removal of the base. On average, each molecule is modified at only one position along its length; every base in the DNA strand has an equal chance of being modified. After the modification reactions, the separate samples are cleaved by specific chemical, which breaks phosphodiester bonds exclusively at the 5′ side of nucleotides whose base has been modified. Since each sample now contains radioactive labelled molecules of various lengths, all with one end in common and with the other end cut at the same type of base. Analysis of the reaction products by electrophoresis is as described for the Sanger method.

To illustrate the procedure, we will follow the 'G' reaction. First, the molecules are treated with dimethyl sulfate, which attaches a methyl group to the purine ring of G *nucleotides*. Only a limited amount of dimethyl sulfate is added, the objective being to modify, on average, just one G per polynucleotide. At this stage the DNA strands are still intact, cleavage not occurring until a second chemical – piperidine – is added. Piperidine removes the modified purine ring and cuts the DNA molecule at the phosphodiester bond immediately upstream of the baseless site that is created. The result is a set of cleaved DNA molecules, some of which are labeled one and some of which are not. The labeled molecules all have one end in common and one end determined by the cut sites, the latter indicating the positions of the G nucleotides in the DNA molecules that were cleaved. In other words, the G family of molecules produced by chemical treatment is equivalent to the G family produced by the chain termination method.

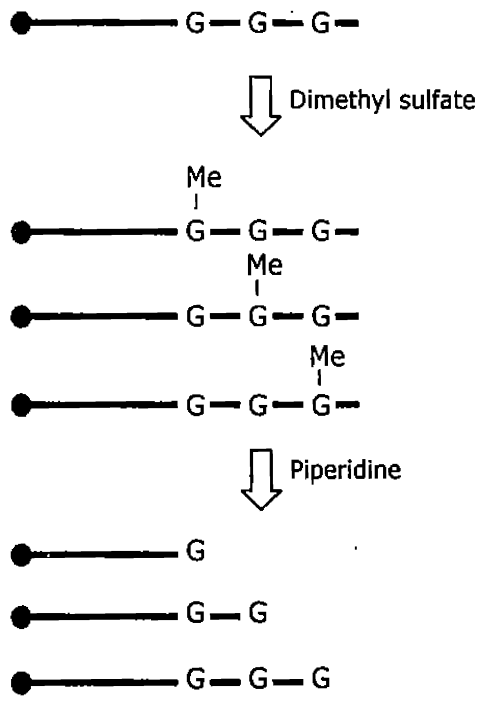


Figure 3.23 : Chemical degradation sequencing.

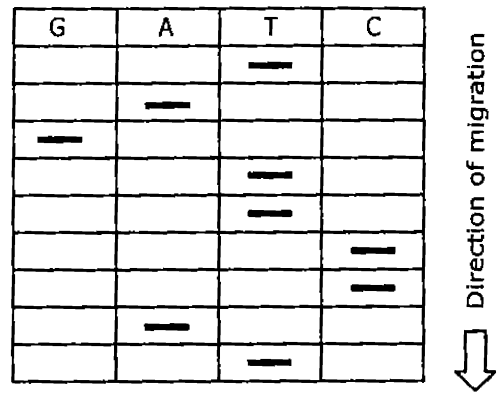
The families of cleaved molecules are electrophoresed in a polyacrylamide gel and the sequence read in a similar way to that described for chain termination sequencing. The only significant difference is that problems have been encountered in developing chemical treatments to cut specifically at A or T, and so the four reactions that are carried out are usually G, A + G, C and C + T. This does not affect the accuracy of the sequence that is read from the gel.

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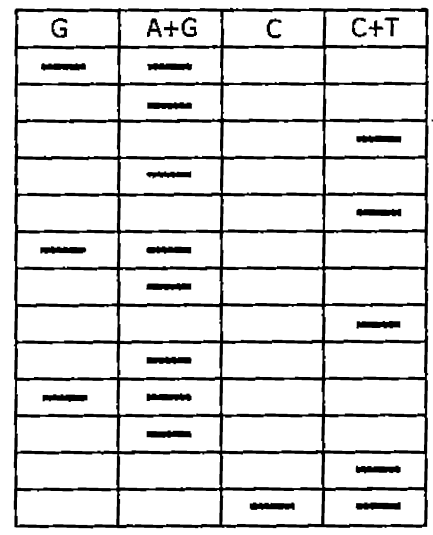
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STUDY QUESTIONS

Q3. a. The following gel pattern was obtained in an attempt to sequence a DNA molecule using the Sanger method. What is the sequence of the DNA molecule in question?



b. A restriction fragment, obtained with a type II endonuclease that recognizes a six base pair site, was subjected to Maxam-Gilbert sequencing with results as shown in the autoradiogram below. Find out the sequence of DNA fragment.



Ans. a. 5′-ATCAAGGTA-3′

b. 5′—CTAGATAGTATAG-3′

Bands appearing in the G and C lanes can be read directly. Bands in the A+G lane that are not duplicated in the G lane are read as A. Bands in the T+C lane that are not duplicated in the C lane are read as T. The sequence is read from the bottom of the gel up.

Table 3.3 : Genome sequencing of some model organisms

Genome sequenced	*Year*	*Genome size*	*comment*
Bacteriophage ϕ X174	1977	5.38 kb	First genome sequenced
Plasmid pBR322	1979	4.3 kb	First plasmid sequenced
Bacteriophage λ	1982	48.5 kb	
Yeast chromosome III	1992	315 kb	First chromosome sequenced
Haemophilus influenzae	1995	1.8 Mb	First genome of cellular organism to be sequenced
Saccharomyces cerevisiae	1996	12 Mb	First eukaryotic genome to be sequenced
Ceanorhabditis elegans	1998	97 Mb	First genome of multicellular organism to be sequenced
Homo sapiens	2000	3000 Mb	First mammalian genome to be sequenced
Arabidopsis thaliana	2000	125 Mb	First plant genome to be sequenced

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3.10 BIOSENSORS

A *sensor* is defined as a measuring device that exhibits a characteristic of an electrical nature when it is subjected to a phenomenon that is not electric. The electrical signal it produces carry the necessary information about the process under investigation. So, a sensor could be regarded as transducer, since it is a system that transform one physical quantity into another. Sensor first recognizes the phenomenon and translates it into a quantifiable property, which is then transformed into an electrical signal by a transducer.

According to the nature of the input signal, sensors can be broadly classified as physical sensors and chemical sensors. Physical sensors include mechanical, magnetic, thermal and optical sensors. Among chemical sensors, the most important is the biosensor. In a *biosensor*, the phenomenon is recognized by a biological system called a bioreceptor. Biosensors transform a biological event into an electrical signal. Technologically. a biosensor is a probe that integrates a biological component, such as a whole bacterium or a biological product (e.g., an enzyme or antibody) with an electronic component to yield a measurable signal. It is used as an analytical tool for the analysis of bio-material samples to gain an understanding of their bio-composition, structure and function by converting a biological response into an electrical signal.

There are three main parts of a biosensor:

1. Biological detection elements, which recognize the substance of interest,
2. A transducer, which converts the biorecognition event into a measurable signal and
3. A signal processing system, which converts the signal into a workable form.

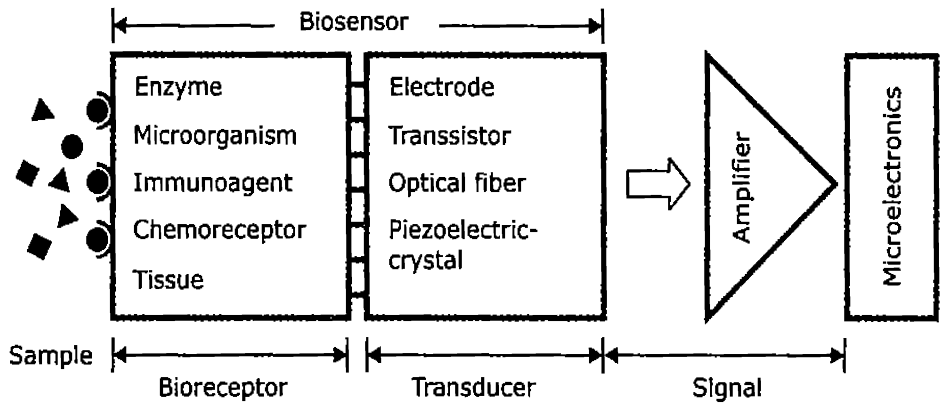


Figure 3.24 : Component of biosensor

Types of biosensors

Biosensors are classified depending upon different criteria like bioreceptors, transducers and different types of physical and chemical interaction. Depending upon type of transducers, biosensors can be classified as

1. Electrochemical
2. Potentiometric/Piezometric
3. Thermal
4. Optical

Optical biosensors detect changes in absorbance or fluorescence of an appropriate indicator and changes in the refractive index. *Piezoelectric sensors* are based on an alternating potential and produce a standing wave in the crystal at a characteristic frequency. This frequency is highly sensitive to the surface properties of the crystal such that, if a crystal is coated with a biological recognition element, the binding of the target analyte to a receptor will produce a change in the resonant frequency. *Electrochemical biosensors* are

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based on enzymatic catalysis of a reaction, which produces ions. The ions produced create a potential that is subtracted from that of the reference electrode to yield a signal. Electrochemical biosensors are usually based on potentiometry and amperometry. *Thermometric biosensors* are constructed by combining enzymes with temperature sensors. When the analyte is exposed to the enzyme, the heat of reaction of the enzyme is measured and is calibrated against the analyte concentration.

Glucose biosensor

Among all the biosensors, the most studied and developed biosensor application is glucose biosensor. In 1962 the American scientist Leland C. Clark first developed glucose biosensor.

The basic operation of glucose biosensor is based on the fact that the enzyme glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. Here the enzyme acts as a biorecognition element, which recognizes glucose molecules. These enzyme molecules are located on an electrode surface, which acts as a transducer. As soon as the enzyme recognizes the glucose molecules, it acts as a catalyst to produce gluconic acid and hydrogen peroxide from glucose and oxygen from the air. The electrode easily recognizes the number of electron transfer due to hydrogen peroxide/oxygen coupling. This electron flow is proportional to the number of glucose molecules present in blood. The glucose oxidation reaction, catalyzed by GOD is

$$\text{Glucose} + H_2O + O_2 = \text{Gluconic acid} + H_2O_2$$

At the electrode:

$$O_2 + 2e^- + 2H^+ = H_2O_2$$

A voltage of −0.7 V is applied between the platinum cathode and the silver anode and this voltage is sufficient to reduce the oxygen. The cell current is proportional to the oxygen concentration and the current is measured (amperometric method of detection has been employed). The concentration of glucose is then proportional to the decrease in current (oxygen concentration).

REVIEW THE CONCEPT

Q1. a. How is the distribution coefficient (K_D) of a protein calculated by using gel filtration chromatography?

b. Give the structure of functional group present in CM cellulose. How will pH effect the binding of a protein to CM cellulose column? **[CSIR-UGC]**

Q2. A protein was purified to homogeneity. It's molecular weight, when determined by using gel-filtration chromatography turns out to be 300 kDa. When subjected to non-reducing SDS-PAGE, it migrated as a single band with a molecular weight of 300 kDa. However, when the protein was treated with beta-mercaptoethanol and then subjected to SDS-PAGE, it resolved into two bands of 100 and 50 kDa with equal intensity.

a. Deduce the subunit composition of the protein justifying your answer.

b. Discuss the nature of interactions responsible for holding the subunits together. **[CSIR-UGC]**

Q3. a. What are the major differences in transmission and scanning electron microscopic applications in biology?

b. What kind of lenses and light source is used in an electron microscope? **[CSIR-UGC]**

Q4. a. Define resolution of a microscopic lens.

b. Explain how immersion oil improves resolution. **[CSIR-UGC]**

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SELF TEST

Q1. The void volume of a gel filtration column, V_0 = 30 mL. A monomeric protein with a known molecular weight of 25 kDa elutes at a volume of 45 mL. The protein that you are trying to purify elutes at a volume of 35 mL. Which of the following is a valid conclusion?

a. your protein is repelled by the gel filtration material
b. your protein Mr = 25 kDa
c. your protein Mr > 25 kDa
d. your protein Mr < 25 kDa

Q2. You have a mixture of proteins with the following properties:

Protein 1 : Mr 12 kD pI = 10
Protein 2 : Mr 62 kD pI = 4
Protein 3 : Mr 28 kD pI = 8
Protein 4 : Mr 9 kD pI = 5

Predict the order of emergence of these proteins when a mixture of the four is chromatographed in DEAE-cellulose at pH 7.0

a. 1, 3, 4, 2
b. 2, 4, 3, 1
c. cannot be predicted
d. 5, 8, 4, 10

Q3. A mixture of three peptides A, B and C is loaded in a C18 reverse phase chromatography column. A mixture of water and acetonitrile is used as the solvent with increased concentration of the latter in a gradient mode. If the hydrophobicities of the peptides are in the order A < B < C, the order in which they will elute from the column would be

a. A followed by B followed by C
b. B followed by C followed by A
c. C followed by B followed by A
d. A followed by C followed by B **[JNU]**

Q4. A mixture containing protein-1,-2,-3,-4, and -5 with molecular weights 5,000, 10,000, 25,000, 65,000 and 100,000, respectively, were separated as Sephadex G-50 column. The order of elution of these proteins from the column will be

a. protein-1, protein-2, protein-3, protein-4, and protein-5
b. protein-5, protein-4, protein-3, protein-2, and protein-1
c. protein-1,-2, and -3 elute first, following by protein-5 and -4
d. protein-4 and -5 elute first, followed by protein-3, -2, and -1 **[GATE]**

Q5. Match the coefficients in group 1 with their corresponding downstream processing steps given in group 2.

Group 1	*Group 2*
P. sedimentation coefficient	1. aqueous two-phase extraction
Q. partition coefficient	2. ultrafiltration
R. rejection coefficient	3. dialysis
S. activity coefficient	4. centrifugation

a. P-3, Q-1, R-4, S-2
b. P-2, Q-1, R-4, S-3
c. P-4, Q-3, R-1, S-2
d. P-4, Q-1, R-2, S-3 **[GATE]**

Q6. Two proteins have the same molecular mass and have identical net charge at pH 7. The best way to separate them would be to use

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a. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
b. native gel electrophoresis
c. cation-exchange chromatography
d. anion-exchange chromatography **[JNU]**

Q7. Chymotrypsinogen in the native state and reduced unfolded states is loaded in wells A and B of a native polyacrylamide gel, respectively. After electrophoresis and staining, one of the following results is likely to be observed
a. protein in well A would have moved longer distance than that in well B
b. protein in well A would have moved shorter distance than that in well B
c. protein in well A and B would have moved the same distance
d. protein in well A and B would not move at all **[JNU]**

Q8. SDS is used while separating proteins by polyacrylamide gel electrophoresis because
a. it helps in solubilization of proteins thereby making it easier to separate
b. it binds to proteins and confers uniform negative charge density thereby making them move during electrophoresis
c. decreases the surface tension of the buffer used for electrophoresis
d. stabilizes the proteins

Q9. One dimensional polyacrylamide gel electrophoresis in the absence of SDS (native gel electrophoresis) resolves proteins based on their mass and
a. charge
b. hydrodynamic volume
c. secondary structure
d. hydrophobicity

Q10. A mixture of Cytochrome–C (MW-11.7 KD) and Myoglobin (MW-17.2 KD) are to be separated by polyacrylamide gel electrophoresis. Their isoelectric pH (pI) values are 9.6 and 7.2 respectively. In which direction will each protein migrate at pH 8.5?
a. myoglobin will migrate to anode and cytochrome–C will migrate to cathode
b. myoglobin will migrate to cathode and cytochrome–C will migrate to anode
c. both will migrate to anode
d. both will migrate to cathode **[JNU]**

Q11. UV-spectroscopy is *not* used to quantitate the compounds based on absorbance
a. nucleic acids have absorbance peak at 260 nm
b. NAD(P)H have absorbance peak at 340 nm
c. aromatic amino acids have absorption maxima about 280 nm
d. sulfur containing amino acids have absorbance at 270 nm

Q12. The dipeptide Lys-Glu on electrophoresis at pH 6.0 would
a. move towards anode (+)
b. move towards cathode (–)
c. remain stationary
d. get degraded **[GATE]**

Q13. Fluorescence microscopy is based on the ability of certain molecules to
a. absorb light of a constant wavelength
b. absorb light of many different wavelengths
c. absorb light of a given wavelength and then emit light of a longer wavelength
d. absorb light of a given wavelength and then emit light at shorter wavelength **[JNU]**

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14. Three-dimensional images of the surface of the cells and tissues could be visualized through
a. transmission electron microscope
b. scanning electron microscope
c. compound microscope
d. fluorescence microscope **[JNU]**

015. From the data given below, identify the protein pair that would (P) give the least mobility band on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) experiment and (Q) elute last on an anion exchange e.g., DEAE column respectively

Protein	pI	Subunit MW	Native MW
A	9	10,000	20,000
B	8	35,000	35,000
C	6	15,000	90,000
D	5	20,000	80,000
E	3	30,000	30,000

a. (P) Protein C (Q) Protein A
b. (P) Protein A (Q) Protein E
c. (P) Protein B (Q) Protein E
d. (P) Protein B (Q) Protein A **[GATE]**

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Chapter - 04

Carbohydrates

Carbohydrates are polyhydroxy aldehydes or polyhydroxy ketones, or compounds that can be hydrolyzed to them. In majority of carbohydrates, H and O present in same ratio as in water, hence also called as *hydrates of carbon*. Carbohydrates are classified into following classes depending upon whether these undergo hydrolysis and if so on the number of products form-

Monosaccharides are simple sugars that cannot be broken down into smaller molecules by hydrolysis.

Oligosaccharides are polymers made up of two to ten monosaccharide units joined together by glycosidic linkages. The most abundant are the disaccharides, with two monosaccharide units.

Polysaccharides are polymers with hundreds or thousands monosaccharide units.

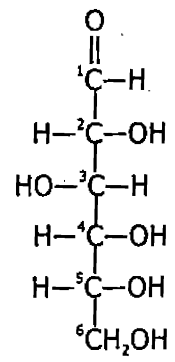
4.1 GENERAL FEATURES OF MONOSACCHARIDE

Monosaccharides consist of a single polyhydroxy aldehyde or ketone unit. Monosaccharides are the simple sugars, which cannot be hydrolyzed further into simpler forms and they have a general formula $C_nH_{2n}O_n$. The most abundant monosaccharide in nature is the D-glucose. Monosaccharides can be further sub classified on the basis of :

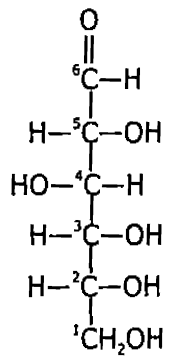
The number of the carbon atoms present

Monosaccharides can be named by a system that is based on the number of carbons with the suffix-*ose* added. Monosaccharides with four, five, six, and seven carbon atoms are called *tetroses, pentoses, hexoses,* and *heptoses,* respectively.

System for numbering the carbons : The carbons are numbered sequentially with the aldehyde or ketone group being on the carbon with the lowest possible number.



Correct



Incorrect

Presence of aldehydes or ketones groups

Aldoses are monosaccharides with an aldehyde group.

Ketoses are monosaccharides containing a ketone group.

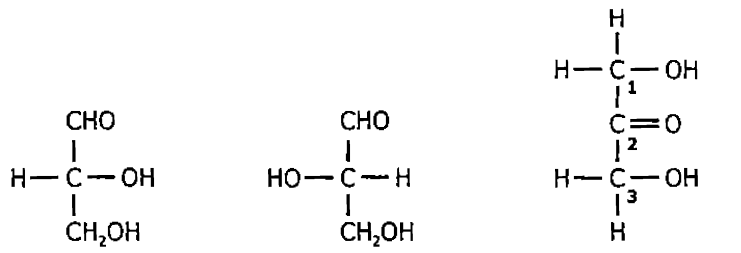
Thus, the sugar **glucose** is an **aldohexose;** that is, it is a six-carbon monosaccharide (-hexose) containing an aldehyde group (aldo-). Similarly **fructose** is a **ketohexose;** that is, it is a six-carbon monosaccharide (-hexose) and containing a ketone group (keto-).

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Trioses are simplest monosaccharides. There are two trioses- dihydroxyacetone and glyceraldehyde. Dihydroxyacetone is called a *ketose* because it contains a keto group, whereas glyceraldehyde is called an *aldose* because it contains an aldehyde group.

Glyceraldehyde has a central carbon (C-2) which is chiral or asymmetrical. Chiral molecules such as glyceraldehyde can exist in two forms or configurations that are nonsuperimposable mirror images of each other. These two forms are called *stereoisomers*, *optical isomers*, or *enantiomers*. A stereoisomer is identified by its absolute configuration. Glyceraldehyde has two absolute configurations. When the hydroxyl group attached to the chiral carbon is on the left in a Fischer projection, the configuration is L; when the hydroxyl group is on the right, the configuration is D.

The absolute configurations of sugar containing more than one chiral centers like hexose are determined by comparing the configuration at the highest-numbered chiral carbon (the chiral carbon farthest from the aldehyde group) to the configuration at the single chiral carbon of glyceraldehyde.

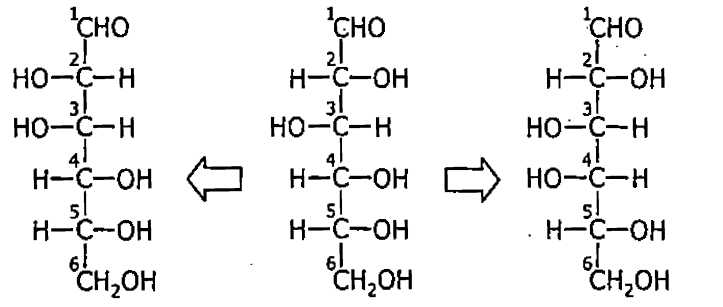


D-Glyceraldehyde L-Glyceraldehyde Dihydroxyacetone

All the monosaccharides except dihydroxyacetone contain one or more chiral carbon atoms and thus occur in optically active isomeric forms. The simplest aldose, glyceraldehyde, contains one chiral center and therefore has two different optical isomers, or enantiomers. As the number of chiral carbon atoms increases, the number of possible isomers also increases. The total number of possible isomers can be determined by using Van't Hoff's rule. A compound with 'n' asymmetric carbon atom has a maximum of 2^n possible stereoisomers.

EPIMERS

Many common sugars are closely related, differing only by the stereochemistry at a single carbon atom. For example, D-glucose and D-mannose differ only at carbon 2. Sugars that differ only by the stereochemistry at a single carbon (other than anomeric carbon) are called epimers. Similarly D-glucose and D-galactose are epimers. D-mannose and D-galactose are not epimers because their configuration differ at more than one carbon.



D-Mannose (epimer at C-2) D-Glucose D-Galactose (epimer at C-4)

Figure 4.1 : D-Glucose and two of its epimers.

CYCLIC FORMS OF MONOSACCHARIDES

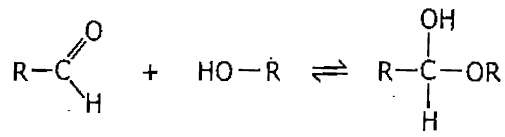
Monosaccharides having 5 or 6 carbons in the chain gives cyclic structure in aqueous solution via internal hemiacetal or hemiketal formation.

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Hemiacetal

In general, an aldehyde can react with an alcohol to form a *hemiacetal*.

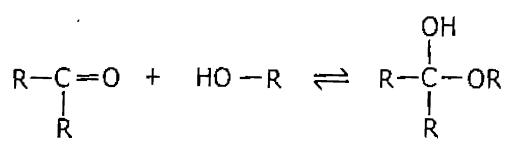


Aldehyde Alcohol Hemiacetal

For an aldohexose such as glucose, the C-1 aldehyde in the open-chain form of glucose reacts with the C-5 hydroxyl group to form an *intramolecular hemiacetal.*

Hemiketal

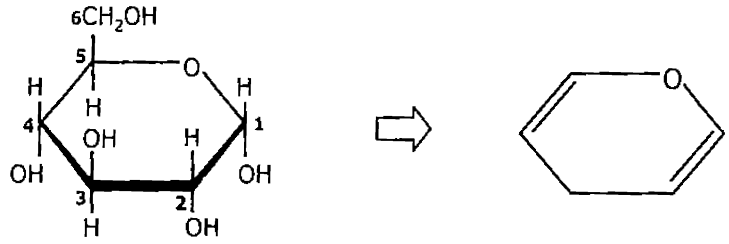
A ketone can react with an alcohol to form a *hemiketal.*



Ketone Alcohol Hemiketal

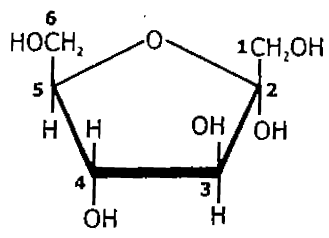
The C-2 keto group in the open-chain form of a ketohexose, such as fructose, can form an *intramolecular hemiketal* by reacting with either the C-6 hydroxyl group to form a six-membered cyclic hemiketal or the C-5 hydroxyl group to form a five-membered cyclic hemiketal.

The cyclic form of glucose is a six-membered ring; such sugars are called **pyranoses** because they resemble *pyran*. Similarly the cyclic form of fructose is a five membered ring called **furanoses** because they resemble *furan*.

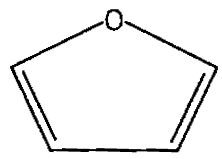


D–Glucopyranose

Pyran



D–Fructofuranose



Furan

Cyclic structure exists in two different configurational forms. If the hydroxyl on the anomeric carbon is below the plane of the ring, it is said to be in the α-position; if above the plane of the ring, it is in the β-position. The two diastereoisomers are called **anomers**. The hemiacetal or hemiketal carbon is known as *anomeric carbon.*

In glucose, C-1 carbon atom is called the *anomeric carbon atom,* and the α and β forms are called *anomers* (as shown in the figure). An equilibrium mixture of glucose contains approximately 37% α-form and 63% β-form and <1% of the open chain form. The two anomers have different physical properties. For example α-D-glucose has a specific rotation of +113° whereas the β-D-glucose form has specific rotation of +19°. When either of these pure substances is dissolved in water, the specific optical rotation of the solution slowly changes until it reaches an equilibrium value of specific rotation of +52°. In aqueous solution the interconversion of α- and β-forms via the open chain structure, to give an equilibrium mixture is known as **mutarotation.**

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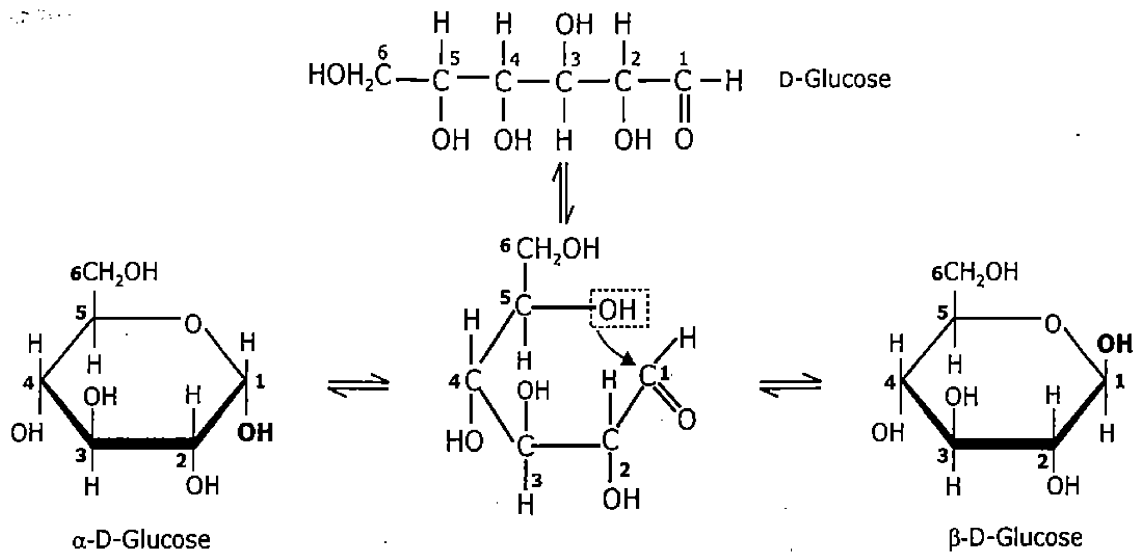


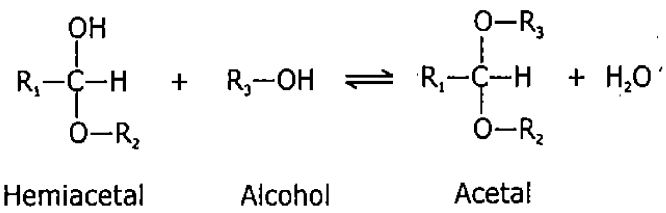
Figure 4.2 : The α and β cyclic isomers of D-glucose can interconvert, with the open-chain structure as the intermediate.

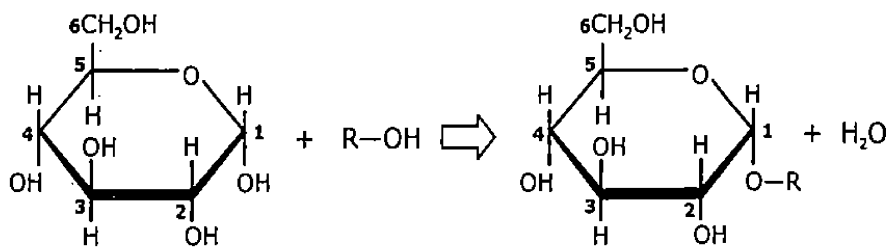
The same nomenclature applies to the furanose ring form of fructose, except that α and β refer to the hydroxyl groups attached to C-2, the anomeric carbon atom. Fructose forms both pyranose and furanose rings. The pyranose form predominates in fructose free in solution, and the furanose form predominates in many fructose derivatives.

4.2 DERIVATIVES OF MONOSACCHARIDE

Glycosides

When hemiacetals react with alcohols, it form acetals and if hemiacetal of sugar reacts with an alcohol to form an acetal, it is known as a *glycoside*. Glycosides are formed by condensation between the hydroxyl group of the anomeric carbon of a monosaccharide, and a second compound that may or may not be another monosaccharide. If the hemiacetal portion is glucose, the resulting compound is *glucoside*; if galactose, a *galactoside*; and so on. Glycosides are widely distributed in nature. A very common glycoside is *ouabain* which inhibits the action of enzyme that pump Na^+ and K^+ ions across cell membrane. Other glycosides include antibiotics such as streptomycin.

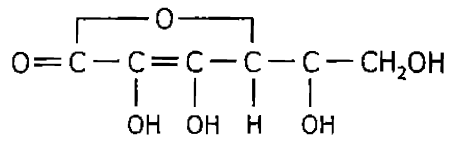




Sugar acids

The aldehyde group in aldoses can be oxidized to produce a class of monosaccharides called **aldonic acids** (if glucose, it is gluconic acid). One important aldonic acid is L-ascorbic acid or vitamin C.

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Ascorbic acid

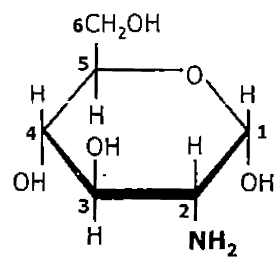
Aldoses can undergo selective oxidation also. If terminal -OH group oxidizes, it produce **uronic acid** (if glucose, it is glucuronic acid). If both the aldehyde group and the terminal -OH oxidizes then produce **aldaric acid** (if glucose, it is glycaric acid).

Sugar alcohols

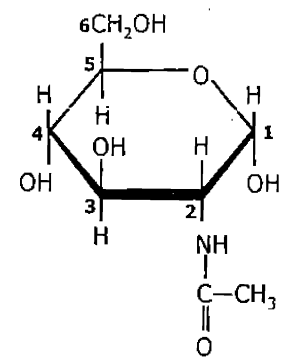
Carbonyl groups in aldoses and ketoses can be reduced to hydroxyl group to form *sugar alcohols* or *alditols*. Sugar alcohols are designated by addition of *-itol* to the name of the parent sugar. The reduction of carbonyl group of glucose and xylose produce polyhydroxy alcohols called *sorbitol* and *xylitol,* respectively. The sugar alcohols are linear molecules that cannot cyclize in the manner of aldoses.

Amino sugars

In amino sugars, a hydroxyl group is replaced by an *amino* or an *acetylamino* group. Most often found as monomer residues in complex oligosaccharides and polysaccharides.



D-Glucosamine

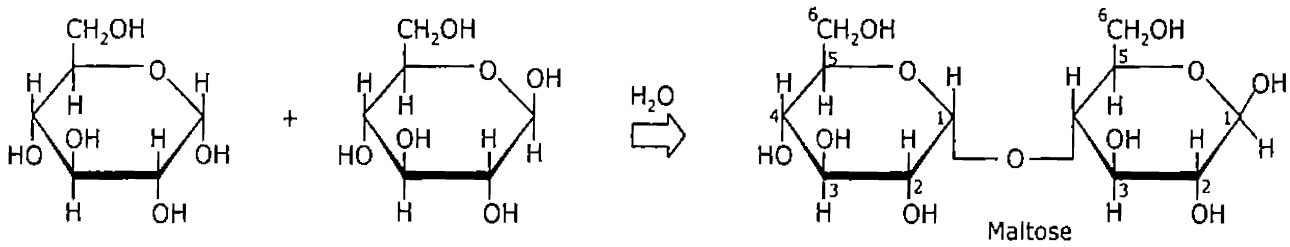


D-Acetylglucosamine

4.3 DISACCHARIDES AND GLYCOSIDIC BOND

A *disaccharide* consists of two sugars joined by an *o*-glycosidic bond. In a disaccharide, the two monosaccharides are joined together by acetal or glycoside formation. The hemiacetal OH of one monosaccharide and an OH of the second monosaccharide, dehydrate to establish the bond called glycosidic bond. A glycosidic bond forms between anomeric carbon and the alkoxy oxygen. By convention, glycosidic linkages are named by reading from left to right.

For example, in maltose two glucose residues are joined by a glycosidic linkage between the α-anomeric form of C-1 on one sugar and the hydroxyl oxygen atom on C-4 of the adjacent sugar. Such a linkage is called an α-1,4-glycosidic bond.



α-D-Glucose β-D-Glucose Maltose

α-D-glucopyranosyl-(1 → 4)-D-glucopyranose

Figure 4.3 : Formation of maltose

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In sucrose anomeric carbon atoms of a glucose unit and α fructose unit are joined through α-1, 2-glycosidic linkage and the configuration of this glycosidic linkage is α for glucose and β for fructose.

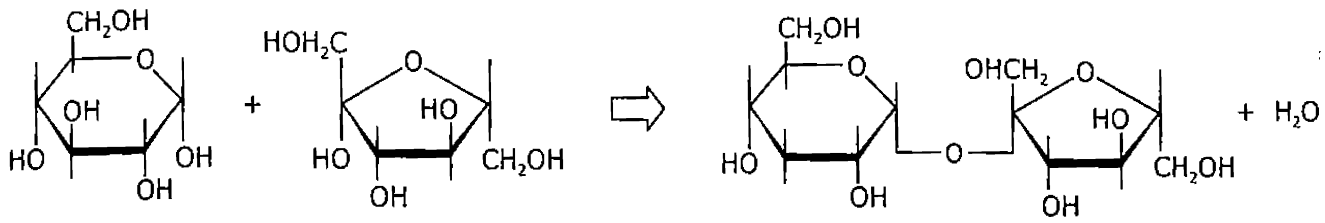


Figure 4.4 : Formation of sucrose

Table 4.1 : Occurrence and biochemical roles of some representative disaccharides

Disaccharides	*Structure*	*Physiological role*
Sucrose	Glucose α(1→ 2) Fructose β	A final product of photosynthesis
Lactose	Galactose β(1→ 4) Glucose	A major animal energy source
Trehalose	Glucose α(1→ 1) Glucose α	A major circulatory sugar in insects; used for energy
Maltose	Glucose α(1→ 4) Glucose	The dimer derived from the starch and glycogen polymers
Cellobiose	Glucose β(1→ 4) Glucose	The dimer of the cellulose polymer
Gentiobiose	Glucose β(1→ 6) Glucose	Constituent of plant glycosides and some polysaccharides

Hydrolysis of sucrose (invert sugar)

Sucrose is dextrorotatory, its specific rotation being +66.5%. Hydrolysis of sucrose with hot dilute acid yields D-glucose and D-fructose. D-glucose is also dextrorotatory, $[\alpha]_D$ = +53°, but D-fructose has a large negative rotation, $[\alpha]_D$ = −92°. Since D-fructose has a greater specific rotation than D-glucose, the resulting mixture is laevorotatory. Because of this the hydrolysis of sucrose is known as the *inversion of sucrose*, and the equimolecular mixture of glucose and fructose is known as invert sugar or invertose.

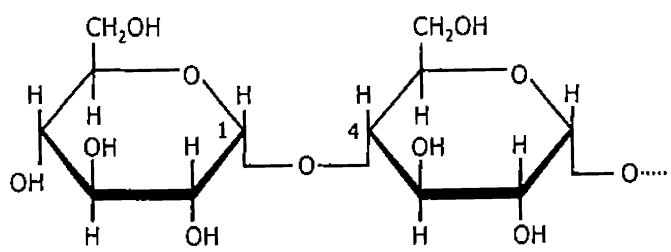
4.4 POLYSACCHARIDES

Polysaccharides are ubiquitous in nature. Polysaccharides are also called *glycans*. They can be classified into two separate groups, based on their *functions* : *Structural* and *storage polysaccharides*. Structural polysaccharides provide mechanical stability to cells, organs, and organisms. Storage polysaccharides serve as carbohydrate stores that release monosaccharides as required.

Polysaccharides may be homopolysaccharides (contain only a single type of monomeric unit) or heteropolysaccharides (contain two or more different kinds of monomeric units).

Homopolysaccharides

Starch is a branched chains of D-glucose units. It is the storage form for glucose in plants. It contains mixture of amylose and amylopectin. **Amylopectin** is a branched polymer of α-D-glucose with α-1, 4-glycosidic linkages and with α-1,6 branching points that occur at intervals of approximately 25 to 30 α-D-glucose residues. **Amylose** is a linear unbranched polymer of α-D-glucose units in a repeating sequence of α-1,4-glycosidic linkages.



Amylose

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Carbohydrates

Glycogen is the major storage form of carbohydrate in animals, found mostly in liver and muscle. It is a highly branched form of amylopectin: α-1, 6 branching points occur every eight to ten D-glucose residues, and the latter are in α-1, 4 linkages.

Cellulose is a Linear, unbranched homopolysaccharide of D-glucose units joined by β-1, 4-glycosidic linkages. Cellulose is a structural polysaccharide of plant cells. Although cellulose forms a part of the human diet (e.g., in vegetables and fruits), it is not hydrolyzed by human enzyme systems.

Chitin is a linear homopolysaccharide composed of N-acetyl-D-glucosamine residues joined by β-1, 4-glycosidic linkages. It is a structural polysaccharide in the exoskeleton of insects and crustaceans.

Heteropolysaccharides

Glycosaminoglycans are negatively charged heteropolysaccharide composed of repeating disaccharide units, $[\text{Acidic sugar} - \text{amino sugar}]_n$. Amino sugar is always either N-acetylglucosamine or N-acetylgalactosamine and the acidic sugar in most cases a uronic acid, usually glucuronic acid. Chondroitin sulfate, keratan sulfate, heparin, heparan sulfate, dermatan sulfate, and hyaluronate are the major glycosaminoglycans. With the exception of hyaluronic acid, all the GAGs contain sulfate groups, either as O-esters or as N-sulfate. All of the glycosaminoglycans except *hyaluronic acid* are found covalently attached to protein forming **proteoglycan.** The covalent attachments between glycosaminoglycan and core protein is O-glycosidic bonds between xylose sugar residue and the hydroxyl group of Ser residue in the protein. A polysaccharide chain is mainly assembled on core protein in the Golgi bodies. A specific *link tetrasaccharide* is first assembled on a serine residue. In most cases it is not clear how the serine residue is selected. The rest of the GAG chain, consisting mainly of a repeating disaccharide unit, is then synthesized, with one sugar residue being added at a time.

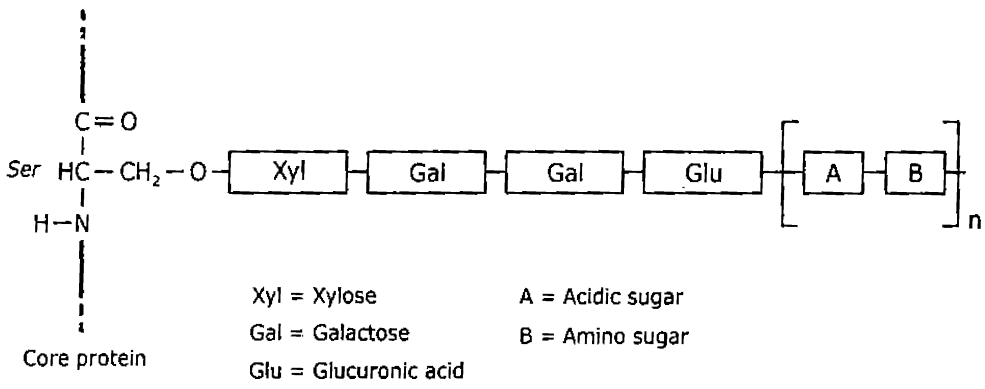


Table 4.2 : Characteristics of Glycosaminoglycans (GAGs)

GAG	Sugar A (Acidic Sugar)	Sugar B (Amino Sugar)	Sulfates	Links to protein
Hyaluronic acid	D-Glucuronic acid	N-Acetyl-D-glucosamine	–	–
Chondroitin sulfate	D-Glucuronic acid	N-Acetyl-D-galactosamine	+	+
Dermatan sulfate	D-Glucuronic acid *or* L-iduronic acid	N-acetyl-D-galactosamine	+	+
Heparan sulfate	D-Glucuronic acid *or* L-iduronic acid	N-acetyl-D-glucosamine	+	+
Heparin	D-Glucuronic acid *or* L-iduronic acid	N-acetyl-D-glucosamine	+	+
Keratan sulfate	D-Galactose (non acidic sugar)	N-acetyl-D-glucosamine	+	+

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Peptidoglycan or *murein* (present in bacterial cell walls) is a heteropolymer of alternating (β1→4) linked N-acetyl-glucosamine (NAG) and N-acetylmuramic acid (NAM) units. Lysozyme (first discovered in 1922 by Flemming) catalyzes the hydrolysis of the β (1–4) glycosidic linkage between NAG and NAM and degrades cell wall.

Table 4.3 : Important polysaccharides

Name	*Monomer*	*Main linkages*	*Branch linkages*
Murein	N-acetyl-glucosamine + N-acetyl-muramic acid	β1,4	—
Dextran	D-glucose	β1,6	β1,3
Cellulose	D-glucose	β1,4	—
Starch (Amylose)	D-glucose	α1,4	—
Starch (Amylopectin)	D-glucose	α1,4	α1,6
Inulin	D-fructose	β2,6	—
Chitin	N-acetyl-glucosamine	β1,4	—
Glycogen	D-glucose	α1,4	α1,6
Callose	D-glucose	β1,3	—

4.5 GLYCOPROTEINS

Carbohydrate groups are covalently attached to many different proteins to form *glycoproteins.* In glycoproteins, sugars are attached either to the amide nitrogen atom in the side chain of asparagine (termed an N-*linkage*) or to the oxygen atom in the side chain of serine or threonine (termed an O-*linkage*).

An asparagine residue can accept an oligosaccharide only if the residue is part of an Asn-X-Ser or Asn-X-Thr sequence, in which X can be any residue. All *N*-linked oligosaccharides have in common a pentasaccharide core consisting of three mannose and two *N*-acetylglucosamine residues. Additional sugars are attached to this core to form the great variety of oligosaccharide patterns found in glycoproteins.

4.6 REDUCING AND NON-REDUCING SUGAR

Sugar capable of reducing ferric (Fe^{3+}) or cupric ion (Cu^{2+}) are called **reducing sugar**. All monosaccharides whether aldoses and ketoses, in their hemiacetal and hemiketal form are reducing sugar. All disaccharides formed from head to tail condensation are also reducing sugar i.e., disaccharides except *sucrose, trehalose* are reducing sugar. All reducing sugar undergo mutarotation in aqueous solution.

Sugars like sucrose, trehalose not capable of reducing ferric or cupric ion are called **non-reducing sugar**. In sucrose and trehalose anomeric carbon becomes involved in a glycosidic bond. So they donot contain free anomeric carbon atom. Sucrose and trehalose are therefore not a reducing sugar, and have no reducing end. So it cannot be oxidized by cupric or ferric ion. In describing disaccharides or polysaccharides, the end of a chain that has a free anomeric carbon (i.e., is not involved in a glycosidic bond) is commonly called the reducing end of the chain.

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4.7 CARBOHYDRATE METABOLISM

GLUCONEOGENESIS

Gluconeogenesis is the synthesis of glucose from non-carbohydrate precursors. Gluconeogenesis is a universal pathway, found in all animals, plants, fungi, and microorganisms.

Site of gluconeogenesis:

In higher animals, gluconeogenesis occurs in liver and to a smaller extent, in kidney cortex. Under normal circumstances, the liver is responsible for 85% to 95% of the glucose that is made. During starvation or during metabolic acidosis, the kidney is capable of making glucose and then may contribute up to 50% of the glucose formed, since, in these condition, the amount contributed by the liver decreases considerably.

Precursors of gluconeogenesis:

Gluconeogenetic precursors include

1. Lactate, pyruvate, glycerol,
2. Citric acid cycle intermediates and
3. Some amino acids (termed glucogenic amino acids). Lysine and leucine are the only amino acids that are not substrate for gluconeogenesis. These amino acids produce only acetyl-CoA upon degradation.

Animal cells can carry out gluconeogenesis from three- and four- carbon precursors, but not from the two acetyl carbons of acetyl-CoA. Animal cells have no way to convert acetyl-CoA to pyruvate or oxaloacetate. Thus, fatty acids are not substrates for gluconeogenesis in animals, because most fatty acids yield only acetyl-CoA upon degradation. Unlike animals, plants and some microorganisms can convert acetyl-CoA derived from fatty acid oxidation to glucose.

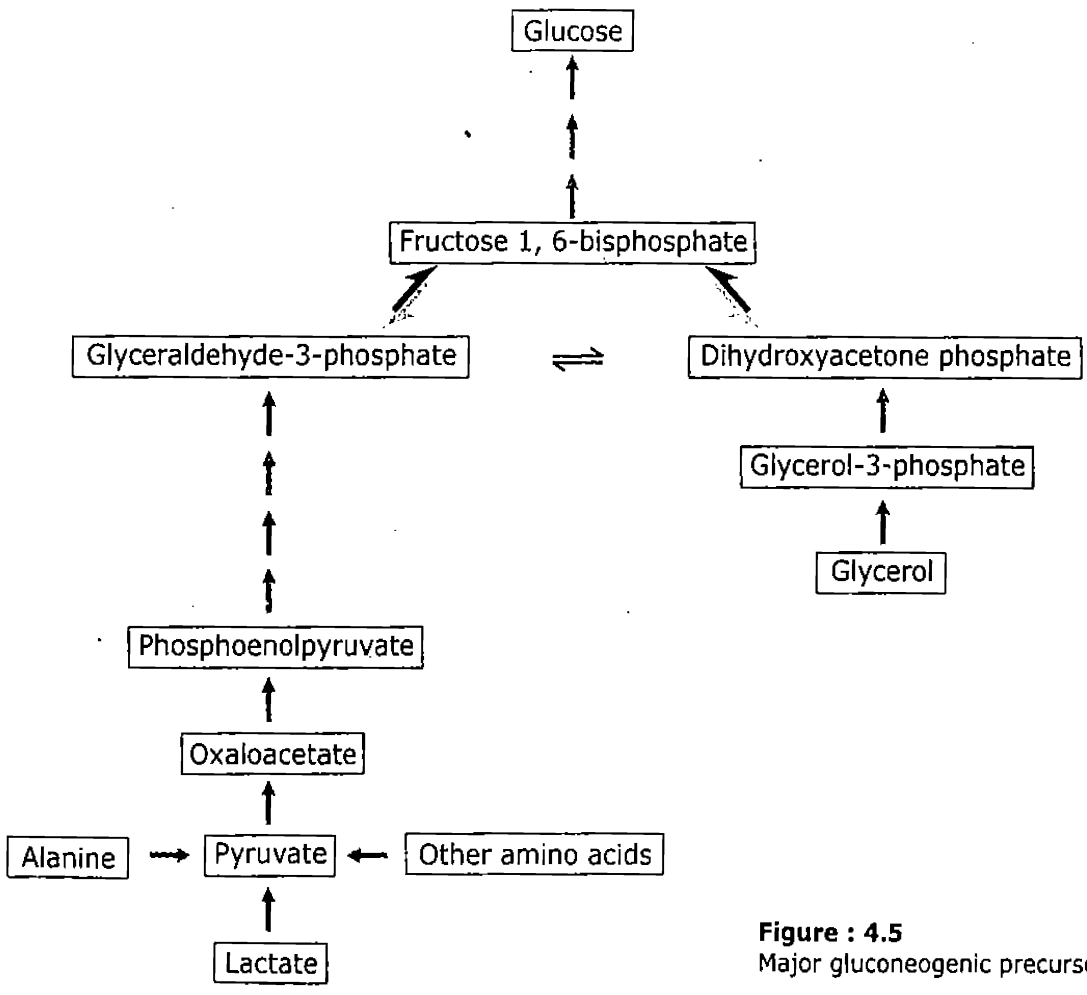


Figure : 4.5
Major gluconeogenic precursors.

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Gluconeogenetic pathway

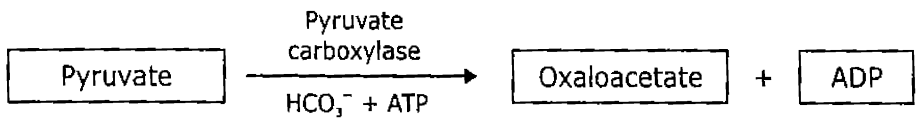
Although glycolysis and gluconeogenesis share several steps but these pathways are not simply the reverse of each other. During gluconeogenesis seven steps are catalyzed by the same enzymes used in glycolysis, these are reversible. Three steps in glycolysis are irreversible. These irreversible steps are:

1. Conversion of glucose to glucose-6-phosphate catalyzed by *hexokinase*.
2. The conversion of fructose 6-phosphate to fructose 1, 6-bisphosphate by *phosphofructokinase* (PFK-1).
3. The conversion of phosphoenolpyruvate to pyruvate by *pyruvate kinase*.

Conversion of pyruvate to phosphoenolpyruvate (PEP)

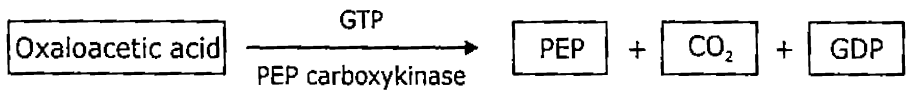
Pyruvate cannot be converted directly to PEP. The conversion requires two reactions that serve to bypass the irreversible pyruvate kinase step of glycolysis. The energy barrier involved in phosphorylating pyruvate to form PEP requires the expenditure of two-energy phosphate bonds.

First reaction for bypassing pyruvate kinase is the conversion of pyruvate to oxaloacetate, catalyzed by *pyruvate carboxylase.* This reaction occurs in the mitochondrial matrix.



Pyruvate Carboxylase is a mitochondrial allosteric enzyme. The prosthetic group of the enzyme is biotin, which functions as a CO_2 carrier. Biotin is covalently bound to the enzyme by an amide linkage between the carboxyl group of its side chain and the ε-amino group of an enzyme lysine residue to form a *biocytin*. The enzyme also requires both Mg^{2+} and Mn^{2+} for activity. Acetyl CoA acts as a allosteric activator of pyruvate carboxylase.

Second reaction for bypassing pyruvate kinase is the conversion of oxaloacetate to phosphoenol pyruvate (PEP), catalyzed by the Mn^{2+} -requiring *PEP carboxykinase.*

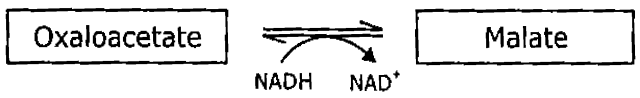


Cellular location of PEP carboxykinase

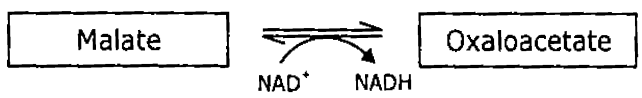
Pyruvate carboxylase is found only in the matrix of the mitochondria. By contrast, PEP carboxykinase may be localized in the cytosol or in the mitochondria or both. In man, PEP carboxykinase is found in both the mitochondria and the cytosol, however in rat and mouse liver it only present in cytosol. In chickens, pigeons, and rabbits, it is purely mitochondrial.

In organisms where PEP carboxykinase is found only in mitochondria, oxaloacetate is converted to PEP, which is then transported to the cytosol for gluconeogenesis. However, in organisms where oxaloacetate is converted into PEP in cytosol, a problem arises.

Oxaloacetate cannot cross the mitochondrial membrane because the mitochondrial membrane has no transporter for oxaloacetate. So, oxaloacetate first reduces to malate by mitochondrial enzyme *malate dehydrogenase*, at the expense of NADH.



Malate leaves the mitochondrion through a specific transporter in the inner mitochondrial membrane and in the cytosol it is reoxidized to oxaloacetate, with the production of cytosolic NADH.



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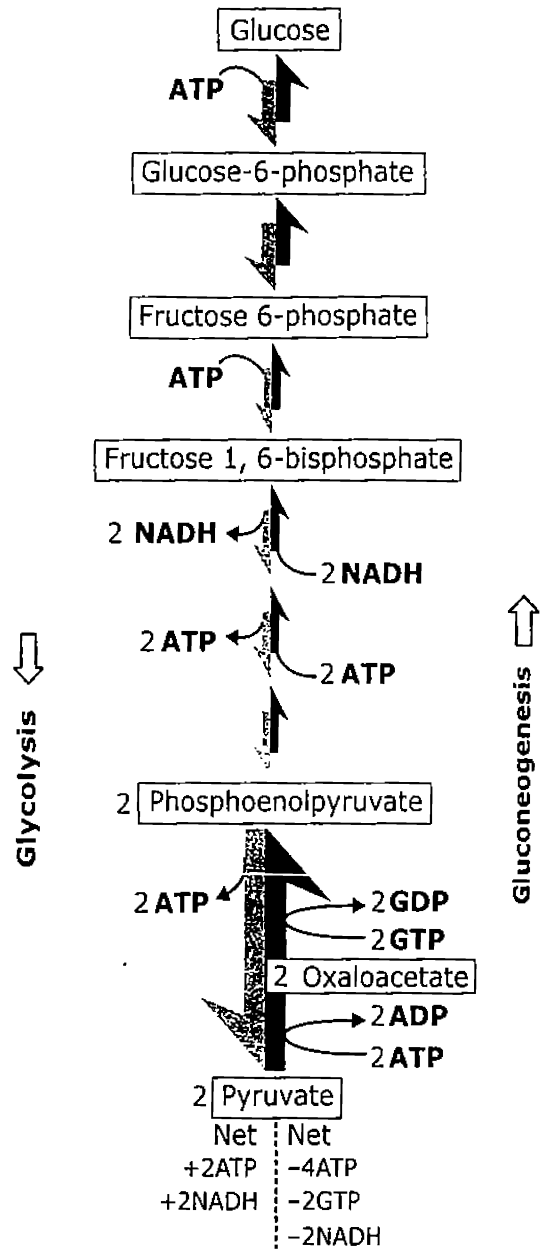


Figure : 4.6
Reactions of glycolysis and gluconeogenesis

CONVERSION OF PEP TO GLUCOSE

This pathway is opposite of glycolysis. However,

1. One step in glycolytic pathway where PFK-1 is involved is irreversible so during gluconeogenesis enzyme fructose 1,6 bisphosphatase acts without using ATP and converts fructose-1,6-bisphosphate to fructose-6-phosphate. Fructose-1,6-bisphosphatase is an allosterically regulated enzyme. Citrate stimulates bisphosphatase activity, but fructose-2,6-bisphosphate is a potent allosteric inhibitor. AMP also inhibits the bisphosphatase.
2. Another step where glucose converted into glucose-6-phosphate during glycolysis is catalyzed by hexokinase and requires ATP. This reaction is also irreversible. During gluconeogenesis conversion of glucose-6-phosphate to glucose requires glucose-6-phosphatase and no ATP is required. This enzyme is present in the membranes of the endoplasmic reticulum of liver and kidney cells, but is absent in muscle and brain. For this reason, gluconeogenesis is not carried out in muscle and brain.

Energetics of gluconeogenetic pathway

$2\ Pyruvate + 2NADH + 4ATP + 2GTP + 6H_2O \rightarrow Glucose + 2NAD^+ + 2GDP + 4ADP$

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Glucose alanine cycle

Pyruvate formed during glycolysis in muscle can undergo transamination with glutamate to yield alanine. The alanine is transported to the liver. In the liver, alanine transaminates with α-ketoglutarate to yield glutamate and pyruvate. The pyruvate is used to produce glucose by the gluconeogenic pathway.

Regulation of gluconeogenesis

Most of the reactions of gluconeogenesis take place in the cytosol, which is also the site of glycolysis. So if metabolic control were not exerted over these (gluconeogenesis and glycolysis) reactions, glycolytic degradation of glucose and gluconeogenetic synthesis of glucose could operate simultaneously with no net benefit to the cell and with considerable consumption of ATP. Hence glycolysis and gluconeogenesis is regulated in such a way, so that glycolysis is inhibited when gluconeogensis is active, and vice versa.

Gluconeogenesis is regulated by allosteric and substrate level control mechanism. The site of regulation along with activators and inhibitors are described in the following diagram.

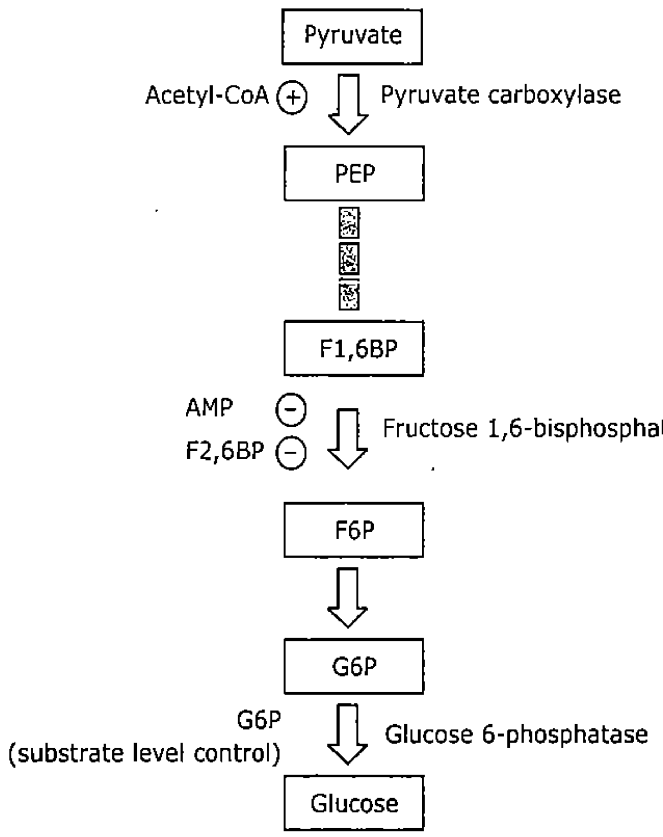


Figure : 4.7
Regulation of gluconeogenesis.
Activators are indicated by plus signs and inhibitors by minus sign.

OAA — Oxaloacetic acid
PEP — Phosphoenol pyruvate
F1,6BP — Fructose 1,6-bisphosphate
F2,6BP — Fructose 2,6-bisphosphate
F6P — Fructose 6-phosphate
G6P — Glucose 6-phosphate

GLYCOGEN METABOLISM

Glycogen structure

Glycogen is a highly branched, very large polymer of glucose molecules linked along its main line by α-1, 4-glycosidic linkages; branches arise by α-1, 6-glycosidic bonds at about every tenth residue. Glycogen occurs in the cytosol as granules, which also contain the enzymes that catalyze its formation and use.

Glycogen storage

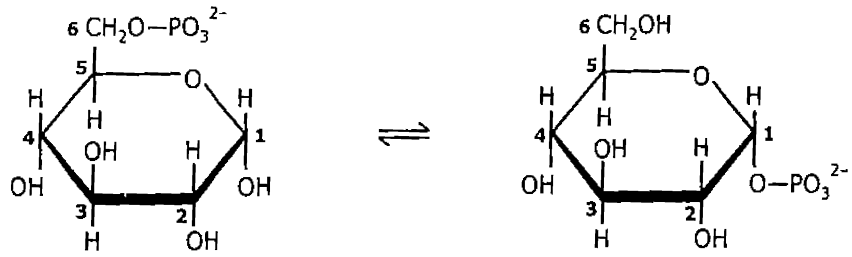
Muscle and liver are the major sites for the storage of glycogen, and although its concentration in the liver is higher, the much greater mass of skeletal muscle stores a greater total amount of glycogen. Liver can mobilize its glycogen for the release of glucose to the rest of the body, but muscle can only use its glycogen for its own energy needs.

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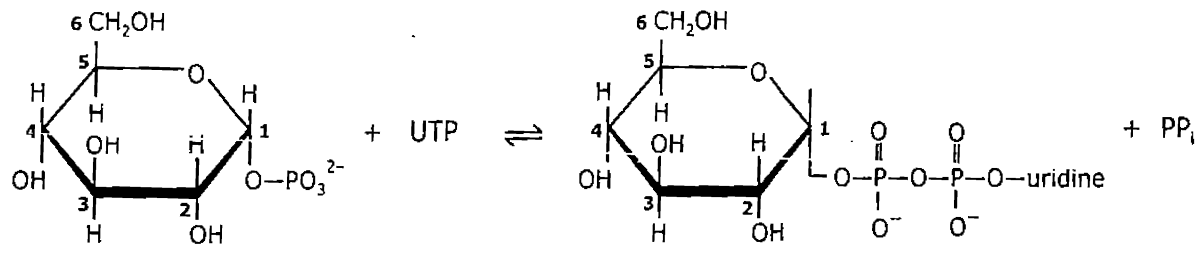
Glycogen synthesis (glycogenesis)

Glycogen is synthesized from glucose 6-phosphate (G6P) mainly in the muscle and liver and stored within these tissues as glycogen granules. The first step in glycogen synthesis is the formation of glucose 1-phosphate (G1P), catalyzed by *phosphoglucomutase*. G6P is isomerized to glucose 1-phosphate.



Glucose 6-phosphate ⇌ Glucose 1-phosphate

The glucose 1-phosphate is then activated to enable its incorporation into glycogen. The activated form is uridine diphosphate glucose (UDP-glucose). UDP-glucose acts as precursor of glycogen and is formed from glucose 1-phosphate and uridine triphosphate (UTP).



Glucose 1-phosphate + UTP ⇌ Uridine diphosphate glucose + PP_i

The reaction is catalyzed by *UDP-glucose pyrophosphorylase*. The C-1 carbon of the glucosyl unit being esterified to the diphosphate moiety of UDP.

Action of glycogen synthase

New glucosyl units are added to the non-reducing terminal residues of glycogen. The activated glucosyl unit, UDP-glucose is transferred to the hydroxyl group at a C-4 terminus of glycogen to form an α-1,4-glycosidic linkage. In the process of elongation, UDP is displaced by the terminal hydroxyl group of the growing glycogen molecule. This reaction is catalyzed by **glycogen synthase**, the key regulatory enzyme in glycogen synthesis.

UDP-glucose	+	Glycogen	⇨	UDP	+	Glycogen
		N residues				N+1 residues

Glycogen synthase can add glucosyl residues only if the polysaccharide chain already contains more than four residues. Thus, glycogen synthesis requires a *primer*. This priming function is carried out by **glycogenin**, a protein composed of two identical subunits, each bearing an oligosaccharide of α-1,4-glucose units. Carbon 1 of the first unit of this chain, the reducing end, is covalently attached to the specific tyrosine in each glycogenin subunit. How is this primer formed? Each subunit of glycogenin catalyzes the addition of eight glucose units to its partner in the glycogenin dimer. UDP-glucose is the donor in this autoglycosylation. After the synthesis of oligosaccharide (a primer), glycogen synthase takes over to extend the glycogen molecule.

Formation of branch chains

Glycogen synthase catalyzes only α–1, 4 glycosidic bond formation to yield α-amylose. Branching is accomplished by a separate enzyme called **branching enzyme**. Branching enzyme (also known as amylo-1,

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4→1,6 transglycosylase) moves a 7-unit segment of α-1,4 residues from a glycogen chain to a C-6 hydroxyl group of a glucosyl residue, that is four residues away from an existing branch.

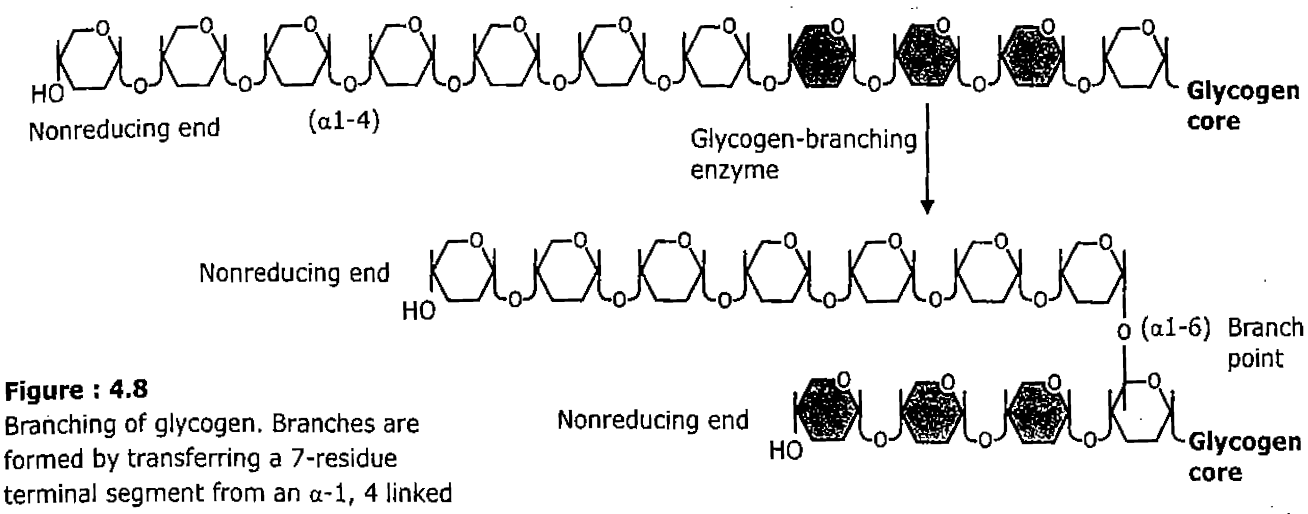


Figure : 4.8
Branching of glycogen. Branches are formed by transferring a 7-residue terminal segment from an α-1, 4 linked glucan chain to the C6—OH group of a glucose residue on the same or another chain.

Overview of glycogenesis

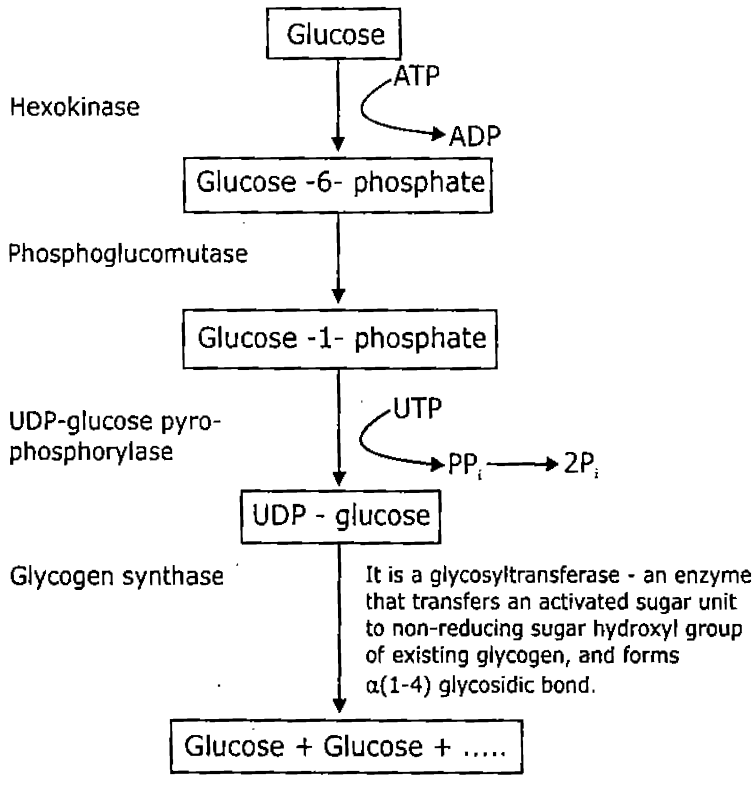


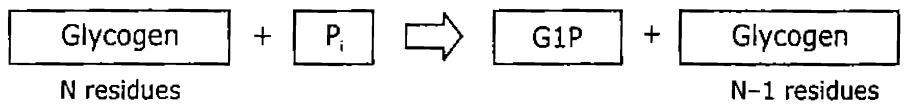
Figure : 4.9
An overview of glycogen synthesis in which glucose is activated to UDP glucose that acts as precursor for glycogen.

GLYCOGENOLYSIS

During glycogenolysis, terminal glucose residue removes as α-D-glucose-1-PO_4^{2-} from the non-reducing end (Phosphorolytic cleavage). This reaction is catalyzed by enzyme **glycogen phosphorylase,** a dimer of identical 842 residue subunits. Glycogen phosphorylase attacks exoglycosidic bonds. *Pyridoxal phosphate is an essential cofactor in the glycogen phosphorylase reaction.* Each catalytic site includes a pyridoxal-phosphate group,

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linked to lysine 680 of the enzyme. Its phosphate group acts as a general acid catalyst, promoting attack by P_i on the glycosidic bond. Glycogen phosphorylase acts repetitively on the non-reducing end of glycogen until it reaches a point 4 glucose residues away from an (α,1 $\rightarrow$ 6) branch point. It is an allosteric enzyme. ATP, G6P and glucose act as allosteric inhibitors and AMP acts as allosteric activator. It is also regulated by covalent modification.



Glycogen phosphorylase is the key enzyme in glycogen breakdown but can carry out this process by itself only to a limited extent because α-1,6-glycosidic bonds at the branch points are not susceptible to cleavage by phosphorylase. Thus further degradation by glycogen phosphorylase occurs only after the action of a glycogen debranching enzyme (α1,4 $\rightarrow$ α1,4) *glucantransferase*, which show two different activities.

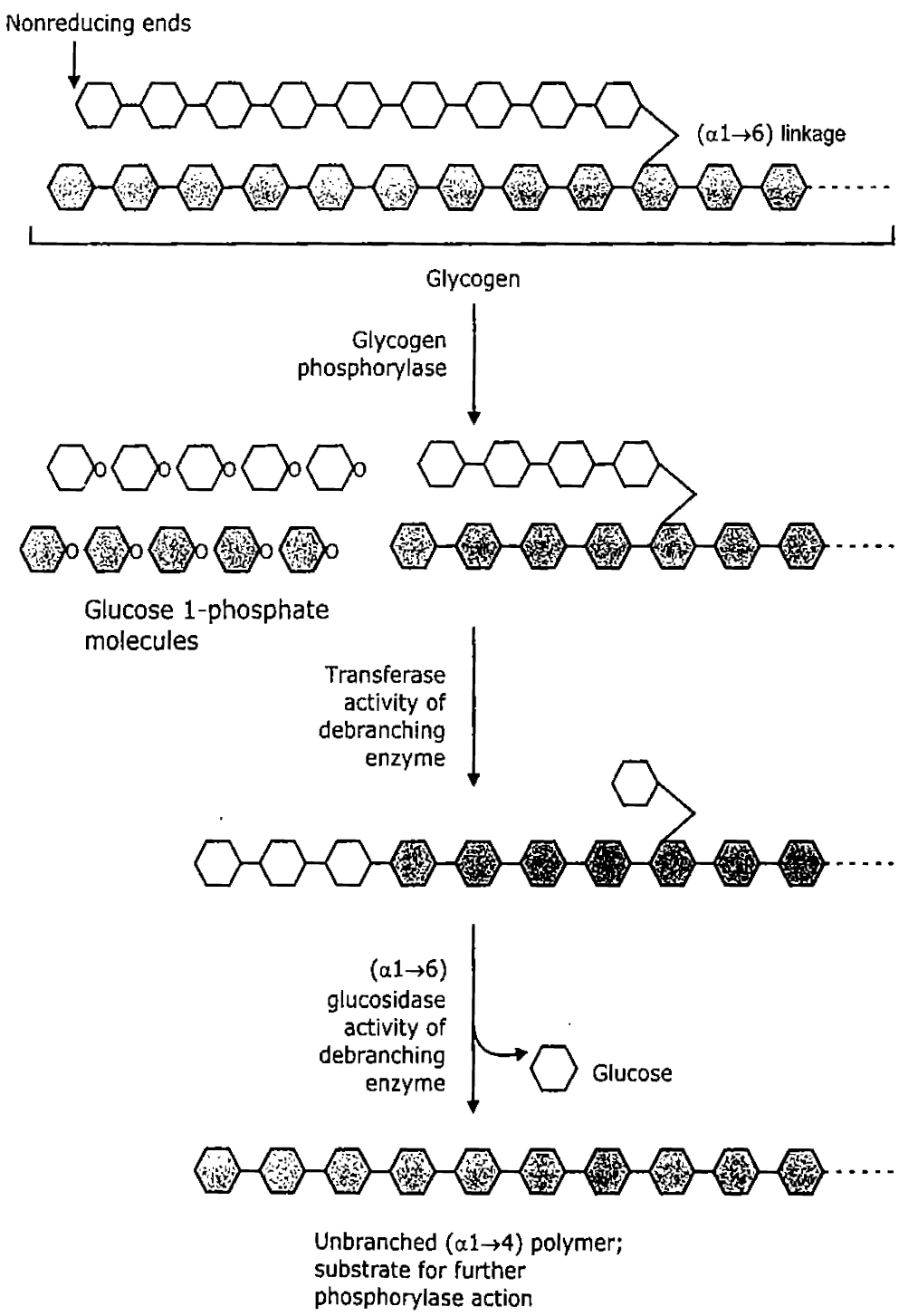


Figure : 4.10
First, alpha-1,4-glycosidic bonds on each branch are cleaved by phosphorylase, leaving four residues along each branch. The transferase shifts a block of three glycosyl residues from one outer branch to the other. The glucose residue is then removed by alpha-1,6-glucosidase, leaving a linear chain with all alpha-1,4 linkages, suitable for further cleavage by phosphorylase.

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First is the *transferase activity*, in which the enzyme removes terminal 3 of remaining glucose residues and transfers this trisaccharide moiety intact to the non reducing end of another branch.

Second, the remaining glucose residue, which is still attached to the main chain is removed by an $\alpha(1,\rightarrow 6)$ *glucosidase activity* of the same debranching enzyme. Thus about 92% of glycogen's glucose residues are converted to G1P. The remaining about 8%, those at the branch points, are converted to glucose.

Effect of glucagon/epinephrine on glycogenolysis

Glucagon and epinephrine markedly stimulate the breakdown of glycogen in muscle and liver. The liver is more responsive to *glucagon whereas muscle is more to* epinephrine. Glycogen phosphorylase, a key enzyme involved in glycogenolysis, is regulated by reversible phosphorylation, which is responsive to hormones such as epinephrine, and glucagon.

Glucagon is a polypeptide hormone secreted by the alpha-cells of the pancreatic islets. Glucagon opposes many of the actions of insulin. Most importantly glucagon acts to maintain blood glucose levels by activation of hepatic glycogenolysis and gluconeogenesis. Glucagon is composed of 29 amino acids arranged in a single polypeptide chain. Unlike insulin, the amino acid sequence of glucagon is the same in all mammalian species examined to date. Glucagon is synthesized as a large precursor molecule that is converted to glucagons through a series of selective proteolytic cleavages.

Epinephrine (adrenaline) is synthesized from the amino acid tyrosine and secreted from adrenal medulla. It is secreted on stimulation by a sympathetic nervous system for meeting an emergency or stress condition, like injury, pain, fear, accident, grief, fall in blood pressure etc. Hence called **emergency hormone.** It increases sugar level in blood by stimulating glycogenolysis in liver and skeletal muscles.

In muscle, epinephrine causes activation of protein kinase A (PKA). PKA promotes glycogen degradation indirectly by phosphorylating and thus activating a kinase, *glycogen phosphorylase kinase*, that in turn phosphorylates and activates *glycogen phosphorylase*, the enzyme that degrades glycogen. The dimeric skeletal muscle *glycogen* phosphorylase exists in two interconvertible forms: *active* **phosphorylase *a*** and *inactive* **phosphorylase *b***. Phosphorylase *b* is converted into phosphorylase *a* when it is phosphorylated at a single serine residue in each subunit. The entire process is reversed when epinephrine is removed and the level of cAMP drops, inactivating PKA.

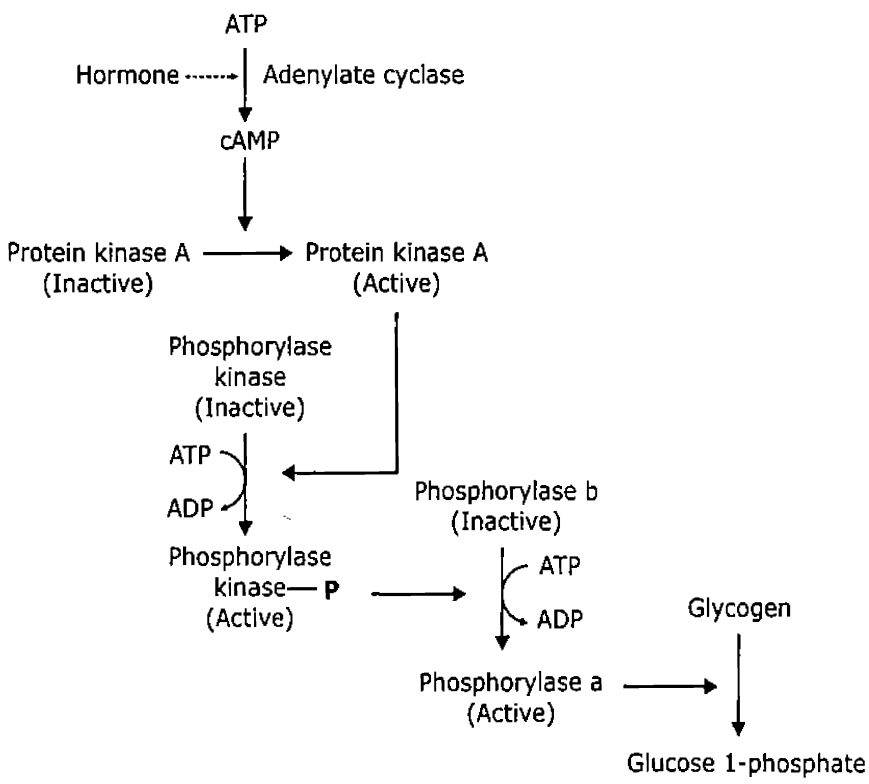


Figure : 4.11
The epinephrine-stimulated increase in cAMP and subsequent activation of PKA enhance the conversion of glycogen to glucose 1-phosphate by *stimulating* glycogen degradation. PKA phosphorylates and thus activates an intermediate kinase, glycogen phosphorylase kinase, that in turn phosphorylates and activates glycogen phosphorylase, the enzyme that degrades glycogen. The entire process is reversed when epinephrine is removed and the level of cAMP drops, inactivating PKA.

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Role of insulin in carbohydrate metabolism

Insulin is a polypeptide hormone produced by the beta-cells of the islets of Langerhans. It favors the synthesis of glycogen, triacylglycerols, and protein. Insulin is composed of 51 amino acids arranged in two polypeptide chains, designated A and B, which are linked together by two disulfide bridges.

Insulin acts to *reduce* glucose concentrations in the blood and therefore stimulates the conversion of glucose to fats, proteins, ribulose 5 phosphate and glycogen. In the liver, insulin decreases the production of glucose by inhibiting gluconeogenesis and the break down of glycogen. In muscle and liver, insulin increases glycogen synthesis. In muscle insulin increases glucose uptake by increasing the number of glucose transporters in the cell membrane. Epinephrine and glucagon have the opposite effects to insulin. It acts to *increase the glucose concentration* in the blood. They therefore stimulate the conversion of fats, glycogen and pyruvate to glucose.

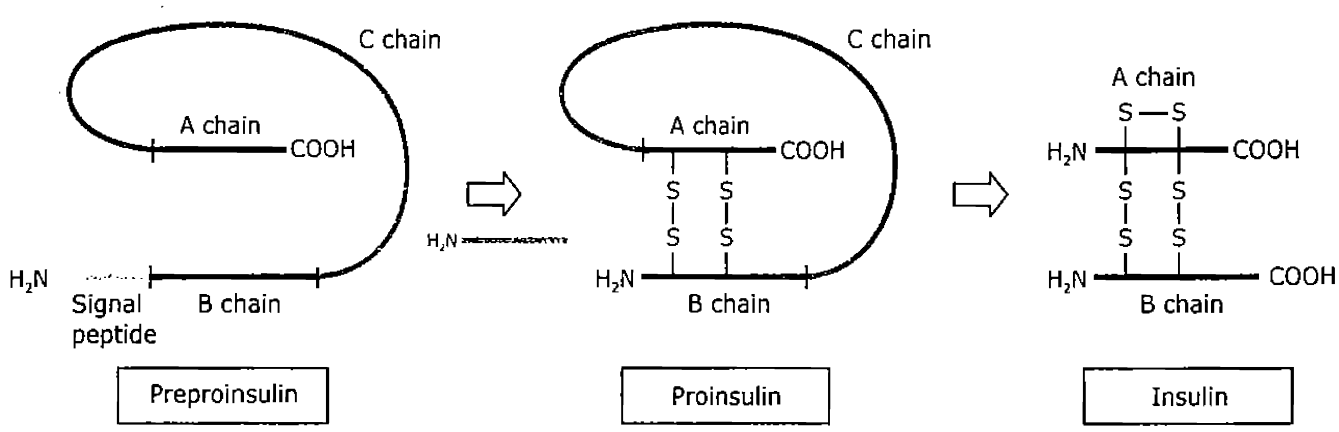


Figure 4.12 : Formation of human insulin from preproinsulin

Table 4.4 : Comparisons of effect of insulin, glucagon and epinephrine on carbohydrate metabolism

Pathway	*Effect of Insulin*	*Effect of Glucagon/Epinephrine*
Glycogenesis	Stimulates	Inhibits
Glycogenolysis	Inhibits	Stimulates
Gluconeogenesis	Inhibits	Stimulates
Glycolysis	Stimulates	Inhibits

Glycogen storage diseases

Glycogen storage diseases are caused by a genetic deficiency of one or another of the enzymes of glycogen metabolism. Many diseases have been characterized that result from inherited deficiency of enzyme. These defects are listed in table.

Table 4.5 : Glycogen storage diseases

Name	*Enzyme Deficiency*
Von Gierke's disease	Liver Glucose 6-phosphatase
Pompe's disease	Lysosomal $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 6$ glucosidase (acid maltase)
Hers' disease	Liver phosphorylase
Tarui's disease	Muscle and erythrocyte phosphofructokinase 1
McArdle's disease	Muscle glycogen phosphorylase
Andersen's disease	Amylo (1,4 $\rightarrow$ 1,6) transglycosylase (Branching enzyme)

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SELF TEST

Q1. Glycogen is a branched polymer of glucose. It has
a. no reducing ends
b. no non-reducing ends
c. one reducing end and several non-reducing ends
d. one non-reducing end and several reducing ends

Q2. One form of glycogen synthetase has a requirements for glucose-6-phosphate. What is the best explanation for this phenomenon?
a. it is an example of negative feedback
b. glucose-6-phosphate is a substrate
c. it is an example of allosteric activation
d. it inhibits an enzyme that hydrolyses UDP-glucose **[JNU]**

Q3. Which one of the following supports glycogen synthesis?
a. high cyclic adenosine monophosphate (cAMP) levels
b. inactive adenyl cyclase
c. active phosphorylase-alpha
d. epinephrine **[JNU]**

Q4. Which of the following statements about glycogen storage are *incorrect*?
P. glycogen is stored in muscles and liver
Q. glycogen is a major source of stored energy in brain
R. glycogen reserves are less rapidly depleted than fat reserves during starvation
S. glycogen storage occurs in the form of dense granules in the cytoplasm of cells
a. P and Q
b. P and R
c. Q and R
d. Q and S

Q5. Starting from a glucose residue in glycogen, how many net ATP molecules will be formed in the glycolysis of the residue to pyruvate?
a. 1
b. 2
c. 3
d. 4

Q6. Which of the following is common features of both glycogen synthesis and glycogen breakdown?
a. both require UDP-glucose
b. both involve glucose 1-phosphate
c. both are driven in part by the hydrolysis of pyrophosphate
d. both use the same enzyme for branching and debranching

Q7. After a meal, blood glucose enters cells and is stored as glycogen, particularly in the liver. Which of the following is the donor of new glucose molecules in glycogen?
a. UDP-glucose-1-phosphate
b. UDP-glucose
c. UDP-glucose-6-phosphate
d. Glucose-6-phosphate

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Q8. Which of the following statements about the hormonal regulation of glycogen synthesis and degradation are *correct*?

P. insulin increases the capacity of the liver to synthesize glycogen

Q. insulin is secreted in response to low levels of blood glucose

R. glucagon and epinephrine have opposing effects on glycogen metabolism

S. glucagon stimulates the breakdown of glycogen, particularly in the liver

a. P and Q

b. Q and R

c. P and R

d. P and S

Q9. Which of the following statements *correctly* describe what happens when acetyl CoA is abundant?

P. pyruvate carboxylase is activated

Q. phosphoenolpyruvate carboxykinase is activated

R. phosphofructokinase is activated

S. if ATP levels are high, oxaloacetate is diverted to gluconeogenesis

a. P and Q

b. Q and R

c. Q and S

d. P and S

Q10. Which of the following statements about gluconeogensis are *true*?

P. it occurs actively in the muscle during periods of exercise

Q. it occurs actively in the liver during periods of exercise or fasting

R. it occurs actively in adipose tissue during feeding

S. it occurs actively in the kidney during periods of fasting

a. P and Q

b. Q and R

c. Q and S

d. P and S

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Chapter - 05

Lipids

5.1 GENERAL FEATURE AND CLASSIFICATION

Biological lipids are chemically diverse group of organic compounds which are insoluble or only poorly soluble in water. They are readily soluble in nonpolar solvents such as ether, chloroform, or benzene. The hydrophobic nature of lipids is due to the predominance of hydrocarbon chains ($-CH_2-CH_2-CH_2-$) in their structures. Unlike the proteins, nucleic acids, and polysaccharides, lipids are not polymers.

Functions

Biological lipids have diverse functions. Four general functions of biological lipids have been identified.

- They serve as a storage form of metabolic fuel.
- They serve as a transport form of metabolic fuel.
- They provide structural components of membranes.
- They have protective functions in bacteria, plants, insects, and vertebrates, serving as a part of the outer coating between the body of the organism and the environment.

Apart from general functions biological lipids serve as *pigments* (carotene), *hormones* (vitamin D derivatives, sex hormones), *signaling molecules* (eicosanoids, phosphatidylinositol derivatives), *cofactors* (vitamin K), *detergents* (bile salt) and many other specialized functions.

5.2 FATTY ACIDS

Fatty acids are the simplest form of lipids and serve as constituents in a large number of complex form of lipids. Fatty acids are water-insoluble long-chain hydrocarbons (4 to 36 carbons long) with one carboxyl group. The chains may be saturated or unsaturated.

Table 5.1 : Predominant fatty acids found in mammalian tissues

Common name	Systematic name	No. of carbon atoms	No. of double bonds
Lauric	Dodecanoic	12	0
Myristic	Tetradecanoic	14	0
Palmitic	Hexadecanoic	16	0
Stearic	Octadecanoic	18	0
Palmitoleic	*Cis*-Δ^9-Hexadecenoic	16	1
Oleic	*Cis*-Δ^9-Octadecenoic	18	1
Linoleic	all *Cis*-Δ^9, Δ^{12}-Octadecadienoic	18	2
Linolenic	all *Cis*-Δ^9, Δ^{12}, Δ^{15}-Octadecatrienoic	18	3
Arachidonic	all *Cis*-Δ^5, Δ^8, Δ^{11}, Δ^{14}-Eicosatetraenoic	20	4

Saturated fatty acids

Saturated fatty acids have no double bonds in the chain. Their general formula is $CH_3-(CH_2)_n-COOH$ where n specifies the number of methylene groups between the methyl and carboxyl carbons. Examples of

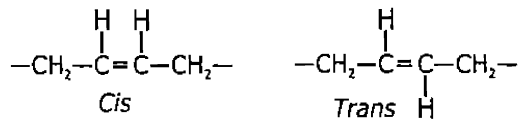
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predominant saturated fatty acids are lauric (dodecanoic), myristic (tetradecanoic), palmitic (hexadecanoic) and others. The systematic name of fatty acid gives the number of carbons, with the suffix - *anoic* appended. Palmitic acid, for example, has 16 carbons and has the systematic name hexadecanoic acid. By an older system, in a fatty acid carbon 2 is referred to as the α-carbon, carbon 3 as the β-carbon with the other carbon atoms lettered accordingly with the end methyl carbon being called ω-carbon.

Unsaturated fatty acids

Unsaturated fatty acids have one or more double bonds, and called monounsaturated or polyunsaturated respectively. Double bonds in naturally occurring fatty acids are generally in a *cis* as opposed to a *trans* configuration.



The most commonly used system for designing the position of double bonds in an unsaturated fatty acid is the delta (Δ) numbering system. The carboxyl carbon is designated carbon 1, and the double bond is given the number of the carbon atom on the carboxyl side of the double bond. For example, palmitoleic acid has 16 carbons and has a double bond between carbons 9 and 10. It is designated as $16:1:\Delta^9$, or 16:1:9.

Nonessential fatty acids

All nonessential fatty acids can be synthesized from acetyl coenzyme A (acetyl-CoA) derived from glucose oxidation. They are nonessential in the sense that they do not have to be obligatorily included in the diet.

Essential fatty acids

Fatty acids of the linoleic and linolenic families, which are the precursors of the prostaglandins, must be obtained from the diet. These fatty acids contain double bonds beyond the ninth carbon atom and there is no human enzyme systems that can introduce a double bond beyond the ninth carbon atom of a fatty acid chain.

Physical properties

1. Fatty acids are detergent-like due to their amphipathic nature; that is, they have both nonpolar (CH_3) and polar (—COOH) ends.
2. The melting point of fatty acids is related to chain length and degree of unsaturation. The longer the chain length, the higher the melting point, and the greater the number of double bonds, the lower the melting point.

STUDY QUESTIONS

Q1. Why unsaturated fatty acids have low melting points?

Ans. Presence of double bonds makes unsaturated chain more rigid. As a result, unsaturated chains cannot pack themselves in crystals efficiently and densely as saturated chain, so, they have lower melting point as compared to saturated fatty acids.

Q2. Cells of cold blooded animals usually have a higher proportion of unsaturated fatty acids than homeothermic (warm blooded) animals, why?

Ans. Body temperature of cold blooded animals become lower in cold condition. Lipid rich in unsaturated fatty acids (which have low melting points) generally remain liquid at temperature lower (usually 5°C or lower) than those rich in saturated fatty acids. This is necessary to maintain the function of plasma membrane, because lipid makes the constituent of plasma membrane.

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5.3 TRIACYLGLYCEROL AND WAX

Triacylglycerols (also called triglycerides) are triesters of fatty acids and glycerol. They are composed of three fatty acids and a glycerol molecule. Triacylglycerols are of two types – **simple** and **mixed** type. Those containing a single kind of fatty acid are called simple triacylglycerols and with two or more different kinds of fatty acids are called mixed triacylglycerols. The general formula of triacylglycerol is given below:

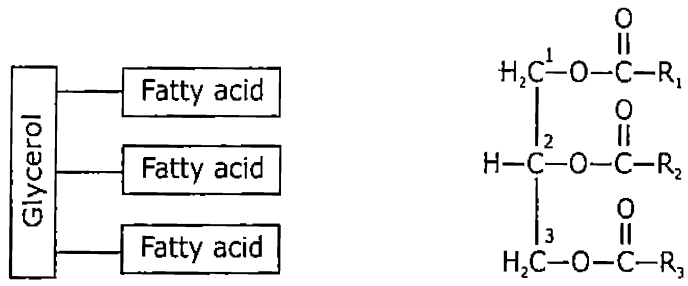


Figure 5.1 : General structure of triacylglycerol

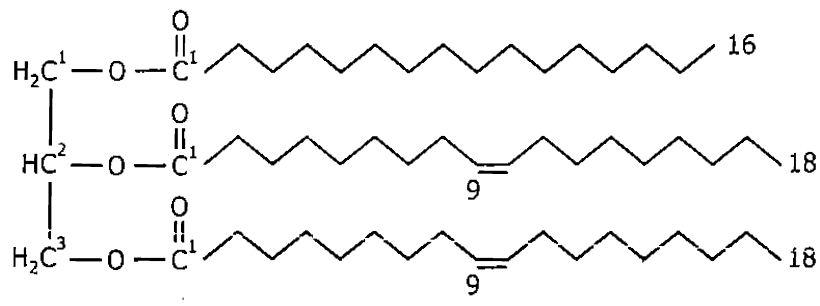


Figure 5.2 : 1-Palmitoyl-2,3-dioleoyl-glycerol

Triacylglycerols are nonpolar, hydrophobic in nature and major form of stored lipid. Because triacylglycerols have no charge (i.e., the carboxyl group of each fatty acid is joined to glycerol through a covalent bond), they are sometimes referred to as **neutral fats**. Most triacylglycerol molecules contain fatty acids of varying lengths, which may be unsaturated, saturated, or a combination of fats or oils. *Fats,* which are solid at room temperature, contain a large proportion of saturated fatty acids. *Oils* are liquid at room temperature because of their relatively high unsaturated fatty acid content. If fats are hydrolyzed with alkalis such as NaOH or KOH, a soap is produced. This process is called **saponification**. The number of milligram of KOH required to saponify one-gram of fat is known as *saponification number.* Saponification number measures the average molecular weight of fats. Similarly, the number of grams of iodine that can be added to 100g sample of fat or oil is called *iodine number*, which is used to determine the degree of unsaturation (i.e., extent of unsaturation).

Waxes

Waxes are formed by esterification of long chain fatty acids (saturated and unsaturated) and long chain alcohols (C14 to C36). The small head group of wax contributes little hydrophilicity, in contrast to the significant hydrophobic contribution of the two long tails.

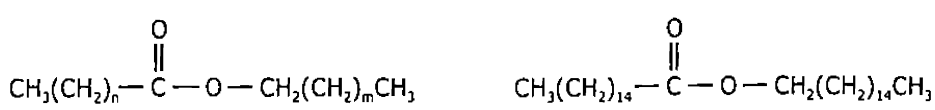


Figure 5.3 : Structure of waxes. The general structure of a wax is shown in which *n* and *m* are usually between 8 and 18.The structure of hexadecyl hexadecanoate (cetylpalmitate), is shown. This is an ester of hexadecanoic (palmitic) acid and hexadecanol (cetyl alcohol).

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5.4 PHOSPHOLIPIDS

Phospholipids are amphipathic molecules. There are two types of phospholipids: phosphoglycerides and sphingophospholipids. **Phosphoglycerides** or **Glycerophospholipids** are molecules that contain glycerol, fatty acids, phosphate, and an alcohol (e.g., choline). Phosphoglycerides are the most numerous phospholipid molecules found in cell membranes.

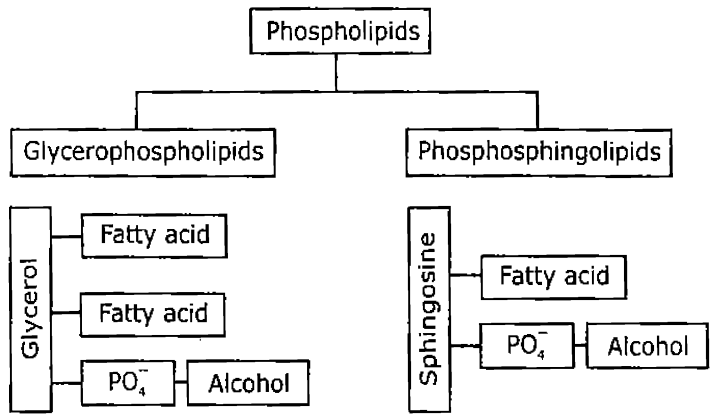
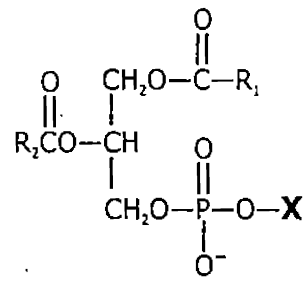


Figure 5.4 : General structure of phospholipids

The simplest phosphoglyceride, **phosphatidic acid**, is the precursor for all other phosphoglyceride molecules. Phosphatidic acid is composed of glycerol-3-phosphate that is esterified with two fatty acids (saturated fatty acid at C-1 and unsaturated fatty acid at C-2 are most common). Phosphoglyceride molecules are classified according to which alcohol becomes esterified to the phosphate group. For example, if the alcohol is choline, the molecule is called phosphatidylcholine (also referred to as *lecithin*) and if serine then it is called phosphotidylserine.



Name of X—OH	Name of Phospholipid	Net Charge (at pH 7)
Water (X=H)	Phosphatidic acid	−1
Choline	Phosphatidylcholine (Lecithin)	0
Ethanolamine	Phosphatidylethanolamine (cephalin)	0
Serine	Phosphatidylserine	−1
Glycerol	Phosphatidylglycerol	−1
Phosphatidylglycerol	Diphosphatidylglycerol (cardiolipin)	−2
Inositol	Phosphatidylinositol	−1

In glycerophospholipids, fatty acids are not only linked with glycerol through ester linkage but also through ether linkage. The ether-linked chain may be saturated, as in the alkyl ether lipids, or may contain a double bond between C-1 and C-2, as in **plasmalogens.** In plasmalogens, the C-1 hydroxyl group of glycerol 3-phosphate is bound to a hydrocarbon chain as a *vinyl ether.*

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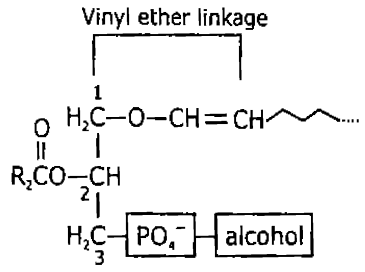
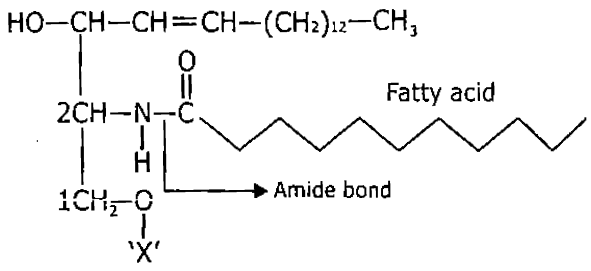


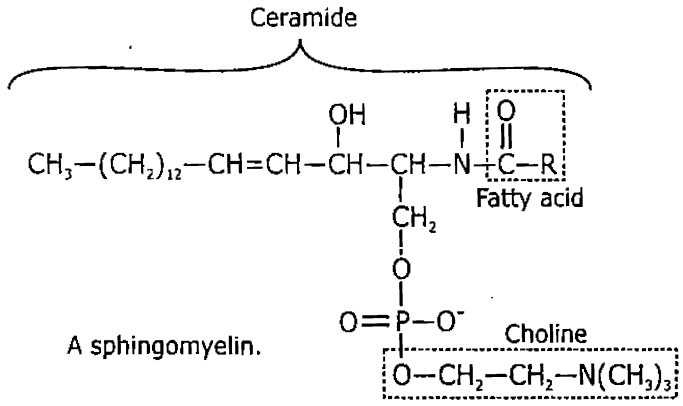
Figure : 5.5
Structure of plasmalogen. One hydrocarbon group is linked to the C-1 position of glycerol as a vinyl ether.

Sphingophospholipid

Sphingophospholipids contain a long chain amino alcohol called **sphingosine** instead of glycerol. When the amino group of sphingosine is acylated with the fatty acid, the product is **ceramide.** Ceramide is the structural parent of all sphingolipids. Addition of groups to the C–1 hydroxyl of sphingosine, leads to a variety of other sphingolipids. An especially important example is **sphingomyelin**, in which a phosphocholine group is attached to the C–1 hydroxyl.



If 'X' = H then it is called ceramide



A sphingomyelin.

5.5 GLYCOLIPIDS

Lipid containing saccharide groups go under the general name of glycolipids. The glycosphingolipid is the most important type of glycolipid and constitute third major class of membrane lipids.

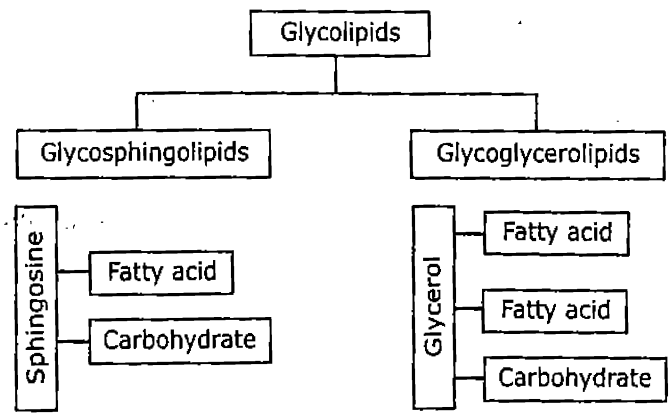


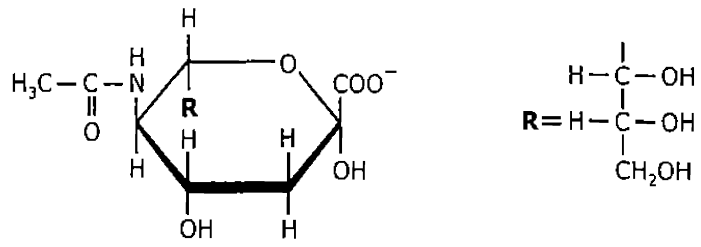
Figure 5.6 : General structure of glycolipids

In glycosphingolipid head group contains one or more sugars connected directly to the —OH at C–1 of the ceramide moiety (do not contain phosphate). It can be

- **Cerebroside** : Have a single sugar linked to ceramide.
- **Globoside** : With two or more sugar linked to ceramide.

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- **Gangliosides** : Globoside that contains complex oligosaccharides as their polar head groups with one or more residues of *N-acetylneuraminic acid*, a sialic acid. More than 60 different gangliosides have been characterized. The normal function of gangliosides is not known.



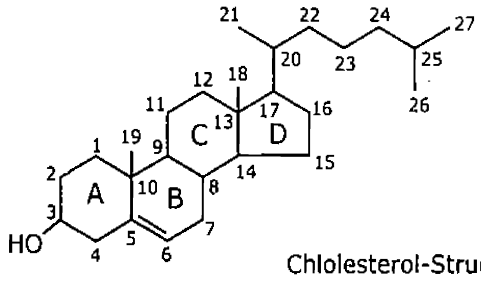
N-Acetylneuraminic acid

Table 5.2 : Selected sphingolipid storage diseases

Disease	*Symptom*	*Accumulating sphingolipid*	*Enzyme deficiency*
Tay-Sachs	Blindness, muscle weakness, seizures, mental retardation	Ganglioside GM2	β-Hexosaminidase A
Gaucher's	Mental retardation, liver and spleen enlargement, erosion of long bones	Glucocerebroside	β-Glucosidase
Niemann Pick	Mental retardation	Sphingomyelin	Sphingomyelinase
Fabry's	Skin rash, kidney failure	Trihexosylceramide	α-Galactosidase A
Krabbe's	Loss of myelin, mental retardation	Deacylated galactocerebroside	Galactocerebrosidase

5.6 STEROIDS

Steroids are complex derivatives of triterpenes. Each type of steroid is composed of four fused rings called **steroids nucleus**. A *sterol* is a class of steroid characterized by a hydroxyl group at C-3. Cholesterol is an example of the sterol and an essential component in animal cell membranes. Cholesterol acts as a precursor for the biosynthesis of all *steroid hormones*, *vitamin D*, and *bile salts*. Cholesterol is usually stored within cells as a fatty acid ester. Sterol that is common in plants are stigmasterol, sitosterol and campesterol.



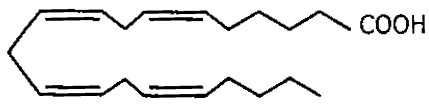
Chlolesterol-Structural formula

5.7 EICOSANOID

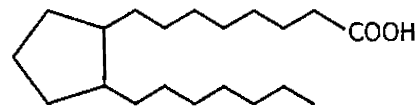
Eicosanoids are family of very potent biological signaling molecules that acts as short range messengers. It includes *prostanoids* and *leukotrienes*. It is known as eicosanoids because of their common origin from 20 carbons polyunsaturated fatty acids, eicosaenoic acids, particularly arachidonic acid. Eicosanoids are potent hormones and act locally instead of being transported in the blood to act on cells of other tissues or organs. Prostanoids and leukotrienes - two major groups of eicosanoids - formed via two distinct pathways, the *cyclooxygenase* and *lipoxygenase* pathways. Prostanoids, which include the *prostaglandins, postacyclin* and *thromboxanes*, are formed through the cyclooxygenase pathway and leukotrienes come from the lipoxygenase pathway.

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Prostaglandins were first identified in human semen. Almost all mammalian cells except RBC produce prostaglandins. Prostaglandins stimulate uterine contraction and lowers blood pressure. Prostaglandins are all derivatives of the hypothetical C_{20} fatty acid **prostanoic acid** in which carbon atoms 8 to 12 comprise a cyclopentane ring.

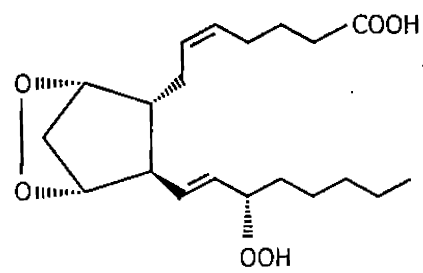


Arachidonic acid



Prostanoic acid

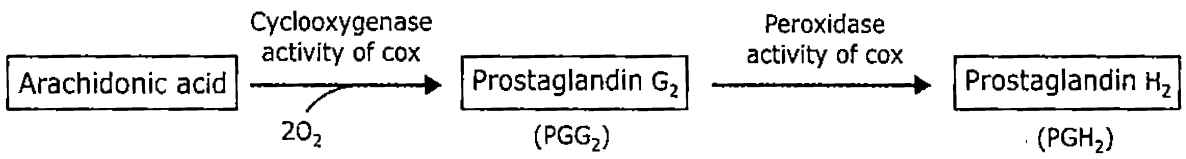
Individual prostaglandins are described by a system of abbreviation in which the name prostaglandin is designated PG, followed by a third letter (A–I) that indicates the nature of the substituents on the cyclopentane ring. A numerical subscript indicates the total number of double bonds in the aliphatic chains.



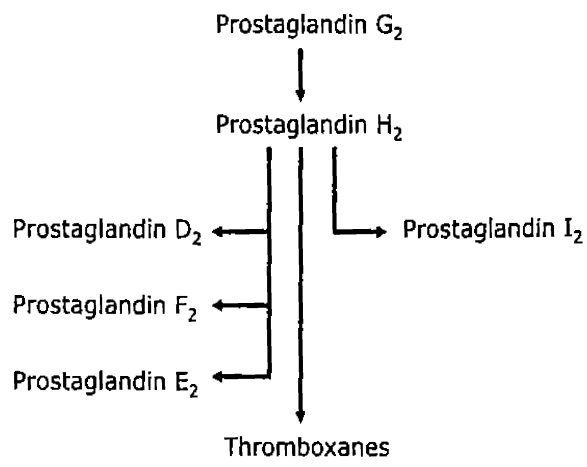
Prostaglandin G_2 (PGG_2)

Synthesis of prostaglandins

In humans, the most prevalent prostaglandin precursor is *arachidonic acid.* Prostaglandins are synthesized as shown in figure from arachidonic acid in a metabolic pathway that begins with plasma membrane phospholipids. The enzyme **cyclooxygenase** (cox), also called *prostaglandin H_2 synthase* catalyzes the introduction of oxygen and the cyclization of the carbon chain of arachidonic acid in the region of the double-bond positions at C-8 and C-11.



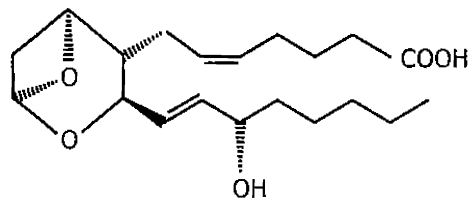
The biosynthesis of the primary prostaglandin, PGG_2, leads to the biosynthesis of a large number of chemically related secondary compounds.



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Prostaglandins have diverse functions. Prostaglandins such as PGE_2 are potent vasodilators, so they increase blood flow and also causes inflammation and pain. **Aspirin** (acetyl salicylate) used as an analgesic (pain-relieving), antipyretic (fever-reducing), and anti-inflammatory agent inhibits the synthesis of prostaglandins. It irreversibly inhibits cyclooxygenase by acetylating a *Ser* residue and blocking the enzymes active site. This inhibition of cyclooxygenase blocks the synthesis of prostaglandins, which in turn reduces the inflammatory response.

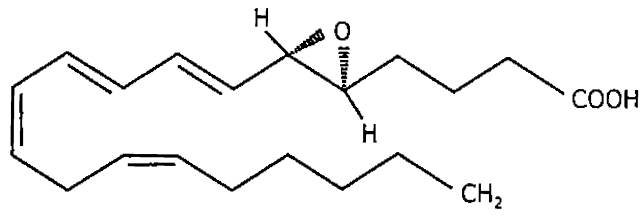
Thromboxanes are derived from prostaglandins in the blood platelets (thrombocytes) by enzyme *thromboxane synthase* which converts PGH_2 to thromboxane A_2 (TxA_2). They act as vasoconstrictor and stimulator of platelet aggregation (an initial step in blood clotting). Thromboxanes have heterocyclic oxane structures. Thromboxane A (TxA) contains an unstable bicyclic oxygenated ring structure, while thromboxane B (TxB) has a stable oxane ring.



TxA_2

Prostacyclins, the endogenous antagonist of thromboxane A_2, are generated from prostaglandin H_2 by a prostacyclin synthase that is particularly abundant in endotheial cells. The biosynthesis of prostacyclin starts with COX-1 and is inhibited by aspirin.

Leukotrienes are hydroxy fatty acid derivatives of arachidonic acid and do not contain a ring structure. Leukotrienes are distinguished by containing a conjugated triene double-bond arrangement. They are involved in chemotaxis, inflammation, and allergic reactions.



Leukotriene A

Biological effects of eicosanoids

Type	*Major functions*
Prostaglandins	Mediation of inflammatory response
	Regulation of nerve transmission
	Inhibition of gastric secretion
	Sensitization to pain
	Stimulation of smooth muscle contraction
Thromboxanes	Platelet aggregation
	Aorta constriction
Prostacyclins	Thromboxane antagonists
Leukotrienes	Bronchoconstriction
	Leukotaxis

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5.8 PLASMA LIPOPROTEINS

Triacylglycerols, phospholipids, cholesterol and cholesterol esters are transported in human plasma in association with proteins as **lipoproteins**. Blood plasma contains a number of soluble *lipoproteins,* which are classified, according to their densities, into four major types. These lipid-protein complexes function as a lipid transport system because isolated lipids are insoluble in blood. There are four basic types of lipoproteins in human blood : *chylomicrons, very low density lipoproteins* (VLDL), *low density liptoproteins* (LDL), and *high density lipoproteins* (HDL). A lipoprotein contains a core of neutral lipids, which includes triacylglyerols and cholesterol esters. This core is coated with a monolayer of phospholipids in which proteins (called *apolipoprotein*) and cholesterol are embedded.

Table 5.3 : Major classes of human plasma lipoproteins : Some properties

Lipoprotein	Density (g/mL)	Protein	Phospho-lipids	Free cholesterol	Cholesterol esters	Triacyl-glycerols	Apolipo-protein
Chylomicrons	<1.006	2	9	1	3	85	A-I, C-I, B-48
VLDL	0.95-1.006	10	18	7	12	50	B-100, C-I, C-II
LDL	1.006-1.063	23	20	8	37	10	B-100
HDL	1.063-1.210	55	24	2	15	4	A-I, A-II, C-I

5.9 MOBILIZATION AND TRANSPORT OF TRIACYLGLYCEROLS

Triacylglycerols are derived from three primary sources:

1. The diet
2. *de novo* biosynthesis, particularly in liver and
3. Storage depots in adipocytes.

Release of fatty acids from stored triacylglycerols of adipose cells

Triacylglycerols in adipose tissue are converted into free fatty acids and glycerol *under the influence of hormones like epinephrine, norepinephrine, glucagon.* A hormone-sensitive lipase (TAG lipase) initiates the process. The hydrolysis of triacylglycerols by lipases is referred to as *lipolysis*.

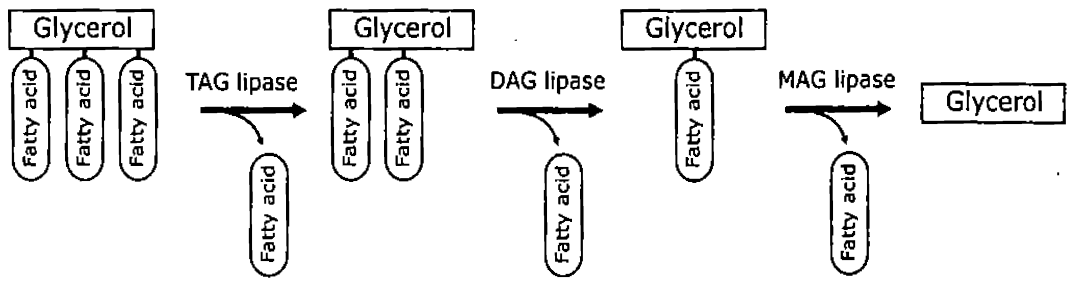


Figure 5.7 : Liberation of fatty acids from triacylglycerols in adipose tissue.

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Release of fatty acids from dietary triacylglycerols

Dietary triacylglycerols are digested by *pancreatic lipases* in the intestinal lumen. Pancreatic lipase cleaves fatty acids from C-1 and C-3 position of triacylglycerol. Resulting monoacylglycerols with fatty acids at C-2 are hydrolyzed by *intestinal lipases*. Before hydrolytic activity of the lipases, emulsification of triacylglycerol occurs. In the intestinal lumen bile salts act on triacylglycerols. *Bile salts* are detergent substances synthesized in liver and stored in the gallbladder. A bile salt molecule is made up of a **bile acid**, such as cholic acid, and an associated cation. The bile salt molecule has both hydrophobic and hydrophilic surfaces. It acts as detergent and emulsifies lipids and yield micelles. Water soluble digestive enzymes digest it and facilitates the absorption of lipid through intestinal mucosal cells. The products of fat digestion comprise a mixture of glycerol, free fatty acids, monoacylglycerols, and diacylglycerols. Less than 10% of the original triacylglycerol also remains unhydrolyzed. During absorption through intestinal mucosal cells, resynthesis of triacylglycerols occurs from the hydrolysis products. This resynthesis occurs in the endoplasmic reticulum and Golgi complex of mucosal cells. Triacylglycerol packaged with cholesterol (dietary) and apolipoproteins (ApoC-II) into lipoprotein aggregates called **chylomicrons**. Chylomicrons moves through the lymphatic and blood vascular system to tissues. *Lipoprotein lipase* in capillary converts triacylglycerols to fatty acids and glycerol. Finally fatty acids enter cells where they are oxidized as fuel or reesterified for storage.

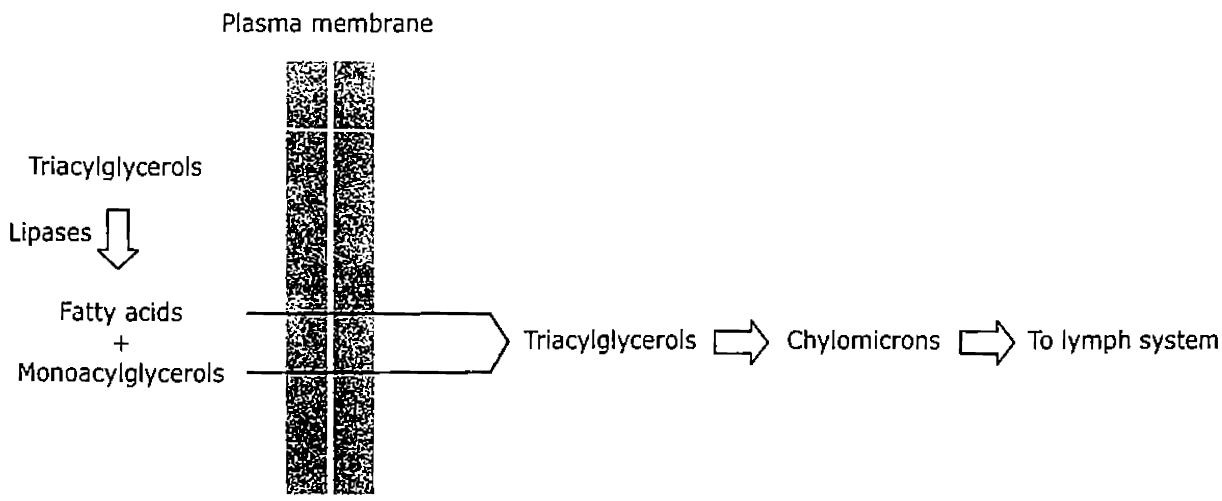


Figure 5.8 : Synthesis of chylomicron

Processing of dietary lipids

1. Emulsification of dietary fat by bile salts in the small intestine.
2. Digestion of triacylglycerol with intestinal lipases.
3. Fatty acids and other breakdown products are absorbed by the intestinal mucosa and reconverted into triacylglycerol.
4. Triacylglycerols are incorporated into chylomicrons.
5. Chylomicrons moves through the lymphatic and blood vascular system to tissues.
6. Lipoprotein lipase in capillary converts triacylglycerols to fatty acids and glycerol.
7. Fatty acids enter cells.

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ATTY ACID METABOLISM

YNTHESIS OF FATTY ACID

lasmic synthesis of saturated fatty acids

y acid synthesis in animal/yeast cells occur in cytosol and in case of plant cells in chloroplast stroma. synthesis of fatty acids involves stepwise addition of two carbon unit. The two carbon units are supplied acetyl-CoA, which in turn is derived from the oxidation of glucose. Acetyl-CoA formed in the matrix of tochondria cannot cross the inner mitochondrial membrane, and an **acetyl CoA shuttle system** is required transport the two-carbon units out of the mitochondria and into the cytosol. Acetyl group passes out of itochondria as citrate. Within the mitochondria, acetyl-CoA reacts with oxaloacetate to form citrate in a eaction catalyzed by *citrate synthase*. Under conditions where acetyl-CoA and ATP are in high concentration, he citrate leaves the mitochondria and in the cytosol, reacts with cytosolic CoA and ATP to form acetyl-CoA nd oxaloacetate in a reaction catalyzed by citrate cleavage enzyme, *citrate lyase*. Oxaloacetate cannot directly return to mitochondrial matrix by crossing mitochondrial membrane so first reduced into malate and hen returns to the mitochondrial matrix.

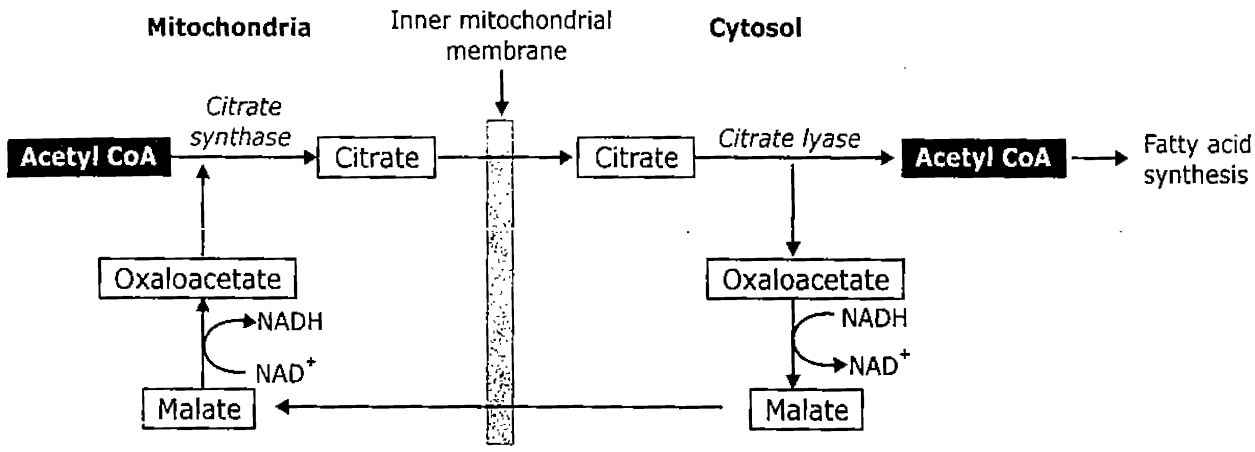


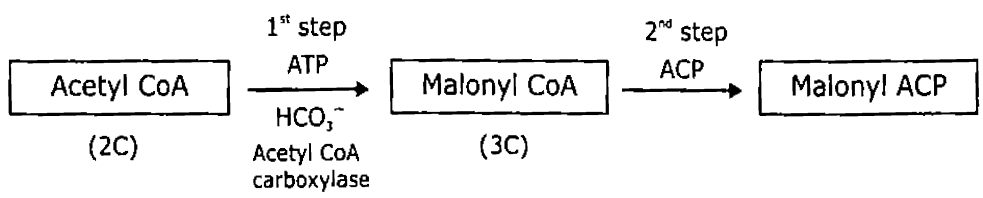
Figure 5.9 : Acetyl-CoA shuttle system

Pathway of synthesis of palmitic acid

Palmitic acid is a 16 carbon saturated fatty acid. The pathway for the biosynthesis of saturated fatty acids is identical in all organisms. A sequence of seven enzyme-catalyzed reactions converts two carbons unit to four carbons unit.

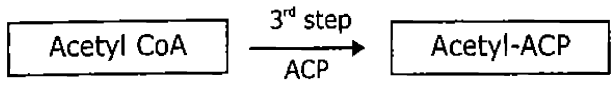
The first committed and irreversible step in fatty acid synthesis is the ATP dependent carboxylation of acetyl CoA to form malonyl CoA . This reaction is catalyzed by *acetyl CoA carboxylase* which has biotin as a prosthetic group. This enzyme is an allosteric enzyme. Palmitoyl CoA, the final product of fatty acid synthesis acts as negative modulator whereas citrate acts as an allosteric activator of this enzyme. Regulatory effects of citrate and palmitoyl CoA are dependent on phosphorylation state of the enzyme. Phosphorylation of the regulatory sites decreases the affinity of the enzyme for citrate and in this case high levels of citrate are required for activation.

The next steps in the biosynthetic pathway are the formation of acetyl-ACP and malonyl-ACP by the enzymes *acetyl transacylase* and *malonyl transacylase* respectively.

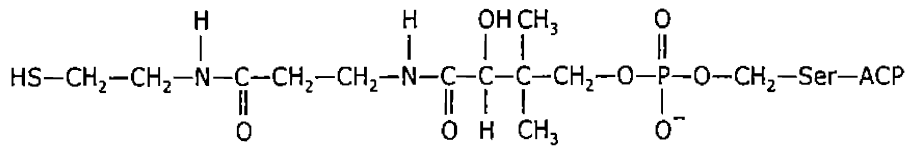


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The activated intermediates of fatty acid biosynthesis are bound to a sulfhydryl group of a *phosphopantetheine* molecule. In prokaryotes, phosphopantetheine group is covalently bound to a small protein consists of a single polypeptide chain called **Acyl Carrier Protein** (ACP). In eukaryotes it is covalently bound to a multienzyme complex called *fatty acid synthase*.



Phosphopantetheine prosthetic group of ACP

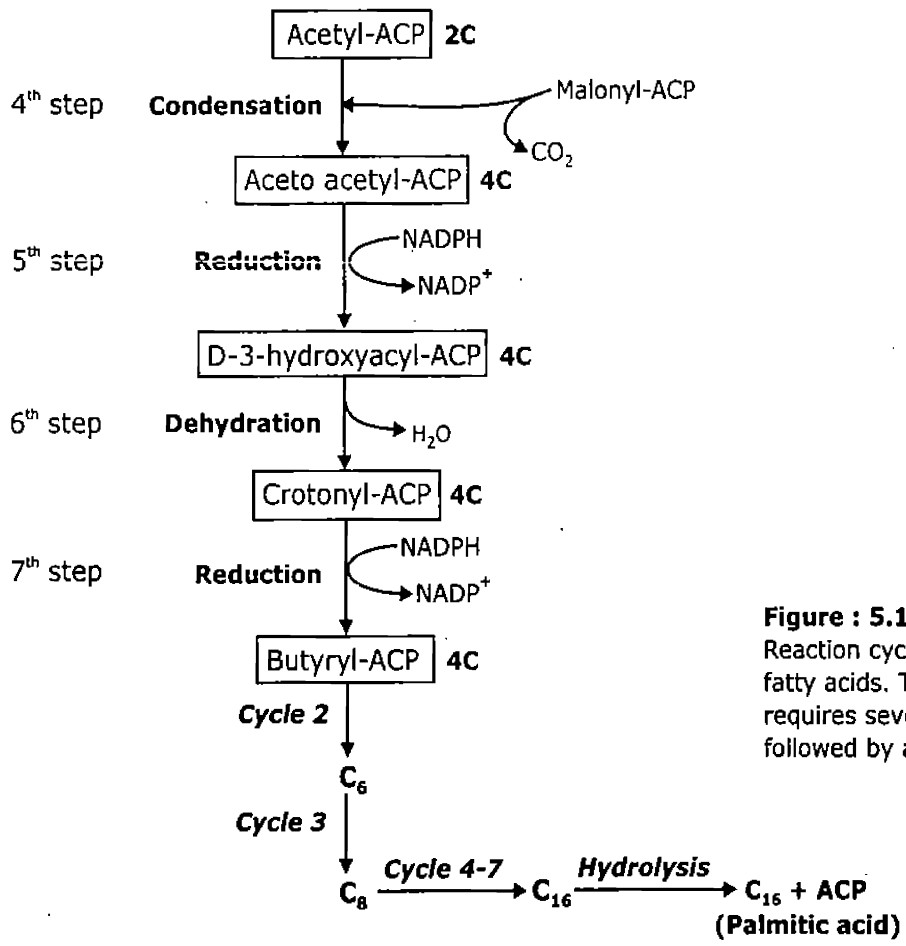


Figure : 5.10
Reaction cycle for the biosynthesis of fatty acids. The biosynthesis of palmitate requires seven cycles of C_2 elongation followed by a final hydrolysis step.

* Steps 4, 5, 6 and 7 used to lengthen a growing fatty acyl chain by 2 carbons.

The last four enzymatic reactions of fatty acid biosynthesis constitute the elongation step. It includes *condensation*, *reduction*, *dehydration* and *reduction*. Repetition of the elongation step, increases chain length by two carbon atoms. In this way the seven elongation cycles produce palmitic acid. Finally *palmitoyl thioesterase* catalyzes hydrolysis of the thioester that binds the acyl chain to the pantothiene-enzyme, releasing free palmitate. In the synthesis of palmitic acid all the carbons have passed through malonyl-CoA except the two donated by original acetyl-CoA. Hence in palmitic acid carbon 16 and 15 are donated by acetyl-CoA and rest by malonyl-CoA.

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In eukaryotes, the enzymes that catalyze reactions from 2 to 7 form a multienzyme complex known as *fatty acid synthase*. The fatty acid synthase from bacteria and plants is a complex of seven different polypeptides. In yeast all seven activities reside in only two polypeptides, and in vertebrates, in a single large polypeptides.

Stoichiometry of the synthesis of palmitate

7 Acetyl CoA + 7 CO_2 + 7 ATP + 7 H_2O → 7 Malonyl CoA + 7 ADP

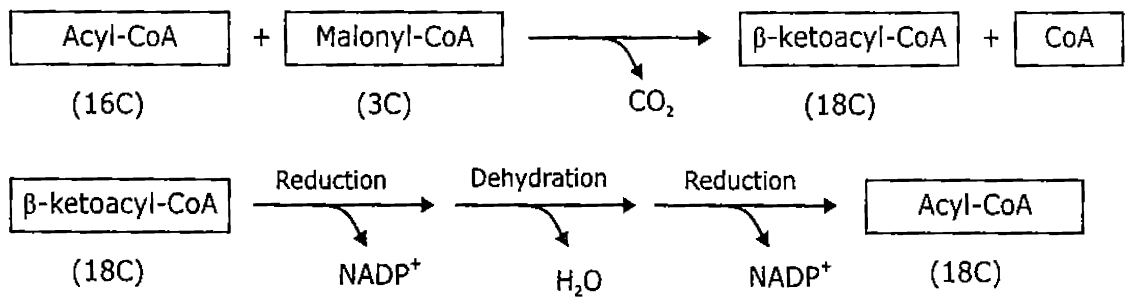
Acetyl CoA + 7 malonyl CoA + 14 NADPH → Palmitate + 7 CO_2 + 14 $NADP^+$ + 6 H_2O

Sum: 8 Acetyl CoA + 7ATP + 14 NADPH + H_2O → palmitate + 7 ADP + 14 $NADP^+$

Elongation of fatty acids

The normal product of the cytosolic fatty acid synthase complex is palmitic acid. Fatty acids longer than 16 carbons can be formed through the addition of two-carbon units by elongation reaction. In eukaryotes fatty acids elongation occurs in both ER and mitochondria.

The most active elongation system is found in the endoplasmic reticulum. It adds malonyl CoA onto palmitate in a manner similar to the action of fatty acid synthase, except that CoA is involved rather than the ACP. Stearic acid (18 carbons) is a common product of this elongation system.



A mitochondrial elongation system uses acetyl-CoA units, rather than malonyl-CoA units, to elongate fatty acids for the synthesis of structural lipids in this organelle.

Unsaturation of fatty acids

Both prokaryotes and eukaryotes are capable of introducing double bond(s) in a newly synthesized fatty acid. In eukaryotes, a microsomal system that contains the flavoenzyme NADH-cytochrome b_5 reductase, Cyt b_5, and an oxygen-dependent desaturase containing a single non-heme iron atom cause desaturation. All components of the desaturase system are integral membrane proteins.

Mammals are unable to introduce double bonds beyond Δ9 in the fatty acid chain. Hence they cannot synthesize either linoleic acid (18 : 2 : Δ^9, Δ^{12}) or linolenic acid (18 : 3 : Δ^9, Δ^{12}, Δ^{15}).

FATTY ACID OXIDATION

β-oxidation

Most fatty acids are degraded by the sequential removal of two-carbon fragments from the carboxyl end of fatty acids. During this process, referred to as *β-oxidation*, acetyl-CoA is formed as the bond between the α- and β-carbon atoms is broken. β-Oxidation is so named because the β-carbon of fatty acids is oxidized. β-oxidation occurs primarily within mitochondria.

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Fatty acid activation and transport across the mitochondrial membrane

Before β-oxidation begins, each fatty acid is activated in a reaction with ATP and CoA. Fatty acids undergo for ATP dependent acylation reaction to form fatty acyl-CoA. This activation process is catalyzed by acyl-CoA synthetase (also called acyl CoA ligase or acyl CoA thiokinase) in the cytosol.

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \rightleftharpoons \text{Fatty acyl - CoA} + \text{AMP} + PP_i$$

Although fatty acids are activated for oxidation in the cytosol, they are oxidized in the mitochondrion. A long chain fatty acyl-CoA cannot directly cross the inner mitochondrial membrane. Because the mitochondrial inner membrane is impermeable to most acyl-CoA molecules, a special carrier called *carnitine* (β-hydroxy γ-trimethyl ammonium butyrate) is used to transport acyl groups into the mitochondrion (as shown in figure). Carnitine-mediated transfer of fatty acyl-CoA into the mitochondrial matrix is accomplished through the following mechanism.

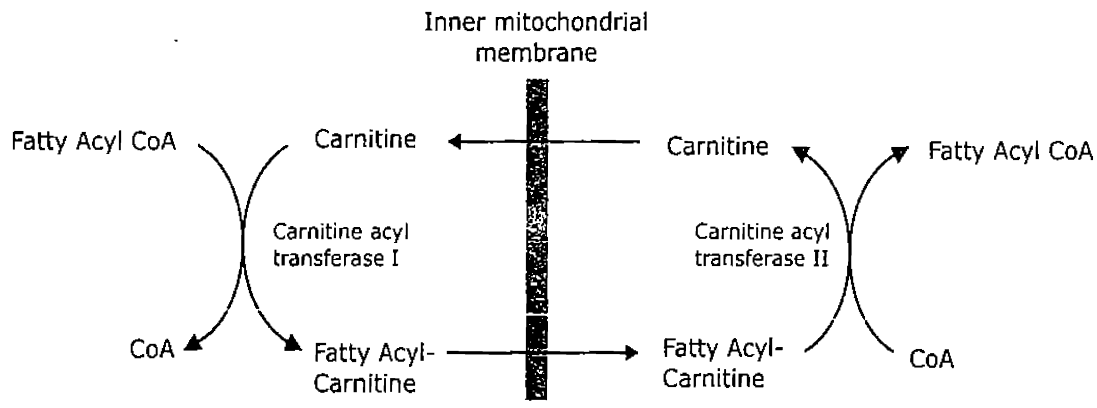


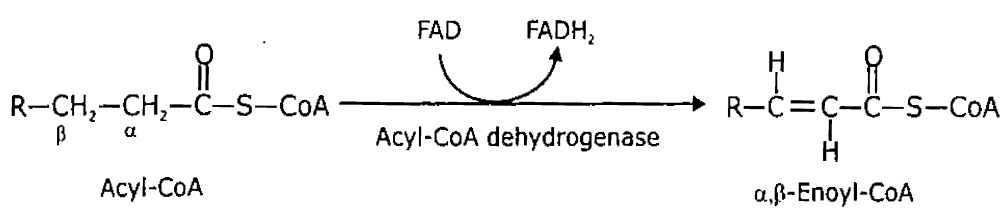
Figure 5.11 : Transport of fatty acids into the mitochondria.

1. Each fatty acyl-CoA molecule is converted to a fatty acyl carnitine derivative by *carnitine acyl transferase I*, located on the outer side of the inner mitochondrial membrane.
2. A carrier protein (carnitine : acyl carnitine translocase) within the mitochondrial inner membrane transfers fatty acyl carnitine into the mitochondrial matrix.
3. Fatty acyl-CoA is regenerated by *carnitine acyl transferase II*, located on the matrix side of inner mitochondrial membrane.
4. Carnitine is transported back into the intermembrane space by the carrier protein. It then reacts with another fatty acyl-CoA.

β-oxidation of saturated fatty acids (e.g., palmitic acid)

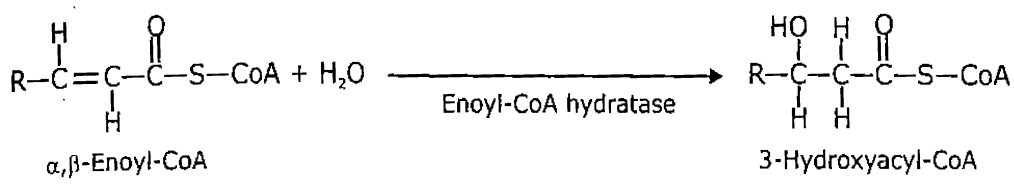
Once inside the mitochondrial matrix, fatty acyl-CoA is oxidized with oxidation of the β–carbon and long-chain fatty acyl-CoA are subjected to a repeated four-step process, which removes two carbons from the chain successively until the last two-carbon fragment is obtained. A summary of the reactions of the β-oxidation of palmitic acid is shown below.

Oxidation

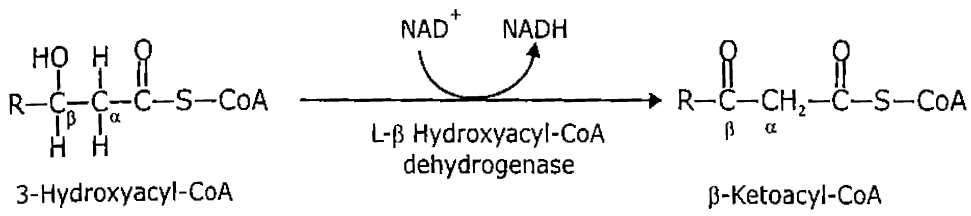


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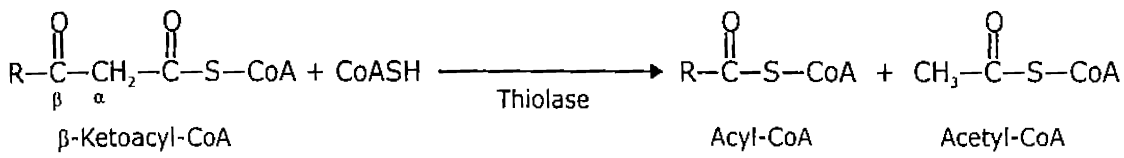
Hydrolysis



Oxidation



Thiolysis



This series of four reactions has yielded a fatty acyl-CoA that has been shortened by two carbons, and one molecule of acetyl-CoA. The shortened fatty acyl-CoA can now go through another β-oxidation cycle. Repetition of this cycle with palmitic acid with an even number of carbons eventually yields 8 molecules of acetyl-CoA.

Stoichiometry of β-oxidation

β-Oxidation of palmitate (16 carbons)

Palmitoyl CoA + 7 CoA + 7 FAD + 7 NAD^+ + 7 H_2O → 8 acetyl-CoA + 7 $FADH_2$ + 7 NADH

Acetyl-CoA is catabolized via TCA cycle, and $FADH_2$ and NADH transfer electrons to the electron transport chain. Thus we can easily compute the metabolic energy yield fatty acid oxidation in terms of moles of ATP synthesized.

Reaction	*ATP yield*	*ATP consumed*
Activation of palmitate to palmitoyl-CoA		-2
Oxidation of 8 acetyl-CoA	8 × 10 = 80	
Oxidation of 7 $FADH_2$	7 × 1.5 = 10.5	
Oxidation of 7 NADH	7 × 2.5 = 17.5	
Net : Palmitate → 16 CO_2 + 130 H_2O	106	

These calculations assume that mitochondrial oxidative phosphorylation produces 1.5 ATP per $FADH_2$ oxidized and 2.5 ATP per NADH oxidized.

STUDY QUESTIONS

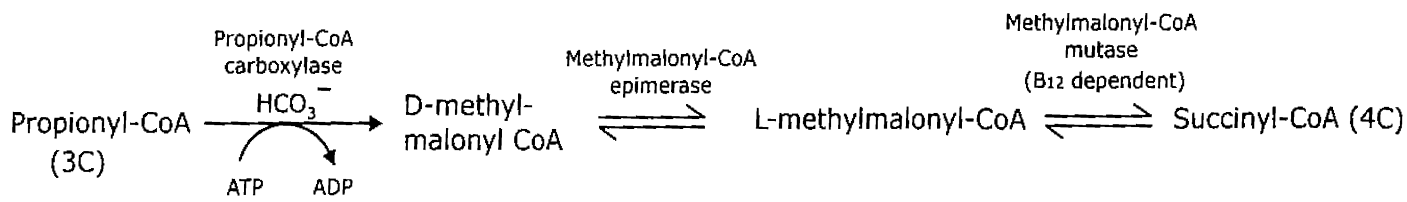
Q3. Calculate the approximate yield in ATP molecules of the complete oxidation of hexanoic acid (C6:0).

Ans. After activation of hexanoic acid to hexanoyl CoA, two rounds of β oxidation are required to produce 3 acetyl CoA molecules. The two cycles also produce 2 $FADH_2$ and 2 NADH molecules. Each acetyl-CoA yields 10 ATP upon complete oxidation, each $FADH_2$ produces 1.5 ATP, and each NADH makes 2.5 ATP—a total of 38 ATP. Activation uses 2 ATP molecules because AMP is one of the products of the reaction, so the net yield is approximately 36 ATP.

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Oxidation of fatty acids with odd numbered carbon chains

Fatty acids with odd numbered carbon chains are only minor component of diet. Oxidation of odd numbered fatty acids chains yield propionyl-CoA, a three carbon compound after the last cycle of β-oxidation. Propionyl-CoA finally gives succinyl-CoA through *biotin dependent carboxylation* and a coenzyme B_{12}-*dependent rearrangement*. Succinyl-CoA can enter the TCA cycle or be converted to acetyl-CoA.



Oxidation of monounsaturated fatty acids

The pathway of β-oxidation of unsaturated fatty acids is same as that for β-oxidation of saturated fatty acids until the double bond is reached. For example, consider the β-oxidation of monounsaturated fatty acids such as palmitoleic acid. Three cycles of β-oxidation of palmitoleoyl CoA produce *cis* Δ^3-enoyl CoA. But further oxidation requires three more enzymes.

First, *isomerase* catalyzes *cis-trans* isomerization and transfer of double bond position. This enzyme is needed because unlike *trans* double bonds introduced during β-oxidation the double bonds of most naturally occurring unsaturated fatty acids have a *cis* configuration.

Second, *hydratase* catalyzes hydration of *trans* double bond.

Third, *epimerase* catalyzes the conversion of R-3-hydroxyacyl CoA to S-3-hydroxyacyl CoA.

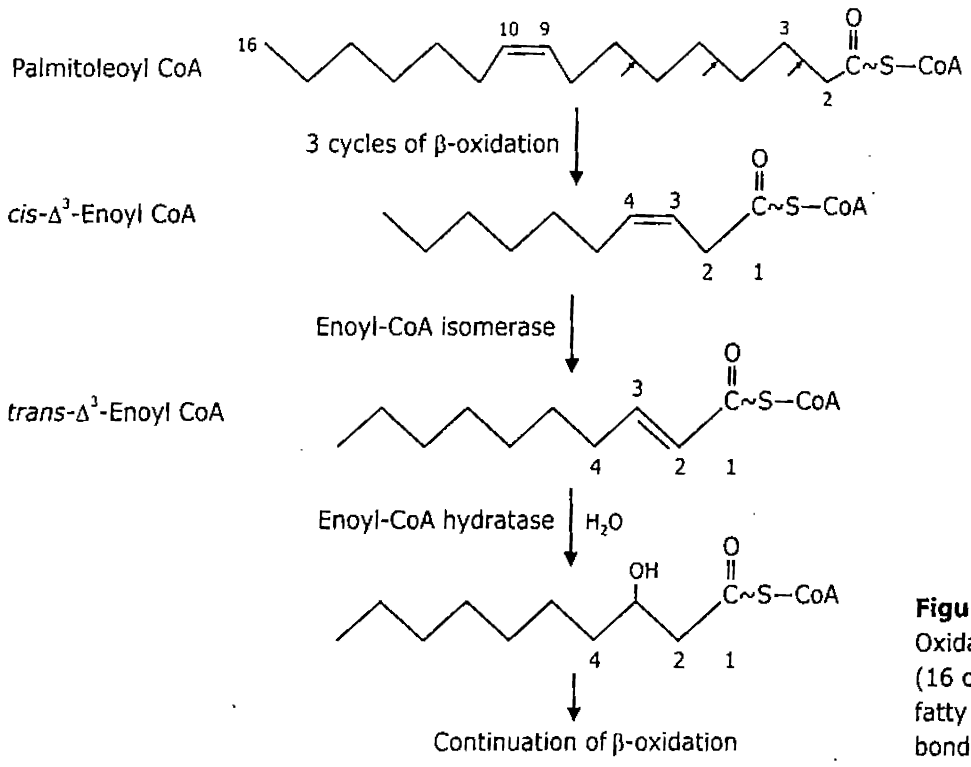


Figure : 5.12
Oxidation of palmitoleate (16 carbon unsaturated fatty acid, which has one double bond between C-9 and C-10)

The energy yield from an unsaturated fatty acid is less as compared to saturated fatty acid. The energy yielding step that is bypassed during oxidation of unsaturated fatty acid is the first dehydrogenation reaction catalyzed by *Acyl-CoA dehydrogenase*. Since this is an FAD-linked dehydrogenation, oxidation of $FADH_2$ by electron transport, which produces 1.5 ATP molecules, is lost. Thus, palmitoleate yields 1.5 less ATP as compared to palmitic acid.

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Oxidation of polyunsaturated fatty acids

Consider linoleate, a C18 polyunsaturated fatty acid with cis-Δ^9 and cis-Δ^{12} double bonds (Figure 5.12). When linoleic acid enters into the process of oxidation, the 1st problem is the *cis* β, γ double bond. *Cis* β, γ double bond is converted into the trans α, β double bond by enzyme *enoyl-CoA isomerase.*

Second problem arises in the fifth round of β-oxidation. The presence of a double bond at an even numbered carbon atom results in the formation of 2,4 dienoyl-CoA which is a poor substrate for enoyl-CoA hydratase. This problem is solved by *2,4-dienoyl-CoA reductase.* NADPH-dependent *2,4-dienoyl-CoA reductase* reduces the double bond and yields *trans*-3-enoyl-CoA, which, to proceed along the β-oxidation pathway, must first be isomerized to *trans*-2-enoyl-CoA by *3,2-enoyl-CoA isomerase.*

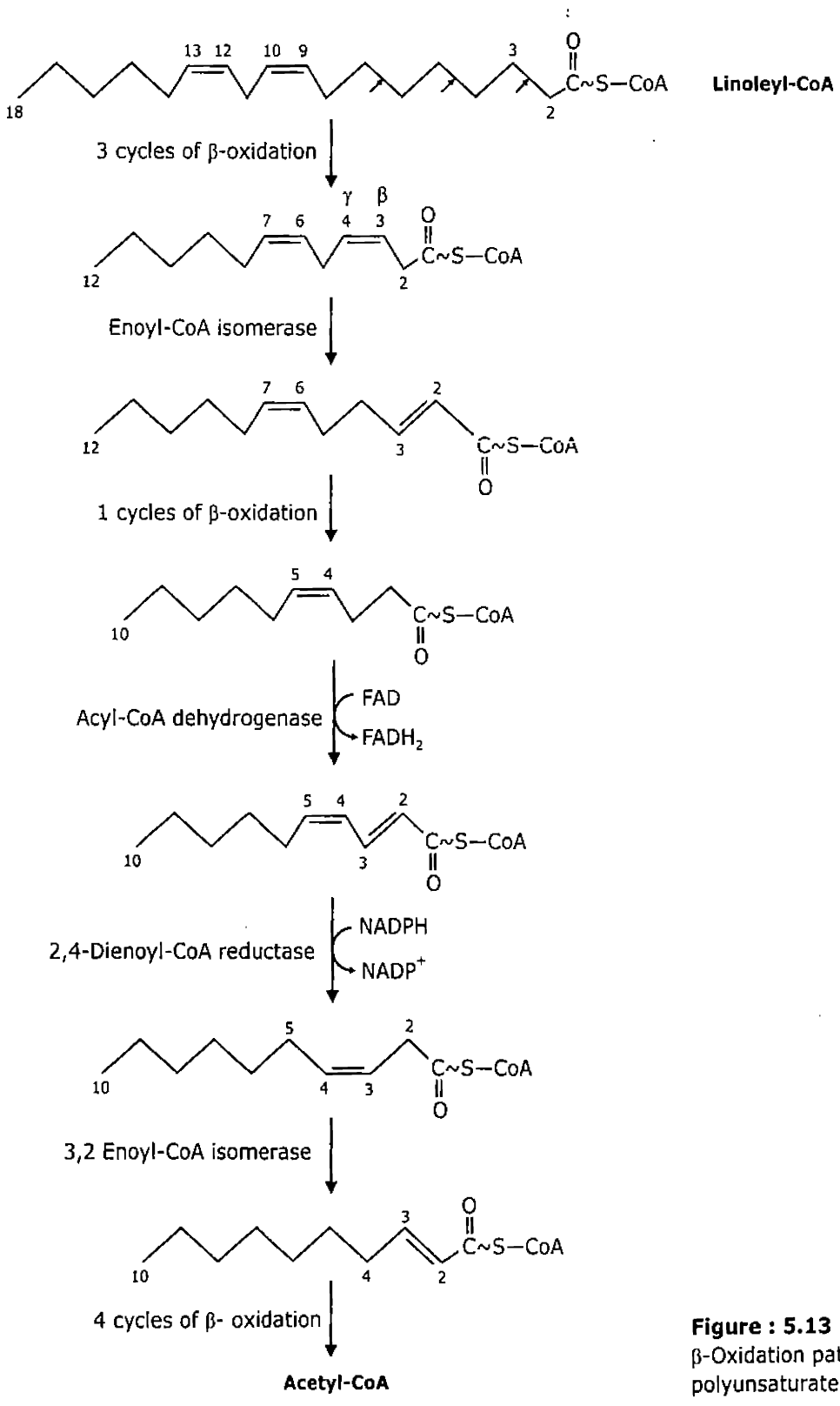


Figure : 5.13
β-Oxidation pathway for polyunsaturated fatty acids.

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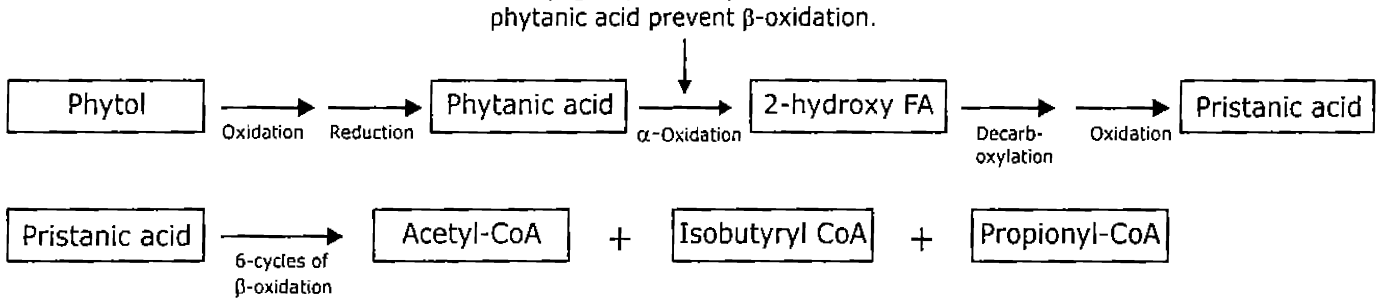
Peroxisomal β-oxidation

Peroxisomal β-oxidation is similar to but not identical with mitochondrial β-oxidation. Peroxisomes, membrane enclosed organelles of animal and plant cells, carry out β-oxidation pathway in which *flavoprotein acyl-CoA oxidase* transfer electrons directly from $FADH_2$ to oxygen. The latter is reduced to H_2O_2. However in mitochondrial β-oxidation, electrons from $FADH_2$ are transferred to respiratory electron transport chain. Because electrons are not shuttled into the respiratory chain, the peroxisomal pathway is not coupled to energy production, but it does generate heat. The NADH formed in the second oxidative step cannot be reoxidized in the peroxisome, so reducing equivalents are exported to the cytosol, eventually entering mitochondria. Plant glyoxysomes also carry the β-oxidation of fatty acid to acetyl-CoA, which is used for succinate synthesis via the *glyoxylate pathway*.

ALTERNATIVE PATHWAYS FOR FATTY ACID OXIDATION

α-Oxidation of fatty acid

α-oxidation is a minor pathway of fatty acid oxidation. In this case the α-carbon undergoes for initial oxidation rather than the β-carbon. α-oxidation is a mechanism for degradation of branched chain and odd chain fatty acid. α-oxidation of phytanic acid (a branched 20 carbon fatty acid) in animal is most common example. Phytanic acid is an oxidation product of phytol of chlorophyll. Pristanic acid forms as a result of oxidation is further degraded through β-oxidation pathway. The α-oxidation pathway is defective in **Refsum's disease** and thus phytanic acid is not converted to a compound that can be degraded. The symptoms of this metabolic disorder include defective night vision, tremors and other neurological abnormalities.



ω-oxidation of fatty acid

ω-oxidation of fatty acids is a minor pathway that involves the oxidation of terminal carbon of a normal fatty acids and lead to the synthesis of small amount of dicarboxylic acids. This process occurs in the endoplasmic reticulum of eukaryotic cells.

Table 5.4 : Comparison of fatty acid synthesis and degradation

	Synthesis	*Degradation*
Major tissue site	Primarily liver	Muscle and liver
Sub cellular site	Primarily cytosol	Primarily mitochondria
Oxidation/reduction cofactor	NADPH	NAD^+, FAD
Two carbon donor/product	Malonyl CoA, donor of one acetyl group	Acetyl CoA; product
Hormonal state favouring pathway	High insulin/glucagon ratio	Low insulin/glucagon ratio

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KETONE BODIES

Most of the acetyl-CoA produced during fatty acid oxidation is used by the citric acid cycle or in fatty acid synthesis. Under normal conditions, fatty acid metabolism is so carefully regulated that only, small amounts of excess acetyl-CoA are produced. However in mitochondria of liver when acetyl-CoA accumulates beyond its capacity to be oxidized or used for fatty acid synthesis it leads to the formation of **ketone bodies.** Formation of acetoacetate, β-hydroxybutyrate, and acetone, a group of molecules called the *ketone bodies* from acetyl-CoA molecules is known as **ketogenesis.**

Ketone body formation, which occurs within the matrix of liver mitochondria, begins with the condensation of two acetyl-CoAs to form acetoacetyl-CoA. Then acetoacetyl-CoA condenses with another acetyl-CoA to form β-hydroxy-β-methyl-glutaryl-CoA (HMG-CoA). In the next reaction, HMG-CoA is cleaved to form acetoacetate and acetyl-CoA. Acetoacetate is then reduced to form β-hydroxybutyrate. Acetone is formed by the spontaneous decarboxylation of acetoacetate when the latter molecule's concentration is high. During starvation or uncontrolled diabetes, accumulation of acetyl-CoA accelerates the formation of ketone bodies. The increased blood levels of acetoacetate and D-β-hydroxybutyrate lower the blood pH, causing the condition known as **acidosis**. Very high concentration of ketone bodies in the blood, **ketonemia** (about 90 mg/100ml as compared to normal level of <3 mg/100ml) and urine, **ketonuria** (about 5000 mg/24 hr as compared to normal rate of less than or equal to 125 mg/24 hr) is known as **ketosis**.

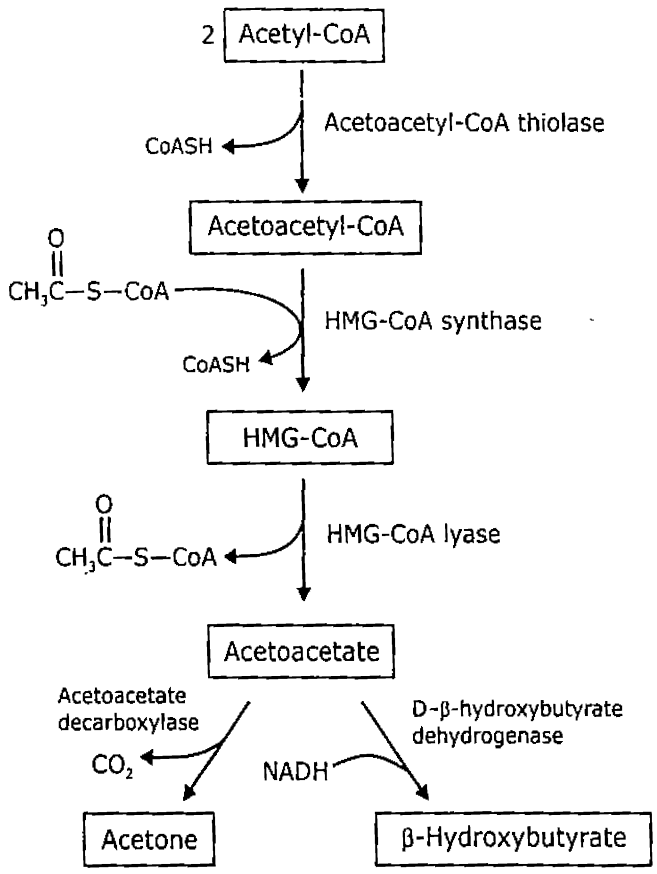


Figure : 5.14
Ketone body formation. When acetyl CoA accumulates, thiolase catalyzes the condensation of two acetyl CoA molecules to acetoacetyl-CoA. Acetoacetyl CoA gives six carbon compound HMG-CoA which in the presence of HMG-CoA lyase gives acetoacetate and acetyl CoA. Acetoacetate is reversibly reduced into β-hydroxybutyrate and also decarboxylated into acetone.

Several tissues, most notably cardiac and skeletal muscle, use ketone bodies to generate energy. During prolonged starvation (i.e. in the absence of sufficient glucose) the brain uses ketone bodies as an energy source. The mechanism by which acetoacetate and β-hydroxybutyrate are converted to acetyl-CoA is illustrated in figure.

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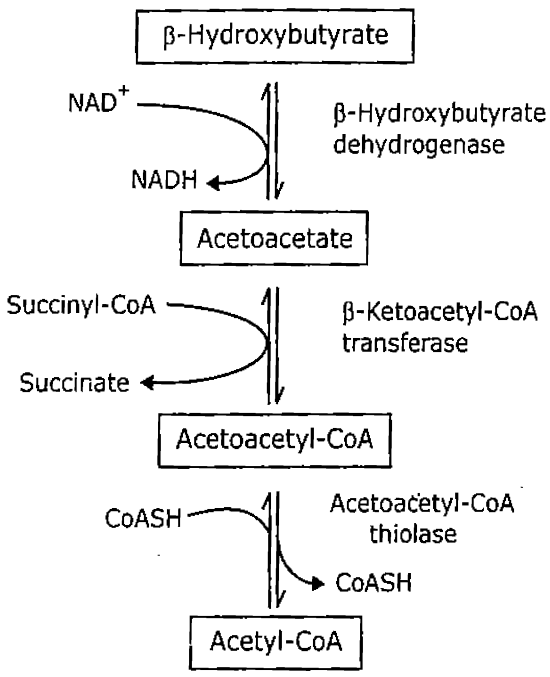


Figure : 5.15
Conversion of Ketone Bodies to Acetyl-CoA. Some organs (e.g. Heart and skeletal muscle) can use ketone bodies as an energy source under normal conditions. During starvation the brain uses them as an important fuel source. Because liver does not have β-ketoacid-CoA transferase, it can not use ketone bodies as an energy source. These reactions are reversible.

5.11 CHOLESTEROL METABOLISM

Synthesis of cholesterol

All 27 carbon atoms in cholesterol is provided by acetate moiety (acetyl CoA). The isoprene units are the essential intermediates in the pathway from acetate to cholesterol. Four basic steps are involved in cholesterol synthesis

1. *Formation of mevalonate from acetate.*

   The first step in cholesterol biosynthesis leads to the intermediate mevalonate. The molecules of acetyl-CoA condense to form acetoacetyl-CoA, which condenses with a third molecule of acetyl-CoA to yield the six-carbon compound β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). The third reaction is the committed and rate-limiting step: reduction of HMG-CoA to mevalonate. This step is catalyzed by an integral membrane protein of the smooth ER called *HMG-CoA reductase*.

2. *Conversion of mevalonate into isopentenyl pyrophosphate.*

   After phosphorylation, mevalonate is decarboxylated to *isopentenyl pyrophosphate, activated* C_5 *isoprenoid compound,* with consumption of ATP.

3. Polymerization of six *isopentenyl pyrophosphate* to form linear structure called *squalene.*

   Isopentenyl pyrophosphate undergoes isomerization to form dimethylallyl diphosphate. The two C5 molecules condense to yield geranyl pyrophosphate, and the addition of another isopentenyl pyrophosphate produces farnesyl pyrophosphate. This can then undergo dimerization, in a *head-to-head reaction,* to yield squalene.

4. *Cyclization of squalene forms steroid nucleus.*

   Squalene, a linear isoprenoid, is cyclized, with O_2 being consumed, to form lanosterol, a C30 sterol. Three methyl groups are cleaved from this in the subsequent reaction steps, to yield the end product cholesterol.

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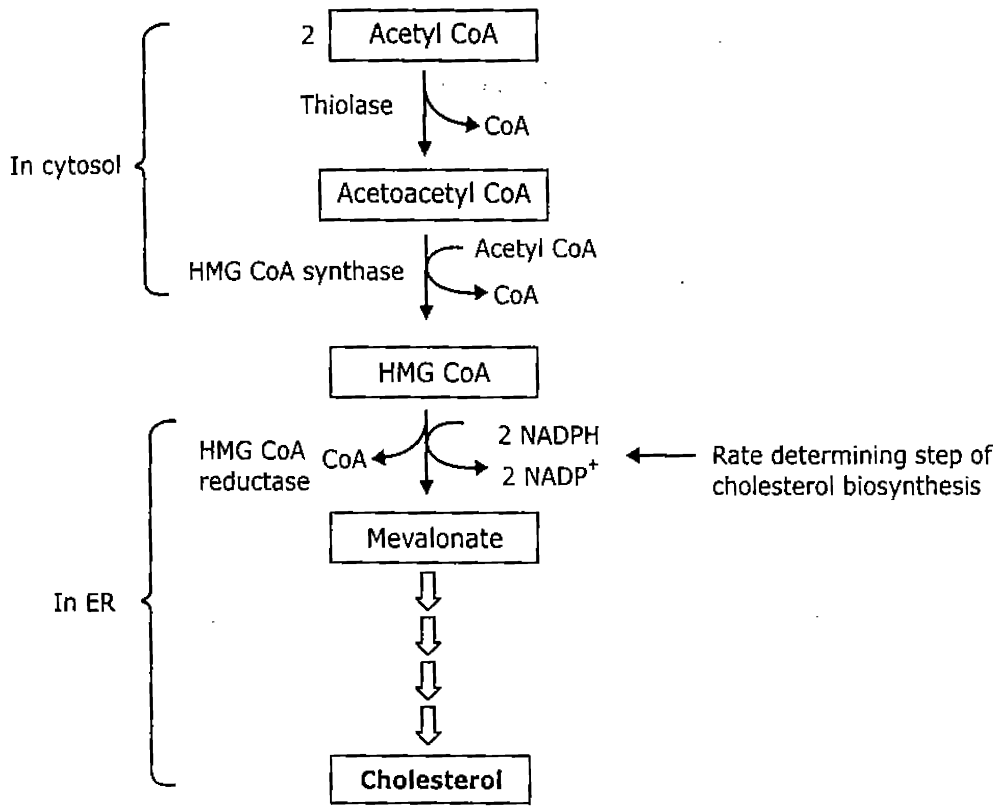


Figure 5.16 : Formation of mevalonate from acetyl-CoA.

5.12 STEROID HORMONES AND BILE ACIDS

Steroid hormones

Cholesterol is the biosynthetic source of all steroid hormones. Synthesis of steroid hormones involves shortening of the hydrocarbon chain of cholesterol and hydroxylation of the steroid nucleus. Biosynthetic pathway of steroid hormones begins with the synthesis of *pregnenolone*. Pregnenolone synthesis occurs in the mitochondria and finally transported into the ER. In ER, a hydroxyl oxidation and migration of the double bond yield progesterone. Progesterone finally acts as precursor for other steroid hormones like cortisol, corticosterone, aldosterone, testosterone and estradiol.

Classes of steroid hormones and their roles:

There are five major classes of steroid hormones:

1. The **progestins** (progesterone), which regulate events during pregnancy and are the precursors to all other steroid hormones.
2. The **glucocorticoids** (cortisol and corticosterone), which promote gluconeogenesis and, in pharmacological doses, suppress inflammation reactions.

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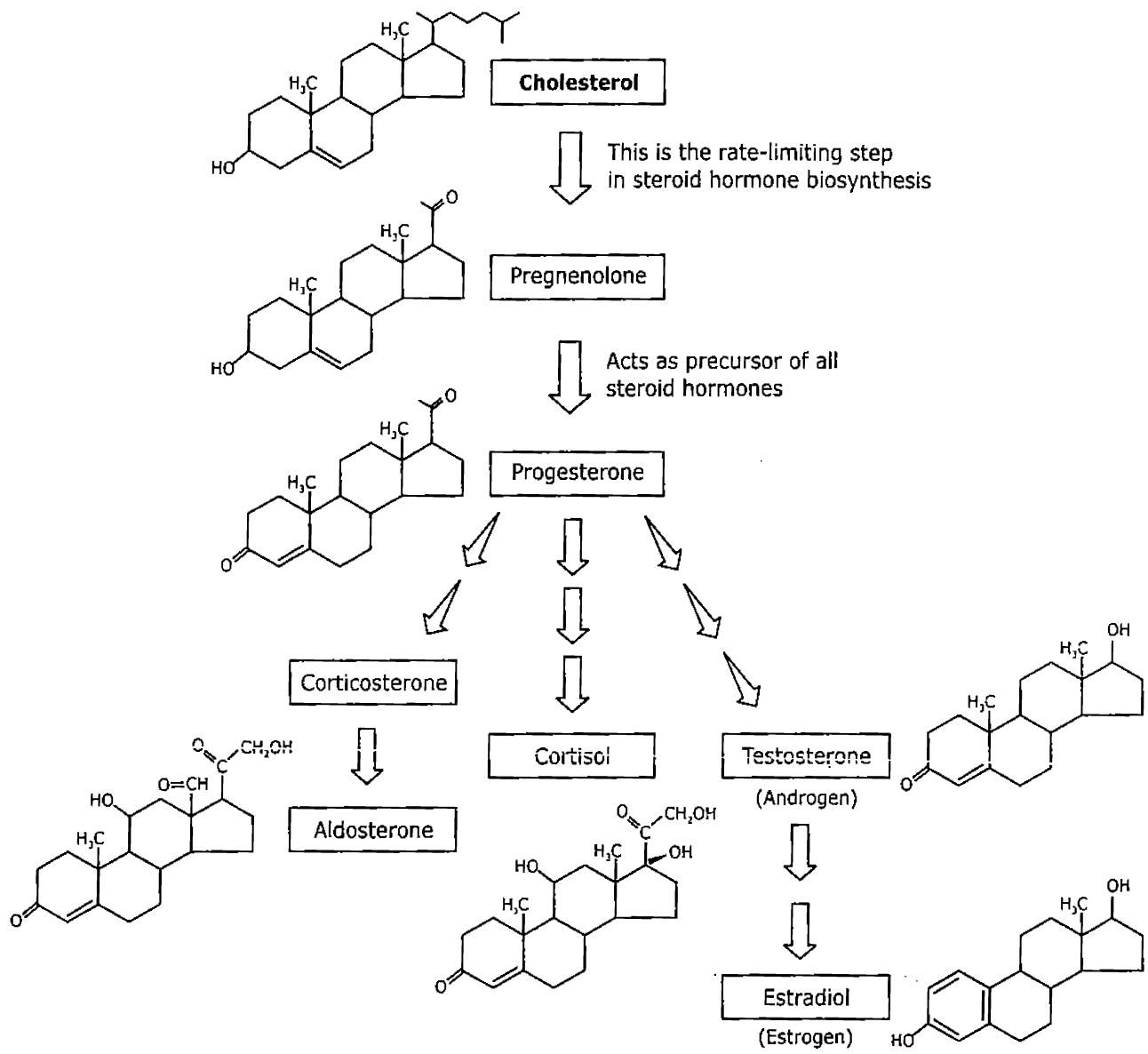


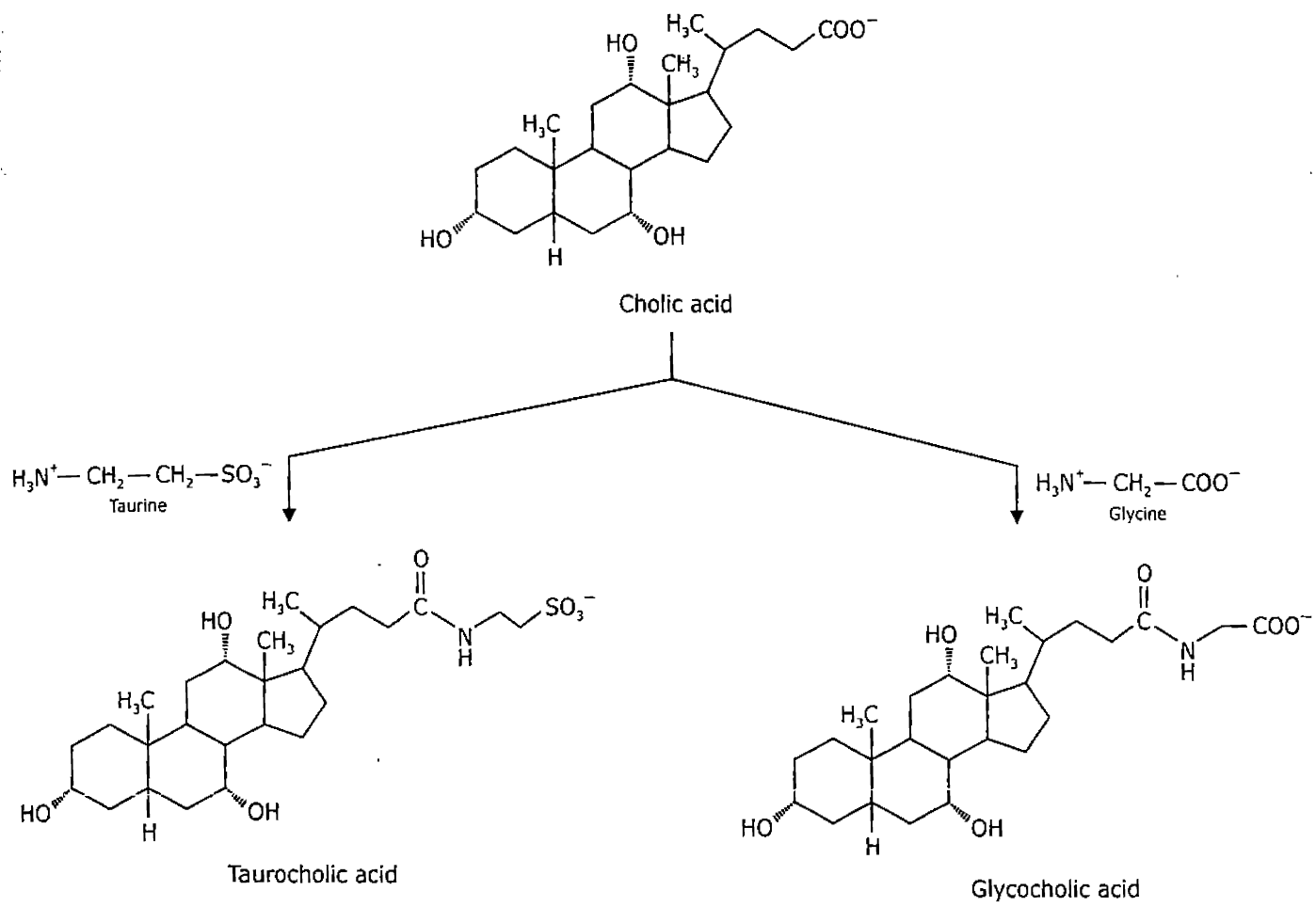
Figure 5.17 : Biosynthetic routes from cholesterol to steroid hormones. Pregnenolone is an intermediate en route from cholesterol to steroid hormones.

3. The **mineralocorticoids** (aldosterone), which regulate ion balance by promoting reabsorption of K^+, Na^+, Cl^-, and HCO_3^- in the kidney.
4. The **androgens** (androstenedione and testosterone), which promote male sexual development and maintain male sex characteristics.
5. The **estrogens** (estrone and estradiol), or female sex hormones, which support female characteristics.

Bile acids

Bile acids are steroid derivatives. They are synthesized in the liver by the metabolism of cholesterol and stored in the gall bladder. Four bile acids - cholic acid, deoxycholic acid, chenodeoxy cholic acid and lithocholic acid-are present in human bile. The most abundant bile acids in humans are **cholic acid** and **chenodeoxycholic acid**. Cholic acid is usually conjugated with the amino acid glycine or taurine, giving compound called *glycocholate* and *taurocholate*, respectively. Bile salts, derivatives of bile acids, cause emulsification of dietary lipids in the intestine and thereby promote fat digestion and absorption. Emulsification is possible becuase of the amphipathic nature of bile salts.

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SELF TEST

Q1. The T_m of phosphatidyl choline A is higher than T_m of phosphatidyl choline B because

a. A has shorter chain fatty acid and more unsaturated fatty acid than B

b. A has longer chain fatty acid and more saturated fatty acid than B

c. A has shorter chain fatty acid than B

d. A has more *cis*-unsaturated fatty acid than B **[GATE]**

Q2. Which of the following fatty acids has the lowest melting point?

a. fatty acids with sites of unsaturation with *cis* double bonds

b. fatty acids with sites of unsaturation with *trans* double bonds

c. fatty acids with *no* sites of unsaturation

d. fatty acids with longer hydrophobic tails

Q3. A structural characteristic common to lipids which allow them to function as good energy stores is

a. they are all hydrophobic

b. they are all hydrophilic

c. they have large numbers of carbon-phosphorus bonds

d. they have large numbers of carbon-hydrogen bonds

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Q4. The nitrogenous base present in lecithin
   a. choline
   b. ethanolamine
   c. inositol
   d. serine

Q5. Which one of the following statements about prostaglandins is *incorrect*?
   a. prostaglandins have a very short half-life
   b. prostaglandins are synthesized only in the liver and the adrenal cortex
   c. prostaglandins generally act locally on or near the tissue that produced them
   d. the common precursor of the prostaglandins is arachidonic acid

Q6. Which one of the following statements about prostaglandin synthesis is *incorrect*?
   a. prostaglandin synthesis is inhibited by glucocorticoids and aspirin.
   b. prostaglandins are derived from an essential fatty acid.
   c. prostaglandins are produced through the action of lipoxygenase.
   d. arachidonic acid is a major precursor of prostaglandins.

Q7. Which of the following is *not* true about fatty acid synthesis
   a. cofactors — NADPH
   b. precursor — two carbon unit
   c. site — cytosol
   d. carrier — carnitine

Q8. Regulation of fatty acid biosynthesis occurs at the enzymatic step catalyzed by
   a. carnitine acyl transferase I
   b. acetyl-CoA carboxylase
   c. pyruvate carboxylase
   d. citrate-malate translocase

Q9. Which of the following is *not* true about beta oxidation of fatty acid containing even number of carbons
   a. end product — acetyl-CoA
   b. cofactor required — NAD^+ and FAD
   c. decreases during starvation
   d. site — mitochondria

Q10. The beta-oxidation of a molecule of palmitic acid
   a. yields 8 molecules of acetyl-CoA
   b. yields 16 molecules of acetyl-CoA only
   c. yields carbon dioxide and water only
   d. does not involve oxygen

Q11. Which of the following is *not* correct about fatty acid degradation and biosynthesis

	Degradation	*Biosynthesis*
a. location of pathway in the cell	mitochondrion	cytosol
b. electron acceptor or donor used	FAD, NAD^+	NADPH
c. participation of CO_2	No	Yes
d. carrier of intermediate acyl groups	ACP-$(SH)_2$	CoA-SH

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Which of the following is *not* true about ketogenesis

a. acetyl-CoA acts as precursor
b. HMG CoA synthase participates during reaction
c. occurs in the liver
d. acetone is the only product

Q13. The physiological roles of bile salts include which of the following

P. they aid in the digestion of lipid
Q. they facilitate the absorption of sugars
R. they facilitate the absorption of lipid
S. they provide a means for excreting cholesterol

a. P and Q
b. P and R
c. Q and S
d. P, Q and S

Q14. If phosphoglyceride A has a higher T_m than phosphoglyceride B, which of the following differences between A and B may exist ? (In each case only one parameter–either chain length or double bonds– is compared)

P. A has shorter fatty acid chains than B
Q. A has longer fatty acid chains than B
R. A has more unsaturated fatty acid chains than B
S. A has more saturated fatty acid chains than B

a. P and Q are correct
b. Only Q is correct
c. Q and S correct
d. R and S are correct

[GATE]

Q15. All of the following statements about acetyl-CoA carboxylase are correct *except*

a. it undergoes protomer-polymer interconversion during its physiological regulation
b. it requires biotin
c. it is inhibited by cAMP-mediated phosphorylation
d. it is activated by both palmitoyl-CoA and citrate

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Chapter - 06

Enzymes and Vitamins

6.1 ENZYMES : GENERAL FEATURES

An enzyme is a biocatalyst that increases the rate of chemical reaction without itself being changed in the overall process. Virtually all cellular reactions or processes are mediated by enzymes. Enzymes have several properties that make them unique.

- Most (but not all) enzymes are proteins.
- Enzymes are highly specific.
- Enzymes exhibit enormous catalytic power. It increases the rate of a reaction by lowering the activation energy. It changes only the rate at which equilibrium is achieved; it has no effect on the position of the equilibrium.

Enzymes can be divided into two general classes : **simple enzymes**, which consist entirely of amino acids and **conjugated enzymes**, contains a non-protein group called a **cofactor**, which is required for biological activity. Removal of the cofactor from a conjugated enzyme produces a simple enzyme, called an *apoenzyme*, which generally is biologically inactive. The complete, biologically active conjugated enzyme (simple enzyme plus cofactor) is called a *holoenzyme*. A cofactor can be linked to the protein portion of the enzyme either covalently or non-covalently. Some cofactors are simple metal ions and other cofactors are complex organic groups, which are also called **coenzymes**. Cofactors which are tightly associated with the protein covalently or non-covalently are called *prosthetic group*.

Table 6.1 : Vitamins and their coenzyme forms

Vitamin	Coenzyme form	Reaction or process promoted
Vitamin B-complex		
Thiamine (B_1)	Thiamine pyrophosphate	Decarboxylation, aldehyde group transfer
Riboflavin (B_2)	FAD and FMN	Redox reaction
Pyridoxine (B_6)	Pyridoxal phosphate	Amino group transfer
Nicotinic acid (niacin)	NAD^+ and $NADP^+$	Redox reaction
Pantothenic acid	Coenzyme A	Acyl group transfer
Biotin	Biocytin	Carboxylation
Folic acid	Tetrahydrofolic acid	One-carbon group transfer
Vitamin B_{12}	Deoxyadenosylcobalamin,	Intramolecular rearrangements
Ascorbic acid (Vitamin C)		Hydroxylation

The vitamins in the human diet that are coenzyme precursors are all water soluble vitamins.

Table 6.2 : Example of some enzymes and their cofactors

Fe^{2+} or Fe^{3+}	Cytochrome oxidase, Catalase, Peroxidase, Xanthine oxidase
Cu^{2+}	Cytochrome oxidase, Lysyl oxidase, Superoxide dismutase
Zn^{2+}	Carbonic anhydrase, Alcohol dehydrogenase, Carboxypeptidase

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Hexokinase, Enolase, Glucose-6-phosphatase
Arginase, Enolase, Pyruvate carboxylase
Pyruvate kinase
Urease
Dinitrogenase, Xanthine oxidase
Glutathione peroxidase

6.2 NAMING AND CLASSIFICATION OF ENZYMES

Many enzymes have common names. For example, trypsin, a proteolytic enzyme, secreted by the pancreas. Common names provide little information about the reactions that enzymes catalyze. Many enzymes are named for their substrates and for the reactions that they catalyze, with the suffix-*ase* added. As for example, ATPase is an enzyme that breaks down ATP, whereas ATP synthase is an enzyme that synthesizes ATP. Because of the confusion that arose from these common names, an International Commission on enzymes was established to create a systematic basis for enzyme nomenclature.

Enzyme Commission has developed a rule for naming enzymes. According to this rule, each enzyme is classified and named according to the type of chemical reaction it catalyzes. The *Enzyme Commission* (EC) has given each enzyme a number with four parts, like EC 2.7.1.2 (Hexokinase). The first three numbers define major *class*, *subclass*, and *sub-subclass*, respectively. The last number is a serial number in the sub-subciass, indicating the order in which each enzyme is added to the list.

Common name and EC numbers of some enzyme

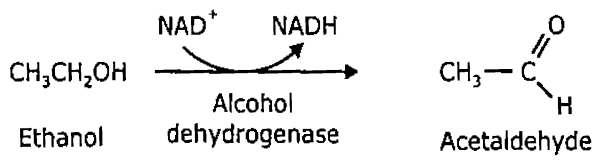
Alcohol dehydrogenase	EC 1.1.1.1
Phosphofructokinase	EC 2.7.1.11
Glutamine synthetase	EC 6.3.1.2
Acetylcholinesterase	EC 3.1.1.7

Systematic classification

The first integer in the EC number designates to which of the six major classes an enzyme belongs. The six major enzyme categories are :

1. **Oxidoreductase**

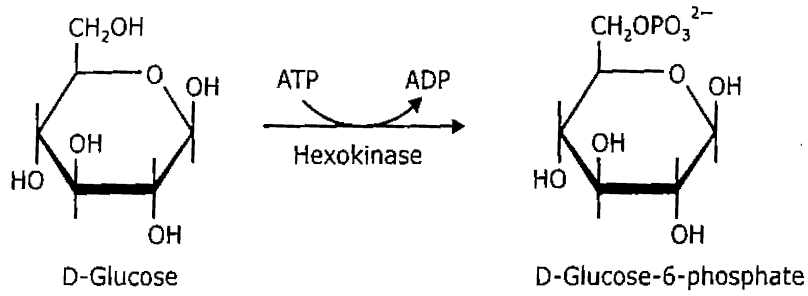
   Oxidoreductase catalyzes oxidation-reduction reactions. Subclasses of this group include the dehydrogenases, oxidases, oxygenases, reductases, peroxidases, and hydroxylases.



2. **Transferases**

   Transferases catalyze reactions that involve the transfer of groups from one molecule to another. Examples of such groups include amino, carboxyl, carbonyl, methyl, phosphoryl and acyl (RC=O). Common trivial names for the transferases often include the prefix *trans*. Examples include the transcarboxylases, transmethylases, and transaminases.

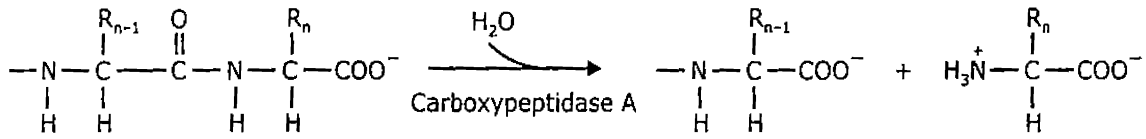
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D-Glucose D-Glucose-6-phosphate

3. **Hydrolases**

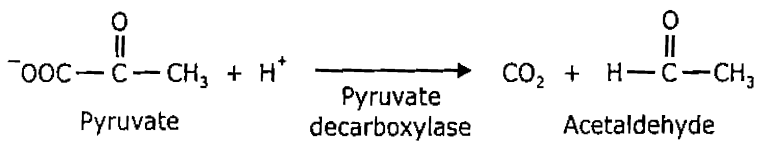
Hydrolases catalyze reactions in which the cleavage of bonds is accomplished by adding water. The hydrolases include the esterases, phosphatases, and peptidases.



C-terminus of polypeptide Shortened polypeptide C-terminal residue

4. **Lyases**

Lyases catalyze reactions in which groups (e.g. H_2O, CO_2, and NH_3) are removed to form a double bond or are added to a double bond. Decarboxylases and synthases are examples of lyases.



Pyruvate Acetaldehyde

5. **Isomerases**

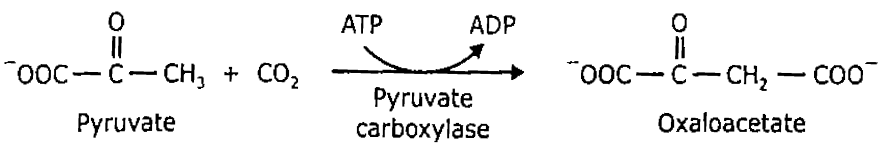
This is a heterogeneous group of enzymes. Isomerases catalyze several types of intramolecular rearrangements and yield isomeric forms. The epimerases catalyze the inversion of asymmetric carbon atoms. Mutases catalyze the intramolecular transfer of functional groups.



Malate Fumarate

6. **Ligases**

Ligases catalyze the formation of C—C, C—S, C—O, and C—N bonds. The energy for these reactions is always supplied by ATP hydrolysis. The names of many ligases include the term synthetase. Several other ligases are called carboxylases.



Pyruvate Oxaloacetate

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WHAT ENZYME DOES?

A chemical reaction between two substances occurs only when an atom, ion, or molecule of one collides with an atom, ion, or molecule of the other. Only a fraction of the total collisions result in a reaction, because usually only a small percentage of the substances interacting have the minimum amount of kinetic energy a molecule must possess for it to react. When the reactants collide, they may form an intermediate product whose chemical energy is higher than the combined chemical energy of the reactants. In order for this transition state in the reaction to be achieved, some energy must enter into the reaction other than the chemical energy of the reactants. The transition state is the one with the highest free energy. The difference in free energy between the transition state and the reactants is called the *Gibbs free energy of activation* or simply the *activation energy*.

An enzyme lowers the activation energy of a reaction, thereby increasing the fraction of molecules that have enough energy to attain the transition state and making the reaction go faster in both directions. In other words, enzymes facilitate the formation of the transition state.

However, the catalyst does not change the relative energies of the initial and final states. The free energy of reaction, $\Delta G°$, remains unchanged in the presence of a catalyst, so the relative amounts of reactants and products at equilibrium are unchanged. In other words, a catalyst does not influence the position of equilibrium. It increases the rate of reaction by lowering the activation energy.

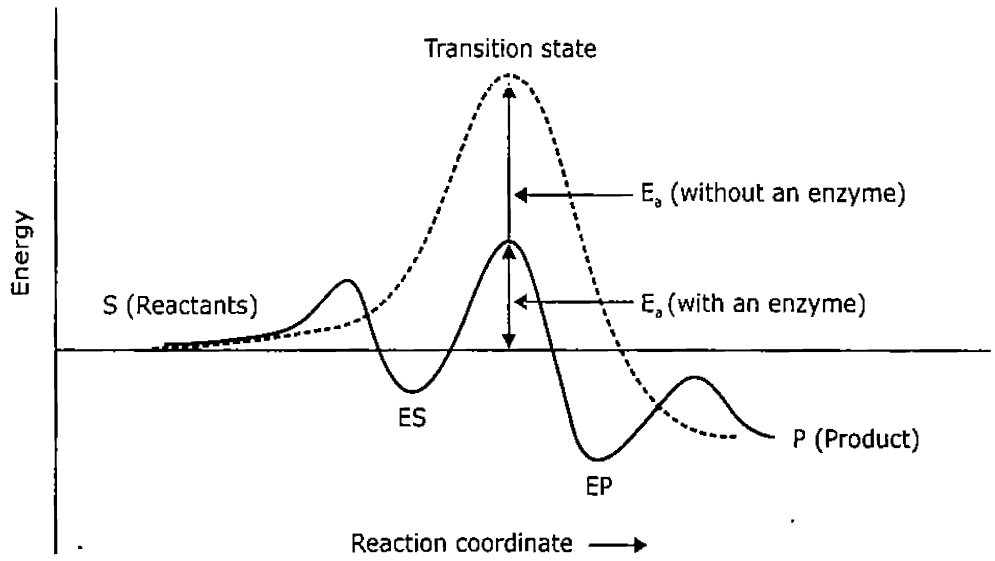


Figure 6.1 : Energy profile of a simple enzyme-catalyzed reaction. The non-enzyme catalyzed reaction proceeds via a higher energy transition state and hence the reaction has a higher activation energy than the enzyme catalyzed reaction.

6.4 HOW ENZYMES OPERATE?

In the first step in catalysis, the enzyme binds to the substrate, the compound to be catalyzed. The substrate binds to the active site of an enzyme by multiple weak non-covalent interactions like hydrophobic, H-bond, ionic interaction or reversible covalent bonds. The free energy released in the formation of a large number of weak interactions between the enzyme and the substrate is termed *binding energy*.

We represent the binding of the substrate to the enzyme by the equilibrium

$$E + S \rightleftharpoons E - S$$

Where E is the enzyme, S is the substrate, and the product E – S is the combined species that is called the *enzyme-substrate complex*. This binding is highly specific. A given enzyme usually binds to only one kind of

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substrate. The specificity of enzyme-substrate interactions arises mainly from hydrogen bonding, which is directional, and the shape of the active site, which rejects molecules that do not have a sufficiently complementary shape. The recognition of substrates by enzymes is accompanied by conformational changes at active sites, and such changes facilitate the formation of the transition state.

In the second step, the substrate is converted into product.

$$E - S \rightleftharpoons E - S$$

Where E – P is a complex of the enzyme and the product.

Enzyme substrate complex

Two important models have been proposed to describe the binding process. The first, the **lock-and-key model**, assumes a high degree of similarity between the shape of the substrate and the geometry of the binding site on the enzyme. Second model takes into account the fact that proteins have some three-dimensional flexibility. According to this **induced-fit model**, the binding of the substrate induces a conformational change in the enzyme that results in a complementary fit once the substrate is bound. The binding site has a different three-dimensional shape before the substrate is bound. When the substrate is bound and the transition state is subsequently formed, the bonds are rearranged. In the transition state, the substrate is bound close to atoms with which it has to react. As bonds are broken and new bonds are formed, the substrate is transformed into product. The product is released from the enzyme.

Catalytic strategies

Enzymes commonly employ one or more strategies to catalyze specific reactions. Among the best characterized mechanisms are *general acid-base catalysis, metal ion catalysis* and *covalent catalysis.* A combination of several catalytic strategies is usually employed by an enzyme. A good example of the use of both covalent catalysis and general acid-base catalysis occurs in chymotrypsin.

1. **Covalent catalysis**

   The process of covalent catalysis involves the formation of a reversible covalent bond between the enzyme and substrate. Although the covalent interactions are temporary, the substrate is bound to the enzyme during the course of catalysis. After the completion of reaction, the enzyme returns to its original unmodified state. Enzymes performing covalent catalysis bear nucleophilic or electrophilic centers. Nucleophilic centers include amines, carboxyl, hydroxyl, imidazole and thiol groups. These groups readily attack electrophilic centers of substrate forming covalent intermediates. Typical electrophilic centers in substrates include phosphoryl groups, acyl groups and glycosyl groups. Covalent electrophilic catalysis is also observed but usually involves coenzyme adducts that generate electrophilic centers. The proteolytic enzyme chymotrypsin provides an excellent example of this mechanism.

2. **General acid-base catalysis**

   In general acid-base catalysis, a molecule other than water plays the role of a proton donor or acceptor. The ionizable functional groups of aminoacyl side chains and of prosthetic groups (if present) contribute to catalysis by acting as acids or bases. For example, chymotrypsin uses a histidine residue as a base catalyst to enhance the nucleophilic power of serine.

3. **Metal ion catalysis**

   Metal ions can function catalytically in several ways. For example, interactions between an enzyme-bound metal and the substrate can help orient a substrate for reaction or stabilize charged reaction transition states. Metals can also mediate oxidation-reduction reactions by reversible changes in the metal ion's oxidation state. A metal ion may serve as an electrophilic catalyst, stabilizing a negative charge on a reaction intermediate.

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6.5 ENZYME KINETICS

Enzyme kinetics is the quantitative study of enzyme catalysis. Kinetic studies measure reaction rates and the affinity of enzymes for substrates and inhibitors. Kinetics also provides insight into reaction mechanisms.

The general principles of chemical reaction apply to enzyme-catalyzed reactions as well. In a chemical reaction of the form A + B → P, where A and B are reactants and P is the product, the rate of reaction can be expressed in terms of either the rate of disappearance of one of the reactants or the rate of appearance of product. The rate of disappearance of A is $-\Delta [A] / \Delta t$, where Δ symbolizes change, [A] is the concentration of A in moles/litre, and t is time. Likewise, the rate of disappearance of B is $-\Delta [B] / \Delta t$, and the rate of appearance of P is $\Delta [P] / \Delta t$. The rate of the reaction can be expressed in terms of any of these changes, because the rates of appearance of product and disappearance of reactant are related by the stoichiometric equation for the reaction.

$$\text{Rate} = -\frac{\Delta[A]}{\Delta t} = -\frac{\Delta[B]}{\Delta t} = \frac{\Delta[P]}{\Delta t}$$

The negative signs for the changes in concentration of A and B indicate that A and B are being used up in the reaction, while P is being produced. It has been established that the rate of a reaction at a given time is proportional to the product of the concentrations of the reactants raised to the appropriate powers.

$$\text{Rate} \propto [A]^f[B]^g$$

or, as an equation,

$$\text{Rate} = k\,[A]^f[B]^g$$

where k is a proportionality constant called the *rate constant*. The exponents *f* and *g must be determined experimentally*. They are *not necessarily* equal to the coefficients of the balanced equation. The exponents in the rate equation are usually small whole numbers such as 1 or 2. (There are also some cases in which the exponent 0 occurs). The values of the exponents are related to the number of molecules involved in the detailed steps that constitute the mechanism. The *overall order* of a reaction is the sum of all the exponents. If, for example, the rate of a reaction A → P is given by the rate equation.

$$\text{Rate} = k[A]^1$$

where k is the rate constant and the exponent for the concentration of A is 1, then the reaction is **first order** with respect to A, and first order overall.

If the rate of a reaction A + B → C + D is given by

$$\text{Rate} = k[A]^1\,[B]^1$$

(where k is the rate constant, the exponent for the concentration of A is 1, and the exponent for the concentration of B is 1), then the reaction is said to be first order with respect to A, first order with respect to B, and **second order** overall.

Many common reactions are first or second order. The possibility exists that exponents in a rate equation may be equal to zero, with the rate for a reaction A → B given by the equation.

$$\text{Rate} = k[A]^0 = k$$

Such a reaction is called zero order, and its rate, which is constant, does not depend on the concentrations of reactants, but depends on other factors such as the presence of catalysts. *Enzyme-catalyzed reactions can exhibit zero-order kinetics when the concentrations of reactants are so high that the enzyme is completely saturated with reactants molecules.*

Measures of enzyme activity

Enzymes are usually present in very small quantities. So, a convenient method of enzyme quantitation is measurement of catalytic activity. Activity is then related to concentration. When enzyme are quantitated relative to their activity rather than a direct measurement of concentration, the unit used to report enzyme levels are *activity units*. There are two standard units to express enzyme activity.

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a. **Enzyme unit** (U) : The amount of enzyme causing transformation of 1 μ mol of substrate per minute at 25°C under optimal conditions of measurement.

b. **Katal** (kat) : The katal is the accepted SI unit of enzyme activity. One katal is that amount of enzyme that catalyzes the transformation of 1 mol of substrate per second.

$$1U = 16.67 \text{ nanokat}$$

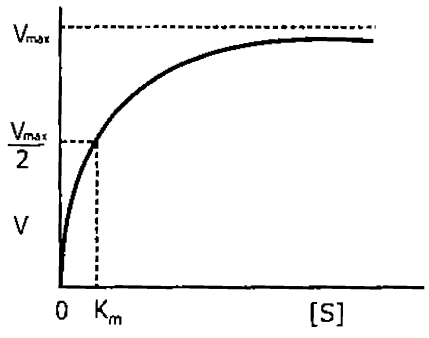
Kinetics of enzyme catalyzed reaction

A particularly useful model for the kinetics of enzyme-catalyzed reactions was devised in 1913 by *Leonor Michaelis* and *Maud Menten* when they studied the reaction in which the enzyme *invertase* hydrolyzes sucrose, a disaccharide, into monosaccharides, glucose and fructose. Michaelis-Menten described the relationship between reaction velocity and substrate concentration. To explain their results on the conversion of sucrose to glucose and fructose by the enzyme invertase, Michaelis and Menten proposed the following scheme of reactions:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \quad (1.1)$$

In this equation k_1 is the rate constant for the formation of the enzyme-substrate complex, ES, from the enzyme, E, and the substrate, S; k_{-1} is the rate constant for the reverse reaction, dissociation of the ES complex to free enzyme and substrate; and k_2 is the rate constant for the conversion of the ES complex to product P and the subsequent release of product from the enzyme.

According to Michalis-Menten approach, when the rate (also called the velocity) of an enzyme catalyzed reaction is measured at varying substrate concentrations, the rate depends on the substrate concentrations [S]. At relatively low concentration of substrate, initial velocity (V) increases almost linearly with increase in a substrate concentration. The reaction reaches a maximum velocity (V_{max}) with increase in substrate concentration and it doesn't increase any further by increasing the concentration of substrate.



The hyperbolic relationship between initial velocity (V) and substrate concentration [S] of an enzyme catalyzed reaction.

Concentration of substrate at which reaction velocity reaches half its maximum velocity is called **Michaelis constant** (k_m). It is the ratio of constants $(k_{-1} + K_2)/k_1$. k_m is expressed as mole of substrate per litre. Lower value of k_m describes the greater affinity of the enzymes for the substrate.

Michaelis and Menten put forward a mathematical equation to establish the mathematical relationship among the quantities [E], [S], V_{max} and k_m.

$$V = \frac{V_{max}\ [S]}{K_m + [S]}$$

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1. When [S] << Km, then V ∝ [S]
2. When [S] >> Km, then V = Vmax
3. When [S] = Km, then $V = \frac{V_{max}}{2}$

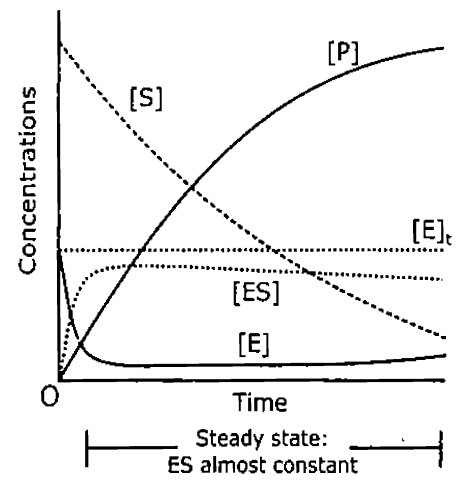
In Michaelis Menten equation, reaction velocity versus substrate concentration shows the *hyperbolic plot*. Michaelis and Menten noted two significant features about the kinetics of this reaction. When the substrate concentration was kept constant and the enzyme concentration was increased, the rate of reaction increased. But when the enzyme concentration was kept constant and the substrate concentration increased, the reaction rate increased to a maximum value and then leveled off. It is caused by the saturation of all the available enzyme binding sites by substrate molecules. Since at low concentration of the substrate, velocity is proportional to substrate concentration [S], the enzyme catalyzed reaction is a *first order reaction*. At high [S], velocity becomes virtually independent of [S] and approaches a maximal limit. Since rate is no longer dependent on [S] at high concentration, the enzyme catalyzed reaction obey *zero order kinetics*.

Michaelis-Menten model is based on the assumptions. The following assumptions are made in driving the Michaelis-Menten rate equation-

1. The concentrations of substrate [S] is much greater than the concentrations of enzyme [E].
2. The rate of formation of ES is equal to that of the breakdown of ES (*the steady state assumption*).
3. Very little accumulation of P, so formation of enzyme-substrate complex from E + P is negligible.

Steady state assumption

The interpretation of Michaelis-Menten model were refined by Briggs and Haldane by an assumption termed the steady state assumption. The steady state hypothesis states that the concentration of enzyme-substrate complex remains constant through much of the reaction. When enzyme and substrate are first mixed, the concentration [ES] will rise rapidly from zero to a so called steady state level as illustrated in the figure below.



For a simple enzyme-catalyzed reaction ($E + S \rightleftharpoons ES \rightarrow E + P$), the graph shows how the concentrations of substrate [S], free enzyme [E], enzyme-substrate complex [ES], and product [P] vary with time. After a very brief initial period, [ES] reaches a steady state in which ES is consumed approximately as rapidly as it is formed. So,

$$\frac{d[ES]}{dt} = 0$$

The amounts of E and ES are greatly exaggerated in the graph for clarity. Note that [E]+[ES]=[E_t], or total enzyme concentration, and that [ES] actually falls very slowly as substrate is consumed, while [E] accordingly rises.

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Significance of K_M and V_{max}

The Michaelis constant, K_M, varies considerably from one enzyme to another, and also with different substrates for the same enzyme. By definition, it is equal to the substrate concentration at half the maximum rate. In another way, K_M represents the substrate concentration at which half the enzyme active sites are filled by substrate molecules. The value of K_M is sometimes equated with the *dissociation constant* (Ks) of the enzyme-substrate complex, ES. However, this is true only when $k_2 << k_{-1}$ so that

$$K_M = \frac{k_{-1}}{k_1} = Ks$$

In general, K_M must be expressed in terms of three rate constants. Nevertheless, K_M (in units of molarity) is customarily reported together with other kinetic parameters for enzyme-catalyzed reactions. To begin with, it is a quantity that can be measured easily and directly. Furthermore, K_M depends on temperature, the nature of the substrate, pH, ionic strength, and other reaction conditions; therefore, its value serves to characterize a particular enzyme-substrate system under specific conditions. Any variation in K_M (for the same enzyme and substrate) is often an indication of the presence of an inhibitor or activator.

The maximum rate, V_{max}, has a well-defined meaning, both theoretically and empirically. It represents the maximum rate attainable; that is, it is the rate at which the total enzyme concentration is present as the enzyme-substrate complex. Mathematically it can be represented as

$$V_{max} = K_2 [E_t] \qquad E_t = \text{Total enzyme concentration}$$

Turn over number (K_{cat})

Turn over number of an enzyme is the number of substrate molecules converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate. The term K_{cat} represent the kinetic efficiency of the enzyme. Its unit is sec^{-1}.

$$\text{At saturating [S]}, \quad V = V_{max} = K_2 [E_t]$$

$$\text{Thus,} \quad K_2 = \frac{V_{max}}{E_t} = K_{cat}$$

Significance of K_{cat} and K_{cat} / K_m

An enzyme with a high K_m requires a higher substrate concentration to achieve a given reaction velocity than an enzyme with a low K_m for the same K_{cat}. K_{cat} is the turnover number for the enzyme. At very low substrate concentrations, where [S] << K_m, most of the enzyme is free. Thus we can think of [E] = [E_0], where E_0 is the total concentration of enzyme. So that the Michaelis-Menten equation becomes

$$V = \frac{K_{cat}}{K_m} [E][S]$$

Thus, the ratio K_{cat} / K_m is equivalent to the rate constant for the reaction between free enzyme and free substrate. K_{cat} / K_m ratio called **specificity constant** is often thought of as a measure of enzyme efficiency. Note that either a large value of K_{cat} (which means rapid turnover) or a small value of Km (which means high affinity for substrate) will make K_{cat} / K_m large. A comparison of K_{cat} / K_m for the same enzyme with different substrates, or for two enzymes with their different substrates, is widely used as a measure of enzyme effectiveness.

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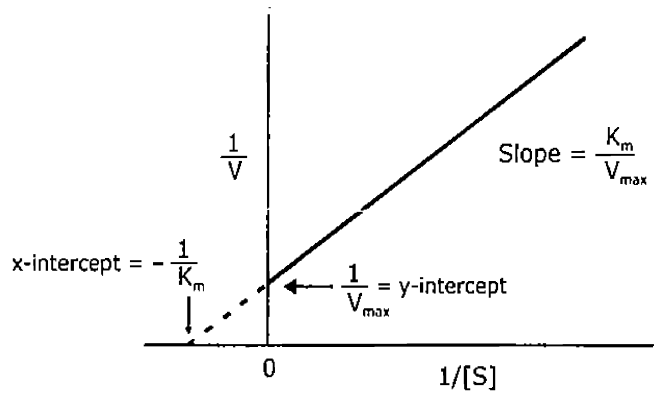
Table 6.3 : Turnover number and specificity constants for some enzymes.

Enzyme	K_{cat}	K_{cat}/K_m
Catalase	4×10^7	4×10^8
Carbonic anhydrase	4×10^5	1.5×10^7
Urease	1.0×10^4	4×10^5

Lineweaver-Burk (or Double reciprocal plot)

It is very difficult to determine the V_{max} directly from a plot of V against [S] and therefore K_m cannot readily be determined in this way either. To overcome these difficulties Michaelis-Menten equation can be rearranged in a number of ways to give convenient graphical representation. If an enzyme obeys Michaelis-Menten kinetics, a plot of the reciprocal of the reaction velocity 1/ V as a function of the reciprocal of the substrate concentration 1/[S] tranforms Michaelis-Menten equation into a straight line equation. Plot between the reciprocal of V and [S] is known as *Lineweaver-Burk* or *double reciprocal plot*.

Lineweaver-Burk equation : $$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$



Lineweaver-Burk graphical procedure for determining the two steady-state kinetic parameters in the Michaelis-Menten equation.

Hanes plot

The Lineweaver-Burk equation may be rearranged to yield the linear equation for the *Hanes plot*.

$$\frac{[S]}{V} = \frac{1}{V_{max}}[S] + \frac{K_m}{V_{max}}$$

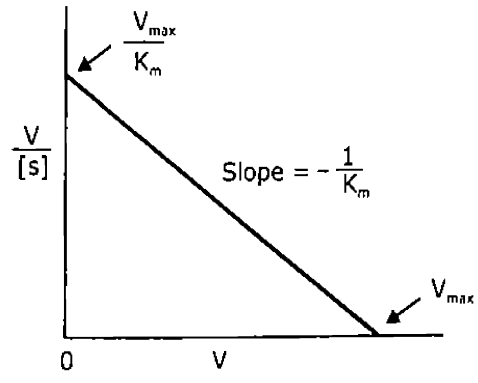
Thus, a plot of [S]/V versus [S] is linear with a slope of $1/V_{max}$. The intercept on the [S]/V axis gives K_m/V_{max}.

Eadie-Hofstee plot

Another widely used linear form of the Michaelis-Menten equation is the *Eadie-Hofstee plot*, which is described by

$$\frac{V}{[S]} = -\frac{V}{K_m} + \frac{V_{max}}{K_m}$$

The plot of V/[S] versus V is linear with slope of $-1/K_m$. The intercept on the v axis gives V_{max}.



An Eadie-Hofstee plot

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EFFECT OF TEMPERATURE AND pH

Effect of temperature

Since most of enzymes are proteins so they unfold and denature at high temperature. This denaturation causes a decrease or loss of catalytic activity, which in turn decreases the rate of reaction. Similarly low temperature also inactivates the enzyme. Within limits, the rate of an enzyme catalyzed reaction increases with increase in temperature because the greater kinetic energy of both enzyme and substrate molecules ensures more frequent collisions. Thus, an enzyme-catalyzed reaction achieves its maximum reaction at an intermediate temperature called *optimum temperature*. The optimum temperature varies greatly from enzyme to enzyme and especially from organism to organism. The reaction rate of most enzymes of homeotherms are maximum at about 40°C. Some enzymes retain activity at unusually high temperature. For example, enzyme like ***Taq* DNA Polymerase** present in thermophilic bacteria *Thermus aquaticus* has optimal temperature 72°C.

Effect of pH

Enzymes are sensitive to pH. This pH dependence is due to the presence of charged amino acids at the active site. Variation in pH cause changes in ovarall charge of amino acids. Thus, each enzyme has its optimal pH at which rapid activity occurs. For example, optimal pH for pepsin (protein digesting enzyme) is 2 and for trypsin it is 8.5. When the activity is plotted against pH, a *bell-shaped curve* is usually obtained.

ENZYME INHIBITION

Inhibition of enzyme activity may be *irreversible* or *reversible*. Irreversible inhibitors usually bound covalently to the enzyme and destroy the functional group in the active site. Most of irreversible inhibitors are toxic substances.

Table 6.4 : Examples of irreversible enzyme inhibitors

Name	*Mode of action*
Cyanide	Reacts with enzyme metal ions (i.e., Fe, Zn, Cu); respiratory chain enzymes are primary targets.
Diisopropyi phosphofluoridate	Inhibits enzymes with active site serine, including acetylcholinesterase.
Sarin (nerve gas)	Like DIPF
Physostigmine	Like DIPF
Parathion (insecticide)	Like DIPF, but especially inhibitory to insect acetylcholinesterase.
Penicillin	Inhibits enzymes responsible for bacterial cell wall synthesis.

In *reversible inhibition*, the inhibitor can dissociate from the enzyme. Reversible inhibitors involve the non-covalent binding with enzymes. Three common types of reversible inhibition are competitive, uncompetitive and noncompetitive inhibition.

1. **Competitive inhibition**

   The structure of a competitive inhibitor closely resembles that of the enzyme's normal substrate. Because of its structure, a competitive inhibitor binds reversibly to the enzyme's active site. The inhibitor forms an enzyme-inhibitor complex (EI) that is equivalent to the ES complex. The effect of a competitive inhibitor on activity is reversed by increasing the concentration of substrate. At high [S], all the active sites are filled with substrate, and reaction velocity reaches the value observed without an inhibitor.

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$$E + S \rightleftharpoons ES \longrightarrow E + P$$
$$+$$
$$I$$
$$\Updownarrow K_I \qquad\qquad K_I = \frac{[E]\,[I]}{[EI]}$$
$$EI$$

In the presence of competitive inhibitor, the Michaelis-Menten equation becomes

$$V = \frac{V_{max}\,[S]}{\alpha K_m + [S]} \qquad \text{where, } \alpha = 1 + \frac{[I]}{K_I}$$

In double-reciprocal form the equation will be

$$\frac{1}{V} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

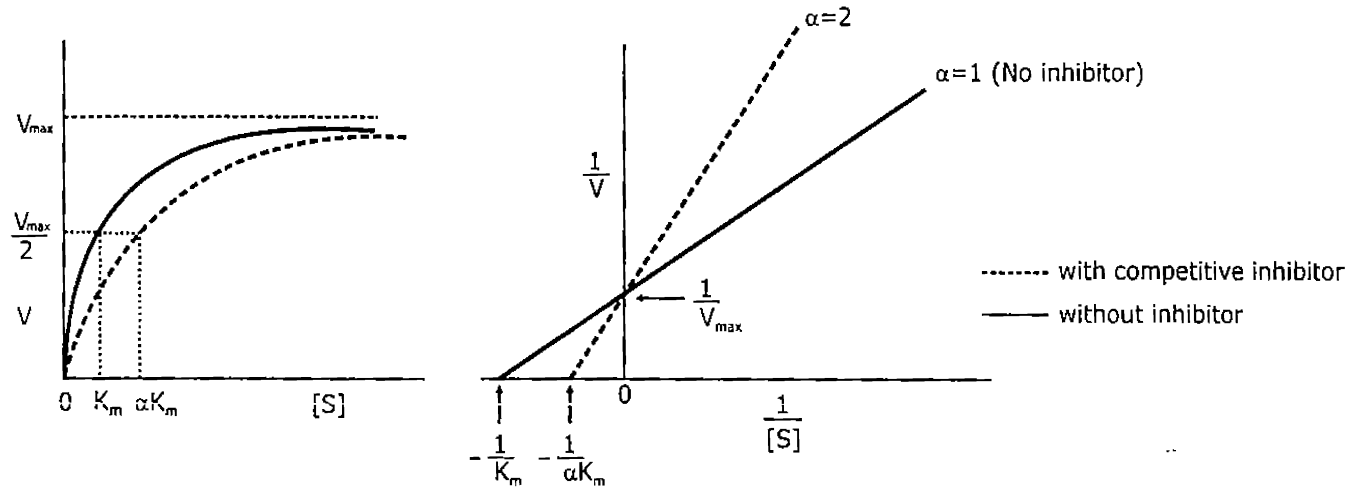


Figure 6.2 : Michaelis-Menten and Lineweaver-Burk plot of uninhibited enzyme activity versus competitive inhibition.

In competitive inhibition, Vmax stays constant and Km increases, but the inhibitor does not affect the turn over number of the enzyme. Clinical treatment of methanol poisoning is a classical example of the exploitation of competitive inhibitory mechanism. In the case of methanol poisoning, methanol in the body is converted to harmful formaldehyde by alcohol dehydrogenase. A high dose of ethanol is used to alleviate the effect of methanol because ethanol competitively binds with the active site of alcohol dehydrogenase.

2. **Non-competitive inhibition**

In non-competitive inhibition (*mixed inhibition*), the inhibitor binds to the enzyme at a site other than the active site. Inhibitor binding alters the enzyme's three-dimensional configuration and blocks the reaction. Inhibitor binds to *either* free enzyme or the ES complex. Noncompetitive inhibition is not reversed by increasing the concentration of substrate. In *non-competitive inhibition, Vmax decreases and Km stays constant.*

$$\begin{array}{ccccc} E & + & S & \rightleftharpoons & ES \longrightarrow E + P \\ + & & & & + \\ I & & & & I \\ \Updownarrow K_I & & & & \Updownarrow K'_I \\ EI & & & & ESI \end{array} \qquad K_I = \frac{[E]\,[I]}{[EI]} \quad \text{and} \quad K'_I = \frac{[ES]\,[I]}{[ESI]}$$

In the presence of non-competitive inhibitor, the Michaelis-Menten equation becomes

$$V = \frac{V_{max}\,[S]}{\alpha K_m + \alpha'\,[S]} \qquad \text{where, } \alpha' = 1 + \frac{[I]}{K'_I} \text{ and } \alpha = 1 + \frac{[I]}{K_I}$$

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In double-reciprocal form the equation will be

$$\frac{1}{V} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}}$$

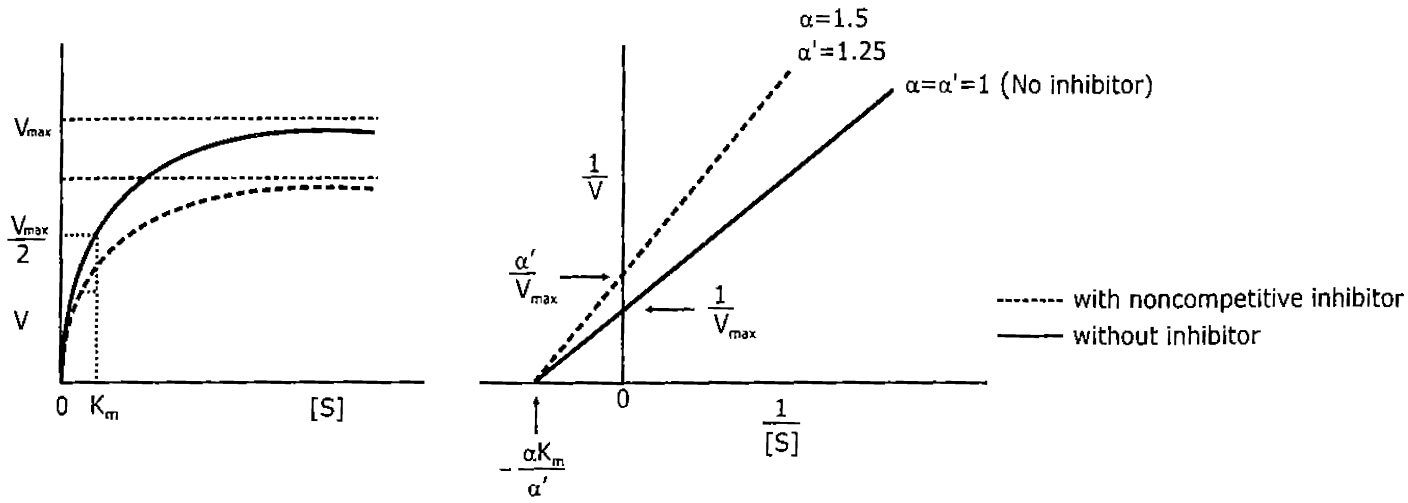


Figure 6.3 : Michaelis-Menten and Lineweaver-Burk plot of uninhibited enzyme activity versus noncompetitive inhibition.

3. Uncompetitive inhibition

In uncompetitive inhibition (rarely encountered) an inhibitor binds at a site distinct from the substrate. However, an uncompetitive inhibitor will bind *only* to the ES complex. On the other hand noncompetitive inhibitor binds to *either* free enzyme or the ES complex. In uncompetitive inhibition, apparent V_{max} and K_m both decreases.

$$E + S \rightleftharpoons ES \longrightarrow E + P$$
$$\quad\quad + I$$
$$\quad\quad \updownarrow K'_I \qquad\qquad K'_I = \frac{[ES]\,[I]}{[ESI]}$$
$$\quad\quad ESI$$

In the presence of uncompetitive inhibitor, the Michaelis-Menten equation becomes

$$V = \frac{V_{max}[S]}{K_m + \alpha'[S]} \qquad \text{where, } \alpha' = 1 + \frac{[I]}{K'_I}$$

In double-reciprocal form the equation will be

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}}$$

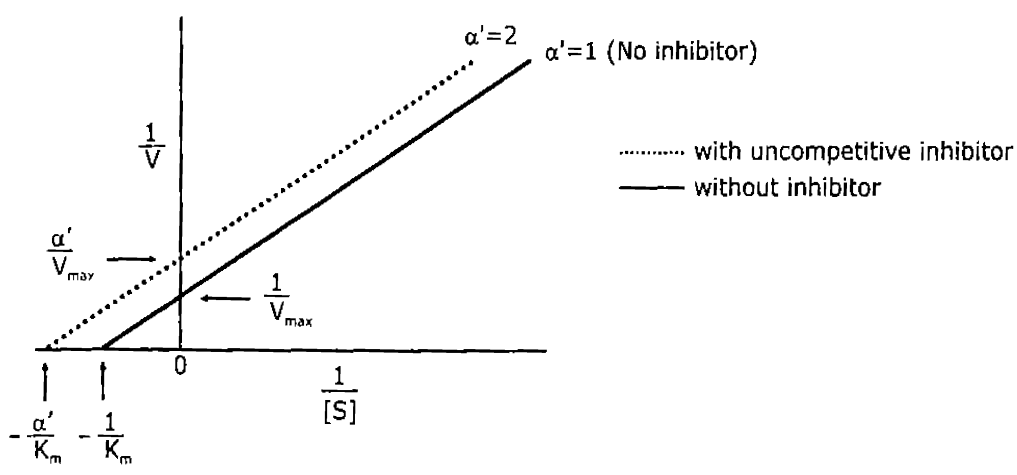


Figure 6.4 : Lineweaver-Burk plot of uninhibited enzyme activity versus uncompetitive inhibition.

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Table 6.5 : Effects of inhibitors on the parameters of the Michaelis-Menten equation

Type of Inhibition	$\mathbf{V_{max}}$	$\mathbf{K_m}$
None	V_{max}	K_m
Competitive	V_{max} (No change)	αK_m (Increased)
Uncompetitive	V_{max}/α' (Decreased)	K_m/α' (Decreased)
Mixed (noncompetitive)	V_{max}/α' (Decreased)	$\alpha k_m/\alpha'$ (No change)

6.6 REGULATORY ENZYMES

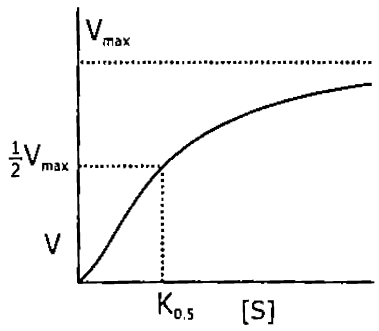
Regulatory enzymes exhibit increased or decreased catalytic activity in response to specific signals. Activities of regulatory enzymes can be modulated through reversible non-covalent binding by regulatory compounds or by reversible covalent modification.

Regulation by reversible non-covalent modification : Allosteric enzymes

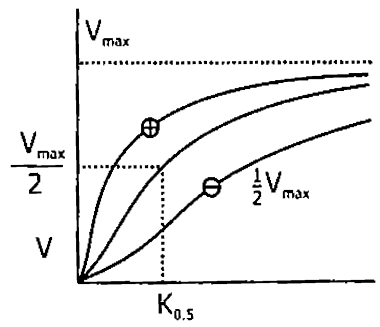
Allosteric enzymes (Greek words: *allo* meaning other and *steric* meaning place or site) are a class of regulatory enzymes which increases or decreases catalytic activities in response to certain signals. Allosteric enzymes function through reversible, noncovalent binding of regulatory compounds called *modulators* or *effectors*. The modulators for allosteric enzymes may be inhibitory or stimulatory. Often the modulator is the substrate itself; regulatory enzymes for which substrate and modulator are identical are called **homotropic**. When the modulator is a molecule other than the substrate, the enzyme is said to be **heterotropic.**

Most allosteric enzymes are oligomeric (consisting of multiple subunits). Allosteric enzymes may have two functionally different binding sites. One of the site, the active site, binds the substrate and catalyzes the reaction. The other type of site, known as *allosteric* or *regulatory site* binds with molecule which is called modulator. Modulator molecules are of two types : positive modulator (called **activators**) that enhance enzyme activity and negative modulator (or **inhibitors**) which inhibit enzyme activity. Binding of modulator causes conformational changes in the allosteric enzymes which influence their catalytic activity. Enzymes with several modulators generally have different specific binding sites for each.

Allosteric enzymes don't obey Michaelis-Menten kinetics. Allosteric enzymes show relationships between V_0 and [s] that differ from Michaelis-Menten kinetics. They do exhibit saturation with the substrate when (S) is sufficiently high, produce a sigmoid saturation rather than the hyperbolic curve typical of non-regulatory enzymes. Sigmoid kinetic behavior generally reflects cooperative interactions between protein subunits. The principles are particularly well illustrated by a nonenzyme; O_2 binding to hemoglobin. Sigmoid kinetic behavior is explained by the *concerted* and *sequential models* for subunit interaction.



The sigmoid curve of a homotropic allosteric enzyme



The effects of a positive modulator (+) and a negative modulator (–) on heterotropic allosteric enzyme. The central curve shows the substrate-activity relationship without a modulator

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Regulation by reversible covalent modification

Activity of some regulatory enzymes is modulated by *reversible covalent modification*. Covalent modification includes phosphorylation, adenylylation, methylation, uridylylation and ADP-ribosylation. Phosphorylation is the most common type of covalent modification. An important example of regulation by phosphorylation occurs in *glycogen phosphorylase* of muscle and liver, which catalyzes the glycogenolysis. Glycogen phosphorylase occurs in two forms : Glycogen phosphorylase *a* (active form) and glycogen phosphorylase *b* (less active form). In glycogen phosphorylase *a*, specific *Ser* residues, one on each subunit, are phosphorylated. Glycogen phosphorylase *a* is converted to the less active glycogen phosphorylase *b* by enzymatic loss of these phosphoryl group. Glycogen phosphorylase *b* can be reconverted to glycogen phosphorylase *a* by the action of phosphorylase kinase.

Table 6.6 : Some enzymes whose activity can be controlled by reversible covalent modification

Enzyme	**Modification**
Glycogen phosphorylase	Phosphorylation
Glycogen synthase	Phosphorylation
Acetyl-CoA carboxylase	Phosphorylation
Glutamine synthetase (*E.coli*)	Adenylylation
Nitrogenase	ADP-ribosylation

6.7 ISOZYMES

Multiple forms of an enzyme that catalyzes the same reaction but differ from each other in their amino acid sequence composition, substrate affinity, V_{max}, and /or regulatory properties are described as *isozymes* (isoenzymes). A common example is Lactate dehydrogenase (LDH) which catalyzes the reversible conversion of pyruvate to lactate. There are five LDH isoenzymes. Each is a tetramer of one or two different types of subunits called H and M. Two different subunits combine randomly with each other forming five isozymes.

H_4	LDH1
H_3M	LDH2
H_2M_2	LDH3
HM_3	LDH4
M_4	LDH5

6.8 ZYMOGEN

An inactive precursor of an enzyme is called *zymogen*. Zymogen is cleaved to form the active enzyme. Many proteolytic enzymes like chymotrypsin, trypsin are initially synthesized as inactive precursor chymotrypsinogen and trypsinogen. Specific cleavage causes conformational changes in the inactive precursor that expose the enzyme active site. In the duodenum, the pancreatic zymogens, *trypsinogen, chymotrypsinogen, proelastase* and *procarboxypeptidase* are converted into active enzymes by enteropeptidase and trypsin, as shown in figure 6.5.

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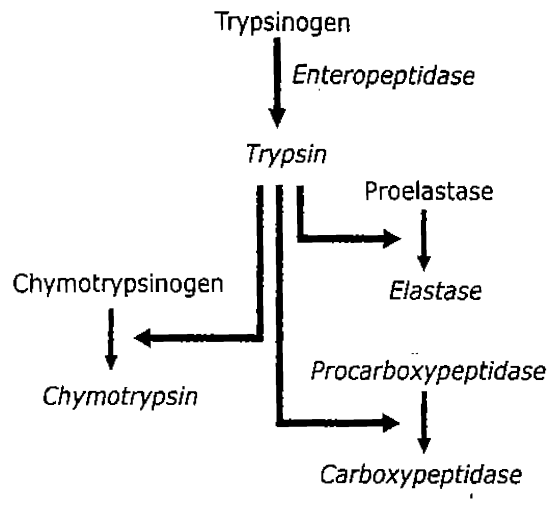


Figure : 6.5
Activation of pancreatic zymogens.

Chymotrypsinogen, a single polypeptide chain of 245 amino acid residues, is converted to alpha-chymotrypsin, which has three polypeptide chains linked by two of the five disulfide bonds present in the primary structure of chymotrypsinogen.

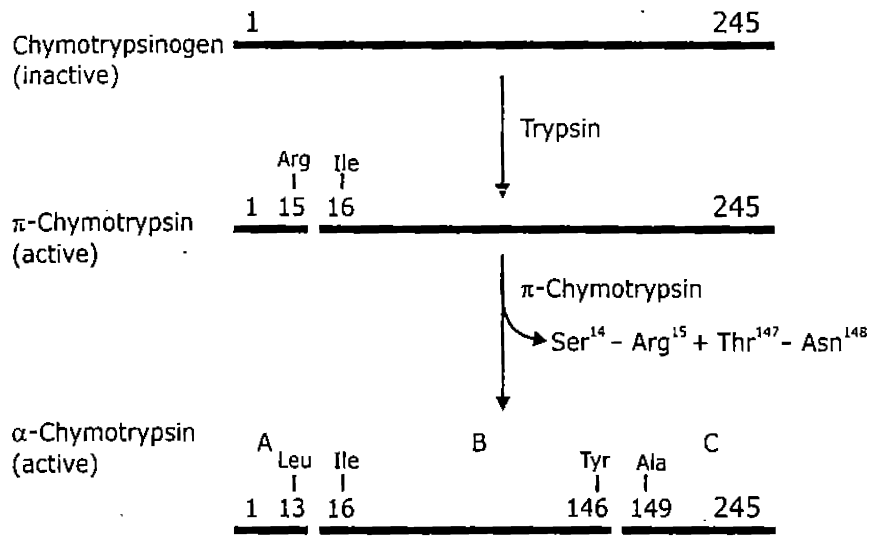


Figure : 6.6
Formation of chymotrypsin (active) from chymotrypsinogen (inactive). The numbering of amino acid residues represents their positions in the primary sequence of the zymogen, chymotrypsinogen. The three polypeptide chains (A, B and C) of chymotrypsin are linked by disulfide bonds.

6.9 RIBOZYME

A ribozyme (from ribonucleic acid enzyme, also called RNA enzyme) is a RNA molecule, that catalyzes a chemical reaction. Before the discovery of ribozymes, proteins were the only known biological catalysts. In 1967, Carl Woese, Francis Crick, and Leslie Orgel were the first to suggest that RNA could act as a catalyst based upon findings that it can form complex secondary structures. The first ribozyme was discovered in the 1980s by Thomas R. Cech, who was studying RNA splicing in the ciliated protozoan *Tetrahymena thermophila*. This ribozyme was found in the intron of an RNA transcript and removed itself from the transcript. Ribozymes often have divalent metal ions such as Mg^{2+} as cofactors. In 1989, Thomas R. Cech and Sydney Altman won the Nobel prize in chemistry for their *discovery of catalytic properties of RNA*.

Although ribozymes are quite rare in the cell, their roles are sometimes essential for life. For example, the functional part of the ribosome, the molecular machine that translates RNA into proteins, is fundamentally a ribozyme. Some known ribozymes include RNase P, Group I and Group II introns, hairpin ribozyme, hammerhead ribozyme, hepatitis delta virus ribozyme, and tetrahymena ribozyme.

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Biochemical reactions catalyzed by ribozymes:

Many natural ribozymes catalyze either their own cleavage or the cleavage of other RNAs, but they have also been found to catalyze the aminotransferase activity of the ribosome. Ribozyme that are known today catalyze two types of biochemical reactions:

1. Cleavage of phosphodiester bonds (RNaseP, group I intron and group II intron)
2. Synthesis of peptide bonds (Peptidyl transferase)

However in the test tube, synthetic RNA molecules have been shown to carry out other biochemical reactions such as synthesis of ribonucleotides and RNA molecules.

6.10 EXAMPLES OF ENZYMATIC REACTIONS

Chymotrypsin

Chymotrypsin is synthesized in the pancreas as a precursor called *chymotrypsinogen* that is enzymatically inactive. The active chymotrypsin generated as a result of cleavage is a three polypeptide molecule interconnected via disulfide bonds. This enzyme catalyzes the hydrolysis of peptide bonds of proteins in the small intestine. It is selective for peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met) on the carboxyl side of this bond. Chymotrypsin also catalyses the hydrolysis of ester bonds.

Chymotrypsin is a **serine protease**. Serine proteases are a class of proteolytic enzymes whose catalytic mechanism is based on an active site serine residue. This family also includes *trypsin*, *elastase*, *thrombin subtilisin*, *acetylcholinesterase* and other related enzymes. Chymotrypsin cleaves peptide bonds by attacking the unreactive carbonyl group with a powerful nucleophile, the Ser^{195} residue located in the active site of the enzyme, which briefly becomes covalently bonded to the substrate, forming an enzyme-substrate intermediate. Treatment with organofluorophosphates such as diisopropylphosphofluoridate (DIPF) inactivates the enzyme irreversibly because DIPF reacts with Ser^{195} present in the active site of enzyme. This *chemical modification reaction* suggested that this unusually reactive serine residue plays a central role in the catalytic mechanism of chymotrypsin. Serine is part of a **catalytic triad** that also includes histidine and aspartic acid. The highly reactive seryl residue, Ser^{195}, participates in a *charge-relay network* with His^{57} and Asp^{102}. The side chain of Ser^{195} is hydrogen bonded to the imidazole ring of His^{57}. The -NH group of this imidazole ring is, in turn, hydrogen bonded to the carboxylate group of Asp^{102}. This catalytic triad act as a **charge relay system.** This grouping has been found in a whole group of enzymes called the serine proteases.

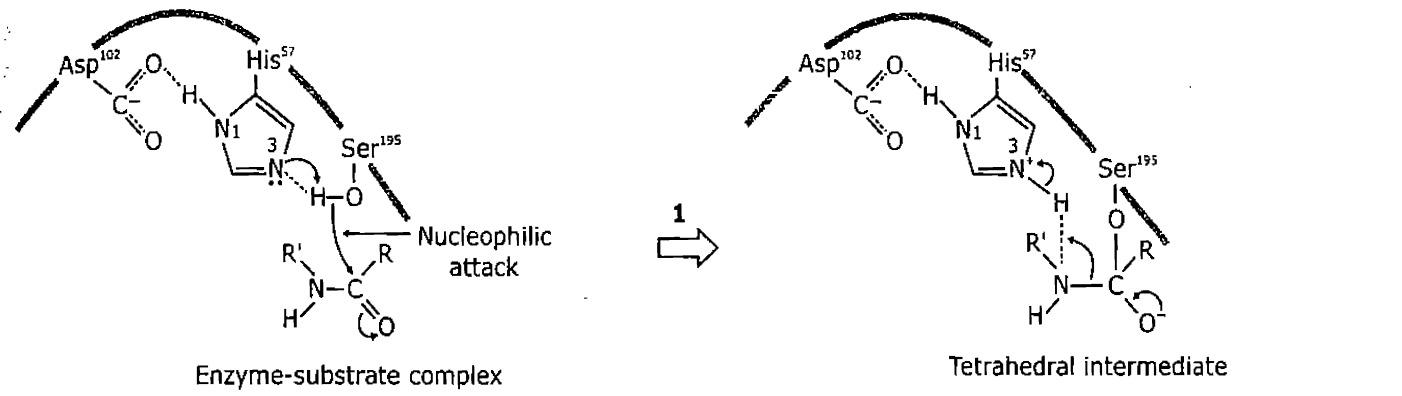
Mechanism of action

Binding of substrate initiates proton shifts that in effect transfer the hydroxyl proton of Ser^{195} to Asp^{102}. The enhanced nucleophilicity of the seryl oxygen facilitates its attack on the carbonyl carbon of the peptide bond of the substrate, forming a covalent acyl-enzyme intermediate. The hydrogen on Asp^{102} then shuttles through His^{57} to the amino group liberated when the peptide bond is cleaved. The portion of the original peptide with a free amino group then leaves the active site and is replaced by a water molecule. The charge-relay network now activates the water molecule by withdrawing a proton through His^{57} to Asp^{102}. The resulting hydroxide ion attacks the acyl-enzyme intermediate and a reverse proton shuttle returns a proton to Ser^{195}, restoring its original state.

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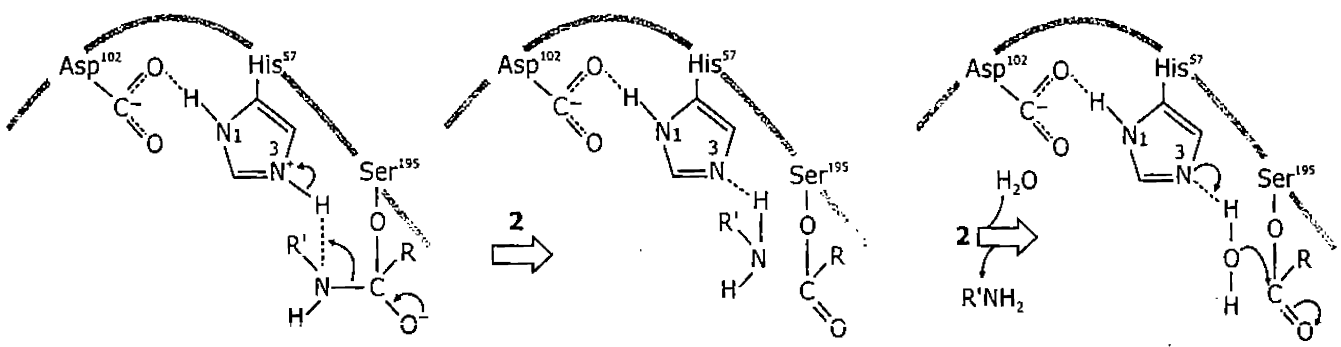
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Catalytic steps :



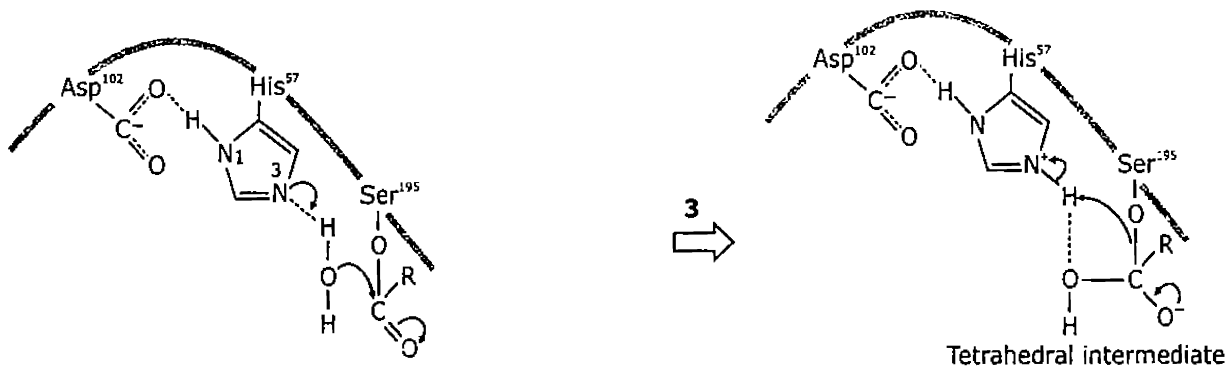
Enzyme-substrate complex — Tetrahedral intermediate

Step 1 : The nucleophilic attack of the active site Ser on the carbonyl carbon atom of the peptide bond to form the tetrahedral intermediate.



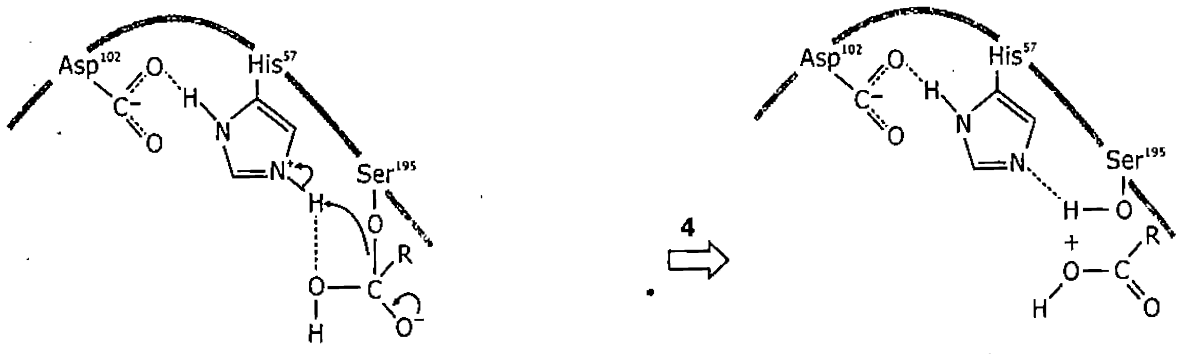
Tetrahedral intermediate — Acyl-enzyme intermediate

Step 2 : The decomposition of the tetrahedral intermediate to the acyl-enzyme intermediate through general acid catalysis by the active site Asp-polarized His, followed by loss of the amine product and its replacement by a water molecule.



Tetrahedral intermediate

Step 3 : The reversal of Step 2 to form a second tetrahedral intermediate.



Tetrahedral intermediate — Active enzyme

Step 4 : The reversal of Step 1 to yield the reaction's carboxyl product and the active enzyme

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RNase A

Ribonucleases or RNases is a group of enzyme that catalyze hydrolysis of phosphodiester bond in RNA molecules. Various types of RNases such as RNase N, RNase II, RNase III, RNase H, RNase A are reported in prokaryotes and eukaryotes. Bovine pancreatic RNase A, the most studied RNase, catalyzes the hydrolysis of phosphodiester bonds (in a ssRNA) between phosphate group of a pyrimidine nucleotide and an adjacent nucleotide to give 3′-phosphate and an RNA fragment with 5′ hydroxyl terminus.

Mechanism of action

The imidazole group of His^{12} acting as a general base catalyst, abstracts a proton from 2′-OH group of sugar, forming a nucleophilic alkoxide anion. This alkoxide anion attacks neighboring phosphorus atom. Cleavage of P-O bond in the substrate 5′-3′ phosphodiester bond yields a cyclic 2′-3′ nucleoside monophosphate intermediate. The released 5′- alkoxide anion picks up a proton from His^{119}. Thus His^{119} acting as general acid catalyst.

The 2′-3′ phosphodiester intermediate hydrolyzes in next step. Role of histidine 119 and 12 are reversed in the hydrolysis reaction. Thus His^{119} whose imidazole group is now unprotonated abstracts a proton from water. The hydroxide ion created in this step attacks the phosphorus atom of the intermediate. The 2′-oxygen, the leaving group in this step retrieves a proton from the imidazolium ion group of His^{12}.

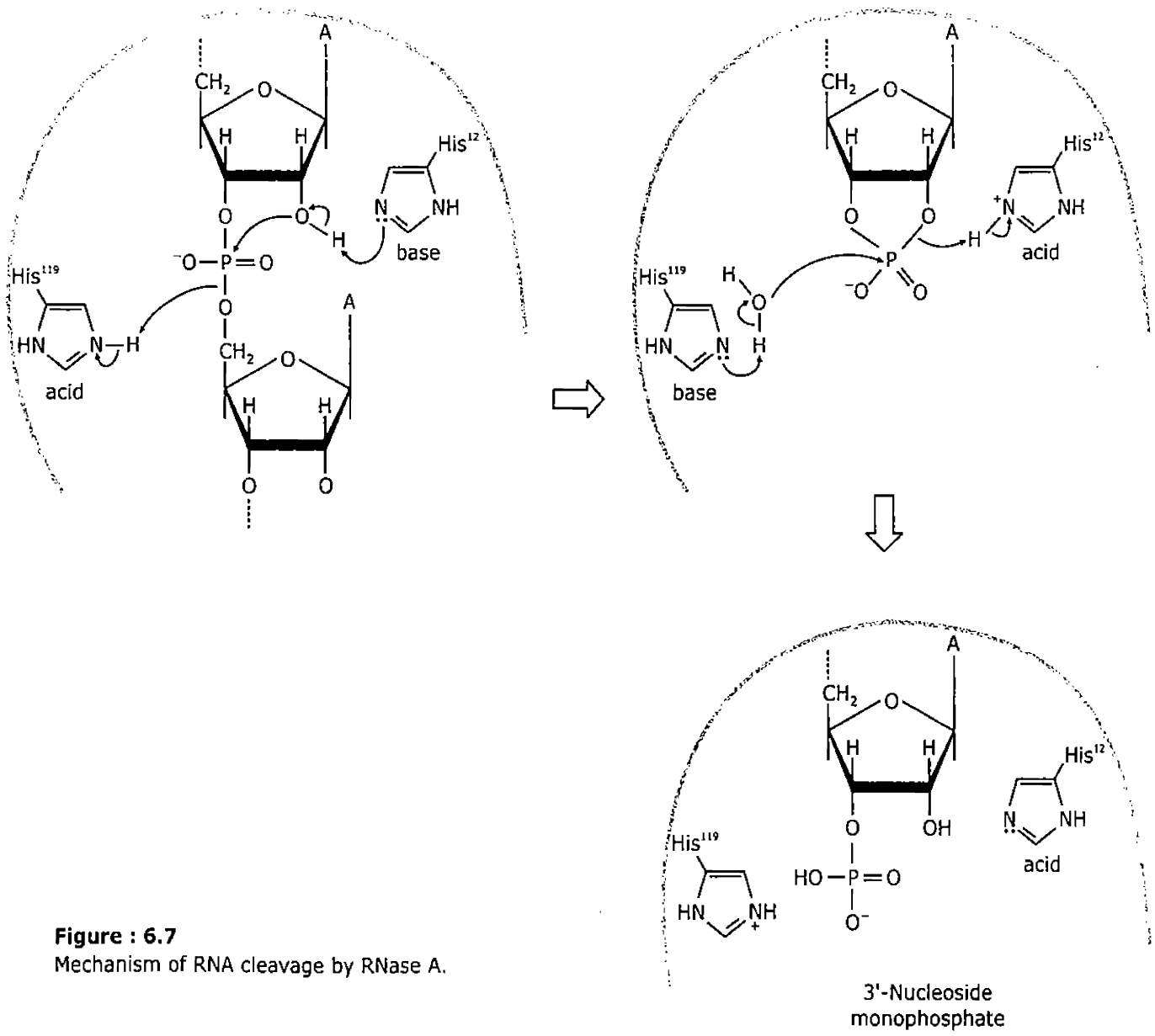


Figure : 6.7
Mechanism of RNA cleavage by RNase A.

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Lysozyme

Lysozyme is an enzyme (EC 3.2.1.17), present in number of secretions such as tears, sweat and also found in egg white. Alexander Fleming, who discovered penicillin, described lysozyme in 1922. Its structure was described by David Chilton Phillips in 1965.

Lysozyme (present in Hen Egg White) is a monomer with 129 amino acid residues. It cleaves the β(1–4) glycosidic bond that connects N-acetyl muramic acid (NAM) with the fourth carbon atom of N-acetylglucosamine (NAG) of peptidoglycan cell wall. It does this by binding to the peptidoglycan molecules in the binding site. The reaction catalyzed by lysozyme is a hydrolysis: a molecule of water is added to a single bond between two adjacent sugar groups in the polysaccharide chain, thereby causing the bond to break. The reaction is energetically favorable because the free energy of the severed polysaccharide chain is lower than the free energy of the intact chain.

The amino acids Glu^{35} and Asp^{52} have been found to be critical to the activity of this enzyme. Glu^{35} acts as a proton donor to the glycosidic bond cleaving the C-O bond in the substrate, whilst Asp^{52} stabilizes the carbonium ion intermediate until it reacts with a free water molecule, extracting a hydroxyl group and freeing a proton that bonds to Glu^{35}, leaving the enzyme unchanged. The following figure 6.8 shows three central steps in this enzymatically catalyzed reaction.

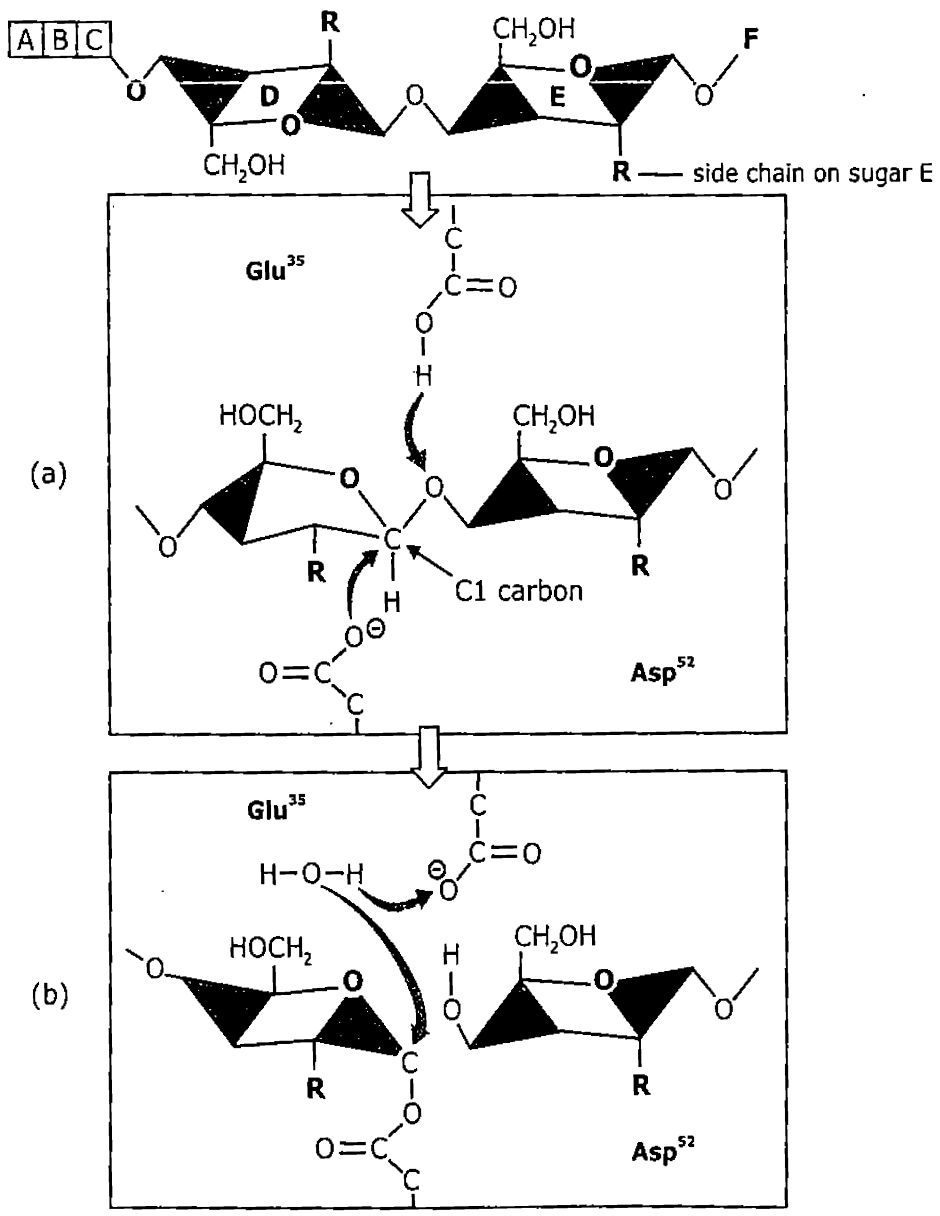


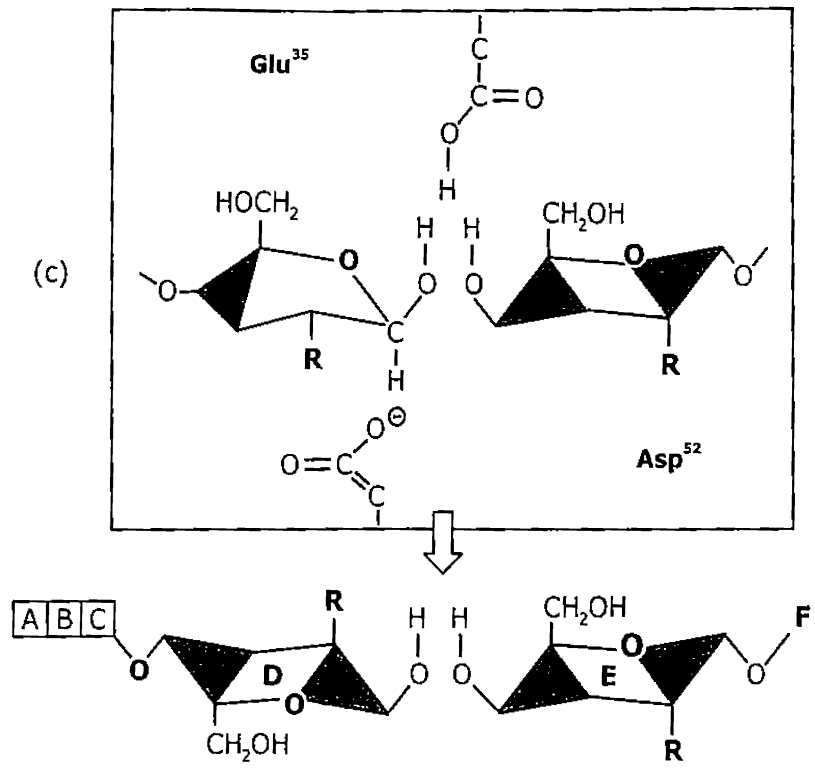
Figure : 6.8
Mechanism of action of lysozyme

(a) In the enzyme-substrate complex (ES), the enzyme forces sugar D into a strained conformation, with Glu^{35} positioned to serve as an acid that attacks the adjacent sugar-sugar bond by donating a proton (H^+) to sugar E, and Asp^{52} poised to attack the C1 carbon atom.

(b) The Asp^{52} has formed a covalent bond between the enzyme and the C1 carbon atom of sugar D. The Glu^{35} then polarizes a water molecule, so that its oxygen can readily attack the C1 carbon atom and displace Asp^{52}.

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(c) The reaction of the water molecule completes the hydrolysis and returns the enzyme to its initial state, forming the final enzyme product complex (EP)



Aspartate proteases

Besides serine proteases, other classes of proteolytic enzyme utilize cysteine (*cysteine proteases*) or aspartate (*aspartate proteases*) to perform their catalytic function. In aspartic proteases, it is actually two aspartic acids that make up the catalytic machinery of the enzyme. The carboxylate groups of these two aspartic acid residues assist in proton transfers from a water molecule that acts as the nucleophile, to attack the peptide bond of the peptide or protein to be cleaved. Aspartic proteases are found in all forms of life. **HIV protease** is a member of the aspartic protease family of proteolytic enzymes. HIV protease plays a critical role in the life cycle of the virus and for this reason has become an important target for drug development in the battle against HIV infection.

6.11 VITAMINS

Vitamins are organic compounds required by the body in trace amounts to perform specific cellular functions. They can be classifieds according to their solubility and their functions in metabolism. The requirement for any given vitamin depends on the organisms. Not all vitamins are required by all organisms. Vitamins are not synthesized by humans, and therefore must be supplied by the diet. Vitamins may be water soluble or fat soluble. Nine vitamins (thiamines, riboflavin, niacin, biotin, pantothenic acid, folic acid, cobalamin, pyridoxine, and ascorbic acid) are classified as water soluble, whereas four vitamins (vitamins A, D, E and K) are termed fat-soluble. Except for vitamin C, the water soluble vitamins are all precursors of coenzymes.

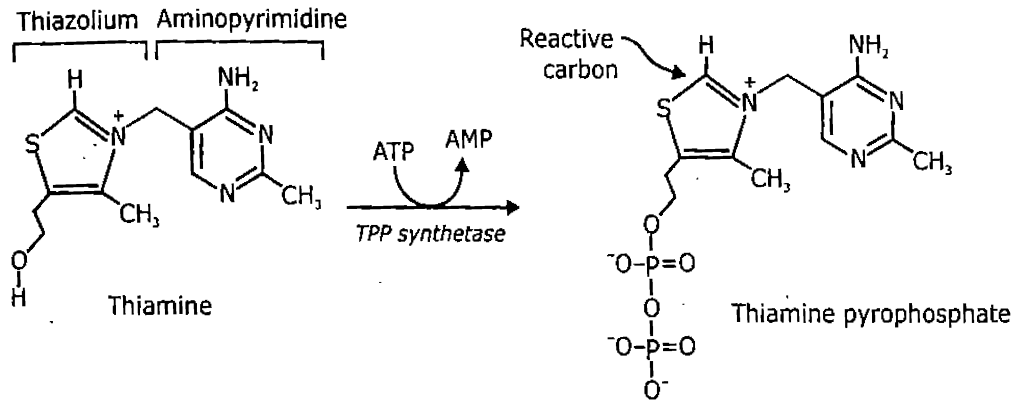
Water-soluble vitamins

Thiamine (vitamin B_1)

Thiamine pyrophosphate (TPP) is the biologically active form of the vitamin, formed by the transfer of a pyrophosphate group from ATP to thiamine. Thiamine is composed of substituted *thiazole ring* joined to a substituted *pyrimidine* by a methylene bridge.

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TPP serves as a coenzyme in the oxidative decarboxylation of α-keto acid, and in the formation or degradation of α-ketols (hydroxy ketones) by transketolase.

$$\text{Pyruvate } (\alpha-\text{keto acid}) \xrightarrow{\textit{Pyruvate decarboxylase}} \text{Acetaldehyde} + CO_2$$

$$\text{Xylulose-5-P} + \text{Ribose-5-P} \xrightleftharpoons{\textit{Transketolase}} \text{Glyceraldehyde-3-P} + \text{Sedoheptulose-7-P}$$

Beri-Beri is a severe thiamine-deficiency syndrome found in areas where polished rice is the major component of the diet.

Riboflavin (vitamin B_2)

Riboflavin is a constituent of flavin mononucleotide (**FMN**) and flavin adenine dinucleotide (**FAD**). FMN is synthesized after the addition of phosphate in riboflavin and FAD formed by the transfer of an AMP moiety from ATP to FMN. FMN and FAD are each capable of reversibly accepting two hydrogen atoms, forming $FMNH_2$ or $FADH_2$. The oxidized form of the isoalloxazine structure absorbs light around 450 nm. The colour is lost, when the ring is reduced.

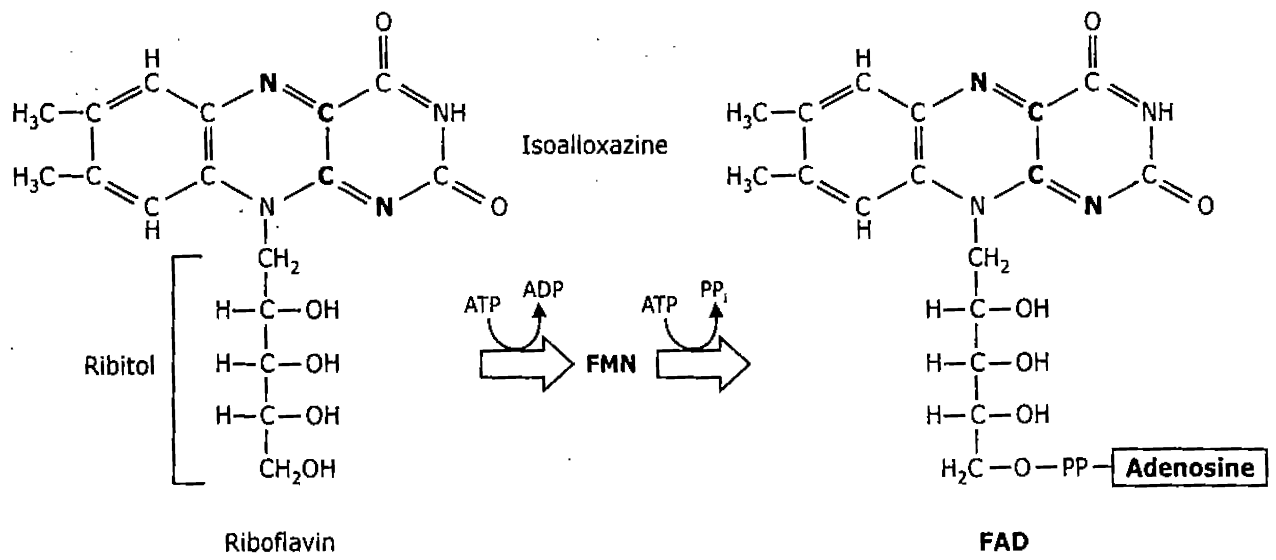
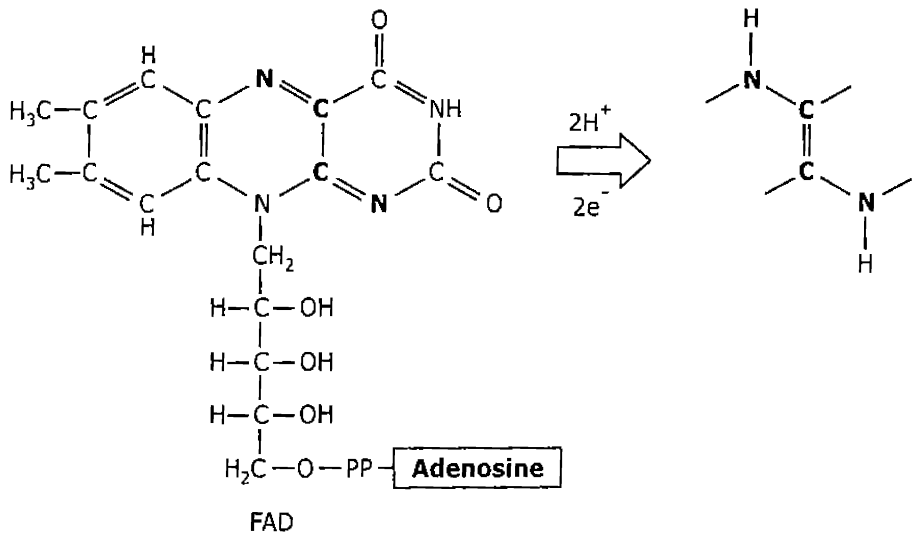


Figure 6.9 : Structure and biosynthesis of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).

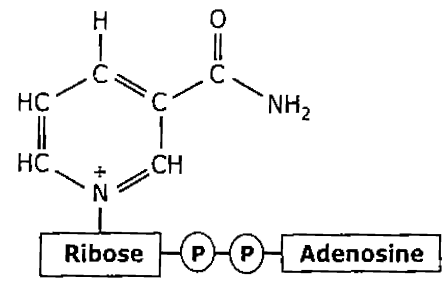
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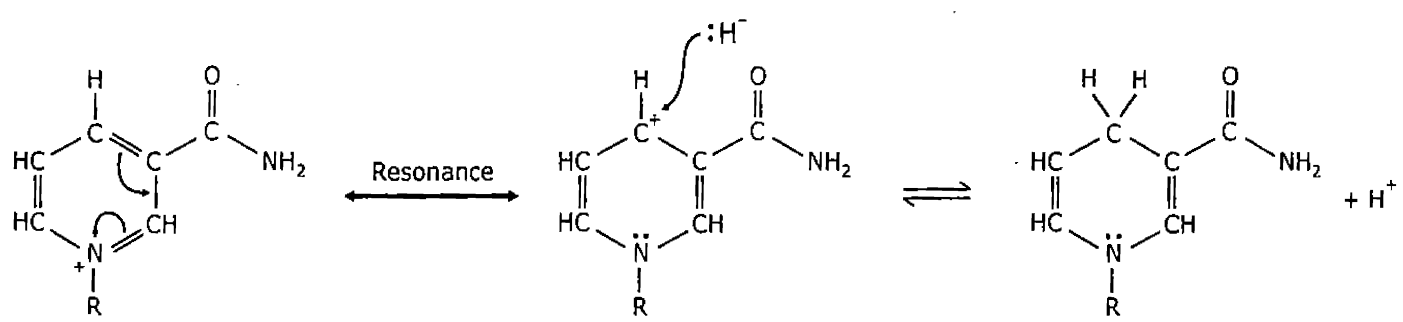
FAD

Niacin

Niacin, or nicotinic acid, is a substituted pyridine derivative. The biologically active coenzyme forms are nicotinamide adenine dinucleotide (**NAD**$^+$) and its phosphorylated derivative, nicotinamide adenine dinucleotide phosphate (**NADP**$^+$). Nicotinamide, a derivative of nicotinic acid that contains an amide instead of a carboxyl group. NAD^+ and $NADP^+$ serve as coenzymes in oxidation-reduction reactions in which the coenzyme undergoes reduction of the pyridine ring by accepting a hydride ion (H^-). The reduced forms of NAD^+ and $NADP^+$ are NADH and NADPH, respectively.



(A)



NAD^+ (oxidized) NADH (reduced)

(B)

Figure 6.10 : (A) Structure of NAD^+ (B) The role of the nicotinamide ring in oxidation-reduction reactions. R is the rest of the molecule. In reactions of this sort, an H^+ is transferred along with the two electrons.

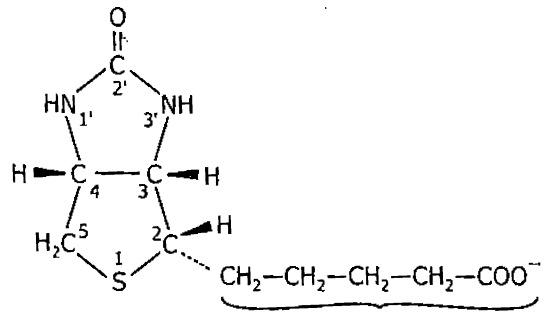
Deficiency of niacin causes **pellagra**, a disease involving the skin and central nervous system. The symptoms of pellagra progress through the three Ds: Dermatitis, Diarrhoea, Dementia, and, if untreated, death.

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Biotin

Biotin is a coenzyme in carboxylation reactions, in which it serves as a mobile carboxyl group carrier. Biotin is covalently bound to the enzyme by an amide linkage between the carboxyl group of its valerate side chain and the ε-amino group of an enzyme *Lys* residue of form a **biocytin** (alternatively, *biotinyllysine*) residues.



Structure of Biotin Valerate side chain

Most biotin-dependent carboxylations use bicarbonate as the carboxylating agent and transfer the carboxyl group to a substrate carbanion. Example of some important biotin-dependent carboxylations are given below.

$$ATP + HCO_3^- + \text{Pyruvate} \xrightarrow{\textit{Pyruvate carboxylase}} \text{Oxaloacetate} + ADP$$

$$ATP + HCO_3^- + \text{Acetyl-CoA} \xrightarrow{\textit{Acetyl-CoA carboxylase}} \text{Malonyl-CoA} + ADP$$

$$ATP + HCO_3^- + \text{Propionyl-CoA} \xrightarrow{\textit{Propionyl-CoA carboxylase}} \text{Methylmalonyl-CoA} + ADP$$

Biotin deficiency does not occur naturally because the vitamin is widely distributed in food. Raw egg white contains a glycoprotein, **avidin**, which tightly binds biotin and prevents its absorption from the intestine. The avidin homolog **streptavidin**, which is secreted by *Streptomyces avidinii*, also has high affinity for biotin.

Pantothenic acid

Pantothenic acid is a component of **coenzyme A**, which is responsible for the transfer of acyl groups. Coenzyme A contains a thiol group that carries acyl compounds as activated thiol esters. Pantothenic acid is also a constituent of *acyl carrier protein* (ACP). Coenzyme A performs two main functions:

- Activation of acyl groups for transfer by nucleophilic attack.
- Activation of the α-hydrogen of the acyl group.

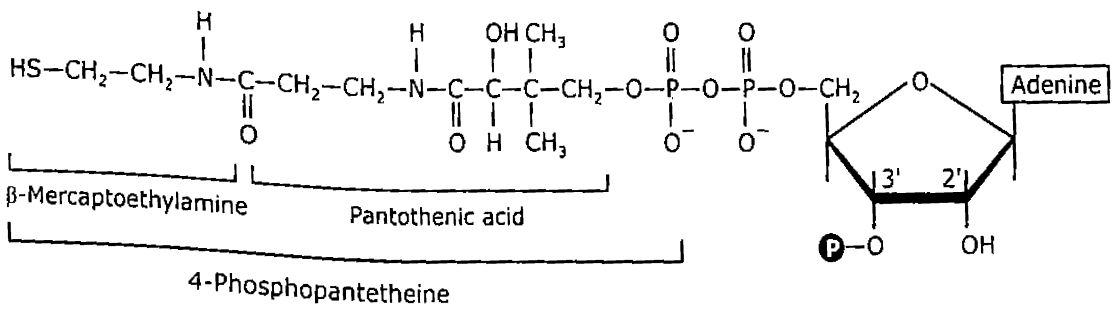


Figure 6.11 : Structure of coenzyme A (CoA-SH)

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Folic acid

Folic acid is composed of a *pterin ring* attached to *p*-aminobenzoic acid (PABA) and conjugated with one or more glutamic acid residues. The biologically active form of folic acid is **tetrahydrofolic acid** (THF). The NADPH-dependent enzyme *dihydrofolate reductase* catalyzes reduction of the 5, 6, 7 and 8 positions of the pterin ring to produce the coenzyme tetrahydrofolate (FH_4). Folic acid (or folate) plays a key role in transfer of one-carbon units for all oxidation levels of carbon except that of CO_2, and is essential for the biosynthesis of certain amino acids, the purines and thymidylate. The one-carbon transfer reaction of FH_4 involve the formation of a covalent bond between N-5 or N-10, or both, of FH_4 and a one carbon unit.

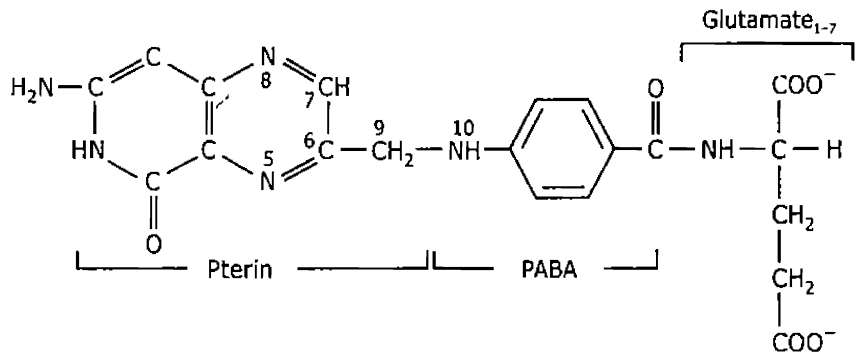
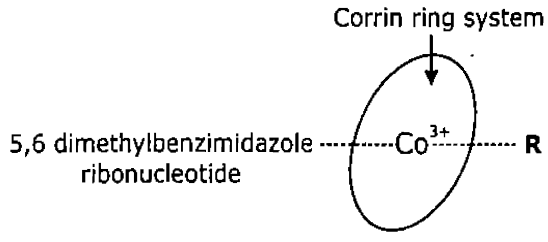


Figure 6.12 : Structure of folate

A primary result of folic acid deficiency is **megaloblastic anemia,** caused by diminished synthesis of purines and thymidine leading to an inability of cells to make DNA and to divide.

Cobalamin (vitamin B_{12})

Cobalamin has two characteristic components: the **corrin ring** system and the 5,6-dimethylbenzimidazole ribonucleotide. Corrin ring system differs from the porphyrins. In corrin ring system, two pyrrole rings are linked directly rather than through a methene bridge. Second the metal that is coordinated by the four pyrrole nitrogens is cobalt rather than iron or magnesium. 5,6-dimethylbenzimidazole ribonucleotide, the second component of vitamin B_{12} is coordinated with cobalt of the corrin ring. Several groups like –CN, –OH, $-CH_3$, deoxyadenosyl group can also bind to the cobalt ion. Cobalamin is converted in the body into two coenzymes : 5′-deoxyadenosylcobalamin, commonly referred to as coenzyme B_{12} and methylcobalamin.



Structure of vitamin B_{12}

— R	*Name*
— CN	Cyanocobalamin
— OH	Hydroxycobalamin
— CH_3	Methylcobalamin
— Deoxyadenosyl	Deoxyadenosylcobalamin

The B_{12} coenzymes participate in two important types of reactions :

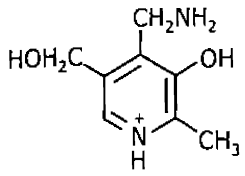
1. Intramolecular rearrangements (isomerization of methylmalonyl CoA)
2. Methyl group transfers (the synthesis of methionine)

Pyridoxine (vitamin B_6)

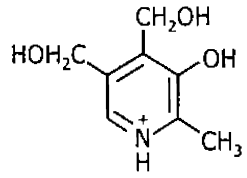
Vitamin B_6 is a collective term for *pyridoxine, pyridoxal* and *pyridoxamine* all derivatives of pyridine, differing only in the nature of the functional group attached to the ring. Pyridoxine occurs primarily in plants, whereas pyridoxal and pyridoxamine are found in foods obtained from animals. All three compounds can serve as precursors of the biologically active coenzyme, **pyridoxal phosphate.**

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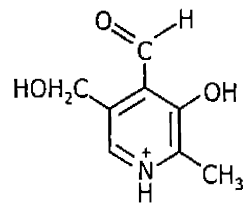
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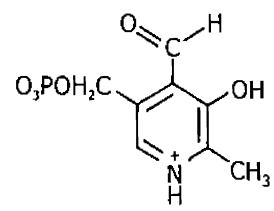
Pyridoxamine



Pyridoxine

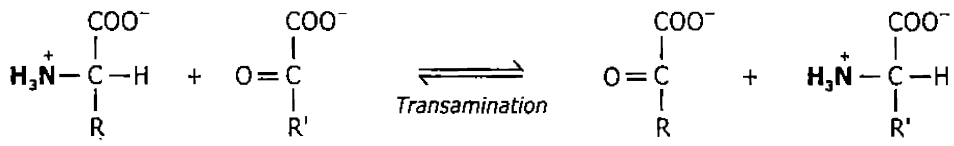


Pyridoxal



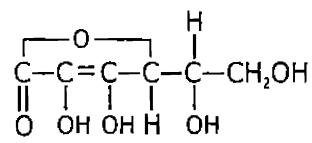
Pyridoxal phosphate

Pyridoxal phosphate participates in the wide variety of enzyme catalyzed reactions including transamination, α and β-decarboxylation, aldol reactions, and β and γ-elimination.



Ascorbic acid (vitamin C)

Ascorbic acid is a strong reducing agent. It is widely distributed in animals and plants. Vertebrates such as human and other primates, certain birds, and some fishes are unable to synthesize it due to lack of enzyme L-*gulono*-γ *lactone oxidase*. Vitamin C has a well documented role as a coenzyme in hydroxylation reactions, for example, hydroxylation of prolyl- and lysyl- residues of collagen. It acts as an important **antioxidant** by virtue of its water solubility and its oxidation to dehydroascorbic acid. A deficiency of ascorbic acid results in **scurvy**, a disease characterized by sore, spongy gums, loose teeth, fragile blood vessels, swollen joints, and anemia. Many of the deficiency symptoms can be explained by a deficiency in the hydroxylation of collagen, resulting in defective connective tissue.



Ascorbic acid

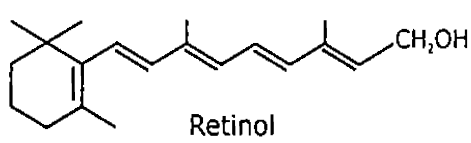
FAT-SOLUBLE VITAMINS

Four vitamins A, D, E and K are termed *fat-soluble*. All are isoprenoid compounds synthesized by the condensation of multiple isoprene unit. In contrast to the water-soluble vitamins, only one of the fat soluble vitamins (vitamin K) has a coenzyme function. These vitamins are released, absorbed, and transported with the fat of the diet.

Vitamin A (retinol)

Vitamin A is an isoprenoid alcohol and functions as hormone as well as visual pigment. It is essential for vision, reproduction, growth, and maintenance of epithelial tissues. *Vitamin A* is often used as collective term for several related biologically active molecules.

Retinol (vitamin A_1) is a primary alcohol containing a β-ionone ring with an unsaturated side chain. Retinol is found in animal tissues as a retinyl ester with long-chain fatty acids.

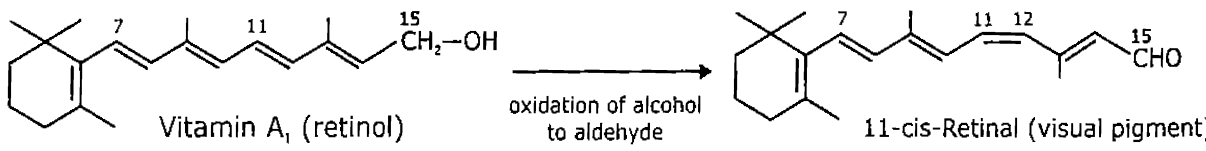


Retinol

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β-carotene is the precursor of vitamin A_1. Plant foods contain β-carotene, which can be oxidatively cleaved in the intestine to yield two molecules of retinol (Vitamin A_1).

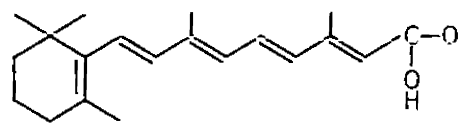
Retinal, a vitamin A derivative, derives from the oxidation of retinol. Oxidation at C-15 convert retinol to the aldehyde, retinal. Retinal and retinol can be readily interconverted. It initiates the response of rod and cone cells of retina to light, producing a neural signal to the brain.



Vitamin A_1 (retinol) → 11-cis-Retinal (visual pigment)

Retinal is a component of the visual pigments of rod and cone cells. **Rhodopsin**, the visual pigment of the rod cells in the retina, consists of *11-cis retinal* specifically bound to the protein **opsin**. In the dark, retinal of rhodopsin is in the *11-cis form*. When rhodopsin is exposed to light, *11-cis retinal* undergoes a series of photochemical reactions that converts it to all trans retinal and release of *all trans retinal* and opsin. This process triggers a nerve impulse that is transmitted by the optic nerve to the brain. Regeneration of rhodopsin requires isomerization of *all trans retinal* back to *11-cis retinal*.

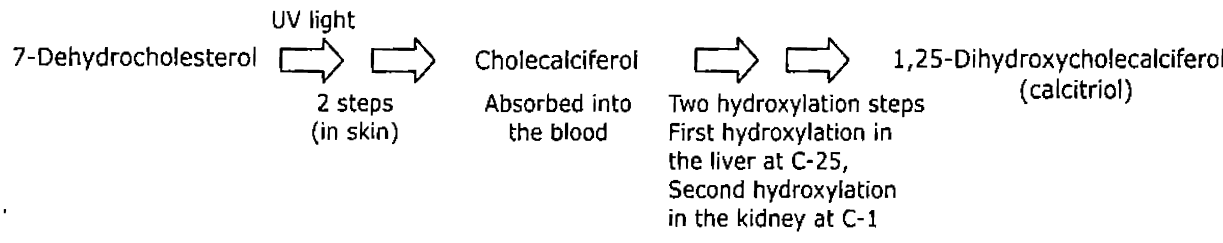
Retinoic acid is a vitamin A derivative, derives from the oxidation of retinal. Retinoic acid cannot be reduced in the body and therefore cannot give rise to either retinal or retinol. It regulates gene expression like steroid hormone.



Structure of Retinoic acid (all-trans)

Vitamin D

Vitamin D serves as hormone precursors. Vitamin D_3 (also called cholecalciferol, calciol) is formed non-enzymatically in skin (dermis and epidermis) from 7-dehydrocholesterol (an intermediate in cholesterol synthesis) in the presence of UV component of sun light. Dietary form of vitamin D are absorbed through the aid of bile salts in the small intestine. Whether absorbed in the intestine or photosynthesized in the skin, cholecalciferol is then transported to then liver by **tanscalciferin**. Vitamin D_3 (not biologically active) converted by enzymes in the liver and kidney to 1, 25 dihydroxycholecalciferol, a hormone that regulates Ca^{2+} uptake in the intestine and calcium levels in kidney and bone. Like steroid hormone 1, 25 dihydroxycholecalciferol, regulates gene expression.

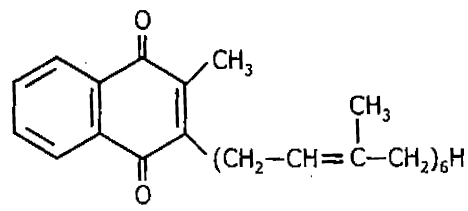


Vitamin D_2 (ergocalciferol) is formed by UV irradiation of the ergosterol. Vitamin D deficiency causes a net demineralization of bone resulting in *rickets* in children and *osteomalacia* in adults.

Vitamin K

The principal role of vitamin K is in the post-translational modification of various blood clotting factors. Vitamin K exists in several forms, for example, in plants as phylloquinone (or vitamin K_1), and in intestinal bacterial flora as menaquinone (or vitamin K_2).

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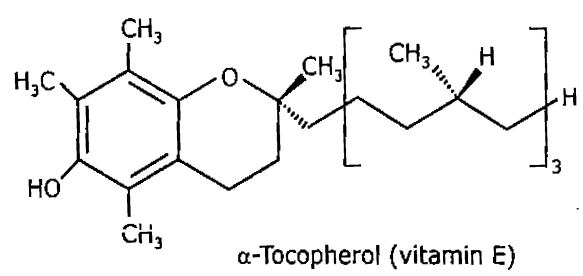


Vitamin K

Warfarin, a structural analog of vitamin K, is a widely used rat poison that inhibits the vitamin K-dependent carboxylation of prothrombin. Warfarin is also clinically used as an anticoagulant. *Dicoumarol* is an other example of anticoagulant.

Vitamin E

The E vitamins consist of eight naturally occurring tocopherols, of which **α-tocopherol** is the most common. The primary function of vitamin E is as an *antioxidant* in prevention of the nonenzymatic oxidation of cell components by molecular oxygen and free radicals. The mechanism of action of vitamin E remain obscure.



α-Tocopherol (vitamin E)

Table 6.7 : Vitamins and their role

Vitamin	**Action**
Water soluble	
Thiamine (Vitamin B_1)	Cofactor in decarboxylations.
Riboflavin (Vitamin B_2)	Constituent of flavoproteins.
Niacin	Constituent of NAD^+ and $NADP^+$.
Pyridoxine (Vitamin B_6)	Forms prosthetic group of certain decarboxylases and transaminases. Converted in body into pyridoxal phosphate and pyridoxamine phosphate.
Pantothenic acid	Constituent of CoA
Biotin	Catalyzes CO_2 "fixation" (in fatty acid synthesis, etc).
Folates (folic acid)	Coenzymes for "1-carbon" transfer; involved in methylating reactions.
Cyanocobalamin (Vitamin B_{12})	Coenzyme in amino acid metabolism.
C	Maintains prosthetic metal ions in their reduced form; scavenges free radicals.
Fat soluble	
A (retinol)	Constituents of visual pigments necessary for fetal development and for cell development throughout life.
D group	Increase intestinal absorption of calcium and phosphate.
E group	Antioxidants; cofactors in electron transport in cytrochrome chain.
K group	Catalyze γ-carboxylation of glutamic acid residues on various proteins concerned with blood clotting.

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Table 6.8 : Vitamins and associated human disease

Vitamin	**Human disease**
Biotin	a
Cobalamin (B_{12})	Pernicious anemia
Folic acid	Megaloblastic anemia
Nicotinamide	Pellagra
Pantothenate	a
Pyridoxine (B_6)	a
Riboflavin (B_2)	a
Thiamine (B_1)	Beri-beri

a–No specific name; deficiency in humans is rare or unobserved.

SELF TEST

Q1. Coenzymes and prosthetic groups are found in all the key metabolic pathways. Which one of the following statements is accurate about these key molecules?

a. carbonic anhydrase contains a tightly bound zinc ion that is essential for its structure but not for its activity

b. some enzymes contain organic molecules called prosthetic groups that are essential for activity; prosthetic groups are vitamins and cannot be synthesised in the body

c. coenzymes are small polypeptides that are involved in transfer reactions catalyzed by enzymes

d. coenzymes carry acyl or phosphate groups in many enzyme-catalyzed reactions that involve the transfer of these groups

Q2. Prosthetic groups are

a. required by all enzymes in the cell

b. loosely bound to enzymes via hydrogen bonds

c. sites on the enzyme molecule that permit allosteric modification of enzyme activity

d. tightly bound to enzymes and are required for their activity

Q3. Metal cations may do all of the following *except*

a. donate electron pairs to functional groups found in the primary structure of the enzyme protein

b. serve of Lewis acids in enzyme

c. participate in oxidation-reduction processes

d. stabilize the active conformation of an enzyme

Q4. The steady-state hypothesis in enzyme kinetics states that

a. there is an equilibrium established between free enzyme (E) and substrate (S) versus the E-S complex

b. there is an equilibrium established between the E-S complex and the products

c. the rate of formation of the E-S complex is the same as the rate of breakdown of this complex either to products or back to reactants

d. the rate of formation of products is the same as the rate of formation of E-S complex **[CSIR-UGC]**

Q5. Which one of the following statements is *correct* concerning the Michaelis constant, Km?

a. is measured in the same units as the rate of the enzyme-catalyzed reaction

b. varies with the amount of enzyme used in measuring the reaction rate

c. can be considered to be a measure of the affinity an enzyme has for its substrate

d. has a low value to indicate a low affinity for substrate

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Q6. Which statement is *not* true about enzyme inhibition?

a. in competitive inhibition, the inhibitor binds to the active site of the enzyme
b. in non-competitive inhibition, the inhibitor binds to the allosteric site of the substrate
c. in irreversible inhibition, a poison binds to the enzyme so that it can never work again
d. none of the above

Q7. An allosteric site on an enzyme is

a. the same as the active site
b. non-protein in nature
c. where ATP attaches and gives up its energy
d. often involved in feedback inhibition

Q8. Heterotropic enzymes

a. are different isoenzymes
b. are modified by their substrate concentration
c. are stimulated or inhibited by an effector or modulator molecule other than their substrate
d. have same V_{max} but different K_m values **[JNU]**

Q9. Which one of the following characteristics best applies to an allosteric effector?

a. competes with substrate for the catalytic site
b. binds to a site on the enzyme molecule distinct from the catalytic site
c. changes the nature of the product formed
d. changes the substrate specificity of the enzyme **[JNU]**

Q10. In a chemical reaction, transition-state species have free energies

a. lower than either the reactants or the products
b. higher than either the reactants or the products
c. lower than the reactants, but higher than the products
d. higher than the reactants, but lower than the products **[JNU]**

Q11. For determining the K_m values of enzyme catalyzed reaction, it is customary to plot the kinetic data in a variety of ways. The plot of V versus V/S yields a straight line and the intercepts on the Y-axis gives

a. K_m/V_{max}
b. V_{max}/K_m
c. $1/V_{max}$
d. Hill coefficient, n **[JNU]**

Q12. Match the transition state or chemical entity of each enzyme that is responsible for their catalytic function.

Column I	*Column II*
P. ribonuclease	1. oxyanion
Q. lysozyme	2. pentacovalent phosphorus
R. chymotrypsin	3. carbonium ion
S. carboxypeptidase	4. mixed anhydride

a. P-3, Q-2, R-4, S-1
b. P-2, Q-3, R-1 S-4
c. P-2, Q-1, R-3, S-4
d. P-4, Q-3, R-2, S-1 **[GATE]**

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Enzymes and Vitamins

Q13. During the reduction of FAD

a. a flavin group is transferred

b. an equivalent of a hydride ion is transferred

c. the isoalloxazine becomes charged

d. two hydrogen atoms are added to the isoalloxazine ring

Q14. Which of the following answer complete the sentence *incorrectly*? NAD^+

a. is a flavin nucleotide

b. is the major electron acceptor used in metabolism

c. contains a nicotinamide ring that accepts a hydride ion during reduction

d. loses a plus charge upon reduction

Q15. Match the function of following cofactors

Column I	*Column II*
P. thiamine pyrophosphate	1. acyl group transfer
Q. coenzyme A	2. transfer of one carbon component
R. pyridoxal phosphate	3. group transfer to / or from amino acid
S. tetrahydrofolate	4. aldehyde transfer

a. P-4, Q-3, R-1, S-2

b. P-4, Q-3, R-2, S-1

c. P-4, Q-1, R-3, S-2

d. P-3, Q-1, R-4, S-2 **[GATE]**

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Chapter - 07

Respiration

7.1 ENERGETICS

Bioenergetics is the quantitative study of the energy transductions that occur in living cells and of the nature and function of the chemical processes underlying these transductions.

Thermodynamics principles

The **First law of Thermodynamics** states that the energy is neither created nor destroyed, although it can be transformed from one form to another i.e., the total energy of a system, including surroundings, remain constant.

Mathematically, it can be expressed as

$$\Delta U = \Delta q - \Delta w$$

ΔU is the change in internal energy,

Δq the heat exchanged from the surroundings,

Δw the work done by the system.

If Δq is positive, heat has been transferred to the system, giving an increase in internal energy. When Δq is negative, heat has been transferred to the surroundings, giving a decrease in internal energy. When Δw is positive, work has been done by the system, giving a decrease in internal energy. When Δw is negative, work has been done by the surroundings, giving an increase in internal energy.

The **Second law of thermodynamics** states that the total entropy of a system must increase if a process is to occur spontaneously. Mathematically, it can be expressed as

where, ΔS is the change in entropy of the system

Entropy is unavailable form of energy and it is very difficult to determine the unavailable form, so a new thermodynamic, term is defined called **free energy**.

Free energy

Free energy or Gibb's free energy indicates the portion of the total energy of a system that is available for useful work (also known as chemical potential). The change in free energy is denoted as ΔG.

Under constant temperature and pressure, the relationship between free energy change (ΔG) of a reacting system and the change in entropy (ΔS) is expressed by following equation

$$\Delta G = \Delta H - T\Delta H$$

Where, ΔH is the change in enthalpy and T is absolute temperature. ΔH is a measure of the change in heat content of the reactants and products. The change in the free energy, ΔG, can be used to predict the direction of a reaction at constant temperature and pressure.

If ΔG is negative, the reaction proceeds spontaneously with loss of free energy (**exergonic**).

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ΔG is positive, the reaction proceeds only if free energy can be gained (**endergonic**).

ΔG is 0, the system is at **equilibrium**; both forward and reverse reactions occur at equal rates.

ΔG of the reaction A $\rightarrow$ B depends on the concentration of the reactant and product. At constant temperature and pressure, the following relation can be derived.

$$\Delta G = \Delta G^\circ + RT \ln \frac{[B]}{[A]}$$

Where, ΔG° is the standard free energy change;

R is the gas constant;

T is the absolute temperature;

[A] and [B] are the actual concentrations of the reactant and product.

Standard free energy change

The actual change in free energy *G* during a reaction is influenced by temperature, pressure, and the initial concentrations of reactants and products and usually differs from *standard free energy change*, ΔG°.

The chemical reaction has a characteristic standard free energy change, ΔG° and it is a constant for a given reaction. It can be calculated from the equilibrium constant of the reaction under standard conditions i.e., at a solute concentration of 1.0M, at temperature of 25°C and at 1.0 atm pressure. The free energy change which corresponds to this standard state is known as standard free energy change, ΔG°.

Relationship between ΔG° and K_{eq}

In a reaction A $\rightarrow$ B, a point of equilibrium is reached at which no further *net* chemical change takes place–that is, when A is being converted to B as fast as B is being converted to A. In this state, the ratio of [B] to [A] is constant, regardless of the actual concentrations of the two compounds:

$$K_{eq} = \frac{[B]_{eq}}{[A]_{eq}}$$

where K_{eq} is the equilibrium constant, and $[A]_{eq}$ and $[B]_{eq}$ are the concentration of A and B at equilibrium. The concentration of reactants and products at equilibrium define the *equilibrium constant*, K_{eq}. The equilibrium constant K_{eq} depends on the nature of the reactants and products, the temperature, and the pressure. Under standard physical conditions (25°C and 1 atm pressure, for biological systems), the K_{eq} is always the same for a given reaction, whether or not a catalyst is present.

If the reaction A $\rightleftharpoons$ B is allowed to go to equilibrium at constant temperature and pressure, then at equilibrium the overall free energy change (ΔG) is zero. Therefore

$$\Delta G^\circ = RT \ln \frac{[B]_{eq}}{[A]_{eq}}$$

So, $\Delta G^\circ = -RT \ln K_{eq}$

This equation allows some simple predictions:

K_{eq}	ΔG°	**Reaction**
> 1.0	Negative	Proceeds forward
1.0	Zero	is at equilibrium
< 1.0	Positive	proceeds in reverse

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As we know, the ionic composition of an acid or base varies with pH. So the standard free energy calculated according to the biochemistry convention are valid only at pH=7. Hence under biochemistry convention, ΔG° is symbolized by $\Delta G^{\circ\prime}$ and likewise, biochemical equilibrium constant is represented by k'_{eq}.

$$\text{So, } \Delta G^{\circ\prime} = -RT \ln k'_{eq}$$

However, all reactions donot take place at standard conditions. A relationship has been worked out by which a distinction can be made between $\Delta G^{\circ\prime}$ and the actual free energy change, ΔG.

$$\Delta G = \Delta G^\circ + RT \ln \frac{[\text{Product}]}{[\text{Reactant}]}$$

If concentration of products and reactants is unity then $\Delta G = \Delta G^{\circ\prime}$. So, standard free energy change, $\Delta G^{\circ\prime}$ is the change in free energy when products and the reactants are present in unit concentration.

Note : In all spontaneous biochemical reaction, the free energy of the reacting system ΔG is negative, but the standard free energy change, $\Delta G^{\circ\prime}$ may be *positive*, *negative* or *zero* depending upon the equilibrium constant of the reaction.

As an example, let's make a simple calculation of the standard free-energy change of the following reaction

$$\text{Glucose 1-phosphate} \rightleftharpoons \text{Glucose 6-phosphate}$$

Chemical analysis shows that whether we start with, say, 20 mM glucose 1-phosphate (but no glucose 6-phosphate) or with 20 mM glucose 6-phosphate (but no glucose 1-phosphate), the final equilibrium mixture at 25°C will be the same : 1 mM glucose 1-phosphate and 19 mM glucose 6-phosphate. From these data we can calculate the equilibrium constant.

$$K'_{eq} = \frac{[\text{glucose 6-phosphate}]}{[\text{glucose 1-phosphate}]} = \frac{19 \text{ mM}}{1 \text{ mM}} = 19$$

From this value of K'_{eq} we can calculate the standard free-energy change:

$$\Delta G^{0\prime} = -RT \ln K'_{eq}$$

$$= -(8.315 \text{ J/mol, K})\ (298 \text{ K})\ (\ln 19)$$

$$= -7.3 \text{ kJ/mol}$$

Standard free energy changes of two consecutive reactions are additive

According to thermodynamics principles, the overall free-energy change for a chemically coupled series of reactions is equal to the sum of the free energy changes of the individual steps.

For example, in the case of two sequential chemical reactions, A $\rightleftharpoons$ B and B $\rightleftharpoons$ C, each reaction has its own equilibrium constant and each has its characteristic standard free energy change, $\Delta G_1^{0\prime}$ and $\Delta G_2^{0\prime}$. As the two reactions are sequential, B cancels out to give the overall reaction A $\rightleftharpoons$ C, which has its own equilibrium constant and thus its own standard free-energy change, $\Delta G^{\circ\prime}_{total}$.

For the overall reaction A $\rightleftharpoons$ C, $\Delta G^{0\prime}_{total}$ is the sum of the individual standard free-energy changes, $\Delta G_1^{0\prime}$ and $\Delta G_2^{0\prime}$, of the two reactions : $\Delta G^{0\prime}_{total} = \Delta G_1^{0\prime} + \Delta G_2^{0\prime}$.

FREE ENERGY CHANGE FOR ATP

ATP is the chemical link between catabolism and anabolism. It is the energy currency of the living cells. It acts as a donor of high energy phosphate. Its central role in energy metabolism was first explained by Fritz

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Lipmann and Herman Kalckar. ATP consists of an *adenosine* moiety to which three *phosphoryl groups* (PO_3^{2-}) are sequentially linked via a *phosphoester bond* followed by two *phosphoanhydride bonds*, referred to as high energy bond. The active form of ATP is usually a complex of ATP with Mg^{2+} or Mn^{2+}.

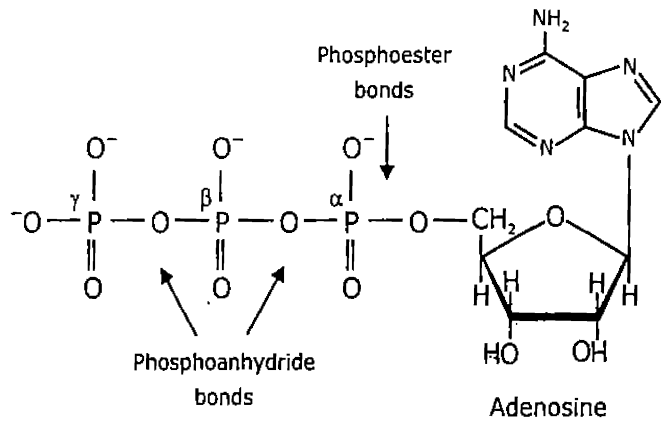


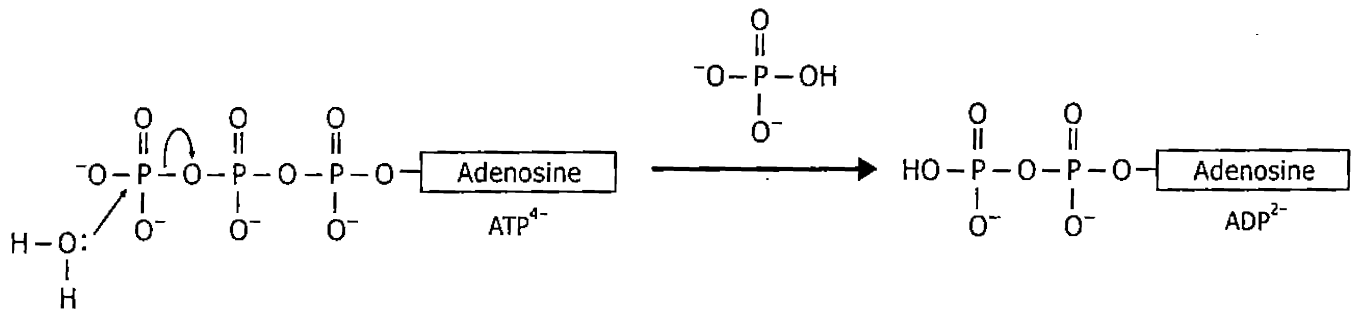
Figure 7.1 : Structure of ATP indicating phosphoester and phosphoanhydride bonds.

Hydrolysis of ATP

In aqueous condition, the phosphoanhydride bonds of ATP cleave with the incorporation of water.

$$ATP + H_2O \rightleftharpoons ADP + P_i \quad (Orthophosphate,\ PO_4^{3-})$$

$$ATP + H_2O \rightleftharpoons AMP + PP_i \quad (Pyrophosphate,\ P_2O_7^{4-})$$



Standard free energy change of ATP hydrolysis:

ATP contains two high energy phosphate groups and ADP contains one, whereas the phosphate in AMP is of the low energy type, since it is a normal ester link. A large amount of free energy is liberated when ATP is hydrolyzed to ADP and P_i or when ATP is hydrolyzed to AMP and PP_i.

Compound	$\Delta G°'$(kJ. mol^{-1})	$\Delta G°'$(kcal. mol^{-1})
$ATP + H_2O \rightleftharpoons ADP + P_i$	-30.5	-7.3
$ATP + H_2O \rightleftharpoons AMP + PP_i$	-32.2	-7.7

The standard free energy change of ATP hydrolysis is large and negative. These negative free energy values are often referred to as *phosphoryl group-transfer potentials* (phosphoryl transfer potential); they are a measure of the tendency of phosphorylated compounds to transfer their phosphoryl groups to water.

ATP has a stronger tendency to transfer its terminal phosphoryl group to water i.e., a higher phosphoryl transfer potential. The structural basis of the high phosphoryl transfer potential of ATP are *resonance stabilization, electrostatic repulsion*, and *stabilization due to hydration*. ADP and, in particular, P_i, have greater resonance stabilization than does ATP.

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Some compounds like phosphoenolpyruvate, 1,3-bisphosphoglycerate, and creatine phosphate in biological systems have a higher phosphoryl transfer potential than that of ATP.

Table 7.1 : Standard Gibb's free energies of hydrolysis of some phosphate compounds at pH 7

Phosphates	Type of phosphate bond	ΔG° for hydrolysis (kcal/mol)
Phosphoenolpyruvate	Enol phosphate bond	-14.8
1,3-bisphosphoglycerate	Anhydride bond to carbon	-11.7
Creatine phosphate	Phosphate bond	-10.3
Adenosine triphosphate	Phosphoanhydride bond	-7.3
Glucose-6-phosphate	Phosphoester bond	-3.3
Glycerol phosphate	Phosphoester bond	-2.2

The transfer of a phosphate group from one molecule, A, to another molecule, B, is energetically favorable if the standard free-energy change (ΔG°) for the hydrolysis of the phosphate bond in molecule A is more negative than that for hydrolysis of the phosphate bond in molecule B.

The actual free energy change of hydrolysis of ATP in living cells is different because the cellular concentration of ATP, ADP and P_i are not only unequal but also much lower than the standard 1.0M concentration. Thus the *actual* free energy of hydrolysis of ATP under intracellular conditions differs from the standard free energy change. The actual free energy change (-52 kJ. mol^{-1}) of hydrolysis of ATP is much larger than the standard free energy change (-30.5 kJ. mol^{-1}). The actual free energy of hydrolysis of ATP under intracellular conditions is given by the relationship.

$$\Delta G = \Delta G^{\circ\prime} + RT \ \ln \frac{[ADP][Pi]}{[ATP]}$$

PHOSPHAGENS

Phosphagens act as storage forms of high energy phosphate and include *creatine phosphate* (in vertebrate skeletal muscle, heart and brain) and *arginine phosphate* (in invertebrate muscle). When ATP is rapidly being utilized as a source of energy, phosphagens permit its concentration to be maintained. But when the ATP/ADP ratio is high, their concentration can increase to act as a source of high energy phosphate. At pH 7, the standard free energy of hydrolysis of creatine phosphate is -10.3 kcal mol^{-1} (-43.1 kJ mol^{-1}), compared with -7.3 kcal mol^{-1} (-30.5 kJ mol^{-1}) for ATP.

Creatine phosphate $\underset{ADP \quad ATP}{\overset{\text{Creatine kinase}}{\rightleftharpoons}}$ Creatine $\quad \Delta G^{\circ\prime} = -10.3$ kcal/mol^{-1}.

7.2 REDOX REACTION

Oxidation-Reduction (Redox or oxidoreduction reaction) involves the transfer of electrons from an electron donor to an electron acceptor.

For example, $Fe^{3+} + Cu^{+} \rightleftharpoons Fe^{2+} + Cu^{2+}$

The electron-donating molecule in an oxidation-reduction reaction is called the *reducing agent* or *reductant*; the electron-accepting molecule is the *oxidizing agent* or *oxidant*. In the above reaction, Cu^{+}, the reductant

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is oxidized to Cu^{2+} while Fe^{3+}, the oxidant, is reduced to Fe^{2+}. Although oxidation and reduction must occur together, it is convenient when describing electron transfers to consider the two halves of an oxidation-reduction reaction separately. Redox reaction may be divided into two half reactions or redox couples, such as

$$Fe^{3+} + e^- \rightleftharpoons Fe^{2+} \quad \text{(Reduction)}$$
$$Cu^{+} \rightleftharpoons Cu^{2+} + e^- \quad \text{(Oxidation)}$$

In cells electrons are transferred from *reducing agent* to *oxidizing agent* in four different ways i.e., transferred directly as *electrons* or in the form of *hydrogen atoms* or a *hydride ion* or direct combination with oxygen.

Free energy changes in Redox reactions

The readiness with which an atom or a molecule *gains* an electron is its **reduction potential** (E). Reduction potentials are measured in volts (V). The value of *E* for a molecule or an atom under standard conditions is its **standard reduction potential** (E_0). In a redox reaction, electrons move spontaneously from atoms or molecules having *negative or less positive* reduction potentials to atoms or molecules having *more positive* reduction potentials. We can use the concept of ΔG as a measure of useful work to show how the free energy change in an oxidation-reduction reaction is related to the voltage developed by the electrochemical cell in which the reaction occurs. The free energy change for a redox reaction is given by

$$\Delta G = -nFE$$

where n is the number of electrons transferred from each molecule of the substance being oxidized to that being reduced, E is the *electromotive force* (in volts) required to prevent the electron transfer, and F is the *Faraday constant*, equal to 96.5 kJ/V/mol. When the components of a redox reaction are in their standard states then

$$\Delta G^\circ = -nFE_0$$

In biological systems, the redox potential is normally expressed at pH 7. The redox potentials of some redox systems are given in the table. The relative positions of redox systems in the table also give the prediction of the direction of flow of electrons from one redox couple to another.

Table 7.2 : Redox potentials of some mammalian redox systems.

System	**E_0 Volts**
H^+/H_2	-0.42
NAD^+/NADH	-0.32
Pyruvate/lactate	-0.19
Oxaloacetate/malate	-0.17
Fumarate/succinate	+0.03
Cytochrome *b*; Fe^{3+}/Fe^{2+}	+0.08
Ubiquinone; ox/red	+0.10
Cytochrome c_1; Fe^{3+}/Fe^{2+}	+0.22
Cytochrome *a*; Fe^{3+}/Fe^{2+}	+0.29
Oxygen/water	+0.82

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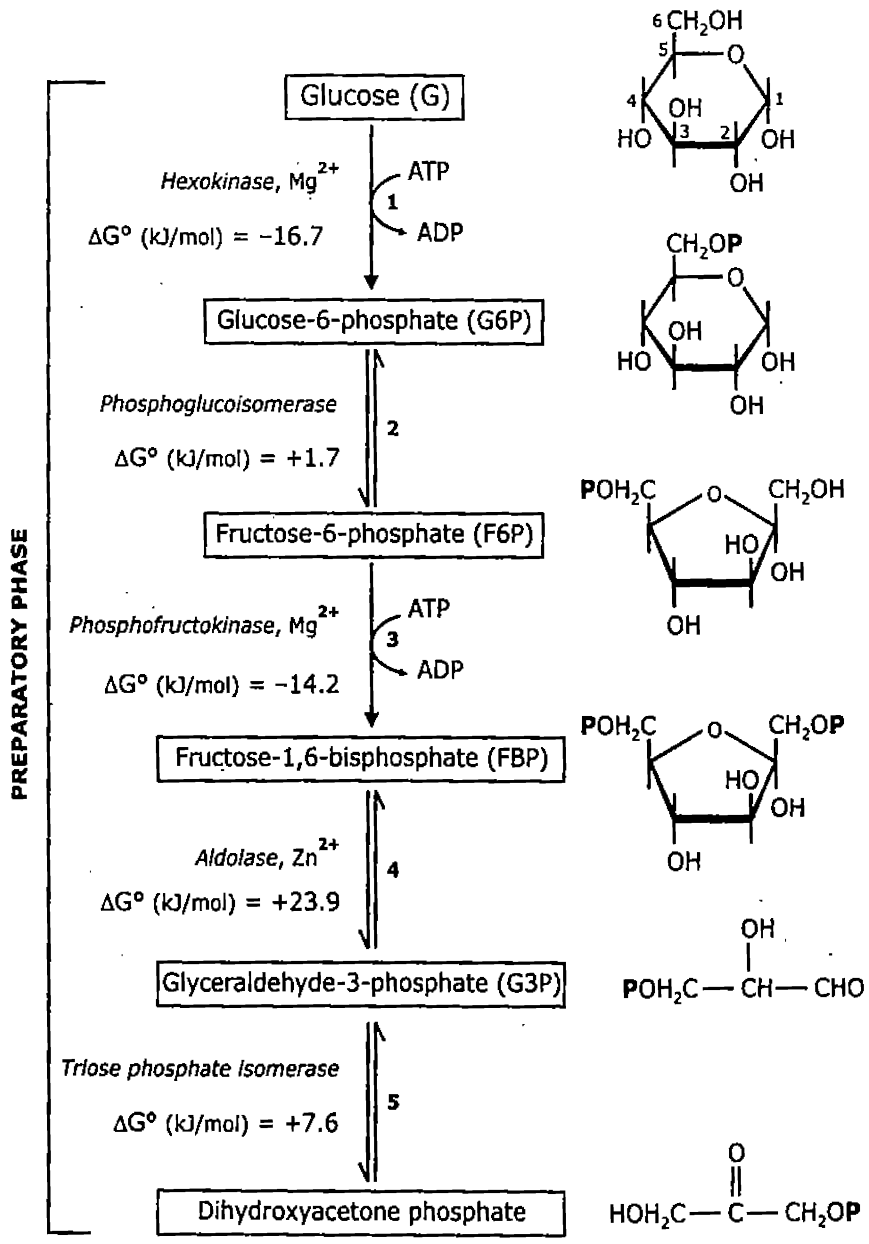
RESPIRATION

Respiration is an oxidative process, in which free energy released from respiratory substrates like glucose, fructose is used in the formation of ATP. When one mole of glucose is completely oxidized into CO_2 and water, approximately 2870 kJ or 686 Kcal energy is liberated. Approximately 32 moles of ATP are generated from per molecule of glucose oxidized to CO_2 and water.

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 686 \text{ Kcal}$$

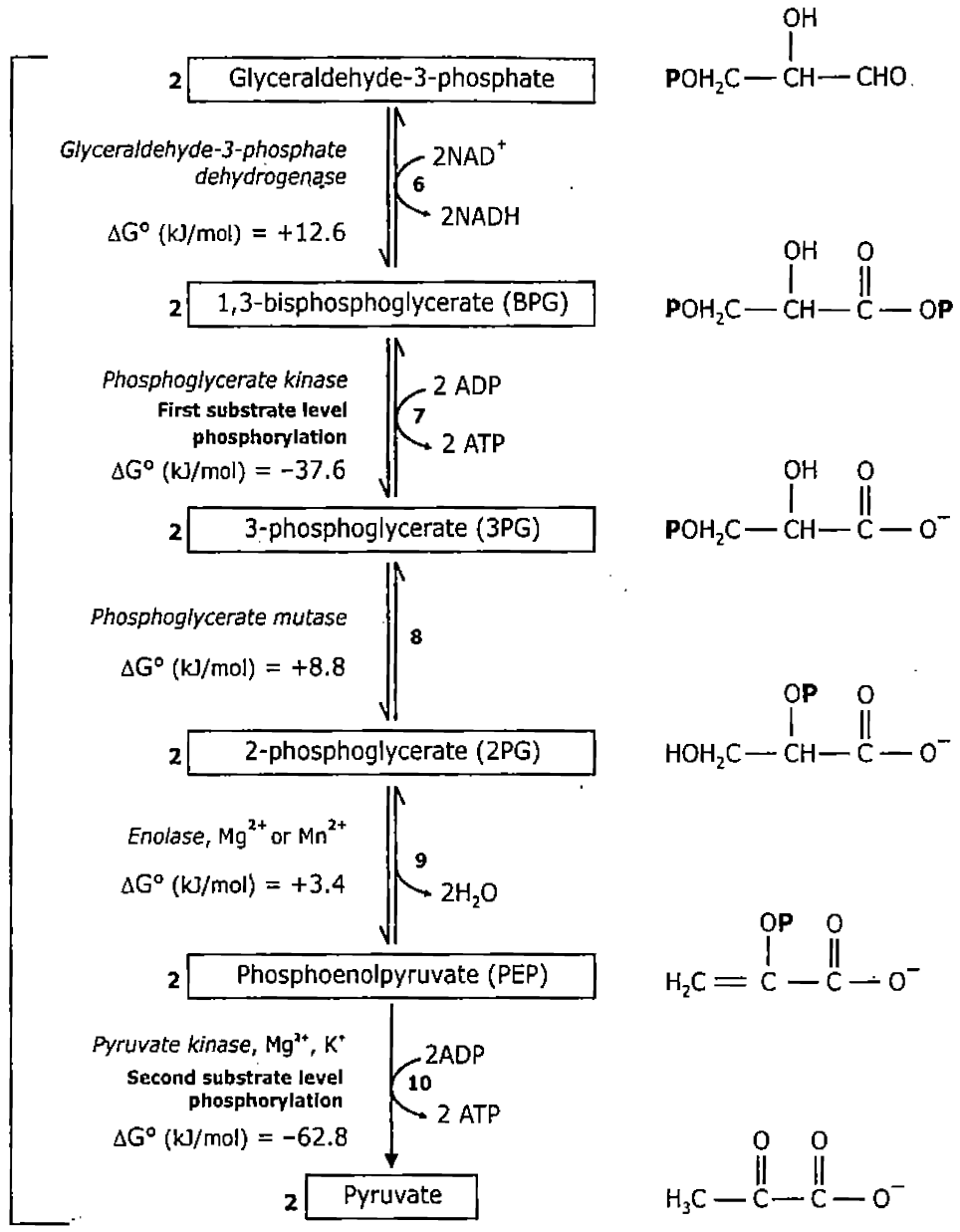
7.3 GLYCOLYSIS

Glycolysis (from the Greek *glykys*, meaning *sweet*, and *lysis*, meaning *splitting*) also known as *Embden-Meyerhof pathway*, is an oxidative process in which one mole of glucose is partially oxidized into the two moles of pyruvate in a series of enzyme-catalyzed reactions. Glycolysis, the major pathway for glucose metabolism, occurs in the cytosol of all cells. It is a unique pathway occurs aerobically as well as anaerobically and does not involve molecular oxygen.



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Step 1 : (**Phosphorylation**) Glucose is phosphorylated by ATP to form a sugar phosphate. The negative charge of the phosphate prevents passage of the sugar phosphate through the plasma membrane, trapping glucose inside the cell. This *irreversible* reaction is catalyzed by *hexokinase*. Hexokinase is present in all cells of all organisms. Hepatocytes also contain a form of hexokinase called **glucokinase** (hexokinase D). Hexokinase and also other kinases, requires divalent metal ion such as Mg^{2+} or Mn^{2+} for activity. Hexokinase and glucokinase are isozymes. Glucokinase is present in liver and beta-cells of pancreas and has a high K_m and V_{max} as compared to hexokinase.

Step 2 : (**Isomerization**) A readily reversible rearrangement of the chemical structure (isomerization) moves the carbonyl oxygen from carbon 1 to carbon 2, forming a ketose from an aldose sugar. Thus, the isomerization of glucose 6-phosphate to fructose 6-phosphate is a conversion of an aldose into a ketose.

Step 3 : (**Phosphorylation**) The new hydroxyl group on carbon 1 is phosphorylated by ATP, in preparation for the formation of two three-carbon sugar phosphates. Fructose 6-phosphate is phosphorylated by ATP to *fructose 1,6-bisphosphate*. The prefix *bis-* in bisphosphate means that two separate monophosphate groups are present, whereas the prefix *di-* means that two phosphate groups are present and are connected by an anhydride bond. This *irreversible* reaction is catalyzed by an allosteric enzyme *phosphofructokinase*.

Step 4 : (**Cleavage**) The six carbon sugar is cleaved to produce two three-carbon molecules- *glyceraldehyde 3-phosphate* (GAP) and *dihydroxyacetone phosphate* (DHAP). This reaction is catalyzed by *aldolase*. Only the glyceraldehyde 3-phosphate can proceed immediately through glycolysis.

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Step 5 : (**Isomerization**) Dihydroxyacetone phosphate is isomerized to form glyceraldehyde 3-phosphate. The isomerization of these three-carbon phosphorylated sugars is catalyzed by *triose phosphate isomerase*.

Step 6 : The two molecules of glyceraldehyde 3-phosphate are oxidized. Enzyme *glyceraldehyde 3-phosphate dehydrogenase* catalyzes the conversion of glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate (1,3-BPG). The reaction occurs in two steps: first the *oxidation* of the aldehyde to a carboxylic acid by NAD^+ and second the *joining* of the carboxylic acid and orthophosphate to form the acyl-phosphate product. **Iodoacetate** is a potent inhibitor of *glyceraldehyde-3 phosphate dehydrogenase* because it forms a covalent derivative of the essential -SH group of the enzyme active site, rendering it inactive.

Step 7 : The transfer of the high-energy phosphate group that was generated in step 6 to ADP form ATP. The formation of ATP is referred to as *substrate-level phosphorylation* because the phosphate donor, 1,3-BPG, is a substrate with high phosphoryl-transfer potential.

Step 8 : The remaining phosphate ester linkage in 3-phosphoglycerate, which has a relatively low free energy of hydrolysis, is moved from carbon 3 to carbon 2 to form 2-phosphoglycerate.

Step 9 : The removal of water from 2-phosphoglycerate creates a high-energy enol phosphate linkage. Enzyme catalyzing this step, *enolase*, is inhibited by **fluoride**.

Step 10 : The transfer of the high-energy phosphate group that was generated in step 9 to ADP forms ATP. This last step in glycolysis is the irreversible transfer of the phosphoryl group from phosphoenolpyruvate to ADP is catalyzed by *pyruvate kinase*. Pyruvate kinase requires either K^+ or Mg^{2+}.

Net reaction : $Glucose + 2NAD^+ + 2ADP + 2H_2PO_4^- \rightarrow 2Pyruvate + 2NADH + 2ATP + 2H_2O$

Glycolysis : An overview

All of the enzymes of glycolysis are found in the cytosol. Certain trypanosomas carry out the first seven reaction of glycolysis in an organized cytoplasmic organelles called *glycosome*. Three glycolytic reactions are irreversible. These reactions are catalyzed by hexokinase, phosphofructokinase and pyruvate kinase.

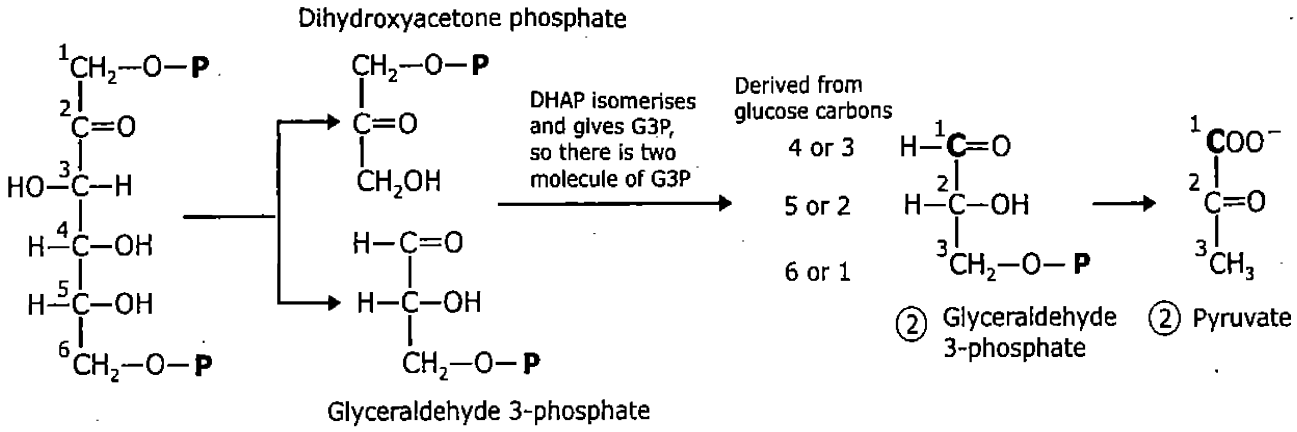
The breakdown of the six-carbon glucose into two molecules of the three-carbon pyruvate occurs in ten steps. The first five steps of this pathway constitute the *preparatory phase*. In this phase energy is consumed as glucose is phosphorylated twice, and is converted to FBP. For both phosphorylations, ATP is the phosphoryl group donor. FBP is split to yield two three-carbon molecules. One of the products, dihydroxyacetone phosphate, is immediately converted to G3P. This yields two molecules of G3P, which are then converted to pyruvate in a five-step process of payoff phase. The energy gain comes in the *payoff phase* of glycolysis. First, G3P is oxidized with NAD^+ as the electron acceptor (to form NADH), and an inorganic phosphate (not by ATP) is simultaneously incorporated to give a high-energy molecule called 1,3-bisphosphoglycerate. The high-energy phosphate on carbon one is subsequently donated to ADP to produce ATP. This synthesis of ATP is called **substrate-level phosphorylation** because ADP phosphorylation is coupled with the exergonic breakdown of a high-energy bond. Thus payoff phase of glycolysis yields ATP and NADH.

The energy yield from the production of two molecules of pyruvate from one molecule of glucose in glycolysis is 2 ATP and 2 NADH. In oxidative phosphorylation, passage of two electrons from NADH to O_2 drives the formation of about 2.5 ATP, and passage of two electrons from $FADH_2$ to O_2 yields about 1.5 ATP.

Fate of the glucose carbons in the formation of pyruvate

Aldolase, catalyzes a reversible aldol condensation. Fructose 1,6-bisphosphate is cleaved to yield two triose phosphates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Only one of the two triose phosphate formed by aldolase, glyceraldehyde 3-phosphate, can be directly degraded in the subsequent steps of glycolysis. The other product, dihydroxyacetone phosphate, is rapidly and reversibly converted to glyceraldehyde 3-phosphate. Each carbon of glyceraldehyde 3-phosphate is derived from either of two specific carbons of glucose.

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Overview of Glycolysis

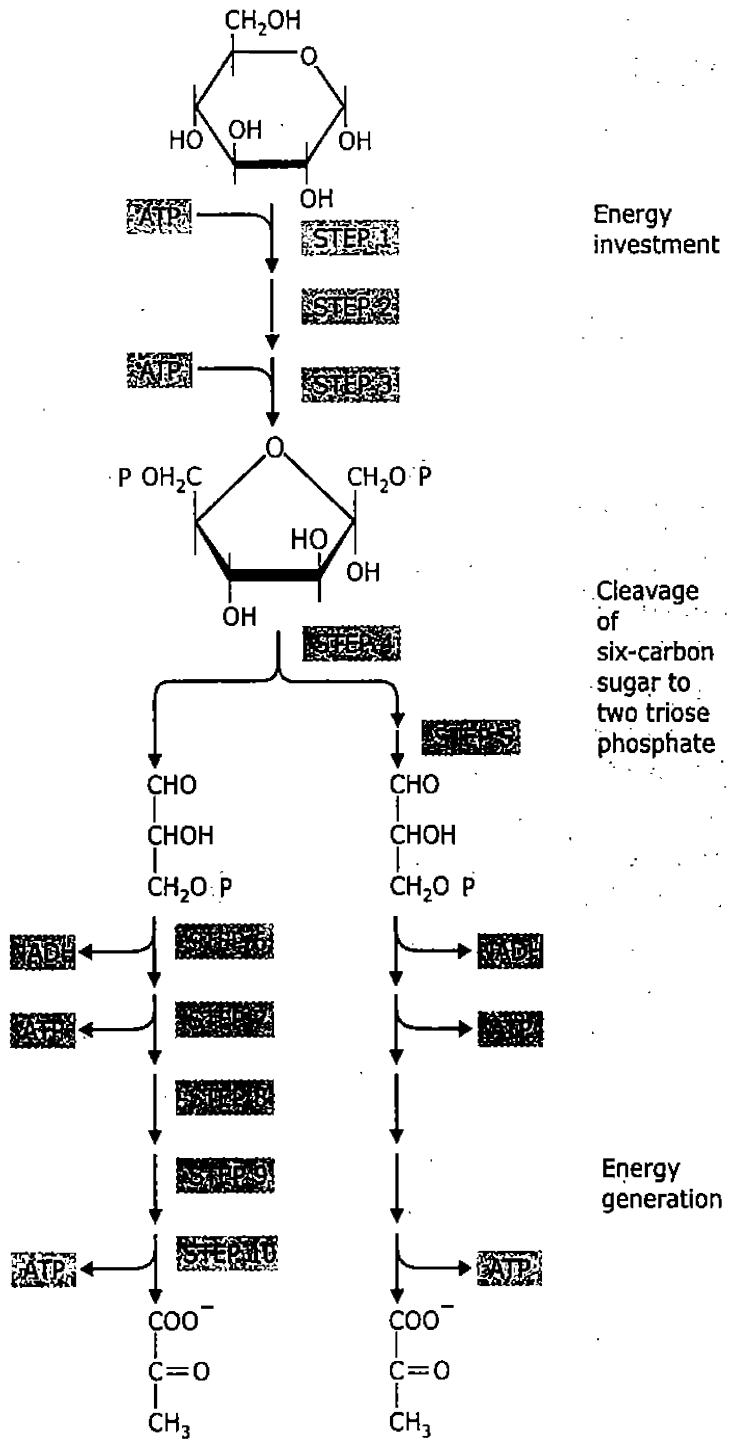


Figure 7.2 : An outline of glycolysis. Adapted and modified from Albert et al, Molecular Biology of Cell, Garland Science.

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REGULATION OF GLYCOLYSIS

The rate at which the glycolytic pathway operates is controlled primarily by allosteric regulation of three enzymes: *hexokinase*, *PFK-1*, and *pyruvate kinase*. The reactions catalyzed by these enzymes are *irreversible*. A high AMP concentration activates PFK-1 and pyruvate kinase. In contrast, a high ATP concentration inhibits both enzymes. Citrate and acetyl-CoA, which indicate that alternative energy sources are available, inhibit PFK-1 and pyruvate kinase, respectively. Other allosteric effectors include fructose-6-phosphate, which stimulates PFK-1 activity and glucose-6-phosphate, which inhibits the hexokinases. Finally, fructose-2,6-bisphosphate stimulates glycolysis by activating PFK-1 and fructose-1, 6-bisphosphate activates pyruvate kinase.

Enzyme	Activator	Inhibitor
Hexokinase	----------	Glucose-6-phosphate
PFK-1	Fructose-2,6-bisphosphate, Fructose-6-phosphate, AMP	Citrate, ATP
Pyruvate kinase	Fructose-1,6-bisphosphate, AMP	Acetyl-CoA, ATP

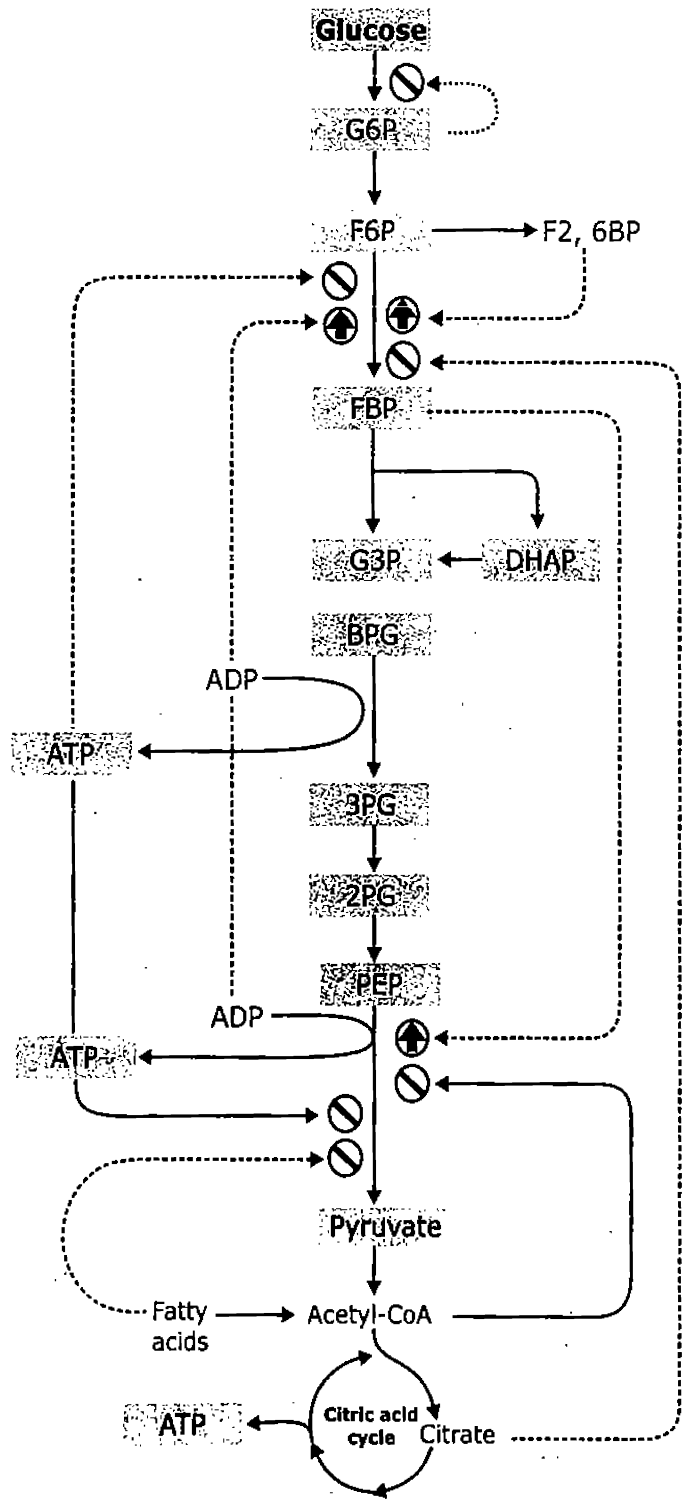


Figure : 7.3
Overview of the regulation of glycolysis. The diagram shows points of coordination with other metabolic pathways. The circled upward arrows identify activation, and the circled downward slashes represent inhibition.

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7.4 FERMENTATION

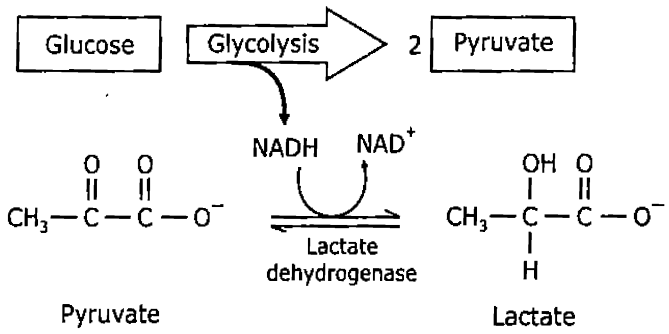
Pyruvate, the end product of glycolysis has two fates. In the absence of oxygen, it undergoes for *anaerobic respiration* or *fermentation* whereas in the presence of oxygen it enters the *aerobic* respiration.

Anaerobic respiration is different from fermentation. In **anaerobic respiration**, final electron acceptor in the electron transport chain is an inorganic molecule other than O_2 like nitrate, sulfate, carbonate whereas **fermentation** is an anaerobic energy yielding process in which final electron acceptor is an organic molecule. Fermentation is a self contained process and no outside electron acceptor is involved. It does not involve an electron transport system.

During fermentation, when O_2 is not present (or the cell cannot use it) NAD^+ is regenerated from NADH by transfering electrons to organic molecules. In this process pyruvate is converted into organic molecules like lactate, ethanol. Fermentations are classified in terms of either the substrate fermented or the fermentation products formed. Some of the major types of fermentations on the basis of products formed are alcoholic, lactic acid, propionic acid, homoacetic acid and butyric acid fermentation.

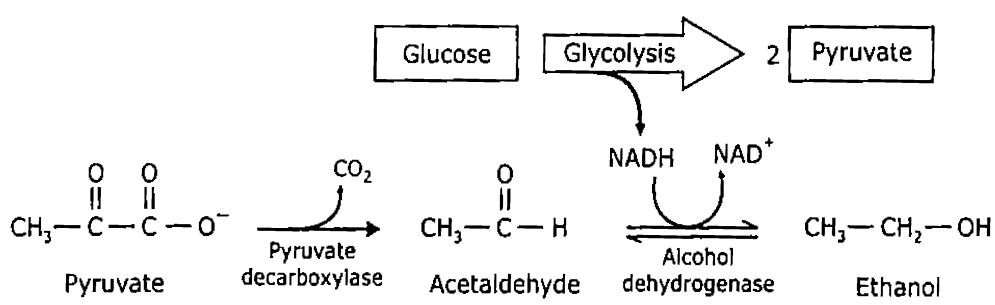
Lactic acid fermentation

Muscle cells and certain bacterial species (e.g., *Lactobacillus*) produce NAD^+ by transforming pyruvate into lactate and known as *lactic acid fermentation*. Lactic acid fermentation is catalyzed by an enzyme, *lactate dehydrogenase* (LDH). LDH exists in animal tissues in multiple molecular forms. Different molecular forms of an enzyme catalyzing the same reaction are called *isoenzymes* or *isozymes*. Most tissues contain five isoenzymes of LDH. It is a tetrameric protein consisting of two types of subunits, called M and H. M and H subunits combine randomly with each other, so that the five major isoenzymes have the compositions M4, M3H, M2H2, MH3, and H4.



Alcoholic fermentation

In *alcoholic fermentation* that occurs in yeast and several bacterial species, pyruvate is converted into ethanol in a two-step pathway. In yeast, pyruvate is decarboxylated to form acetaldehyde, which is then reduced by NADH to form ethanol. The non-oxidative decarboxylation of pyruvate to acetaldehyde is catalyzed by *pyruvate decarboxylase* and NADH-dependent reduction of acetaldehyde to ethanol is catalyzed by *alcohol dehydrogenase*. Pyruvate decarboxylase requires thiamine pyrophosphate as a coenzyme. This coenzyme, derived from vitamin B1, participates in a number of group transfer reactions involving an activated aldehyde moiety.



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Table 7.3 : Comparison of aerobic respiration, anaerobic respiration, and fermentation

Aerobic respiration

Growth condition : Aerobic

Final hydrogen (electron) acceptor : Molecular oxygen

Type of phosphorylation used to generate ATP : Substrate-level and oxidative

Anaerobic respiration

Growth condition : Anaerobic

Final hydrogen (electron) acceptor : An inorganic substance such as nitrate, sulfate but not oxygen

Type of phosphorylation used to generate ATP : Substrate-level and oxidative

Fermentation

Growth condition : Aerobic/anaerobic

Final hydrogen (electron) acceptor : An organic substance

Type of phosphorylation used to generate ATP : Substrate-level

Pasteur effect

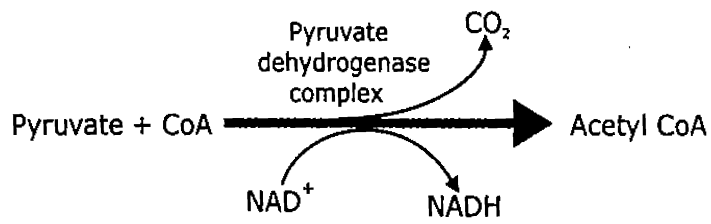
Louis Pasteur observed that when yeast are exposed to aerobic conditions, their glucose consumption and ethanol production drop. Reason for decrease in consumption of glucose is that fermentation results in the production of 2 ATPs per glucose whereas oxidative phosphorylation yields 32 ATPs per glucose. This accounts for Pasteur's observation that yeast consumes far more sugar when growing anaerobically than when growing aerobically (called *Pasteur effect*).

7.5 AEROBIC RESPIRATION

In the presence of oxygen, further oxidation of pyruvate occurs in the mitochondrial matrix (cytosol in case of prokaryote). In the mitochondrial matrix, the pyruvate first oxidizes into acetyl-CoA.

Pyruvate oxidation

The conversion of pyruvate to acetyl-CoA, catalyzed by highly organized multienzyme **pyruvate dehydrogenase complex**, is an oxidative decarboxylation process. In the overall reaction the carboxyl group of pyruvate is lost as CO_2, while the remaining two carbons form the acetyl moiety of acetyl-CoA. The reaction is highly exergonic and is essentially irreversible in vivo.



Pyruvate dehydrogenase complex is an assembly of three individual enzymes: *Pyruvate dehydrogenase* (E_1), *Dihydrolipoyl transacetylase* (E_2) and *Dihydrolipoyl dehydrogenase* (E_3). The oxidation of pyruvate to acetyl-CoA involves the coenzymes thiamine pyrophosphate (TPP), lipoic acid, FAD, NAD^+, and co-enzyme A (CoA), acting in association with E_1, E_2 and E_3 in the pyruvate dehydrogenase complex. In eukaryotes, this enzyme complex also contains small amounts of two regulatory enzymes as well-a *kinase* that phosphorylates serine residues and a *phosphatase* that removes those phosphates. Arsinite and mercuric ions inhibits the activity of pyruvate dehydrogenase.

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Table 7.4 : *E.coli* pyruvate dehydrogenase complex

Enzyme	*Function*	*No. of polypeptide*	*Cofactors*
Pyruvate dehydrogenase (E_1)	Decarboxylates pyruvate	24	TPP
Dihydrolipoyl transacetylase (E_2)	Catalyzes transfer of acetyl group to CoA	24	Lipoic acid, CoA
Dihydrolipoyl dehydrogenase (E_3)	Reoxidizes dihydrolipoamide	12	NAD^+, FAD

Mechanism of action of the pyruvate dehydrogenase complex

E_1 accepts a two-carbon aldehyde group from pyruvate and binds it to TPP, forming hydroxyethyl-TPP. The aldehyde group is transferred by E_1 to the lipoamide on E_2 and simultaneously oxidized to an acetyl group. E_2 transfers the acetyl group to the CoA, producing acetyl-CoA. E_3 oxidizes the reduced lipoamide by transferring two hydrogens to FAD. The reduced FAD is oxidized by NAD^+.

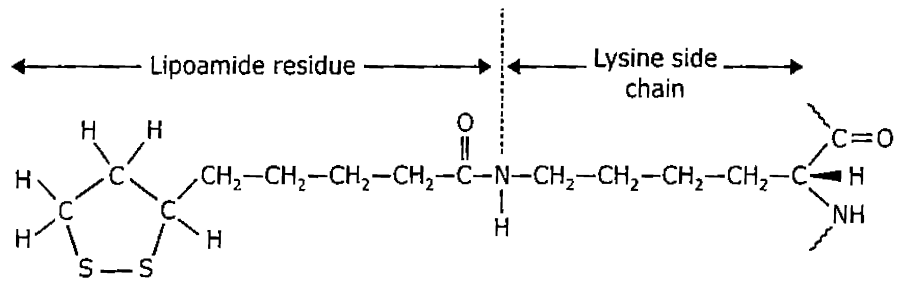


Figure 7.4 : Structure of dihydrolipoamide.

Pyruvate dehydrogenase complex is regulated by two processes. One *allosteric inhibition* by NADH and acetyl-CoA and other is *covalent modification*, occurs only in eukaryotes, by phosphorylation/dephosphorylation. E_2 is inhibited by acetyl-CoA and activated by CoA and E_3 is inhibited by NADH and activated by NAD^+. ATP also is an allosteric inhibitor of the complex, and AMP is an activator. E_1 subunit of enzyme complex undergoes for reversible phosphorylation/dephosphorylation.

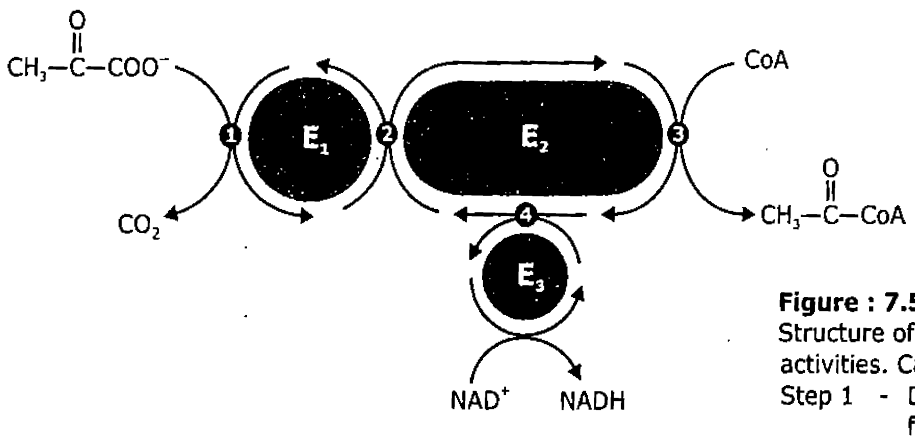


Figure : 7.5
Structure of pyruvate dehydrogenase and its catalytic activities. Catalytic activities occurs in four steps-
Step 1 - Decarboxylation of pyruvate occurs with formation of hydroxy ethyl - TPP
Step 2 - Transfer of the two carbon unit to lipoic acid
Step 3 - Formation of Acetyl-CoA
Step 4 - Lipoic acid is re-oxidized.

KREB'S CYCLE

Kreb's cycle (also called as citric acid cycle or tricarboxylic acid cycle) was discovered by H. A. Kreb, German born British Biochemist who received Nobel prize in 1953. This cycle occurs in matrix of mitochondria (cytosol in prokaryotes). Whole cycle is explained in the following figure. The net result of Kreb cycle is that for each acetyl group entering the cycle as Acetyl-CoA, two molecules of CO_2 are produced.

Step 1 : The citric acid cycle begins with the condensation of an oxaloacetate (four carbons unit), and the acetyl group of acetyl CoA (two-carbon unit). Oxaloacetate reacts with acetyl-CoA and H_2O to yield citrate and CoA. This reaction, which is an aldol condensation followed by a hydrolysis, is catalyzed by *citrate synthase.*

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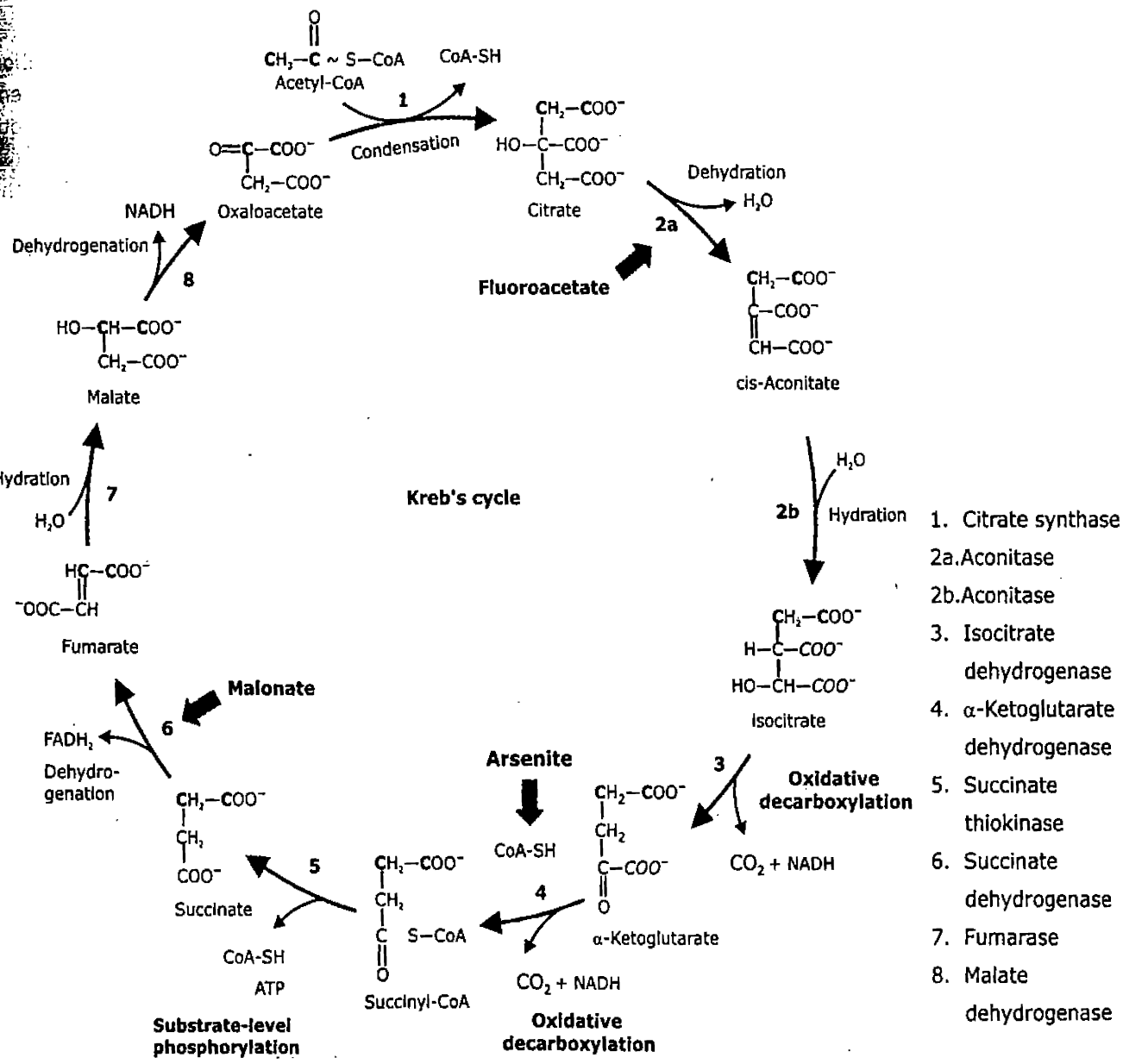
...d 2b : An isomerization reaction, in which water is first removed and then added back, moves ...yl group from one carbon atom to its neighbour. Enzyme catalyzing this step, *aconitase* (nonheme ...in), is the target site for the toxic compound **fluoroacetate** (used as a pesticide). Fluoroacetate ...e citric acid cycle by its metabolic conversion of *fluorocitrate*, which is a potent inhibitor of aconitase.

...Isocitrate is oxidized and decarboxylated to a*lpha*-Ketoglutarate. In the first of four oxidation ...the cycle, the carbon carrying the hydroxyl group is converted to a carbonyl group. The immediate ...is unstable, losing CO_2 while still bound to the enzyme. The oxidative decarboxylation of isocitrate ...yzed by *isocitrate dehydrogenase.*

... : A second oxidative decarboxylation reaction result in formation of succinyl CoA from a*lpha*-...lutarate. A*lpha-ketoglutarate dehydrogenase* catalyzes this oxidative step and produces NADH, CO_2, ... high-energy thioester bond to coenzyme A (CoA).

...p 5 : The cleavage of the thioester bond of succinyl CoA is coupled to the phosphorylation of a purine ...cleoside diphosphate, usually GDP (substrate level phosphorylation). This reaction is catalyzed by *succinyl ...oA synthetase* (succinate thiokinase). This is the only step in the citric acid cycle that directly yields a ...mpound with high phosphoryl transfer potential through a substrate-level phosphorylation.

...tep 6 : In the third oxidation step in the cycle, FAD removes two hydrogen atoms from succinate. Enzyme ...talyzing this step, succinate dehydrogenase, is strongly inhibited by **malonate**, a structural analog of ...uccinate and a classic example of a competitive inhibitor.



1. Citrate synthase
2a. Aconitase
2b. Aconitase
3. Isocitrate dehydrogenase
4. α-Ketoglutarate dehydrogenase
5. Succinate thiokinase
6. Succinate dehydrogenase
7. Fumarase
8. Malate dehydrogenase

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Step 7 : The addition of water to fumarate places a hydroxyl group next to a carbonyl carbon.

Step 8 : In the last of four oxidation steps in the cycle, the carbon carrying the hydroxyl group is converted to a carbonyl group, regenerating the oxaloacetate needed for step 1.

Overall reaction:

Acetyl CoA + $3NAD^+$ + FAD + GDP^{3-} + $3H_2O \rightarrow 2\ CO_2$ + 3NADH + $FADH_2$ + GTP^{4-} + H_2O

The energy yield from two pyruvate molecules when oxidized to 6 CO_2 via the pyruvate dehydrogenase complex and the citric acid cycle, and the electrons are transferred to O_2 via oxidative phosphorylation, as many as 25 ATP are obtained.

Fate of carbon in the citric acid cycle

Acetyl-CoA entering the citric acid cycle is highlighted (in bold) to show the fate of its two carbons through reaction 4. After reaction 5, the carbon atoms most recently entered are no longer highlighted, because succinate is a symmetrical molecule. Carboxyl groups that leave the cycle as CO_2 in reactions 3 and 4 are shown in bold letter.

STUDY QUESTION

Q1. Using pyruvate, labeled with ^{14}C in its keto group, via the pyruvate dehydrogenase reaction and the TCA cycle, where would the carbon label be at the end of one turn of the TCA cycle? Where would the carbon label be at the end of the second turn of the cycle?

Ans. The labeled keto carbon of pyruvate becomes the labeled carboxyl carbon of acetyl–CoA. After condensation with oxaloacetate, the first carboxyl group of citrate is labeled. This label is retained through subsequent reactions to succinate. However, succinate is a symmetrical compound to the enzyme, so, in effect, both carboxyl group of succinate are labeled. This means that the oxaloacetate regenerated is labeled in both carboxyl groups at the end of one turn (actually, half the molecules are labeled in one carboxyl and half in the other but this cannot be distinguished experimentally.

Regulation of the citric acid cycle

There are four major regulatory enzymes in the citric acid cycle. These are *citrate synthase*, *isocitrate dehydrogenase*, *α-ketoglutarate dehydrogenase*, and *succinate dehydrogenase*. Activators and inhibitors of these regulatory enzymes involved in citric acid cycle is shown in the following figure. The most important factor controlling citric acid cycle activity is the intra-mitochondrial ratio of NAD^+ to NADH.

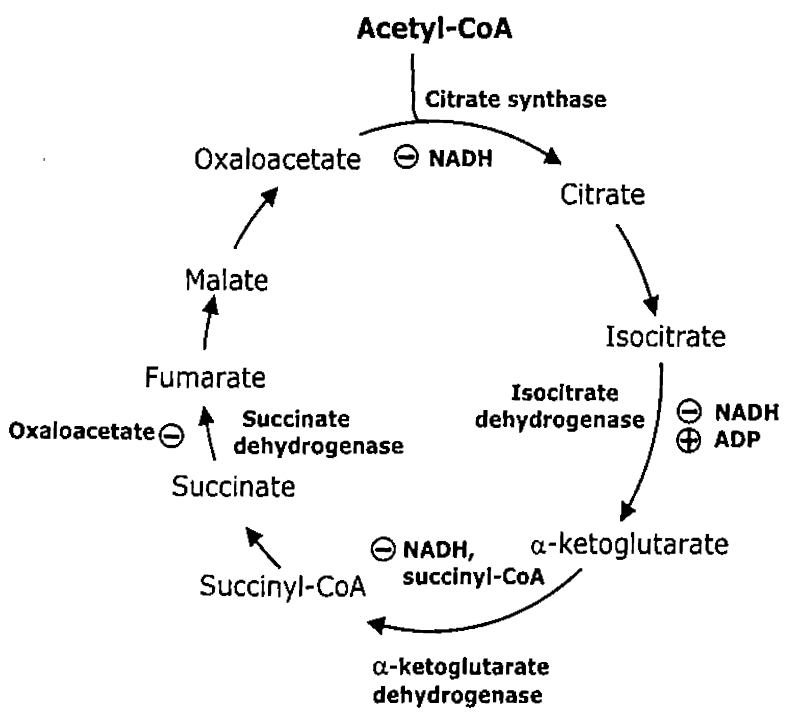


Figure : 7.6
The four regulatory steps in the citric acid cycle.
⊕ = activation; ⊖ = inhibition

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plerotic reaction

ric acid cycle intermediates are used in biosynthetic pathways as well. So, the anabolic nature of the ebs cycle has raised the question of how the level of intermediates can be replenished when, certain ntermediates are removed for anabolic purposes. Kornberg has proposed the term *anaplerotic* for these eplenishing or *filling up* reactions.

n animals, the most important anaplerotic reaction is catalyzed by *pyruvate carboxylase*. Pyruvate arboxylase, a mitochondrial enzyme, converts pyruvate to oxaloacetic acid.

$$\text{Pyruvate} \xrightarrow[\text{ATP}]{CO_2} \text{Oxaloacetic acid}$$

n plants and bacteria, an alternative route leads directly from phosphoenolpyruvate to oxaloacetate. This reaction is catalyzed by *phosphoenolpyruvate carboxylase*.

$$\text{Phosphoenolpyruvate} \xrightarrow{HCO_3^-} \text{Oxaloacetic acid}$$

A related enzyme, phosphoenolpyruvate carboxykinase, also converts phosphoenolpyruvate to oxaloacetate.

In addition to pyruvate carboxylase and phosphoenolpyruvate carboxylase, a third anaplerotic process is provided by an enzyme commonly known as *malic enzyme* but more officially as *malate dehydrogenase*. The malic enzyme catalyzes the reductive carboxylation of pyruvate to give malate.

$$\text{Pyruvate} \xrightarrow[\text{NADPH}]{HCO_3} \text{L-Malate} + NADP^+ + H_2O$$

STUDY QUESTION

Q2. What factors determine whether pyruvate carboxylase acts primarily as an anaplerotic or a gluconeogenic enzyme?

Ans. The main factor is the intracellular location of pyruvate—if it accumulates in mitochondria, the anaplerotic role predominates, whereas pyruvate in cytosol is used primarily as a gluconeogenic precursor. The levels of other intermediates are also important. For example, if intramitochondrial levels of oxaloacetate are adequate, then pyruvate can be either oxidized by pyruvate dehydrogenase or exported for gluconeogenesis. Oxaloacetate in mitochondria can also be converted by transamination to asparate, however, which can be exported to the cytosol for gluconeogenesis.

7.6 SHUTTLE SYSTEMS

The glycolytic pathway is a primary source of NADH formation. NADH synthesized during the glycolytic process finally transfer the electrons to electron transport chain. But NADH cannot cross the inner mitochondrial membrane. So, two different shuttle systems help in the transfer of electrons from NADH to the electron transport chain. Within the mitochondria the electrons of NADH can be coupled to ATP production during the process of *oxidative phosphorylation*.

The **malate/aspartate shuttle** is the principal mechanism for the movement of reducing equivalents (in the form of NADH) from the cytoplasm into the mitochondrial matrix.

The electrons are "carried" into the mitochondrial matrix in the form of malate. Cytoplasmic *malate dehydrogenase* (MDH) reduces oxaloacetate to malate while oxidizing NADH to NAD^+. Malate then enters the mitochondrial matrix where the reverse reaction is carried out by mitochondria MDH and regeneration of NADH occurs.

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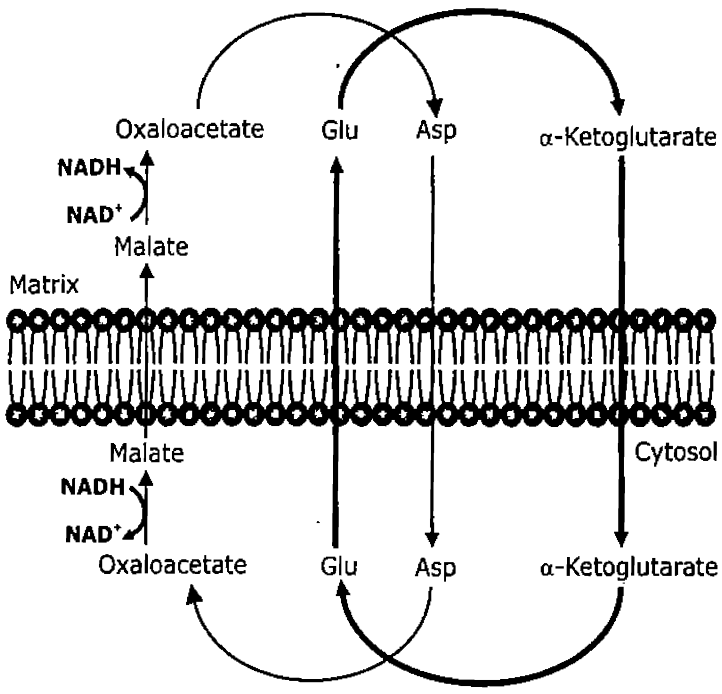


Figure : 7.7
The malate-aspartate shuttle for transporting reducing equivalents, NADH, from cytosol into the mitochondrial matrix. NADH in the cytosol transfers electrons to oxaloacetate, producing malate. Malate is transported across the inner membrane by the help of transporter. In the matrix, malate passes two reducing equivalants to NAD^+; the resulting matrix NADH is oxidized by the mitochondrial respiratory chain.

In **Glycerol 3-Phosphate Shuttle** electrons from NADH can enter the mitochondrial electron transport chain by being used to reduce dihydroxyacetone phosphate to glycerol 3-phosphate. Glycerol 3-phosphate is reoxidized by electron transfer to an FAD prosthetic group in a membrane-bound glycerol 3-phosphate dehydrogenase. Subsequent electron transfer to Q to form QH_2 allows these electrons to enter the electron-transport chain.

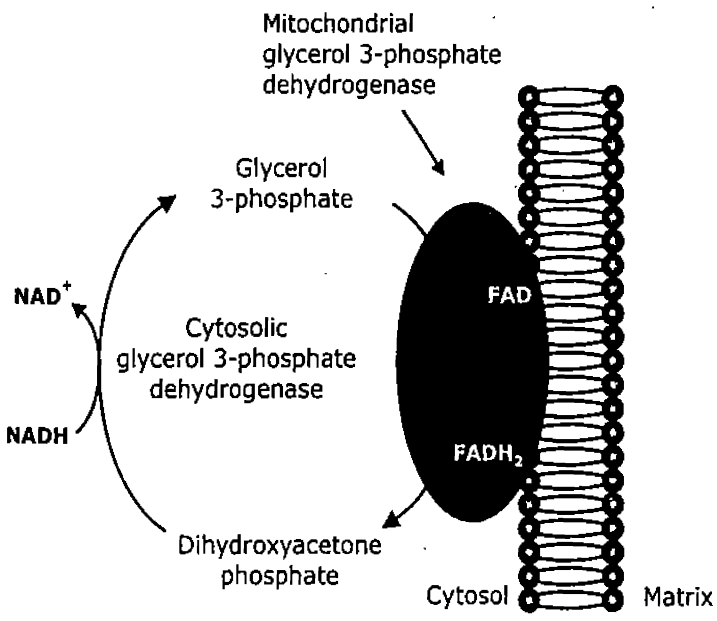


Figure : 7.8
Glycerol 3-Phosphate Shuttle. Electrons from NADH can enter the mitochondrial electron transport chain by being used to reduce dihydroxyacetone phosphate to glycerol 3-phosphate. Glycerol 3-phosphate is reoxidized by electron transfer to an FAD prosthetic group in a membrane-bound glycerol 3-phosphate dehydrogenase.

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...ECTRON TRANSPORT CHAIN AND OXIDATIVE PHOSPHORYLATION

...the free energy released during the oxidation of glucose to CO_2 is retained in the reduced coenzymes ...and $FADH_2$, generated during glycolysis and TCA cycle. Electrons are released from NADH and $FADH_2$ ...eventually are transferred to O_2, forming H_2O.

$$NADH + H^+ + 1/2\ O_2 \rightarrow NAD^+ + H_2O$$

$$FADH_2 + 1/2\ O_2 \rightarrow FAD + H_2O$$

...free energy change for these exergonic reaction are –52.6 Kcal/mol (NADH) and –43.14 Kcal/mol ($FADH_2$). ...large amount of free energy released during the oxidation of NADH and $FADH_2$ is used in the formation of ... For this reason, the term *oxidative phosphorylation* is used to describe this energy conversion process.

...ectrons are transferred from NADH/$FADH_2$ to O_2 through a series of electron carriers present on inner ...itochondrial membrane. The process of electron transport begins when the hydride ion is removed from ...ADH and is converted into a proton and two electrons. Most of the proteins (electron carriers) involved are ...rouped into four large *respiratory enzyme complexes*, each containing transmembrane proteins that hold ...he complex. The electrons start with very high energy and gradually lose it as they pass along the chain. Each complex in the chain has a greater affinity for electrons than its predecessor, and electrons pass sequentially from one complex to another until they are finally transferred to oxygen, which has the greatest affinity of all for electrons.

The four major respiratory enzyme complexes of electron transport chain in the inner mitochondrial membrane :

NADH–CoQ reductase (Complex I)

Succinate–CoQ reductase (Complex II)

$CoQH_2$–cytochrome c reductase (Complex III)

Cytochrome c Oxidase (Complex IV)

All the four multiprotein complexes which act as electron carriers comprise prosthetic groups, such as flavins, heme, Fe-S clusters, and copper. The table below lists the prosthetic group in each multiprotein complex.

Table 7.5 : Name of multiprotein complex and their prosthetic groups

Enzyme (multiprotein) **Complex**	**Prosthetic Groups**
NADH–CoQ reductase (Complex I)	FMN, FeS
Succinate–CoQ reductase (Complex II)	FAD, FeS
$CoQH_2$–cytochrome *c* reductase (complex III)	Heme b_{566}, Heme b_{562}, Heme c_1, FeS
Cytochrome *c* oxidase (Complex IV)	Heme a, Heme a_3, Cu_a^+, Cu_a^+

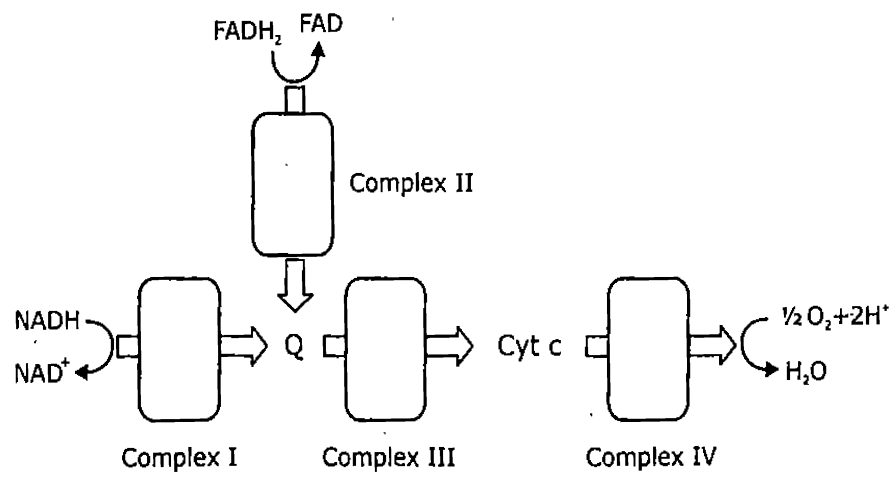


Figure 7.9 : Overview of electron flow through the respiratory chain (Q, co-enzyme Q or ubiquinone; Cyt, cytochrome)

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Electrons are transferred along the electron transport chain by the reversible reduction and oxidation of iron-sulfur clusters, ubiquinone, cytochromes and copper ions. Each carrier accepts an electron or electron pair from a carrier with a less positive reduction potential and transfers the electron to a carrier with a more positive reduction potential. Thus, reduction potentials of electron carriers favour unidirectional electron flow from NADH and $FADH_2$ to O_2.

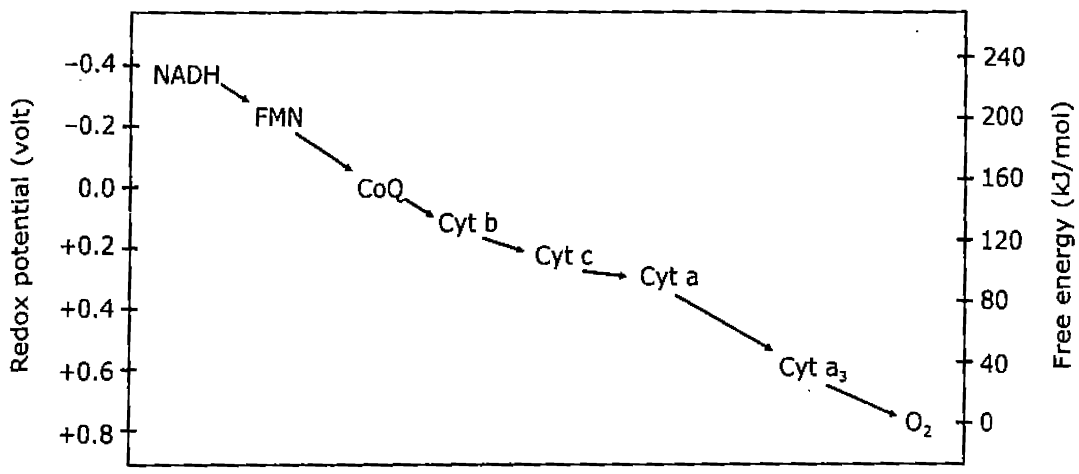


Figure 7.10 : Changes in redox potential and free energy during stepwise flow of electrons through the respiratory chain.

Complex I

Complex I (also called *NADH dehydrogenase*) is a large, multisubunit complex with about 40 polypeptide chains passes electron from NADH to CoQ. It contains one molecule of flavin mononucleotide (FMN) and six to seven iron-sulfur clusters that participate in the electron-transport process. During transport of each pair electrons from NADH to coenzyme Q, complex I pumps four protons across the inner mitochondrial membrane.

The reaction catalyzed by this enzyme appears to be

$$NADH + Q + 5H^+_{matrix} = NAD^+ + QH_2 + H^+_{cytosol}$$

Iron-sulfur centers consists of nonheme iron complexed to sulfur. There are two very common types of iron-sulfur centers : designated [2Fe-2S] and [4Fe - 4S]. These iron-sulfur centers consist of equal numbers of iron and sulfide ions and are both coordinated to four *Cys* sulfhydryl groups.

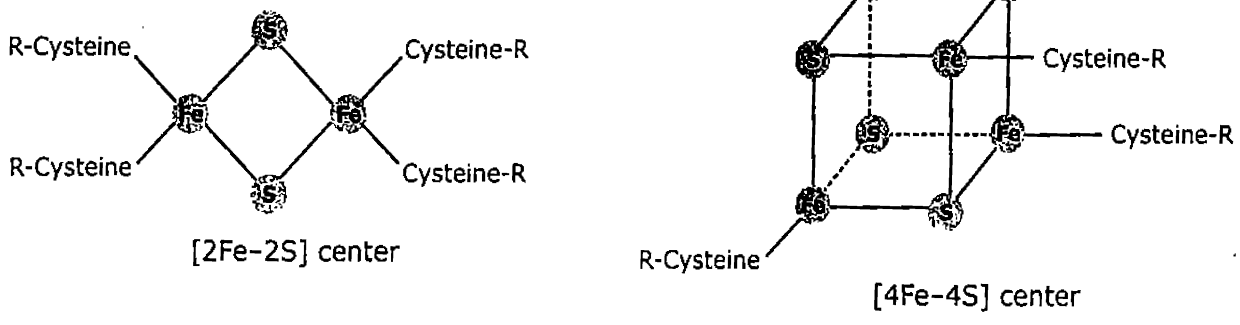


Figure 7.11 : Arrangement of the iron-sulfur centers of nonheme iron-sulfur proteins. The cysteine linkages are from the protein portion of the molecule. Iron-sulfur proteins typically carry electrons only.

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...me Q (CoQ, also known as **ubiquinone**) is a benzoquinone linked to a number of isoprene units. ...me-ubiquinone is for the ubiquitous nature of the quinone. CoQ is the only electron carrier in the ...on transport chain that is not a protein bound prosthetic group. It is a carrier of hydrogen atoms, ...s protons plus electrons.

...plex II

...inate dehydrogenase, a inner mitochondrial membrane bound enzyme, is an integral component of ...succinate-CoQ reductase complex. It converts succinate to fumarate during kreb cycle. The two electrons ...eased in conversion of succinate to fumarate are transferred first to FAD, then to an iron-sulfur center, ...finally to CoQ. Thus, CoQ draws electrons into the respiratory chain, not only from NADH but also from ...cinate. No protons are translocated across the membrane by the succinate-CoQ reductase complex.

...mplex III

...complex I or complex II donates two electrons to the complex III and regenerates oxidized CoQ. Concomi...ntly it releases two protons picked up on the cytosolic face into the intermembrane space generating ...proton gradient. Within complex III, the released electrons are transferred to an iron-sulfur center and then to two *b*-type cytochromes (b_{566} or b_L and b_{562} or b_H) or cytochrome c_1. Finally, the two electrons are transferred to two molecules of the oxidized form of cytochrome c. Two additional protons are translocated from the mitochondrial matrix across the inner mitochondrial membrane for each pair of electrons transferred. This transfer of protons involve the proton-motive **Q cycle.**

Cytochromes are heme proteins having distinctive visible-light spectra. The major respiratory cytochromes are classified as *b*, *c* or *a*, depending on the wavelengths of the spectral absorption peaks. Within each class, the cytochromes are distinguished by smaller spectral differences. In the respiratory electron carriers there are two *b*-type cytochromes, cytochromes *c* and c_1, and cytochromes *a* and a_3.

The heme prosthetic groups of a and b cytochromes are tightly, but not covalently, bound to their associated proteins; whereas heme groups of c-type cytochromes are covalently attached through Cys residues. Cytochrome c present in all aerobic organisms. The degree of sequence similarity in cytochrome c among species has been used as a measure of the evolutionary distances that separate species.

Q-cycle

The mechanism of the participation of ubiquinone in the electron transport process was proposed by Peter Mitchell and termed as proton motive *Q-cycle*. Ubiquinones are hydrophobic and uncharged and hence can migrate along the hydrophobic core of the membrane. Diffusion takes place of one UQH_2 to the Q_p binding site adjacent to the iron-sulfur protein at the P face of the mitochondrial membrane. One electron is transferred to Fe-S protein and the second electron is transferred to the b_{566} heme (b_L) and two protons are released to the P face. The Fe-S protein transfers the electron along the chain to Cyt c_1 and cytochrome oxidase. The electron moves from b_L to b_{562} heme (b_H). UQ then binds to b_H at the Q_n site and electron from the reduced b_H forms UQ at this site. Now a second UQH_2 molecule is oxidized at the Q_p site, the process follows as described above and the second electron formed completes the reduction of UQ^- to UQH_2. Two protons are taken from the matrix for this purpose and released to the P face. The reduced ubiquinone then goes back to the pool and thus the Q-cycle is completed.

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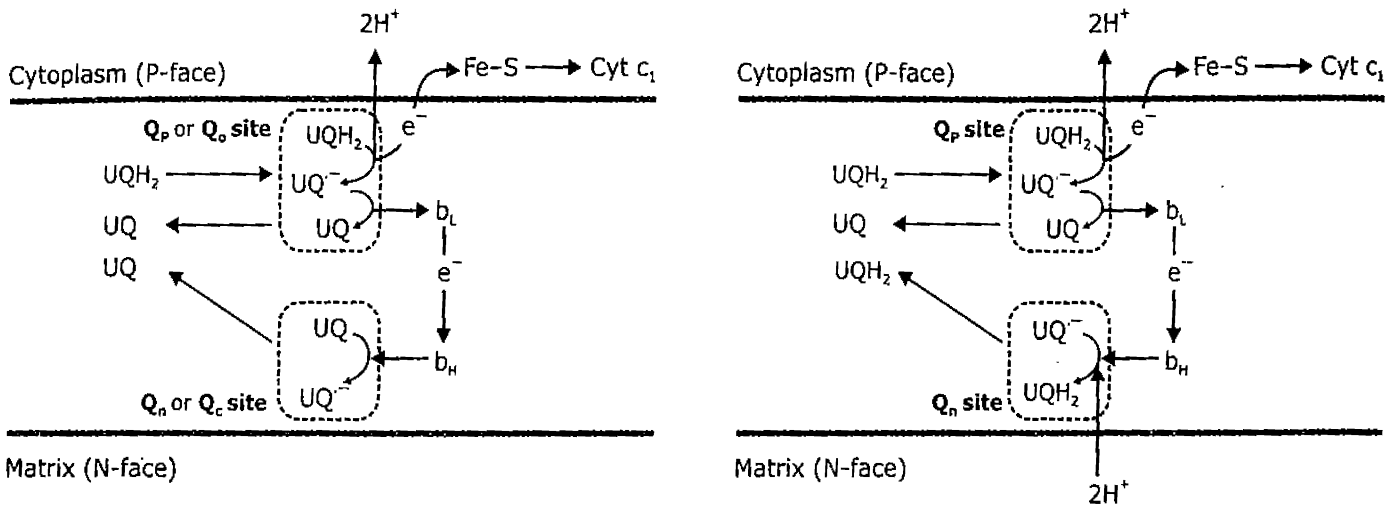
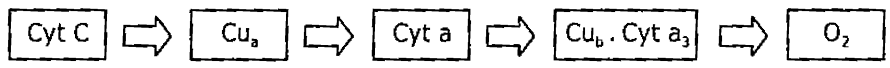


Figure 7.12 : A simplified outline of Q-cycle in mitochondria.

Complex IV

Cytochrome *c* transports electrons, one at a time, to the complex IV. Within this complex, electrons are transferred, first to a pair of copper ions called Cu_a^{2+}, then to Cyt a, next to a complex of another copper ion (Cu_b^{2+}) and Cyt a_3, and finally to O_2, the ultimate electron acceptor, yielding H_2O.



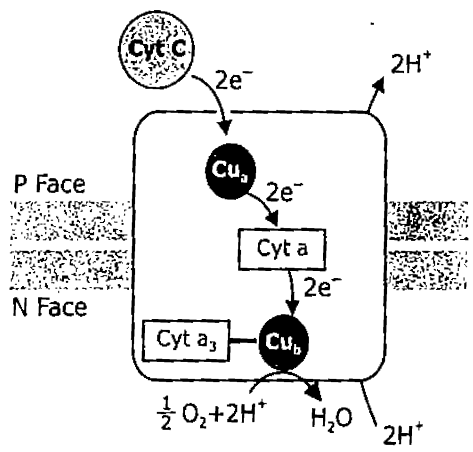


Figure : 7.13
The electron transfer pathway for cytochrome oxidase. Cytochrome c binds on the cytosolic side, transferring electrons through the copper and heme centers to reduce O_2 on the matrix side of the membrane.

Two electrons, sequentially released from two molecules of reduced cytochrome *c*, together with two protons from the matrix, combine with one O atom to form one water molecule. Additionally, for each electron transferred from cytochrome *c* to oxygen, one proton is transported from the matrix to the intermembrane space, or a total of four electrons are transferred for each O_2 molecule reduced to two H_2O molecules.

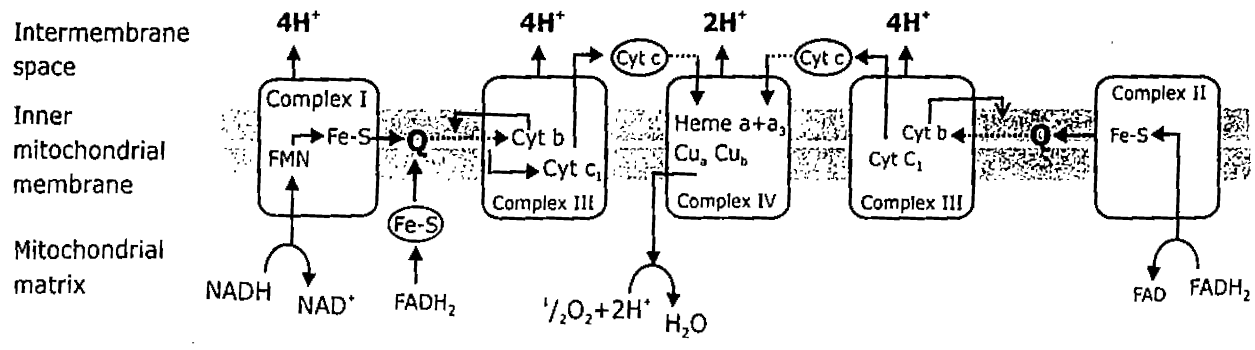


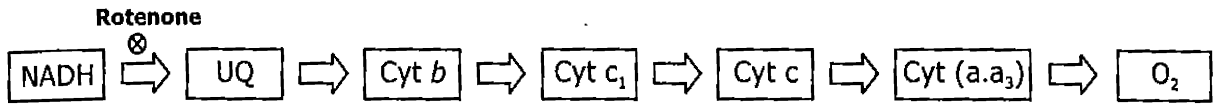
Figure 7.14 : Flow of electrons through the respiratory chain complexes, showing the entry points for reducing equivalents from important substrates. Q and cyt c are mobile components of the system as indicated by the dotted arrow. Fe-S, iron sulfur protein; ETF, electron transferring flavoprotein; Q, co-enzyme Q or ubiquinone; C, cytochrome.

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Inhibitors of electron transport

Site-specific inhibitors of electron transport have been identified. These compounds prevent the passage of electrons by binding to a component of the chain, blocking the oxidation/reduction reaction.

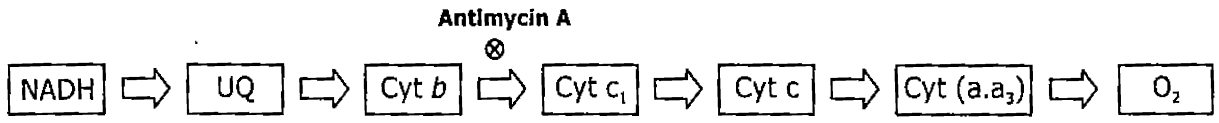
Rotenone, a plant product, inhibits the transfer of electrons through the NADH–CoQ reductase complex. It is used as a fish poison and as an insecticide.



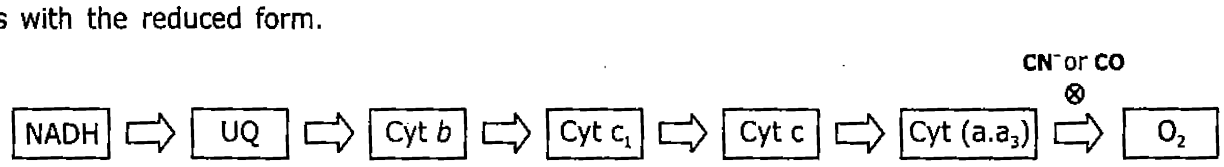
Amobarbital (Amytal) also acts at the same site and inhibits electron transport through the NADH-CoQ reductase complex.

Piericidin A, an antibiotic, blocks the transfer of electrons at the NADH-CoQ reductase complex by competing with Q. The electrons from the complex are transferred to piericidin A instead of Q.

Antimycin A, also an antibiotic, blocks electron transport at the level of the $CoQH_2$–cytochrome c-reductase (complex III).



Cyanide, azide and **carbon monoxide** binds with cytochrome oxidase complex and inhibits the terminal transfer of electrons to oxygen. Cyanide and azide react with the oxidized form of the cytochrome and CO reacts with the reduced form.



Plants also have alternative mechanisms for oxidizing NADH. To regenerate NAD^+, electrons from NADH directly transfer to ubiquinone and from ubiquinone directly to O_2, bypassing complexes III and IV. This process also does not involve H^+ pumping. So, the energy in NADH is dissipated as heat. Because transfer of electrons does not involve complex IV, hence NADH oxidation by this pathway is *cyanide resistant*.

STUDY QUESTION

Q3. Rotenone, a toxic natural product from plants, strongly inhibits NADH dehydrogenase of insect and fish mitochondria. Antimycin A, a toxic antibiotic, strongly inhibits the oxidation of ubiquinol.

a. Explain why rotenone ingestion is lethal to some insect and fish species.

b. Explain why antimycin A is a poison.

c. Given that rotenone and antimycin A are equally effective in blocking their respective sites in the electron transport chain, which would be a more potent poison? Explain.

Ans. a. Inhibition of NADH dehydrogenase by rotenone decreases the rate of electron flow through the respiratory chain, which in turn decreases the rate of ATP production. If this reduced rate is unable to meet the organism's ATP requirements, the organism dies.

b. Antimycin A strongly inhibits the oxidation of Q in the respiratory chain, reducing the rate of electron transfer and leading to the consequences described in (a).

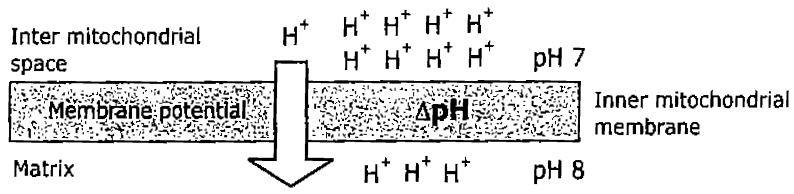
c. Because antimycin A blocks all electron flow to oxygen, it is a more potent poison than rotenone, which blocks electron flow from NADH but not from $FADH_2$.

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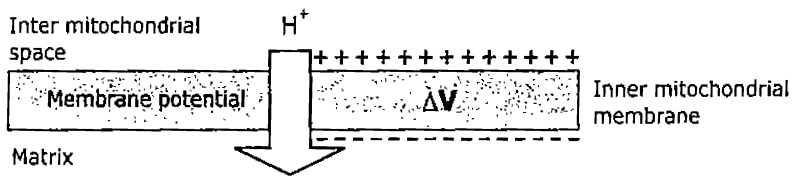
Electrochemical proton gradient

Transfer of electrons through electron transport chain is accompanied by pumping of protons across inner mitochondrial membrane, from the mitochondrial matrix to inter mitochondrial space. A total of $10H^+$ ions are translocated from the matrix across the inner mitochondrial membrane per electron pair flowing from NADH to O_2. This movement of H^+ generates:

a. pH gradient across the inner mitochondrial membrane (with the pH higher in the matrix than in the cytosol).



b. Voltage gradient (membrane potential) across the inner mitochondrial membrane (with the inside negative and outside positive).



The pH gradient (ΔpH) and voltage gradient together constitute **electrochemical proton gradient.** The electrochemical proton gradient exerts a **proton motive force** (pmf). A mitochondrion actively involved in aerobic respiration typically has a membrane potential of about 160mV (negative inside matrix) and a pH gradient of about 1.0 pH unit (higher on the matrix side). A difference of one pH unit represents a ten fold difference in H^+ concentration and a pH gradient of one unit across a membrane is equivalent to an electric potential of 59 mV (at 20°C). The total proton-motive force across the inner mitochondrial membrane consists of a large force due to the membrane potential and a smaller force due to the H^+ concentration gradient (pH gradient). In a typical cell, the proton motive force across the inner mitochondrial membrane of a respiring mitochondrion is about 220 mV.

Determination of electric potential and pH gradient

Because mitochondria are very small so the electric potential and pH gradient across the inner mitochondrial membrane cannot be determined by direct measurement. However, the inside pH can be measured by trapping fluorescent pH-sensitive dyes inside vesicles formed from the inner mitochondrial membrane.

Similarly the electric potential can be determined by adding radioactive K^+ ions and a trace amount of valinomycin to a suspension of respiring mitochondria. Valinomycin is an *ionophore.* Although the inner membrane is normally impermeable to K^+ ions but valinomycin selectively binds K^+ ions in its hydrophilic interior and carries it across through the impermeable membranes. In the presence of valinomycin, K^+ ions equilibrates across the inner membrane of isolated mitochondria in accordance with the electric potential; the more negative the matrix side of the membrane, the more K^+ ions will accumulate in the matrix. At equilibrium, the value of electric potential can be calculated from the Nernst equation.

Calculation of proton motive force

The proton-motive force, pmf, can be calculated by following mathematical equation,

$$pmf = \psi - \left(\frac{RT}{F}\right) \times \Delta pH = \psi - 59\ \Delta pH$$

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where R is the gas constant of 1.987 cal/(degree·mol), T is the temperature (in degrees Kelvin), F is the Faraday constant [23,062 cal/(V·mol)], and Ψ is the transmembrane electric potential; Ψ and pmf are measured in millivolts. Measurements on respiring mitochondria have shown that the electric potential (Ψ) across the inner membrane is ~160 mV (negative inside matrix) and that ΔpH is ~1.0 (equivalent to ~60 mV). Thus the total pmf is ~220 mV, with the transmembrane electric potential responsible for about 73 percent.

Mechanism of oxidative phosphorylation : Chemiosmotic theory

What is the actual mechanism by which energy released from respiration is used to drive the synthesis of ATP? In 1961, Peter Mitchell, a British biochemist, proposed a mechanism of **chemiosmotic coupling** to answer this question.

This model proposes that energy from electron transport drives an active transport system, which pumps protons out of the mitochondrial matrix into the intermitochondrial space. This action generates an electrochemical gradient for protons, with a lower pH value outside the inner mitochondrial membrane than inside. The protons on the outside have a thermodynamic tendency of flow back into the matrix so as to equalize pH. When protons do flow back into the matrix, the energy is dissipated, some of it being used to drive the synthesis of ATP.

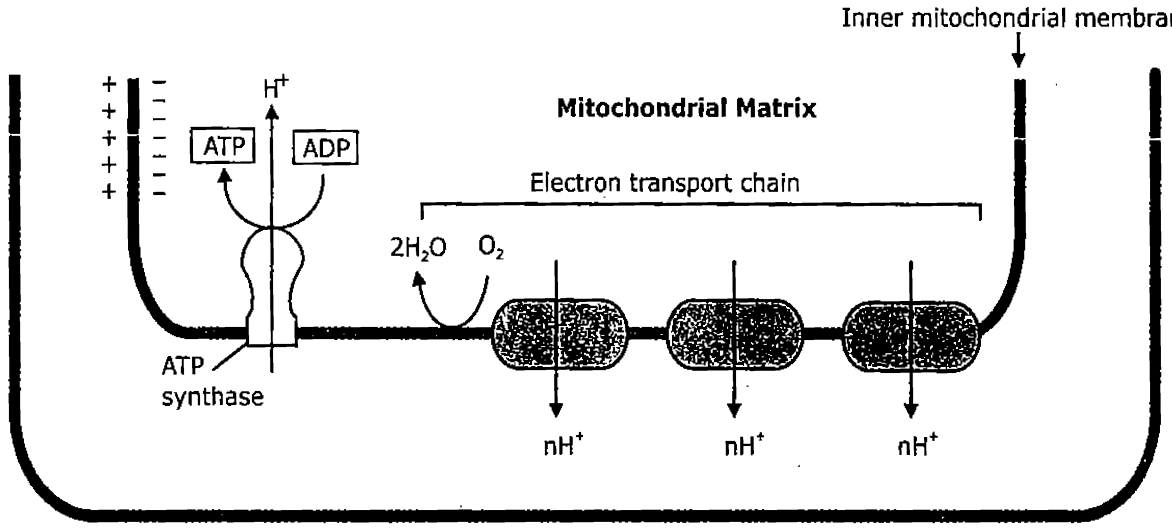


Figure 7.15 : The general mechanism of oxidative phosphorylation. As a high-energy electron is passed along the electron-transport chain, some of the energy released is used to drive three respiratory enzyme complexes that pump H^+ out of the matrix space. The resulting electrochemical proton gradient across the inner membrane drives H^+ back through the ATP synthase, a transmembrane protein complex that uses the energy of the H^+ flow to synthesize ATP from ADP and P_i in the matrix

Experimental proof of chemiosmotic hypothesis

Experimental proof of chemiosmotic hypothesis was provided by Andre Jagendorf and Ernest Uribe in 1966. In an elegant experiment isolated chloroplast thylakoid vesicles containing F_0F_1 particles were equilibrated in the dark with a buffered solution at pH 4.0. When the pH in the thylakoid lumen became 4.0, the vesicles were rapidly mixed with a solution at pH 8.0 containing ADP and Pi. A burst of ATP synthesis accompanied the transmembrane movement of protons driven by electrochemical proton gradient. In similar experiments using *inside-out* preparations of submitochondrial vesicles, an artificially generated membrane electric potential also resulted in ATP synthesis.

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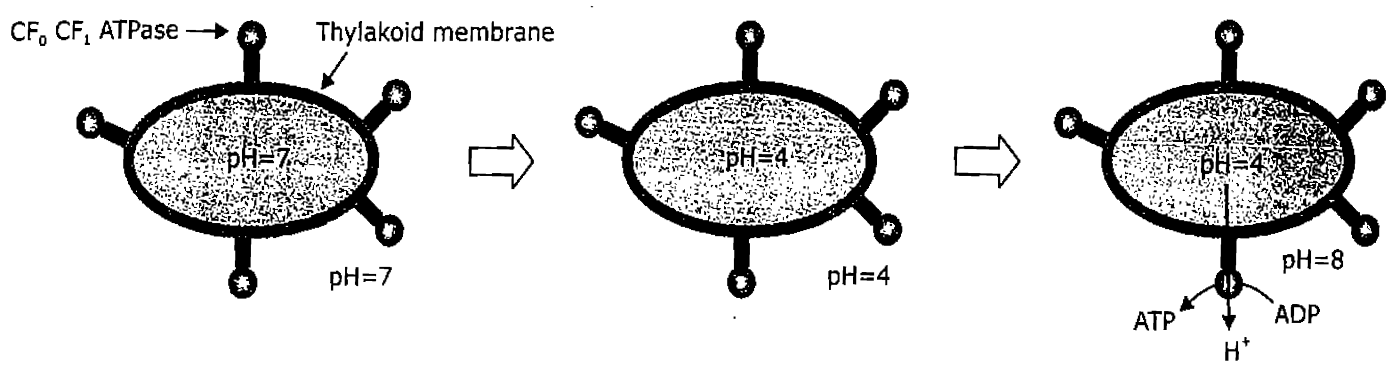


Figure 7.16 : Synthesis of ATP by F_0F_1 depends on a pH gradient across the membrane.

ATP Synthase

The use of proton motive force for ATP synthesis is catalyzed by ATP synthase. The multiprotein ATP synthase or F_0F_1 complex or complex V catalyzes ATP synthesis as protons flow back through the inner membrane down the electrochemical proton gradient. It consists of two components – F_0 component and F_1 ATPase. The F_0 component is embedded in the inner mitochondrial membrane. F_0 contains one 'a' subunit, two 'b' subunits, and 9-12 'c' subunits. The c subunit consists of two α helices that span the membrane. An aspartic acid residue in the second helix lies on the center of the membrane. F_0 is a transmembrane complex that forms a regulated H^+ channel. Thus an antibiotic **oligomycin** completely blocks ATP synthesis by blocking the flow of protons through F_0 of ATP synthase.

F_1 ATPase (made up of 3α, 3β, γ, δ and ε) is tightly bound to F_0 and protrudes into the matrix; it contains three β-subunits that are the sites of ATP synthesis. At the center of F_1 ATPase is the γ subunit. The γ subunit extends through F_1 and interacts with F_0.

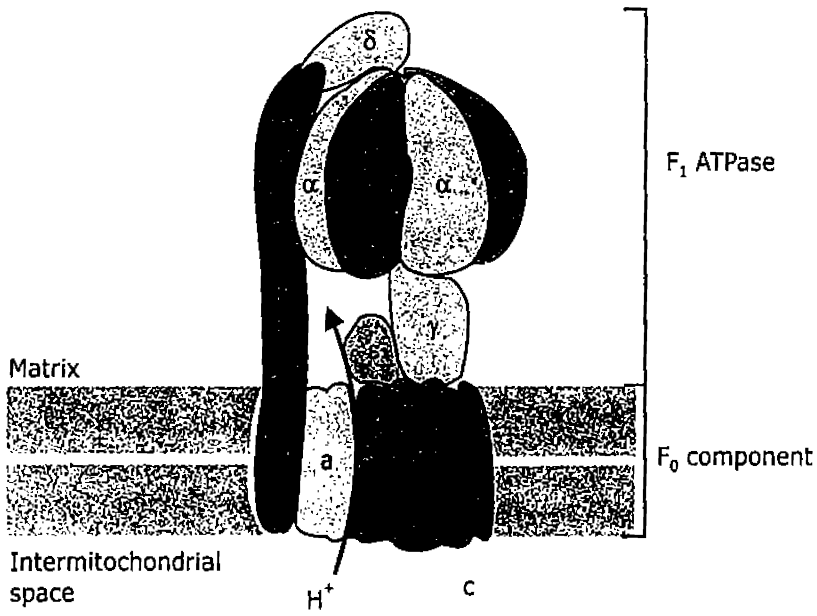


Figure : 7.17
The enzyme complex consists of an F_0 component and F_1 ATPase. Proton passing through the disc of 'C' units cause it and the attached γ subunit to rotate. The γ subunit fits inside the F_1 ATPase of a three α and three β subunits, which are fixed to the membrane and do not rotate.

F_1 ATPase was first extracted from the mitochondrial inner membrane and purified by Efraim Racker and his colleagues. F_1 cannot synthesize ATP from ADP and P_i; because it can catalyze the hydrolysis of ATP. Thus the enzyme was originally called F_1ATPase. The complete F_0F_1 complex, like isolated F_1, can hydrolyze ATP to ADP and P_i, but its biological function is to catalyze the condensation of ADP and P_i to form ATP. The F_0F_1 complex is therefore more appropriately called *ATP synthase*.

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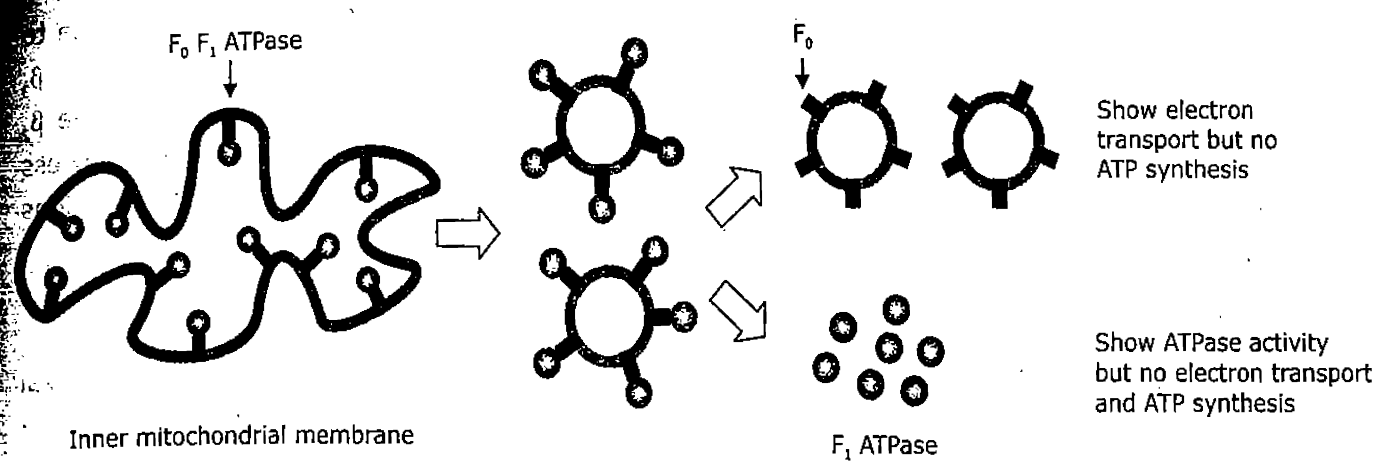


Figure 7.18 : F_1 particles are required for ATP synthesis, but not for electron transport. Submitochondrial vesicles from which F_1 is removed by mechanical agitation cannot catalyze ATP synthesis. Because F_1 separated from membranes is capable of catalyzing ATP hydrolysis, it has been called the F_1 ATPase.

ATP Synthesis

The *binding change mechanism* is a widely accepted model of ATP synthesis. Paul Boyer developed the binding change, or flip-flop mechanism, which postulated that ATP synthesis is coupled with a conformational change in the ATP synthase generated by rotation of the gamma subunit. Proton translocation through F_0 powers the rotation of the γ-subunit of F_1 ATPase, leading to changes in the conformation of the nucleotide-binding sites in the F_1 β-subunits (as described below). By means of this binding change mechanism, the F_0F_1 complex harnesses the proton-motive force to power ATP synthesis.

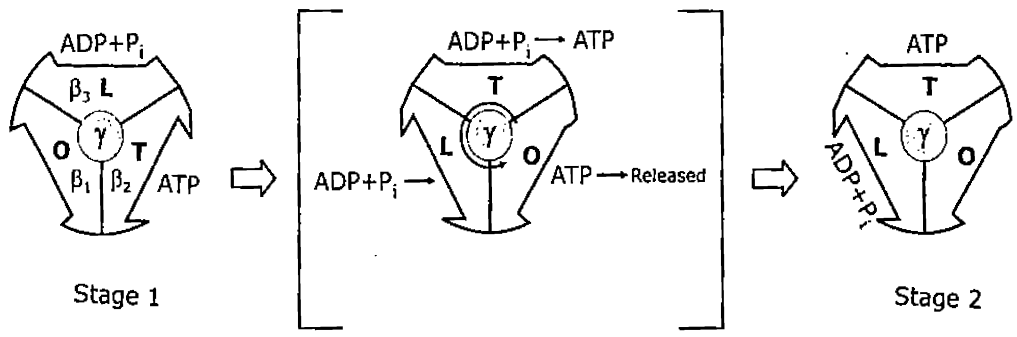


Figure 7.19 : The binding-change mechanism of ATP synthesis from ADP and P_i by the F_0F_1 complex. The molecule contains three binding sites, which interconvert between three conformational states as the molecule rotates. The diagram shows one stage of the active cycle. The three αβ-dimers have three different states. In 1, the open state O is empty; the loose state L contains ADP + P_i ; and the tight state T contains ATP. In logical intermediate stage (bracketed), rotation of the γ within the $(\alpha\beta)_3$ hexamer converts the L state to a T state, the T state to an O, and the O state to an L. The L state can accept a new charge of substrate, the T state can form ATP. At stage 2, the ATP has fallen out of the O state, new ADP + P_i have bound to the L state, and ATP has been synthesized in the T state.

The three $F_1\beta$ subunits alternate between three conformational states that differ in their binding affinities for ATP, ADP and P_i : one that binds substrates and products loosely (L state), one that binds them tightly (T state), and one that does not bind them at all (open or O state). The free energy released on proton translocation is harnessed to interconvert these three states. The phosphoanhydride bond of ATP is synthesized only in the T state and ATP is released only in the O state.

Suppose ADP and P_i bind to one of the three β subunits, for example β_1 subunit whose nucleotide-binding site is in the O (open) conformation. Proton flux powers a 120° rotation of the γ subunit. This causes an conformational change in β_1 subunit. It changes from O to T state. It increases the binding affinity of the β_1 subunit for ADP and P_i. There is an increase in the binding affinity of the β_3 subunit for ADP and P_i due to

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change in conformation from L to T, and a decrease in the binding affinity of the β_2 subunit for ATP due to change from T to O, causing release of the bound ATP. In step 2, the ADP and P_i in the T site (here the β_3 subunit) form ATP, a reaction that does not require an input of energy, and ADP and P_i bind to the β_2 subunit, which is in the O state. This generates an F_1 complex identical with that which started the process except that it is rotated 120°. Step 1 now occurs again, and the cycling of the $O \rightarrow L \rightarrow T \rightarrow O$ conformations of each β subunit continues.

Calculation of free energy change

The standard free energy change for movement of protons across the membrane along the electrochemical proton gradient can be calculated from the Nernst equation:

$$\Delta G \text{ (cal/mol)} = -n\mathcal{F}\Delta E$$

We can calculate the amount of free energy released by the passage of one mol of protons down an electrochemical gradient of 220mV from above equation.

$$\Delta G = -n\mathcal{F}\Delta E$$
$$= 23{,}062 \times 0.22 \text{ V} \quad (n=1)$$
$$= -5074 \text{ cal/mol or } -5.1 \text{ kcal/mol}$$

The most widely accepted experimental value for number of protons required to drive the synthesis of one ATP molecule is four. Hence if 10 protons are pumped out per NADH, four must flow in to produce one ATP, the proton-based P/O ratio (the ratio of ATP synthesized to oxygen reduce to water molecule) is 2.5 for NADH (1.5 for $FADH_2$).

Uncoupling agents

Uncoupling agents uncouple oxidation from phosphorylation. Electron transport continues unabated, but ATP synthesis stops. Most common uncoupling agents are *2,4,dinitrophenol* (DNP), *dicoumarol* and carbonylcyanide-p-trifluoromethyoxyphenylhydrazone (FCCP). DNP is weak acid that is soluble in lipid bilayer both in their protonated neutral forms and in their anionic states. DNP in anionic state pick up protons in the inter mitochondrial space and diffuse readily across mitochondrial membranes. After entering the matrix in the protonated form, they can release a proton, thus dissipating the proton gradient and thus inhibits ATP synthesis. Dicoumarol and FCCP act in the same way. Similarly, **thermogenin** is a physiological uncoupler found in brown adipose tissue that function to generate body heat, particularly for the new born and during hibernation in animals.

Role of ionophore

Ionophores are lipophilic molecules that complex specific cations and facilitate their transport through membrane. Ionophore uncouple electron transfer from oxidative phosphorylation by dissipating the electrical contribution to the electrochemical gradient across the mitochondrial membrane. **Valinomycin**, an antibiotic is an example of ionophore. It allows inorganic ions (K^+) to pass easily through membranes.

ATP-ADP exchange across the inner mitochondrial membrane

Most of the ATP generated by oxidative phosphorylation in mitochondria is exported to the cytoplasm. Thus mitochondria must contain mechanisms to export ATP and import ADP and P_i. This is mediated by two proteins in the inner membrane: a *phosphate transporter* (HPO_4^{2-}/OH^- antiporter) and an *ATP/ADP antiporter*. The proton-motive force across the inner mitochondrial membrane also powers the exchange of ATP formed inside the mitochondrion for ADP and P_i in the cytosol. Similarly, pyruvate, the end product of aerobic glycolysis, is transported into mitochondria via an electrically neutral counterexchange with OH^-, and its transport, like that of P_i, is linked to ΔpH.

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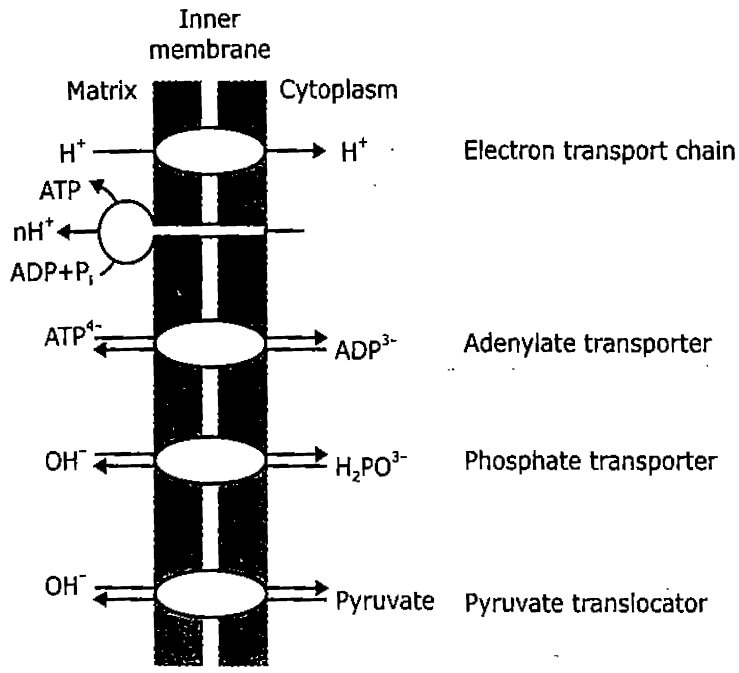


Figure : 7.20
The phosphate and ATP/ADP transport system in the inner mitochondrial membrane.

Summary of ATP synthesis from the oxidation of one molecule of glucose

Glycolysis (cytoplasm)	**NADH**	**$FADH_2$**	**ATP**
Glucose → Glucose-6-phosphate			-1
Fructose-6-phosphate → Fructose-1,6-bisphosphate			-1
2 Glyceraldehyde-3-phosphate → 2 Glycerate-1,3-bisphosphate	+2		
2 Glycerate-1,3-bisphosphate → 2 Glycerate-3-phosphate			+2
2 Phosphoenolpyruvate → 2 Pyruvate			+2
Mitochondrial reactions			
2 Pyruvate → 2 Acetyl-CoA	+2		
Citric acid cycle			
2 Isocitrate to 2 α-ketoglutarate	+2		
2 α-ketoglutarate to 2 succinyl CoA	+2		
2 Succinyl CoA to 2 succinate			+2
2 Succinate to 2 Fumarate		+2	
2 Malate to Oxaloacetate	+2		
	10 NADH	**2 $FADH_2$**	**4ATP**

Total yield of ATP from the complete oxidation of one molecule of glucose (via glycolysis, pyruvate dehydrogenase compelx reaction, TCA and oxidative phosphorylation) = 32 (or 30). This is calculated as 2.5 ATP per NADH and 1.5 ATP per $FADH_2$. Total number is either 30 or 32 depending on the mechanisms used to shuttle NADH equivalents from the cytosol to the mitochondrial matrix.

P/O ratio

Under aerobic condition, each mole of NADH or $FADH_2$ carriers $2e^-$ and each $2e^-$ reduce one atom of O. Therefore under aerobic conditions ATP production from oxidative phosphorylation with O_2 as the electron acceptor can be equated to electron released or atoms of O consumed. The ratio of ATP production to e^-

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released or atoms of O consumed may be designated the P/O or P/2e⁻ ratio, under aerobic conditions. The P:O ratio is equal to the product of ($H^+/2e^-$) and ($\sim P/H^+$). Note that the P:O ratio is identical with the (P:2e-) ratio.

Estimation of the number of ATP molecules formed during aerobic state can be calculated from the P/O ratio. During transport of two electrons from NADH to oxygen, about 10 protons are transported into the intermembrane space, while from $FADH_2$, the number is only six. ATP synthase probably requires three H^+ to synthesize one ATP, so that maximum P/O ratio of around 3 (in case of NADH) or 2 (in case of $FADH_2$) are possible. This implies a yield of up to 38 ATP per mol of glucose. However, the actual value is lower. It needs to be taken into account that the transport of specific metabolites into the mitochondrial matrix and the exchange of ATP^{4-} for ADP^{3-} are also driven by the proton gradient. The P/O ratio for the oxidation of NADH and $FADH_2$ are therefore 2.5 and 1.5, respectively.

7.8 RESPIRATORY SUBSTRATE AND RESPIRATORY QUOTIENT (RQ)

Catabolism or breakdown of dietary fuels occur in three stages.

Stage 1 : Breakdown of large molecules of food to simple building blocks. Carbohydrates in the form of polysaccharides are broken down to simple sugars such as glucose. Proteins are converted to amino acids. Neutral fats are converted to free fatty acids and glycerol.

Stage 2 : Breakdown of simple building blocks to acetyl-CoA.

Stage 3 : Complete oxidation of acetyl-CoA to CO_2 and water with the production of ATP.

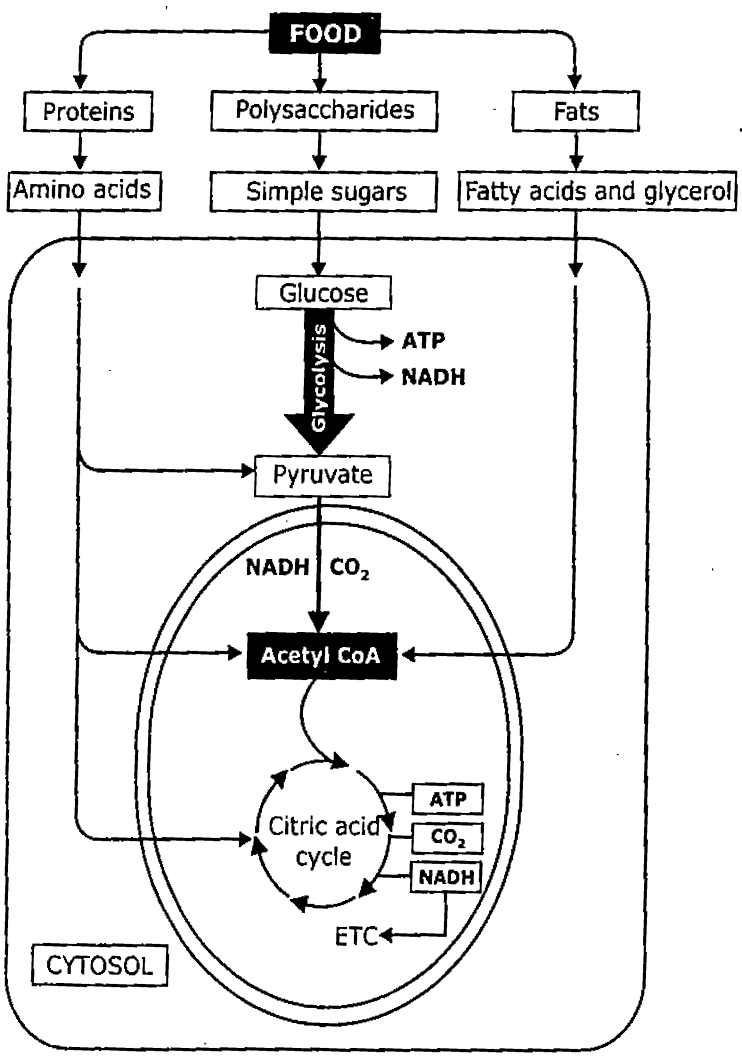


Figure : 7.21
Simplified diagram showing the fate of respiratory substrates

Respiratory Quotient is defined as the ratio of moles of CO_2 produced to the moles of oxygen consumed during complete oxidation of a metabolic fuel to CO_2 and H_2O. For carbohydrates, such as glucose, the RQ is given by

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$$C_nH_{2n}O_n + nO_2 \rightarrow nCO_2 + nH_2O$$

The number of molecules of O_2 consumed to the molecules of CO_2 produced. We have a ratio of 1 to 1, since there are n O_2 and n CO_2 molecules. So, RQ = n / n = 1 and for glucose, RQ = 6 / 6 = 1.

RQ is less than one when respiration is aerobic but the respiratory substrate is either fat or protein. RQ is about 0.7 for most of common fats and about 0.9 in case of proteins.

For palmitate, $$RQ = \frac{16 \text{ mol of } CO_2 \text{ produced}}{23 \text{ mol } O_2 \text{ consumed}} = 0.7$$

RQ is more than unity when organic acids such as oxalic acid, malic acid are broken down as respiratory substrate under aerobic condition.

7.9 GLYOXYLATE CYCLE

The glyoxylate cycle is a cyclic pathway that converts two molecules of acetyl-CoA, to one molecule of succinate.

$$2 \text{ AcetylCoA} + NAD^+ + 2H_2O \longrightarrow \text{Succinate} + NADH + 3H^+ + 2CoA-SH$$

This process occurs in some bacterial and plant cells. In plant cells this process occurs in the **glyoxysome** (present only in plant cells); a specialized organelle that carries out both β-oxidation of fatty acids to acetyl-CoA and utilization of that acetyl-CoA in the glyoxylate cycle.

The glyoxylate cycle consists of five reactions. The succinate produced (as described below in diagram) is transported to the mitochondria, where it enters the TCA cycle and is converted to malate, which has two alternative fates:

1. It can be converted to oxaloacetate in mitochondria.
2. It can be transported to the cytosol where it is converted to oxaloacetate for entry into gluconeogenesis.

The glyoxylate cycle allows plants and bacteria to carry out net conversion of fat to carbohydrate, bypassing CO_2-generating reactions of the citric acid cycle.

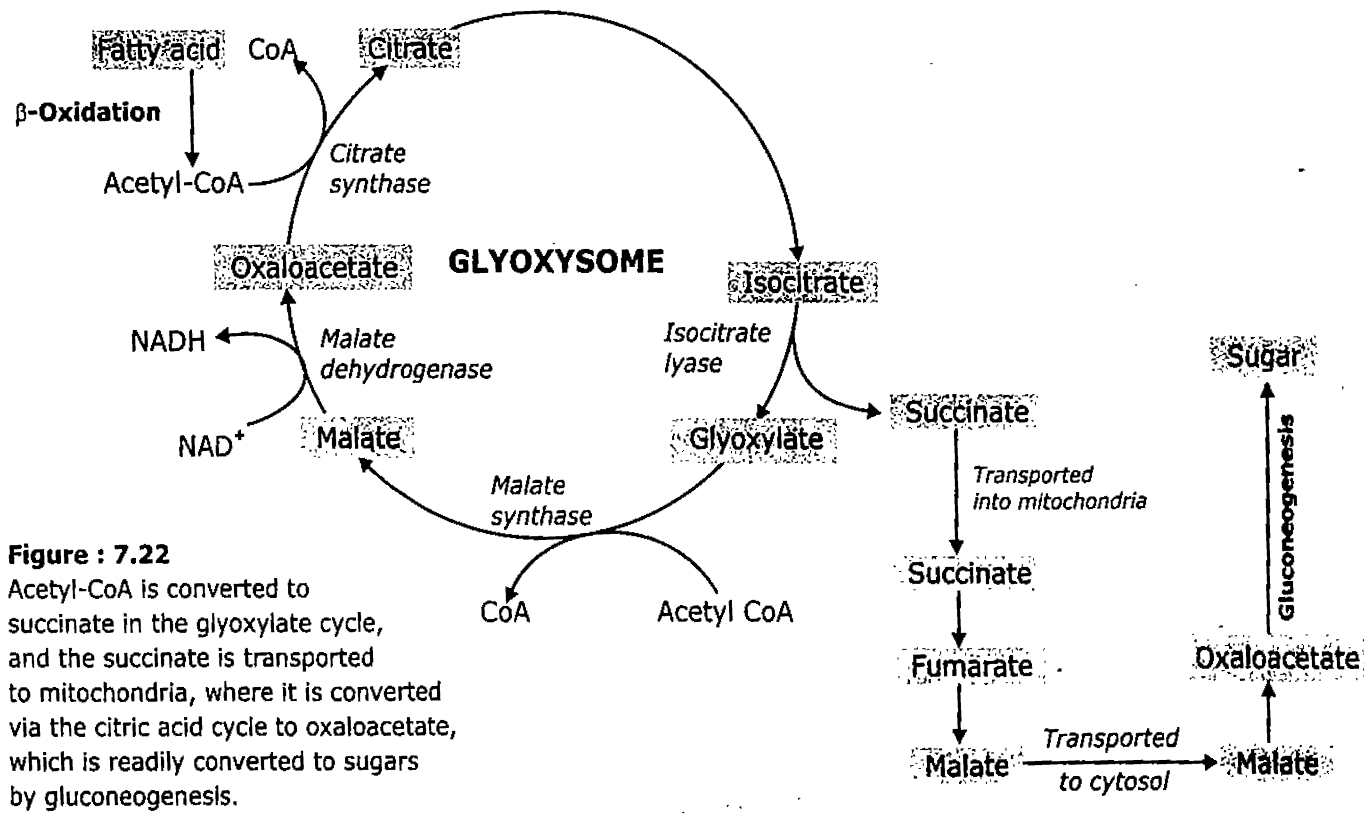


Figure : 7.22
Acetyl-CoA is converted to succinate in the glyoxylate cycle, and the succinate is transported to mitochondria, where it is converted via the citric acid cycle to oxaloacetate, which is readily converted to sugars by gluconeogenesis.

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7.10 PENTOSE PHOSPHATE PATHWAY

Hexose monophosphate shunt or pentose phosphate pathway or phosphogluconate pathway is an alternative pathway to glucose oxidation. The enzymes of HMP shunt are located in the cytosol. Hexose monophosphate shunt has two primary functions

1. To provide NADPH for reductive biosynthesis and
2. To provide ribose-5-phosphate for nucleotide and nucleic acid biosynthesis.

The sequence of reactions of HMP shunt is divided into two phases–oxidative and non-oxidative phase.

Oxidative phase (Irreversible)

Glucose 6-phosphate dehydrogenase (G6PD) is an NADP-dependent enzyme that converts glucose 6-phosphate to 6-phosphogluconolactone. The latter is then hydrolysed by the gluconolactonase to 6-phosphogluconate. The next reaction involving the synthesis of NADPH is catalyzed by 6-phosphogluconate dehydrogenase to produce 3-keto 6-phosphogluconate which then undergoes decarboxylation to give ribulose 5-phosphate.

The net result of the oxidative phase is generation of 2 moles of NADPH, oxidation of one carbon to CO_2, and synthesis of 1 mole of pentose phosphate.

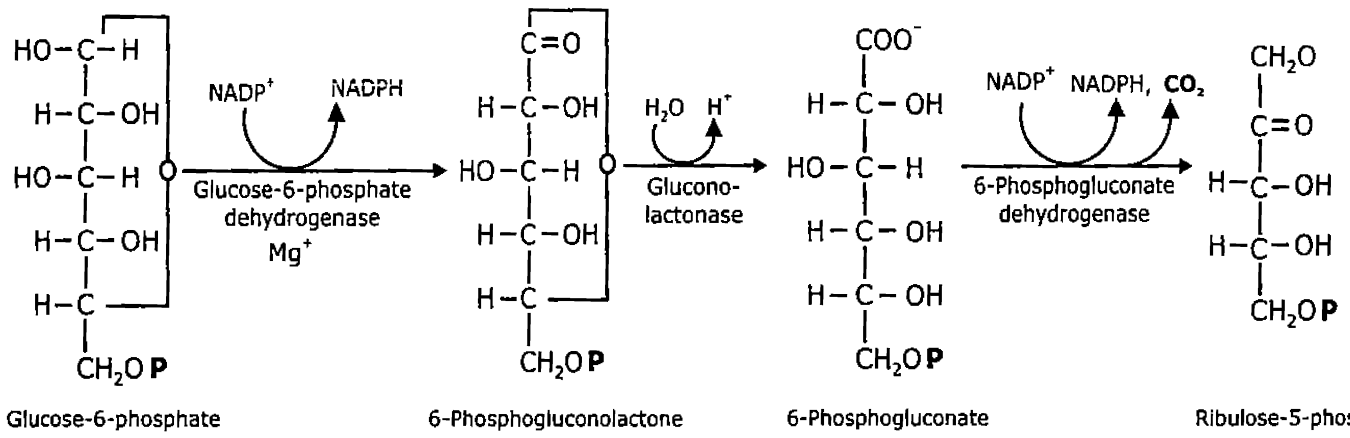
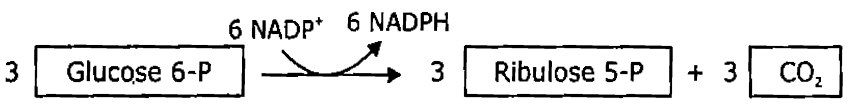


Figure 7.23 : Oxidative phase of the pentose phosphate pathway. The three reactions of the oxidative phase include two oxidations, which produce NADPH.



Note : In pentose phosphate pathway, oxidation of glucose-6-Phosphate utilizes $NADP^+$ rather than NAD^+ and produces CO_2. Whereas glycolytic process utilizes NAD^+ and does not produce CO_2. Similarly no ATP is generated in the pentose phosphate pathway, where as it is a major product of glycolysis.

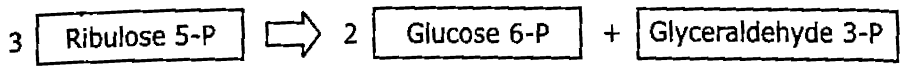
Non-oxidative phase (Reversible)

In oxidative phase, three molecules of glucose-6-phosphate (G6P) give rise to three molecules of CO_2 and three five carbon sugars. These are rearranged to regenerate two molecules of glucose-6-phosphate and one molecule of glyceraldehyde-3-phosphate (G3P) in non-oxidative phase.

The non-oxidative reactions are concerned with the interconversion of three, four, five and seven carbon monosaccharides. Ribulose 5-phosphate (Ru5P) is acted upon by an *epimerase* to produce xylulose 5-phosphate (Xu5P) while ribose 5-phosphate ketoisomerase converts ribulose 5-phosphate to ribose

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5-phosphate. In the non-oxidative phase, some of the Ru5P produced in the oxidative phase is converted to ribose-5-phosphate (R5P) by *phosphopentose isomerase.*



Overview of pentose phosphate pathway

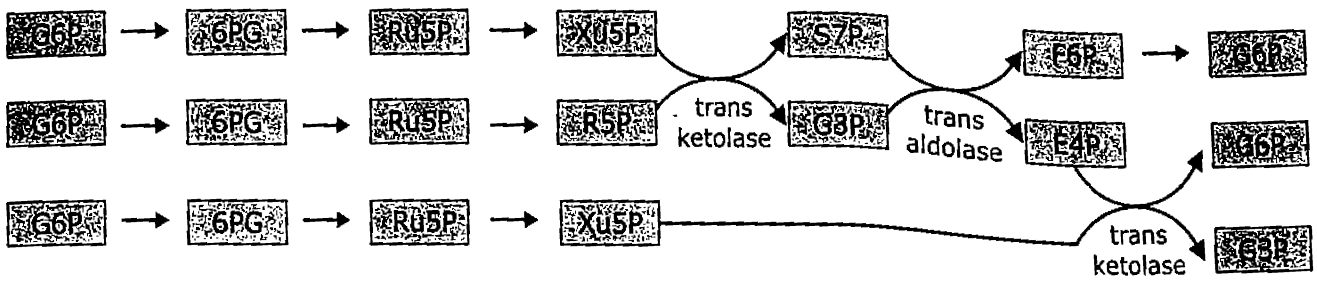


Figure 7.24 : Flowchart of pentose phosphate pathway.

G6P (Glucose 6-phosphate), 6PG (6-Phosphogluconate), Ru5P (Ribulose-5-phosphate) Xu5P (Xylulose 5-phosphate), R5P (Ribose 5-phosphate), G3P (Glyceraldehyde 3-phosphate), E4P (Erythrose 4-phosphate), S7P (sedoheptulose 7-phosphate)

Function of pentose phosphate pathways

1. NADPH from the pentose phosphate pathway serves as a source of electrons for the reduction of molecules during biosynthesis.
2. The pathway synthesizes four- and five-carbon sugars for a variety of purposes. The four-carbon sugar erythrose 4-phosphate is used to synthesize aromatic amino acids and vitamin B_6 (pyridoxal). The pentose ribose 5-phosphate is a major component of nucleic acids, and ribulose 1,5-bisphosphate is the primary CO_2 acceptor in photosynthesis.
3. Intermediates in the pentose phosphate pathway may be used to produce ATP. Glyceraldehyde 3-phosphate from the pathway can enter the three-carbon stage of the glycolytic pathway and be converted to ATP and pyruvate.

Glucose-6-Phosphate Dehydrogenase (G6PD) deficiency

G6PD deficiency is an X-linked inherited disease. G6PD deficiency impairs the ability to form NADPH. NADPH is required for several reductive processes in addition to lipid biosynthesis. For example, erythrocyte membrane integrity requires a plentiful supply of reduced glutathione (GSH), a cys-containing tripeptide (γ-glutamyl-cysteinylglycine). A major function of GSH in the erythrocyte is to eliminate H_2O_2 and organic hydroperoxides. H_2O_2, a toxic product of various oxidative processes, reacts with double bonds in the fatty acid residues of the erythrocyte cell membrane to form organic hydroperoxides. These, in turn, results in premature cell lysis. Peroxides are eliminated through the action of glutathione peroxidase, yielding glutathione disulfide (GSSG).

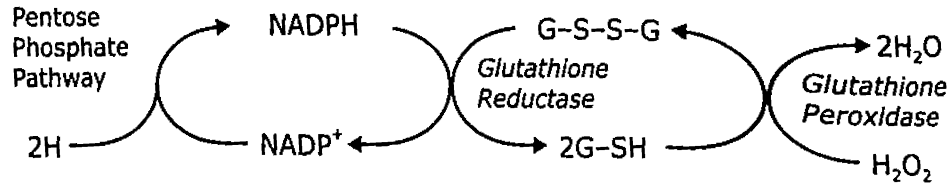


Figure 7.25 : Role of the pentose phosphate pathway in the reduction of oxidized glutathione.

So, G6PD deficiency results in hemolytic anemia caused by the inability to detoxify oxidizing agents.

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7.11 ENTNER-DOUDOROFF PATHWAY

Entner-Doudoroff pathway is an alternative pathway that catabolize glucose to pyruvate using a set of enzymes different from those used in either glycolysis or the pentose phosphate pathway. This pathway was first reported by Michael Doudoroff and Nathan Entner. This pathway occurs only in prokaryotes, mostly in gram-negative bacteria such as *Pseudomonas aeruginosa, Azotobacter, Rhizobium.*

In the Entner-Doudoroff pathway, glucose phosphate is oxidized to 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) which is cleaved by *2-keto-3-deoxyglucose-phophate aldolase* to pyruvate and glyceraldehyde-3-phosphate. The latter is oxidized to pyruvate by glycolytic pathway where in 2 ATP are produced by substrate level phosphorylations. This process yields 1 ATP as well as 1 NADH and 1 NADPH for every from one glucose molecule.

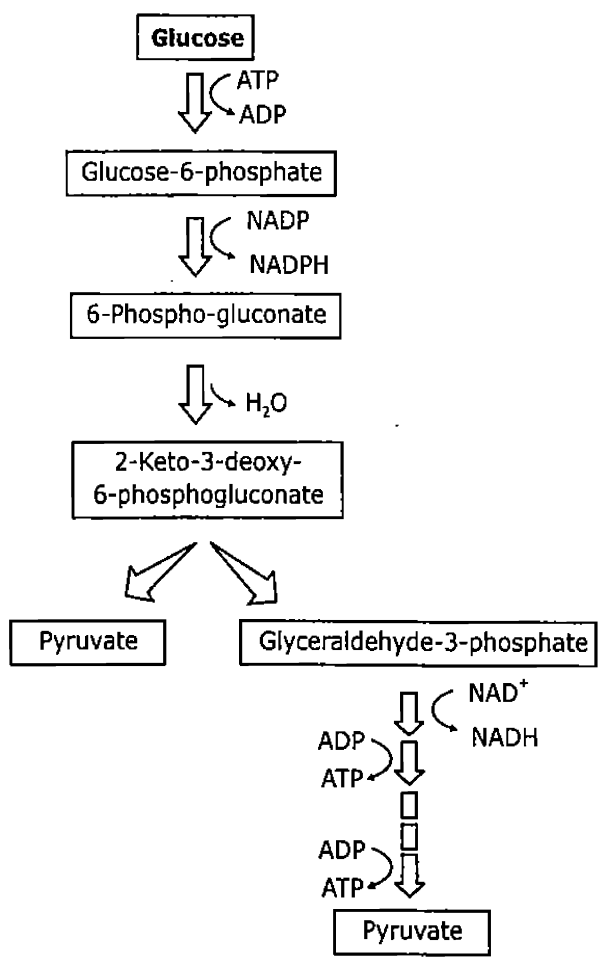


Figure 7.26 : Entner-Doudoroff pathway.

STUDY QUESTIONS

Q4. When O_2 is added to an anaerobic suspension of cells consuming glucose at a high rate, the rate of glucose consumption declines greatly as the O_2 is used up, and accumulation of lactate ceases. This effect, first observed by Louis Pasteur in the 1860s, is characteristic of most cells capable of both aerobic and anaerobic glucose catabolism.

a. Why does the accumulation of lactate cease after O_2 is added?

b. Why does the presence of O_2 decrease the rate of glucose consumption?

Ans. a. Because NADH is reoxidized via electron transfer instead of lactic acid fermentation.

b. Oxidative phosphorylation is more efficient and release more energy from 1 mole of glucose.

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Q5. How are the protons and electrons produced during glycolysis important in generating energy for the cell, and what relationship do they have with mitochondria?

Ans. The four electrons and two of the four protons produced in glycolysis are used to reduce the electron carrier NAD^+ to NADH. The electrons of NADH are transferred via an electron shuttle to the mitochondrial matrix, from which they can enter the electron transport chain in the inner mitochondrial membrane. Thus the energy of these electrons is used to produce the proton-motive force and is ultimately converted into ATP by the F_0F_1 ATP synthase.

Q6. Although both pyruvate dehydrogenase and glyceraldehydes 3-phosphate dehydrogenase use NAD^+ as their electron acceptor, the two enzymes do not compete for the same cellular NAD^+ pool. Why?

Ans. Pyruvate dehydrogenase is located in mitochondria; glyceraldehydes 3-phosphate dehydrogenase in the cytosol. The NAD^+ pools are separated by the inner mitochondrial membrane.

Q7. When pure reduced cytochrome c is added to carefully prepared mitochondria along with ADP, P_i, antimycin A, and oxygen, the cytochrome c becomes oxidized, and ATP is formed, with a P/O ratio (the ratio of ATP synthesized to O atoms reduced) approaching 1.0.

a. Indicate the probable flow of electrons in this system.

b. Why was antimycin A added?

c. What does this experiment tell you about the location of coupling sites for oxidative phosphorylation?

Ans. a. cyt c $\rightarrow$ cyt a $\rightarrow$ cyt $a_3 \rightarrow O_2$

b. To block oxidation of endogenous substrates.

c. One site associated with cytochrome oxidase.

Q8. If protons are to be transported from outside of the inner mitochondrial membrane to the inside, i.e., the matrix, in the presence of (i) a proton gradient whose outside concentration is 10^2 fold higher compared to the inside concentration and (ii) an electrical gradient whose value is 0.2 volt (the electrical potential being more negative inside the matrix). Calculate the value to free energy change that is associated with this electrochemical gradient at 27°C.

Given, Faraday constant , F = 96,500 joules/(volt-mole),

Gas constant, R = 8.3 joules/mole

Ans. $\Delta G = 2.303\ RT \log_{10} \dfrac{[C_2]}{[C_1]} + ZF\ \Delta\alpha$

where ΔG = free energy change associated with the translocation of protons;

F = Faraday constant = 96,500 joules/volt-mole;

R = 8.3 joules/mole;

$\Delta\alpha$ = membrane potential in volts = −0.2

z = charge on the molecule (+1 for H^+)

$$\frac{[C_2]}{[C_1]} = \frac{C_{inside}}{C_{outside}} = \frac{1}{10^2} = 10^{\square}$$

T = 273 + 27 = 300 kelvin

$$\therefore\ \Delta G = 2.303 \times 8.3 \times 300 \times \log\left(\frac{1}{10^2}\right) + 1 \times 96{,}500 \times (-0.2)$$

$$= -11{,}469 - 19{,}300 = -30{,}769 \text{ joules/mole}$$

Q9. Each of the cytochromes in the mitochondria contain a heme prosthetic group, which permits these proteins to transport electrons in the electron transport system.

a. Explain how heme prosthetic groups function as electron carriers.

b. What property of the various cytochromes assures unidirectional electron flow along the electron transport chain?

c. As electrons flow through the electron transport chain, they lose energy. How is much of this energy utilized?

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Ans. a. Each of the heme prosthetic groups present in cytochromes contains an iron atom that accepts an electron as it is reduced and releases an electron as it is oxidized. Because the heme ring has numerous resonance forms, the second electron is delocalized to the heme carbon and nitrogen atoms.

b. The various cytochromes in the electron transport chain contain heme prosthetic groups with different axial ligands. As a result, each cytochrome has a different reduction potential, so that electrons can move only in a single order through the electron carriers.

c. Much of the energy lost by electrons moving through the electron transport chain is used to pump protons from the matrix to the intermembrane space, thus generating the proton-motive force.

Q10. D-Ribose 5-phosphate is produced from glucose in the pentose phosphate pathway. Which of the following enzyme cofactors require D-ribose 5-phosphate for their synthesis: ATP, thiamine pyrophosphate, CoA, FAD, pyridoxamine, biotin, NAD^+?

Ans. ATP, CoA, FAD, and NAD^+ contain the D-ribose 5-phosphate moiety.

SELF TEST

Q1. Which statement is *true* of glucokinase?

a. it catalyzes the phosphorylation of fructose

b. it has a lower K_m for glucose than does hexokinase

c. it is found in liver

d. it is inhibited by glucose-6-phosphate **[JNU]**

Q2. Pyruvate kinase transfers a phosphate group from phosphoenolpyruvate to ADP, forming pyruvate and ATP. The reaction catalyzed by this enzyme is essentially irreversible. Which of the following is the best explanation for the irreversible nature of this reaction?

a. the binding of pyruvate to the active site is very weak relative to the binding of phosphoenolpyruvate.

b. the reaction is coupled to the pyruvate dehydrogenase reaction

c. the hydrolysis of ATP is highly favorable

d. the change in free energy for the overall reaction is large and negative

Q3. Incubation of a cell extract containing all enzymes of glycolysis with [γ^{32}P] ATP and unlabeled inorganic phosphate, results in the formation of which of the following labeled compounds (assume that pyruvate kinase is inactivated)?

1. Glucose-6-^{32} phosphate
2. (3-^{32}P)-phosphoglycerate
3. (1-phospho-3-^{32}phospho)-bisphosphoglycerate
4. (1-phospho-6-^{32}phospho)-fructose bisphosphate

a. 1 and 3

b. 1, 2 and 3

c. 2 and 4

d. only 4 **[GATE]**

Q4. One of the carbon atoms of a glucose molecule is [^{14}C]-labeled. If $^{14}CO_2$ is released during the conversion of pyruvate to acetyl coenzyme-A, which carbon atom of glucose was radio labeled?

a. C3 but not C4

b. C3 or C4

c. C1 or C6

d. C1 but not C6 **[GATE]**

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Q5. Iodoacetamide irreversively inhibits few enzymes by reacting with the amino acid residue at the active site having the functional group.

a. –SH
b. –COOH
c. –OH
d. $-NH_2$ **[CSIR-UGC]**

Q6. Which of the following statements about the enzyme complexes of the electron transport system is *correct*?

a. they interact with one another via mobile electron carriers
b. they are located in the mitochondrial matrix
c. they can not be separated from one another in a functional form
d. they all have cytochromes **[GATE]**

Q7. Plant mitochondria were treated with antimycin A and sodium azide. This would lead to

a. increased cyanide resistant respiration and reduced ATP synthesis
b. increased cyanide resistant respiration and increased ATP synthesis
c. increased cytochrome oxidation
d. reduced nuclear-mitochondria interaction **[JNU]**

Q8. Thermogenin, the natural uncoupler in brown fat mitochondria, generates heat based on its ability to

a. inhibit electron transport by binding to b-type cytochromes
b. allow protons to re-enter into the mitochondrial matrix
c. inhibit ATP production by binding to the ATP synthase
d. block electron transport to flavoproteins

Q9. 2,4-dinitrophenol uncouples electron transport from oxidative phosphorylation in mitochondria by

a. causing dissipation of the proton gradient generated by the electron transport
b. allowing the proton translocating function of ATP synthase while inhibiting its ATP synthesizing activity
c. activating a second proton pump that sends back the protons into the mitochondrial matrix
d. neutralizing the proton gradient by absorbing the protons generated during the electron transport

Q10. The number of protons translocated by the various vectorial localized on the inner mitochondrial membrane of an organism was cytochrome aa_3 complex: 4 and F_1F_0-ATPase: 3. One proton is also required for the transport of inorganic phosphate into the mitochondrial matrix.

I. The number of ATP molecules that can be synthesized by the oxidation of one NADH molecule is

a. 2
b. 2.5
c. 3
d. 3.3

II. If the cytosolic NADH is transported to the matrix by the glycerol 3-phosphate shuttle, then the number of ATPs synthesized is

a. 1.5
b. 2
c. 2.5
d. 3.3 **[GATE]**

Q11. More free energy is released during the citric acid cycle than during glycolysis, but only 1 mole of ATP is produced for each mole of acetyl CoA that enters the cycle. What happens to most of the remaining free energy that is produced during the citric acid cycle?

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a. it is used to synthesize GTP
b. it is used to reduce electron carriers
c. it is lost as heat
d. it is used to reduce pyruvate

Q12. The following 4 different solutions are prepared by mixing the components of electron transport chain. Which among them is expected to cause a net transfer of electrons to cytochrome c?

a. reduced ubiquinone and reduced cytochrome c
b. reduced ubiquinone, cytochrome b-c_1 complex and reduced cytochrome c
c. oxidized ubiquinone and oxidized cytochrome c
d. reduced ubiquinone, cytochrome b-c_1 complex and oxidized cytochrome c **[GATE]**

Q13. Choose the mismatch

Inhibitor	*Function*
a. Malonate	Prevents oxidation of succinate
b. Cyanide	Inhibits cytochrome oxidase
c. Oligomycin	Inhibits ATP synthase
d. Rotenone	Blocks $CoQH_2$-cyt C oxidoreductase

Q14. Treatment of intact mitochondria with an uncoupler, such as 2,4-dinitrophenol, in the presence of ADP, P_i, succinate, and oxygen would have which of the following effects on the rates of electron transport and ATP synthesis? (+ = stimulation; 0 = no effect; - = inhibition)

	Rate of electron transport	Rate of ATP synthesis
a.	+	+
b.	–	–
c.	+	–
d.	–	+

Q15. In the pentose phosphate pathway

a. only the C-1 carbon of glucose is oxidized to CO_2
b. all the carbons of glucose is oxidized to CO_2
c. no decarboxylation occurs
d. C-4 and C-5 of glucose is oxidized to CO_2

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Chapter - 08

Photosynthesis

8.1 CHLOROPLAST AND PHOTOSYNTHETIC PIGMENTS

Photosynthesis is a process by which photosynthetic organisms convert light energy into chemical energy in the form of reducing power (as NADPH) and ATP, and use these chemicals to drive carbon dioxide fixation and reduction to produce sugars. In oxygenic photosynthetic organisms, including higher plants, the source of reducing equivalents is water, releasing oxygen as a by-product. The overall reaction of oxygenic photosynthesis can be represented as

$$nCO_2 + nH_2O \xrightarrow[chl]{Light} (CH_2O)_n + nH_2O + nO_2$$

Chloroplast is a double membrane bound organelle. The outer membrane is believed to be eukaryotic in origin. The inner membrane is, according to the endosymbiont theory, the cell membrane of an ancient prokaryote that became an endosymbiont. The fluid inside this double-membrane organelle is called the **stroma**. The stroma is, according to the endosymbiont theory, the cytoplasm of the prokaryotic endosymbiont. Floating in the stroma are tiny membrane sacs. These are called *thylakoids*. The sacs are stacked in groups. Each group is called a *granum*. There are many grana in each chloroplast (usually 10 to 100 grana) which are interconnected by unstacked *stromal lamellae*. The lipids of the thylakoid membrane have a distinctive composition. About 80% lipids are uncharged mono- and digalactosyl diacylglycerol and only about 10% are phospholipids. The thylakoid membranes are the site of the photosynthetic light reactions. The thylakoid is thus the location for light absorption and ATP synthesis. The stroma uses these ATP to store the trapped energy in carbon-carbon bonds of carbohydrates. Chloroplast is a *semiautonomous* structure and divides by the process of *binary fission* as occur in bacteria. As one would suspect it has a nucleoid region to house the circular, dsDNA. It also holds 70S (prokaryotic-type) ribosomes.

Light-harvesting photosynthetic pigment molecules

The solar energy needed for photosynthesis is captured by pigment molecules. Different types of pigments described as photosynthetic pigment participate in this process. The major photosynthetic pigment is the chlorophyll.

Chlorophylls

Chlorophyll contains a polycyclic, planar tetrapyrrole ring, with a structure similar to that of the prosthetic heme group of hemoglobin and cytochromes, and is biosynthetically derived from protoporphyrin IX. It has following important features:

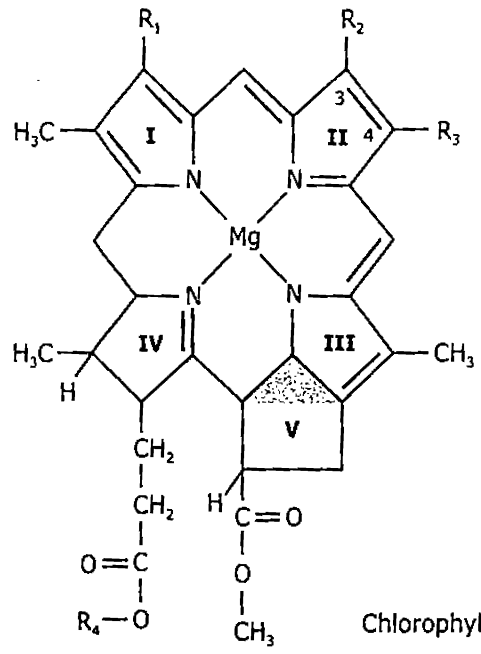
1. The central metal ion in Chl is Mg^{2+}, not Fe^{2+} or Fe^{3+} as in the hemes.
2. Chl has a cyclopentanone ring (ring V) with an attached carboxylic ester group, fused to pyrrole ring III.
3. Pyrrole ring IV of Chla and b is reduced, i.e., it contains two extra hydrogen atoms. In bacteriochloropylls a and b (BChla and BChlb), rings II and IV are reduced.
4. The propionyl group on ring IV of Chla and b and BChlb is esterified to a long-chain tetraisoprenoid alcohol. In chl a and b as well as Bchl b it is phytol. In BChla the propionyl group is esterified by either phytol or the all-trans geranylgeraniol.

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Photosynthetic organisms contain different types of chlorophyll molecules like Chla, BChla, Chlb, Chlc, Chld. These chlorophyll molecules differ by having different substituent groups on the tetrapyrrole ring.

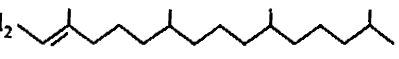
Table 8.1 : Differences between Chlorophyll *a* and Chlorophyll *b*

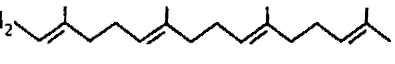
Chlorophyll a	*Chlorophyll b*
1. It is $C_{55}H_{72}O_5N_4Mg$.	1. It is $C_{55}H_{70}O_6N_4Mg$.
2. In pure state, chlorophyll *a* is blue-green.	2. In pure state, chlorophyll *b* is olive-green.
3. It is essential photosynthetic pigment.	3. It is accessory photosynthetic pigment.
4. Pyrrole ring II contains methyl ($-CH_3$) group.	4. Pyrrole ring II contains aldehyde (—CHO) group.
5. It absorbs more red wavelength than violet-blue wavelength of light.	5. It absorbs more violet-blue wavelength than red wavelength of light.



Chlorophyll structures

	R_1/C-2	R_2/C-3	R_3/C-4	R_4
Chlorophyll *a*	– CH = CH_2	– CH_3	– CH_2CH_3	P
Chlorophyll *b*	– CH = CH_2	– CHO	– CH_2CH_3	P
Bacteriochlorophyll *a*	– (C = O) – CH_3	– CH_3	– CH_2CH_3	P or G
Bacteriochlorophyll *b*	– (C = O) – CH_3	– CH_3	= CH – CH_3	P

P = $-CH_2$  Phytyl side chain

G = $-CH_2$  Geranylgeranyl side chain

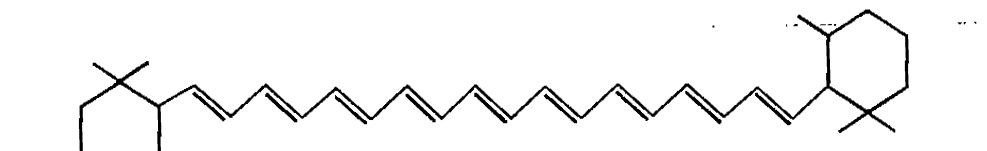
Accessory pigments

Besides the major light-absorbing chlorophyll molecules, there are two groups of accessory pigments which absorb light in the wavelength region where chlorophylls do not absorb strongly. The two types of accessory pigments are the **carotenoids** and **phycobilins**.

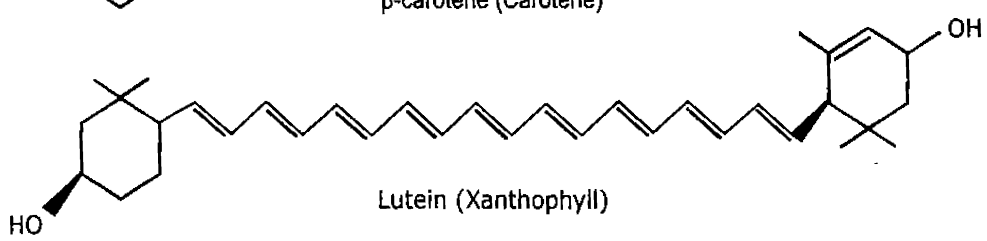
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Carotenoids are long-chain, conjugated hydrocarbons containing a string of isoprene residues and distinguished from one another by their end groups. Carotenoids are lipid soluble pigments.

The characteristic absorption spectrum of carotenoids is responsible for the yellow color of leaves in autumn and the orange color of carrots, for which this group of pigments is named. Carotenoid absorbance extends from the UV to about 550nm in the visible region. The upper wavelength limit of absorption by carotenoids depends on the number of conjugated double bonds, the isomeric configuration, and also on the nature of the end groups.



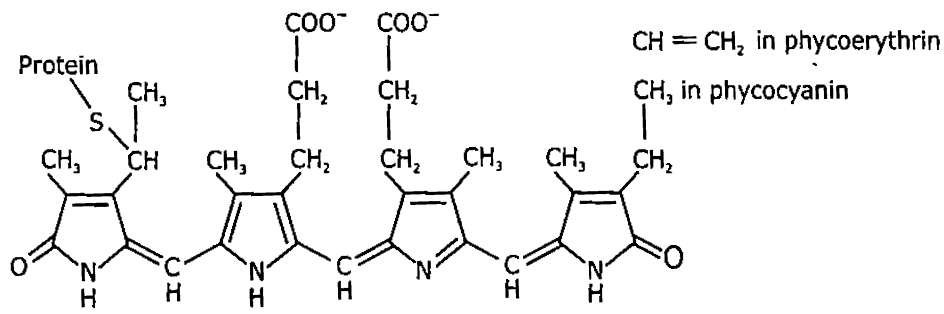
β-carotene (Carotene)



Lutein (Xanthophyll)

Function: The first role is as an accessory light-harvesting pigment. The other role of carotenoids is as a *quencher* of triplet chlorophyll to provide protection against photo-oxidative damage (called **solarization**).

The second group of non-chlorophyll, light-harvesting pigments are the **phycobilins**, which are abundant in red algae and cyanobacteria. Phycobilins are non-cyclic, linear tetrapyrroles, i.e., structures that may be considered as a chlorophyll tetrapyrrole ring opened up and the Mg^{2+} ion discarded. Phycobilins are soluble in water.



General structure of phycobilin

Absorption spectrum

Photosynthetic pigments absorb electromagnetic radiation in the visible region. A graph showing the degree of absorption of light by a pigment as a function of the wavelength is referred to as the *absorption spectrum* for that pigment. Chlorophylls absorb strongly in the blue-violet and red regions of the spectrum while carotenoids absorb in the blue and green regions. The colour of the carotenoids (yellow to orange and red) is usually masked by that of the chlorophylls, which are present in larger quantities.

Pigment	Chl *a*	Chl *b*	BChl *a*	BChl *b*	BChl *c*
Absorption maxima (nm)	430, 660	463, 643	364, 770	373, 795	434, 666

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Similarly, a graph showing the degree to which different wavelengths affect photosynthesis is called the **action spectrum** for photosynthesis. The action spectrum for photosynthesis is closely related with the action spectra for chlorophylls a and b and the carotenoids.

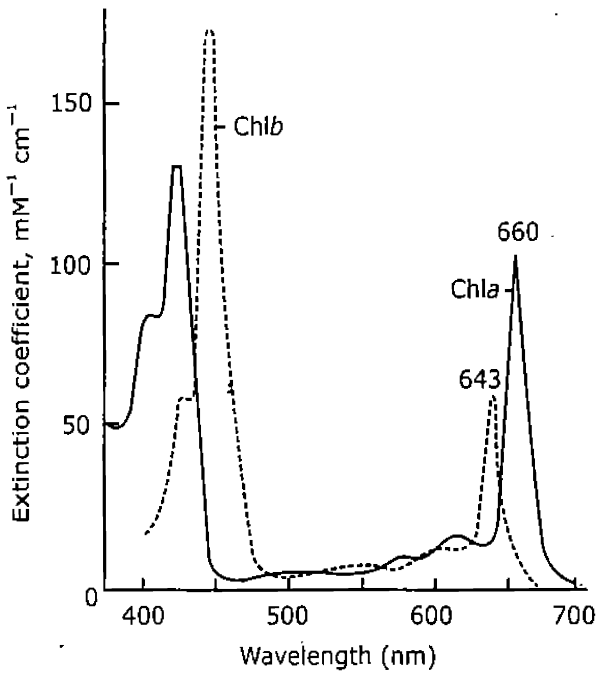
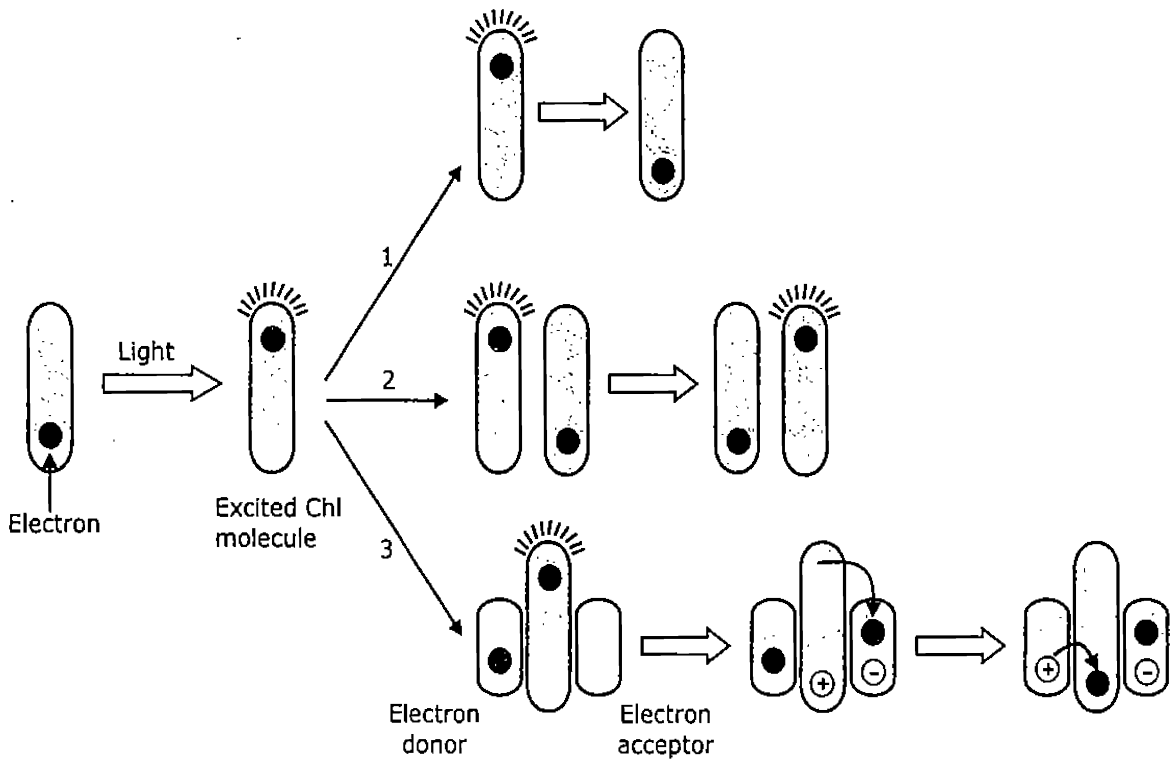


Figure 8.1 : Absorption spectra of Chl*a* and Chl*b* in ether.

FATE OF LIGHT ENERGY ABSORBED BY PHOTOSYNTHETIC PIGMENTS

Each photon represents a quantum of light energy. A molecule of chlorophyll on the absorption of light becomes excited to a higher energy state. An excited chlorophyll molecule is not stable and it returns rapidly to their ground level (unexcited state), in three possible ways:



Adapted and modified from *Molecular biology of the cell*, Alberts et. al, Garland Science

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converting the extra energy into heat or to some combination of heat and light of a higher wave th (**fluorescence**). An excited molecule in a singlet state usually has a maximum lifetime of ~10^{-8} due to an inherent tendency to fluoresce but can undergo various modes of nonradiative as well radiative decay, the latter generally termed *luminescence*. The fate of the excited molecule depends the pathway taken by the excitation energy. Depending on the mode of radiative decay, luminescence ay also be called fluorescence, phosphorescence, etc. Once the excited molecule has returned to e of the lower vibrational sublevels of the first excited singlet state, it can return to one of the wer vibrational sublevels of the ground state by emitting a photon as *fluorescence*.

nother important energy pathway is called *intersystem crossing*, which refers to the conversion of an xcited singlet state to an excited *triplet state*, followed by a radiative or nonradiative decay of the excited triplet state to the ground singlet state. The light emitted by the radiative, triplet-to-ground tate transition is called **phosphorescence**.

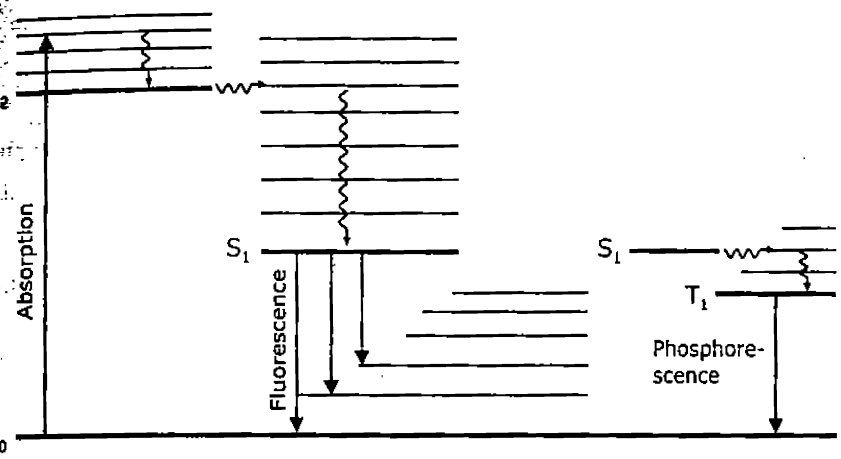


Figure : 8.2
Excited states of Chl. S_0 represents the ground state, S_1 and S_2 represent the lowest vibrational energy level of the first and second excited singlet states, respectively, and T_1 represents the lowest vibrational energy level of the triplet state. The other horizontal lines represent vibrational energy levels associated with each electronic state.

2. By transferring the energy but not the electron directly to a neighbouring chlorophyll molecule by a process called **resonance energy transfer** (also known as forster transfer). The excitation energy is trapped by the reaction center because the excited state of its Chl has a lower energy than that of the antenna molecules.

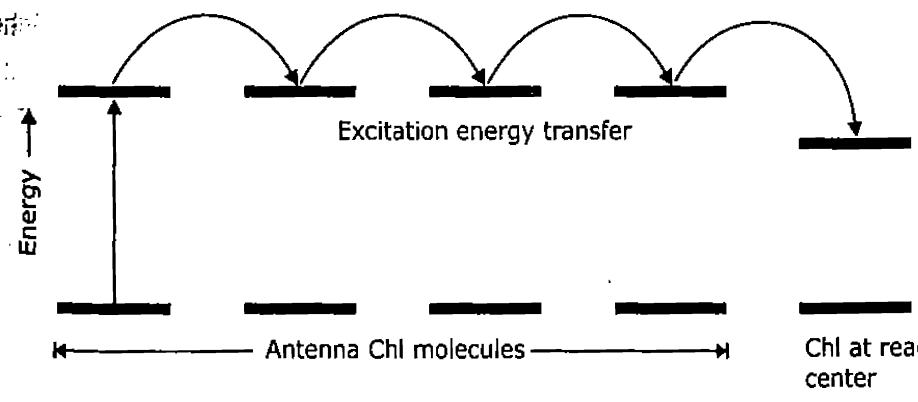


Figure : 8.3
Energy level diagram illustrating excitation transfer. The excitation energy is trapped by the reaction center because the excited state of its Chl has a lower energy than that of the antenna molecules.

3. By transferring the high energy electron to another nearby molecule (*electron acceptor*) and then returning to its original state by taking up a low energy electron from some other molecule (*an electron donor*) i.e., photooxidation.

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8.2 CONCEPT OF PHOTOSYNTHETIC UNIT

In 1932, Robert Emerson and William Arnold suggested that not all the chlorophyll molecules in a chloroplast were directly involved in the conversion of light energy into chemical energy. Using suspensions of the alga *Chlorella* and flashing lights of very short duration (~10μsec) at saturating intensity, they determined the minimum amount of light needed to produce maximal oxygen production during photosynthesis. Based on the number of chlorophyll molecules present in the preparation, they calculated that one molecule of oxygen was being released during a brief flash of light for every two thousand five hundred molecules of chlorophyll present. It was later shown that a minimum of 8 photons must be absorbed to produce one molecule of oxygen and for this about 300 or so chlorophyll molecules act together as one **photosynthetic unit** in which only one member of the group- the *reaction center chlorophyll*- actually transfers electrons to an electron acceptor.

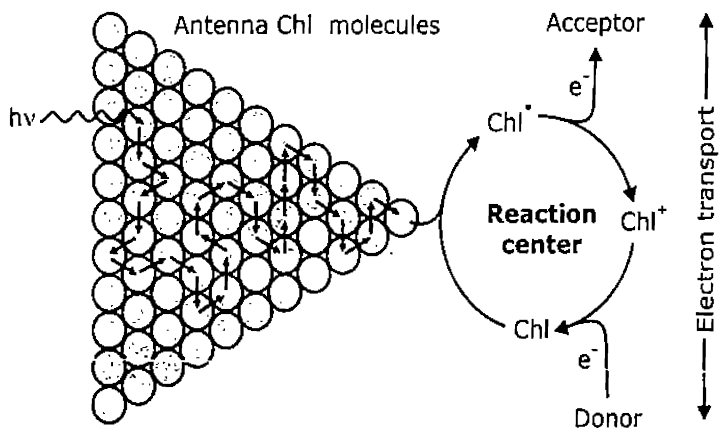


Figure : 8.4
Simplified representation of the photosynthetic unit consisting of the light-harvesting antenna chlorophyll molecules and the reaction center, small arrows in the Chl antenna represent migration of electronic excitation; Chl* is electronically excited chlorophyii in the reaction enter.

8.3 HILL REACTION

In 1937, Robert Hill, separated photosynthetic particles of green leaves. Hill's chloroplast did not evolve O_2 when illuminated but did so when suitable electron acceptor (oxidants) like potassium ferrioxalate or potassium ferricyanide was added to the illuminated suspension. This phenomenon is known as *Hill reaction*. It is a light driven transfer of electron from water to non-physiological oxidants (Hill reagent). Later S. Ochoa showed that $NADP^+$ is the biological electron acceptor in the chloroplast.

$$2H_2O + 2NADP^+ \xrightarrow{\text{Light}} 2NADPH + 2H^+ + O_2$$

8.4 OXYGENIC AND ANOXYGENIC PHOTOSYNTHESIS

Cornelis van Neil, Dutch microbiologist, found that photosynthetic bacteria *Chromatium vinosum* assimilates CO_2 in light without evolving O_2. Such bacteria use H_2S, instead of water as electron donor and forms sulphur instead of O_2.

$$CO_2 + 2H_2S \xrightarrow[\text{Bchl}]{\text{Light}} (CH_2O) + H_2O + 2S$$

The chemical similarity between H_2S and H_2O led van Niel to propose that the general photosynthetic reaction is

$$CO_2 + 2H_2A \xrightarrow{\text{light}} (CH_2O) + 2A + H_2O$$

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where H_2A is H_2O in green plants and cyanobacteria and H_2S in photosynthetic sulfur bacteria.

The validity of van Niel's hypothesis was established in year 1941 by Ruben and Kamen. They directly demonstrated by isotopic study using ^{18}O labeled water and unlabeled CO_2 on green alga *chlorella* that the source of oxygen formed in photosynthesis is water.

$$CO_2 + 2H_2{}^{18}O \xrightarrow[Chl]{Light} (CH_2O) + H_2O + {}^{18}O_2$$

The photosynthetic organism may be **oxygenic** (cyanobacteria and eukaryotic photosynthetic organisms) or **anoxygenic** (green and purple photosynthetic bacteria) on the basis of evolution of oxygen during photosynthesis.

Table 8.2 : Example of oxygenic and anoxygenic photosynthetic organisms

Organisms	*Reductant*	*Reaction*
Plants, algae, cyanobacteria	H_2O	$CO_2 + 2H_2O \rightarrow [CH_2O] + H_2O + O_2$
Green sulfur bacteria	H_2S	$CO_2 + 2H_2S \rightarrow [CH_2O] + H_2O + 2S$
Purple sulfur bacteria	$[HSO_3^-]$	$CO_2 + H_2O + 2[HSO_3^-] \rightarrow [CH_3O] + 2[HSO_4^-]$
Nonsulfur photosynthetic bacteria	H_2 or many other reductants, such as lactate	$CO_2 + 2H_2 \rightarrow [CH_2O] + H_2O$

8.5 CONCEPT OF PIGMENT SYSTEM

In 1943, Robert Emerson and Charlton Lewis examined the action spectrum in the visible region for oxygen evolution in the green algae *Chlorella pyrenoidosa*. They found the quantum yield remained fairly constant upto 685nm, beyond which it declined sharply. This drop in quantum yield in the long red region of the spectrum was called the *red drop phenomenon*. **Quantum yield** is the number of oxygen molecules produced per photon absorbed. Reciprocal of quantum yield is *quantum requirement* i.e., number of photons needed for each oxygen molecule produced.

In the another experiment Emerson and his colleagues set up two beams of light- one in the region of 650-680nm and the other in the region of 700-720nm. Emerson found that when the two beams were applied simultaneously, the rate of photosynthesis was 2-3 times greater than the sum of the rates obtained with each beam separately. This phenomenon is known as **Emerson enhancement effect**. The enhancement effect suggests that photosynthesis involves two photosystems—one driven by long wavelength light greater than 680nm and one driven by short wavelength light less than or equal to 680 nm.

Pigment systems

In all natural photosynthetic systems, pigment molecules are bound to polypeptides forming pigment protein complexes called **photosystem** or **pigment system**. The photosystems have two components :

Photochemical reaction center that carries out photochemical charge separation and electron transport. Reaction center contains the special pair of chlorophyll a molecules that participates in photochemical reaction.

Antenna complex : It consists of a number of distinct pigment - protein complexes (known as light harvesting complexes, LHC). The antenna complex captures light energy and feeds it to the reaction center by a process called resonance energy transfer.

Pigment systems are of two types in oxygenic photosynthetic organisms like plants, cyanobacteria, on the basis of differences in their light absorption maxima by reaction center chlorophyll molecules.

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Photosystem I (PSI)

PSI is driven by light of wavelength 700nm or less. In PSI, photochemical reaction center is designated as **P700** because it absorbs wavelength maximum at 700nm. The two photosystems are distributed differently in thylakoid membrane. PSI found almost exclusively in stroma lamellae (stroma thylakoid) and at the edges of the grana lamellae (grana thylakoid) and a high ratio of Chl*a* to Chl*b*.

Photosystem II (PSII)

PSII is driven by light of wavelength shorter than 690nm. Its absorption maxima is light of wavelength 680nm. Photochemical reaction center designated **P680**. PSII located predominantly in grana lamellae and its antenna complex has nearly equal amount of Chl a and Chl b and carotenoid pigments.

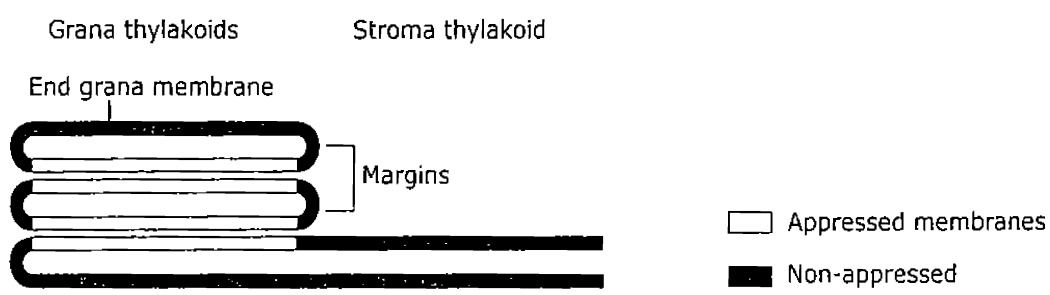


Figure 8.5 : The outer membrane surface of the thylakoids can be divided into two structurally different regions: the appressed membranes of the grana thylakoids whose surface is in contact with other membranes, and the non appressed membranes of the stroma thylakoids and the ends and margins of the grana thylakoids that are also exposed to the stroma.

8.6 STAGES OF PHOTOSYNTHESIS

Photosynthesis is a two stage process - one stage is dependent on the light and other is independent of it. The **Light reactions**, a *light-dependent reactions* which occur in the grana, and require the direct energy of light to make energy-carrier molecules that are used in the dark reaction. The light energy is trapped by chlorophyll to make ATP (called *photophosphorylation*) and NADPH.

The **Dark reaction**, a *light-independent reaction* which occurs in the stroma of the chloroplasts, when the products of the light reaction, ATP and NADPH, are used to make carbohydrates from carbon dioxide (reduction); initially glyceraldehyde 3-phosphate (a 3-carbon atom molecule) is formed.

LIGHT REACTIONS : Photophosphorylation

Photophosphorylation is a process of formation of ATP from ADP and inorganic phosphate by utilizing light energy. In oxygenic photosynthetic organisms, photophosphorylation is of two types: *cyclic* as well as *non-cyclic*.

A. NONCYCLIC PHOTOPHOSPHORYLATION

A noncyclic photophosphorylation, in which the formation of ATP is coupled with a light-induced electron transport from water to ferredoxin and a concomitant evolution of oxygen. It involves a collaboration of two photoreactions: a short-wavelength photoreaction belonging to pigment system II and a long-wavelength photoreaction belonging to pigment system I. Electrons move from H_2O through PS II to PSI and then on to $NADP^+$. Noncyclic photophosphorylation involves three processes - Absorption of light; electron transport leading to formation of O_2 from H_2O, reduction of $NADP^+$ to NADPH, and generation of a proton-motive force; and synthesis of ATP. All these processes are tightly coupled and controlled and carried out by proteins in the thylakoid membrane.

The diagram in figure 8.6 often called the **Z scheme** because of its overall form, outlines the pathway of electron flow between the two photosystems and the energy relationship in the light reactions.

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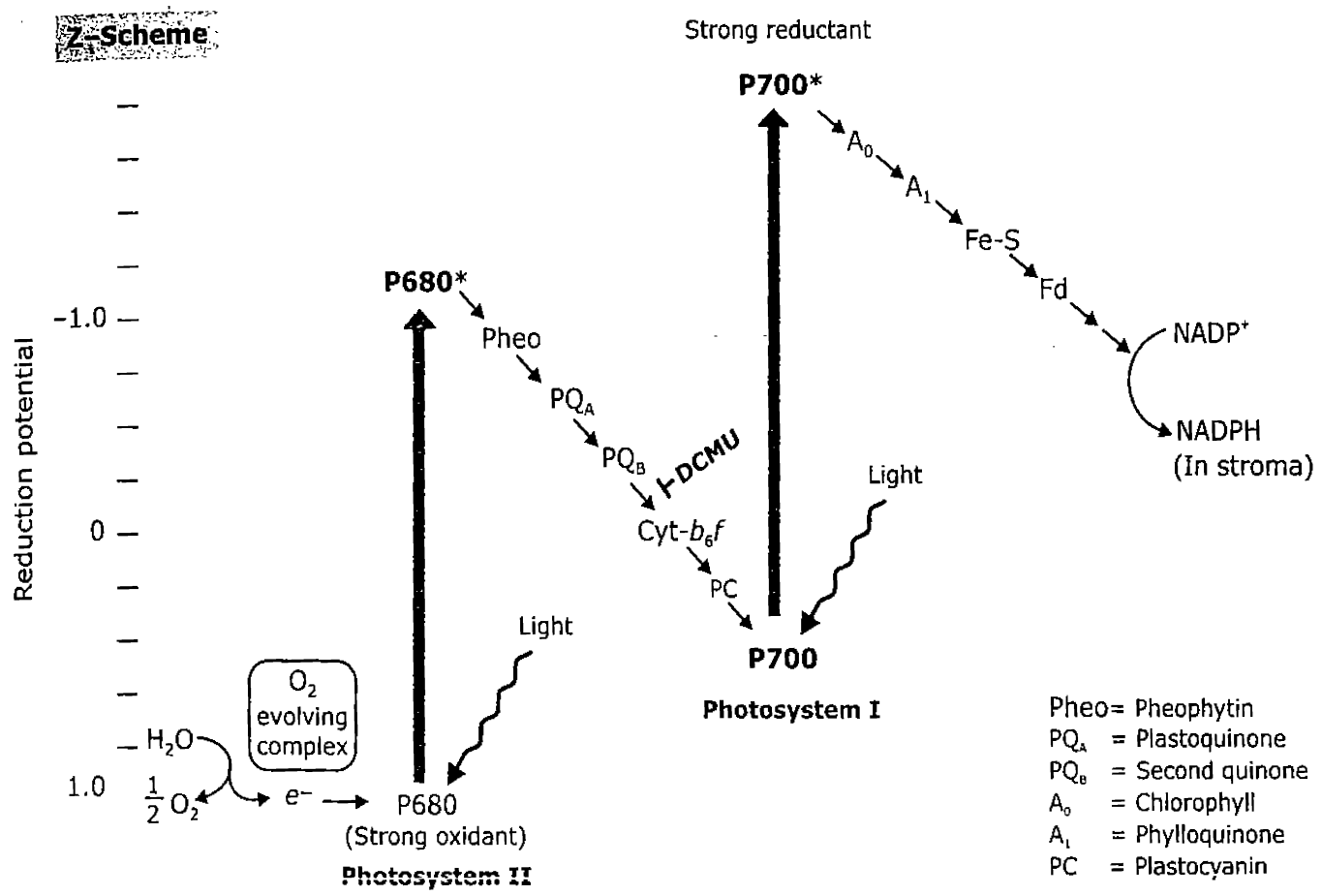
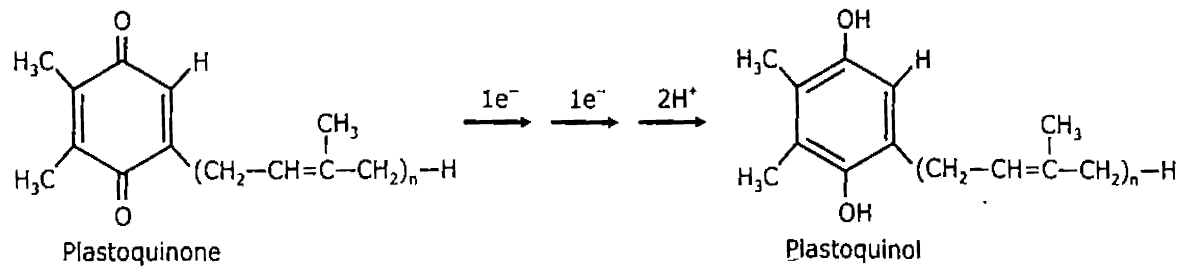


Figure 8.6 : The Z-scheme for photosynthesis in plants and cyanobacteria. Two photosystems, PSI and PSII, function to drive electrons from H_2O to NADPH. The reduction potential increase downward so that electron flow occurs spontaneously in this direction. The herbicide DCMU blocks photosynthetic electron transport from PSII to cytochrome *f*.

Electron transport and generation of proton motive force

Noncyclic photophosphorylation begins with absorption of a photon by PSII, causing an electron to move from a P_{680} Chl*a* to an acceptor plastoquinone, Q_B and synthesis of molecular oxygen from water. Photochemical reaction centre of PS II contains a special pair of Chl*a* molecules known as *P680*. Upon absorption of a photon, P680 is excited to P_{680}*, which rapidly transfers an electron to a nearby *pheophytin* (chlorophyll lacking the Mg^{2+} ion) within a few picoseconds. The plastoquinone, Q_A accepts the electron from the pheophytin and transfers it to the plastoquinone, Q_B. After two reductions by Q_A, the Q_B is converted into a plastoquinol, which is replaced by another *new* plastoquinone.

Plastoquinone is a photosynthetically active *quinone* present in photosystem II. Other photosynthetically active quinones are *ubiquinone* and *menaquinone* in green and purple photosynthetic bacteria, and *phylloquinone* in photosystem I. Plastoquinone is present in green-plant photosystem II both as a tightly-bound and a loosely-bound electron carrier, designated Q_A and Q_B, respectively. Q_A is photoreduced only to the semiquinone (PQ) but Q_B can accept two electrons, forming the plastohydroquinone or plastoquinol (PQH_2).



Plastoquinone Plastoquinol

Figure 8.7 : The acceptance of two electrons and two protons reduces PQ (plastoquinone) to PQH_2 (plastoquinol).

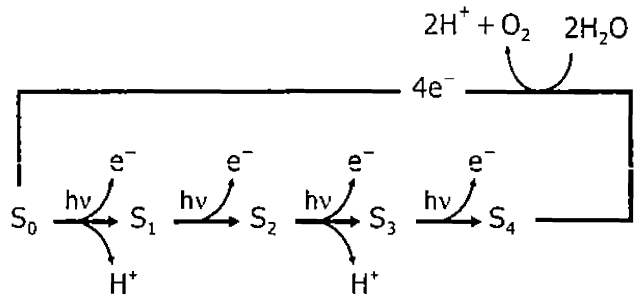
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The positively charged Chl*a*$^+$, a strong oxidizing agent attracts an electron from an electron donor on the luminal surface to regenerate the original Chl*a*. In oxygenic photosynthetic organisms H_2O acts as electron donor. Splitting of two water molecules yield four electrons, four protons and molecular oxygen.

$$2H_2O \rightarrow 4H^+ + 4e^- + O_2$$

The splitting of water is catalyzed by a protein complex, the **oxygen-evolving complex**, located on the luminal surface of the thylakoid membrane. The oxygen-evolving complex contains four manganese together with a Ca^{2+} ion and one or two Cl^- ion. The four electrons abstracted from photolysis of two water molecules do not pass directly to P_{680}^+, which can accept only one electron at a time. The oxygen-evolving complex oxidizes water and passes four electrons one at a time to P_{680}^+. The oxygen-evolving complex cycles through a series of oxidation states, while abstracting protons and electrons from the H_2O molecules, and finally releases O_2 into the inner thylakoid space.

Manganese ions in the oxygen-evolving complex cycles through five different states, S_0 through S_4. Each of the transitions between S_0 and S_4 is a photon-driven redox reaction; that from S_4 to S_0 results in the release of O_2. Three of these five steps release protons into the thylakoid lumen. The five reaction steps release a total of four water-derived protons into the inner thylakoid space (lumen) in a stepwise manner.



The electrons released from H_2O are transferred, one at a time, via the Mn ions and nearby tyrosine side chain on the D1 subunit, to the reaction-center P_{680}^+, where they regenerate the reduced chlorophyll, P680.

Plastoquinol diffuses in the membrane and binds with the Q_0 site on a Cyt b_6f complex. The Cyt b_6f complex lying between PS II and PS I in the electron-transport system. Its function is to oxidize the plastohydroquinone formed by PS II and to transfer these electrons to plastocyanin. **DCMU** (3-(3,4 dichlorophenyl)-1,1 dimethylurea), a herbicide, abolishes photosynthetic oxygen production because it competes with plastoquinone for the Q_B binding site of PSII and blocks electron flow from PSII to Cyt b_6f complex.

Cytochromes are electron-transfer proteins containing a *heme*, as the prosthetic group. According to their characteristic absorption spectra and chemical composition, cytochromes may be divided into three groups, namely, cytochromes-*a*, -*b* and -*c*. Cytochromes transfer electrons with a change in the oxidation state between Fe (III) and Fe (II) in the heme. In photosynthetic organisms, mainly *b*- and *c*-type *cytochromes* are found. It is present in the form of Cyt b_6f complex that resembles the Cyt bc_1 complex of mitochondria and anoxygenic photosynthetic bacteria. Cyt b_6f complex possess one Rieske iron-sulfur protein, two *b*-hemes of Cyt b_6 and Cyt *f*.

When the plastohydroquinone becomes fully oxidized at the lumenal surface of the membrane, it loses two electrons and also releases two protons to the lumen. Thus accompanying the reduction of one plastoquinone molecule by PS II and its subsequent reoxidation, there is a net transfer of two protons from the stroma into the lumen. Thus, with the splitting of two water molecules by PS II to form one oxygen molecules, four protons are translocated across the membrane.

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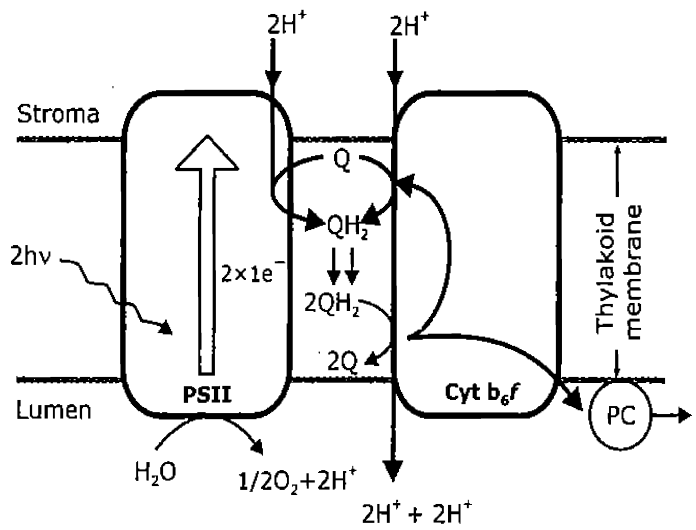
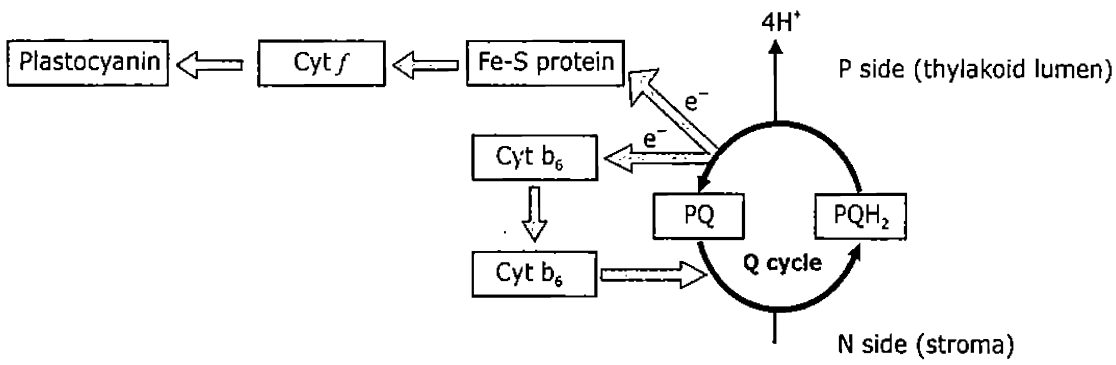


Figure 8.8 : Proton pumping stoichiometry of Cyt b_6f complex.

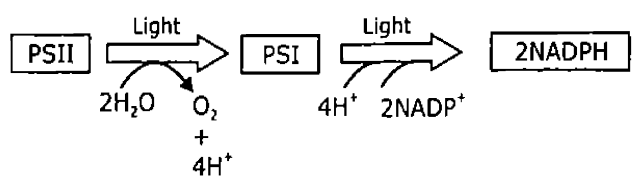
Oxidation of two water molecules also releases an additional four protons into the lumen. Thus water splitting and plastoquinone reduction/re-oxidation result in the generation of eight protons and the creation of a proton gradient across the thylakoid membrane.

The plastohydroquinone is first oxidized by the Rieske FeS to a semiquinone, which is then oxidized by cytochrome *f*, which then releases the electron to the copper protein plastocyanin. After loss of one electron by the plastohydroquinone, the resulting semiquinone loses an electron to the two *b*-hemes in series. The *b*-hemes operate in the so-called **Quinone cycle** or **Q-cycle**, similar to that in the mitochondria and provide a translocation of additional protons across the membrane into the lumen.



At the core of PS I is a special pair of Chl*a* molecules known as P700. Upon absorption of a photon, P700 is excited to P700*, which is sufficiently reducing to transfer the electron to A_0, a modified chlorophyll molecule resulting in the formation of $P700^+A_0^-$. The resulting oxidized $P700^+$ is reduced by an electron passed from the PSII reaction center via the Cyt b*f* complex and plastocyanin. Reduced plastocyanin diffuses in the thylakoid lumen, carrying the electron to $P700^+$ in PSI.

Phylloquinone, known as A_1, then accepts the electron from the modified chlorophyll A_0. From here, the electron moves to **ferredoxin**, an iron-sulfur protein containing a 2Fe-2S center. Ferredoxin undergoes one-electron oxidation and reduction reactions. The fourth electron carrier in the chain is the flavoprotein *ferredoxin: NADP⁺ oxidoreductase,* which transfers electrons from reduced ferredoxin to $NADP^+$.



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ATP synthesis

During this process of photophosphorylation, ATP is generated by the photosystem II electron transport chain and chemiosmosis. According to the *chemiosmotic theory*, as the electrons are transported down the electron transport chain, some of the energy released is used to pump protons across the thylakoid membrane from the stroma of the chloroplast to the thylakoid interior space producing a proton gradient or proton motive force. It is estimated that the transport of the four electrons through the Cyt b_6f complex occurs with the translocation of eight protons from the stroma to the thylakoid lumen. As the accumulating protons in the thylakoid interior space pass back across the thylakoid membrane to the stroma through ATP synthase complexes (CF_0-CF_1 ATPase), this proton motive force is used to generate ATP from ADP and P_i According to most estimates, chloroplast ATP synthase produces one ATP for every three protons it transports out of the thylakoid lumen. Non-cyclic electrons transport therefore results in the production ~4 molecules of ATP per molecule of O_2 evolved.

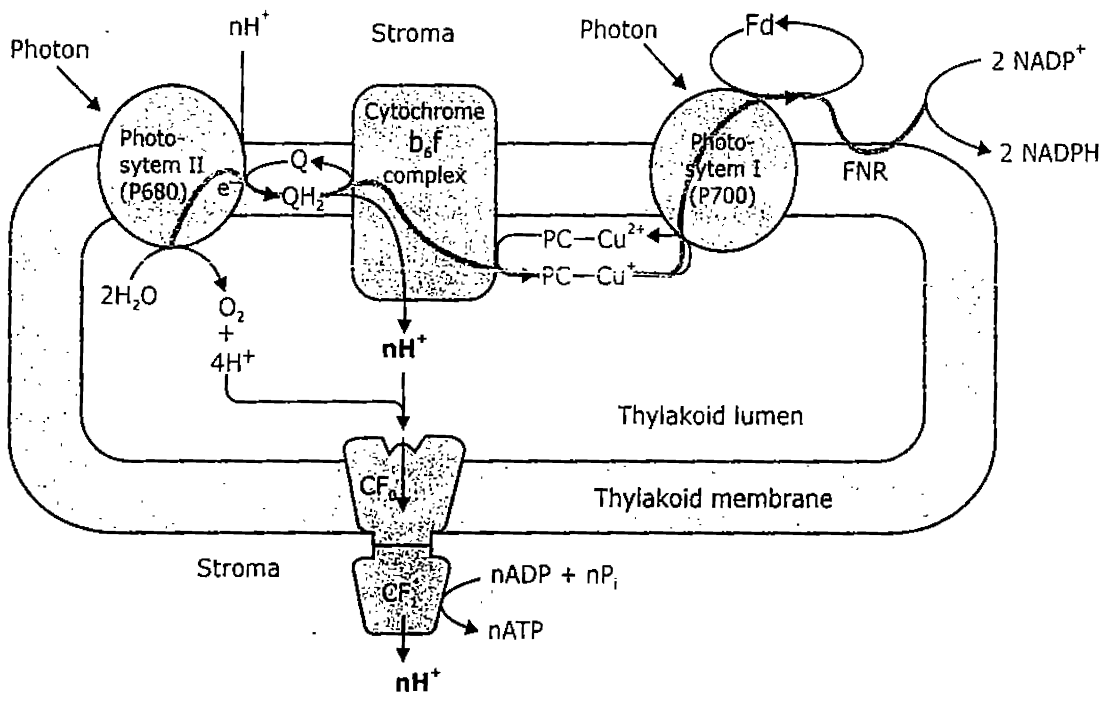


Figure 8.9 : Summary view of the light reactions as they occur in the thylakoid

B. CYCLIC PHOTOPHOSPHORYLATION

A cyclic photophosphorylation which yields *only* ATP and produces no net change in the oxidation-reduction state of any electron donor or acceptor. The cyclic electron pathway begins after PS I pigment complex absorbs solar energy. High-energy electrons leave PS I reaction-center chlorophyll a molecule but eventually return to it. Before they return, the electrons enter and travel down an electron transport system. Electrons pass from a higher to a lower energy level. Energy released is stored in form of a proton gradient. When protons flow down their electrochemical gradient through ATP synthase complexes, ATP production occurs. Some photosynthetic bacteria utilize cyclic electron pathway only; pathway probably evolved early. In plants, the cyclic flow of electrons is utilized only when the reductive power (NADPH) that it needed to synthesize new carbon skeletons is sufficient, but still needs ATP to power other activities in the chloroplast.

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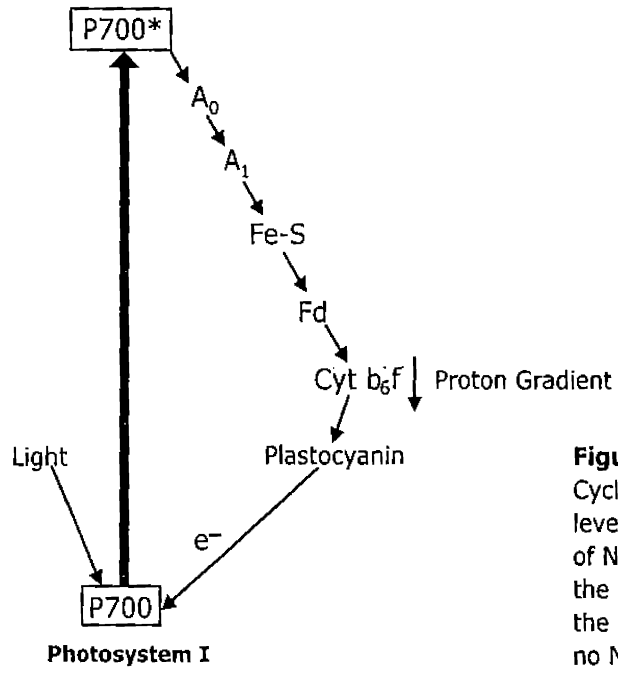


Figure : 8.10
Cyclic photophosphorylation. When levels of $NADP^+$ are low and levels of NADPH are high, electrons from the P700 center are returned to it via the cytochrome b_6f complex. There is no $NADP^+$ reduction, but protons are pumped across the membrane and therefore ATP is generated.

Overview of operation of pigment systems

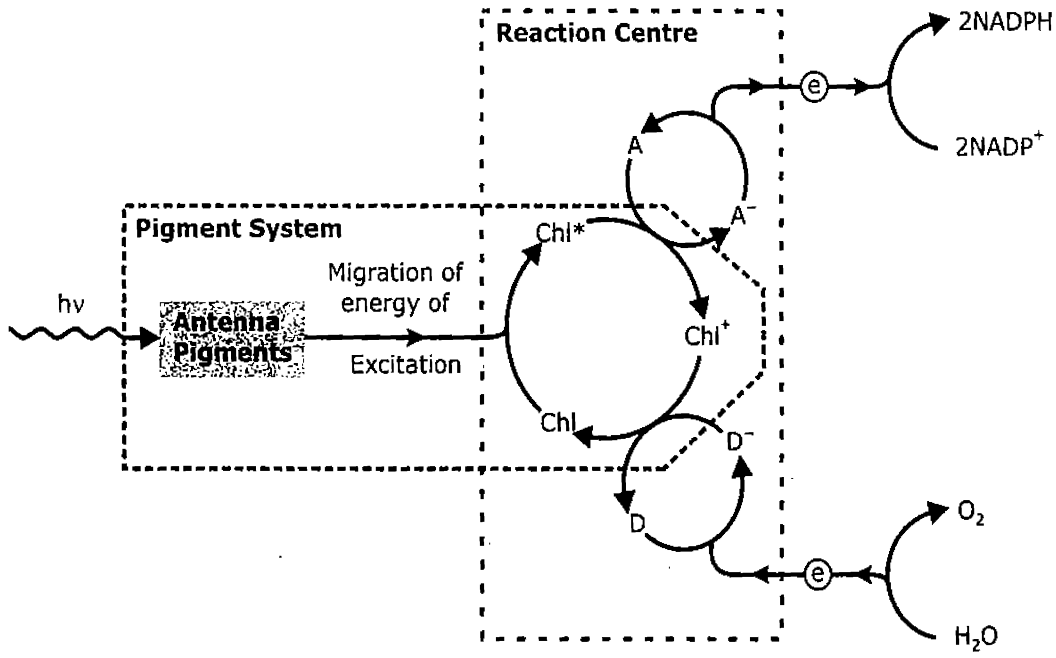


Figure : 8.11
Simple concept of the operation of Pigment systems I and II. PS I and PS II are inserted at two different points in the chain of redox systems carrying electrons from H_2O to $NADP^+$. They each have a different acceptor (A/A^-) and donor (D/D^-) redox system.

Prokaryotic photosynthesis

There are three groups of photosynthetic bacteria – the purple bacteria, the green bacteria, and the cyanobacteria. The cyanobacteria differ most fundamentally from the green and purple photosynthetic bacteria is being able to carry out *oxygenic photosynthesis*. Similar to plants they have PSI and PSII, use water as an electron donor, and generate oxygen during photosynthesis. In contrast, purple and green bacteria have only one photosystem and use *anoxygenic photosynthesis*.

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Table 8.3 : Comparison of oxygenic and anoxygenic photosynthesis

Property	*Oxygenic photosynthesis*	*Anoxygenic photosynthesis*
Photosynthetic pigment	Chl a	BChl
Photosynthetic electron donors	H_2O	H_2, H_2S, S, organic matter
O_2 production	Present	Absent
Primary products of energy conversion	ATP + NADPH	ATP
Carbon source	CO_2	Organic compound and/or CO_2

Photochemical reaction center in anoxygenic photosynthetic bacteria

Anoxygenic photosynthetic bacteria have relatively simple phototransduction machinery, with one of two general types of reaction center :

- Pheophytin-Quinone reaction center is found in purple bacteria
- Fe-S reaction center is found in green sulfur bacteria

Pheophytin-Quinone reaction center of purple bacteria, *Rhodobacter viridis* consists of 3 transmembrane proteins, H, L, M, four molecules of Bchlb, two molecules of bacteriopheophytin and two quinones (one ubiquinone and other menaquinone). A C-type cytochrome containing four heme is also forms an integral component of reaction center of *R. viridis*. The reaction center of other purple photosynthetic bacteria like *Rhodobacter sphaeroides*, and *Rhodospirillum rubrum*, contain only the H, M, and L subunits.

Light drives the *cyclic* flow of electrons from the reaction center P_{865} through pheophytin, quinone and the cytochrome bc_1 complex, then through cytochrome c_2 back to the reaction center.

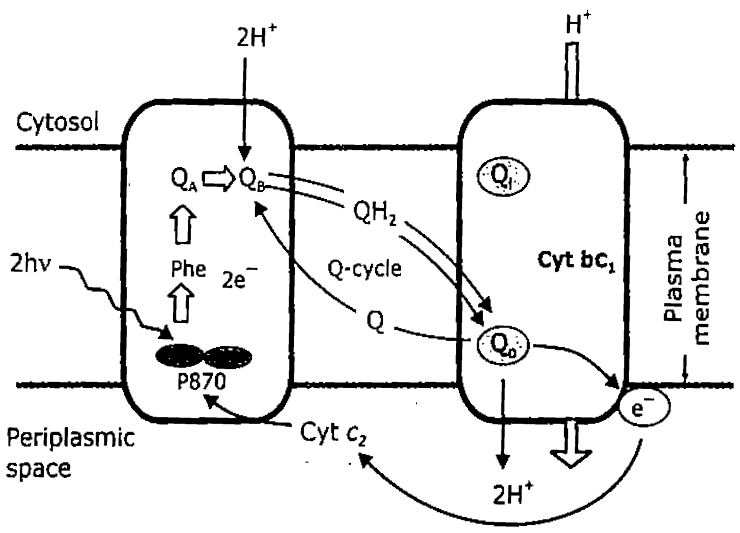


Figure : 8.12
Cyclic electron flow in the single photosystem of purple bacteria, *R. sphaeroides*

Green sulfur bacteria (containing Fe-S reaction center) have two routes for electrons driven by excitation of P_{840} – a *cyclic* route passes through a quinone to the Cyt bc_1 complex and back to the reaction center via Cyt *c*, and a *non-cyclic* route from the reaction center through the Fe-S protein ferredoxin, then to NAD^+.

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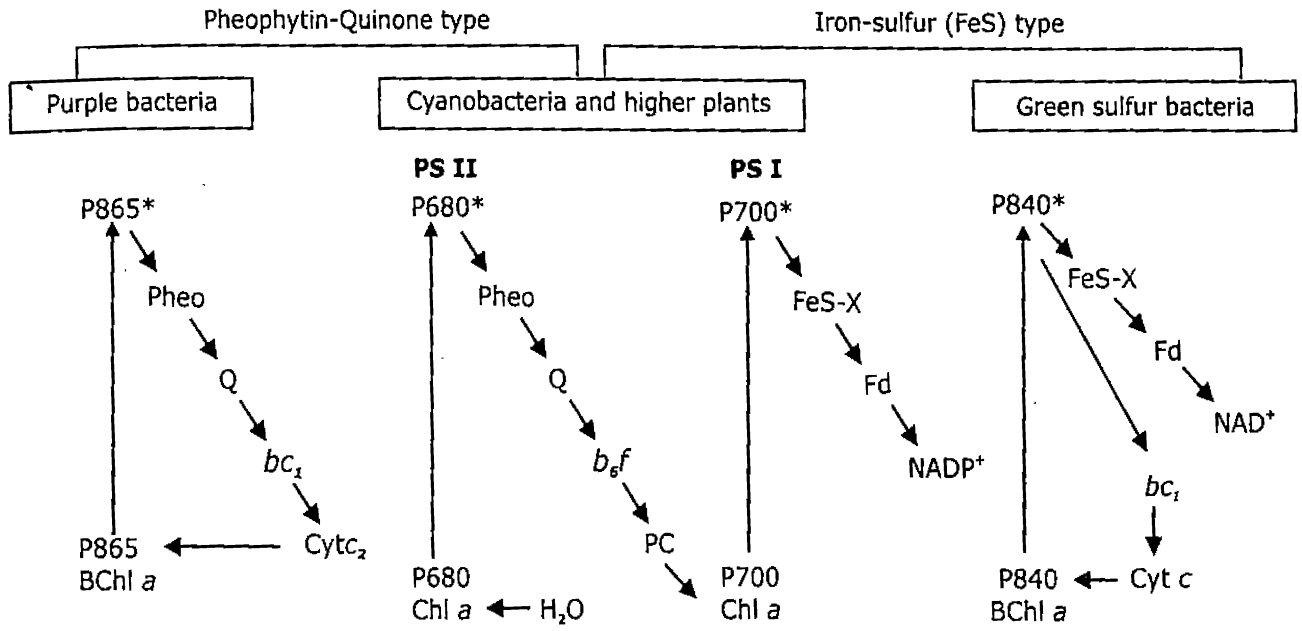


Figure 8.13 : Comparison of photosynthetic reaction centers and their electron-transfer scheme.

Characteristics of gram-negative *anoxygenic* photosynthetic bacteria

Green Sulfur

Major photosynthetic pigments	:	Bacteriochlorophylls a plus c, d, or e
Location of photosynthetic pigments	:	Chlorosomes and plasma membrane
Photosynthetic electron donors	:	H_2, H_2S, S
Sulfur deposition	:	Outside of the cell
Representative genera	:	*Chlorobium*

Green Non-sulfur

Major photosynthetic pigments	:	Bacteriochlorophylls a and c
Location of photosynthetic pigments	:	Chlorosomes and plasma membrane
Photosynthetic electron donors	:	Variety of sugars, amino acids, and organic acids; H_2S, H_2
Representative genera	:	*Chloroflexus*

Purple Sulfur

Major photosynthetic pigments	:	Bacteriochlorophylls a or b
Location of photosynthetic pigments	:	Plasma membrane and chromatophore (lamellar membrane complexes that are continuous with the plasma membrane)
Photosynthetic electron donors	:	H_2, H_2S, S
Sulfur deposition	:	Inside the cell
Representative genera	:	*Chromatium*

Purple Non-sulfur

Major photosynthetic pigments	:	Bacteriochlorophylls a or b
Location of photosynthetic pigments	:	Plasma membrane and chromatophore
Photosynthetic electron donors	:	Usually organic molecules; sometimes reduced sulfur compounds or H_2
Sulfur deposition	:	Outside of the cell in few cases
Representative genera	:	*Rhodopseudomonas, Rhodospirillum*

Non-chlorophyll based photosynthesis

Oxygenic and anoxygenic photosynthesis are chlorophyll based photosynthesis. Pigment chlorophyll or bacteriocholorophyll is the major pigment used to absorb light and initiate the conversion of light energy to chemical energy. However, some archaea instead of using chlorophyll, use a membrane protein called

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bacteriorhodopsin. One such archaeon is the halophile *Halobacterium salinarum*. Similar to the rhodopsin found in the mammalian eye, bacteriorhodopsin functions as a light-driven proton pump. Bacteriorhodopsin has two distinct features - a chromophore that is a derivative of retinal which is covalently attached to the protein by a Schiff base with the amino group of lysine and seven membrane-spanning domains. When retinal absorbs light, the double bond between carbons 13 and 14 changes from a *trans* to a *cis* configuration and the Schiff base loses a proton. Protons move across the plasma membrane to the periplasmic space during these alternations. The light driven proton pumping generates a pH gradient that can be used to power the synthesis of ATP by a chemiosmotic mechanism.

DARK REACTION : Calvin cycle

The Calvin cycle (also known as *reductive pentose phosphate cycle*) is a series of biochemical reactions taking place in the stroma of chloroplasts of eukaryotic photosynthetic organisms (cytosol in prokaryotic organism). It was discovered by Melvin Calvin and Andy Benson at the University of California, Berkeley.

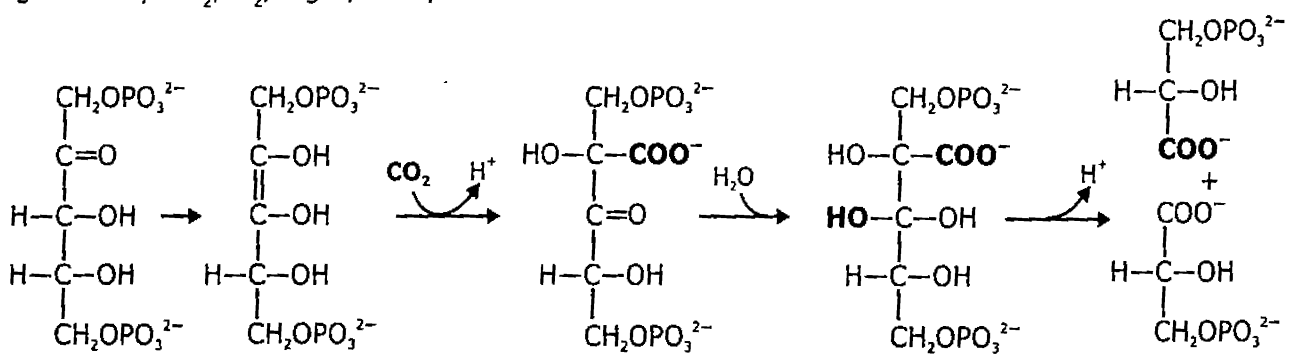
The net equation for the Calvin cycle is

$$3\,CO_2 + 6\,NADPH + 9\,ATP \longrightarrow \text{Glyceraldehyde}-3-\text{Phosphate} + 6\,NADP^+ + 9\,ADP_i$$

For every three molecules of CO_2 that are incorporated into carbohydrate molecules, there is a net gain of one molecule of glyceraldehyde-3-phosphate. The reactions of the cycle can be divided into three phases:

1. Carboxylation

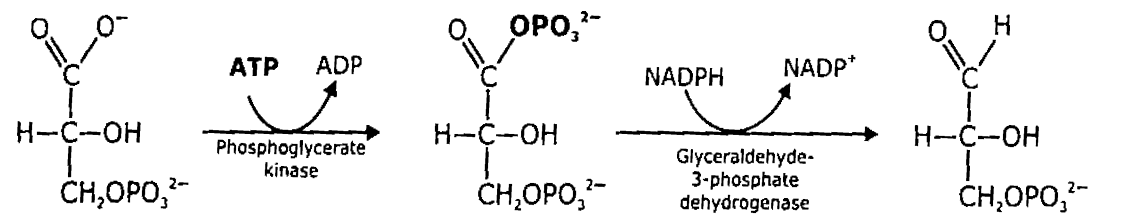
Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) catalyzes the carboxylation of ribulose-1,5-bisphosphate to form two molecules of glycerate-3-phosphate. RuBisCo (a complex molecule composed of eight large subunits (56 kD) and eight small subunits (14 kD), is the pacemaker enzyme of the Calvin cycle. Large subunits are encoded by the chloroplast genome whereas smaller subunits are encoded by nuclear genome. It makes up about 50% soluble protein in chloroplasts. Its activity is regulated by CO_2, O_2, Mg^{2+}, and pH.



Ribulose-1,5-bisphosphate | Enediol intermediate | 2-Carboxy-3-keto-D-arabinitol-1,5-bisphosphate | Hydrated intermediate | 2 Molecules of 3-phosphoglycerate

2. Reduction

The next phase of the cycle consists of two reactions. Six molecules of glycerate-3-phosphate are phosphorylated at the expense of six ATP molecules to form glycerate-1,3-bisphosphate. The latter molecules are then reduced by NADP-glyceraldehyde-3-phosphate dehydrogenase to form six molecules of glyceraldehyde-3-phosphate. These reactions are similar to reactions in gluconeogenesis. Unlike the dehydrogenase in gluconeogenesis, the Calvin cycle enzyme uses NADPH as a reducing agent.



3-Phosphoglycerate | 1,3-Bisphosphoglycerate | Glyceraldehyde-3-phosphate

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3. Regeneration

As noted previously, the net production of fixed carbon in the Calvin cycle is one molecule of glyceraldehyde-3-phosphate. The other five glyceraldehyde-3-phosphate molecules are processed in the remainder of the Calvin cycle reactions to regenerate three molecules of ribulose-1,5-bisphosphate as summarized below. This is accomplished in a series of reactions. The intermediates in this pathway include three-, four-, five-, six-, and seven carbon sugars.

$C_3 + C_3 - C_6$

$C_3 + C_6 - C_5 + C_4$

$C_3 + C_4 - C_7$

$C_3 + C_7 - C_5 + C_5$

Overall : $5C_3 - 3C_5$

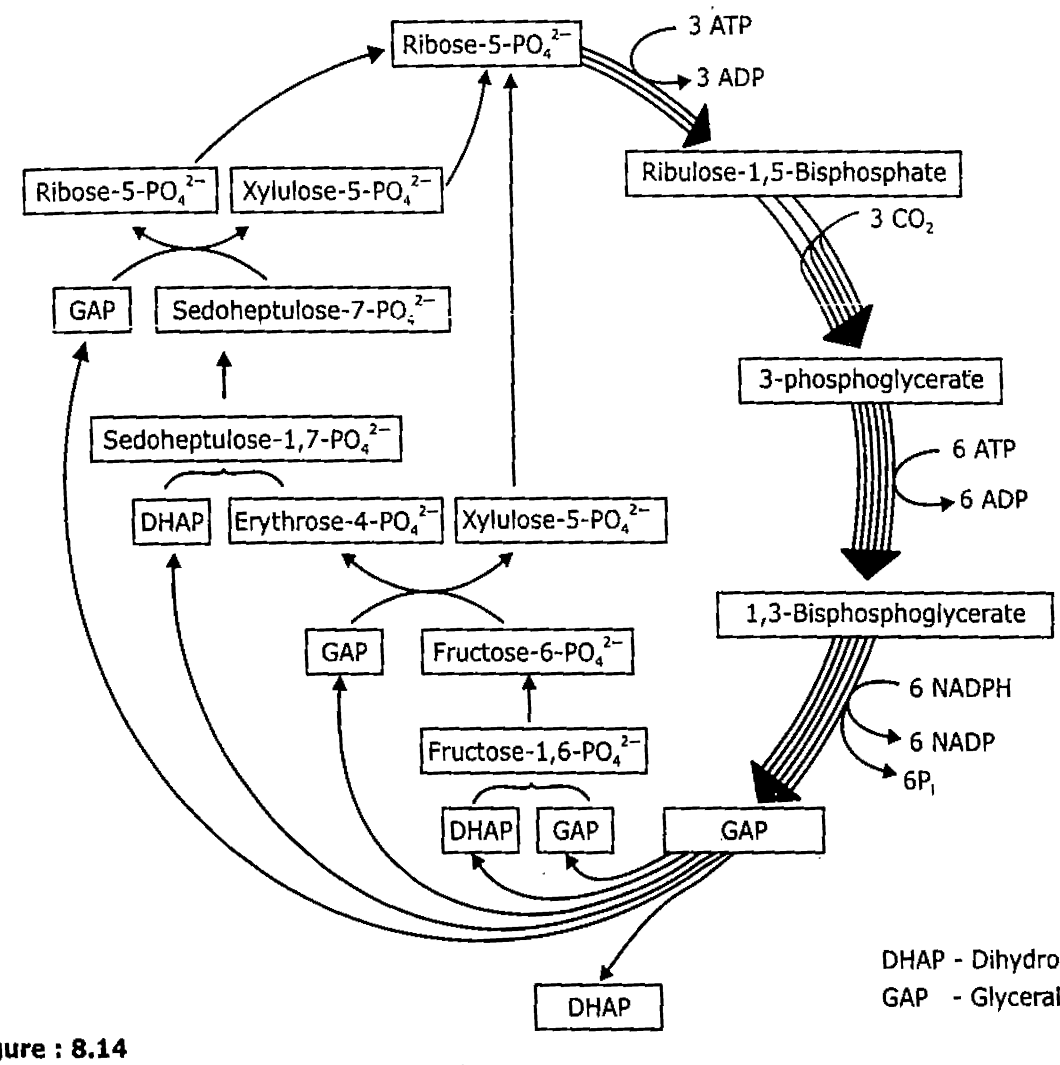


Figure : 8.14

The cycle can be divided into three phases. The first phase is carboxylation and second is the reductive phase. The third phase is regeneration of the acceptor, ribulose-1,5-Bisphosphate in the *sugar phosphate shuffle*, in which five C_3 units are converted into three C_5 units. One of the six molecules of triose-phosphate, deriving from three CO_2 molecules incorporated by Rubisco, is available for product synthesis or for regeneration. *Aldolase* converts both DHAP and GAP into fructose-1,6-Bisphosphate, and DHAP and erythrose-4-phosphate to the seven carbon sugar, sedoheptulose-1,7-Bisphosphate. *Fructose* and *sedoheptulose bisphosphatases* convert the bisphosphates into fructose-6-phosphate and sedoheptulose-7-phosphate, and *transketolase* catalyzes the reaction of these with GAP to generate pentose-phosphate and erythrose-4-phosphate. Interconversion of pentose-phosphate is then catalyzed by *ribose-phosphate isomerase* (ribose-5-phosphate to ribulose-5-phosphate) and *ribulose-5-phosphate 3-epimerase* (xylulose-phosphate to ribulose-5-phosphate). Finally, *ribulose-5-phosphate kinase* catalyzes the ATP-dependent conversion of ribulose-5-phosphate into ribulose-1,5-Bisphosphate. *Glyceraldehyde-phosphate dehydrogenase*, the bisphosphatases and product synthesis recycle the phosphate required for continued ATP synthesis. *Adapted from Plant Biochemistry*, Dey and Harborne, Eds. Academic Press, 1997

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Schematic view of the Calvin cycle

The cycle may be divided into two stages :

In stage I, CO_2 is fixed and glyceraldehyde-3-phosphate is produced. Part of this G3P is used to make hexose phosphates and eventually polysaccharides. Another fraction of the G3P is used in stage II to generate the acceptor molecule, ribulose-1,5-bisphosphate.

For three molecules of ribulose-1,5-bisphosphate (total of 15 C) that are carboxylated, cleaved, phosphorylated, reduced, and dephosphorylated, six molecules of glyceraldehyde-3-phosphate are produced (total of 18 C). Of these, one glyceraldehyde-3-phosphate (3 C) exits the pathway as product and five of the 3-C molecules (total of 15 C) are recycled back into three 5-C molecules of ribulose-1, 5-bisphosphate. The overall biochemistry of photosynthesis for the formation of one glucose molecule from six CO_2 molecules may be written as:

$$6CO_2 + 12\ H_2O + 18\ ATP + 12\ NADPH \longrightarrow C_6H_{12}O_6 + 18\ ADP + 12\ NADP^+ + 6\ O_2\uparrow$$

The reduction of one mole of CO_2 molecule to glucose requires 12 kcal and thus the efficiency of photosynthetic reaction is 112/384, or ~30%. The actual efficiency may be lower, given that not all photons may be effective.

Control of Calvin cycle

Carbon dioxide fixation (dark reaction) occurs during the day time and is strictly dependent on the light reaction, i.e., the formation of reductive power as NADPH. It does not occur literally in the dark (at night). The metabolic activity at night is mainly shifted to carbohydrate oxidation, when plants consume oxygen rather than producing it. The control of the Calvin cycle therefore means the control of regulation between light and dark reaction. The enzymes of the Calvin cycle are sensitive to the proton concentration of the chloroplast stromal compartment. The optimum pH for Rubisco activity lies around pH 8. The proton pump driven during light absorption moves protons from the stroma to the lumenal side to the thylakoid membrane increasing the stromal pH. This activates Rubisco and thus the Calvin cycle. The proton level control is coupled to magnesium interaction (chelation) with carbamates. Magnesium stabilizes carbamate formation and thus activates Rubisco. The key enzymes *fructose biphosphatase* and *sedoheptulose biphosphatase* of the Calvin cycle are also under pH control. In addition, they are sensitive to the redox potential of the chloroplast stromal compartment. Biphosphatases are active in their *reduced state* only sensing the oxidation state of photosystem I. The mediator molecule is the small protein **thioredoxin**. Thioredoxin is coupled to ferredoxin by ferredoxin-thioredoxin reductase. Thioredoxin also inhibits *phosphofructokinase*. In plant cells, light stimulates the Calvin cycle enzymes (PSI being the strong reductant) and inhibits glycolysis.

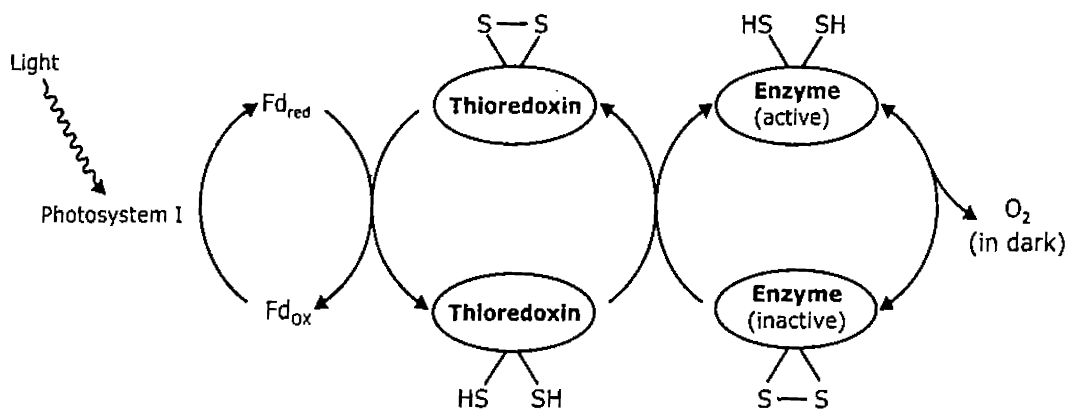


Figure 8.15 : Light-driven reactions lead to electron transfer from water to ferredoxin. Reduced ferredoxin regulate enzymes from the Calvin cycle. One key protein in these regulatory processes is thioredoxin. In chloroplasts, oxidized thioredoxin is reduced by ferredoxin in a reaction catalyzed by ferredoxin-thioredoxin reductase. Reduced thioredoxin activates certain Calvin cycle enzymes by cleaving regulatory disulfide bonds.

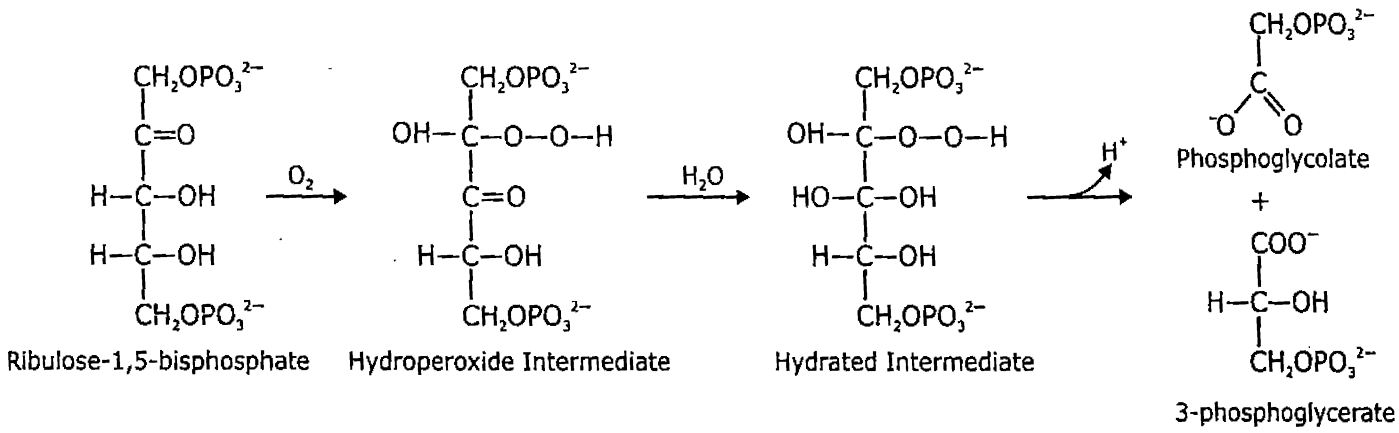
Tl e light activation is mediated by thioredoxin. In the light, thioredoxin is reduced by electrons moving from photosystem I through ferredoxin (Fd) then thioredoxin reduces critical disulfide bonds in each of the enzymes

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sedoheptulose 1,7-bisphosphatase, fructose 1,6-bisphosphatase, ribulose 5-phosphate kinase, and glyceraldehyde 3-phosphate dehydrogenase, activating these enzymes. In the dark, the –SH groups undergo reoxidation to disulfides, inactivating the enzymes.

8.7 PHOTORESPIRATION

Otto Warburg made an observation that O_2 inhibits photosynthesis in C3 plants. This phenomenon, originally known as the **Warburg effect**, was later recognized as the light-dependent release of CO_2 due to oxygenase activity of Rubisco. Rubisco is a bifunctional enzyme. It catalyzes both the carboxylation and the oxygenation of RuBP. Oxygenation of RuBP leads to the production of one molecule of *glycerate-3-phosphate* and one of *glycolate-2-Phosphate*. The principal factors influencing the enzyme's oxygenase activity are the ambient concentrations of O_2, CO_2 and temperature. High temperatures promote oxygenation, and hence photorespiration, because the solubility of CO_2 in water declines more rapidly than that of O_2 as the temperature is increased. Second the specificity factor of Rubisco decreases with increasing temperature.



Photorespiration pathway (glycolate pathway)

Glycolate-2-Phosphate produced by Rubisco when it oxygenates RuBP cannot be utilized within the Calvin cycle. The pathway by which glycolate-2-Phosphate is further metabolized is described as **glycolate pathway**.

This pathway involves three subcellular compartments, the chloroplasts, peroxisomes and mitochondria. The key features of this pathway are the conversion of the two-carbon molecule, glycolate-2-Phosphate, to glycine and decarboxylation of two molecules of glycine to serine, CO_2 and NH_3. The three-carbon molecule, serine, is then converted into glycerate-3-Phosphate, which re-enters the Benson–Calvin cycle.

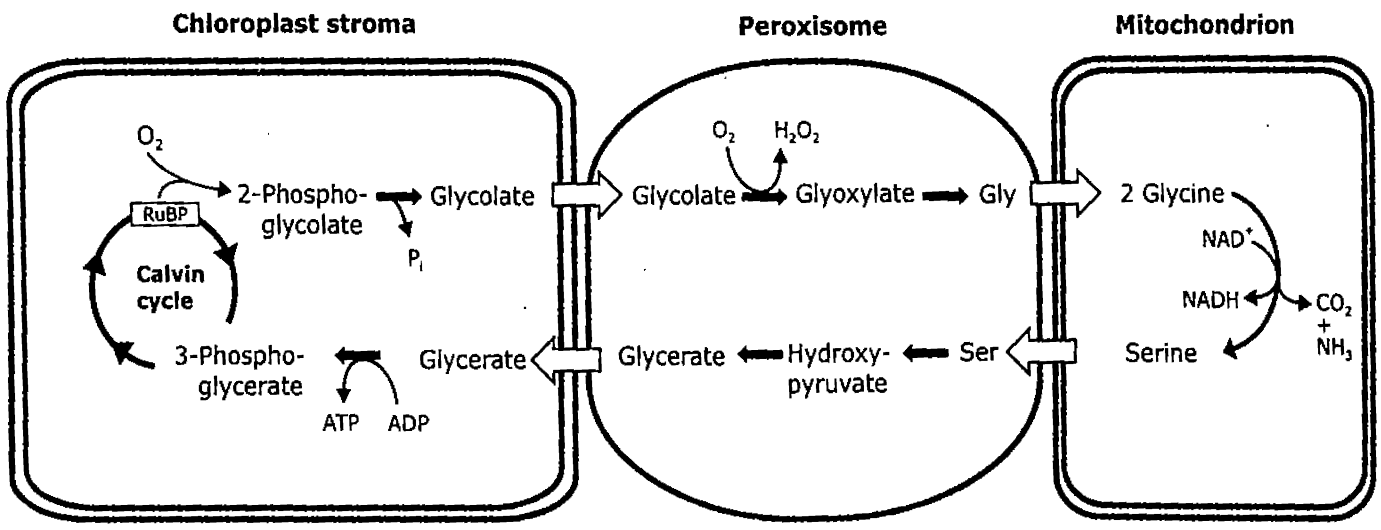


Figure : 8.16 : Photorespiration. Ribulose-1,5-bisphosphate (RuBP) can be diverted from the Calvin cycle, especially when the concentration of CO_2 is low. RuBP carboxylase/oxygenase catalyzes the oxidation of RuBP to form phosphoglycolate. In the reactions that follow, more O_2 is used, CO_2 is generated, and ATP is hydrolyzed, as metabolites pass from the chloroplast to near by peroxisomes and mitochondria and then back into the chloroplast.

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8.8 C_4 PHOTOSYNTHESIS

The C_4 pathway is an adjunct to the Benson–Calvin cycle. The C_4 pathway occurs only in conjunction with structural modifications which allow it to operate as a CO_2 concentrating mechanism. In all C_4 plants (are called so because initial CO_2 fixation product is four carbon acid), phosphoenolpyruvate (PEP) is carboxylated to C_4 acids and these are the first products of photosynthesis, in contrast to glycerate-3-P in C_3 plants. These C_4 acids are formed in one compartment, the mesophyll cells, and are then transferred, by diffusion, to the bundle-sheath, where they are decarboxylated. This generates a high concentration of CO_2 within the bundle-sheath. Rubisco and the majority of the enzymes of the Benson–Calvin cycle are confined to the bundle-sheath. The CO_2 concentration in the bundle-sheath is sufficient to suppress the oxygenase activity of Rubisco. A C_3 compound returns to the mesophyll from the bundle-sheath. In a plant such as maize, the net reaction catalyzed by the C_4 pathway is to transfer CO_2 from the mesophyll to the bundle-sheath at the expense of 2ATP per CO_2 transferred. It is, therefore, an ATP-driven CO_2 pump.

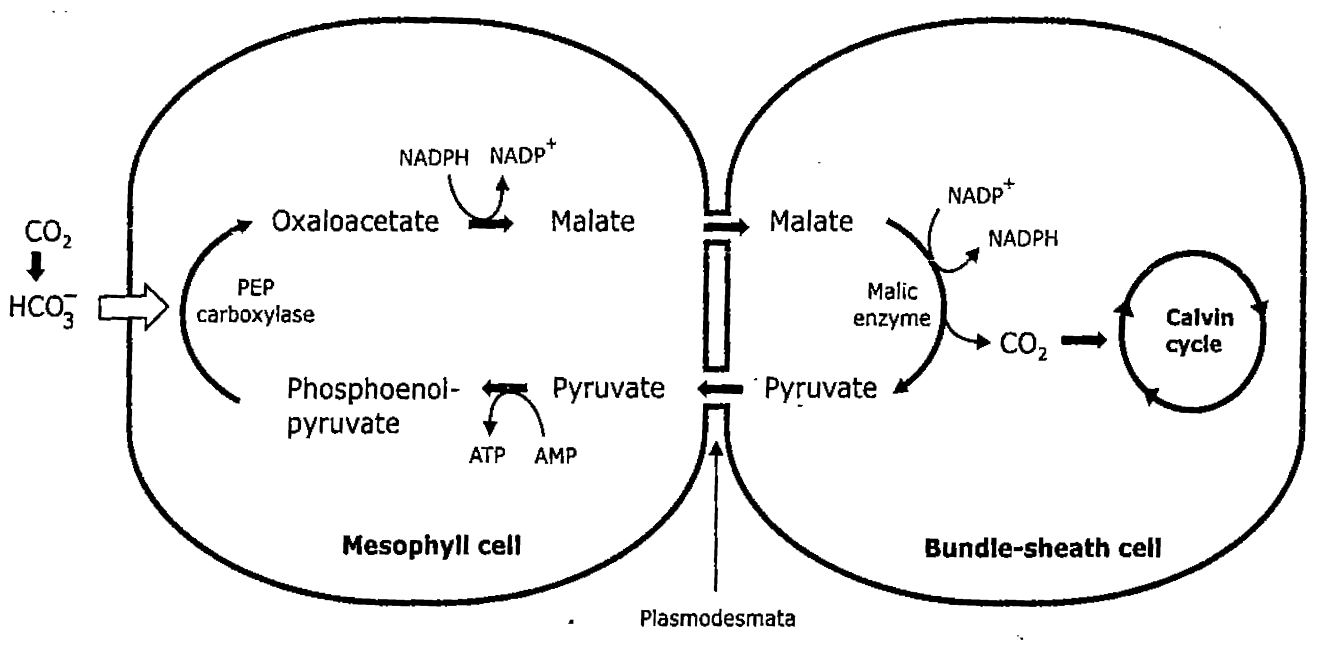


Figure 8.17 : Reactions of the C_4 cycle CO_2 is transported from mesophyll cells to the bundle sheath cells by coupling it to phosphoenolpyruvate, forming oxaloacetate. Oxaloacetate is then reduced to malate, which is passed to bundle sheath cells and decarboxylated. The pyruvate product is returned to the mesophyll cells, where it is phosphorylated to regenerate phosphoenolpyruvate.

Leaf anatomy of C_4 plants

C_4 plants are unique in possessing two types of photosynthetic cell. A layer of cells surrounding the vascular bundle, the bundle-sheath, is a common structural feature, but only in C_4 plants it contains chloroplasts. The bundle-sheath is thick-walled, sometimes suberized and there is no direct access from the intercellular spaces of the mesophyll. The appearance of a wreath of cells surrounding the vasculature gives rise to the term 'Kranz' (German: wreath) anatomy.

The distance between bundle-sheath cells is normally only two or three mesophyll cells, so that no mesophyll cell is more than one cell away from a bundle-sheath cell. Mesophyll cells are also connected to bundle-sheath cells by large numbers of plasmodesmata.

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8.9 CAM PATHWAY

Crassulacean acid metabolism (CAM) is a photosynthetic adaptation in succulent plants. This pathway allows gas-exchange to occur at night, when air temperatures are cooler and water vapor pressure deficits are lower. About 10% of vascular plants have developed CAM photosynthesis. Although it is characteristically found in succulent plants in many arid regions of the world, such as the cacti and euphorbias, it is also a feature of many tropical epiphytes that also experience erratic supplies of water, such as bromeliads and the orchids. The principal metabolic feature of CAM plants is assimilation of CO_2 at night into malic acid which is stored in the vacuole. Malate is generated in the reaction catalyzed by PEP carboxylase and PEP is in turn, generated by degradation of starch or soluble sugars. During the day, malate is released from the vacuole and is decarboxylated to provide CO_2 for fixation in the Benson—Calvin cycle behind closed stomata. The cycle of carboxylation and decarboxylation is thus spread out over time, rather than spatially, as in C4 plants.

Table 8.4 : Difference between C3, C4 and CAM plants

Characteristics	C3 Plant	C4 Plant	CAM Plant
1. Kranz Anatomy	Absent	Present	Absent
2. CO_2 : ATP : $NADPH_2$ ratio	1 : 3 : 2	1 : 5 : 2	1 : 6.5 : 2
3. Carboxylating Enzyme	RuBP Carboxylase	*In mesophyll* - PEP carboxylase *In bundle sheath* - RuBp carboxylase	*In dark* - PEP carboxylase *In light* - RuBp carboxylase
4. Initial CO_2 acceptor	RuBP	PEP	PEP
5. First stable product	3-PGA	Oxaloacetate	Oxaloacetate
6. Optimum temperature for photosynthesis	15-25°C	30-40°C	>40°C
7. CO_2 compensation point (ppm)	30-70	0-10	0-5 in dark
8. Photorespiration	Present	Not easily detectable	Detectable in afternoon
9. Calvin cycle operative	Alone	Along with the Hatch and Slack cycle	Both C_3, Hatch and Slack

STUDY QUESTION

Q1. The substance dichlorophenyldimethylurea (DCMU) is an herbicide that inhibits photosynthesis by blocking electron transfer between plastoquinones in photosystem II.

a. Would you expect DCMU to interfere with cyclic photophosphorylation?

b. Normally, DCMU blocks O_2 evolution, but addition of ferricyanide to chloroplasts allows O_2 evolution in the presence of DCMU. Explain.

Ans. a. No, because plastoquinones are not involved in this process.

b. Addition of ferricyanide as an electron acceptor allows a Hill reaction.

Q2. Blue-green algae (cyanobacteria) form heterocysts when deprived of ammonia and nitrate. In this form, the algae lack nuclei and are attached to adjacent vegetative cells. Heterocysts have photosystem I activity but are entirely devoid of photosystem II activity. What is their role?

Ans. They carry out nitrogen fixation. The absence of photosystem II provides an environment in which O_2 is not produced. Recall that the nitrogenase is very rapidly inactivated by O_2.

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Q3. If a algae are exposed to $^{14}CO_2$ for a brief period while illuminated, the labeled carbon is initially found almost entirely in the carboxyl group of 3-phosphoglycerate. However, if illumination is continued after the label pulse, other carbon atoms become labeled. Explain.

Ans. Most of the 3PG that is initially labeled is reused in replacing RuBP, which then re-enters the cycle.

Q4. $^{14}CO_2$ was bubbled through a suspension of liver cells that was undergoing gluconeogenesis from lactate to glucose. Which carbons in the glucose molecule would become radioactive?

Ans. None. The CO_2 that is fixed comes off in the PEP carboxykinase reaction.

Q5. Sedoheptulose 1,7-bisphosphate is an intermediate in the Calvin cycle but not in the pentose phosphate pathway (PPP). What is the enzymatic basis of this difference?

Ans. Aldolase participates in the Calvin cycle, whereas transaldolase participates in the PPP.

Q6. An illuminated suspension of Chlorella is actively carrying out photosynthesis. Suppose that the light is suddenly switched off. How would the levels of 3-phosphoglycerate and ribulose 1,5-bisphosphate change in the next minute?

Ans. The concentration of 3-phosphoglycerate would increase, whereas that of RuBP would decrease.

Q7. It is believed that the ratio of cyclic photophosphorylation to non-cyclic photophosphorylation changes in response to metabolic demands. In each of the following situations, would you expect the ratio to increase, decrease, or remain unchanged?

a. Chloroplasts carrying out both the Calvin cycle and the reduction of nitrite (NO_2^-) to ammonia (This process does not require ATP).

b. Chloroplasts carrying out not only the Calvin cycle but also extensive active transport

c. Chloroplasts using both the Calvin cycle and the C_4 pathway

Ans. a. Decrease

b. Increase

c. Increase

SELF TEST

Q1. A carotenoid-less mutant plant was grown under normal sunlight, it will experience

a. increased photosynthetic rate

b. increased chlorophyll biosynthesis

c. reduced photorespiration

d. increased chlorophyll oxidation and necrosis **[JNU]**

Q2. Plastocyanin is a mobile electron carrier between

a. PSII and cytochrome bf complex

b. cytochrome bf complex and PSI

c. oxygen evolving complex and PSII

d. PSI and ferredoxin **[JNU]**

Q3. Which of the following statement is *not* true with respect to the Photosystem II found in the chloroplasts?

a. PS II is located in the grana

b. Chl molecule in the reaction centre for the PS II is bound to a 110 Kd integral membrane protein

c. a complex of cytochromes *b* and *f* transports electrons from PS II and PS I

d. PS II reaction centre is bound to two Mn^{2+} ions **[GATE]**

Q4. Dibromothymoquinone, an inhibitor of cytochrome bf complex of photosynthetic electron transport chain was added to the green alga *Chlamydomonas*. After illumination it was found that in the alga

a. plastiquinone was in reduced state

b. P700 was in reduced state

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c. plastocyanin was in reduced state
d. both plastoquinone and plastocyanin were in oxidized state [JNU]

Q5. What is the strongest reducing agent in photosynthetic electron-transfer reactions?
a. plastoquinone
b. P680*
c. P700*
d. P700

Q6. When chlorophyll a is in its oxidized state
a. it fluoresces in the thylakoids
b. O_2 is generated from sucrose
c. it directly reduces $NADP^+$
d. it results in protons being generated from H_2O

Q7. DCMU (Dichlorophenyl dimethyl urea) is a herbicide which kills the plant by
a. inhibition of respiration
b. destroying the chloroplasts
c. inhibiting flow of electrons from water to $NADP^+$
d. inhibiting PSI and photolysis of water

Q8. A mutant plant lacked plastocyanin. Upon illumination of this plant
a. plastoquinol will remain in oxidized state
b. plastoquinol will be in reduced state
c. ferredoxin will be in the reduced state
d. NAD will be in reduced state [JNU]

Q9. Assume a thylakoid is somehow punctured so that the interior of the thylakoid is no longer separated from the stroma. This damage will have the most direct effect on which of the following processes?
a. the flow of electrons from photosystem II to photosystem I
b. the reduction of $NADP^+$
c. the splitting of water
d. the synthesis of ATP

Q10. F_1F_0-ATPase in chloroplasts is located on the
a. inner chloroplast membrane with F1 facing the stroma
b. inner chloroplast membrane with F1 facing the inter-membrane space
c. thylakoid membrane with F1 facing the stroma
d. thylakoid membrane with F1 facing the thylakoid lumen [GATE]

Q11. The large subunit of Rubisco is coded by
a. chloroplast genome
b. nuclear genome
c. mitochondrial genome
d. both mitochondrial and nuclear genome [JNU]

Q12. In which of the following organelles glycolate is oxidized to glyoxylate during photorespiration
a. chloroplast
b. peroxisome
c. mitochondria
d. endoplasmic reticulum [JNU]

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Photosynthesis

Q13. In photorespiration, glycolate and glyoxylate are produced sequentially in the following organelles. Choose the *correct* sequence

a. chloroplast and mitochondria
b. chloroplast and peroxisome
c. peroxisome and mitochondria
d. peroxisome and chloroplast **[GATE]**

Q14. In C4 plants, phosphoenol pyruvate carboxylase is located in

a. cytosol
b. chloroplast
c. peroxisome
d. mitochondrion

Q15. Which of these statements about thioredoxin is *correct*?

a. it contains a heme that cycles between two oxidation states
b. its oxidized form predominates while light absorption is taking place
c. it activates some biosynthetic enzymes by reducing disulfide bridges
d. it activates some degradative enzymes by reducing disulfide bridges

Q16. Suppose the interior of the thylakoids of isolated chloroplasts were made acidic and then transferred in the dark to a pH-8 solution. What would be likely to happen?

a. the Calvin cycle will be activated
b. the isolated chloroplasts will make ATP
c. cyclic photophosphorylation will occur
d. only a and b will occur

Q17. When sunlight is on the chloroplast, pH is lowest in the

a. stroma
b. space enclosed by the inner and outer membranes
c. spaces enclosed by the thylakoid membranes
d. cytosol

Q18. DCMU inhibits photosynthesis at photosystem during

a. Phe $\rightarrow Q_A$
b. $Q_A \rightarrow Q_b$
c. Cyt $bf_b \rightarrow$ PC
d. $Q_B \rightarrow$ Cyt bf_b **[CSIR-UGC]**

Q19. Cyanobacteria differ from purple and green photosynthetic bacteria because they

a. have a membrane-enclosed nucleus
b. use H_2S as an electron donor
c. do not require light
d. produce oxygen during photosynthesis

Q20. Which of the following statement about photosystem II is *incorrect?*

a. it is a multimolecular transmembrane assembly containing several polypeptides, several chlorophyll molecules, a special pair of chlorophyll (P680), pheophytin, and plastoquinones
b. it transfers electrons to photosystem I via the cytochrome *bf* complex
c. it uses light energy to create a separation of charge whose potential energy can be used to oxidize H_2O and to produce a reductant, plastoquinol
d. it uses an Fe^{2+}-Cu^{+} center as a charge accumulator to form O_2 without generating potentially harmful hydroxyl radicals, superoxide anions, or H_2O_2

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Chapter - 09

Cell Structure and Functions

9.1 ORGANIZATION AND DIVERSITY OF CELLS

All organisms more complex than viruses consist of cells, aqueous compartments bounded by membranes, which under restricted conditions are capable of existing independently. All cells are derived by cell division from other cells. All cellular organisms can be subdivided into two major classes, prokaryotes and eukaryotes, on the basis of the architecture of their cells.

Prokaryotes lack a defined nucleus and have a relatively simple internal organization. Under the electron microscope they appear relatively featureless. They comprise two kingdoms of life: **eubacteria** which include most of the bacteria; and the **archaea**, rather poorly understood organisms that superficially resemble bacteria and often grow in unusual environments, such as in acid hot springs, saturated brines, etc. **Eukaryotes** are thought to have first appeared about 1.5 billion years ago. There is only one kingdom of eukaryotic organisms - the eukarya - but this includes *Protists, Fungi, Animals and Plants*. Eukaryotes have a much more complex intracellular organization with internal membranes, membrane-bound organelles including a nucleus, and a well-organized cytoskeleton as compared to prokaryotes.

STRUCTURE OF EUKARYOTIC CELLS

9.2 PLASMA MEMBRANE

Plasma membrane (PM) is a dynamic, fluid structure and forms the external boundary of cells. It acts as a selectively permeable membrane and regulates the molecular traffic across the boundary. Different models were proposed to explain the structure of plasma membranes. In 1972, Jonathan Singer and Garth Nicolson proposed **fluid-mosaic model** which is now the most accepted model. In this model, membranes are viewed as quasi-fluid structures in which proteins are inserted into lipid bilayers. It describes both the *mosaic* arrangement of proteins embedded throughout the lipid bilayer as well as the *fluid* movement of lipids and proteins alike.

Chemical constituents of plasma membrane

All plasma membranes, regardless of source, contain proteins as well as lipids. The ratio of protein to lipid varies enormously depending on cell types. For example, the plasma membrane of human RBC contains 43% lipid and 49% protein by weight whereas plasma membrane of mouse liver cells contain 54% lipid and 46% protein by weight. Carbohydrates are also present in plasma membranes and comprises only 5 to 10% of membrane mass. Carbohydrates bound either to proteins as constituents of glycoproteins or to lipids as constituents of glycolipids. Carbohydrates are especially abundant in the plasma membranes of eukaryotic cells.

Phospholipid bilayer

The basic structure of the plasma membrane is the phospholipid bilayer. This bilayer is composed of two *leaflets* of amphipathic phospholipids molecules whose polar head groups are in contact with the intra- or extracellular aqueous phase, whereas their non-polar tails face each other, constituting the hydrophobic interior of the membrane.

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The primary physical forces for organizing lipid bilayer are *hydrophobic interactions*. These interactions result in the formation of a phospholipids bilayer, a sheet containing two layers of phospholipid molecules whose polar head groups face the surrounding watery surface while the fatty acyl chains form a continuous hydrophobic interior.

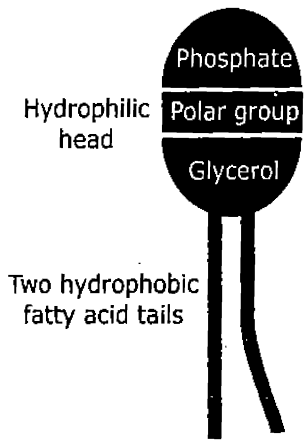


Figure 9.1 : Phospholipid structure

Two types of phospholipids occur in bilayer : *glycerophospholipids,* in which the hydrophobic regions are composed of two fatty acids joined to glycerol and *sphingophospholipids*, in which a single fatty acid is joined to sphingosine. Glycerophospholipids are the most abundant class of the lipids in most membranes. The plasma membrane of animal cells contain four major phospholipids, such as *phosphatidylcholine* (the most abundant Glycerophospholipids in the plasma membrane), *phosphatidylserine*, *phosphatidylethanolamine*, and *sphingomyelin*. At neutral pH, the polar head group may have no net charge (phosphatidylcholine) or it may have net negative charges (phosphatidylglycerol, cardiolipin, phosphatidylserine). Rarer phospholipids have a net positive charge. The lipid bilayer is not composed exclusively of phospholipids. In addition to the phospholipids, the plasma membranes also contain **glycolipids** and **sterol.**

Glycolipids contain sugar residues covalently attached to the lipid. These can derive from either glycerolipids or sphingolipids. The simplest glycolipid, called a *cerebroside*, contains a single sugar residue, either glucose or galactose. *Gangliosides* are more complex glycolipids, containing a branched chain of as many as seven sugar residues. The glycolipids are found exclusively in the outer leaflet of the plasma membrane, with their carbohydrate portions exposed on the cell surface.

Sterols are structural lipids present in the membranes of most eukaryotic cells. Basic structure of sterol is a *steroid nucleus,* consisting of four fused rings, three with six carbons and one with five. It is planar, and relatively rigid structure. *Cholesterol* is the major sterol present in the plasma membrane of animal cells. With rare exceptions, bacteria lack sterols. Plant cells also lack cholesterol, but they contain sterols like stigmasterol.

Table 9.1 : Major lipid components of plasma membranes

Source	PC	PE + PS	SM	Cholesterol
Plasma membrane (human RBC)	21	29	21	26
Plasma membrane (*E. coli*)	0	85	0	0
Myelin membrane (human neurons)	16	37	13	34

Composition in mol %

PC - phosphatidylcholine; PE - phosphatidylethanolamine; PS - phosphatidylserine; SM - sphingomyelin.

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Lipids are not randomly mixed in each leaflet of a bilayer. Certain lipids in the plasma membrane, particularly cholesterol and sphingolipids, are organized into aggregates, called **lipid rafts**. Lipid rafts are membrane microdomains that are enriched with cholesterol and glycosphingolipids. These microdomains also contain specific proteins. In mammalian cells, lipid rafts termed *caveolae* are marked by the presence of *caveolin* proteins. The rafts in cells appear to be heterogeneous both in terms of their protein and their lipid content, and can be localized to different regions of the cell. Lipid rafts have been implicated in processes as diverse as signal transduction, endocytosis and cholesterol trafficking.

When amphipathic lipids are mixed with water, three types of lipid aggregates can form. In the case of fatty acid salt, which contain only one fatty acid chain, the molecules form a small and spherical micellar structure (diameter usually <20nm) in which the hydrophobic fatty acid chain are hidden inside the **micelle.** A second type of lipid aggregate in water is the bilayer, in which two lipid monolayers combine to form a two-dimensional sheet. In third type of lipid aggregate lipid bilayer form a hollow sphere called a **liposome**. Liposomes are closed, self sealing, solvent filled vesicles that are bounded by only a single bilayer.

Motion of lipid molecules

Lipid molecules can rotate freely around their long axis (**rotational motion**) and diffuse laterally (**lateral motion**) within each leaflet. The rapid lateral movement of membrane proteins has been visualized by means of fluorescence microscopy through the use of the technique of *fluorescence recovery after photobleaching* (FRAP). The transition of a molecule from one membrane surface to the other is called transverse diffusion (**flip-flop**).

The thermodynamic barrier to passive lipid *flip-flop* prevents rapid spontaneous transbilayer diffusion of phospholipids and is therefore a rare event. The half time for phospholipid flip-flop is approximately several hours to days and depend on the nature of the lipid and the membrane. Flip-flop rates are also dependent on phospholipid acyl chain length and degree of unsaturation.

Asymmetry of lipid bilayer

The phospholipid in plasma membranes are asymmetrically distributed across the bilayer; the amine-containing phospholipids are enriched on the cytoplasmic surface of the plasma membrane, while the choline-containing and sphingolipids are enriched on the outer surface. This asymmetry is usually not absolute, except for glycolipids. In the human erythrocyte, for example, the phospholipids, such as sphingomyelin and phosphatidylcholine are mostly found in the extracytoplasmic leaflet whereas phosphatidylserine and phosphatidylethanolamine are preferentially located on the cytoplasmic face.

The maintenance of transbilayer lipid asymmetry is essential for normal membrane function. Once lipid asymmetry has been established, it is maintained by a combination of slow transbilayer diffusion, protein-lipid interactions, and protein-mediated transport.

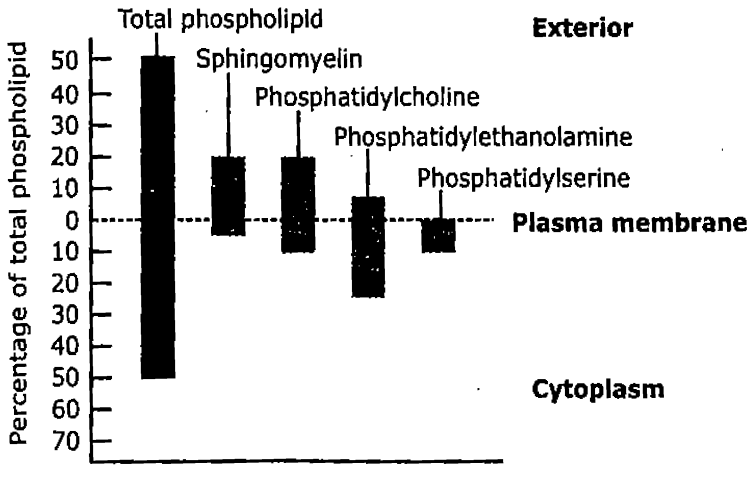


Figure : 9.2
Distribution of phospholipids on the two faces of the erythrocyte plasma membrane.

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Role of flippase and floppases

Although the barrier to rapid spontaneous flip-flop contributes to the maintenance of lipid asymmetry, other mechanisms must be responsible for the regeneration of lipid asymmetry. Perhaps the most significant contributors to the maintenance and dissipation of transbilayer lipid asymmetry are proteins that catalyze the movement of lipids across the membrane. These activities are classified according to substrate specificity, requirements for energy, and direction of transport. Two classes of transport activities have been described that are responsible for the *ATP-dependent transport* of lipids. The most well-characterized activity, catalyzed by **flippase**, transports glycerophospholipid from the outer monolayer to the cytoplasmic surface of the plasma membrane. A second *ATP-dependent* activity, catalyzed by **floppases**, transports lipids in the opposite direction.

Fluidity of a lipid bilayer

The fluidity of the membrane, the optimal value of which is a prerequisite for normal cell growth and function, depends on lipid composition and temperature. In general, an increase in the temperature tends to elevate membrane fluidity. Lowering of temperature causes a freely flowing, low-viscosity, fluid like membrane structure to a more rigid, gel-like organization. This change is described as **phase transition** and the temperature at which this happens is called the **transition temperature**.

Fluidity of lipid bilayer also depends on chain length and the degree of saturation of its component fatty acid residues. Lipids with short or unsaturated fatty acyl chains undergo the phase transition at lower temperatures than lipids with long or saturated chains. Short chains have less surface area with which to form van der Waals interactions with each other. Hence a shorter chain length reduces the tendency of the hydrocarbon tails to interact with one another. Unsaturated fatty acyl chains have kinks, thus tend to adopt a more random, fluid state and form less stable van der Waals contacts with other lipids. So, increased proportion of unsaturated to saturated fatty acids in the membrane increases the fluidity of bilayer at the reduced temperature.

Cholesterol is also a major determinant of membrane fluidity. At high temp, cholesterol interferes with the movement of the phospholipid fatty acid chains, making the membrane less fluid but at low temp it interferes with interactions between fatty acid chains and prevents membranes from freezing. At the high concentrations found in eukaryotic plasma membranes, cholesterol tends to make the membrane, overall, less fluid at growth temperatures near 37°C.

MEMBRANE PROTEINS

Many of the proteins associated with membrane can be released from the membrane by relatively gentle extraction procedures, such as exposure to solutions of very high or low ionic strength or of extreme pH, which interfere with protein-protein interactions but leave the lipid bilayer intact; these proteins are referred to as **peripheral proteins** (or extrinsic proteins). Proteins that are held in the bilayer by unusually tight binding to other proteins or lipids and cannot be released by relatively gentle extraction procedures are called **integral membrane proteins** (or intrinsic proteins).

Peripheral membrane proteins do not interact with the hydrophobic core of the phospholipid bilayer. Instead they are usually bound to the membrane indirectly by interactions with integral membrane proteins or directly by interactions with lipid polar head groups. Most peripheral proteins are soluble in aqueous solution. *Spectrin* and *ankyrin* are very common example of peripheral proteins present in the membrane of RBCs. Spectrin and ankyrin membrane proteins help to determine the shape and flexibility of the RBC.

Integral membrane proteins, have one or more segments that are embedded in the phospholipid bilayer. Most integral proteins contain residues with hydrophobic side chains that interact with fatty acyl groups of the membrane phospholipids, thus anchoring the protein to the membrane. These proteins may be transmembrane proteins or lipid linked proteins.

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Transmembrane proteins

Most integral proteins span the entire phospholipid bilayer. Transmembrane proteins may be *single pass* (monotopic) or *multi pass* (polytopic). *Glycophorin* is a major single pass transmembrane protein of RBC. It is a small glycoprotein of 131 amino acid residues. It was the first membrane protein for which the complete amino acid sequence was determined. Despite their abundance in the plasma membrane of RBC their function remains unknown.

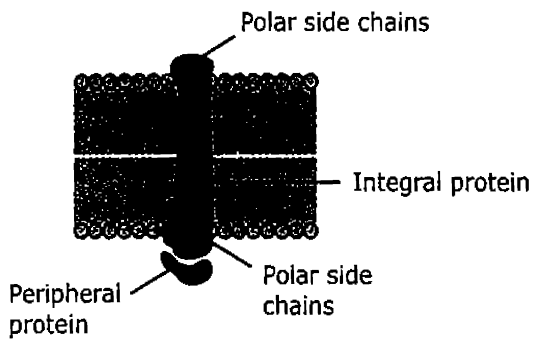


Figure : 9.3
Schematic outline of the interaction of a typical integral membrane protein and peripheral protein with a lipid bilayer.

Another prominant protein present on the plasma membrane of RBC is *band 3 protein* or *chloride-bicarbonate exchanger*, a 95 kDa multipass membrane protein. It is responsible for mediating the electroneutral exchange of chloride for bicarbonate.

Transmembrane proteins are characterized by having transmembrane α-helices. They contain a stretch of 21 to 26 hydrophobic amino acid residues coiled into an α-helix that is believed to facilitate the spanning of a membrane bilayer. In a few membrane proteins, notably the **porins**, the transmembrane portion comprises β-barrel made of antiparallel β-strands. The β-barrel is stabilized by extensive hydrogen bonding between the β-strands. The interior of the barrel is hydrophilic.

Lipid linked protein

Lipid linked proteins form covalent attachments with three classes of lipids: isoprenoid groups such as farnesyl and geranylgeranyl resiudes; fatty acyl groups such as myristoyl and palmitoyl residues; and glycoinositol phospholipids (GPIs).

An isoprenoid group that contains 15 (farnesyl) or 20 (geranylgeranyl) carbon atoms attaches to a cysteine residue at the C-terminus via a *thioether* linkage.

A myristic acid (14-carbon, saturated fatty acid) molecule attaches to a protein through *amide* linkage to the alpha-amino group of an N-terminal *Gly* residue. A palmitic acid (16-carbon, saturated fatty acid) molecule attaches to a *Cys* residue close to the N- or C-terminus via a *thioester* linkage.

A glycosyl phosphatidyl inositol molecule (GPI) attaches to the C-terminal amino acid via an amide linkage. GPI-linked proteins occur on the exterior surface of the plasma membrane.

Table 9.2 : Characteristics of lipid linked proteins

Lipid anchor	*Attachment site*	*Subcellular location*
Isoprenyl (Farnesyl/Geranylgeranyl)	Cysteine residue at C-terminus	Intracellular
Myristoyl	Glycine residue at N-terminus	Intracellular
Palmitoyl	Cysteine residue near N- or C-terminus	Intracellular
GPI linked proteins	Various residues at C-terminus	Cell surface

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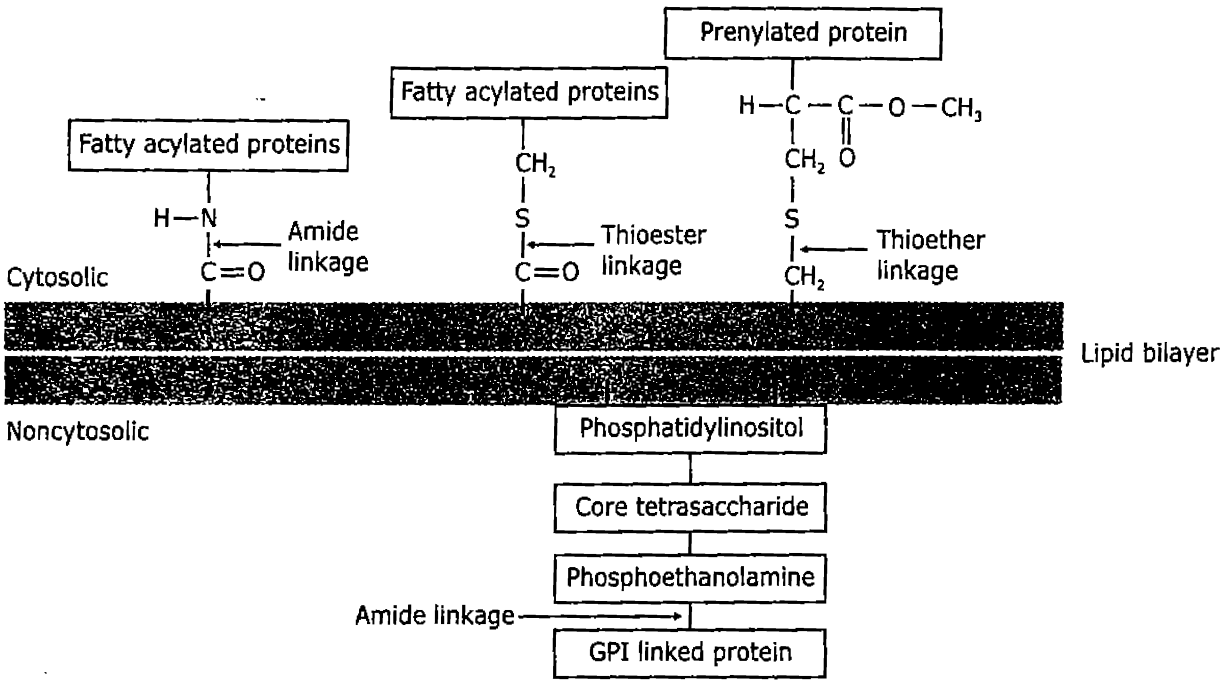


Figure 9.4 : Lipid linked membrane proteins are attached to the bilayer either through a fatty acid chain or prenyl group in the cytoplasmic monolayer or, less often, via an oligosaccharide, to a minor phospholipid, phosphatidylinositol, in the noncytoplasmic monolayer. A fatty acid chain, myristic acid, is attached via an amide linkage to an amino-terminal glycine. The palmitic acid is joined to a protein in thioester linkage to a specific Cys residue. A prenyl group (either farnesyl or a longer geranylgeranyl group) is attached via a thioether linkage to a cysteine residue that is four residues from the carboxyl terminus. GPI anchors are derivatives of phosphatidylinositol in which the inositol bears a short oligosaccharide covalently joined to the carboxyl-terminal residue of a protein through phosphoethanolamine.

Transport proteins

Membrane proteins can also be classified on the basis of function like catalytic proteins, structural proteins and transport proteins. Transport proteins, responsible for selective transport of molecules may be **carrier** or **channel proteins**.

Carrier mediated transport maybe active or passive but channel mediated transport is always passive and transport through channel proteins occurs at a much faster rate than mediated by carrier proteins. There are three general classes of carrier mediated transport systems that differ in the number of solutes (substrates) transport and the direction in which each is transported. Carrier proteins that transport single solid from one side of membrane to the other are called **uniporters**. These single noncoupled fluxes are called uniports. Some carrier proteins mediate coupled transport. Coupled transport involves either the simultaneous transfer of a second solute in the same direction, called **symport** or the transfer of second solute in the opposite direction, called **antiport**.

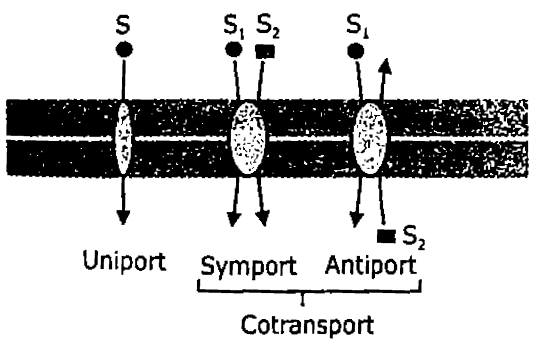


Figure : 9.5
Three types of carrier mediated transport involving carrier proteins functioning as uniporters, symporters and antiporters.

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Freeze-fracture technique

This technique is used to visualize the distribution of proteins in membranes. In this technique a membrane specimen is rapidly frozen to near liquid nitrogen temperatures (-196°C) to immobilize the sample. The specimen is then fractured with a cold microtome knife, which often splits the bilayer into monolayer and exposes the interior of the lipid bilayer and its embedded proteins.

The freeze fracture method involves the following steps :

- Cryofixation of the specimen by rapid freezing.
- Fracturing of the specimen by cleaving it with a microtome knife or breaking it.
- Coating of freeze-fracture plane by heavy metals under high vacuum.
- Removal of the specimen from the replica.
- Observation of the replicas with a TEM.

Freeze etching is a slight modification of freeze fracturing through the process of controlled sublimation of water vapour (transition from the solid state to the gas state, bypassing the fluid state) to expose membrane surfaces or macromolecules just below the fracture plane.

Cell coat or **glycocalyx**

Carbohydrates are present on the surface of all eukaryotic cells. These carbohydrates as oligosaccharides occur both as *glycoproteins* and *glycolipids*. Plasma membranes also contain integral *proteoglycan molecules* with surface-exposed polysaccharide chains. The term *cell coat*, or *glycocalyx* is used to describe the carbohydrate coating on the cell surface. *Cell coat* helps to protect the cell surface from mechanical and chemical damage and also mediate specific, transient, cell-cell adhesion events.

ABO blood group

The ABO antigens are complex oligosaccharides present in most cells of the body. On membranes of RBCs, the oligosaccharides that determine the specific natures of the ABO antigens joined either to ceramide where the substances are glycosphingolipids, or to the polypeptide backbone of a protein via a serine or threonine residue where the substances are glycoproteins. In the formation of A and B antigens, H molecule acts as a precursor. H molecule is the blood group substance found in persons of type O. In case of *A antigen*, H contains an additional *N-acetylgalactosamine* (GalNAc). Similarly in case of *B antigen* an additional *galactose* (Gal) is linked to H molecule. Anti-A antibodies are directed to the additional GalNAc residue found in the A substance, and anti-B antibodies are directed towards the additional Gal residue found in the B substance. The addition of the terminal GalNAc to the H molecule is catalyzed by *GalNAc transferase*, a product of A gene. Similarly, the product of the B gene is the *Gal transferase* adding the Gal residue to the H molecule. Individuals of type AB possess both enzymes and thus have two A and B antigens and individuals of type O contains only H molecule.

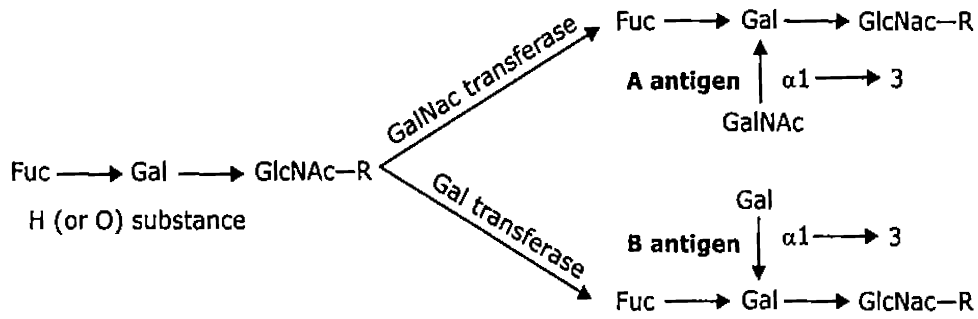


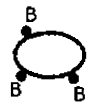
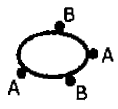
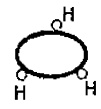
Figure 9.6 : Diagrammatic representation of the structures of the H, A, and B blood group substances. R represents a long complex oligosaccharide chain, joined either to ceramide or to the polypeptide backbone of a protein. The H, A, and B antigens each contain two of their respective short oligosaccharide chains shown above.

Fuc =Fucose; Gal = Galactose ; GalNAc = N-Acetylgalactosamine ; GlcNAc = N-Acetylglucosamine

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Blood group : ABO system

Blood group	**A**	**B**	**AB**	**O**
Genotype	AA and AO	BB and BO	AB	OO
Antibodies (in blood)	Anti B	Anti A	—	Anti A Anti B
Antigen				

9.3 TRANSPORT ACROSS PLASMA MEMBRANE

The internal composition of the cell is maintained because the plasma membrane is a selectively permeable structure. Plasma membrane forms a barrier that blocks the free exchange of molecules between the cytoplasm and the external environment of the cell.

Most biological molecules are unable to diffuse through the protein-free phospholipid bilayer (or synthetic lipid bilayer). Small non-polar molecules, such as O_2 and CO_2, readily dissolve in synthetic lipid bilayers and therefore diffuse rapidly across them. Small uncharged polar molecules, such as water or urea, also diffuse across a synthetic lipid bilayer. By contrast, synthetic lipid bilayers are highly impermeable for charged molecules (as shown in the figure 9.7).

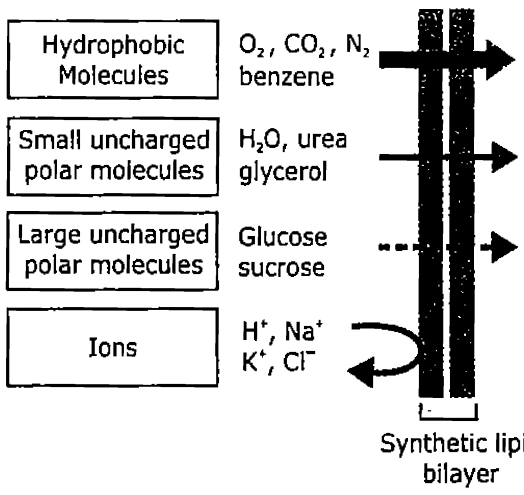


Figure : 9.7
The relative permeability of different classes of molecules across synthetic lipid bilayer. Bilayer is permeable to small hydrophobic molecules and small uncharged polar molecules, slightly permeable to water and urea, and essentially impermeable to ions.

However the plasma membrane unlike synthetic lipid bilayer allow the passage of various polar molecules such as ions, sugars, amino acids and others. Special classes of transport proteins (carrier and channel) are responsible for transferring such solutes across cell membrane. Thus the transport across plasma membrane can occur either through the lipid bilayer or through the transport proteins. The characteristics of lipid-phase diffusion and protein mediated transport are basically different. Transport across the plasma membrane is of two types :

1. PASSIVE TRANSPORT

It occurs along the concentration gradient and without use of metabolic energy. It may be-

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a. **Simple diffusion**

During simple diffusion, a molecule simply dissolves in the phospholipid bilayer, diffuses across it. No membrane proteins are involved and the direction of transport is determined simply by the relative concentrations of the molecule inside and outside of the cell.

The relative diffusion rate of any substance across a *pure* phospholipid bilayer is proportional to its *concentration gradient* across the layer and to its *hydrophobicity* and *size.* The hydrophobicity of a substance is measured by its *partition coefficient*. It is the equilibrium constant for partition of the molecule between oil and water. The higher the substance partition coefficient, the more lipid soluble it is.

Movement of solutes by diffusion is always from a higher to a lower concentration, and the rate is described by *Fick's law of diffusion*.

$$J = -D\left(\frac{\Delta C}{\Delta x}\right)$$

Where J is the flux per unit area, D is the *diffusion coefficient* (usually expressed as cm^2/sec), and ΔC is the difference in concentration between two regions separated by a distance Δx (membrane thickness in case of membrane transport).

Simple diffusion is a *nonselective* process by which any molecule able to dissolve in the phospholipid bilayer is able to cross the plasma membrane and equilibrate between inside and outside of the cell. The rate of diffusion of the molecule will be proportional to its hydrophobicity. Gases (such as O_2 and CO_2), hydrophobic molecules (such as benzene), and small polar but uncharged molecules (such as H_2O and ethanol) are able to diffuse across the plasma membrane.

b. **Facilitated diffusion**

Facilitated diffusion, like passive diffusion, involves the movement of molecules in the direction determined by their relative concentrations inside and outside of the cell. However, passage is mediated by transport protein called **permease** and is selective in nature.

The rate of transport of the molecule across the membrane is far greater in facilitated diffusion as compare to simple diffusion. Facilitated diffusion allows polar and charged molecules, such as carbohydrates, amino acids, nucleosides, and ions, to cross the plasma membrane.

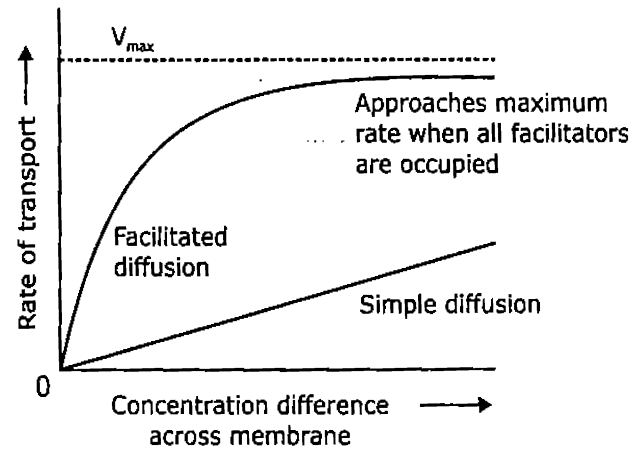


Figure 9.8 : Facilitated and simple diffusion.

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Classes of proteins that mediate facilitated diffusion:

Carrier proteins mediated

Carrier proteins (called *transporters* or *permeases*) non-covalently binds specific molecules to be transported on one side of the membrane. They then undergo conformational changes that allow the molecule to pass through the membrane and be released on the other side. A classic example is the movement of glucose mediated by carrier protein, **glucose transporter** (GLUT). There are various isoforms of the glucose transporter (GLUT) family. These carriers are composed of ~500 amino acids and possess 12 transmembrane segments. Individual members differ in their kinetic properties, tissue distribution, and regulation. The GLUT-1 (erythrocyte), GLUT-2 (liver), GLUT-3 (brain), and GLUT-5 (small intestine) isoforms are constitutively expressed on the cell surface, whereas GLUT-4, the predominant isoform of skeletal muscle and adipose tissue, is stored in intracellular vesicles, which upon insulin stimulation fuse with the plasma membrane, thereby increasing the rate of sugar uptake by several-fold.

The rate of transport (V) can be well described with the following equation:

$$V = V_{max}/(1+K_m/C)$$

Where V_{max} is the maximal transport velocity, K_m is the substrate (glucose) concentration at which the half-maximal transport rate is attained, and C is the concentration of the substrate. This relationship is formally equivalent with the Michaelis-Menten equation, which relates the velocity of enzyme catalyzed reactions to the substrate concentration. The K_m values, which can be viewed as the affinity of the isoforms for glucose.

The *chloride-bicarbonate exchanger*, (also called anion exchange protein) mediates the transport of two anions simultaneously HCO_3^- ion moves in one direction and Cl^- moves in opposite direction. The exchange is electroneutral because of no net transfer of charge.

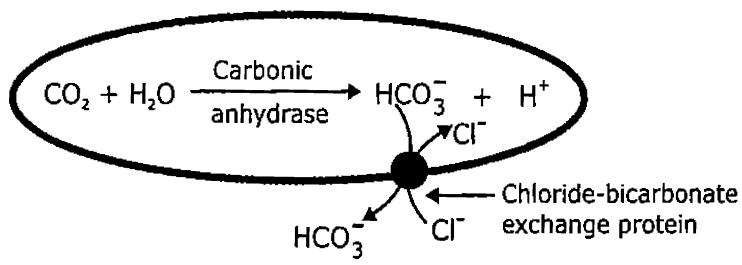


Figure : 9.9
The chloride-bicarbonate exchange protein of the erythrocyte membrane allows the entry and exit of HCO_3^-. For each HCO_3^- ion that moves in one direction, one Cl^- ion moves in the opposite direction. The result of this paired movement of two monovalent anions is no net change in the charge or electrical potential across the erythrocyte membrane.

Channel proteins mediated

Channel proteins form open pores through the membrane, allowing the free diffusion of any molecule of the appropriate size and charge. Rate of diffusion mediated by channel protein is higher than those mediated by carriers. Channels typically show less stereospecificity than carriers and are usually non-saturable. Channel proteins concerned specifically with inorganic ions transport are called **ion channels.** Many animal cells also contain specialized *water channel* in their plasma membrane to facilitate osmotic water flow called **aquaporins.** Ion channels are highly selective. Most of the ion channels are not permanently open. Some channels (called **voltage gated channels**) open in response to change in electric potential; others (called **ligand gated channels**) open in the response to the binding of ligands.

The properties of ion channels can be studied by means of the **patch clamp technique**. In this technique a very fine pipette (with an opening of about 0.5 μm) is pressed against the plasma membrane and the plasma membrane can be pulled away from the cell and the preparation placed in a test solution of desired composition. Current flow through a single ion channel can then be measured. This technique has provided lots of valuable information about ion channels that its inventors, Erwin Neher and Bert Sakmann, were awarded Nobel Prize in 1991.

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Ionophores

Ionophores are small hydrophobic lipid-soluble molecules that dissolve in lipid bilayers and increase their permeability to specific inorganic ions. Ionophores shield the charge of the ion to be transported, enabling it to penetrate the hydrophobic interior of the lipid bilayer. There are two classes of ionophores - *mobile ion carriers* and *channel formers*.

Valinomycin (*isolated from Streptomyces fulvissimus*) is an example of a mobile ion carrier. It transports K^+ down its electrochemical gradient. **Gramicidin A** (produced by the bacterium *Bacillus brevis*) is a 15-residue peptide with alternating D- and L-amino acids. In membranes it forms a channel. The channel permits the passage of water and univalent cations but not anions.

2. ACTIVE TRANSPORT

Active transport occurs against the concentration gradient and is mediated by carrier proteins. Metabolic energy is used to move ions or molecules against concentration gradient. Active transport results in the accumulation of solute on one side of membrane. Active transport is different from carrier proteins mediated facilitated diffusion. Comparison of facilitated and active transport is given in the following table.

Table 9.3 : Comparison of facilitated diffusion and active transport

Facilitated diffusion	*Active transport*
Selective	Selective
Passive	Active
Occurs along the conc. gradient	Occurs against the conc. gradient
Transport protein involved	Transport protein involved
Saturable	Saturable
Entropy increases	Entropy decreasès

Active transport is of two types:

a. Primary (direct) active transport

It is coupled directly to a metabolic source of energy, such as ATP hydrolysis, oxidation-reduction reaction (electron transport chain of mitochondria and chloroplast) or absorption of light by carrier protein (in halobacteria). Example, virtually every animal cell maintains lower Na^+ concentration and high K^+ concentration than is found in its surrounding. This imbalance is established and maintained by a active transport system in PM, involves the enzyme **Na^+ -K^+ ATPase** coupled with breakdown of ATP (discovered by Jens Skou in 1957). For each molecule of ATP hydrolyzed, two K^+ moves inward and three Na^+ ions move outwards. It is a heterodimer composed of an alpha-subunit and a glycoprotein beta-subunit. The alpha-subunit is responsible for ATP hydrolysis and ion pumping while the beta-subunit is important for the correct assembly of the ATPase in the endoplasmic reticulum. The alpha-subunit contains 10 transmembrane segments.

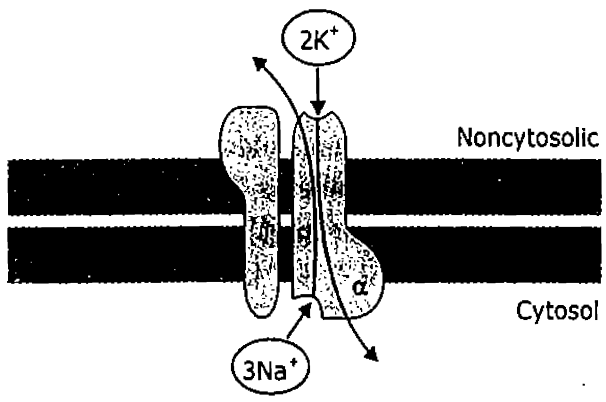


Figure : 9.10
Structure of Na^+-K^+ ATPase. The Na^+K^+ATPase is primarily responsible for setting and maintaining the intracellular concentrations of $Na^+K^+$and for generating the transmembrane electrical potential, which it does by moving $3Na^+$ out of the cell for every $2K^+$it moves in.

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Certain steroids like *Digitalis* and ouabain, which are known as **cardiotonic steroids** are potent inhibitors of the Na^+-K^+ pump. These compounds inhibit the dephosphorylation of Na^+-K^+ pump when applied on the extracellular face of the membrane. Digitalis is used in the treatment of congestive heart diseases because it increases the force of contraction of heart muscle by inhibiting the Na^+-K^+ pump.

The active transport of Ca^{2+} across the plasma membrane is driven by a Ca^{2+} pump that is structurally related to the Na^+-K^+ pump and is similarly powered by ATP hydrolysis. The Ca^{2+} pump transports Ca^{2+} out of the cell, so intracellular Ca^{2+} concentrations are extremely low.

Transport ATPase

Transport ATPase are the ATP powered pumps, which transport ions and various small molecules against their concentration gradients. Three major types of ATPase are associated with membranes that couple ATP hydrolysis to the translocation of specific ions across a membrane: P-, V-, and F-ATPases.

P-ATPases have a simple polypeptide composition and are phosphorylated during catalysis. Examples of P-ATPases are the Na^+ K^+-ATPase of the plasma membrane of animal cells; the H^+-ATPase of the plasma membranes of fungi, and plants; and the Ca^{2+}-ATPase of the sarcoplasmic reticulum. The plasma membrane Na^+, K^+-ATPase and H^+-ATPase function to generate and maintain the plasma membrane electrical potential difference (inside negative).

V-ATPases are found on the membranes within the cells (endomembranes), including membranes from vacuoles (from which the 'V' is derived) and not phosphorylated during catalysis.

F-ATPases (also known as F_0-F_1 ATPase) have a complex polypeptide composition and, as in V-ATPases, there is no phosphorylated intermediate in the reaction mechanism. In nonphotosynthetic eukaryotes, the F-ATPase is found exclusively on the inner membrane of mitochondria, whereas in green plants and algae there are two distinct F-ATPases; one in mitochondria and the other on the thylakoid membrane of chloroplasts. In bacteria, F-ATPase is present in the plasma membrane. These ATPases couple the flow of protons to ATP hydrolysis and synthesis.

ABC Transporter

ABC (*A*TP *B*inding *C*assette) transporters constitute a large family of ATP dependent transporters that pump wide range of molecules, including ions, sugars, and amino acids. ABC transporters utilize energy derived from ATP hydrolysis to transport molecules. ABC proteins share a conserved domain architecture. A functional transporter requires the cooperation of two transmembrane domains (TMD) and two cytosolic nucleotide binding domains (NBDs, also called ATP-binding cassettes). Eukaryotic ABC proteins are either full transporters combining all required domains in one polypeptide (2 TMDs and 2 NBDs), or half-transporters consisting of 1 TMD and 1 NBD that need to form homo- or heterodimers to constitute a functional pump.

In eukaryotic cells, the first ABC transporter was discovered was **MDR** (multidrug resistance) transporter that makes cancer cells resistant to a variety of drugs used in chemotherapy. The protein, energized by ATP hydrolysis can export a variety of drugs against their concentration gradient, and in addition, it also seems to inhibit their influx. However transport exhibits very little substrate specificity. The only common characteristic of the accepted substrates appears to be their high hydrophobicity that is, work as a *hydrophobic vacuum cleaner*.

CFTR (*c*ystic *f*ibrosis *t*ransmembrane *c*onductance *r*egulator) also belongs to ABC transporter family. It functions as a Cl^- channel. Most ABC transporters act as pumps, but at least some members of the superfamily act as ion channels that open and closed by ATP hydrolysis. The CFTR Channel is unusual in that it require both ATP hydrolysis and cAMP-dependent phosphorylation. Defective Cl^- transport is responsible for disease *cystic fibrosis*.

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b. Secondary (indirect) active transport

Secondary active transport occurs when endergonic (uphill) transport of one solute is coupled to the exergonic (downhill) flow of a different solute that was originally pumped uphill by primary active transport. Secondary active transport is either symport or antiport, depending on whether the two solutes move in the same or opposite directions.

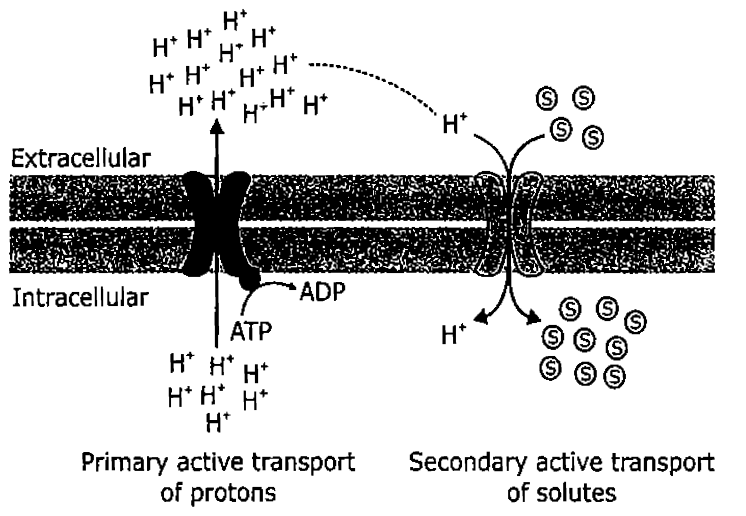


Figure 9.11 : Comparison of primary and secondary active transport.

Example, the transmembrane protein **Na^+/glucose transporter**, acts as a carrier, allows sodium ions and glucose to enter the cell together. The sodium ions flow down their concentration gradient while the glucose molecules are pumped up theirs. Later the sodium is pumped back out of the cell by the Na^+/K^+ ATPase.

Thermodynamics of transport

Free energy change, ΔG, for transporting one mole of a substance from a region in which its concentration is C_1 to a place where its concentration is C_2 is given by

$$\Delta G = RT \ln \frac{C_2}{C_1} \quad \ldots\ldots\ldots\ldots (1)$$

According to this equation, if C_2 is less than C_1, ΔG is negative, and the process is thermodynamically favorable. As more and more substance is transferred C_1 decreases and C_2 increases, until $C_2 = C_1$. At this point $\Delta G = 0$, and the system is in equilibrium. A substance that can traverse the membrane will eventually reach the same concentration on both sides.

However if an ion of charge Z moves then the free energy change for transport across a cell membrane involves two contributions: the normal concentration term, as given in equation (1), plus a second term describing the energy change involved in moving a mole of ions across the potential difference. We consider a process in which ions are transported from outside to inside.

$$\Delta G = RT \ln \frac{C_{in}}{C_{out}} + ZF\,\Delta\psi$$

Here F is the Faraday constant (96.5 kJ $mole^{-1}$ V^{-1}), and $\Delta\psi$ is the change in membrane potential in volts. If $\Delta\psi$ is negative and Z is positive, the ZF $\Delta\psi$ term in equation makes a negative contribution to $\Delta\psi$. That is, the transport of cations into the cell is favored. For anions, of course, the opposite is true; they will be driven out. If the potential difference is somehow maintained, the equilibrium state ($\Delta\psi = 0$) will not correspond to the same concentration of ions on the two sides of the membrane.

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STUDY QUESTIONS

Q1. The parietal cells of the stomach contain membrane 'pumps' that transport hydrogen ions from the interior of the cell (pH 7.0) to the surrounding medium to make gastric juice (pH 1.0). Calculate the free energy required to transport 1 mol of hydrogen ions.

Ans. The free energy required to transport 1 mol of H^+ from the interior of the cell, where $[H^+]$ is 10^{-7} M (C_1), across the membrane to where $[H^+]$ is 10^{-1} M(C_2) is

$$\Delta G = 2.303\ RT \log (C_2/C_1) = 2.303(1.98)(298) \log \frac{10^{-1}}{10^{-7}} = 8150 \text{ cal/mol} = 8.2 \text{ kcal/mol}$$

Q2. Glucose is frequently administered intravenously to patients as a food source. Given that the transformation of glucose to glucose 6-phosphate consumes ATP, why not administer glucose 6-phosphate instead?

Ans. The phosphate group on glucose 6-phosphate is completely ionized at pH 7, giving the molecule an overall negative charge. Since membranes are generally impermeable to molecules with an electric charge, glucose 6-phosphate could not pass from the bloodstream to the tissue and hence could not enter the glycolytic pathway and generate ATP. That is why glucose once phosphorylated in the cell cannot escape.

9.4 MEMBRANE POTENTIAL

Electrical character of ion transport may be **electroneutral** i.e.,electrically silent either by symport of the oppositely charged ions or antiport of similarly charged ions or **electrogenic** i.e., result in charge separation across the membrane. Electrogenic transport affects and can be affected by the membrane potential. For example, the Na^+/K^+ pump imports $2K^+$ and simultaneously exports $3Na^+$; that is, it moves 1 positive charge out of the cell. Its electrogenic operation directly contributes to the negative inside membrane potential, which is evidenced by the fact that stopping the pump using an alkaloid inhibitor, *oubain*, causes an immediate, slight depolarization of the cell membrane.

Electrical potentials across cell membranes are a function of the electrolyte concentrations in the intracellular and extracellular solutions and of the selective permeabilities of the ions. Active transport of ions by ATP-driven ion pumps, generate and maintain ionic gradients. In addition to ion pumps, which transport ions against concentration gradients, plasma membrane contains channel protein that allow ions to move through it at different rates down their concentration gradient. Ion concentration gradients and selective movements of ions create a difference in electric potential or voltage across the plasma membrane. This is called **membrane potential**. Typical membrane potentials are between -30 and -70mv (*minus sign indicates that inside of cells always negative with respect to the outside*). In nerve and muscle cells, membrane potential in resting state is referred as **resting potential**.

For many cells, the principal diffusing ions are sodium, potassium, and chloride. The exact quantitative expression for membrane potential is given by *Goldman-Hodgkin-Katz equation*, which relates the membrane potential to the intra- and extracellular concentration of these ions and their permeability coefficient.

$$\Psi = -\frac{RT}{F} \ln \frac{P_{K^+}[K^+]_{in} + P_{Na^+}[Na^+]_{in} + P_{Cl^-}[Cl^-]_{out}}{P_{K^+}[K^+]_{out} + P_{Na^+}[Na^+]_{out} + P_{Cl^-}[Cl^-]_{in}}$$

The equation provides a quantitative relation for predicting the membrane potential of resting cells, where R is the universal gas constant, T is the absolute temperature, and F is the Faraday constant; this relation requires only the permeabilities P and the concentrations of sodium, potassium, and chloride ions inside and outside the cell.

In most cells the resting membrane potential is dictated predominantly by the K^+ gradient, and its value is not far from the K^+ equilibrium potential. The reason for this is that at rest K^+ is the most permeable ion across the cell membrane. In contrast, Na^+ and Ca^{2+} ions are very far from their equilibrium, and they are attracted inward both by the negative membrane potential and by their concentration gradient. However,

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their contribution to the resting potential is very modest (in the case of Ca^{2+} it is negligible) because at rest the Na^+ and Ca^{2+} channels are closed, and therefore the membrane permeability is very small for these ions.

In all animal cells, the electric potential across the plasma membrane is generated primarily by movement of cytosolic K^+ ions through resting K^+ ions channels to the external medium. Movement of K^+ ions across membrane down its concentration gradient leaves an excess negative charge on the cytosolic face and deposits a positive charge on the axoplasmic face. In nerve cells, the resting potential is –70 mV, with the inside of the cell at a negative potential relative to the external medium. This is close to the potassium equilibrium potential and is largely due to a much higher potassium than sodium permeability across the membrane. In plants and fungi, the membrane potential is maintained by the ATP driven pumping of protons from the cytosol across the membrane.

NEURONS AND TRANSMISSION OF NERVE IMPULSES

Neurons

Neurons (nerve cells) are specialized cells that have the ability to respond to a stimulus and convert it into an action potential (possess *electrical excitability*). Most neurons have three parts:

1. A cell body,
2. Dendrites, and
3. An axon.

The **cell body** (perikaryon) contains a nucleus surrounded by cytoplasm that includes typical cellular organelles such as lysosomes, mitochondria, and a Golgi complex. Neuronal cell bodies also contain free ribosomes and prominent clusters of rough endoplasmic reticulum, termed *Nissl bodies*.

A nerve fiber is a general term for any neuronal process or extension that emerges from the cell body of a neuron. Most neurons have two kinds of processes: multiple **dendrites** and a single **axon**. Dendrites are the receiving or input portions of a neurons. They usually are short, tapering, and highly branched.

The single axon of a neuron propagates nerve impulses towards another neuron, a muscle fiber, or a gland cell. An axon is a long, thin, cylindrical projection that often joins the cell body at a cone-shaped elevation called the *axon hillock*. An axon contains mitochondria, microtubules, and neurofibrils. Because rough endoplasmic reticulum is not present, protein synthesis does not occur in the axon. The cytoplasm of an axon, called **axoplasm**, is surrounded by a plasma membrane known as the *axolemma* (lemma = sheath or husk). Along the length of an axon, side branches called *axon collaterals* may branch off, typically at a right angle to the axon. The axon and its collaterals end by dividing into many processes called *axon terminals* (telodendria).

Transmission of nerve impulses

A *nerve impulse* is an electrical signal produced by the flow of ions across the plasma membrane of a neuron. It propagates (travels) along the surface of the membrane of a neuron at speeds ranging from 0.5 to 130 meters per second. Propagation of nerve impulse in nerve cell (axon) changes the permeability of membrane. This change in permeability causes change in the potential across the axon membrane. This change in resting potential is called **action potential**. An action potential is generated when the membrane is depolarized (i.e. decrease in polarity between the two sides of the membrane). In the resting state, the membrane potential is -60 mV. In depolarized state the membrane potential becomes positive and attains a value of about +30. *Voltage-gated channels* are responsible for the generation of action potential. Two kinds of voltage-sensitive channels, one selectively permeable to Na^+ and the other to K^+ is responsible for transient changes in the permeability. Opening of voltage-gated Na^+ channels increases the permeability of membrane to Na^+ ions. The increase in Na^+ conductance increases the number of positive ions inside the axon. Similarly voltage-gated K^+ channels increase the K^+ ion conductance outside the axon.

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Depolarization at one point along an axon opens Na^+ channels locally. Sodium ions begin to flow into the cell along the electrochemical gradient across the plasma membrane. This leads to a very rapid and large change in membrane potential from about -60 mV to +30 mV in a millisecond. The resulting inward current flows passively along the axon depolarizing the adjacent region of the axon. As the action potential spreads, the membrane potential repolarizes due to K^+ channel opening and Na^+ channel inactivation. Due to opening of K^+ channel potassium ions flow outward, and so the membrane potential returns to a negative value. The resting level of -60 mV is restored in a few milliseconds as the K^+ conductance decreases to the value characteristic of the unstimulated state.

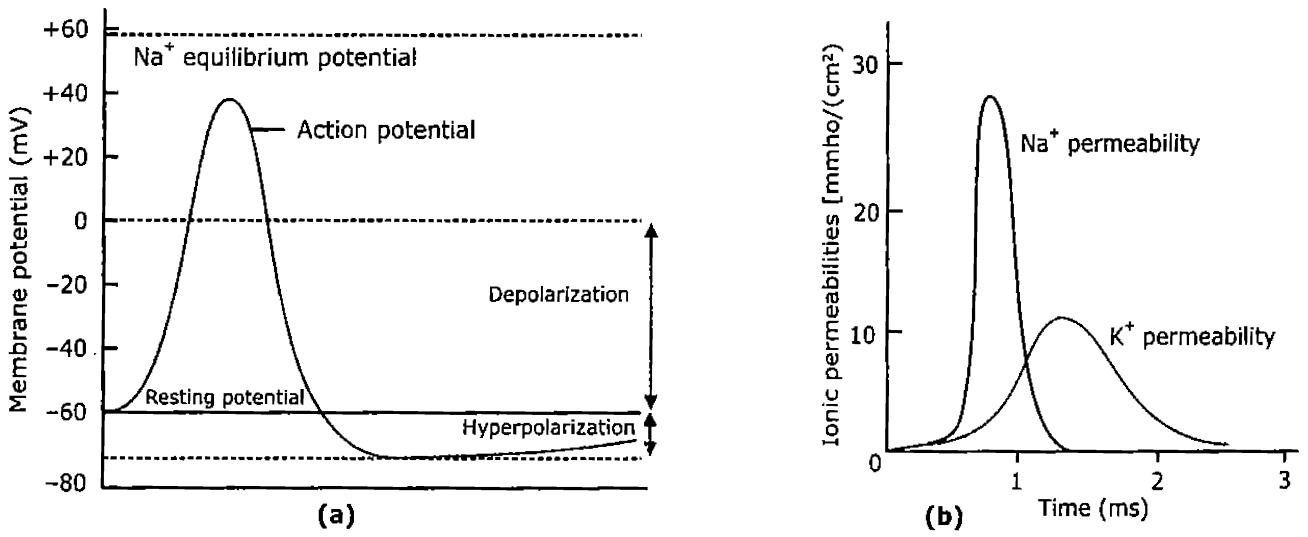


Figure 9.12 : Time course of an action potential. **a.** The axon membrane undergoes rapid depolarization, followed by a nearly as rapid hyperpolarization and then a slow recovery to its resting potential. **b.** The depolarization is caused by a transient increase in Na^+ permeability (conductance), whereas the hyperpolarization results from a more prolonged increase in K^+ permeability that begins in a fraction of a millisecond later.

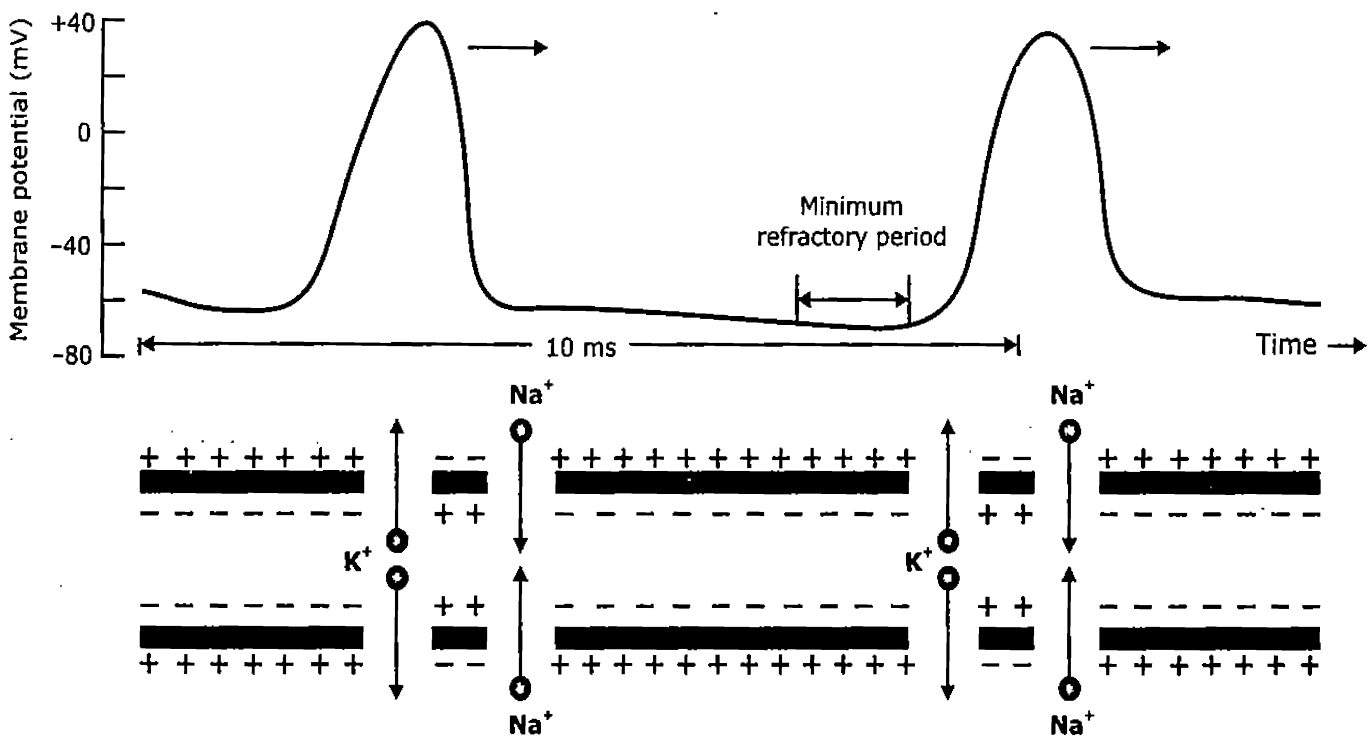
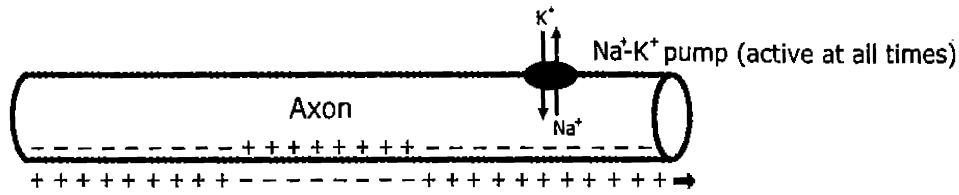


Figure 9.13 : Action potential propagation along an axon. Membrane depolarization at the leading edge of an action potential triggers an action potential at the immediately downstream portion of the axon membrane by inducing the opening of its voltage-gated Na^+ channels. As the depolarization wave moves farther downstream, the Na^+ channels close and the K^+ channels open to hyperpolarize the membrane. After a brief refractory period, during which the K^+ channels close and the hyperpolarized membrane recovers its resting potential, a second impulse can follow.

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State of axon	Resting	Active	Recovering
Type of potential	Resting pot.	Action pot.	Resting potential
Membrane	Polarised	Depolarised	Repolarising
Ionic movement	Mainly due to movement of K^+ ion through K^+ leaky channels	Increased permeability to Na^+ ions through voltage gated Na^+ channels	Increased permeability to K^+ ions through voltage gated K^+ channels
Internal potential	Negative	Positive	Negative
External potential	Positive	Negative	Positive

Figure 9.14 : Comparison between the resting and active state of axon.

How is information transferred from one neuron to the next? Neurons communicate at their meeting points, called **synapses**; the small gaps separating the neurons are referred to as the **synaptic cleft**. These synapses are not merely gaps but are functional links between the two neurons. Signals are transferred in only one direction across the synapse.

Synapses can be either **chemical** or **electrical**. An electrical synapse is what is often called a *gap junction*, in which the membranes of two neurons are continuous at tiny spots, making the cells electrically contiguous. Gap junctions, which are not unique to neurons, allow for even more rapid communication. No chemical intermediary is involved in an electrical synapse.

CHEMICAL SYNAPSES

The space between the pre- and postsynaptic neurons is called the *synaptic cleft*. The key feature of all chemical synapses is the presence of small, membrane-bound organelles called *synaptic vesicles* within the presynaptic terminal. These spherical organelles are filled with one or more *neurotransmitters*, the chemical signals secreted from the presynaptic neuron, and it is these chemical agents acting as messengers between the communicating neurons that gives this type of synapse its name. There are many kinds of neurotransmitters, the best studied example being acetylcholine.

Transmission at chemical synapses occur when an action potential invades the terminal of the presynaptic neuron. The change in membrane potential caused by the arrival of the action potential leads to the opening of *voltage-gated calcium channels* in the presynaptic membrane. The opening of these channels causes a rapid influx of Ca^{2+} into the presynaptic terminal, with the result that the Ca^{2+} concentration of the cytoplasm in the terminal transiently rises to a much higher value. Elevation of the presynaptic Ca^{2+} concentration, in turn, allows synaptic vesicles to fuse with the plasma membrane of the presynaptic neuron. The Ca^{2+}-dependent fusion of synaptic vesicles with the terminal membrane causes their contents, most importantly neurotransmitters, to be released into the synaptic cleft.

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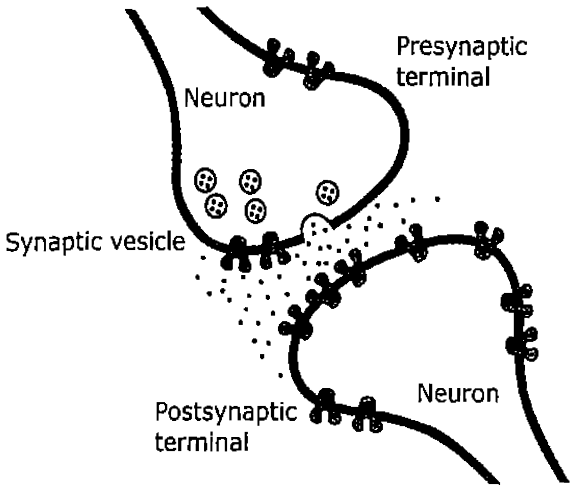


Figure : 9.15
Transmission at chemical synapses. Synaptic cleft separates the plasma membranes of the presynaptic and postsynaptic cells. Arrival of action potentials at a synapse causes release of neurotransmitters by the presynaptic cell, their diffusion across the synaptic cleft, and their binding by specific receptors on the plasma membrane of the postsynaptic cell. Generally these signals depolarize the postsynaptic membrane (making the potential inside less negative), tending to induce an action potential in it.

Following exocytosis, transmitters diffuse across the synaptic cleft and bind to specific receptors on the membrane of the postsynaptic neuron. The binding of neurotransmitter to the receptors causes channels in the postsynaptic membrane to open (or sometimes to close), thus changing the ability of ions to flow into (or out of) the postsynaptic cells. The resulting neurotransmitter-induced current flow alters the conductance and usually the membrane potential of the postsynaptic neuron, increasing or decreasing the probability that the neuron will fire an action potential. In this way, information is transmitted from one neuron to another.

Types of chemical synapses - Excitatory or **Inhibitory**

Action of the neurotransmitter may promote or inhibit the generation of an action potential in the postsynaptic cell. Binding of a neurotransmitter to an **excitatory receptor** opens a channel that admits Na^+ ions or both Na^+ and K^+ ions. These non-voltage-gated ion channels can be part of the receptor protein or can be a separate protein that opens in response to a cytosolic signal generated by the activated receptor. Channel opening leads to depolarization of the postsynaptic plasma membrane, promoting generation of an action potential. In contrast, binding of a neurotransmitter to an **inhibitory receptor** on the postsynaptic cell causes opening of K^+ or Cl^- channels. The resulting membrane hyperpolarization inhibits generation of an action potential in the postsynaptic cell.

Neurotransmitters are usually small molecules, such as amino acids, amines, neuropeptides. Some neurotransmitters stimulate neurons to fire, while others inhibit firing. The effect of the neurotransmitter comes out by its binding with receptor proteins on the membrane of the postsynaptic neuron. Each neurotransmitter binds specifically in a lock-and-key mechanism to its type of receptor.

Table 9.4 : The major known neurotransmitter

Neurotransmitter	**Functional Class**
Ester	
Acetylcholine (Nicotenic)	Excitatory
Acetylcholine (muscarintic)	Inhibitory
Biogenic Amines	
Norepinephrine	Excitatory or inhibitory
Dopamine	Generally excitatory; may be inhibitory at some sites
Serotonin (5-hydroxy tryptamine)	Generally inhibitory

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Amino acids	
GABA (Gamma aminobutyric acid)	Inhibitory
Glycine	Inhibitory
Glutamate	Excitatory
Aspartate	Excitatory
Neuropeptides	
Substance P	Excitatory
Met-enkephalin	Generally inhibitory

Acetylcholine and acetylcholine receptor

Acetylcholine is a *colinergic* neurotransmitter synthesized from choline and acetyl-CoA by enzyme *choline acetyl transferase*. It is synthesized in the axonal terminal bulbs. After synthesis it is released by the process of exocytosis in the synaptic cleft. Enzyme *acetylcholinesterase* presents in synaptic cleft form choline and acetyl–CoA from acetylcholine. Acetylcholinesterase is a serine esterase. Insectisides like malathion, parathion, nerve gas like sarin and DIPF inhibit the activity of acetylcholinesterase. Acetylcholine can produce an excitatory response in some postsynaptic cells and an inhibitory response in others. Response is mediated by the binding of acetylcholine with **acetylcholine receptor** presents on postsynaptic cells. The acetylcholine receptor is the best-understood ligand-gated channel. Acetylcholine receptors have been divided into two main types : *Nicotinic* and *muscarinic* acetylcholine receptor.

The Nicotinic acetylcholine receptor (ionotropic receptor) is a ligand gated ion channel. It is a pentamer of four kinds of membrane-spanning glycoprotein subunits α, β, γ, and δ. Each α subunit has a binding site for acetylcholine. It has a central pore and function as ligand gated channel for both $Na^+$and K^+. Na^+ rushes in while K^+ streams out, but, because the Na^+ gradient across this membrane is steeper than that of K^+, the Na^+ influx greatly exceeds the K^+ efflux. The net influx of Na^+ ion depolarizes the postsynaptic membrane.

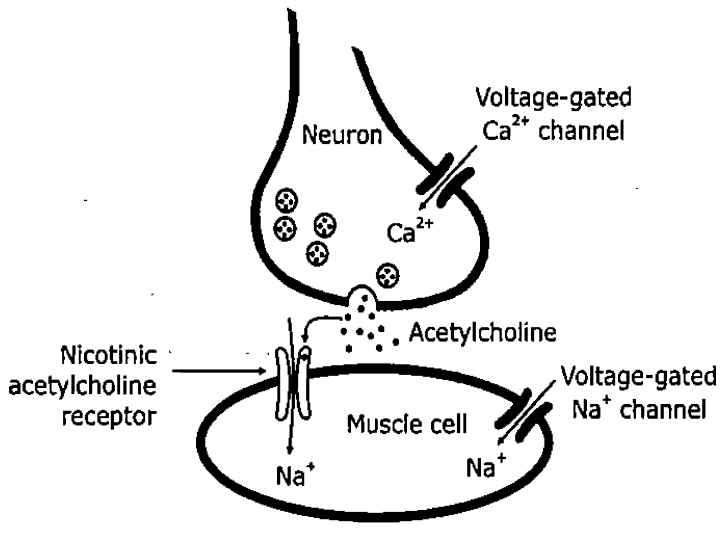


Figure : 9.16
Release of acetylcholine triggers the opening of the ligand-gated acetylcholine receptors in the muscle plasma membrane. The resulting influx of Na^+ produces a localized depolarization of the membrane, leading to opening of voltage-gated Na^+ channels and generation of an action potential.

Opening of acetylcholine receptor channels following binding of acetylcholine produces a net depolarization to about -15 mV from the muscle resting potential of -85 to -90 mV. This localized depolarization of the muscle plasma membrane triggers opening of voltage-gated Na^+ channels, leading to generation and conduction of an action potential in the muscle plasma membrane. Snake neurotoxins such as *alpha-bungarotoxin* and *cobra toxin* bind specifically to acetylcholine receptors and block the transmission of impulses between nerve and muscle.

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The *muscarintic acetylcholine receptors* (metabotropic receptor) are transmembrane proteins that interact with G- proteins. Activation of muscarintic receptors (by binding with acetylcholine) results in several effects, including the inhibition of adenylate cyclase, the stimulation of phospholipase C and opening of K^+ channels. Opening of K^+ channels and the subsequent efflux of K^+ ions causes hyperpolarization of the plasma membrane. Antagonist like atropine from *Atropa belladona* binds with muscarintic receptor and inhibits the function. It is used for pupil dilation.

Neurotoxins

Many plants and animals contain neurotoxins. Some work by blocking transmission of nerve impulse, other by blocking synaptic transmission. Example of some neurotoxins that inhibit the function of Na^+ and K^+ channels are given below.

Tetrodotoxin : Produced by puffer fish also known as blow fish or swell fish, binds with Na^+-channel and blocks all ion movement.

Saxitoxin : Produced by marine dinoflagellates such as *Gonyaulax catenella* and prevents the voltage gated Na^+ channel from opening.

Veratridine : Isolated from seeds of sabadilla and binds with Na^+ channel and makes it permanently open.

Batrachotoxin : Produced by Colombian frog, *Phyllobates aurotaenia* and makes Na^+-channel permanently open.

Dendrotoxin : Produced by *dendroaspis polylepsis* and binds with K^+ channel and blocks all ions movement.

9.5 TRANSPORT OF MACROMOLECULES ACROSS PLASMA MEMBRANE

Endocytosis

The term endocytosis was coined by Christain de duve in year 1963. Endocytosis is the process whereby eukaryotic cells internalize material from their surrounding environment. Internalization is achieved by the formation of membrane-bound vesicles at the cell surface that arise by progressive invagination of the plasma membrane, followed by pinching off and release of free vesicles into the cytoplasm.

Classically, endocytosis has been divided into **phagocytosis** (cellular eating) and **pinocytosis** (cellular drinking). *Phagocytosis* (first reported by Metchinkoff) describes the internalization of large particles following particle binding to specific plasma membrane receptors and by formation of large endocytic vesicles (generally > 250 nm in diameter) called **phagosomes**. Phagocytosis occurs in specialized mammalian cells (macrophage, monocytes, neutrophils). It is an active and highly regulated process involving signalling cascades mediated by Rho-family GTPases.

Pinocytosis (also termed as **fluid-phase endocytosis**) involves the ingestion of fluid and solutes via small vesicles (<150 nm in diameter). Uptake of material dissolved in extracellular fluid during pinocytosis occurs both specifically as well as non-specifically. *Receptor-mediated endocytosis* is an example of ligand specific pinocytic process.

Receptor-mediated endocytosis

In *receptor-mediated endocytosis*, a specific receptor on the cell surface binds tightly to the extracellular macromolecule (the ligand) that it recognizes. The plasma-membrane region containing the receptor-ligand complex then undergoes endocytosis, becoming a transport vesicle. Receptor ligand complexes are selectively incorporated into the intracellular transport vesicles. Receptor-mediated endocytosis may be a clathrin-dependent and a clathrin-independent.

Clathrin dependent endocytosis

In case of clathrin dependent endocytosis, macromolecules bind to cell-surface receptors, accumulate in clathrin-coated pits, and enter the cell as receptor and ligand complexes in clathrin-coated vesicles. **Clathrin**

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consists of three copies each of heavy chain and light chain, forming a three-legged structure called a *triskelion*. Clathrin triskelions are the assembly units of the polygonal lattice. Clathrin assembles into coats on the cytoplasmic side of the plasma membrane by interacting with its *adaptor proteins* (comprising AP1, AP2, AP3 and AP4). Each of these four classes are localised to different intracellular compartments and vary in their receptor specificity.

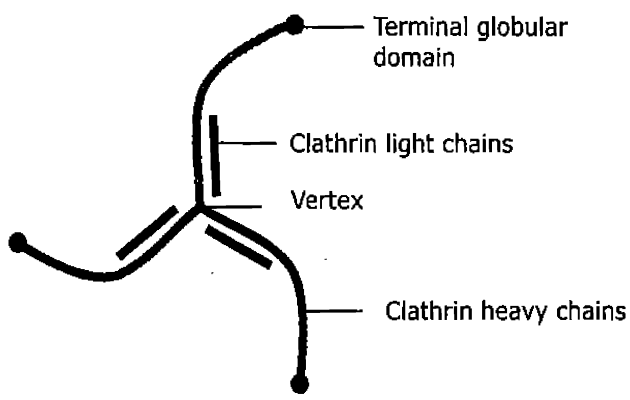


Figure : 9.17
Structure of clathrin triskelion. Each triskelion consists of three clathrin heavy chains radiating from a central vertex, with a terminal globular domain at the tip of each triskelion leg and a clathrin light chain bound to the inner half of each leg.

A common example of clathrin-mediated endocytosis is the uptake of cholesterol-containing particles called **low-density lipoprotein** (LDL). LDL is a sphere of 20-25 nm in diameter. It has an outer phospholipid monolayer containing a large protein called **apo B100**. It contains free cholesterol, cholesterol ester, and triacylglycerol.

Receptor mediated endocytosis of LDL occurs via clatherin/AP2 coated pits and vesicles. AP-2 is hetero-tetramer consists of two large α and $\beta 2$, one medium $\mu 2$, and one small $\sigma 2$ subunit.

Michael Brown and Joseph Goldstein demonstrated that the uptake of LDL by mammalian cells requires the binding of LDL to a specific cell surface receptor that is concentrated in clatherin-coated pits and internalized by endocytosis. When a cell needs cholesterol for membrane synthesis, it makes transmembrane receptor proteins for LDL called **LDL receptor** and inserts them into its plasma membrane. The LDL receptor, made up of 839 amino acids residues, is a single pass transmembrane glycoprotein. LDL receptors have four-residue sequence, *Asn–Pro–X–Tyr* (X = any amino acid) in the cytosolic domain that is crucial for internalization. Many cell surface receptors can be directed into forming clathrin/AP2 pit by different cytosolic sorting signals like *Tyr-X-X-ø*, where X can be any amino acid and ø is a bulky hydrophobic amino acid, such as Phe, Leu, or Met residue and *Leu-Leu*, signal.

Any LDL particles bound to LDL receptors in the clathrin/AP2 coated pits are rapidly internalized in clathrin/AP2 coated vesicles. Clathrin-coated vesicles are 50–100 nm in diameter. These coated vesicles from the plasma membrane move to endosomes. *Dynamin*, a cytosolic protein, is essential for release of complete vesicles. Dynamin polymerizes around the neck portion and then hydrolyzes GTP. The energy derived from GTP hydrolysis is essential for the final pinching off of a completed clathrin-coated vesicle.

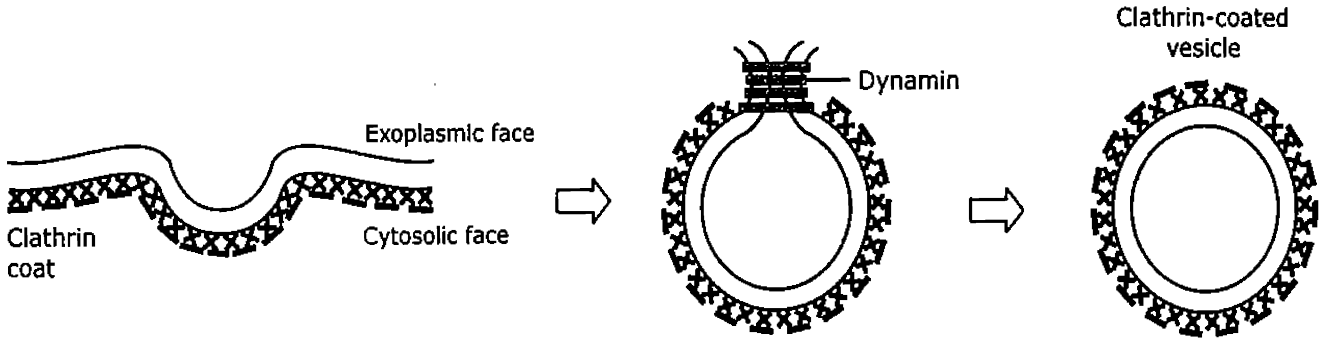


Figure 9.18 : Dynamin-mediated pinching off of clathrin coated vesicles. When clathrin polymerizes on a donor membrane, it does so in association with adaptor protein, which assemble between the clathrin lattice and the membrane. After a vesicle bud forms, dynamin polymerizes over the neck. Dynamin-catalyzed hydrolysis of GTP leads to release of the vesicle from the donor membrane. Adapted from K. Takel et al., 1995, Nature 374:186.

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Endocytic vesicles derive from the plasma membrane are directed to fuse with and deliver their membrane and content to the intracellular organelles of the endocytic pathway, the **endosomes** and **lysosomes.** Endosomes are single membrane bound heterogenous structure (act as sorting compartment). It is of two types – *Early endosome* (closer to plasma membrane) and *late endosome* (closer to nucleus). Late endosomes are more acidic then early endosomes.

After shedding their clathrin coats, the vesicles deliver their contents to early endosomes, which are located near the cell periphery. Once the LDL and LDL receptors encounter the low pH in the endosomes, LDL is released from its receptor and is delivered via late endosomes to lysosomes. Lysosomes are usually regarded as the graveyard of the cell, the site where ligands internalized from the cell surface and destined for degradation are delivered and hydrolyzed.

Clathrin-independent endocytosis

Various *clathrin-independent endocytic* mechanisms have also been identified, including uptake through **caveolae** and **macropinosomes**. While our understanding of the machinery regulating and driving clathrin mediated endocytosis has progressed dramatically, including the elucidation of the structure of individual components and partial *in vitro* reconstitution, the role of caveolae as alternative endocytic carriers still remains elusive.

Caveolae mediated endocytosis

Caveolae (Latin for *little caves*) are flask shaped invaginations on the cell surface in many vertebrate cell types, especially in endothelial cells and adipocytes. These flask-shaped structures are rich in proteins as well as lipids such as cholesterol and sphingolipids. Formation and maintenance of caveolae is primarily due to the protein **caveolin**. This protein has both a cytoplasmic C-terminus and a cytoplasmic N-terminus, linked together by a hydrophobic hairpin that is inserted into the membrane. The presence of caveolin leads to the local change in morphology of the membrane. Caveolae contain some receptor proteins and are used for certain types of receptor-mediated endocytosis. However, receptor-mediated endocytosis generally occurs via clathrin-coated pits and vesicles.

Exocytosis

Transport vesicles destined for the plasma membrane undergo fusion with the plasma membrane and release the contents outside the cell in the process called **exocytosis**. It may be *constitutive secretory pathway* (carried out by all cells) or *regulated secretory pathway* (carried out by specialized cells). Regulated secretory pathway is found mainly in cells specialized for secreting products rapidly on demand such as hormones, neurotransmitters, or digestive enzymes.

FATE OF RECEPTOR

The fate of a receptor-ligand complex depends upon its response to the acidic environment of the endosome. Exposure to low pH changes the conformation of the external domain of the receptor, causing its ligand to be released, and/or changes the structure of the ligand. Four possible fates for a receptor-ligand complex are described below:

Receptor recycled and ligand degraded

The classic example is the LDL receptor, whose ligands are the plasma low density lipoproteins (LDL). LDL binds with LDL receptor and the LDL is released from its receptor in the endosome. The receptor recycles to the surface to be used again and the LDL is sent on to the lysosome, where it is degraded.

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Receptor and ligand both recycled

The transferrin receptor provides the classic example of this pathway. Most cells acquire iron from *transferrin* by a *receptor* mediated process, and the transferrin receptor controls iron uptake. The transferrin receptor is a glycosylated homodimer membrane protein linked by two intramembranous disulfide bonds. At the pH of the extracellular space (~7.4), iron-bearing transferrin preferentially binds transferrin receptor. Binding of transferrin to its receptor is rapidly followed by internalization of the transferrin-transferrin receptor complex to a *clathrin-coated* vesicle, which soon matures to an ATP-driven proton-pumping endosome. The acid environment of endosome causes transferrin to release the iron. The iron-free ligand, called apo-transferrin, remains bound to the transferrin receptor, and recycles to the plasma membrane. In the neutral pH of the plasma membrane, apo-transferrin dissociates from the receptor. This leaves apotransferrin free to bind another iron.

Receptor and ligand both are degraded

The epidermal growth factor (EGF) receptor binds its ligand and internalize it. Although EGF and its receptor appear to dissociate at low pH, they are both carried on to the lysosome, where they are degraded.

Transcytosis

Transcytosis is a process of transcellular transport which combines both endocytosis and exocytosis. It is employed to import an extracellular ligand from one side of a cell, transport it across the cytoplasm, and secrete it from the plasma membrane at the opposite side. An example of transcytosis is the movement of maternal immunoglobulins (antibodies) across the intestinal epithelial cells of the newborn human.

9.6 RIBOSOME

The ribosomes are large *ribonucleoproteins* consisting of RNAs and proteins, ubiquitous in all cells, that translates genetic information stored on the messenger RNA into polypeptides. The ribosome are approximately globular structure, its average diameter ranging from 2.5 nm (*Escherichia coli*) to 2.8 nm (mammalian cells). The functional ribosomes consist of two subunits of unequal size, known as the large and small subunits. Ribosomes are consist of *rRNA* and *r-proteins*. The names of the r-proteins are composed of L or S (depending on whether the protein is from the large or small subunit).

Table 9.5 : Ribosome structure and chemical composition

Property	**Prokaryote**	**Eukaryote**
Overall size	70S	80S
Small subunit	30S	40S
Number of proteins	~21	~30
RNA size (number of bases)	16S (1500)	18S (2300)
Large subunit	50S	60S
Number of proteins	~34	~50
RNA size (number of bases)	23S (2900) 5S (120)	28S (4200) 5.8S (160) 5S (120)

rDNA organization

In prokaryotes such as *Escherichia coli*, there are three ribosomal RNAs (16S, 23S and 5S), which are organized as a single transcription unit. In all eukaryotes studied so far, the organization of the ribosomal

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RNA genes is recognizably similar to that of prokaryotes, but with major differences; the size of the small subunit RNA has increased from 16S to 18S, and that of the large subunit from 23S and 28S; a new small 5.8S rRNA has become interspersed between the 18S and the 28S rRNA and the 5S rRNA has become separated from the other RNAs in a different transcription unit. The former transcription unit is generally simply referred to as the rRNA gene or the ribosomal DNA (rDNA). 5S genes are transcribed by a different RNA polymerase from rRNA genes (RNA polymerase III rather than RNA polymerase I). There are generally more repeats of the 5S sequences than of the rDNA. The human genome contains about 100 rDNA copies per haploid set. Many other species, including most plants, have several thousand copies. The rRNA-containing region is transcribed to give a precursor the 45S pre-rRNA, which is processed in a series of post-transcriptional modifications to the mature rRNA species.

Table 9.6 : Different types of ribosomes and their rRNA

Ribosome source	**Sedimentation coefficient**	**rRNA** (large sub/small sub)
Bacterial	70S	5S, 23S/16S
Chloroplast	70S	5S, 23S/16S
Mitochondria (human)	55S	16S/12S
Archaebacteria	70S	5S,23S/16S
Eukaryotes (cytoplasm)	80S	5S, 5.8S, 28S/18S

Eukaryotic ribosomes

Most eukaryotes contain two distinct types of ribosomes: cytoplasmic and organellar. The ribosomes of eukaryotic cells (other than mitochondrial and chloroplast ribosomes) are substantially larger and more complex than bacterial ribosomes. Cytoplasmic ribosomes show marked variation with the complexity of the organism: the yeast ribosome is relatively small and globular, resembling a eubacterial ribosome except for its greater size, while higher eukaryotes have larger and more ellipsoidal ribosomes.

Organelle ribosomes are distinct from the ribosomes of the cytosol and take varied forms. Organellar ribosomes from mitochondria (found both in animals and plants) and plastids (found in plants only) are smaller than cytoplasmic, in general, and bear some resemblance to the eubacterial ribosomes, consistent with the hypothesis that these organelles have a eubacterial origin (endosymbiosis). While chloroplast ribosomes have been measured at 67-68S, mitochondrial ribosomes show a wide variation, from 55S (mammalian) to 77S (yeast).

In eukaryotic cells, proteins are synthesized in cytosol and within organelles (mitochondria or chloroplasts). In the cytosol, proteins are synthesized on *membrane-bound* or *membrane free* ribosomes. Proteins that are imported into cytoplasmic organelles are synthesized on membrane free ribosomes in the cytosol. Whereas proteins that are imported into the ER-Golgi system are synthesized on ER membrane-bound ribosomes.

Table 9.7 : Proteins synthesized by different classes of cellular ribosomes

Location of ribosome	*Classes of protein synthesized*
Membrane free ribosomes	Soluble cytosolic proteins
	Extrinsic membrane proteins localized to the cytoplasmic surface (e.g., actin, spectrin)
	Mitochondrial proteins encoded by nuclear DNA
	Chloroplast proteins encoded by nuclear DNA
	Peroxisomal proteins

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ER membrane bound ribosomes	Secreted proteins
	Integral plasma membrane proteins
	Lysosomal proteins
	ER proteins
	Golgi complex proteins
	Extrinsic membrane proteins localized to the extracytoplasmic surface (e.g., fibronectin)

Protein targeting and translocation

Fate of proteins synthesized by cytosolic ribosomes depend on their amino acid sequence, which can contain *sorting signals* that direct their transport from the cytosol into the nucleus, the ER, mitochondria, plastids or peroxisomes. Proteins without sorting signal remain in the cytosol as permanent residents.

Protein translocation describes the movement of a protein across a membrane. Within the cell, translocation of cytosolic proteins occur in three different ways:

1. *Gated transport* : Protein movement between the cytosol and nucleus through the nuclear pore.
2. *Transmembrane transport* : Transport of proteins across a membrane from the cytosol into the ER lumen, mitochondria, plastids or peroxisomes.
3. *Vesicular transport* : Transport of proteins from one compartment to another through transport vesicles. The transfer of soluble proteins from the ER to the Golgi apparatus, for example, occur in this way.

Protein translocation may occur co-translationally or post-translationally. Proteins synthesized by membrane bound ribosomes are translocated co-translationally. The membrane free ribosomes synthesize all proteins that are translocated post-translationally.

9.7 ENDOPLASMIC RETICULUM

Endoplasmic reticulum (ER) is the largest intracellular compartment with an extensive array of interconnecting membrane tubules and cisternae that extend throughout the cell including the nuclear envelope. ER membranes are physiologically active, interact with the cytoskeleton, and contain differentiated domains specialized for distinct functions.

ER membranes are differentiated into rough and smooth regions (RER and SER, respectively), depending on whether ribosomes are associated with their cytoplasmic surfaces. The membranes and luminal spaces of the ER are normally continuous throughout the cell and that RER and SER form an interconnected membrane system. When cells are disrupted by homogenization, the ER breaks into fragments and reseals into small vesicles called **microsomes**. Microsomes derived from RER are studded with ribosomes on outer surface and are called *rough microsomes*. Microsomes lacking attached ribosomes are called *smooth microsome*.

The RER is the site of co-translational membrane insertion of proteins, whereas the SER is thought to be the site of lipid biosynthesis, detoxification, and calcium regulation. In the lumen of the RER, five principal modifications occur before they reach their final destinations: formation of disulfide bonds, proper folding, addition and processing of carbohydrates, specific proteolytic cleavages, assembly into multimeric proteins.

Translocation of proteins across the ER membrane

Proteins synthesized by membrane bound ribosomes include soluble and membrane proteins that reside in the ER itself, resident proteins in the lumen of golgi complex and lysosomes, integral proteins in the

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membrane of these organelles and the plasma membrane and proteins that are secreted from the cell. Proteins destined to be secreted move through the **secretory pathway** in the following order: rough ER → ER-to-Golgi transport vesicles → Golgi cisternae → secretory or transport vesicles → cell surface.

Synthesis of secretory proteins begin on an unattached ribosome in the cytosol. Ribosomes engaged in the synthesis of secretory proteins are then targeted to the ER by a **signal sequence** (a short sequence of hydrophobic amino acids) at the N-terminus of growing polypeptide chain. These signal sequences are cleaved from the polypeptide chain during its transfer into the ER lumen. In 1971, D. Sabatini and G. Blobel first proposed this hypothesis; known as *signal hypothesis*. A **signal-recognition particle** (SRP), a cytosolic particle binds to the signal sequence of a nascent protein chain, and the complex of SRP, nascent polypeptide, and ribosome then binds to the α subunit of the SRP receptor in the ER membrane. SRP receptor contains two polypeptide subunits: a transmembrane β-subunit and a peripheral α-subunit.

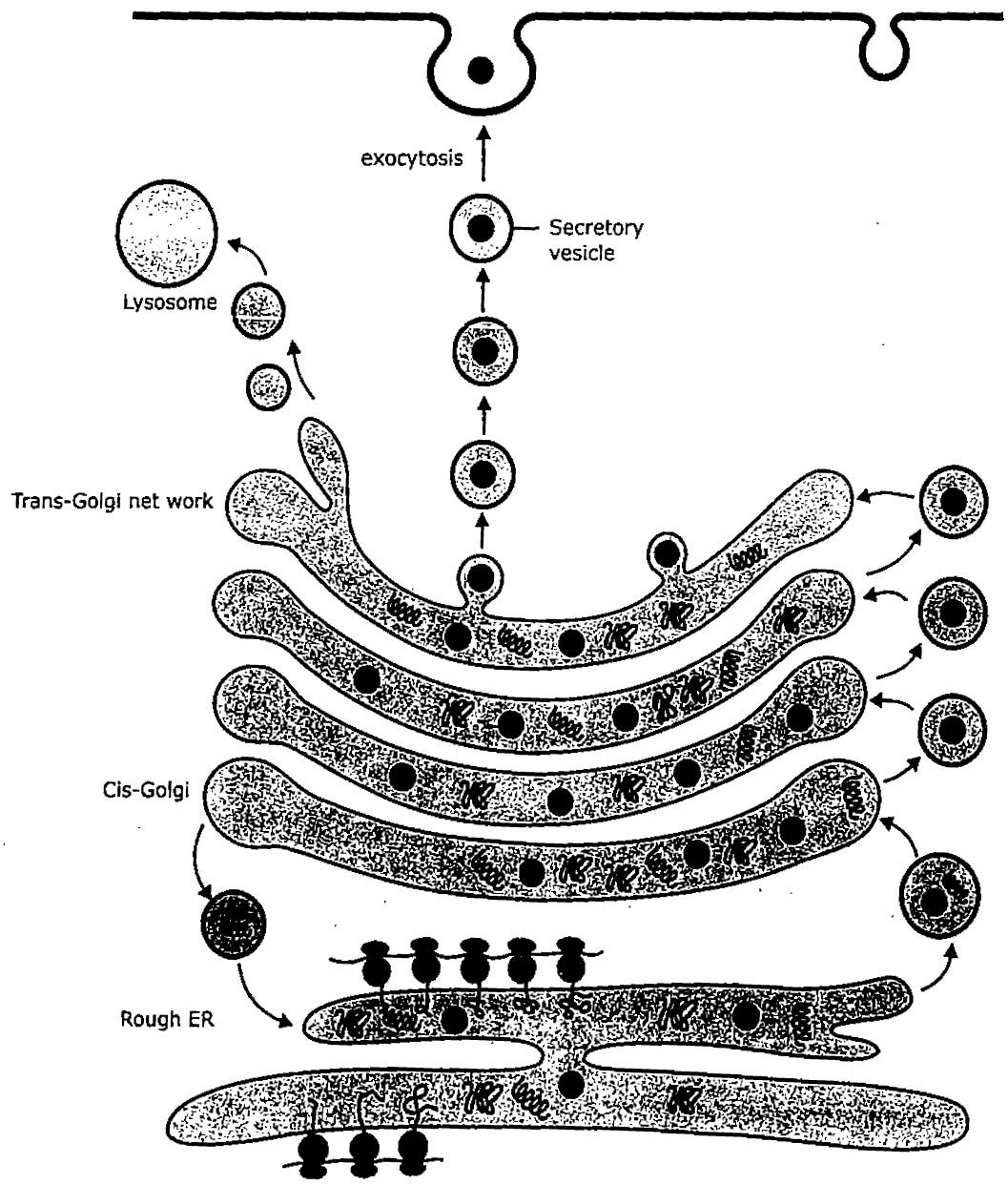


Figure 9.19 : Diagrammatic representation of secretory pathway. Newly synthesized proteins are inserted into the lumen of the ER. Those proteins that are transported out of the ER may then pass through the various sub-compartments of the Golgi until they reach the *trans*-Golgi network, the exit side of the Golgi. In the *trans*-Golgi network, proteins are segregated and sorted. Proteins destined for the plasma membrane or those that are secreted in a constitutive manner are carried out to the cell surface in transport vesicles. Some proteins enter late endosomes and are selectively transferred to lysosomes.

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Mammalian SRPs are composed of 7SL scRNA of ~300 nucleotides in length, which is complexed with six proteins having molecular weights of 9, 14, 19, 54, 68, and 72 kDa, to form an 11S RNP particle. The 7SL scRNA is an abundant transcript of RNA polymerase III. It is a member of a class of mammalian intermediate-repeat sequences called short interspersed elements (SINES). The 7SL scRNA has a remarkable sequence homology with the *Alu* family of repetitive sequences in primates and rodents.

SRP has two binding sites - one end of the SRP binds to an signal sequence on a growing polypeptide chain, while the other end binds to the ribosome itself and stops translation. The 7SL scRNA in the particle may mediate an interaction with ribosomal RNA. The SRP directs both the binding of the ribosome to the ER membrane and the insertion of the nascent protein into the transmembrane channel. Interaction of the SRP along with ribosome complex with the SRP receptor is promoted when GTP is bound by both the P54 subunit of SRP and the α-subunit of the SRP receptor.

The peptide chain continues to elongate and is extruded into the ER lumen through the **translocon** (translocation channel), a gated channel (*co-translational translocation*). In mammal, translocon is made up of 3-proteins called the *Sec 61 complex* (Sec 61α, Sec 61β, Sec 61γ). Hydrolysis of GTP of SRP and the SRP receptor is essential to drive the insertion of nascent protein into the translocon.

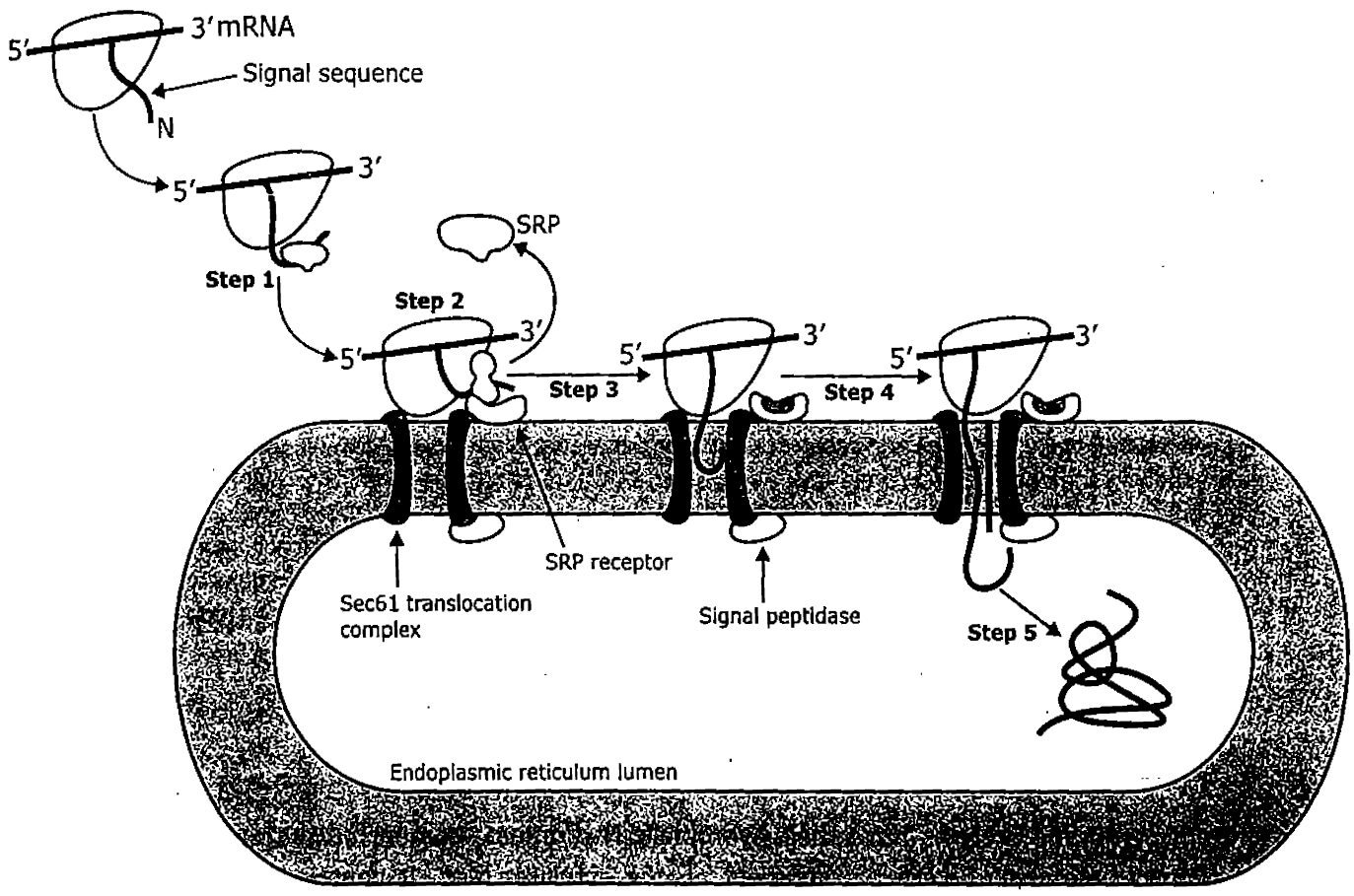


Figure 9.20 : Synthesis of secretory proteins and their co-translational translocation across the ER membrane

The polypeptide chain elongates; then the signal sequence is cleaved by a **signal peptidase** (transmembrane ER protein associated with the translocon is a protease, recognizes a sequence on the C-terminal side of the hydrophobic core of the signal peptide and cleaves the chain) in the ER lumen and is rapidly degraded. Driving force for unidirectional translocation across the ER membrane is provided by a protein complex called Sec 63 complex and a member of Hsp 70 family of molecular chaperones known as BiP (BiP for *bi*nding *p*rotein). The Sec 63 protein and BiP are associated with the Sec 61 translocation channel. BiP has a peptide binding domain and an ATPase domain. It binds and stabilizes unfolded proteins. ATP driven cycle of BiP binding and release pulls the protein into the lumen. Transport from the ER back into the cytosol occurs by

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a reversal of the usual process of import. This is called *reverse translocation* (also sometimes called retrotranslocation or dislocation).

Sequence of events during co-translational targeting of secretory proteins to the ER:

Step 1: Signal sequence emerges from the ribosome, it is recognized and bound by the SRP.

Step 2: The SRP escorts the complex to the ER membrane, where it binds to the SRP receptor.

Step 3: The SRP is released, the ribosome binds to a membrane translocation complex of Sec 61 proteins, and the signal sequence is inserted into a membrane channel.

Step 4: Translation resumes, and the growing polypeptide chain is translocated across the membrane.

Step 5: Cleavage of the signal sequence by signal peptidase releases the polypeptide into the ER lumen.

Insertion of integral proteins into the ER membrane

Integral proteins located in ER, Golgi, lysosomal membranes and in plasma membrane are synthesized on the RER. These proteins are initially inserted into the ER membrane instead of being released into the lumen. From the ER membrane, they proceed to their final destination along the same pathway as that of secretory proteins : ER → Golgi → Plasma membrane or lysosome. During this transport the orientation of a membrane protein is preserved. Integral membrane protein are embedded in the membrane by hydrophobic region that spans the lipid bilayer. Integral membrane proteins have four topological classes:

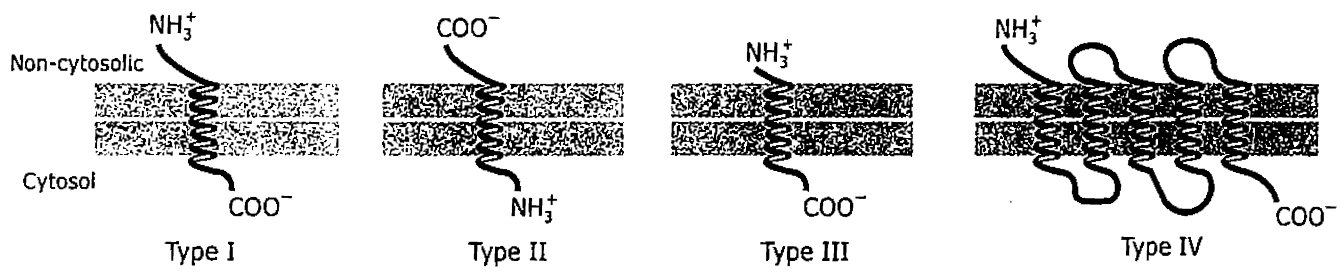


Figure 9.21 : Major topological classes of transmembrane proteins synthesized on the rough ER. The hydrophobic segments of the protein chain embedded in the membrane bilayer. The regions outside the membrane are hydrophilic. Type I proteins contain a cleaved signal sequence and a single internal stop transfer sequence. Type II and type III proteins contain a single internal signal-anchor sequence. The difference in the orientation of these proteins depend largely on whether there is a high density of positively charged amino acids on the N-terminal side of the internal signal-anchor sequence (type II) or on the C-terminal side of the internal signal-anchor sequence (type III). All type IV proteins have multipass transmembrane proteins. Nearly all multipass proteins lack a cleavable signal sequence.

TYPE I

Single-pass transmembrane proteins have their hydrophilic N-terminal segment on the exoplasmic face (non cytosolic) and their hydrophilic C-terminal segment on the cytosolic face. It contains internal **stop transfer signal** and cleavable N-terminal signal sequence. Common examples of type I proteins are LDL receptor, insulin receptor, and growth hormone receptor.

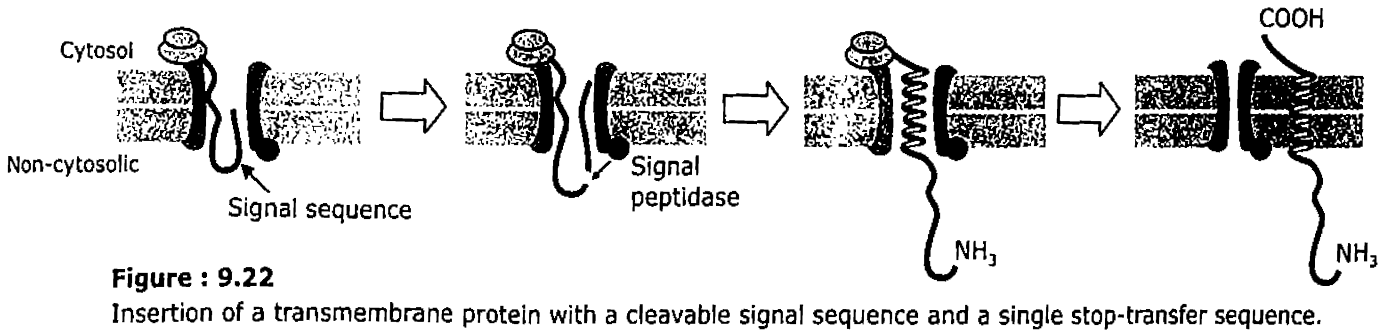


Figure : 9.22
Insertion of a transmembrane protein with a cleavable signal sequence and a single stop-transfer sequence.

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TYPE II

Single-pass transmembrane proteins have their N-terminus on the cytosolic face and the C-terminus on the exoplasmic (*luminal* in case of ER) face. Proteins with this orientation have a single noncleavable internal signal sequence. Example of type II proteins are transferrin receptor and Golgi galactosyl transferase.

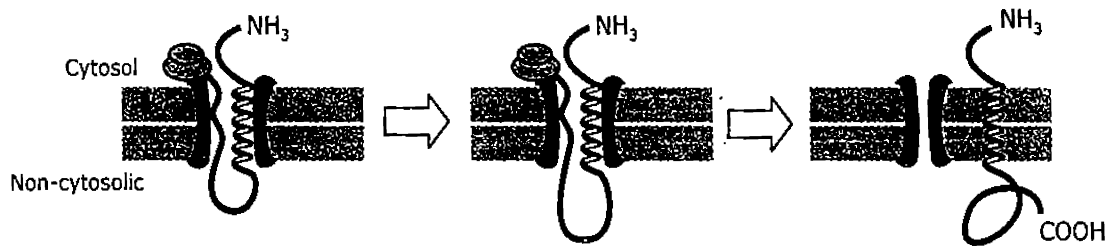


Figure 9.23 : Insertion of a transmembrane protein with a internal noncleavable signal sequence.

TYPE III

Single pass transmembrane proteins similar to type I but donot contain a cleavable signal sequence. N-terminal on the exoplasmic side and C-terminal region on the cytosolic side. Example - Cyt P450

TYPE IV

Multipass transmembrane proteins having both its N-terminal and C-terminal oriented towards the same side of the membrane or two ends have opposite orientations. These proteins contain internal signal sequence and many stop transfer sequence. Example – β-adrenergic receptor, CFTR, GLUT1

GPI linked cell surface proteins

After insertion in the ER membrane, some proteins are transferred to a *glycosylphosphatidylinositol* (GPI) anchor. After the completion of protein synthesis, the nascent protein remains anchored in the ER membrane by C-terminal sequence of ~20 amino acid residues; the rest of the protein is in the ER lumen. Enzyme present in the ER cuts the protein free from its membrane bound C-terminus and simultaneously attaches the new C-terminus to an amino group on a GPI. GPI helps to direct these proteins to cell membranes.

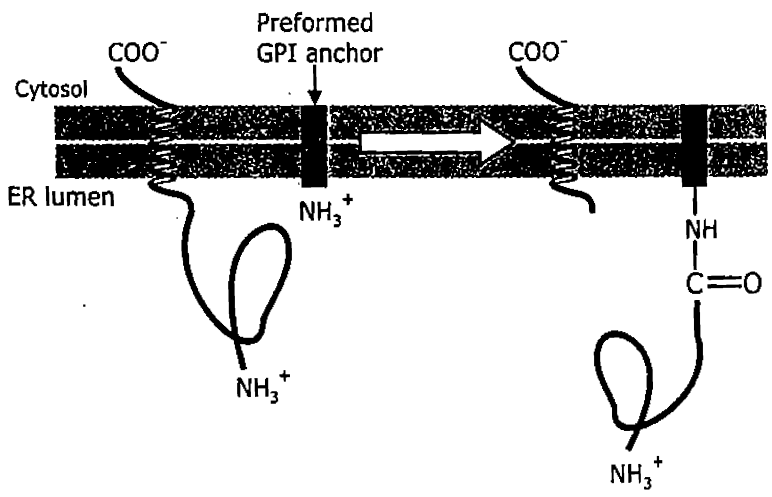


Figure : 9.24
GPI are added to polypeptides anchored in the membrane by a carboxy-terminal membrane spanning region. The membrane-spanning region is cleaved, and the new carboxy terminus is joined to the NH_2 group after translocation is completed leaving the protein attached to the membrane by the GPI anchor.

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N-Linked glycosylation of many proteins

Biosynthesis of all *N*-linked oligosaccharides begins in the rough ER with addition of a large preformed oligosaccharide precursor. This precursor oligosaccharide is linked by a pyrophosphoryl residue to *dolichol*, a long-chain (75-95 carbon atoms) polyisoprenoid lipid that is firmly embedded in the ER membrane and acts as a carrier for the oligosaccharide. The structure of *N*-linked oligosaccharide is the same in plants, animals, and single-celled eukaryotes - a branched oligosaccharide, containing three glucose (Glc), nine mannose (Man), and two *N*-acetylglucosamine (GlcNAc) molecules which is written as (Glc_3 Man_9 $(GlcNAc)_2$).

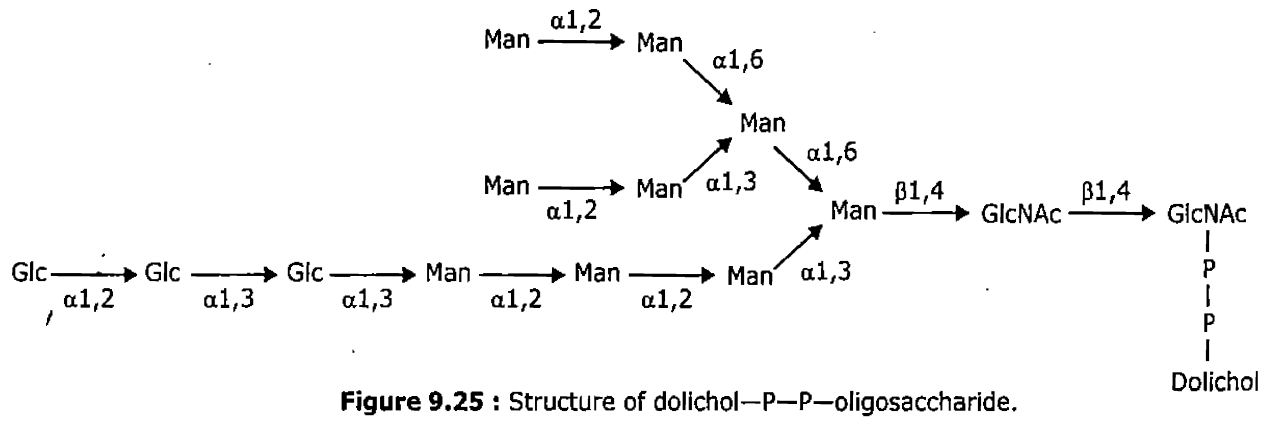


Figure 9.25 : Structure of dolichol—P—P—oligosaccharide.

Biosynthesis of oligosaccharide begins on the cytoplasmic face of the ER membrane with the transfer of N-acetyl glucosamine. Formation of the first sugar-to sugar glycosidic bond occurs upon transfer of a residue of N-acetylglucosamine. Five residues of mannose (GDP mannose) are added sequentially and the lipid-linked oligosaccharide is reoriented to the lumenal side of the membrane. Further addition of sugar molecules (mannose and glucose) occurs in the lumenal side. **Tunicamycin** inhibits N-Glycosylation by blocking the first step in the biosynthesis of oligosaccharide-P-P-dolichol.

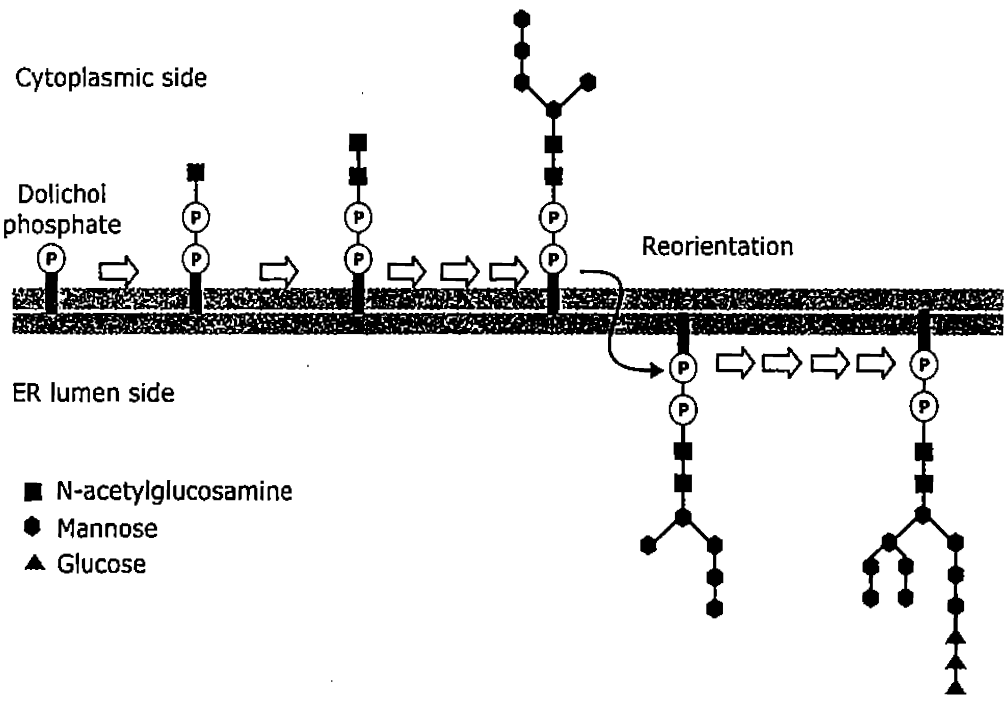


Figure 9.26 : Biosynthesis of N-linked oligosaccharides at the surface of the ER.

The entire Glc_3 Man_9 $(GlcNAc)_2$ oligosaccharide is transferred *en bloc* from the dolichol carrier to an asparagine residue on a nascent polypeptide, a reaction catalyzed by *oligosaccharide-protein transferase*. Only asparagine residues in the tripeptide sequences *Asn-X-Ser* and *Asn-X-Thr* (where X is any amino acid except proline) are substrates for this transferase.

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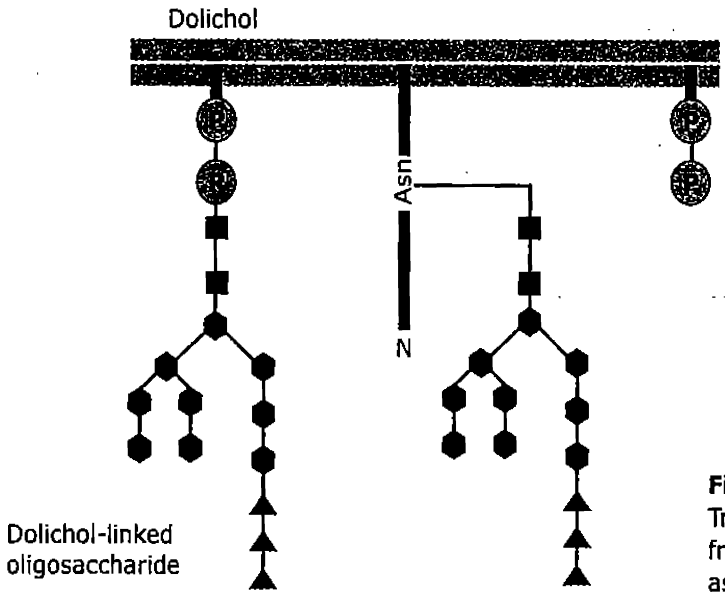


Figure : 9.27
Transfer of an oligosaccharide from the dolichol carrier to a asparagine residue on a protein

Immediately after the oligosaccharide is transferred to a nascent polypeptide, all three glucose residues and one particular mannose residue are removed by different enzymes. The three glucose residues, which are the last residues added in synthesis of $(Glc)_3\,(Man)_9\,(GlcNAc)_2$ on the dolichol carrier, appear to act as a signal that the oligosaccharide is complete and ready to be transferred to a protein. Five of its 14 residues are conserved in the structures of all *N*-linked oligosaccharides on secretory and membrane proteins. Further modifications of *N*-linked oligosaccharides occur in golgi complex.

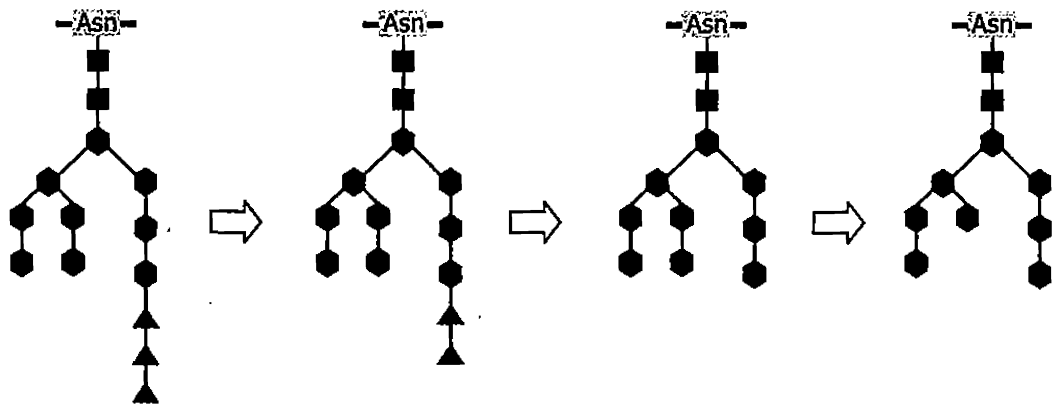


Figure 9.28 : Oligosaccharide processing in the ER. In three separate reactions, first one glucose residue, then two glucose residues, and finally one mannose residue are removed

Folding

It is assisted by chaperone and other ER proteins like BiP. Chaperone prevents segments of a nascent chain from misfolding. Two other chaperones in ER apart from BiP, **calnexin** and **calreticulin**, prevent premature, incorrect folding of segments of a newly made protein. Calnexin and calreticulin are calcium binding lectins (carbohydrate-binding proteins) located in the ER membrane and ER lumen respectively. Calnexin and calreticulin bind selectively to certain *N*-linked oligosaccharides on growing nascent chains. The ligand for these two lectins, which contains a single glucose residue, is generated in the ER lumen. Binding of calnexin and calreticulin to unfolded nascent chains prevent premature, incorrect folding of a newly made protein.

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Formation of disulfide bond in ER

Disulfide bonds donot form in the cytosol due to reducing environment. Reducing environment maintains cysteine residues in their reduced (-SH) state. Disulfide bonds formation in ER lumen is facilitated by the enzyme **protein disulfide isomerase** (PDI). PDI also catalyzes rearrangement of disulfide bond. In bacterial cells, S-S bonds are formed in the periplasmic space. Thus S-S bonds are found only in secretory proteins and in the exoplasmic domains of membrane proteins. Cytoplasmic proteins and organelle proteins synthesized on free ribosome lack disulfide bonds.

Synthesis of membrane lipid

The SER produces most of membrane lipids, including both phospholipids and cholesterol. The phospholipid synthesis occurs exclusively in the cytosolic half of the ER bilayer. Phospholipids are amphipathic molecules and their synthesis from fatty acyl CoAs and glycerol 3-phosphate take place at the interface between a membrane and the cytosol and are catalyzed by membrane associated enzymes. Fatty acids are not directly incorporated into phospholipids; rather, they are first converted into CoA esters. Fatty acids from fatty acyl CoA are esterified to the phosphorylated glycerol backbone, forming phosphatidic acid, whose two long hydrocarbon chains anchor the molecule to the membrane. A phosphatase converts phosphatidic acid into diacylglycerol. Finally, a polar head group (e.g., phosphorylcholine) is transferred from CDP-choline to the exposed hydroxyl group.

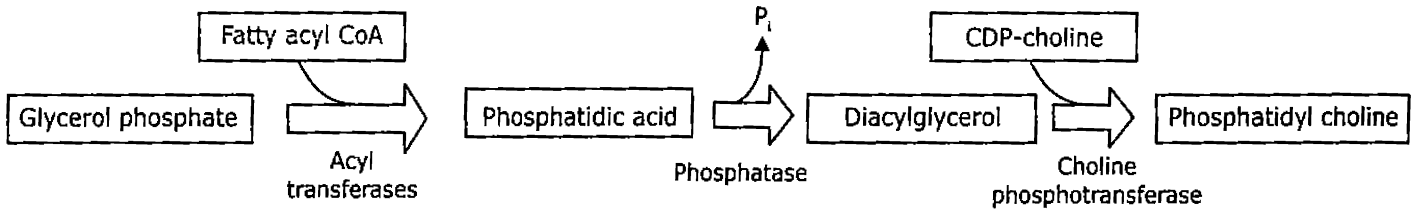


Figure 9.29 : Phospholipid (phophatidyl choline) synthesis

As phospholipid synthesis takes place in the cytosolic half of the ER bilayer, they need enzymes that transfers some of the newly formed phospholipid molecules to the other half of the bilayer. Two membrane bound enzymes, **flippase** and **scramblase**, mediate this transbilayer movement. *Scramblase* is required to transfer lipid molecules from the cytosolic lipid layer to the non-cytosolic (lumenal side) lipid layer to ensure that both monolayers remain equally populated with lipids.

Detoxification of xenobiotic compounds

SER in the hepatic cells are the major site for removal of xenobiotics. Cytochrome P-450 present in the SER catalyze this process. Cytochromes P-450 are hemeproteins and monooxygenases. Monooxygenases (mixed-function oxidases) catalyze the incorporation of a single atom of molecular oxygen into a substrate with the concomitant reduction of the other atom to water. Microsomal cytochromes P-450 are membranebound and accept electrons from a microsomal NADPH-cytochrome P-450 reductase, containing FAD and FMN. Cytochrome P-450 systems catalyze reactions as diverse as hydroxylation; dealkylation; sulfoxidation; epoxidation; deamination; desulfuration; dehalogenation and peroxidation.

Transport of proteins from ER to *cis* Golgi

Following the ER-specific folding, oligomerization and processing, secreted proteins are exported from the ER through budding of transport vesicles followed by the targeting and fusion of these vesicles to the *cis* Golgi network, the first compartment of the Golgi apparatus. Most of the protein components in transport vesicle are highly specific in order to maintain organelle distinction.

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To be transported from one secretory compartment to another, protein products must be packaged into transport vesicles. Transport vesicles arise from specialized coated regions of membranes within the secretory apparatus. These membranes are surrounded by a coat of proteins covering the cytosolic face so that these membranes eventually bud off as *coated transport vesicles*. Prior to fusing with the target membrane, this protein coat is discarded to allow the membranes to fuse directly.

Mainly three types of coated vesicles are known, each with a different type of protein coat and each formed by reversible polymerization of a distinct set of protein subunits. In addition to coat proteins, various adaptor proteins and small GTP-binding proteins are required for formation of coated vesicles. Each type of vesicle transports proteins from particular parent organelles to particular destination organelles.

Clathrin coated : Clathrin forms multiple complexes based on its association with different adaptor protein (APs). Clathrin that is associated with AP1 and AP3 forms vesicles for transport from the trans-Golgi network to the lysosome. Clathrin that is associated with AP2 forms vesicles from the plasma membrane during endocytosis that transport to the early endosomes.

COPI coated : COPI forms vesicles for both intra-Golgi transport and retrograde transport from the Golgi to the endoplasmic reticulum.

COPII coated : COPII forms vesicles for anterograde transport from the endoplasmic reticulum to the Golgi.

Table 9.8 : Coated vesicles found within eukaryotic cells

Coated vesicle	*Coat proteins*	*Transport*
Clathrin	Clathrin, AP1	Golgi complex to Endosome
Clathrin	Clathrin, AP2	Plasma membrane to Endosome
COPI	COPI	Golgi complex to ER or Golgi complex
COPII	COPII	ER to Golgi complex

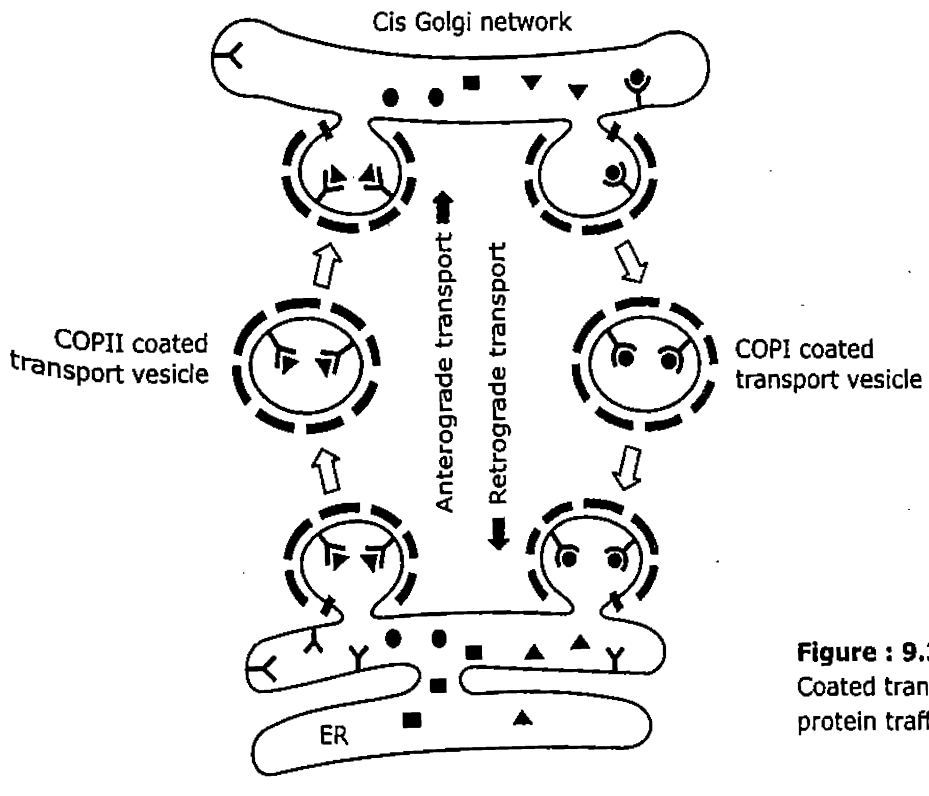


Figure : 9.30
Coated transport vesicle-mediated protein trafficking between ER and *cis* Golgi

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ER-resident proteins often are retrieved from the *Cis*-Golgi

Proteins entering into the lumen of ER are of two types- soluble *resident* proteins such as BiP and *export* proteins such as lysosomal proteins. How, then, are resident proteins retained in the ER lumen to carry out their work?

The answer lies in a specific C-terminal sequence present in resident ER proteins and in a receptor that recognizes this sequence. Most ER-resident proteins have a *Lys-Asp-Glu-Leu* (**KDEL** in the one-letter code) sequence at their C-terminus. Several experiments demonstrated that this *KDEL sequence,* acts as sorting signal, is both necessary and sufficient for retention in the ER.

The KDEL sorting signal is recognized and bound by the *KDEL receptor* found on the ER and the *cis*-Golgi. The KDEL receptor acts mainly to retrieve proteins with the KDEL recognition sequence that have escaped to the *cis*-Golgi network and return them to the ER. The finding that most KDEL receptors are localized to the membranes of small transport vesicles shuttling between the ER and the *cis*-Golgi and to the membranes of the *cis*-Golgi reticulum also support this concept. The KDEL receptor acts mainly to retrieve soluble proteins containing the KDEL sequence. The retention of transmembrane proteins in the ER is carried out by short C-terminal sequences that contain two lysine residues (KKXX sequences).

Clearly, the transport of newly synthesized proteins from the RER to the Golgi cisternae is a highly selective and regulated process; the selective entry of proteins into membrane-bounded transport vesicles is an important feature of protein targeting as we will encounter several times in our study of the subsequent stages in the maturation of secretory and membrane proteins.

9.8 GOLGI COMPLEX

Golgi complex was first discovered in 1897 by an Italian physician named Camillo Golgi. The Golgi complex, also called Golgi body or Golgi apparatus and found universally in both plant and animal cells, is typically comprised of a series of five to eight flattened membrane-bound sacs called **cisternae**. In some unicellular flagellates, however, as many as 60 cisternae may combine to make up the Golgi apparatus. Similarly, the number of Golgi bodies in a cell varies according to its function. This complex is usually located close to the cell nucleus.

Each Golgi stack has two distinct faces: a ***cis* face** (or forming face) and a ***trans* face** (or maturing face). Both *cis* and *trans* faces are closely associated with special compartments, each composed of a network of interconnected tubular and cisternal structures: the ***cis* Golgi network** (CGN) and the ***trans* Golgi network** (TGN), respectively. Proteins and lipids enter the *cis* Golgi network in vesicular tubular clusters arriving from the ER and exit from the *trans* Golgi network. Both networks are thought to be important for protein sorting. As we have seen, proteins entering the CGN can either move onward in the Golgi apparatus or be returned to the ER. Similarly, proteins exiting from the TGN can either move onward and be sorted according to whether they are destined for lysosomes, secretory vesicles, or the cell surface, or be returned to an earlier compartment.

The Golgi apparatus is especially prominent in cells that are specialized for secretion, such as the goblet cells of the intestinal epithelium, which secrete large amounts of polysaccharide-rich mucus into the gut. In such cells, unusually large vesicles are found on the *trans* side of the Golgi apparatus, which faces the plasma membrane domain where secretion occurs.

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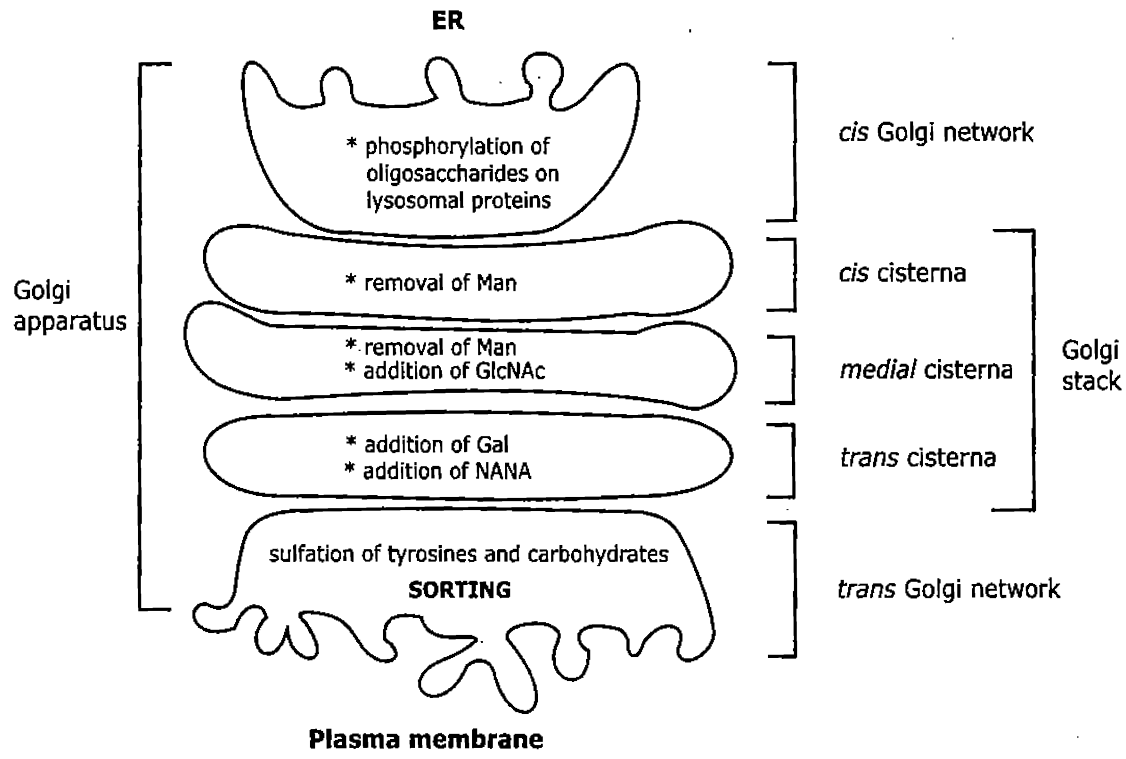


Figure 9.31 : The functional compartmentalization of the Golgi apparatus

The Golgi apparatus is often considered the distribution and shipping department for the cell's chemical products. It modifies proteins and lipids (fats) that have been built in the endoplasmic reticulum and prepares them for export outside of the cell or for transport to other locations in the cell. Proteins and lipids built in the smooth and rough endoplasmic reticulum bud off in tiny bubble-like vesicles that move through the cytoplasm until they reach the Golgi complex. The vesicles fuse with the Golgi membranes and release their internally stored molecules into the organelle. Once inside, the compounds are further processed by the golgi apparatus, which adds molecules or chops tiny pieces off the ends. When completed, the product is extruded from the golgi apparatus in a vesicle and directed to its final destination inside or outside the cell.

The modifications to molecules that take place in the Golgi apparatus occur in an orderly fashion. Substances from ER enter into the Cis face of a Golgi stack, the end of the organelles, for processing and exit in the from of smaller detached vesicles from trans face. Consequently, the cis face is found near the endoplasmic reticulum and the trans face is positioned near the plasma membrane of the cell. The chemical make-up of each face is different and the enzymes contained in the lumens (inner open spaces) of the cisternae between the faces are distinctive.

Glycosylation of proteins

N-linked oligosaccharide chains on proteins are altered as the proteins pass through the Golgi cisternae *en route* from the ER. Some proteins undergo ***O*-linked glycosylation** in the Golgi cisternae. O-linked oligosaccharides are linked to the hydroxyl group of *serine* or *threonine* via N-acetylgalactosamine (GalNac) or (in collagens) to the hydroxyl group of *hydroxylysine* via galactose. O-linked oligosaccharide are generally short, often containing only one to four sugar residues. O-linked sugars are added one at a time, and each sugar transfer is catalyzed by a different glycosyltransferase enzyme. Typical N-linked oligosaccharides, in contrast, always contain mannose as well as N-acetylglucosamine and usually have several branches each terminating with a negatively charged sialic acid residue.

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Lipid and polysaccharide metabolism

In addition to its activities in processing and sorting glycoproteins, the Golgi apparatus functions in lipid metabolism—in particular, in the synthesis of glycolipids and sphingomyelin. Ceramide, which is synthesized in the ER, is converted either to sphingomyelin or to glycolipids in the Golgi apparatus.

Golgi apparatus is also acts as a major site of carbohydrate metabolism. In plant cells, the Golgi apparatus acts as the site where complex polysaccharides of the cell wall are synthesized. Complex cell wall polysaccharides such as hemicelluloses and pectins are synthesized in the Golgi apparatus and then transported in vesicles to the cell surface. In animals, most of the glycosaminoglycans of the extracellular matrix are synthesized in Golgi.

Transport of proteins through cisternae

Several models have been proposed to explain the ordered transport of proteins through the cisternae. Two most accepted models are *cisternal progression model* and *Vesicular transport model*.

According to **cisternal progression model,** stacks of cisternae are dynamic. The *cis*-most cisterna is assembled by the fusion of transport vesicles from the ER, carrying newly synthesized cargo. Anterograde transport through the golgi occurs from the *cis*- to the *medial*-golgi and from the *medial*- to the *trans*-golgi. In this case each golgi cisterna matures as it migrates outward through a stack and at each stage, the golgi resident proteins that are carried forward in a cisterna are moved backward to an earlier compartment. In this way each cisterna progress into the next one.

Vesicular transport model postulates a stable stack of cisternae containing a fixed and ordered array of processing enzymes. The cargo is delivered to each cisterna is turn by vesicles that bud from one cisterna and fuse with the next in the stack. There is still controversy as to whether either model, or a hybrid one, is correct.

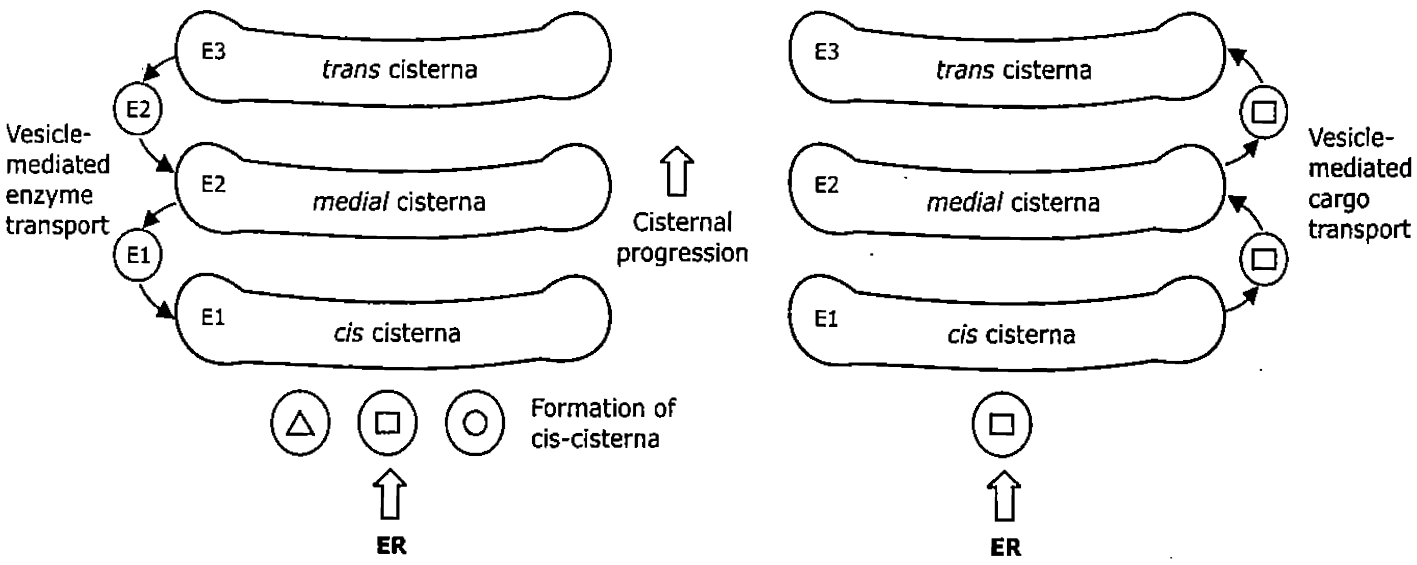


Figure 9.32 : Models for transport through the Golgi stack. (a) Cisternal progression envisages a dynamic stack of cisternae in which each cisterna, carrying a mixture of cargo molecules, matures into the next one through the retrograde transport of Golgi enzymes. (b) Vesicular transport envisages a stable stack of cisternae through which a mixture of cargo molecules passes, in sequence, transported from cisterna to cisterna by vesicles.

Mannose 6-phosphate residues target proteins to lysosomes

Some *N*-linked oligosaccharides help in targeting lysosomal enzymes to lysosomes and prevent their secretion. The addition and initial processing of the preformed *N*-linked oligosaccharide precursor in the rough ER is the

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same for lysosomal enzymes as for membrane and secretory proteins. In the *cis*-Golgi, one or more mannose residues in the resulting $Man_8(GlcNAc)_2$ oligosaccharides of lysosomal enzymes become phosphorylated.

The many mannose 6-phosphate (M6P) residues that are formed then bind to *mannose 6-phosphate receptors (M6P)*, which are found primarily in the *trans*-Golgi network. The luminal domain of this transmembrane protein contains a region that binds M6P very tightly and specifically. Transport vesicles containing the M6P receptor and bound lysosomal enzyme bud from the *trans*-Golgi network and then fuse with a sorting vesicle, *late endosome*, which has an internal pH of ~5.5. Because M6P receptors can bind M6P at the slightly acidic pH (between 6.5 to 7) of the *trans*-Golgi network but not at a pH less than ~6, the bound lysosomal enzymes are released within late endosomes. Furthermore, a phosphatase within late endosomes generally removes the phosphate from lysosomal enzymes, preventing their rebinding to the M6P receptor.

Two types of vesicles bud from late endosomes. One type contains lysosomal enzymes but not the M6P receptor; after these vesicles bud from late endosomes, they fuse with lysosomes, delivering the lysosomal enzyme to their final destination. The other type of vesicle recycles the M6P receptor back to the *trans*-Golgi network or, on occasion, to the cell surface.

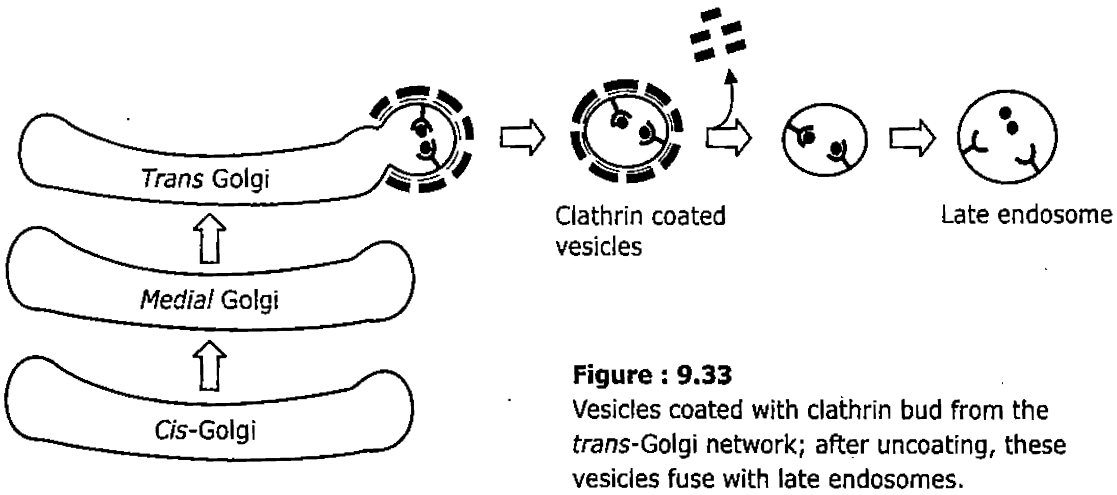


Figure : 9.33
Vesicles coated with clathrin bud from the *trans*-Golgi network; after uncoating, these vesicles fuse with late endosomes.

Table 9.9 : Examples of sorting signals that direct secreted and membrane proteins to specific transport vesicles

Signal sequence	Type of protein	Transport step	Vesicle type
Lys-Asp-Glu-Leu (KDEL)	Secreted	Golgi to ER	COP I
Lys-Lys-X-X (KKXX)	Membrane	Golgi to ER	COP I
Di-acidic (e.g., Asp-X-Glu)	Membrane	ER to Golgi	COP II
Mannose 6-phosphate (M6P)	Secreted	*Trans*-Golgi and plasma membrane to late endosome	Clathrin

X = any amino acids

Adapted from Molecular Cell Biology, fifth edition, Lodish et. al., W. H. Freeman and Company

VESICLE FUSION

The fusion of a transport vesicle with its target involves two types of events. First, the transport vesicle must specifically recognize the correct target membrane and second, the fusion of vesicle and target membranes. Vesicle fusion is mediated by specific recognition between a vesicle and its target by interactions between unique pairs of transmembrane proteins, followed by fusion between the phospholipid bilayers of the vesicle and target membranes. Rothman and his colleagues proposed a general model, called the **SNARE hypothesis**, to explain vesicle fusion. According to this model, vesicle fusion with target is mediated by interactions between specific pairs of proteins, called SNAREs, on the vesicle and target membranes (v-SNAREs and t-SNAREs, respectively). v-SNAREs, integral membrane proteins pair with cognate t-SNAREs

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in the target membrane. In neurons, v-SNARE present on synaptic vesicles is designated *synaptobrevin* and t-SNARE are designated *syntaxin* and *SNAP 25*.

Recognition step is controlled by members of a family of monomeric GTPases called **Rab** proteins, which check that the interaction between a v-SNARE and a t-SNARE is correct. Rab proteins make attachment with the surface of vesicle. When a vesicle encounters the correct target membrane, the binding of v-SNARE to t-SNARE causes the vesicle to remain bound for long enough to allow the Rab protein to hydrolyze its bound GTP, which locks the vesicle onto the target membrane. Cells contain many types of Rab proteins, each associated with membrane of particular organelle involved in the secretory or endocytic pathways.

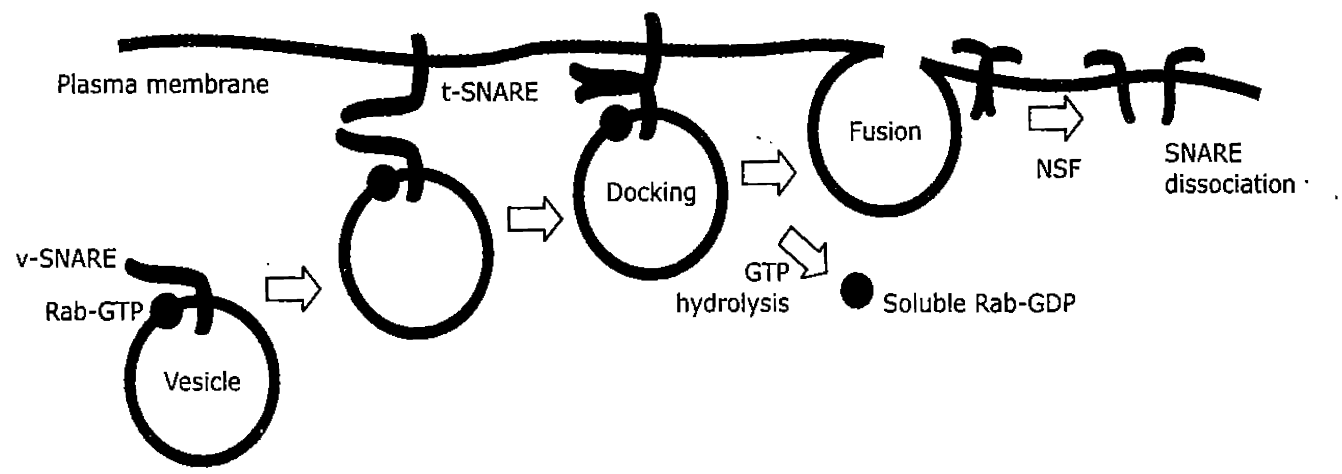


Figure 9.34 : Complementary sets of vesicle-SNAREs (v-SNAREs) and target-membrane SNAREs (t-SNAREs) determine the selectivity of transport-vesicle docking. v-SNAREs of transport vesicles from the donor membrane, bind to complementary t-SNAREs in the target membrane. The Rab protein hydrolyzes its bound GTP, locking the vesicle onto the target membrane and releasing Rab-GDP into the cytosol, from where it can be reused in a new round of transport. The vesicle then fuses with the target membrane. NSF is an ATPase that hydrolyzes ATP to release the complex once it has done its job.

After the v-SNAREs and t-SNAREs have mediated the fusion of a vesicle on a target membrane, the NSF binds to the SNARE complex via adaptor proteins, **SNAPs** (soluble NSF attachment factor) proteins and hydrolyzes ATP to dissociate the SNAREs apart.

9.9 LYSOSOMES

Lysosomes are membrane-enclosed compartments filled with hydrolytic enzymes that are used for the controlled *intracellular* digestion of macromolecules. They contain about 40 types of hydrolytic enzymes, including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, and sulfatases. All are *acid hydrolases* because for optimal activity they require an acid environment and the lysosome provides this by maintaining a pH of about 5.0 in its interior. A H^+ pump in the lysosomal membrane uses the energy of ATP hydrolysis to pump H^+ into the lysosome, thereby maintaining the acidic pH of lumen. Lysosomes greatly vary in size and shape. There are two types of lysosomes : *Primary lysosomes* (do not contain particle or membrane for digestion) and *Secondary lysosomes* (contain particles or membranes in the process of being digested).

Lysosomes are responsible for the degradation of large particles taken up by phagocytosis and for the gradual digestion of the cell's own components by autophagy. On this bases lysosome can be divided into

Heterophagic vacuole (or heterolysosome or phagolysosome)

They are formed by the fusion of primary lysosome with cytoplasmic vacuoles containing *extracellular* substances brought into the cell by an endocytic process.

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Autophagic vacuoles (or autolysosomes)

Autophagic vacuoles contain particles isolated from the cells own cytoplasm including mitochondria, microbodies etc.

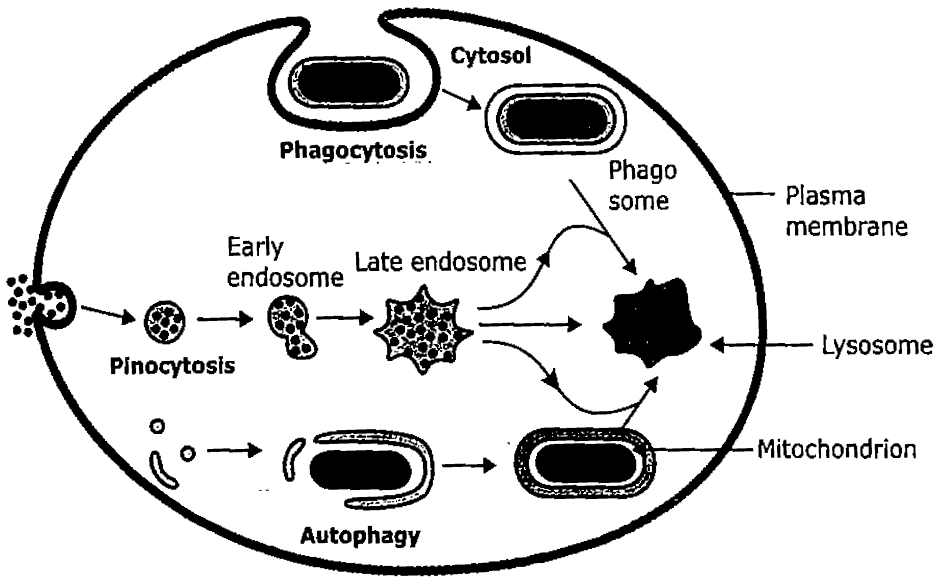


Figure : 9.35
Schematic overview of three pathways by which materials are moved to lysosomes :endocytic pathway, phagocytic pathway and autophagic pathway

Table 9.10 : Acid hydrolases present in lysosomes

Enzyme	**Natural substrate**
Phosphatases	
Acid phosphatase	Most phosphomonoesters
Acid phosphodiesterase	Oligonucleotides and other phosphodiesters
Nucleases	
Acid ribonuclease	RNA
Acid deoxyribonuclease	DNA
Polysaccharide/mucopolysaccharide hydrolyzing enzymes	
β-Galactosidase	Galactosides
α-Glucosidase	Glycogen
α-Mannosidase	Mannosides, glycoproteins
β-Glucuronidase	Polysaccharides and mucopolysaccharides
Lysozyme	Bacterial cell walls and mucopolysaccharides
Hyaluronidase	Hyaluronic acids; chondroitin sulfates
Arylsulfatase	Organic sulfates
Proteases	
Cathepsin(s)	Proteins
Collagenase	Collagen
Peptidases	Peptides
Lipid-degrading enzymes	
Esterase(s)	Fatty acyl esters
Phospholipase(s)	Phospholipids

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Most plant and fungal cells (including yeasts) contain one or several very large, fluid-filled vesicles called **vacuoles**. Vacuoles are related to the lysosomes of animal cells, containing a variety of hydrolytic enzymes, but their functions are remarkably diverse. Like a lysosome, the lumen of a vacuole has an acidic pH, which is maintained by similar transport proteins in the vacuolar membrane. The plant vacuole can act as a storage organelle for both nutrients and waste products, as a degradative compartment, and as a controller of turgor pressure. Different vacuoles with distinct functions (e.g., digestion and storage) are often present in the same cell.

9.10 MITOCHONDRIA

Mitochondria are *energy-converting organelles*, which are present in virtually all eukaryotic cells. Mitochondria are double membrane-bound mobile as well as plastic organelle. The outer membrane is fairly smooth. But the inner membrane is highly convoluted; forming folds called *cristae* and is highly impermeable to small ions due to having a very high content of a phospholipid called *cardiolipin*. The cristae greatly increase the inner membrane's surface area. The outer membrane protects the organelle, and contains specialized transport proteins such as *porin* which allows free passage for various molecules into the intermitochondrial space (the space between the inner and outer membranes) of the mitochondria.

The matrix (large internal space) contains several identical copies of the mitochondrial DNA genome, special mitochondrial ribosomes, tRNAs, and various enzymes required for expression of the mitochondrial genes. Mitochondrial ribosomes of different species vary considerably in their sedimentation coefficients, ranging from 55S-80S, while cytoplasmic ribosomes are uniformly 80S.

Mitochondria are semi autonomous organelle and are supposed to have evolved from endosymbiotic association of purple photosynthetic bacteria about 1.5×10^9 years ago.

Mitochondrial genome

Mitochondrial genome is circular (linear DNA molecule in Paramecium, Tetrahymena and others) dsDNA. The size of mitochondrial DNA varies greatly among different species.

Organisms	**Size** (kb)
Human	16.6
Xenopus (frog)	18.4
Drosophila (fruit fly)	18.4
Saccharomyces (yeast)	75.0
Arabidopsis (mustard plant)	367.0

Table 9.11 : Features of human mitochondrial genomes

Size	16.6 kbp
Nature of DNA molecule	ds circular DNA molecule
Number of DNA molecules	More than one
Number of genes	37
Repetitive DNA	Very little
Introns	Absent
% of coding DNA	93%
Recombination	Not evident
Inheritance	Maternal

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Individual mitochondria contain several copies of mtDNA and without histone protein. mtDNA is larger in plants than in animals (10 to 150 times). In humans, mtDNA encodes two ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), as well as 13 polypeptides essential for the oxidative respiratory functions of the organelle.

Mitochondrial proteins

Mitochondrial proteins are synthesized by cytosolic as well as matrix ribosomes. Proteins synthesized by cytosolic ribosomes are translocated into mitochondria **post-translationally**. Proteins imported into mitochondria may be located in the outer membrane, the intermembrane space, the inner membrane, or the matrix. Precursor proteins synthesized in the cytosol that are destined for the matrix of mitochondria contain N-terminal *matrix-targeting* sequences. They are rich in hydrophobic amino acids, positively charged basic amino acids (arginine and lysine), and hydroxyl group containing amino acids (serine and threonine), but lack negatively charged acidic residues. Generally, this sequence is cleaved once it reaches the matrix. Mitochondrial protein import requires membrane receptors and translocons. Import of mitochondrial protein is initiated by the binding of a matrix targeting sequence to an *import receptor* in the outer mitochondrial membrane.Two distinct *translocation complexes* that mediate translocation are situated in the outer and inner mitochondrial membranes, referred to as **TOM** (translocase-of-the outer membrane) and **TIM** (translocase-of-the-inner membrane). Proteins remain in the unfolded state to pass through the complexes, and this is carried out by ATP-dependent binding of chaperone proteins such as Hsp70. Soon after the N-terminal matrix-targeting sequence of a protein enters the mitochondrial matrix, it is removed by a protease that resides within the matrix. Translocation of proteins across mitochondrial membranes is an active process. An electrochemical gradient across the inner mitochondrial membrane is also required in addition to ATP hydrolysis. However, it is still uncertain how the electrochemical gradient contributes to protein translocation.

9.11 CHLOROPLAST

The chloroplast is an organelle where photosynthesis occurs in photosynthetic eukaryotes. It is surrounded by a double membrane. The inner membrane encloses a fluid-filled region called the **stroma** that contains enzymes for the Calvin cycle, light-independent reactions of photosynthesis. Embedded in the stroma is a complex network of stacked sacs. Each stack is called a **granum** and each of the flattened sacs which makes up the granum is called a **thylakoid**. The thylakoid membrane, that encloses a fluid-filled thylakoid interior space, contains chlorophyll and other photosynthetic pigments as well as electron transport chains. The light-dependent reactions of photosynthesis occur in the thylakoids.

Chloroplast is a semi-autonomous organelle. The stroma contains chloroplast DNA, 70S ribosomes, tRNAs, and various enzymes required for expression of the chloroplast genes. Chloroplast DNAs vary in length from 120-190 kb. There are multiple copies of the genome per organelle, typically 20-40 in a higher plant. The chloroplast genome codes for all the rRNA and tRNA species needed for protein synthesis. The chloroplast genome codes for ~50 proteins.

Chloroplast differentiates from *proplastids*. Proplastids also differentiate into *leucoplasts* and *chromoplasts*. Chromoplasts are mainly the site for pigment synthesis and storage. Whereas, leucoplasts may differentiate into more specialized forms:

- Amyloplasts: for starch storage
- Elaioplasts: for storing fat
- Proteinoplasts: for storing and modifying protein

A collective term for these different kinds of organelles, all derived from proplastids, is *plastid*. Like mitochondria, all plastids come from the division of pre-existing plastids.

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9.12 PEROXISOMES

Peroxisomes are ubiquitous organelles in eukaryotes. Peroxisomes were discovered by *Christian de Duve* in 1965. They are small organelles, approximately 0.2-1 μm in diameter. Peroxisomes lack DNA and ribosomes and are lined by a single membrane. Thus all peroxisomal proteins are encoded by nuclear genes, synthesized on ribosomes free in the cytosol, and then incorporated into pre-existing peroxisomes. Peroxisomes divide, forming new ones, similar to mitochondria and chloroplasts.

Peroxisomes contain oxidative enzymes, such as *catalase* and *urate oxidase.* Like the mitochondrion, peroxisomes contain enzymes that use molecular oxygen to oxidize various substrates. Some peroxisomal enzymes use molecular oxygen to remove hydrogen atoms from specific organic substrates in an oxidative reaction that produces H_2O_2. Enzyme catalase efficiently decomposes H_2O_2 into H_2O.

$$H_2O_2 + H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Another enzyme that destroys hydrogen peroxide is *peroxidase*, which differs from catalase. Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen. Peroxidases are a large family of enzymes that typically catalyze a reaction of the form:

$$ROOR' + 2e^- + 2H^+ \longrightarrow ROH + R'OH$$

Substrates for peroxidase can be hydrogen peroxide, lipid peroxides and others and it requires a reductant for activity, usually NADH.

A major oxidative reactions carried out in peroxisomes is the β-*oxidation.* β-oxidation in mammalian cells occur both in mitochondria and peroxisomes; in plant cells, however, this essential reaction is exclusively found in peroxisomes. Peroxisomes also have two important roles in plants - *photorespiration* and *glyoxylate cycle*. In *photorespiration,* glycolate-2-phosphate produced by oxygenase activity of rubisco is metabolized into serine, CO_2 and NH_3.This pathway involves three subcellular compartments, the chloroplasts, peroxisomes and mitochondria. *Glyoxylate cycle* occurs in specialized peroxisomes called *glyoxysomes.* In the glyoxylate cycle two molecules of acetyl CoA produced by β-*oxidation* are used in synthesis of succinic acid.

Targeting of peroxisomal proteins

Proteins that are required for peroxisome formation are called **peroxins.** Transport of proteins to peroxisomes occur *post-translationally*. Peroxisomal proteins syhthesized on cytosolic ribosomes are generally fold into their mature conformation in the cytosol before import into the organelle. Proteins that are imported into the peroxisome have *peroxisomal targeting sequences*–PTS1 and PTS2. The PTS1 is a tri- or tetrapeptide at the C-terminus. It was first characterized in catalase as a **Ser-Lys-Leu** sequence (SKL in one-letter code) at the very C-terminus. The PTS2 signal is a sequence of nine amino acids and can be located near the N-terminus or internally.

The importance of import process in peroxisomes is dramatically demonstrated by the inherited human disease, **Zellweger syndrome.** It is a rare, congenital disorder, characterized by the reduction or absence of peroxisomes due to defect in importing proteins into peroxisomes.

9.13 CYTOSKELETON

Cytoskeleton, *an intracellular network of protein filaments,* involved in determining cell shape, cell movement and intracellular movement. There are three types of cytoskeletal fibers—**microfilaments, intermediate filaments**, and **microtubules**. All these fibers are polymers built from small protein subunits held together by noncovalent bonds.

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Microtubules

Microtubules are hollow, cylindrical structure, approximately 25 nm in diameter. They are present in virtually all eukaryotic cells and play critical roles in the determination of cell shape in the organization of the cells cytoplasm and in many forms of cellular motility. The basic building block unit of the microtubule is **tubulin**, a dimeric protein composed of two 50-kDa subunits known as α-tubulin and β-tubulin. A large number of additional cellular molecules, called **microtubule-associated proteins** (MAP), bind to the surface of the microtubule and determine and control its many functions. It is widely accepted that many of these MAPs play critical roles in the regulation of microtubule polymerization and dynamics, in the organization of microtubule arrays, and in the functional interactions of microtubules with other cellular components.

Microtubule structures

Microtubule polymers are composed of αβ-tubulin heterodimers arranged in a uniformly repeated head-to-tail fashion. Each linear array of tubulin heterodimers is called a *protofilament*, and in cells generally 13 protofilaments arranged into a tube structure called *microtubules*. However, many examples exist in which microtubules have a different number of protofilaments (from 11 to as many as 15). The 13 protofilaments are aligned in parallel with the same polarity. β-tubulin subunits of all protofilaments face one end of the microtubule, and the α-subunits face the opposite end. The ends are designated either plus or minus, based upon their different dynamic and structural properties. β-subunits are exposed at the plus ends (fast-growing) while the α-subunits are exposed at the minus ends (slow-growing).

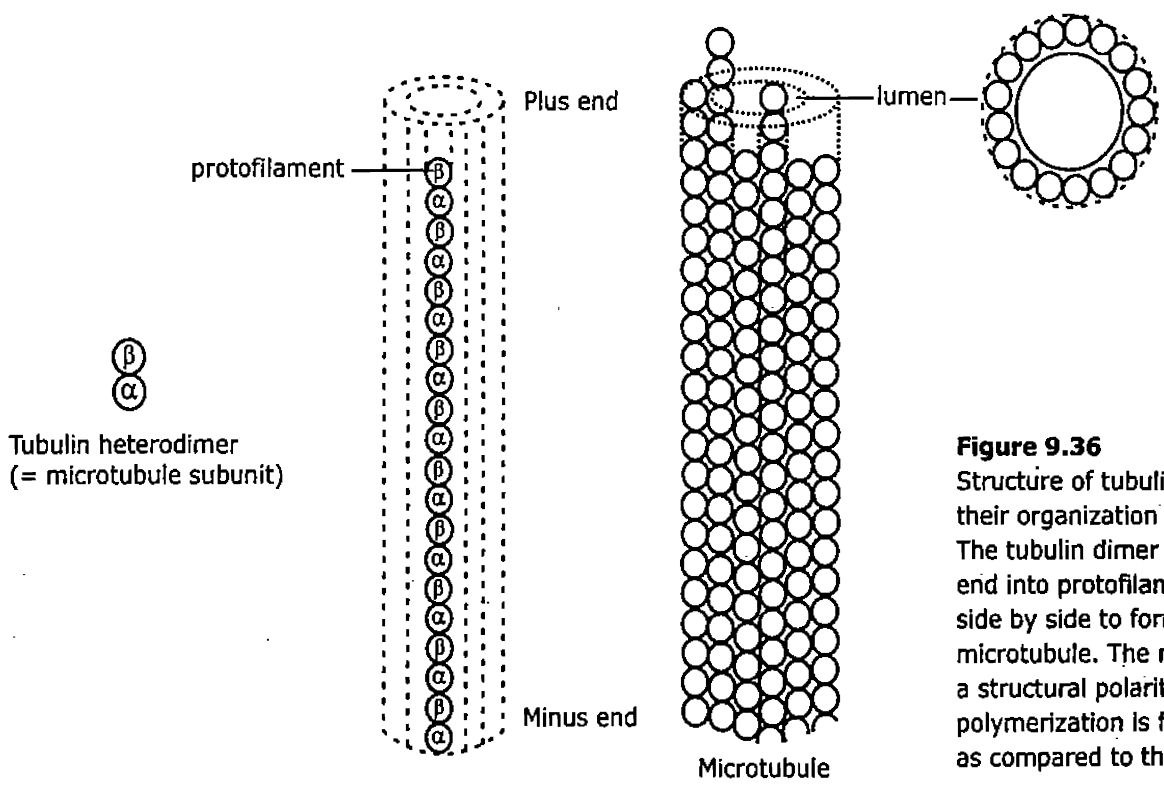


Figure 9.36
Structure of tubulin monomers and their organization in microtubules. The tubulin dimer are aligned end to end into protofilaments, which pack side by side to form the wall of the microtubule. The microtubule displays a structural polarity. The rate of polymerization is faster at the plus end as compared to the minus end.

Microtubules in cells are usually organized at **microtubule organizing center** (MTOC). In Interphase it is typically located to one side of the nucleus, close to the outer surface of the nuclear envelope. In animal cells MTOC contains a pair of centrioles and known as **centrosome.** The minus ends are embeded at the MTOC and the plus ends are away from it. One of the known proteins of the MTOC is a form of tubulin called γ-tubulin, which does not form microtubules itself and does not become incorporated into microtubules, but does appear to function in the nucleation of microtubule formation. The MTOCs in cells from higher plants do not contain centrioles, but they still function as microtubule organizing centers.

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Single microtubules in cytosol is very common but many specialized microtubule arrays also exist in which microtubules are fused along their lengths into doublet or triplet microtubule structures. *Doublet microtubules* consist of 10 or 11 protofilaments of a second microtubule fused to an entire 13-protofilament first microtubule, and *triplet microtubules* have an additional 10 or 11 protofilaments of a third microtubule fused to the second microtubule. Doublet microtubules occur in the long shafts of motile cell appendages called *cilia* and *flagella*. Triplet microtubules occur in the basal body structures that in most animal cells are situated at the base of cilia and flagella and in centrioles, which are situated at the center of animal cell centrosomes.

Microtubule polymerization and dynamics

Microtubules are dynamic structures that are constantly being polymerized and depolymerized. Its polymerization occurs by a nucleation-elongation mechanism, in which the formation of a short microtubule *nucleus* is followed by growth of the microtubule at both its ends by the reversible noncovalent addition of tubulin subunits. Whole course of polymerization is divided into three phases –

The *lag phase* corresponds to time taken for nucleation.

The *growth phase* occurs as monomers add to the exposed ends of the growing filament, causing filament elongation.

The *steady phase* is reached when the growth of the filament due to monomer addition (polymerization) is exactly balanced by depolymerization from the ends. The number of monomers that add to the polymer per second depends on the concentration of the free subunits. The concentration of free subunits left at this point that is, steady state, is called the **critical concentration**.

GTP hydrolysis

Soluble tubulin binds GTP reversibly at a site on the β-subunit, and the GTP becomes hydrolyzed to GDP and P_i as, or shortly after, the tubulin polymerizes onto a growing microtubule ends. The α-tubulin subunit also carries GTP, but this cannot be exchanged for free GTP and is not hydrolyzed. Hydrolysis of the bound nucleotide reduces the binding affinity of the subunit for neighboring subunits and makes it more likely to dissociate from each end of the filament. Thus the normal role of GTP hydrolysis is apparently to allow microtubules to depolymerize by weakening the bonds between tubulin subunits in the microtubule.

Treadmilling

The rate of subunit addition at the end of a filament is the product of the free subunit concentration. The rate of polymerization as well as depolymerization is much faster for the plus end of a filament than for the minus end. When the αβ-tubulin concentration is higher than the critical concentration at the *plus-end* but lower than the critical concentration at the *minus-end*, microtubules can treadmill by adding subunits to one end and dissociating subunits from the opposite end. This special state in polymer dynamics, known as **treadmilling**, keeps the polymer length unchanged.

Dynamic instability

Dynamic instability is characterized by phase transitions, or switching, between phases of relatively slow growing and rapid shortening at the ends of individual microtubules. Dynamic instability is suppose to be a result of the hydrolysis of GTP bound to β-tubulin shortly after assembly. The kinetics of GDP-tubulin are different from those of GTP tubulin. GDP-bound tubulin subunit is prone to depolymerization. Since tubulin adds onto the end of the microtubule only in the GTP-bound state, there is generally a cap of GTP-bound tubulin at the tip of the microtubule, protecting it from disassembly. When hydrolysis catches up to the tip of the microtubule, it begins a rapid depolymerization and shrinkage. This switch from growth to shrinking is called a **catastrophe**. Addition of GTP-bound tubulin to the tip of the microtubule again, provides a new cap and protects the microtubule from shrinking. This is referred to as **rescue**.

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Microtubule motor proteins - Kinesins and Dyneins

Kinesin is a motor protein that moves along microtubules. It carries membrane-enclosed organelles toward the *plus* end of microtubules. Kinesin is structurally similar to myosin II in having two heavy chains and two light chains per active motor, two globular head motor domains, and an elongated coiled-coil responsible for heavy chain dimerization. Like myosin, kinesin is a member of a large protein superfamily, for which the motor domain is the only common element. Many of the kinesin superfamily members have specific roles in mitotic and meiotic spindle formation and chromosome separation during cell division.

Dyneins are a family of *minus-end* directed microtubule motors proteins. They are composed of two or three heavy chains (that include the motor domain) and a large and variable number of associated light chains. Dyneins are the largest of the known molecular motors, and they are also among the fastest.

The dynein family can be classified into two major groups :

Cytoplasmic dyneins are probably found in all eukaryotic cells, and they are important for vesicle trafficking, as well as for localization of the Golgi apparatus near the center of the cell.

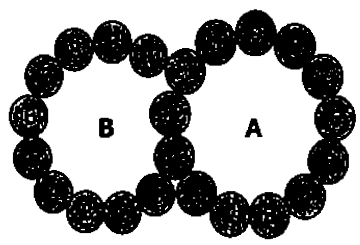
Axonemal dyneins, highly specialized for the rapid and efficient sliding movements of microtubules that drive the beating of cilia and flagella.

CILIA AND FLAGELLA : STRUCTURE AND MOVEMENT

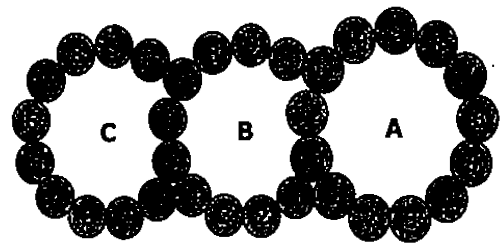
Cells contain two types of microtubules: stable, long-lived microtubules and unstable, short-lived microtubules. Stable microtubules are found in *cilia* and *flagella*. In contrast to permanent, stable microtubules, unstable microtubules undergo fast polymerization and depolymerization. For example, the cytosolic microtubule network characteristic of interphase cells and spindle fibers of mitotic cells.

All eukaryotic cilia and flagella are remarkably similar in their organization, possessing a central bundle of microtubules, called the **axoneme,** in which nine outer doublet microtubules surround a central pair of singlet microtubules (9 + 2 arrangement). Each doublet microtubule consists of A and B tubules. The A tubule is a complete microtubule with 13 protofilaments, while the B tubule contains 10 protofilaments. The junction between A and B tubules of one doublet is probably strengthened by the protein *tektin,* a highly α-helical protein that is similar in structure to intermediate-filament proteins. The bundle of microtubules comprising the axoneme is surrounded by the plasma membrane.

At its point of attachment to the cell, the axoneme connects with the **basal body** or **kinetosome**. Like centrioles, basal bodies are cylindrical structures which contain nine triplet microtubules. Each triplet contains one complete 13-protofilament microtubule, the A tubule, fused to the incomplete B tubule, which in turn is fused to the incomplete C tubule. The A and B tubules of basal bodies continue into the axonemal shaft, whereas the C tubule terminates within the transition zone between the basal body and the shaft.



Doublet microtubule



Triplet microtubule

Within the axoneme, the two central singlet and nine outer doublet microtubules are continuous for the entire length of the structure. Permanently attached to the A tubule of each doublet microtubule is an inner and an outer row of *dynein* arms. These dyneins reach out to the B tubule of the neighboring doublet.

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A linker made up of protein *nexin,* joins adjacent outer doublet microtubules. A second linker *radial spokes,* which radiate from the central singlets to each A tubule of the outer doublets, form the third linkage system.

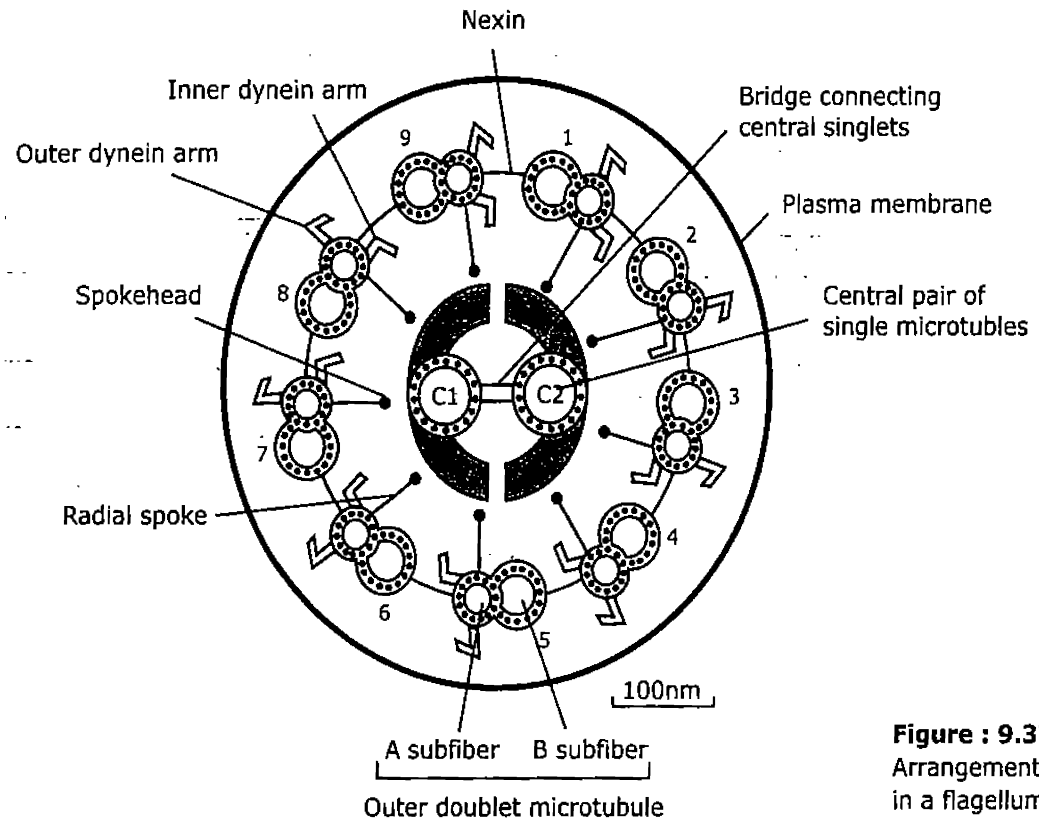


Figure : 9.37
Arrangement of microtubules in a flagellum.

The basis for axonemal movement is the sliding of protein filaments relative to one another. In cilia and flagella, the filaments are the doublet microtubules, all of which are arranged with their (+) end at the outer tip of the axoneme. Axonemal bending is produced by forces that cause sliding between pairs of doublet microtubules. The active sliding occurs all along the axoneme, so that the resulting bends can be propagated without damping. Dynein arms generate the sliding forces in axonemes.

The dynein arms on the A tubule of one doublet 'walk' along the adjacent doublet's B tubule toward its base, the (-) end. The force producing active sliding requires ATP and is caused by successive formation and breakage of cross-bridges between the dynein arm and the B tubule. Successive binding and hydrolysis of ATP causes the dynein arms to successively release from and attach to the adjacent doublet.

CENTRIOLE

A **centriole** is a barrel shaped microtubule structure found in most animal cells, cells of fungi and algae but not frequently in plants. The walls of each centriole are composed of nine triplet microtubules (9 + 0) arrangement or, in the case of *Drosophila*, nine doublet microtubules. Two centrioles are arranged perpendicularly in the centre of the **centrosome**.

The **centrosome** is the principal *microtubule-organizing center* in animal cells. It consists of a cloud of amorphous material (called the *centrosome matrix* or *pericentriolar material)* that surrounds a pair of *centrioles*. During interphase, the centrosome matrix nucleates a *cytoplasmic array* of microtubules, with their fast-growing plus ends projecting outward toward the cell perimeter and their minus ends associated with the centrosome. The matrix contains a great variety of proteins, including microtubule-dependent motor proteins, coiled-coil proteins that are thought to link the motors to the centrosome, structural proteins, and components of the cell-cycle control system. Most important, it contains the **γ-tubulin ring complex**, which is the component mainly responsible for nucleating microtubules.

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Although the localization of centrioles within the centrosome together with their precise duplication prior to mitosis has suggested a role in bipolar spindle assembly or function, the actual role of centrioles in cell division remains unclear and controversial. Cells from which centrioles and centrosomes are ablated can still form bipolar spindles via a centrosome-independent self-organization process.

Centriole replication

During interphase duplication of the centrosome occurs. The process of centrosome duplication and separation is known as the **centrosome cycle**. During interphase of each cell cycle, the centrioles and other components of the centrosome are duplicated. At a certain point in G1 phase the two centrioles separate. During S phase a daughter centriole begins to grow near the base of each old centriole and at a right angle to it. The elongation of the daughter centriole is usually completed by G2 phase. The two centriole pairs remain close together in a single centrosomal complex during the interphase. In the beginning of M phase the centrosome splits in two and the two halves begin to seperate.

ACTIN FILAMENTS

Actin filament (microfilament) is made up of G-actins. The G-actin is a globular protein and like tubulin, it has a binding site for a nucleotide, but the nucleotide is ATP (or ADP) rather than GTP (or GDP). As for tubulin, the G-actin subunits assemble head-to-tail to generate F-actins with a distinct structural polarity. Two parallel F-actins twist around each other in a *right-handed helix* to from an *actin filament*. The structural polarity of actin filament is created by the regular, parallel orientation of all of their subunits.

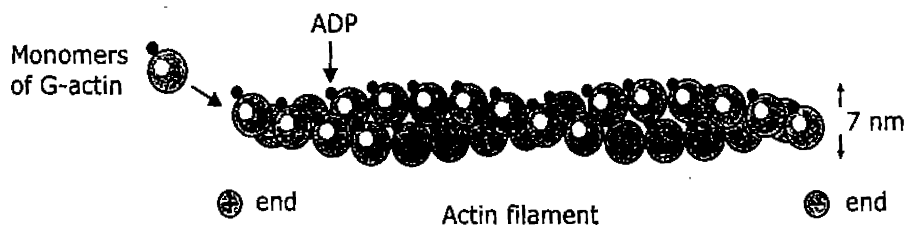


Figure 9.38 : Actin filament made up of two strings of monomeric G actin subunits coiled about each other in a right-handed helix.

In a structurally polar filament, the kinetic rate constants for association and dissociation k_{on} and k_{off}, respectively are often much greater at one end than at the other. Thus, if an excess of purified subunits is allowed to assemble onto marked fragments of preformed filaments, one end of each fragment elongates much faster than the other. If filaments are rapidly diluted so that the free subunit concentration drops below the critical concentration, the fast-growing end also depolymerizes fastest. The more dynamic of the two ends of a filament, where both growth and shrinkage are fast, is called the **plus end**, and the other end is called the **minus end**.

Table 9.12 : Properties of microtubules and microfilaments

	Microtubules	*Microfilaments*
Structure	Hollow cylindrical structure consisting of 13 protofilaments	Two intertwined chains of F-actin
Diameter	Outer : 25 nm, Inner : 15 nm	7 nm
Monomers	α-tubulin, β-tubulin	G-actin
Polarity	(+) and (−) ends	(+) and (−) ends
Nucleotide bound	GTP	ATP

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Drugs that affect microtubules and actin filament

Microtubule and actin filaments are the target of a variety of *drugs* that act by interfering with the polymerization and depolymerization of subunits. Example of some common drugs that affect microtubules and actin filament:

Actin filament

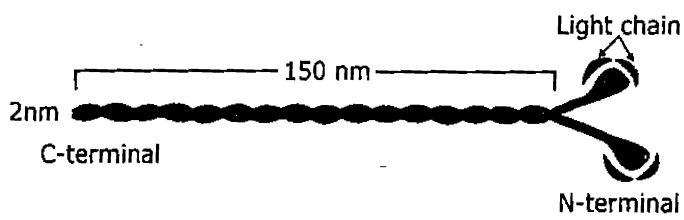
Cytochalasin D	:	Binds plus ends of actin filaments and prevents elongation.
Latrunculin	:	Binds G actin monomers and prevents them from polymerizing into filaments.
Phalloidin	:	Binds tightly all along the sides of actin filaments and stabilizes them against depolymerization.
Jasplakinolide	:	Induces actin polymerization by stimulating actin filament nucleation; stabilizes F-actin.
Swinholide	:	Severs filaments

Microtubules

Nocodazole	:	Causes microtubules to depolymerize to tubulin subunits.
Colchicine	:	Binds tubulin subunits and prevents their polymerization
Colcemid	:	Binds tubulin subunits and prevents their polymerization
Taxol	:	Binds to and stabilizes microtubules, preventing their depolymerization.
Vinblastine	:	Binds β-tubulin to prevent polymerization.
Vincristine	:	Binds free tubulin to prevent polymerization

MYOSIN

Myosin is a motor protein. The form of myosin found in muscle and first identified was myosin-II. Each myosin-II molecule is an oligomer composed of one pair of identical heavy chains and two pairs of non-identical light chains. Each of the heavy chains has a globular head domain at its N-terminus that contains an actin-binding site and a catalytic site that hydrolyzes ATP. Globular head domain is followed by a very long amino acid sequence that forms an extended coiled-coil that mediates heavy chain dimerization. The two light chains bind close to the N-terminal head domain. Each myosin head binds and hydrolyses ATP, using the energy of ATP hydrolysis to walk toward the *plus* end of an actin filament.



The long coiled-coil tail bundles itself with the tails of other myosin molecules. These tail-tail interactions result in the formation of large bipolar *thick filaments* that have several hundred myosin heads, oriented in opposite directions at the two ends of the thick filament.

When myosin is treated with the proteases trypsin, it is cleaved into two fragments called *light meromyosin* and *heavy meromyosin*. Light meromyosin consists of most of the alpha-helical rod of the myosin molecule. Heavy meromyosin consists of the globular heads with the light chains and the remaining rod section. Subsequent treatment of heavy meromyosin with papain releases two globular protein heads called S_1 subfragments and a rod-like section called an S_2 subfragment.

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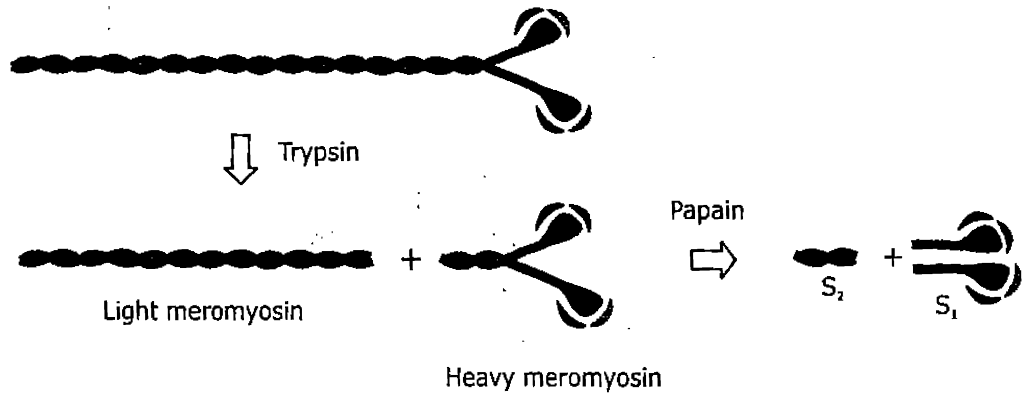


Figure 9.39 : Cleavage of myosin by the proteases trypsin and papain

In addition to myosin-II (conventional two-headed myosin), several other types of myosin (I, III, IV..... XIV) are found in nonmuscle cells. In contrast to myosin-II, these unconventional myosins do not form filaments and therefore are not involved in contraction. They may, however, be involved in a variety of other kinds of cell movements, such as the transport of membrane vesicles and organelles along actin filaments, phagocytosis, and extension of pseudopods in amoebae.

MUSCLE CONTRACTION

Molecular structure of skeletal muscle and sarcomere

Skeletal muscles are usually attached to bone by tendons composed of connective tissue. This connective tissue also ensheaths the entire muscle and is called *epimysium*. Skeletal muscles consist of numerous bundles called *fascicles*. Each fasciculus is surrounded by a layer of connective tissue called the *perimysium*. Each fasciculus is composed of numerous **muscle fibers** (or muscle cells) and each individual muscle cell is surrounded by connective tissue called the *endomysium*. Muscle fibers are long, cylindrical, striated fibers with many peripherally located nuclei. The cell membrane of a muscle fiber is called the *sarcolemma*. The sarcoplasm is the specialized cytoplasm of a muscle fiber that contains a modified ER known as the sarcoplasmic reticulum. Transverse (T)-tubules invaginate the sarcolemma, allowing impulses to penetrate the cell and activate the sarcoplasmic reticulum. Sarcoplasm contains fine thread like contractile structure called *myofibrils*.

In each myofibril, actin thin filaments and myosin thick filaments are organized into a linear chain of highly ordered structures, called **sarcomeres**, the basic functional units of a myofibril. One end of the thin filaments is attached at their plus ends to the Z disc, the demarcation between adjacent sarcomeres. Alpha-actinin anchors actin to Z disc and stabilizes actin filaments. The capped minus ends of the actin filaments extend towards the middle of the sarcomere, where they overlap with thick filaments. Thin filaments contain the proteins *actin*, *nebulin*, *tropomodulin*, *tropomyosin* and *troponin*. Tropomyosin is a fibrous molecule that consists of two chains, alpha and beta, that attach to F-actin in the groove between its filaments. **Troponin**, a complex of three polypeptides, troponins T, I, and C (named for their tropomyosin-binding, inhibitory, and Ca^{2+}-binding activities, respectively). *Troponins T* binds to tropomyosin as well as to the other two troponin components. *Troponin I* binds to actin as well as to troponin T and inhibits the F-actin-myosin interaction. *Troponin C* is a calcium-binding polypeptide that is structurally and functionally analogous to **calmodulin**. Four molecules of calcium ion bind per molecule of troponin C. **Nebulin**, a repeating 35-amino-acid actin-binding motif stretches from the Z disc to the minus end of each thin filament and acts as a *molecular ruler* to dictate the length of the filament. The minus ends of the thin filaments are capped and stabilized by **tropomodulin**. Thus, the actin filaments in sarcomeres are remarkably stable.

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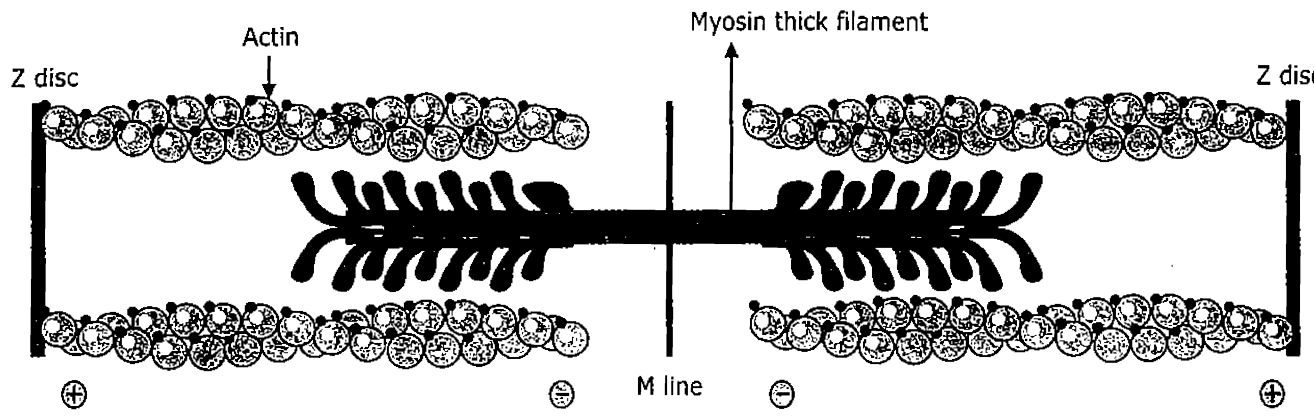


Figure 9.40 : The arrangement of thick myosin and thin actin filaments in the relaxed state. The plus ends of actin filaments are attached to the Z discs.

Thick filaments are composed of several hundred myosin molecules. Myosin molecules associate by ordered tail-to-tail interactions to form thick filament up to 1.5 μm long. The thick filaments are positioned midway between the Z discs by opposing pairs of an even longer template protein, called **titin**, acts as a molecular spring. Titin, the largest protein in body, may have role in relaxation of muscle.

Table 9.13 : Major proteins associated with sarcomere

Protein	*Function*
Actin	Component of thin filaments
Myosin	Component of thick filaments
Tropomyosin	Binds along the length of thin filaments
Troponin	Binds along thin filaments
Titin	Acts as molecular spring
Nebulin	Acts as molecular ruler
Myomesin	Myosin-binding protein present at the M line
-actinin	Bundles actin filaments and attaches them to Z line
CapZ	Attaches actin filaments to Z line
Tropomodulin	Maintains thin filament length and stability

Bipolar thick filaments walk toward the plus ends of two sets of thin filaments of opposite orientations, driven by dozens of independent myosin heads that are positioned to interact with each thin filament. Sarcomere shortening is caused by the myosin filaments sliding past the actin thin filaments, with no change in the length of either type of filament. The thick and thin filaments of sarcomeres form a pattern of alternating light and dark bands. The light bands, called **I bands** (*isotropic*) contain only thin filaments. The dark bands of sarcomeres are called **A-bands** (*anisotropic*). A-band contains both thin and thick filaments. A narrow **H-zone** in the center of each A-band contains thick but not thin filaments. Supporting proteins that hold the thick filaments togather at the center of the H-zone form the **M-line**, so named because it is at the middle of the sarcomere.

When muscle contracts, the I-band shorten. The length of each sarcomere of a myofibril decreases when muscle contracts. However thick and thin filaments donot shorten during muscle contraction. On the basis of this observation, two different groups, Andrew Huxley and Ralph Niedergerke and Hugh Huxley and Jean Hanson, concluded that thick filaments slide along the thin filaments during muscle contraction, which is described as *sliding filament model*.

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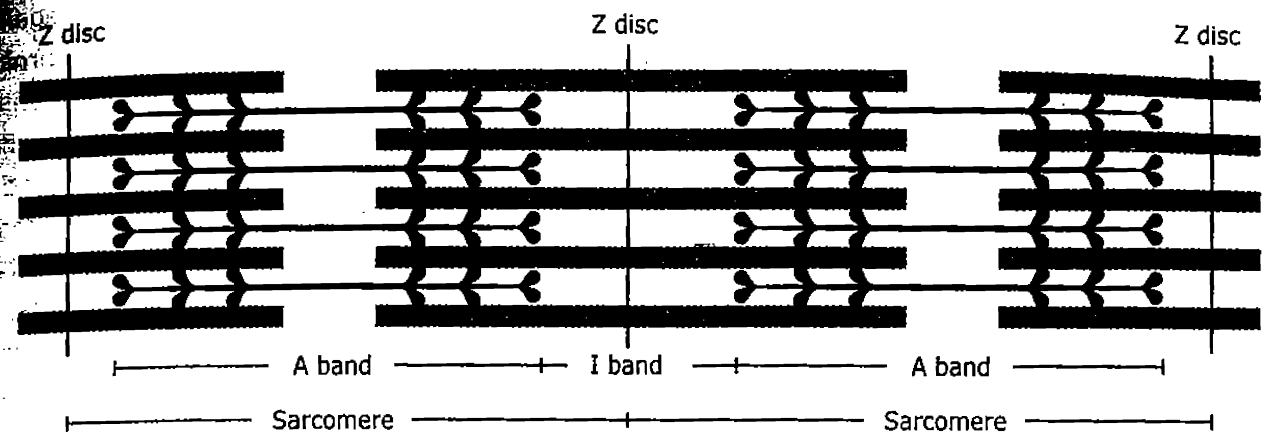


Figure 9.41 : Diagram of a sarcomere. The (+) ends of actin filaments are attached to the Z discs.

Biochemical events occurring during one cycle of muscle contraction

The signal from the nerve triggers an action potential in the sarcolemma. The action potential propagates through the *T-tubules* (transverse tubules). T-tubules extend inward from the sarcolemma around each myofibril. T-tubule membrane contains voltage sensitive proteins, which are activated by the incoming action potential. Activated voltage sensitive proteins trigger the opening of Ca^{2+} release channels in the sarcoplasmic reticulum. Increase cytosolic Ca^{2+} initiates the contraction of each myofibril. Because the signal from the sarcolemma is passed within millisecond to every sarcomere in the cell, all of the myofibrils in the cell contract at the same time. The increase in Ca^{2+} concentration is transient because the Ca^{2+} is rapidly pumped back into the sarcoplasmic reticulum by an abundant, Ca^{2+} ATPase, in its membrane. Typically, the cytoplasmic Ca^{2+} concentration is restored to resting levels within 30 m sec, allowing the myofibrils to relax.

The Ca^{2+} dependence of skeletal muscle contraction is due to presence of tropomyosin and troponin. Each tropomyosin presents along the groove of thin filament. In resting muscle, troponin I and troponin T interact with tropomyosin to keep tropomyosin away from the groove of thin filament. This prevents the binding of myosin heads with thin actin filaments, thereby preventing any force generating interaction. When the level of Ca^{2+} is raised, troponin C which binds up to four molecules of Ca^{2+} causes troponin I to release its hold on actin. This allows the tropomyosin molecules to slip back into their normal position so that the myosin heads can walk along the actin filaments.

Steps in contraction

1. Release of neurotransmitter at motor end-plate
2. Binding of neurotransmitter to receptors
3. Generation of end-plate potential
4. Generation of action potential in muscle fibers
5. Inward propagation of depolarization along T tubules
6. Release of Ca^{2+} from sarcoplasmic reticulum
7. Binding of Ca^{2+} to troponin C, uncovering myosin binding sites on actin
8. Formation of linkages between actin and myosin and sliding of thin on thick filaments

Coupling of actin-myosin and ATP hydrolysis

Myosin head without ATP locks tightly on to an actin filament. Binding of ATP to myosin head causes a slight change in the conformation of the actin binding site. This reduces the affinity of the head for actin and allows it to move along the filament. Hydrolysis of ATP causes the head to be displaced along the filament by a distance of about 5 nm. However, product of ATP hydrolysis, ADP and P_i remain tightly bound to the myosin head. Release of P_i causes binding of the myosin head to a new site on the actin filament. This

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release triggers the *power stroke* (the force generating change in shape during which the head regains its original conformation). In the course of the power stroke, the head loses its bound ADP and returning to the start of a new cycle.

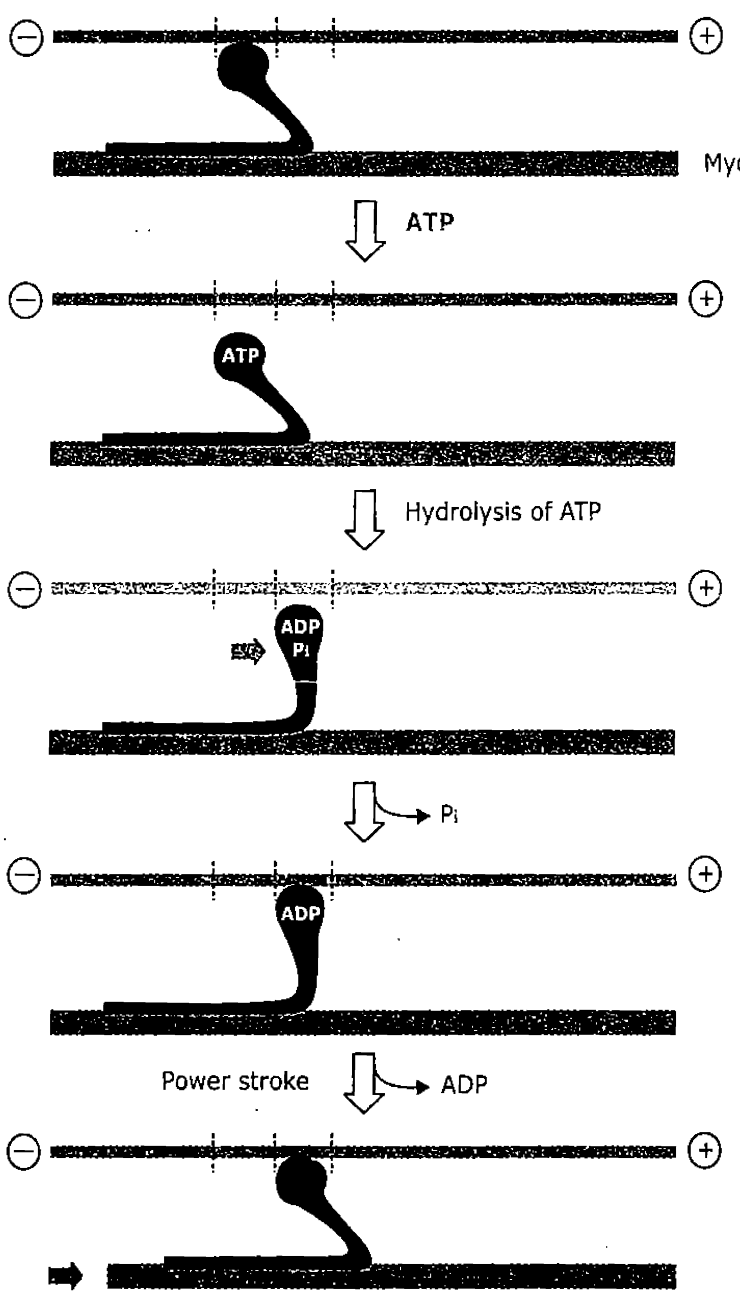


Figure : 9.42
Coupling between ATP hydrolysis and conformational changes for myosin. ATP binding with myosin releases the head from the filament. ATP hydrolysis occurs while the myosin head is detached from the filament, causing the head to assume different conformation. When the head rebinds to filament, the release of phosphate, followed by the release of ADP, trigger the power stroke that moves the filament relative to the motor protein

INTERMEDIATE FILAMENTS

Intermediate filaments are alpha-helical rods that assemble into ropelike filaments of about 10-nm diameter. Intermediate filaments are found in nearly all animals but not in plants and fungi. Unlike the actin and tubulin proteins, the intermediate filament proteins are chemically heterogenous and show species-dependent variations in molecular weight. Principal function of intermediate filaments are structural to reinforce cells and to organize cells into tissues. Unlike microfilaments and microtubules, intermediate filaments do not participate in cell motility. Intermediate filament proteins are classified into four major types based on their sequences and tissue distribution : *lamins, keratins, type III intermediate filaments* and *neurofilaments.*

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Lamins are the most ubiquitous group of intermediate filaments and found exclusively in the nucleus. **Keratin** (acidic and basic) express in epithelial cells. The keratins are the most diverse classes of intermediate filament proteins. Four proteins **vimentin**, **desmin**, **glial fibrillary acidic protein** (GFAP), and **peripherin** are classified as type III intermediate filament proteins. Unlike the lamins and keratins, the type III proteins can form both homo- and heteropolymeric intermediate filaments. The most widely distributed of all intermediate filament proteins is vimentin, which is typically expressed in leukocytes, blood vessel endothelial cells, some epithelial cells, and mesenchymal cells such as fibroblasts. The other type III intermediate filament proteins have a much more limited distribution. Desmin filaments in muscle cells are responsible for stabilizing sarcomeres in contracting muscle. Glial fibrillary acidic protein forms filaments in the glial cells that surround neurons and in astrocytes. Peripherin is found in neurons of the peripheral nervous system, but little is known about it. Neurofilaments form primary cytoskeletal component in mature nerve cells. In mammals, three different neurofilament proteins have been recognized: NF-L, NF-M, and NF-H, for low, middle, and high molecular weight, respectively, all three are usually found in each neurofilament.

9.14 CELL JUNCTIONS

Many cells in tissues are linked to one another and to the extracellular matrix at specialised contact sites called cell junctions. Cell junctions can be classified into three functional groups.

1. OCCLUDING JUNCTIONS

Seal cells together in an epithelium in a way that prevent even small molecules from leaking from one side of the sheet to the other.

Tight junctions or **zonula occludens**

Tight Junctions are cell-cell occluding junctions mediated by two major transmembrane proteins *claudins and occludin.* Tight junctions are the closest known contact between adjacent cells. Tight junctions form seals that prevent the free passage of molecules (including ions) across an epithelial sheet in the spaces between cells. They also maintain the polarity of epithelial cells by preventing the diffusion of molecules between the apical and the basolateral regions of the plasma membrane.

2. ANCHORING JUNCTIONS

Anchoring junctions mechanically attach cells (and their cytoskeletons) to their neighbors or to the extracellular matrix and perform the key task of holding cells together into tissues. It includes two main types of junctions - *adherens junction* and *desmosome.*

Adherens junctions

Adherens junctions connect bundles of actin filaments from cell to cell or from cell to extracellular matrix.

Adhesion belt (or zonula adherens) : It is a cell to cell junction. It is mediated by actin filaments and proteins belong to the *cadherin family*. Adhesion belts are usually located near the apical surface, just below the tight junctions.

Focal contact (or adhesion plaque) : It is a cell-matrix junction which is mediated by transmembrane adhesion proteins of the *integrin family and* by actin filament.

Desmosomes

Desmosomes are buttonlike points of intercellular contact which bond neighboring cells together. It has a dense cytoplasmic plaque (composed of a mixture of intracellular attachment proteins, including *plakoglobin* and *desmoplakins*) responsible for connecting the cytoskeleton to the transmembrane linker

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proteins of the cadherin family of cell-cell adhesion molecules, bind to the plaques and interact through their extracellular domains to hold the adjacent membranes together by a Ca^{2+}-dependent mechanism. Desmosomes contain two specialized cadherin proteins, desmoglein and desmocollin. Each plaque is associated with a thick network of keratin filaments (in most epithelial cells) and *desmin filaments* (in heart muscle cells), which are attached to the surface of the plaque.

Hemidesmosomes, or half-desmosomes, resemble desmosomes but instead of joining adjacent epithelial cell membranes, they connect the basal surface of epithelial cells to the underlying *basal lamina*- a specialized mat of extracellular matrix at the interface between the epithelium and connective tissue. The transmembrane linker proteins in hemidesmosomes belong to the integrin family of extracellular matrix receptors, rather than to the cadherin family of cell-cell adhesion proteins used in desmosomes. The intracellular attachment proteins in hemidesmosomes are also different from those in desmosomes.

Table 9.14 : Characteristics of anchoring junctions

Junction type	*Transmembrane protein*	*Intracellular linkage*
Zonula adherens	Cadherin	Actin filaments
Focal contact	Integrin	Actin filaments
Desmosome	Cadherin	Intermediate filaments
Hemi-desmosome	Integrin	Intermediate filaments

3. COMMUNICATING JUNCTIONS

Communicating junctions mediate the passage of chemical or electrical signals from one interacting cell to its partner.

Gap junction

The channels allow inorganic ions and other small water-soluble molecules to pass directly from the cytoplasm of one cell to the cytoplasm of the other, thereby coupling the cells both electrically and metabolically. Gap junctions serve as direct connections between the cytoplasms of adjacent cells. They provide open channels through the plasma membrane, allowing ions and small molecules to diffuse freely between neighboring cells, but preventing the passage of proteins and nucleic acids. In electrically excitable cells, such as heart muscle cells, the direct passage of ions through gap junctions couples and synchronizes the contractions of neighboring cells. Gap junctions also allow the passage of some intracellular signaling molecules, such as cAMP and Ca^{2+}, between adjacent cells, potentially coordinating the responses of cells in tissues.

Gap junctions are constructed of transmembrane proteins called **connexins**. Six connexins assemble to form a cylinder with an open aqueous pore in its center called **connexon**. When the connexons in the plasma membranes of two cells in contact are aligned, they form a continuous aqueous channel called **gap junction**, which connects the two cell interiors.

Plasmodesmata

Adjacent plant cells communicate with each other through cytoplasmic connections called *plasmodesmata* (singular, plasmodesma), which function analogously to animal cell gap junctions.

Despite their similarities in function, plasmodesmata are structurally unrelated to gap junctions. At each plasmodesma the plasma membrane of one cell is continuous with that of its neighbor, creating an open channel between the two cytosols. An extension of the smooth endoplasmic reticulum called **desmotubule** passes through the pore, leaving a ring of surrounding cytoplasm through which ions and small molecules are able to pass freely between the cells.

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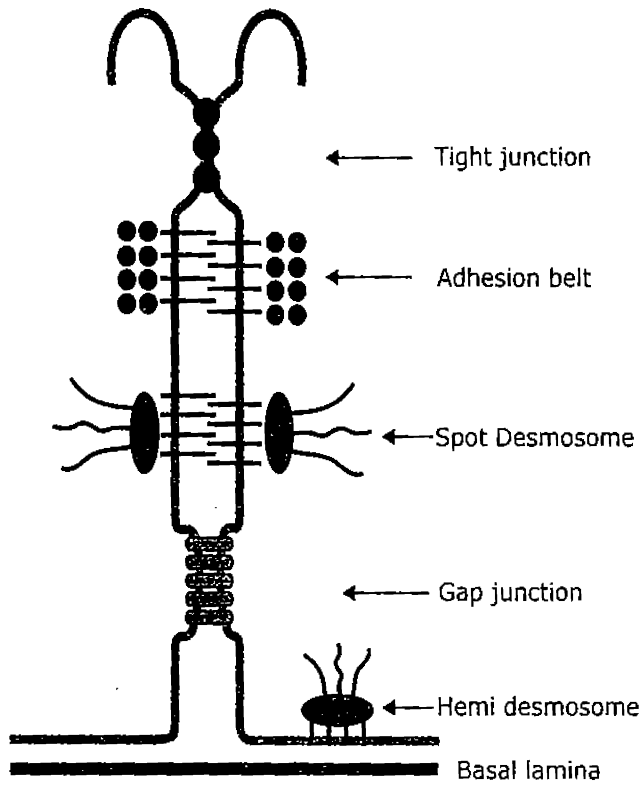


Figure 9.43 : Summary of cell junctions.

CELL ADHESION MOLECULES (CAMS)

Cells adhere to each other and to the extracellular matrix through cell surface proteins called cell adhesion molecules. CAMs include transmembrane proteins that fall into two categories cell-cell adhesion molecules or cell-matrix adhesion molecules.

Cadherins are major cell-cell adhesion molecules. The cadherins are calcium dependent proteins which hold cells together by homophilic interactions (molecules on one cell bind to other molecules of the same kind on adjacent cell). Most cadherins are single pass transmembrane glycoprotein. Two other families of transmembrane cell-cell adhesion molecules are **selectins** and **immunoglobulin superfamily**. Selectin also depends on extracellular calcium to function in cell adhesion whereas immunoglobulin superfamily mediates calcium independent cell-cell adhesion. Selectins mediate cell-cell adhesion by heterophilic binding (the molecule on one cell binds to the molecule of a different kind on adjacent cells) whereas immunoglobulin family members undergo both homophilic and heterophilic adhesion. **Integrins** are the principal transmembrane cell matrix adhesion molecules. They are also calcium dependent adhesion proteins and undergo heterophilic interaction.

9.15 EXTRACELLULAR MATRIX

PLANT CELL WALLS

Many cells are surrounded by an insoluble array of secreted macromolecules. Cells of bacteria, fungi, many protists and plants are surrounded by rigid cell walls, which are an integral part of the cell. In contrast to bacteria, the cell walls of eukaryotes (including fungi, plants) are composed principally of polysaccharides. The basic structural polysaccharide of fungal cell walls is *chitin* (a polymer of *N*-acetylglucosamine residues). The cell walls of plant cells are composed principally of cellulose, which is the single most abundant polymer on Earth. Cellulose makes up 15–30% of the dry mass of primary and up to 40% of secondary cell walls. Cellulose is a linear polymer of glucose residues. The glucose residues are joined by $\beta(1 \rightarrow 4)$ linkages.

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Several chains then associate in parallel with one another to form 30 nm diameter *microfibrils*. The microfibrils are lined up parallel to each other and consist of crystalline regions, within which cellulose molecules are tightly packaged. Cellulose is synthesized by a plasma membrane enzyme complex - *cellulose synthase*. In expanding cells, the newly synthesized cellulose microfibrils are deposited parallel to cortical microtubules underlying the plasma membrane.

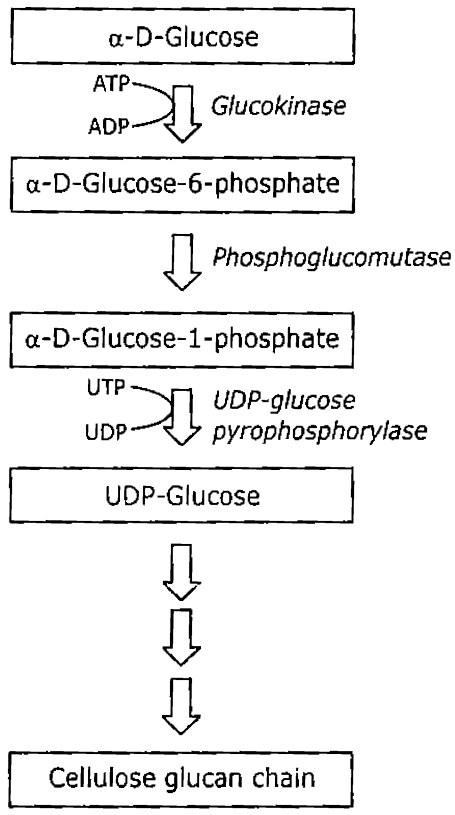


Figure : 9.44
Cellulose biosynthesis. First, *glucokinase* utilizes water soluble α-D-glucose and one phosphate molecule of an ATP to produce α-D-glucose-6-phosphate, which is converted by *phosphoglucomutase* to α-D-glucose-1-phosphate. Following this step, *UDP-glucose pyrophosphorylase* removes one organic phosphate from α-D-glucose-1-phosphate to produce UDP-glucose, which is soluble in the cytoplasm and is the precursor for the generation of microcrystalline cellulose. There are three more steps associated with polymerization of UDP glucose and formation of glucan chains: chain initiation, chain elongation and chain termination.
In chain initiation, *UDP-glucosyltransferase* transfer a glucose residue from UDP-glucose into a sitosterol molecule on the cytoplasmic face of the plasma membrane, forming sitosterol β-glucoside and releasing an UDP. Sitosterol β-glucoside functions as a primer for cellulose biosynthesis in plants.

Within the primary cell wall, cellulose microfibrils are embedded in a matrix consisting of proteins and two other types of polysaccharides: *hemicelluloses* and *pectins*. Hemicelluloses are highly branched polysaccharides that are hydrogen-bonded to the surface of cellulose microfibrils. Pectins are branched polysaccharides containing a large number of negatively charged galacturonic acid residues. In the cell wall, the pectins form a gel-like network that is interlocked with the crosslinked cellulose microfibrils. Pectin has roles in forming connections between plant cells, adjusting pH and ion balance, recognizing foreign molecules to alert the cell to the presence of microbes and establishing cell wall porosity. Matrix components, including hemicelluloses and pectins, are synthesized in the Golgi apparatus and secreted.

Lignin is a major constituent of secondary cell walls, and accounts for about 10–25% of total plant dry matter. Lignin is composed of a complex of phenylpropanoids (aromatic compounds). The lignin biosynthetic pathway is not well understood. Lignin has an important role in protecting the plants against invasion by pathogens and insects, and lignin deposition is thought to be increased it response to attack by these invaders.

EXTRACELLULAR MATRIX OF ANIMALS

The cells of multicellular organisms are embedded in an extracellular matrix consisting of secreted proteins and polysaccharides. The extracellular matrix fills the spaces between cells and binds cells and tissues together. One type of extracellular matrix is sheet like *basal laminae,* or basement membranes, upon which

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layers of epithelial cells rest. Extracellular matrix is most abundant in connective tissues. Extracellular matrices are composed of tough fibrous proteins embedded in a gel-like polysaccharide ground substance.

The components of the extracellular matrix can be divided into several groups :

- Structural proteins such as collagens and elastins.

  The major structural protein of the extracellular matrix is **collagen**, which is the single most abundant protein in animal tissues. Connective tissues also contain *elastic fibers*, composed principally of a protein called **elastin** (*collagens and elastins are discussed in chapter amino acids and proteins*).

- Polysaccharide **glycosaminoglycans** which are usually found covalently linked to protein in the form of proteoglycans. Proteoglycans differ from glycoproteins in that sugar residues in the former represent up to 95% of the total weight of the molecule. The fibrous structural proteins of the extracellular matrix are embedded in gels formed from polysaccharides called glycosaminoglycans, or GAGs, which consist of repeating units of disaccharides. One sugar of the disaccharide is either *N*-acetylglucosamine or *N*-acetylgalactosamine, which in most cases is sulfated and the second is usually acidic (either glucuronic acid or iduronic acid) sugar. GAGs are highly negatively charged. There are four main groups of GAGs : *hyaluronan, chondroitin sulfate* and *dermatan sulfate, heparan sulfate* and *heparin,* and *keratan sulfate.* Hyaluronan is the simplest of the GAGs. Except hyaluronan, all of the other GAGs are linked to proteins in the form of proteoglycans.

- **Adhesion proteins**, such as laminins and fibronectins.

  Adhesion proteins, the third class of extracellular matrix constituents, are responsible for linking the components of the matrix both to one another and to the surfaces of cells.

  Laminins are extracellular heterotrimeric matrix glycoproteins which consist of alpha, beta and gamma chains. Laminins play the central role in organizing and establishing the basement membrane. Eight genetically distinct laminin chains are known so far. Their primary role is in cell-matrix attachment, but many additional biological activities, including promoting cell growth and migration, tumour growth and metastases, nerve regeneration, and wound repair have been demonstrated.

  Fibronectin is an extracellular matrix glycoprotein that binds to receptor proteins called integrins. In addition to integrins, fibronectin also binds extracellular matrix components such as collagen, fibrin and heparan sulfate proteoglycans. Fibronectin exists as a dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds.Fibronectin plays a major role in cell adhesion, growth, migration and differentiation, and it is important for processes such as wound healing and embryonic development.

Table 9.15 : Comparision of extracellular matrix of animals and plants

	Animals	*Plants*
Chemical nature	Protein rich	Carbohydrate rich
Structural fiber	Collagens and elastins	Cellulose
Components of hydrated matrix	Proteoglycans	Hemicelluloses
Adhesive molecules	Fibronectins and laminins	Pectins

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9.16 NUCLEUS

The nucleus is the center for genetic inheritance and gene expression within the cells of eukaryotes. Most eukaryotic cells contain one nucleus, but there are many examples of multinucleated cells. Conversely, mature mammalian red blood cells lack nuclei. Nuclei differ in size depending on the cell type. Most nuclei are spherical, but multilobed nuclei are also common, such as those found in polymorphonuclear leukocytes or mammalian epididymal cells. A nucleus has four components: *Nuclear envelope, nucleolus, nucleoplasm* and *chromatin*.

Nuclear envelope

The nucleus is bordered by a double-membrane nuclear envelope that contains **nuclear pores** for macromolecular import and export from and to the cytoplasm. The outer membrane is continuous with that of the endoplasmic reticulum. The space between the two nuclear membranes, the **perinuclear space**, is continuous with the lumen of the endoplasmic reticulum. Network of intermediate filaments present on the nuclear side of the inner membrane is known as **nuclear lamina**. Nuclear lamina is made up of intermediate filament protein called *lamins*. The lamina lends support and shape to the nucleus. Each nuclear pore is formed from an elaborate structure termed the **nuclear pore complex**. The inner and outer membranes fuse at the nuclear pore complexes. Nuclear pore complexes is made up of multiple copies of some 50 to 100 different proteins called **nucleoporins**.

Transport through nuclear pores

Bidirectional transport of molecules occurs through nuclear pore between the cytosol and the nucleus. RNA molecules and ribosomal subunits are exported to the cytosol, while all of the proteins that function in the nucleus are synthesized in the cytosol and are then imported. Molecules upto 50 kD can enter the nucleus by passive diffusion (but they may also be actively transported). Proteins >50 kD in size do not enter the nucleus by passive diffusion. These molecules are actively transported into and out of the nucleus.

Proteins imported to or exported from the nucleus through nuclear pores contain a specific amino acid sequence that functions as a *nuclear-localization signal* or a *nuclear-export signal*.

Nuclear localization signals direct nuclear proteins to the nucleus. It acts as a binding site for an *importin*. Nuclear localization signals were first identified in the viral protein called *T-antigen*, which is encoded by the SV40 virus and is needed for viral DNA replication in the host cell nucleus. A nuclear protein containing nuclear localization signal is transported through the nuclear pores post-translationally and in a fully folded conformation. Nuclear localization signals are not cleavable. *Nuclear export signal* is a short sequence motif on a protein that acts as a binding site for an *exportin* and thereby targets the protein to the cytomplasm.

Several different types of nuclear localization and export signal have been identified. Each type of nuclear-transport signal interact with a specific receptor protein described as *exportin* or *importin*. Importins bind the molecules in the cytoplasm and release it in the nucleus. Exportins bind the molecules in the nucleus and release it in the cytoplasm. The two types of receptor proteins are highly homologous in sequence and belongs to the *beta importin family* or **karyopherins**.

Another important protein called **Ran**, small monomeric nuclear GTPases, plays a critical regulatory role in the interaction of the complex with the nuclear pore complexes and in its translocation through the nuclear pore complexes. Ran-GTP occurs in the nucleus and Ran-GDP in the cytosol. The reason for this difference is an asymmetric distribution of two proteins–Guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP). The nucleus contains Ran-GEF, which stimulates replacement of GDP by GTP, thus converting Ran-GDP to Ran-GTP. The cytoplasm contains Ran-GAP, which causes the GTP to be hydrolyzed to GDP. The Ran-GAP is localized on the surface of the cytoplasmic side of the nuclear pore complex.

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Export complexes are stable in the presence of Ran-GTP, whereas import complexes are stable in the presence of Ran-GDP. So export complexes are driven to form in the nucleus and dissociate in the cytosol whereas the reverse is true of import complexes. Thus the state of the guanine nucleotide bound to Ran controls directionality of nuclear import and export.

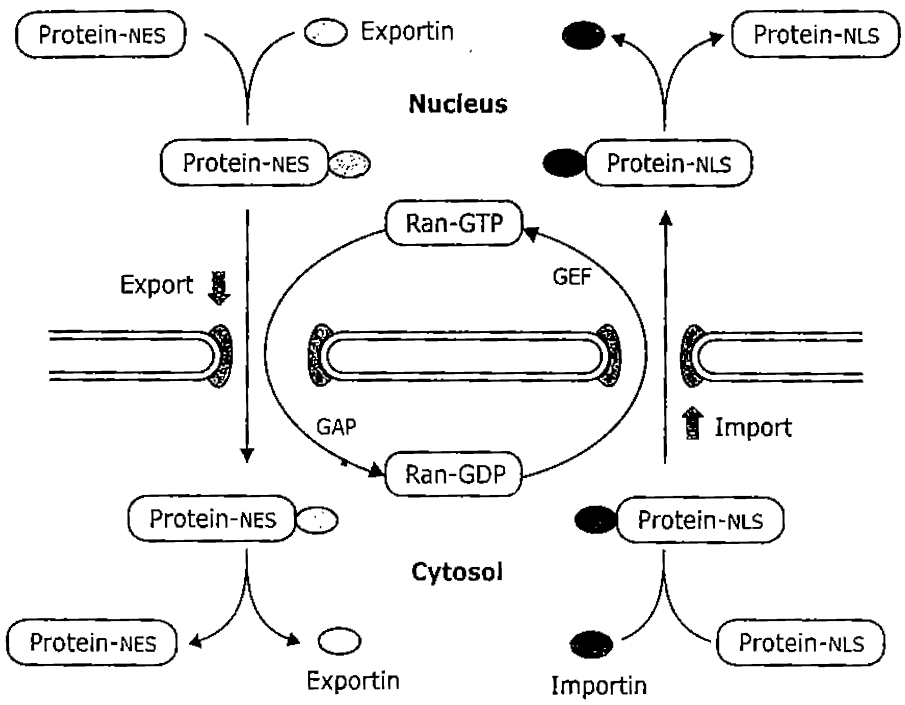


Figure 9.45 : Transport through the nuclear pore complex. Ran shuttles across the nuclear envelope through nuclear pores, but is concentrated in the nucleus because of nuclear transport factor-2 mediated active import. In the nucleus, a high concentration of Ran-GTP is generated by nucleotide exchange. This is catalyzed by chromatin-bound GEF. Ran-GTP causes the dissociation of imported complexes, which contain proteins that carry a nuclear localization signal (NLS), by binding to importin and ejecting the cargo. Ran-GTP also promotes the assembly to export complexes containing proteins with a nuclear export signal (NES). In the cytoplasm, Ran-GTP meets GAP, which stimulates GTP hydrolysis and the export complexes dissociate. The importins and exportins are recycled by transport back across the pore (not shown).

NUCLEOLUS

Nucleolus (discovered by Fontana) is a non membrane bound *dynamic body* which disappears in the late prophase and reappears at nucleolar organizing region of chromosome in the telophase stage of cell division. Each nucleolus is produced by a **Nucleolar-Organizing Region** (NOR) presents on a nucleolar-organizing chromosome. All eukaryotic cells contain at least one such chromosome. Its number may be one or more (several hundred per nucleus), but 1 to 4 being the most common. The number of nucleoli per nucleus also differ. The yeast cell contains one relatively large nucleolus with respect to its nuclear volume. At the other extreme, *Xenopus oocytes* contain over 1000 nucleoli per nucleus.

It is a site of transcription of ribosomal RNA and assembly of ribosome. So, nucleolus has a high concentration of rRNA and proteins. Once the ribosomal subunits mature they come out from nucleus and released into the cytoplasm through nuclear pore.

Nucleolus structure : It consists of three major regions.

Fibrillar centers : Containing rRNA genes in the form of partly condensed chromatin.

Fibrillar component : Surrounds the fibrillar centers, which contains RNA molecules in the process of transcription.

Granular regions : Outermost regions having mature ribosomal precursor particles.

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Ribosomal subunit production:

The nucleolus is the most conspicuous structure within the interphase nucleus. It is responsible for synthesizing a large nascent precursor ribosomal RNA (pre-rRNA) that is 45S in mammals and the processing (cleavage and base modification) of this RNA to yield mature ribosomal RNA of 18S, 5.8S and 28S. The concomitant assembly of these RNAs with incoming ribosomal proteins generates small and large ribosomal subunits that then pass into the cytoplasm.

Nucleolar organizer:

Nucleolar organizers are secondary constrictions within the mitotic chromosomes of eukaryotes. The primary constriction occurs at the centromere. Historically, Heitz concluded that the number of secondary constrictions within certain chromosomes of *Drosophila funebris* is proportional to the number of nucleoli. He called these regions *sine acid thymonucleinico*. McClintock actually coined the term *nucleolar organizer* to describe the satellite segment at the tip of maize chromosome 6 that organizes the assembly of the nucleolus beginning in the telophase. As the name implies, the nucleolar organizer gives rise to the nucleolus in the telophase of mitosis. During the late telophase, RNA polymerase I begins to transcribe rRNA genes once again, and the newly synthesized rRNA likely nucleates the assembly of the nucleolus by recruiting prenucleolar bodies.

CHROMOSOME

A *chromosome* is an organized structure of DNA and protein that is found in the nucleus of eukaryotic cells. It is a single piece of coiled DNA containing coding and noncoding sequences. The word *chromosome* comes from the Greek word *chroma*, color and *soma*, body due to their property of being very strongly stained by particular dyes. A chromosomal DNA molecule contains three specific nucleotide sequences:

Centromere

The constricted region of linear chromosomes is known as the centromere. Although this constriction is called the centromere, it usually is not located exactly in the center of the chromosome and, in some cases, is located almost at the chromosome's end. The regions on either side of the centromere are referred to as the chromosome's arms. Centromeres help to keep chromosomes properly aligned during the complex process of cell division. The centromeres serve both as the sites of association of sister chromatids and as the attachment sites for microtubules of the mitotic spindle.

Telomere

Telomeres are repetitive stretches of DNA located at the ends of linear chromosomes. They protect the ends of chromosomes in a manner similar to the way the tips of shoelaces keep them from unraveling.

Origin of replication

The origin of replication (also called the *replication origin*) is a particular sequence in a chromosome at which replication is initiated.

Chromosome number

All eukaryotic cells have multiple linear chromosomes. Every cell maintains a characteristic number of chromosomes. Depending on the eukaryotic organism, the number of chromosomes typically varies from 2 to less than 50, but in rare instances are reach thousands. The majority of eukaryotic cells are diploid; that is, they contain two copies of each chromosome.

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Table 9.16 : Chromosome number in different eukaryotic organisms.

Species	Haploid number of chromosome
Saccharomyces cerevisiae (budding yeast)	16
Schizosaccharomyces pombe (fission yeast)	03
Caenorhabditis elegans	06
Arabidopsis thaliana	05
Drosophila melanogaster	04
Tetrahymena thermophilus	Micronucleus 5, Macronucleus 225
Mus musculus (mouse)	19 + X and Y
Homo sapiens	22 + X and Y

9.17 CELL SIGNALING

Signal transduction is a process of converting extracellular signal into cellular responses. All cells receive and respond to signals from 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 extracellular signal molecules.

Signal molecules

Signal molecules are chemically heterogenous compounds. These molecules are divided into two categories – membrane bound and secretory signal molecules. Some signal molecules remain bound to the surface of the cells and mediate contact dependent signaling. In most cases, signal molecules are secreted by signaling cells. Secreted signal molecules are divided into three general categories based on the distance over which signals are transmitted : *endocrine, paracrine* and *autocrine* signal molecules.

Endocrine signaling molecules (hormones) are secreted by specialized endocrine cells and carried through the circulation to act on target cells at distant body sites.

Paracrine signaling molecules released by one cell acts on neighbouring target cells. An example is provided by the action of neurotransmitters in carrying signals between nerve cells at a synapse.

Autocrine signaling molecules produce effect on same cell that produce it. One important example of such is the response of cells of the vertebrate immune system to foreign antigens. Certain types of T lymphocytes respond to antigenic stimulation by synthesizing a growth factor that drives their own proliferation, thereby increasing the number of responsive T lymphocytes and amplifying the immune response.

Signal molecules canbe further classified into *extracellular* and *intracellular* molecules. Extracellular signal molecules are synthesized and released by signaling cells and bind to either cell surface receptors or intracellular receptors. Extracellular signaling molecules are synthesized and released by signaling cells and produce a specific response only in target cells that have receptors for the signaling molecules. Most of the extracellular signaling molecules bind to receptors expressed on the target cell surface called *cell surface receptors.* Cell-surface receptors belong to three major classes :

1. G protein coupled receptors
2. Ion-channel receptors and
3. Enzyme linked receptors

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Binding of extracellular signaling molecules to cell surface receptor leads to increase (or decrease) of low molecular weight intracellular signaling molecules termed **secondary messengers**. These low-molecular-weight signaling molecules include cyclic AMP (cAMP), cyclic GMP (cGMP), diacylglycerol (DAG); inositol 1,4,5-trisphosphate (IP_3), phosphoinositides and calcium.

Some extracellular signaling molecules like steroids, retinoids spontaneously diffuse through the plasma membrane and bind to *intracellular receptors* present in cytoplasm or nucleus. By virtue of their hydrophobic nature, they traverse the plasma membrane and exert their effects within the cell, where they control the activities of specific genes. These regulatory effect occur at the level of transcription of responsive genes. Steroid and related hormones bind specific receptor proteins within the cell which interact with **hormone response element** (HRE). Binding of the complex to HRE affects transcription rates of nearby genes.

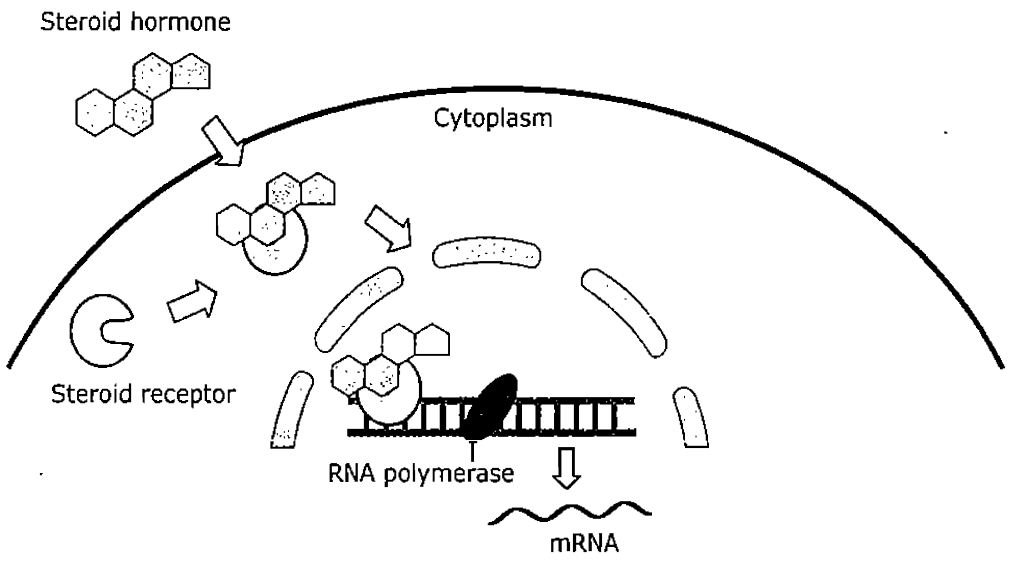


Figure 9.46 : Steps in the activation of a gene by a steroid hormone, such as the glucocorticoid.

Example of signal molecules

Signal molecules that interact with cell surface receptor

Epinephrine, Non-Epinephrine, Glucagons, Insulin, Gastrin, Secretin, Cholecystokinin, ACTH

Signal molecules (hormones) that interact with cytosolic or nuclear receptor

Steroid hormone (Progesterone, Estradiol, Testosterone, Cortisol, Corticosterone, Aldosterone), Steroid like hormone (α-ecdysone) and non-steroid hormone (Thyroxine, Retenoid, derived from Retinol)

Agonists and antagonists

Structural analogs of signaling molecules fall into two classes: *agonists* and *antagonists*. Structural analogs which mimic the function of a signaling molecule by binding to its receptor and causing the normal response are called *agonists*. Whereas *antagonists* are structural analogs which bind to the receptor but induces no response. Hence an antagonist acts as an inhibitor of the natural signaling molecule (or agonist) by competing for binding sites on the receptor, thereby blocking the physiological activity of the signaling molecule. For example alprenolol and isoproterenol act as antagonists and agonist to the epinephrine receptor, respectively.

G proteins coupled receptors (GPCR) and G-protein

GPCRs are the largest family of cell surface receptors that transmits signals to intracellular targets via the intermediary action of guanine nucleotide-binding proteins called **G-protein**. GPCRs are present in all eukaryotes. It includes the receptors for many neurotransmitters, neuropeptides, and peptide hormones.

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In addition, the G protein-coupled receptor family includes a large number of receptors that are responsible for smell, light, and taste. Despite the chemical and functional diversity of the signal molecules that bind to them, all GPCRs have a similar structure. GPCRs consists of single polypeptide of multipass nature. They crosses the lipid bilayer seven times and are therefore sometimes called *serpentine* receptors. An example of GPCR that responds to chemical signals is the *β-adrenergic receptor.* This protein receptor binds a hormone, epinephrine (also called adrenaline).

GPCRs are linked to trimeric G proteins (simply G-protein). G proteins are **GTPase switch proteins**. There are two classes of GTPase switch proteins: **trimeric G proteins** and **monomeric G protein**. These proteins are turned *on* when bound to GTP and turned *off* when bound to GDP. In the absence of a signal, G protein is bound to GDP, signals activate the release of GDP, and the subsequent binding to GTP over GDP is favoured by the higher concentrations of GTP in the cell. The intrinsic GTPase activity of these GTP-binding proteins hydrolyzes the bound GTP to GDP and p_i, thus converting the active form back to the inactive form.

G-proteins are attached to the cytoplasmic face of plasma membrane. Ligand binding to GPCR leads to activation of an associated signal-transducing G protein. All G proteins contain three subunits: α, β and γ. α and γ subunits of G-proteins are anchord to the membrane lipids covalently. The β subunit is always complexed with the γ subunit, forming the heterodimeric structure $G_{\beta\gamma}$. The α subunit is a GTPase switch protein that alternates between an active state with bound GTP and an inactive state with bound GDP. Binding of a trimeric G protein to an activated receptor leads to dissociation of GDP, binding of GTP to G_α, and dissociation of G_α–GTP from $G_{\beta\gamma}$. G_α–GTP and $G_{\beta\gamma}$ can specifically interact with effector proteins leading to changes in their activity. The intrinsic GTPase activity of G_α inactivates G_α–GTP by catalyzing GTP hydrolysis. G_α–GDP then dissociates from its effector and reassociates with $G_{\beta\gamma}$. The GTPase activity of the G_α determines the length of time that the signal remains on. In many cases, a protein termed *RGS* (*r*egulator of *G* protein *s*ignaling) accelerates GTP hydrolysis by the G_α subunit.

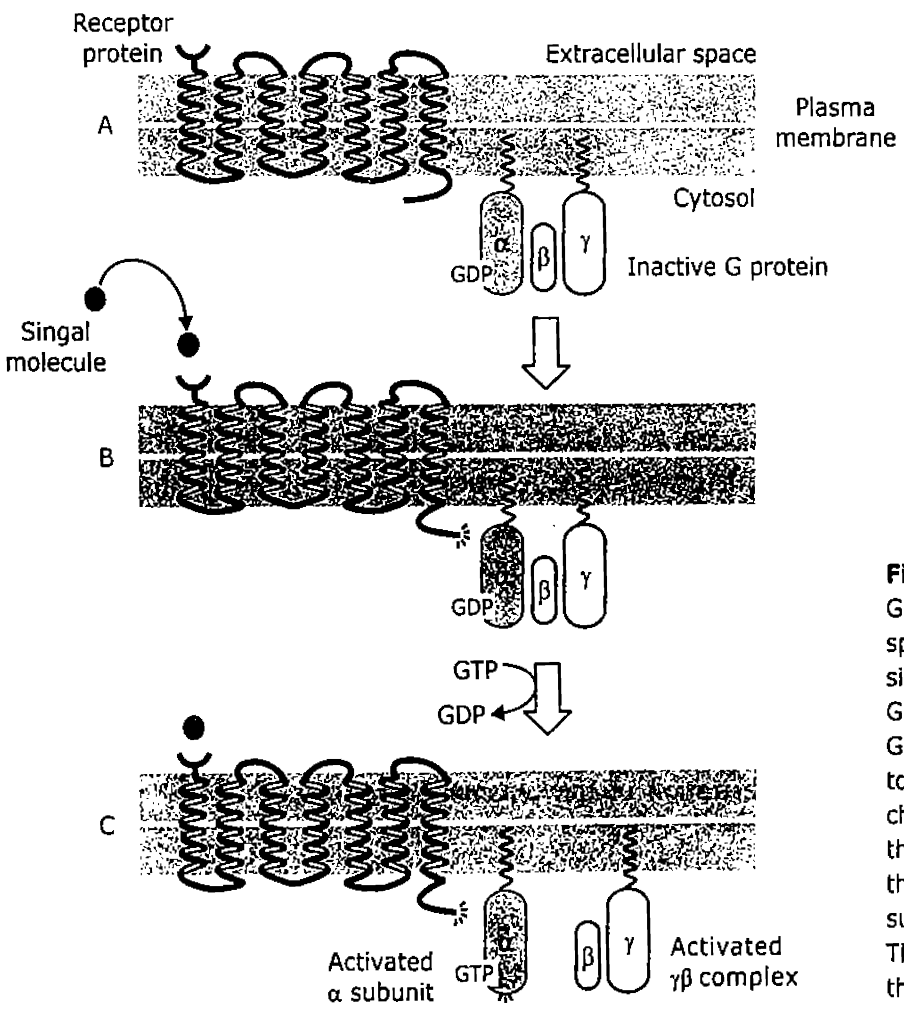


Figure : 9.47
GPCR typically have seven membrane-spanning domains. In the absence of signal molecule, the heterotrimeric G-protein complex is in an inactive GDP-bound form. On binding of hormone to the receptor, there is a conformational change of the receptor and activation of the G-protein complex. This results from the exchange of GDP with GTP on the α subunit, after which α and $\beta\gamma$ dissociate. The α or $\beta\gamma$ subunit binds to and activates the effector.

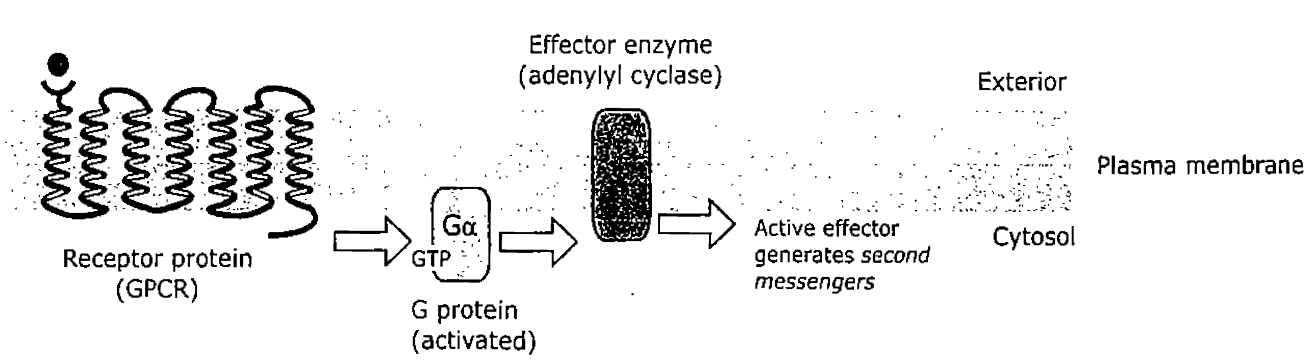
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There is a large variety of G proteins which are activated by a wide variety of receptors. The activation of an individual G protein may cause it to stimulate or to inhibit a particular effector. Two of the classic G proteins are Gs, which stimulates adenylate cyclase, and G_q, which stimulates phospholipase-C-β.

Table 9.17 : Classes of trimeric G proteins and their function

Family members	*Action mediated by*	*Function*
G_s	α	activates adenylyl cyclase; activates Ca^{2+} channels
G_{olf}	α	activates adenylyl cyclase in olfactory sensory neurons
G_t	α	inhibits adenylyl cyclase
	βγ	activates K^+ channels
G_0	βγ	activates K^+ channels; inactivates Ca^{2+} channels
	α and βγ	activates phospholipase C-β
G_t	α	activates cyclic GMP phosphodiesterase
G_q	α	activates phospholipase C-β

In the case of Gs, the Gsα subunit activates effector, adenylate cyclase. Because this G protein is involved in enzyme *activation,* it is called stimulatory G protein (Gs). The Gsα subunit binds and hydrolyzes GTP and activates plasma-membrane-bound enzyme adenylate cyclase. Adenylate cyclase is a large multipass transmembrane protein with its catalytic domain on the cytosolic side of the plasma membrane. Activated adenylate cyclase synthesizes cAMP from ATP.



An best-studied example of a GPCR that responds to epinephrine is the β-*adrenergic receptor.* β-adrenergic receptor is coupled to G proteins Gs, which activate *adenylate cyclase*. Once activated, adenylate cyclase catalyzes synthesis of cAMP. The epinephrine-stimulated increase in cAMP and subsequent activation of protein kinase A enhance the conversion of glycogen to glucose 1-phosphate by stimulating glycogen degradation **protein kinase A** promotes glycogen degradation indirectly by phosphorylating and thus activating an kinase, glycogen phosphorylase kinase, that in turn phosphorylates and activates glycogen phosphorylase, the enzyme that degrades glycogen. The entire process is reversed when epinephrine is removed and the level of cAMP drops.

The effector protein for some of G proteins is a Na^+ or K^+ channel. For example, the *muscarinic acetylcholine receptors* in cardiac muscle are GPCRs. These receptors are linked via a trimeric G protein (Gi) to K^+ channels. Binding of acetylcholine triggers activation of the Giα subunit that leads to opening of associated K^+ channels. The subsequent efflux of K ions causes hyperpolarization of the plasma membrane.

cAMP and protein kinase A

cAMP directly activates *cAMP-dependent protein kinase* termed protein kinase A, **PKA**. cAMP also induces opening of certain types of ion channels in the plasma membrane of some highly specialized cells. PKA catalyzes the transfer of the terminal phosphate group from ATP to specific serines or threonines of selected

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target proteins, thereby regulating their activity. In the inactive state, PKA consists of a complex of two catalytic subunits and two regulatory subunits. The binding of cyclic AMP to the regulatory subunits alters their conformation, causing them to dissociate from the complex. The released catalytic subunits are thereby activated to phosphorylate specific substrate protein molecules. The active catalytic subunits phosphorylate a number of target proteins (cytosolic as well as nuclear) on serine and threonine residues.

One of the major nuclear targets for PKA is the transcription factor **CREB** (cAMP Response Element Binding Protein), which is activated by phosphorylation. Phosphorylation at a single *Ser* residue greatly increases the activity of CREB bound to the **CRE** (cAMP Response Element), which is found in genes whose transcription is induced by cAMP.

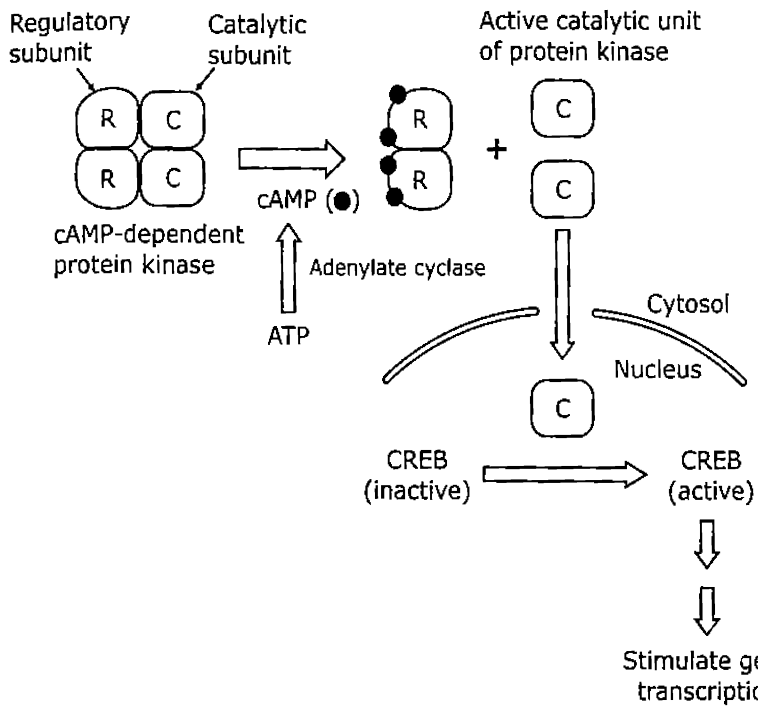
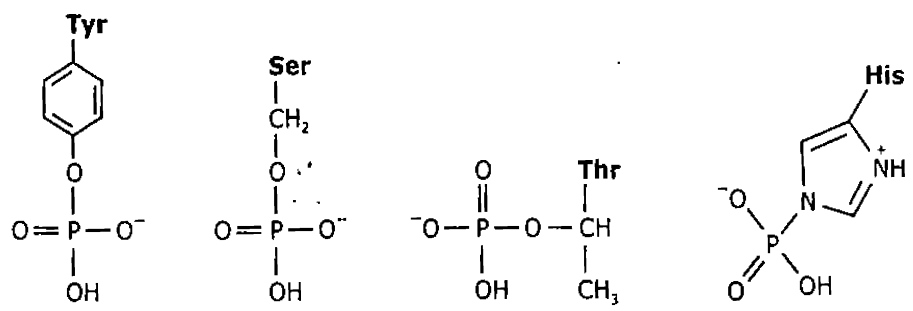


Figure : 9.48
The activation of cyclic-AMP-dependent protein kinase (PKA). The binding of cyclic AMP to the regulatory subunits induces a conformational change, causing these subunits to dissociate from the complex, thereby activating the catalytic subunits. Each regulatory subunit has two cyclic-AMP-binding sites. Once the catalytic subunits are freed and active, they can migrate into the nucleus (where they can phosphorylate gene regulatory proteins), while the regulatory subunits remain in the cytoplasm.

Protein kinases

Activation of all cell-surface receptors leads to changes in protein phosphorylation through the activation of protein kinase. There are many types of protein kinases involved in signal transduction. They all have the same basic catalytic activity: they add a phosphate group to an amino acid in a target protein. The phosphate is provided by ATP. Protein kinases can be classified by the types of amino acids that they phosphorylate in the protein target. Four groups of protein kinases are distinguished by the types of amino acid targets:

- Protein serine/threonine kinases (phosphorylate either serine or threonine in the target protein)
- Protein tyrosine kinases (phosphorylate tyrosine in the target protein)
- Dual specificity kinases (phosphorylate target proteins on either tyrosine or serine/threonine)
- Histidine kinases (phosphorylate target proteins on histidine)



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Protein kinases may be membrane bound or cytosolic on the basis of their location in the cell. *Cytosolic protein kinases* are most often Ser/Thr protein kinases. The activity of protein kinases is opposed by the activity of protein *phosphatases*, which remove phosphate groups from specific substrate proteins.

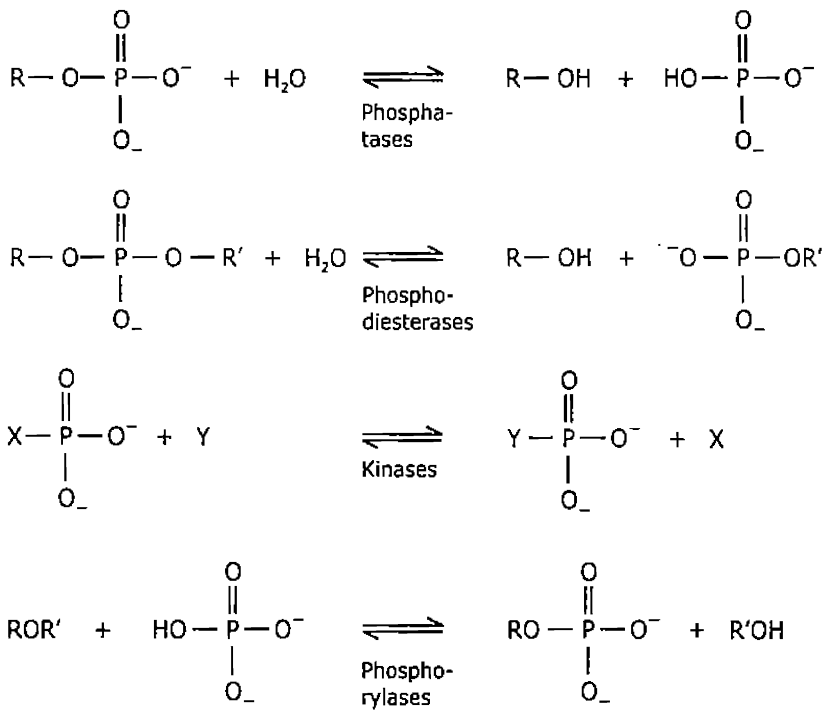
Differences betweeen *phosphatase, phosphodiesterase, kinase* and *Phosphorylases*

A **phosphatase** is an enzyme that removes a phosphate group from its substrate by hydrolysing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group.

A **phosphodiesterase** is an enzyme that breaks a phosphodiester bond.

A **kinase**, alternatively known as a *phosphotransferase*, is an enzyme that transfers phosphate groups from high-energy donor molecules, such as ATP, to substrates.

A **phosphorylase** is an enzyme that catalyze the addition of an inorganic phosphate to an acceptor.



G_q and phospholipase C-β

Many G-protein-coupled receptors exert their effects via G proteins like G_q that activate the plasma-membrane-bound enzyme **phospholipase C-β** (PLC-β). The activated phospholipase C cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two secondary messenger : *inositol 1,4,5-trisphosphate* (IP_3) and *diacylglycerol* (DAG). The membrane phospholipid PIP_2 is a minor component of the plasma membrane, localized to the inner leaflet of the phospholipid bilayer. One secondary messenger 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 endoplasmic reticulum. 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, which affects the activities of a variety of target proteins, including protein kinases and phosphatases. Diacylglycerol together with phosphatidylserine and Ca^{2+}, helps to activate the enzyme **protein kinase C** (PKC), which is recruited from the cytosol to the cytosolic face of the plasma membrane. When activated, PKC phosphorylates specific serine or threonine residues on target proteins.

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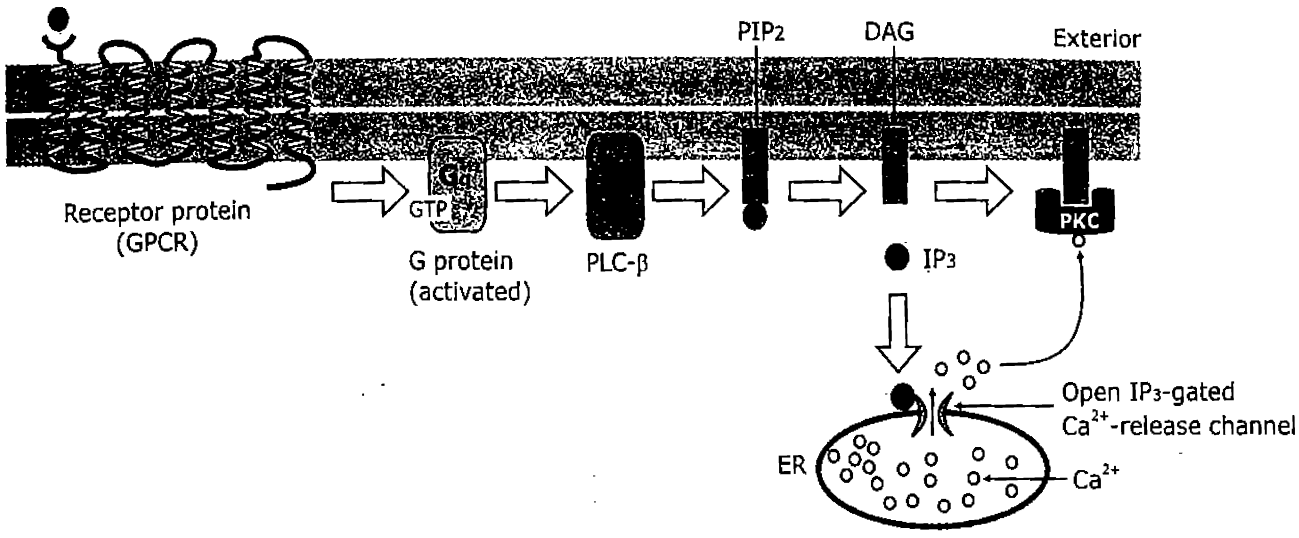


Figure 9.49 : The activated receptor binds to a specific trimeric G protein (G_q), causing the α subunit to dissociate and activate phospholipase C-β. Two intracellular mediators are produced when PIP_2 is hydrolyzed by phospholipase C-β: IP3, which diffuses through the cytosol and releases Ca^{2+} from the ER, and DAG, which remains in the membrane and helps activate the enzyme protein kinase C.

Mode of action of cholera and pertussis toxin

Cholera toxin is a protein complex secreted by the bacterium *Vibrio cholerae*. The cholera toxin is an oligomeric complex made up of six protein subunits: a single copy of the A subunit, and five copies of the B subunit. The A subunit has catalytic property that ADP-ribosylates G proteins. The pentameric protein binds to the surface of the intestinal epithelium. The A subunit ribosylates the *Arg* residue of alpha subunit of G_s protein. ADP ribose is provided by the intracellular NAD^+. This ADP ribosylation alters the a subunit so that it can no longer hydrolyze its bound GTP, causing it to remain in an active state that stimulates adenylyl cyclase indefinitely. The resulting prolonged elevation in cyclic AMP levels within intestinal epithelial cells causes a large efflux of Cl^- and water into the gut, thereby causing the severe diarrhea that characterizes cholera.

Pertussis toxin, an exotoxin that causes pertussis (whooping cough), catalyzes the ADP ribosylation of the *Cys* residue of α subunit of G_i. G_i is an inhibitory G protein. In activated state $G\alpha_i$ binds GTP and dissociate from the βγ complex. The released α_i inhibits the adenylate cyclase.The ADP ribosylation of $G\alpha_i$ prevents the G_i complex from interacting with receptors. So the complex remains bound to GDP and is unable to inhibit adenylate cyclase.

Ca^{2+}/CALMODULIN COMPLEX

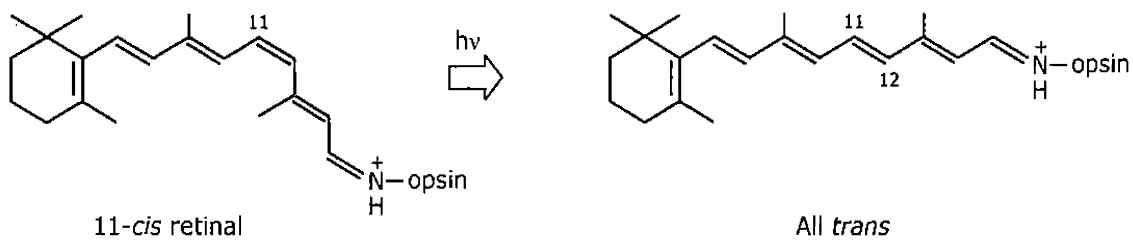
Many of the effects of Ca^{2+} are mediated by the protein calmodulin, which is activated by Ca^{2+} binding when the concentration of cytosolic Ca^{2+} increases. Ca^{2+}/calmodulin then binds to a variety of target proteins, including protein kinases. Calcium activates the regulatory protein calmodulin, which stimulates many enzymes and transporters. Calmodulin (17 KDa protein) is a small, highly conserved calcium binding cytosolic acidic protein found in all eukaryotic cells. It binds up to four calcium ions and acts as an important intracellular receptor for regulatory calcium signals. Ca^{2+} binds to calmodulin in a cooperative fashion. Thus a small change in the level of cytosolic Ca^{2+} leads to a large change in the level of active calmodulin. As it binds calcium, calmodulin undergoes conformational changes which can increase its affinity for target proteins. It acts both directly, through interaction with key target enzymes, and indirectly, via specific kinases. Calmodulin participates in the regulation of several biological processes including energy and biosynthetic metabolism, cell motility, exocytosis, cytoskeletal assembly, and intracellular modulation of both cAMP and calcium concentrations.

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Rhodopsin and Gt

Vision depend on G-protein-coupled receptors that regulate cyclic-nucleotide-gated ion channels. The first step in vision is absorption of light energy by rhodopsin. **Rhodopsin** is an integral protein with seven membrane-spanning alpha helices, present in the rod cells. The light-absorbing pigment (chromophore) **11-*cis*-retinal** is covalently attached to **opsin,** the protein component of rhodopsin. When a photon is absorbed by the retinal component of rhodopsin, the energy causes a photochemical change; 11-cis-retinal is converted to **all-*trans*-retinal**. This change in the structure of the chromophore causes conformational changes in the rhodopsin molecule.



11-*cis* retinal All *trans*

In its excited conformation, rhodopsin interacts with a second protein, **transducin**. Transducin (Gt) is a heterotrimeric GTP-binding protein. It can bind either GDP or GTP. In the dark, GDP is bound, all three subunits of the protein (α, β and γ) remain together, and no signal is sent. When rhodopsin is excited by light, it interacts with transducin, catalyzing the replacement of bound GDP by GTP from the cytosol. Transducin then dissociates into $G_{t\alpha}$ and $G_{t\beta\gamma}$ and the $G_{t\alpha}$-GTP carries the signal from the excited receptor to the effector molecule *cGMP phosphodiesterase* (PDE). PDE enzyme converts cyclic GMP to 5'-GMP. The cyclic GMP (cGMP) levels in the cytosol fall. This drop in cyclic GMP concentration leads to a decrease in the amount of cyclic GMP bound to the plasma membrane Na^+ channels, allowing more of these highly cyclic-GMP-sensitive channels to close. Closure of these channels inhibits the influx of Na^+. These channels are kept open in the dark by cyclic GMP that has bound to them. In this way light causes a hyperpolarization rather than a depolarization of the plasma membrane. So, the activation of rhodopsin molecules by light results in hyperpolarization.

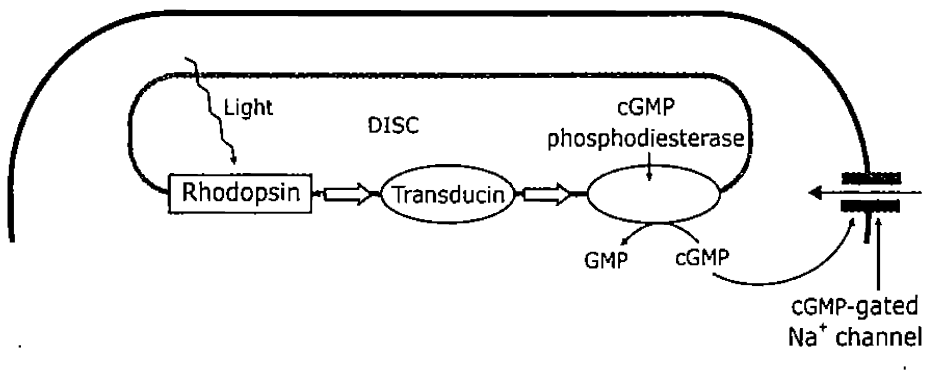


Figure : 9.50
Light activates rhodopsin, which activates transducin to bind GTP. This activates phosphodiesterase, which catalyzes the conversion of cGMP to GMP. The resulting decrease in the cytoplasmic cGMP concentration causes cGMP-gated ion channels to close.

A number of mechanisms operate in rods to allow the cells to revert quickly to a resting, dark state. First, the Na^+ channels that close in response to light are also permeable to Ca^{2+}, so that when they close, the normal influx of Ca^{2+} is inhibited, causing the Ca^{2+} concentration in the cytosol to fall. The decrease in Ca^{2+} concentration stimulates guanylyl cyclase to replenish the cyclic GMP, rapidly returning its level to where it was before the light was switched on. A specific Ca^{2+}-sensitive protein mediates the activation of guanylyl cyclase in response to a fall in Ca^{2+} levels. In contrast to calmodulin, this protein is inactive when Ca^{2+} is bound to it and active when it is Ca^{2+}-free.

Second, a *rhodopsin-specific kinase,* a serine kinase, phosphorylates the cytosolic tail of activated rhodopsin

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and partially inhibits the ability of the rhodopsin to activate transducin. An inhibitory protein called **arrestin** then binds to the phosphorylated rhodopsin, further inhibiting rhodopsin's activity. At the same time as rhodopsin is being shut off, a *regulator of G protein signaling* (RGS) protein, which acts like GTPase activating protein, binds to activated transducin, stimulating the transducin to hydrolyze its bound GTP to GDP, which returns transducin to its inactive state.

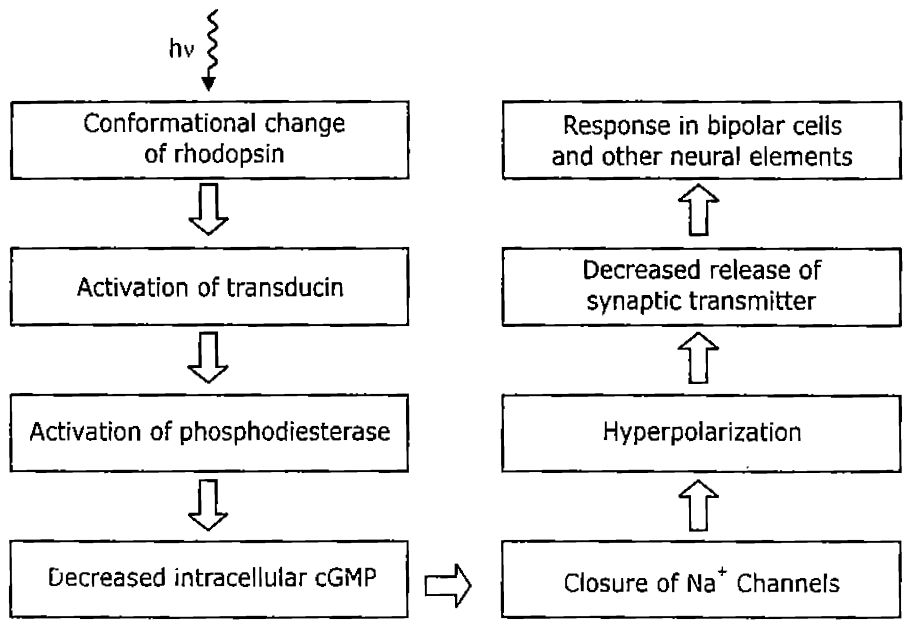
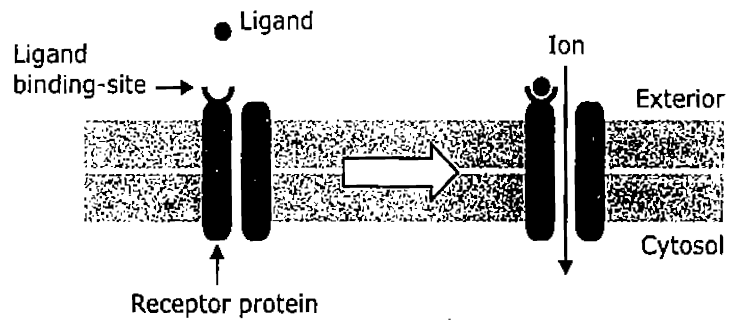


Figure 9.51 : Sequence of events involved in phototransduction in rods.

ION-CHANNEL-LINKED RECEPTORS

Ion-channel-linked receptors, also known as *transmitter-gated ion channels* or *ionotropic receptors*, are involved in rapid synaptic signaling between electrically excitable cells. This type of signaling is mediated by a small number of neurotransmitters that transiently open or close an ion channel formed by the protein to which they bind, briefly changing the ion permeability of the plasma membrane and thereby the excitability of the postsynaptic cell. The ion-channel-linked receptors belong to a large family of homologous, multipass transmembrane proteins.



ENZYME-LINKED RECEPTORS

Enzyme-linked receptors are a second major type of cell-surface receptor. Like G-protein-linked receptors, enzyme-linked receptors are transmembrane proteins with their ligand-binding domain on the outer surface of the plasma membrane. Instead of having a cytosolic domain that associates with a monomeric G protein, however, their cytosolic domain either has an intrinsic enzyme activity or associates directly with an enzyme.

Several classes of enzyme-linked receptors have thus far been identified like Receptor tyrosine kinases (the most numerous of the enzyme-linked receptors), Tyrosine-kinase-associated receptors, Receptor like tyrosine phosphatases, Receptor serine/threonine kinases, Receptor guanylyl cyclases and others.

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A. Receptor tyrosine kinases

The receptor tyrosine kinases (RTKs) are the second major type of cell-surface receptors. The ligands for RTKs may be insulin, Epidermal growth factor (EGF), Transforming growth factor-α (TGFα), Platelet-derived growth factor, (PDGF), Fibroblast growth factor (FGF) and Colony stimulating factor-1 (CSF-1).

All RTKs comprise an extracellular domain containing a ligand-binding site, a single hydrophobic transmembrane α–helix, and a cytosolic domain that includes a region with protein-tyrosine kinase activity. Most RTKs are monomeric, and ligand binding to the extracellular domain induces formation of receptor dimers.

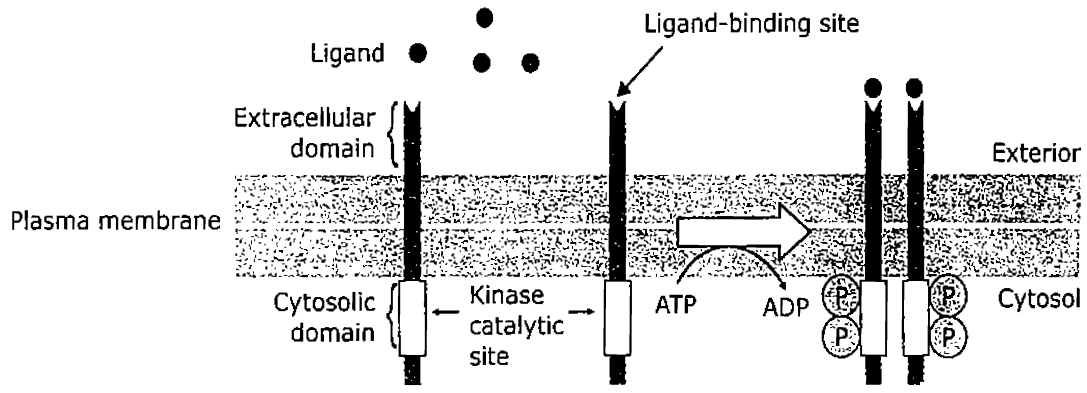


Figure 9.52 : General structure and ligand-induced activation of receptor tyrosine kinases (RTKs). The cytosolic domain of RTKs contains a protein tyrosine kinase catalytic site. Ligand binding causes a conformational change that promotes formation of a functional dimeric receptor, bringing together two intrinsic or associated kinases, which then phosphorylate each other on a tyrosine residue in the cytosolic domain. The activated kinase then phosphorylates other tyrosine residues in the receptor's cytosolic domain. The resulting phosphotyrosines function as docking sites for various signal-transduction proteins.

Ligand binding leads to activation of the intrinsic protein-tyrosine kinase activity of the receptor and *autophosphorylation* of tyrosine residues in its cytosolic domain. The activated receptor also can phosphorylate other protein substrates. Several types of proteins with which the activated receptor may interact. Some of these proteins are a GTPase activating protein (GAP), phospholipase C-γ (PLC-γ), and the *Src*-like nonreceptor protein tyrosine kinases. Three major pathways can transduce a signal from the activated, RTK :

1. Ras–MAP kinase pathway;
2. IP_3/DAG pathway;
3. PI-3 kinase pathway.

Ras–MAP kinase pathway

Ligand-induced activation of an RTK stimulates its tyrosine kinase activity, which subsequently stimulates several signal-transduction pathways including the *Ras–MAP kinase pathway*. *Ras* is an intracellular monomeric GTPase switch protein and functions in transducing signals from many different RTKs. *Ras* belongs to Ras superfamily which also includes Rho, Rab and Ran.

Ras superfamily

Subfamily	Function
Ras	Regulates cell growth through serine-threonine protein kinases
Rho	Reorganizes cytoskeleton through serine-threonine protein kinases
Rab	Plays a key role in endocytotic pathways
Ran	Functions in the transport of RNA and protein into and out of the nucleus

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Ras cycles between an inactive GDP-bound form and active GTP-bound form. Ras cycling requires the assistance of two proteins, GEF (guanine nucleotide exchange factor) and GAP (GTPase-activating protein). Ras activation is accelerated by a protein called GEF, which binds to the Ras–GDP complex, causing dissociation of the bound GDP. Because GTP is present in cells at a higher concentration than GDP, GTP binds spontaneously to *empty* Ras molecules, with release of GEF. The average lifetime of a GTP bound to Ras is about 1 minute. GAP, which binds to Ras–GTP and accelerates its intrinsic GTPase activity. Binding of a ligand to an RTK lead to activation of Ras. Two cytosolic proteins - adaptor protein and GEF provide the key links between RTK and Ras.

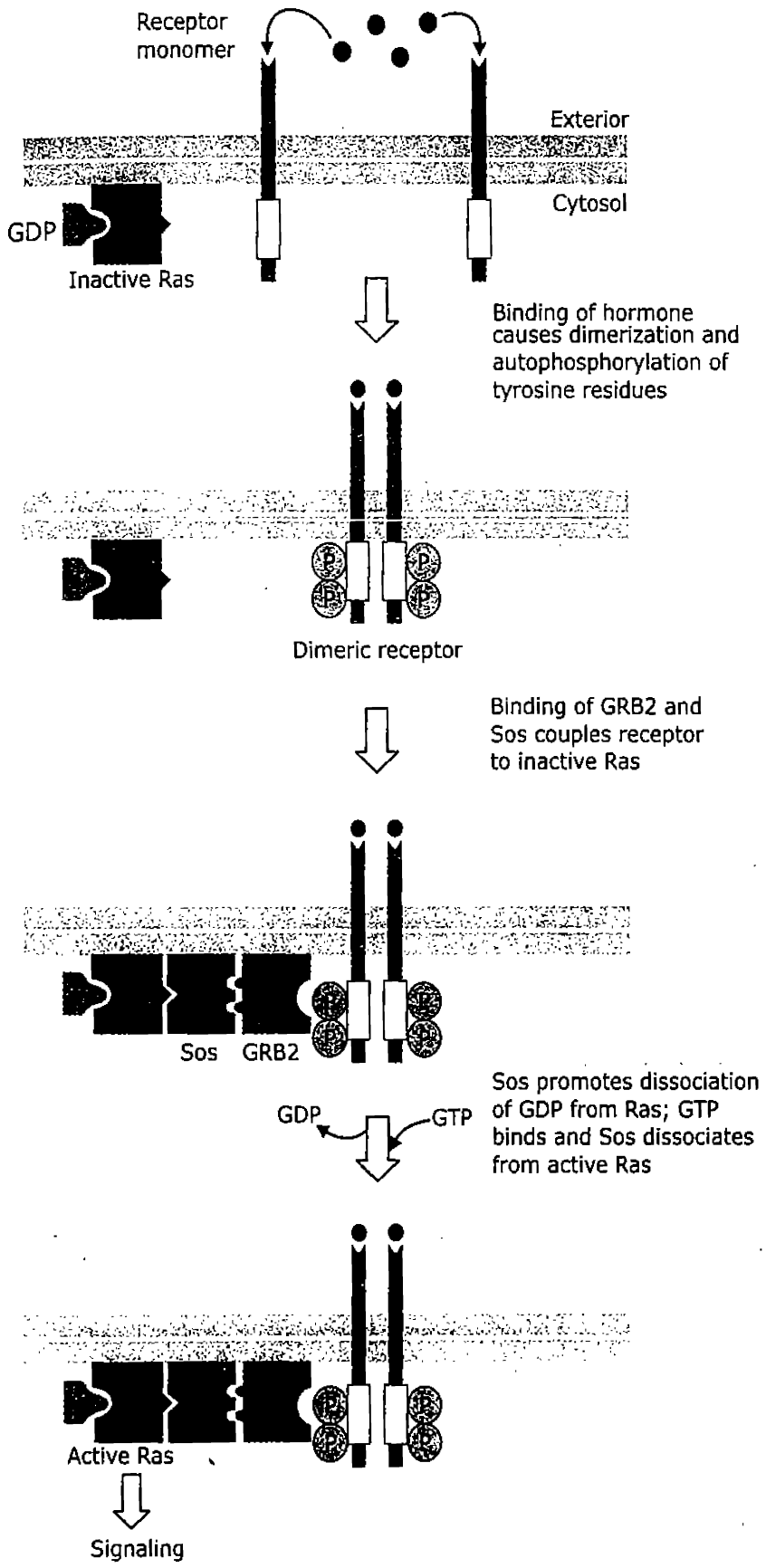


Figure : 9.53
Activation of Ras. An adapter protein, GRB2, and guanine nucleotide-exchange factor, Sos link most activated receptor tyrosine kinases to Ras. The SH2 domain in GRB2 binds to a phosphotyrosine in activated RTKs, while its two SH3 domains bind Sos, thereby bringing Sos close to membrane-bound RasGDP and activating its nucleotide exchange activity. Binding of Sos to inactive Ras causes a large conformational change that permits release of GDP and binding of GTP, forming active Ras. GAP, which accelerates GTP hydrolysis, is localized near RasGTP by binding to activated RTKs.

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Adaptor proteins bind to an activated receptor, and then other proteins(s) bind to them, and may therefore become substrates for the receptor. Adaptor has no catalytic activity of its own, but serves merely to bring other proteins to the receptor. The cytosolic adaptor protein GRB2 binds to a specific phosphotyrosine on an activated, ligand-bound receptor and to the cytosolic GEF, Sos protein. Sos then promotes formation of active RasGTP. An Src homology domain, *SH2 domain,* in GRB2 binds to a specific phosphotyrosine residue in the activated receptor. The SH2 domain was originally identified as a conserved amino acid sequence in the non-catalytic region of tyrosine kinases encoded by viral oncogenes such as v-*src*. GRB2 also contains two *SH3 domains,* which bind to and activate Sos. SH3 binds to proline-rich motifs.

MAP Kinase pathways

Activation of a receptor tyrosine kinase may itself trigger multiple pathways: for example, activation of EGF receptor activates the Ras dependent pathway as well as Ras-independent pathways involving second messengers. The activation of Ras triggered by activated receptor tyrosine kinases are short-lived. *Ras* activates a downstream serine/threonine phosphorylation cascade that includes a **MAP-kinase** (*m*itogen-*a*ctivated *p*rotein kinase). Activated Ras induces a *kinase cascade* that culminates in activation of MAP kinase. This serine/threonine kinase, which can translocate into the nucleus, phosphorylates many different proteins including transcription factors that regulate expression of important cell-cycle and differentiation-specific proteins. Activation of MAP kinase in two different cells can lead to similar or different cellular responses, as can activation in the same cell by stimulation of different RTKs.

Many serine/threonine kinases participate in this phosphorylation cascade, but three of them constitute the core module of the cascade. The last of the three is called MAP-kinase. Downstream serine/threonine phosphorylation cascade follow the following pattern:

1. Activated Ras binds to the N-terminal domain of **Raf** (MAP-kinase-kinase-kinase), a Ser/Thr kinase.
2. Raf activity leads to the activation of **Mek** (MAP-kinase-kinase). Raf directly phosphorylates Mek, which is activated by phosphorylation on two serine residues. Mek is an unusual enzyme with dual specificity, which can phosphorylate both threonine and tyrosine. Its target is the **Erk** (MAP kinase).
3. MEK phosphorylates and activates MAP kinase. The MAP kinases are serine/threonine kinases. After this point in the pathway, all the activating events take the form of serine/threonine phosphorylations. MAP kinase phosphorylates many different proteins, including nuclear transcription factors, that mediate cellular responses.

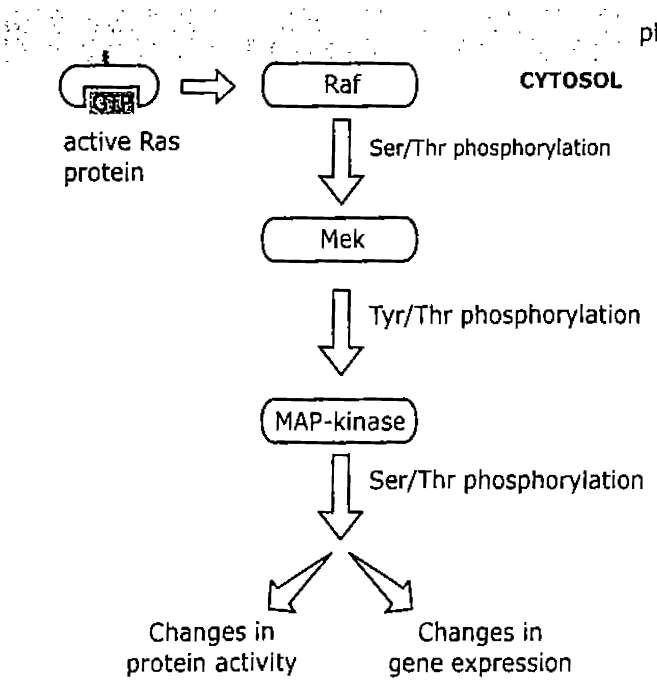


Figure : 9.54
MAP kinase pathway. Activated Ras triggers the downstream kinase cascade, culminating in activation of MAP kinase. Interaction of the Raf N-terminal regulatory domain with RasGTP leads to activation of Raf kinase activity. Note that in contrast to many other protein kinases, activation of Raf does not depend on phosphorylation. Hydrolysis of RasGTP to RasGDP releases active Raf, which phosphorylates and thereby activates *Mek*. Active Mek then phosphorylates and activates MAP kinase, another serine/threonine kinase. MAP kinase phosphorylates many different proteins, including nuclear transcription factors, that mediate cellular responses.

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The ultimate effect of the MAP kinase pathway is a change in the pattern of transcription. In the classic MAP kinase pathway, MAP kinase moves into the nucleus, where it phosphorylates target transcription factors. In alternative pathway, it phosphorylates a cytoplasmic factor; this may be a transcription factor that then moves to the nucleus or a protein that regulates a transcription factor (for example, by releasing it to go to the nucleus).

IP3/DAG pathway

Many RTKs can initiate the IP3/DAG pathway by activating another isoform of phospholipase C, the phospholipase C-γ. The SH2 domains of PLC-γ bind to specific phosphotyrosines of the activated receptors, thus positioning the enzyme close to its membranebound substrate PIP2. Membrane-bound PLC-γ after activation cleaves PIP2 to generate two second messengers, DAG and IP3. Signaling via the IP3/DAG pathway leads to an increase in cytosolic calcium and to activation of protein kinase C.

PI-3 kinase pathway

In addition to initiating the IP3/DAG pathway, some activated RTKs also initiate another phosphoinositide pathway, the *PI-3 kinase pathway*. PI-3 kinase binds to phosphotyrosine residues in the cytosolic domain of activated RTKs. PI-3 kinase catalyzes the formation of PI 3,4-bisphosphate or PI 3,4,5-trisphosphate which act docking sites for various signal-transducing proteins.

A primary binding target of PI 3,4,5-trisphosphate is **protein kinase B** (PKB), a serine/threonine kinase. Besides its kinase domain, PKB contains a PH domain that tightly binds the 3-phosphate in PI 3,4,5-trisphosphate. In an inactive form protein kinase B present in the cytosol. In case of rise in PI 3,4,5-trisphosphate, protein kinase B binds to them and is localized at the cell surface. PI 3,4,5-trisphosphates also recruits kinase, PDK1, to the plasma membrane via binding of its PH domain. Active PDK1 causes phosphorylation of protein kinase B. Fully activated protein kinase B then dissociates from the plasma membrane and phosphorylate its many target proteins.

Insulin signaling pathways

The insulin receptor is a dimeric (linked together by disulphide bonds) receptor tyrosine kinase. Binding of the hormone activates the receptor, which then phosphorylates itself and other proteins at various tyrosine residues. The phosphorylated insulin receptor next phosphorylates *insulin receptor substrates* (IRS) on tyrosine residues. Phosphorylated IRS binds to the SH2 domains of a variety of proteins that are directly involved in mediating different effects of insulin.

Phosphorylated IRS molecule acts as docking site for the lipid kinase, PI-3 kinase that leads to the activation of protein kinase B (PKB). Other signal transduction proteins interact with IRS including Grb2, an adaptor protein that contains SH3 domains, which in turn associates with the Sos and elicits activation of the G protein, **Ras**. Ras activates the MAP kinase pathway leading to mitogenic responses. These pathways act in a coordinated manner to regulate glucose, lipid and protein metabolism.

B. Tyrosine kinases activated receptors (cytokine receptors)

Rather than possessing intrinsic enzymatic activity, many enzyme linked receptors act by stimulating intracellular protein-tyrosine kinases with which they are noncovalently associated. This family of receptors includes the receptors for most cytokines (e.g., interleukin-2 and erythropoietin) and for some polypeptide hormones (e.g., growth hormone) whose cytosolic domains are closely associated with a member of a family of cytosolic protein tyrosine kinases, the *JAK kinases*. Like RTK, the cytokine receptors contain N-terminal extracellular ligand-binding domains, single transmembrane *alpha* helices, and C-terminal cytosolic domains. However, the cytosolic domains of the cytokine receptors are devoid of any known catalytic activity. Instead, the cytokine receptors function in association with nonreceptor protein-tyrosine kinases, which are activated as a result of ligand binding.

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The first step in signaling from cytokine receptors is thought to be ligand-induced receptor dimerization and cross-phosphorylation of the associated nonreceptor protein-tyrosine kinases. These activated kinases then phosphorylate the receptor, providing phosphotyrosine-binding sites for the recruitment of downstream signaling molecules. The nonreceptor protein-tyrosine kinases associated with the cytokine receptors fall into two major families. Many of these kinases are members of the *Src* family, which consists of Src and closely related proteins. Src possess protein-tyrosine kinase activity. In addition to Src family members, the cytokine receptors are associated with nonreceptor protein-tyrosine kinases belonging to the Janus kinase, or **JAK**, family. Members of the JAK family appear to be universally required for signaling from cytokine receptors. In contrast, members of the Src family play key roles in signaling from antigen receptors on B and T lymphocytes but do not appear to be required for signaling from most cytokine receptors.

JAK-STAT pathway

The JAK kinases take their name (originally Janus kinases) from the characteristic presence of two kinase domains in each molecule. Several members of the family are known (JAK1,2,3, etc.); each associates with a specific set of cytokine receptors. The JAK kinases are tyrosine kinases whose major substrates are transcription factors called STATs (Signal transducers and activators of transcription). There are several types of STATs; each STAT is phosphorylated by a particular set of JAK kinases. All STAT proteins contain an N-terminal SH2 domain that binds to a phosphotyrosine in the receptor's cytosolic domain, a central DNA-binding domain, and a C-terminal domain with a critical tyrosine residue.

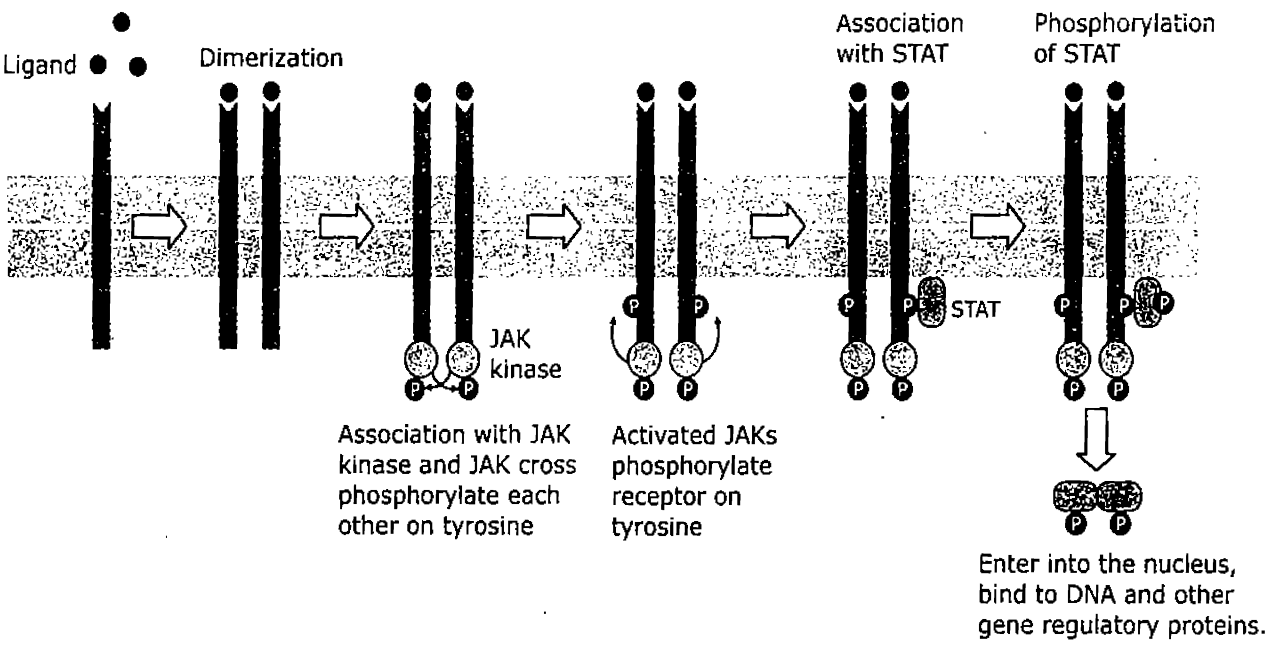


Figure 9.55 : Cytokine receptors associate with and activate JAK kinases. STATs bind to the complex and are phosphorylated. They dimerize and translocate to the nucleus. The complex binds to DNA and activates transcription.

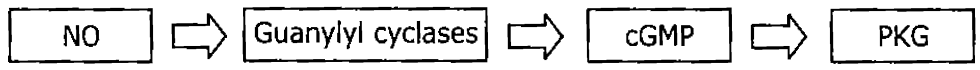
Once a STAT is bound to the receptor, the C-terminal tyrosine is phosphorylated by an associated JAK kinase. STAT phosphorylation leads to the formation of both homodimers and heterodimers. The basis for dimerization is a reciprocal interaction between an SH2 domain in one subunit and a phosphorylated Tyr in the other subunit. The STAT dimers move into the nucleus, where they bind to specific *enhancer* sequences controlling target genes.

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Nitric oxide

Nitric oxide (NO) is a paracrine signaling molecule. Like the steroid hormones, NO is able to diffuse directly across the plasma membrane of its target cells. Nitric oxide is synthesized from the amino acid *arginine* by the enzyme *nitric oxide synthase.* Once synthesized, NO diffuses out of the cell and can act locally to affect nearby cells. One well-characterized example of NO action is the dilation of blood vessels. The acetylcholine, a neurotransmitter acts on endothelial cells to stimulate NO synthesis. NO then diffuses to neighboring smooth muscle cells where it interacts with iron bound to the active site of the enzyme guanylyl cyclase. This increases enzymatic activity, resulting in synthesis of the second messenger cyclic GMP, which induces muscle cell relaxation and blood vessel dilation.



Effect of Viagra

Concentration of cGMP decreases because a specific phosphodiesterase convert cGMP to the inactive 5′-GMP. Sildenafil (Viagra) causes cGMP levels to remain high by inhibiting the activity of phosphodiesterase.

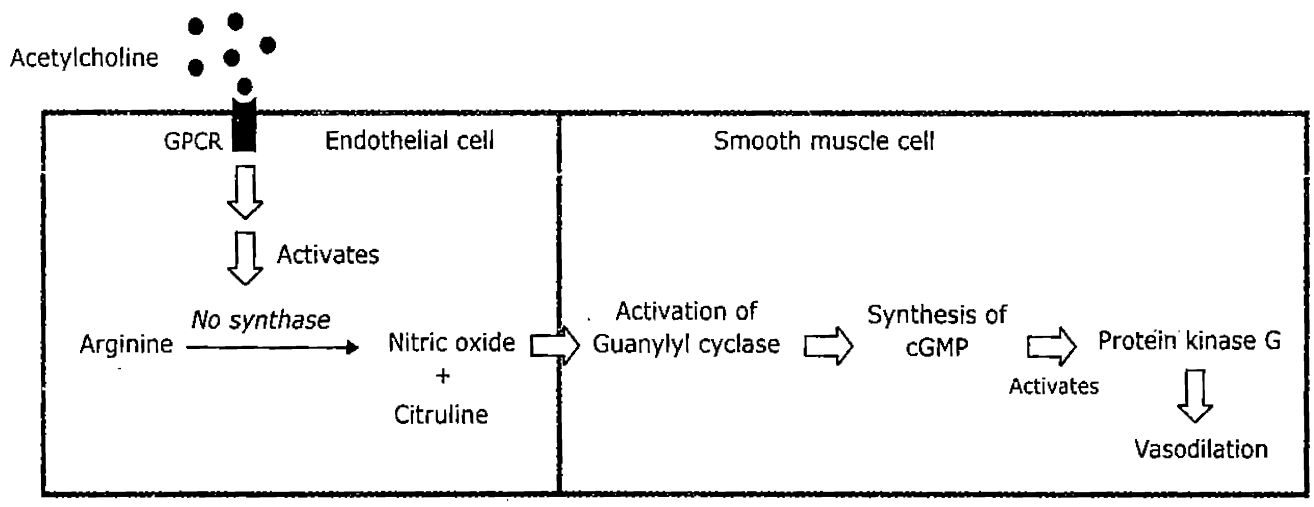


Figure 9.56 : Action of nitric oxide

TWO-COMPONENT SIGNALING SYSTEMS

Two-component signaling system is the most common form of signaling pathway that responds to extracellular events in bacteria and plants. A two component system consists of a *sensor* that is an auto-phosphorylating histidine kinase and an *effector* that can catalyze transfer of phosphate from the sensor to itself.

The sensor protein is a *histidine kinase* that is located in the membrane. It can be activated by binding a ligand that is in the extracellular medium. Activation causes the kinase to autophosphorylate. The reaction transfers the phosphate from ATP on to a histidine residue in the kinase.

The sensor interacts with an effector protein (also called a *response regulator*). The effector protein has two domains - regulatory domain and effector domain. The regulatory domain catalyzes transfer of the phosphate group from the histidine on the sensor to an aspartic acid residue in its own domain. This activates the effector domain. The usual end target of a two-component pathway is the regulation of gene transcription.

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CHEMOTAXIS IN BACTERIA

Movement of organisms in specific directions in response to chemical stimulus is called *chemotaxis*. Chemotaxis in bacteria depends on a signaling pathway that terminates at the flagellar motor.

Bacterial flagellar motion is rotatory in nature. Membrane proteins MotA and MotB along with FliG create a proton channel that drives rotation of the flagellum. The rotation of flagella can be *clockwise* or *counterclockwise*. When the flagella rotate clockwise, forward motion ceases and the cells *tumble*. Whereas counterclockwise rotations of flagella impart forward motion (*run*).

In the absence of a chemical gradient, bacteria (*E.coli*) move in a random fashion that includes runs, where the cell is swimming forward and tumbles when the cell stops. Following a tumble, the direction of the next run is random. However, if a gradient of a chemical attractant is present, these random movements become biased. As the bacteria senses that it is moving toward higher concentrations of the attractant runs become longer and tumbles less frequent. If the bacteria is sensing a repellent, the same general mechanism applies, although in this case it is the decrease in concentration of the repellent that promotes runs.

Bacterial chemotaxis is mediated by a transmembrane receptors and a phosphorylation relay system. It depends on a two component signaling pathway activated by *histidine kinase* associated receptors. The chemotaxis receptors are methylated during adaptation and so are also called *methyl-accepting chemotaxis proteins (MCPs)*.

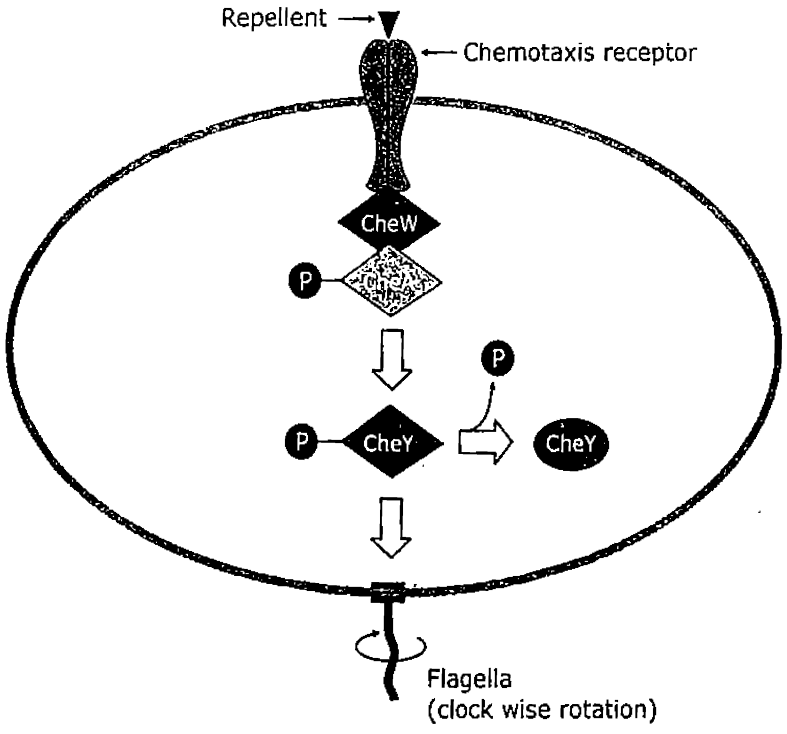


Figure : 9.57
The two-component signaling pathway that enables chemotaxis receptors to control the flagellar motor during bacterial chemotaxis.

The phosphorylation relay system enables the chemotaxis receptors to control the flagellar motor. The binding of a repellent increases the activity of the receptor, which binds CheW (adaptor protein) and CheA (histidine kinase), thereby stimulating CheA to phosphorylate itself on histidine. CheA quickly transfers its covalently bound, high-energy phosphate directly to aspartate of CheY to generate CheY-phosphate. The phosphorylated CheY dissociates from the receptor, diffuses through the cytosol and binds to the flagellar motor and causes it to rotate clockwise, resulting in *tumbling*. The binding of an attractant has the opposite effect. It decreases the activity of the receptor and therefore decreases the phosphorylation of CheA and CheY, which results in counterclockwise flagellar rotation and run. CheZ accelerates the dephosphorylation of CheY-phosphate, thereby inactivating it.

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QUORUM SENSING

Bacteria use a variety of means to communicate with one another and with their eukaryotic hosts. In some cases, social interactions allow bacteria to synchronize the behavior of all of the members of the group and thereby act like multicellular organisms. Bacteria are also able to measure the concentration of the molecules within a population. Thus accumulation of signaling molecules enable a single cell to sense the number of bacteria (cell density). This sensing phenomenon is termed as *Quorum Sensing*.

Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density. Bacteria that use quorum sensing constantly produce and secrete certain signaling molecules (called *autoinducers*). These bacteria also have a receptor that can specifically detect the signaling molecule. The detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression. Both gram-positive and gram-negative bacteria use quorum sensing communication circuits to regulate a diverse array of physiological activities. These processes include symbiosis, virulence, competence, conjugation, antibiotic production, motility, and sporulation. In general, Gram-negative bacteria use *N-acyl homoserine lactones* as autoinducers, and Gram-positive bacteria use processed *oligo-peptides* to communicate.

Examples

The first incidence of this biological phenomenon came to light with the discovery of luminescence produced by marine bacteria *Vibrio fischeri*. These bacteria, when free-living in sea water (i.e. at low cell density) are non-luminescent. However, when grown to high cell densities it bioluminisces with a blue-green light. This bacterium commonly forms symbiotic relationships with some fishes and squid species. These marine animals carry a specialized organ called the light organ, in which bacteria luminescent appearance in dark environments due to the maintenance of a high-density *V. fischeri* population in the light organ. In the marine environment, the bacteria only luminesce when colonising the light organs and do not emit light when in the free-living state. Research carried out to answer the question of how *V. fischeri* regulates bioluminescence led to the discovery of bacterial quorum sensing via *N*-acyl-L-homoserine lactones (AHLs).

Synthesis of AHL is catalyzed by an enzyme called ALH synthase, the product of the *luxI* gene. The luxI gene is subject to positive autoregulation i.e., transcription of *luxI* increases as AHL accumulates in the cell. This is accomplished through a transcriptional activator, LuxR, which is active only when it binds AHL. Thus without AHL-activated LuxR, the *luxI* gene will be transcribed only at basal level. AHL freely diffuses out of the cell and accumulates in the environment. When cell density increases, concentration of AHL also increases. Greater concentration of AHL flows back into the cell and activate high level transcription of *luxI* and other genes whose products are needed for bioluminescence.

SCATCHARD PLOT

The Scatchard plot is a graphical method of analyzing equilibrium ligand binding data. It is used to determine the number of ligand-binding sites on a receptor, whether these sites show cooperative interactions, whether more than one class of site exists, and the respective affinities of each site.

Ligand binds specifically and non-covalently with the receptor. A receptor may possess one binding site per molecule or more then one independent binding sites. Receptor-Ligand binding is described by the equation;

$$R(\text{Receptor}) + L(\text{Ligand}) \rightleftharpoons R-L\ (\text{Receptor-Ligand complex})$$

The equilibrium constant for this association reaction K_a, is

$$K_a = \frac{[RL]}{[R][L]}$$

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$$\text{and} \quad K_d = \frac{[R][L]}{[RL]} \qquad \ldots(1)$$

Now, the *fractional saturation* of sites

$$Y = \frac{\text{Concentration of L bound to R}}{\text{Total concentration of R}} = \frac{[RL]}{[R]+[RL]} \qquad \ldots(2)$$

The value of Y ranges from zero, when [RL] = 0, to 1, when [R] = 0.
To determine the value of K_d, we can rearrange the equation (1),

$$[RL] = \frac{[R][L]}{K_d}$$

Substituting the expression for [RL] in equation (2), we obtain

$$Y = \frac{[R][L]/K_d}{[R]+[R][L]/K_d}$$

$$Y = \frac{[L]}{[L]+K_d} \qquad \ldots(3)$$

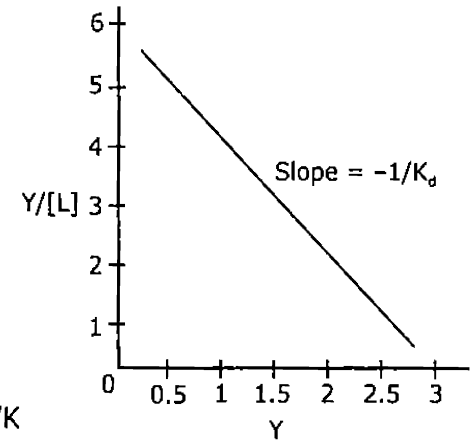
By taking the reciprocal of equation (3), we get

$$\frac{1}{Y} = 1 + \frac{K_d}{[L]}$$

Alternatively it can be rearrange to

$$\frac{Y}{[L]} = \frac{1}{K_d} - \frac{Y}{K_d}$$

A plot of Y/[L] versus Y gives a straight line of slope $-1/K$



If receptor has *n* equivalent binding sites for ligand

Now consider the case in which a macromolecule has *n* equivalent sites; that is each binding sites has the same K_d value, regardless of whether other sites on the same molecule are occupied.

In this case, the equation will be

$$Y = \frac{n[L]}{[L]+K} \qquad \ldots(4)$$

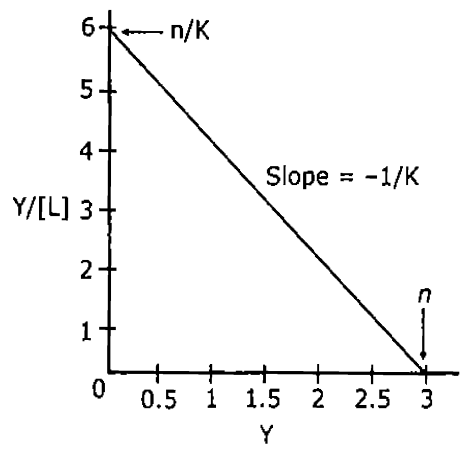
For *n* equivalent sites, we have $K = (K_1 K_2 K_3 \ldots\ldots\ldots K_n)^{1/n}$

From equation (4),

$$Y[L] + KY = n[L]$$

$$\frac{YL}{K} + Y = \frac{n[L]}{K}$$

$$Y = \frac{n[L]}{K} - \frac{Y[L]}{K}$$



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$$\frac{Y}{[L]} = \frac{n}{K} - \frac{Y}{K} \qquad \ldots(5)$$

Equation (5) is known as the *Scatchard equation* and the plot between Y/[L] versus Y is known as *Scatchard plot*. Nonlinear Scatchard plots are diagnostic of several more complex types of ligand-receptor interaction. A plot that is concave downward indicates positive homotropic cooperativity between binding sites on an allosteric receptor. A second type of nonlinear Scatchard plot arises when multiple classes of binding sites with distinct affinities are present on a receptor.

STUDY QUESTIONS

Q3. The incorporation of cholesterol into membranes has two closely related effects:

a. The temperature at which the membrane turns from a frozen to a fluid state is lowered and

b. The membrane becomes more rigid.

Suggest an explanation for these two effects in terms of the structure of cholesterol.

Ans. Cholesterol is a relatively planar, rigid, non-polar molecule. The hydrophobic cholesterol is incorporated between the hydrocarbon chains of fatty acids, impairing lipid crystallization and lowering the temperature at which the lipid melts. The rigid, planar nature of cholesterol increases the stiffness of the membrane.

Q4. Sec61 is a critical component of the protein channel through the ER membrane. In Sec61 mutant cells, what is the fate of proteins that are normally localized to the Golgi apparatus?

Ans. These proteins are unable to enter the ER and therefore remain in the cytosol.

Q5. You have generated a recombinant cDNA in which a signal sequence has been added to the amino terminus of a nuclear protein. Where would you predict the protein encoded by this cDNA to be localized?

Ans. The signal sequence would direct the growing polypeptide chain to the ER and the protein would then be secreted via the bulk flow pathway.

Q6. Skeletal muscle, eukaryotic cilia, and bacterial flagella use different strategies for the conversion of free energy into coherent motion. Compare and contrast these motility systems with respect to

a. The free-energy source and

b. The number of essential components and their identity.

Ans. a. Skeletal muscle and eukaryotic cilia derive their free energy from ATP hydrolysis; the bacterial flagellar motor uses a protonmotive force.

b. Skeletal muscle requires myosin and actin. Eukaryotic cilia require microtubules and dynein. The bacterial flagellar motor requires MotA, MotB, and FliG, as well as many ancillary components.

Q7. Under certain stable concentration conditions, actin monomers in their ATP form will polymerize to form filaments that disperse again into free actin monomers over time. Provide an explanation.

Ans. Above its critical concentration, ATP-actin will polymerize. The ATP will hydrolyze through time to form ADP-actin, which has a higher critical concentration. Thus, if the initial subunit concentration is between the critical concentrations of ATP-actin and ADP-actin, filaments will form initially and then disappear on ATP hydrolysis.

Q8. Would cytochalasin affect the movement of cells that extend pseudopodia or that utilize flagella for locomotion?

Ans. Cytochalasin inhibits actin polymerization. It would therefore inhibit the movement of cells by pseudopodia but would not affect the microtubule-based movement of flagella.

Q9. Why is the polarity of actin filaments important to muscle contraction?

Ans. The polarity of actin filaments defines the direction of myosin movement. If actin filaments were not polar, the unidirectional movement of myosin that results in the sliding of actin and myosin filaments could not take place.

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Q10. Which aspect of cell division would be affected by colchicine: chromosome segregation or cytokinesis?

Ans. Colchicine inhibits microtubule polymerization, so it would affect chromosome segregation. It would not affect cytokinesis, which is driven by an actin-myosin contractile ring.

Q11. The table below lists some of the properties and functions associated the different cytoskeletal fibers. For each type of fiber, fill in the blanks with *yes* or *no* indicate whether or not it exhibits each listed property or function.

Function/property	*Microfilament*	*Microtubule*
1 Can generate motion by assembly	—	—
2. Can generate motion by sliding filaments	—	—
3. Contain bound nucleotide	—	—
4. Have functional association with ATPases	—	—
5. Are stable in detergent and high salt	—	—

Ans. 1. Yes, Yes
2. Yes, Yes
3. Yes, Yes
4. Yes, Yes
5. No, No

Q12. Glucose is mobilized for ATP generation in muscle in response to epinephrine, which activates G_α. Cyclic AMP phosphodiesterase is an enzyme that converts cAMP into AMP. How would inhibitors of cAMP phosphodiesterase affect glucose mobilization in muscle?

Ans. G_α stimulates adenylate cyclase, leading to the generation of cAMP. This signal then leads to glucose mobilization. If cAMP phosphodiesterase were inhibited, then cAMP levels would remain high even after the termination of the epinephrine signal, and glucose mobilization would continue.

Q13. Platelet-derived growth factor (PDGF) is a dimer of two polypeptide chains. What would be the predicted effect of PDGF monomers on signaling from the PDGF receptor?

Ans. PDGF monomers would not induce receptor dimerization. Since this is the first critical step in signaling from receptor protein-tyrosine kinases, they would be unable to stimulate the PDGF receptor.

Q14. Dominant negative mutants of both Ras and Raf block growth factor-stimulated cell proliferation. The inhibitory effects of dominant negative Ras are overcome by expression of activated Raf. Would you expect activated Ras similarly to overcome the inhibitory effects of dominant negative Raf? How about activated MEK?

Ans. Raf acts downstream of Ras in the MAP kinase pathway. Activated Raf can therefore bypass the effects of dominant negative Ras, but activated Ras cannot overcome the inhibitory effects of dominant negative Raf. MEK acts downstream of Raf, so activated MEK can overcome the effects of dominant negative Ras or Raf.

REVIEW THE CONCEPT

Q1. a. In what ways do channels, carriers and pumps differ in the mechanism of solute transport across plant cell membranes?

b. Name three mineral nutrients involved in redox reactions, and explain the mechanism of transport of any one. **[CSIR-UGC]**

Q2. What do you understand by Signal hypothesis? It is known that all proteins destined for export are synthesized on membrane bound ribosomes of the rough endoplasmic reticulum. Discuss the pathways of export of membrane proteins and secretory proteins pointing out the salient differences in these pathways. **[CSIR-UGC]**

Q3. a. Explain the molecular basis of sliding filament mechanism during muscle contraction.

b. Explain the role of calcium during muscle contraction. **[CSIR-UGC]**

Q4. a. Where is Ca^{2+} stored in muscle cells and how is it released during muscle contraction?

b. What is the role of Ca^{2+} in muscle contraction? **[CSIR-UGC]**

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Q5. The cytoskeleton provides a structural framework for the cell, serving as a scaffold that determines cell shape and general organization of the cytoplasm. Moreover, the cytoskeleton is responsible for cell movements.

a. What are the different kinds of protein filaments associated with cytoskeleton and what is their structural composition?

b. Would cytochalasin affect the movement of cells that extend pseudopodia or utilize flagella locomotion?

c. Which aspects of cell division would be affected by colchicines-chromosome segregation or cytokinesis?

d. Why is the polarity of actin filament important for muscle contraction? **[CSIR-UGC]**

SELF TEST

Q1. All of the following statements about the fluid mosaic model of biological membranes are true except

a. lipid molecules in the membrane readily undergo lateral diffusion

b. integral membrane proteins can undergo lateral diffusion

c. protein molecules in the membrane readily undergo transverse (flip-flop) diffusion

d. the transition temperature of a membrane is sensitive to the composition of the lipid molecules in the membrane

Q2. Which of the following peptide sequences is likely to be present in the plasma membrane?

a. Asp–Glu–Asp–Glu–Asp–Glu

b. Val–Leu–Ile–Val–Leu–Ile

c. Arg–His–Lys–Arg–His–Lys

d. Ser–Thr–Ser–Thr–Ser–Thr **[JNU]**

Q3. Longer fatty acids make membranes less fluid because

a. the long chains tend to get interwined and tangled

b. this allows greater hydrogen-bonding between the methyl groups

c. longer fatty acids bind rather tenaciously to membrane proteins

d. there are more van der Waals interactions between the chains **[JNU]**

Q4. Which of the following is *not* correct about an embedded segment of an integral membrane protein

a. a typically an α-helical segment long enough about 20-25 amino acids, to span the lipid bilayer

b. typically has polar C=O and NH groups in contact with the fatty acyl chains of phospholipids

c. typically has any charged residues facing the interior of the molecule

d. often contains hydrophobic amino acids, flanked by positively charged residues that interact with phospholipid head groups

Q5. Digitoxigenin, a cardiotonic steroid that inhibits ATPase when applied on extra cellular face of membrane, helps in accumulation of Ca^{2+} inside the cardiac muscle cells by

a. activating Na^+-K^+ pump and blocking Na^+-Ca^{2+} exchanger

b. inhibiting Na^+-K^+ pump and blocking Na^+-Ca^{2+} exchanger

c. having no effect on Na^+-K^+ pump

d. increasing passive diffusion

Q6. Which of the following statement is *correct*?

P. the plasma membrane is highly impermeable to all charged molecules

Q. all membrane transport proteins so far known traverse the lipid bilayer, and their polypeptide backbones generally extend back and forth across the membrane a number of times

R. ionophores operates by shielding the charge of the transported ion so that it can penetrate the hydrophobic interior of the lipid bilayer

S. since the Na^+-K^+ ATPase exchanges equal numbers of Na^+ and K^+ ions in each pumping cycle, it is electrically neutral

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a. P, Q
b. Q, R
c. R, S
d. P, S

Q7. Why do glycolipids exist almost exclusively on the exterior side, but not the cytoplasmic side, of the cell membrane?
a. the inner layer of the membrane is not thick enough to accommodate carbohydrates
b. carbohydrates are added only to lipids on the lumen side of the ER and golgi membranes
c. flippases move the glycolipids to this side of the membrane
d. carbohydrates are removed from the cytoplasmic side by glycosylase enzymes

Q8. What characteristic domain is found in eukaryotic proteins that enable them to enter the endoplasmic reticulum?
a. stop transfer domain
b. signal sequence
c. signal sequence receptor
d. signal recognition protein

Q9. Although multiple disulfide bonds are possible during the formation of the tertiary structure of some secretory proteins, only the correct ones are found in the secreted product. This is primarily due to the fact that
a. incorrectly folded proteins are degraded by lysosomes
b. processing and folding is continued in the endosomes
c. a protein facilitates the formation of correct disulfide bonds in the endoplasmic reticulum
d. only correctly folded proteins are translated in the endoplasmic reticulum

Q10. Which of the following is *not* correct about secreted proteins
a. are all present in one type of vesicle
b. are often synthesized as a precursor and cleaved before release
c. may be continuously exocytosed
d. may require an acidic compartment for maturation

Q11. Targeting of enzymes to lysosomes involves which of the following steps?
1. dissociation of ligand from receptor
2. binding to a mannose-6-phosphate receptor
3. decrease in pH
4. fusion with late endosome
5. addition of phosphate to mannose
a. 2 and 5
b. 2, 3 and 4
c. 2, 4 and 5
d. 1, 2, 3, 4 and 5

Q12. Which one of the following statements *incorrectly* describes lysosomes.
a. hydrolytic enzymes move to the lysosomes via vesicular transport
b. materials phagocytosed into the cell go to the lysosomes
c. the pH of lysosomes is more acidic than that of the cytoplasm
d. hydrolytic enzymes are transported to lysosomes directly from the cytosol

Q13. What mechanism is responsible for movements of eukaryotic cilia and flagella?
a. kinesin moving on microfilaments
b. dynein moving on microfilaments
c. dynein moving on microtubules
d. myosin moving on microfilaments

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Q14. Cytochalasins are drugs that interfere with actin polymerization into microfilaments. If you add cytochalasin to cultured mammalian cells that have just begun mitosis, what is most likely to happen?

a. the cells will arrest at mitotic metaphase
b. the cells will cease metabolism and die
c. the cells will complete mitosis and arrest at cytokinesis
d. the cell will arrest at mitotic anaphase

Q15. Identify the correct set of three statement for cytoskeletal protein filaments from the following list

A. actin filament is about 8 nm wide
B. actin filament is 25 nm wide
C. intermediate filaments have size intermediate between actin filaments and microtubules
D. protofilaments of microtubules are composed of alpha/beta tubulin heterodimer
E. colchicine binds to the tubulin subunits in spindle microtubule causing disassembly to free units

a. C, D, E
b. B, C, D
c. A, C, D
d. A, B, C

Q16. The common pathway of entry into the endoplasmic reticulum (ER) of secretory, lysosomal, and plasma membrane proteins is best explained by which of the following?

a. binding of their mRNAs to a special class of ribosomes attached to the ER
b. addition of a common sorting signal to each type of protein after completion of synthesis
c. addition of oligosaccharides to all three types of proteins
d. presence of a signal sequence that targets each type of protein to the ER during synthesis

Q.17. Membrane carrier proteins differ from membrane channel proteins by which of the following characteristics?

a. carrier proteins are glycoproteins, while channel proteins are lipoproteins
b. carrier proteins transport molecules down their electrochemical gradient, while channel proteins transport molecules against their electrochemical gradient
c. carrier proteins can mediate active transport, while channel proteins cannot
d. carrier proteins are synthesized on free cytoplasmic ribosomes, while channel proteins are synthesized on ribosomes bound to the endoplasmic reticulum.

Q18. The KDEL sequence, found on luminal proteins of the ER, is responsible for

a. translocation of proteins into the ER lumen
b. insertion of proteins into the membrane of the ER
c. recognition by signal peptidase of the signal sequence
d. retrieval of ER luminal proteins from the Golgi

Q19. Treatment of root tip meristem cells with the microtubule inhibitor colchicine results in all of the following *except*

a. induction of polyploidy
b. prevention of cytokinesis
c. inhibition of mitotic spindle assembly
d. cessation of DNA replication

Q20. Which of the following best supports the endosymbiotic theory of the evolutionary origin of mitochondria?

a. Mitochondria, chloroplasts, and prokaryotes contain electron carriers
b. Genes for mitochondrial pyruvate dehydrogenase subunits are found in the nuclear DNA
c. Mitochondrial and bacterial ribosomal functions are inhibited by the same antibiotics
d. The outer mitochondrial membrane contains the protein porin

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Q21. All of the following processes occur in the pathway leading to regulated protein secretion in animal cells *except*
a. formation of transport vesicles from the rough endoplasmic reticulum
b. an increase in the concentration of cytosolic calcium ions prior to secretion
c. synthesis of an amino-terminal signal sequence
d. phosphorylation of a mannose residue in a glycoprotein

Q22. Paracrine hormones are those which
a. act on the same cells which produce them
b. act on the cells in the immediate vicinity of the cells which produce them
c. act on the cells which are distantly located from the cells where they are produced
d. do not require any second messenger to transmit their respective signals into the target cell **[JNU]**

Q23. Which of the following statements are *true* for steroid hormones?
P. increase the enzymatic activity of pre-existing target enzyme
Q. act at cell nucleus
R. interact with the plasma membrane receptors of target cells
S. form a complex with receptor and acts as trasncriptional enhancers
a. PR
b. QS
c. PQ
d. RS **[GATE]**

Q24. Response of a tissue almost stopped when exposed for prolonged time to a chemical. After washing and leaving it for sometime, the tissue started responding to the same chemical at the same concentration. The reason for the reduced response is likely to be
a. increased apoptosis of the cells
b. increased necrosis of the cells
c. desensitization/down-regulation of the receptors
d. fixation of the cells **[JNU]**

Q25. Which one of the following statements does *not* correctly describes G-proteins?
a. energy for their activity comes from GTP molecules
b. they become activated by interaction with '7-pass' receptors
c. G-proteins are membrane associated proteins
d. G-protein activation always increase the concentration of cAMP

Q26. After interacting with a cell-surface receptor that signals activation to a trimeric G-protein, the first event that happens is
a. GTP is hydrolyzed to produce GDP
b. tyrosines on the G-protein become phosphorylated
c. the G-protein dissociates into a β- and $\alpha\gamma$-subunits
d. GTP replaces GDP in the α-subunit

Q27. Cholera toxin exerts its harmful effects in gut cells by
a. inhibiting the receptor part of the CFTR protein
b. inducing the onset of apoptosis
c. stimulating the production of cyclic AMP
d. blocking the chloride channel in the cell membrane

Q28. Which of the following is *not* true about NO
a. acts as intracellular signaling molecule as well as neurotransmitter
b. regulates the vasodilation

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c. synthesized from L-arginine
d. induces adenylate cyclase, which catalyzes cAMP formation **[IISc]**

Q29. IP3 initially causes Ca^{2+} to be released into the cytoplasm from
a. mitochondria
b. lysosome
c. the endoplasmic reticulum
d. the plasma membrane (from extracellular to intracellular)

Q30. Match the biological functions mentioned in Column A with the enzymes given in Column B.

Column A	*Column B*
P. diacylglycerol synthesis	1. protein kinase A
Q. CREB phosphorylation	2. ras
R. GTP hydrolysis	3. phospholipase
	4. phospholipase D
	5. protein kinase G

a. P – 3, Q – 1, R – 5
b. P – 4, Q – 1, R – 2
c. P – 3, Q – 1, R – 2
d. P – 3, Q – 5, R – 2 **[GATE]**

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Chapter - 10

Cell Cycle and Cancer

The process of cell division is the culmination of a series of events. The period between two mitotic divisions defines the *somatic cell cycle*. The time from the end of one mitosis to the start of the next is called **interphase**. The period of actual division, corresponding to the visible mitosis, is called **M phase**. The interphase is divided into the G1, S and G2 periods. Cells may withdraw from the cycle into G0 or re-enter from it. The timing of the four stages varies from species to species, from organ to organ within a species. In most cells the whole of M phase takes only about an hour, which is only a small fraction of the total cycle time.

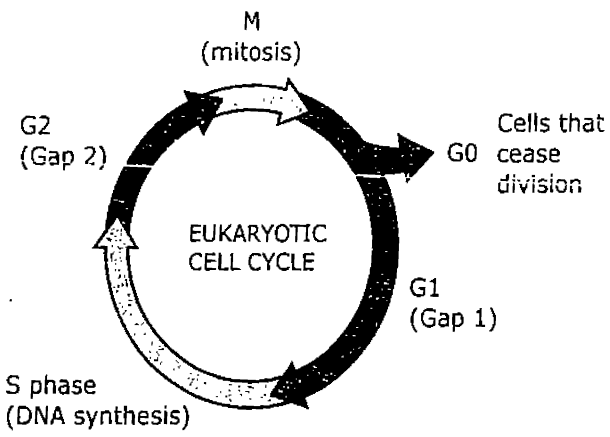


Figure : 10.1
The four successive phases of a standard eukaryotic cell cycle. During interphase the cell grows continuously; during M phase it divides. DNA replication is confined to the part of interphase known as S phase. G1 phase is the gap between M phase and S phase; G2 is the gap between S phase and M phase. Cells in G1, if they have not yet committed themselves to DNA replication, can pause in their progress around the cycle and enter a specialized resting state, often called G0, where they can remain for days, weeks, or even years before resuming proliferation.

10.1 CELL CYCLE

Cell cycle is an ordered series of events. There are four main stages of the cell cycle: *M phase* - mitosis, and three stages that are components of *interphase* - G1, the gap period between the end of mitosis and the start of DNA replication; S, the period during which DNA synthesis occurs; and G2, the gap period following DNA replication and preceding the initiation of the mitotic prophase.

The engines that drive progression from one step of the cell cycle to the next are a series of protein complexes composed of two subunits: a **cyclin** and a **cyclin dependent protein kinase** (abbreviated CDK). Cyclin is regulatory component whereas CDK is catalytic and acts as protein kinase. Cyclins are so called because they undergo a cycle of synthesis and degradation in each division cycle of the cell. In every eukaryote, there is a family of structurally and functionally related cyclin proteins. There are three major classes of cyclins, each defined by the stage of the cell cycle at which they bind CDKs and function : **G_1-cyclins**, **S-cyclins** and **M-cyclins**. Cyclins bind to CDK molecules and control their ability to phosphorylate appropriate target proteins.

Cyclin-dependent protein kinases also constitute a family of structurally and functionally related proteins. *Kinases* are enzymes that add phosphate groups to target substrates; for *protein kinases* such as CDKs, the substrates are proteins. CDKs are so named because their activities are regulated by cyclins and because they catalyze the phosphorylation of specific serine and threonine residues of specific target proteins.

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The target proteins for CDK phosphorylation are determined by the associated cyclin. Because different cyclins are present at different phases of the cell cycle so different phases of cell cycle are characterized by the phosphorylation of different target proteins. The phosphorylation events are transient and reversible. When the cyclin CDK complex disappears, the phosphorylated substrate proteins are rapidly dephosphorylated by protein *phosphatases*. There are three major classes of cyclin-CDK complexes that control passage through the cell cycle: the *G1, S-phase,* and *mitotic cyclin-CDK complexes*.

In yeast cells, a single CDK protein binds all classes of cyclins and drives all cell-cycle events by changing cyclin partners at different stages of the cycle. In vertebrate cells, by contrast, there are more than one CDKs to interact with G1-cyclins, S-cyclins, and M-cyclins.

Table 10.1 : Examples of selected cyclins and cyclin-dependent kinases (CDKs)

Organisms	**Cyclin**	**CDKs**
S. pombe	Cdc13	Cdc2 (CDK1)
S. cerevisiae	G1 cyclin : Cln1, Cln2, Cln3	Cdc28 (CDK1)
	S-phase cyclins : Clb5, Clb6	Cdc28 (CDK1)
	Mitotic cyclins : Clb1, Clb2, Clb3, Clb4	Cdc28 (CDK1)
Vertebrates	G1 cyclin : Cyclins D	CDK4, CDK6
	Late G1 and S-cyclin : Cyclin E	CDK2
	S-cyclin : cyclin A	CDK2
	Mitotic cyclin : Cyclin B	CDK1

Note: The original name of Cdk1 was Cdc2 in both vertebrates and fission yeast, and Cdc28 in budding yeast.

When cells are stimulated to divide, G1 cyclin-CDK complexes are expressed first. These prepare the cell for the S phase by inducing enzymes synthesis required for DNA replication and S-phase cyclins and CDKs. In higher organisms, control of the cell cycle is achieved primarily by regulating the synthesis and activity of G1 CDK complexes.

Mitotic CDK complexes or *Maturation promoting factor* (MPF) or *M phase promoting factors* are synthesized during the S phase and G2. Mitotic Cdk complexes induce chromosome condensation, breakdown of the nuclear envelope, assembly of the mitotic spindle apparatus, and alignment of condensed chromosomes at the metaphase plate. After the proper association of all chromosomes with spindle microtubules has occurred, the mitotic CDK complexes activate the **anaphase-promoting complex** (APC). This multiprotein complex is a *ubiquitin ligase* that directs the *ubiquitin-mediated proteolysis* of anaphase inhibitors leading to inactivation of the protein complexes that connect sister chromatids at metaphase. Degradation of these inhibitors thus permits the onset of anaphase. The destruction of cyclin is as important for exit from mitosis as its synthesis is for entry. The APC also directs proteolytic degradation of the mitotic cyclins.

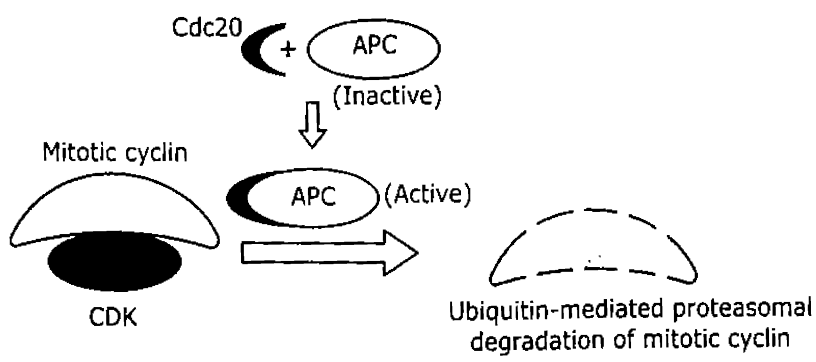


Figure : 10.2
Mitotic cyclin ubiquitination is performed by APC, which is activated in late mitosis by the addition of an Cdc20. APC contains binding sites that recognize specific amino acid sequences of the target protein.

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The resulting decrease in mitotic CDK activity permits the separated chromosomes to decondense, the nuclear envelope to re-form around daughter-cell nuclei during telophase, and the cytoplasm to divide at cytokinesis, yielding the two daughter cells.

Passage through three critical cell-cycle transitions, G_1 to S phase, metaphase to anaphase, and anaphase to telophase and cytokinesis, is irreversible because these transitions are triggered by the regulated degradation of proteins. As a consequence, cells are forced to traverse the cell cycle in one direction only.

Regulation of activity of cyclin-CDK complex

Cyclin/CDK activity is governed by three mechanisms:

- Cyclin expression and degradation,
- Phosphorylation/dephosphorylation reaction,
- Binding of CDK inhibitor proteins (CKIs)

The rise and fall of cyclin levels is the primary determinant of CDK activity during the cell cycle. The protein kinase activity of the cyclin-CDK complex also depends on the phosphorylation state of the catalytic CDK subunit. The CDK activity is inhibited by protein-tyrosine kinase, **Wee1** catalyzed phosphorylation of tyrosine. This inhibitory phosphate is removed by the **Cdc25** protein phosphatase. A decrease in Wee1 activity and increase in Cdc25 activity, resulting in activation of the cyclin-CDK complex. Full activation of the cyclin-CDK complex occurs when a separate kinase, the **CDK-activating kinase** (CAK), phosphorylates an activating *threonine* residue near the CDK active site. This causes a small conformational change that further increases the activity of the CDK, allowing the kinase to phosphorylate its target proteins effectively.

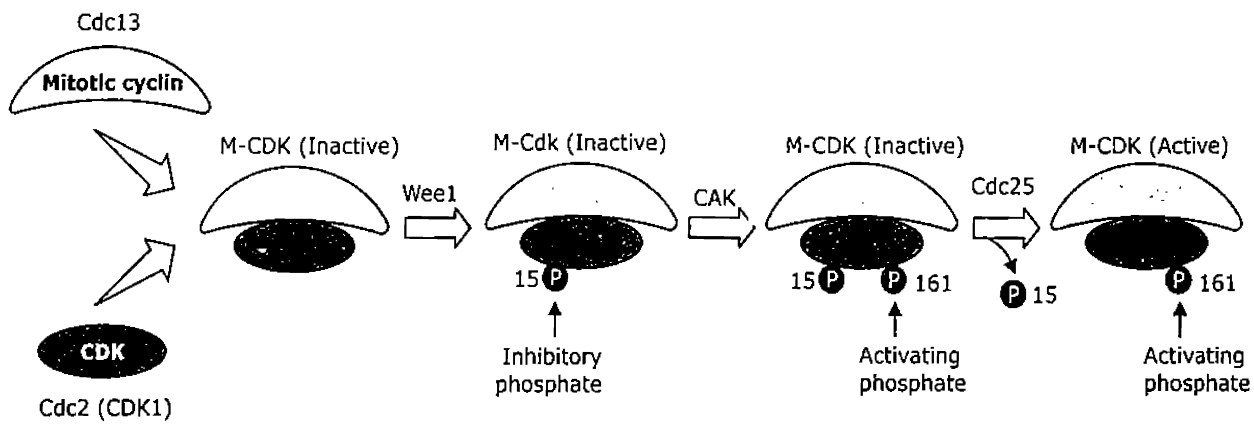


Figure 10.3 : Regulation of activity of M-CDK complex in *S. pombe*. Interaction of mitotic cyclin with cyclin-dependent kinase forms M-CDK complex. The CDK subunit can be phosphorylated at two regulatory sites: by Wee1 at *tyrosine-15* and by CAK at *threonine-161*. Removal of the phosphate on *tyrosine-15* by Cdc25 phosphatase yields active M-CDK complex.

Cyclin-CDK complexes can also be regulated by the binding of **CDK inhibitor proteins (CKIs)**. CKIs binding make the cyclin-CDK complex inactive. There are variety of CKI proteins like, p27, p21, p16 and they are primarily employed in the control of G_1 and S phase.

Cell cycle checkpoints

The cell cycle is a highly regulated and a dependent series of events, mediated by a number of *checkpoints*. Cell cycle checkpoints function to ensure that incomplete or damaged chromosomes are not replicated and passed on to daughter cells. In most cells there are several checkpoints in the cell cycle at which the cycle can be arrested if previous events have not been completed. There are 3-major checkpoints in the cell cycle :

- G_1 check point (towards the end of G_1 phase)
- G_2 check point (towards the end of G_2 phase)
- Metaphase checkpoint (M-phase)

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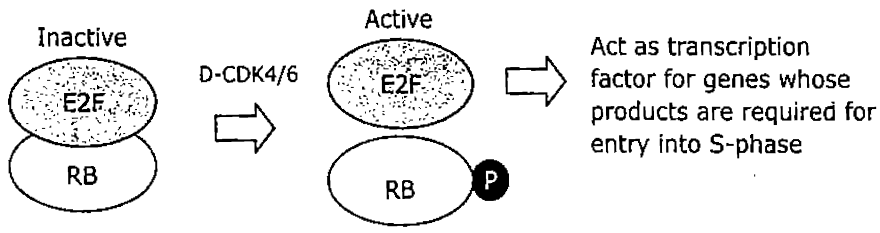
In most of the cells, the major cell cycle checkpoint is in late G_1 (commitment to chromosome replication). This G_1 check point is called *start* (in yeast cell) or *restriction point* (in animal cell). G_1 checkpoint blocks progression into S phase. G_2, called the *mitotic entry checkpoint* is most important in fission yeast.

Role of Rb protein in cell cycle regulation

Rb protein is a product of retinoblastoma (Rb) gene, a **tumor suppressor gene** (genes whose inactivation contributes to the development of a tumor are called tumor-suppressor genes). Loss-of-function mutations in Rb genes are associated with the disease *retinoblastoma.* The Rb gene is located on chromosome 13q14.2. The Rb protein encoded by the retinoblastoma gene is a 110-kDa polypeptide chain, also known as p105RB, which can be phosphorylated.

How the product of RB gene regulates cell division?

RB is a substrate for CDK-cyclin D complexes, and exerts its effects during the part of G1 that precedes the restriction point. It acts as a break in mammalian G_1 cells. E2F, a transcription factor, binds to specific DNA sequence and induces many genes to transcribe. High levels of E2F activates transcription of the *cyclin A* gene. The products of these genes are required for S-phase of cycle. E2F activity is controlled by Rb protein. When Rb protein binds to E2F its activity is suppressed and transcription of gene involved in S-phase stops. Phosphorylation of Rb protein, reducing it affinity for E2F and allow E2F to activate S-phase genes expression. Phosphorylation of Rb protein is initiated by cyclin D-CDK4 and cyclin D-CDK6 in mid G1. Un or hypo-phosphorylated RB proteins blocks cell cycle progression. In homozygous null *rb* cells, Rb protein is permanently inactive. Thus, E2F is always able to promote S phase, and the arrest of normal cells in late G1 does not occur in retinoblastoma cells.



Retinoblastoma : Retinoblastoma is a childhood tumor of the retina. The gene whose loss is critical for development of the cancer is called the retinoblastoma, or *RB,* gene. Retinoblastoma arises when both copies of the *RB* gene are inactivated. There are two forms of the disease, one hereditary, the other not. Hereditary form affects both eyes whereas in the non-hereditary form only one eye is affected. In the hereditary form all cells lack one of the normal two functional copies of a tumor suppressor gene, and tumors occur where the remaining copy is lost or inactivated by a somatic mutation. In the non-hereditary form (sporadic form) all cells initially contain two functional copies of the gene, and the tumor arises because both copies are lost or inactivated through the coincidence of two somatic mutations in one cell.

Role of p53 in cell cycle regulation

DNA-damage blocks progression through the cell cycle until the damage is repaired. Damage to DNA can result from chemical and physical agents. Tumor-suppressor protein, p53, contributes to arrest of cells with damaged DNA *at DNA-damage checkpoint*. Cells with functional p53 arrest in G1 and G2, whereas cells lacking functional p53 do not arrest in G1.

p53 gene codes for p53 protein is a well-characterized **tumor suppressor gene** that encodes a phosphorylated protein with an apparent molecular weight of 53 kDa. It is located on human chromosome 17p13.1 and encodes a polypeptide chain of 375 amino-acid residues. p53 protein is a *transcription factor* that regulates the expression of cell-cycle regulatory proteins. p53 is not required for normal cell division. Thus under normal conditions it is extremely unstable. The proteasomal degradation of p53 results from its

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polyubiquitination by a ubiquitin ligase called *Mdm2.* In case of DNA damage, the rapid degradation of p53 is inhibited by ATM, which phosphorylate p53 at a site that interferes with binding to Mdm2.

In response to DNA damage, p53 levels increase and arrest the cell at the G1-phase of the cell cycle. If all of the repairs have been made to the DNA, the cell divides normally and completes the cell cycle. However, if the cell still contains mutated or duplicated DNA sequences, it dies by a suicidal *apoptotic* mechanism to prevent its expansion. In those cells that have mutated or lost p53 genes, the arrest at G1 does not occur, and the cells that have mutated genomes proliferate and become cancerous.

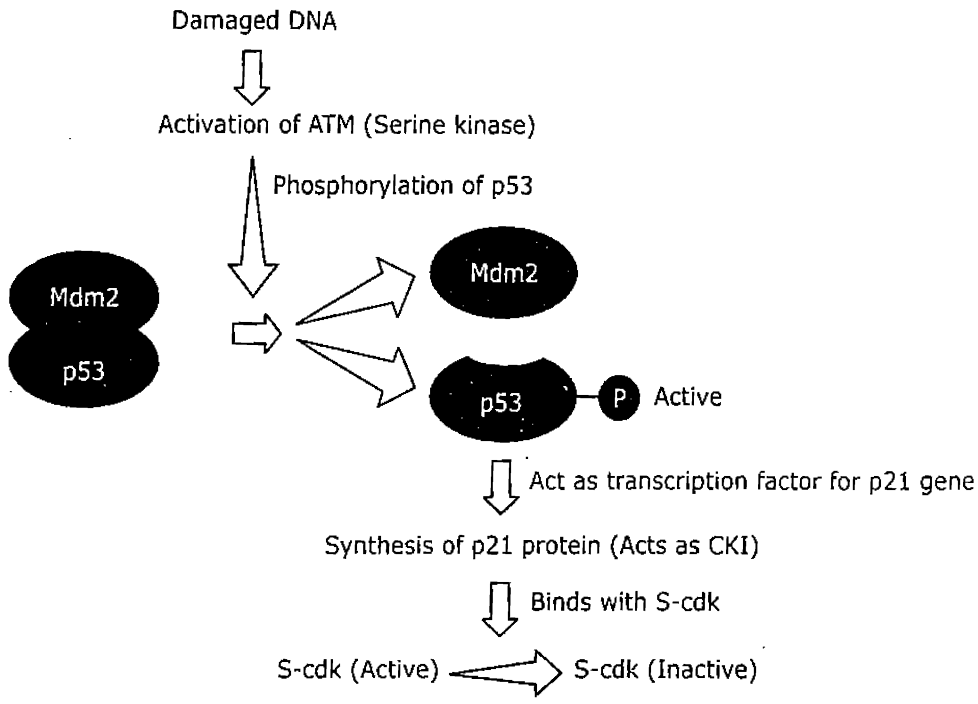


Figure 10.4 : How DNA damage arrest the cell cycle in G1

10.2 CELL DIVISION

In eukaryotes, two types of cell divisions partition the genetic material into offspring, or daughter cells. In one process called **mitosis**, parent cell divides into two daughter cells and each receive an exact copy of the genetic material in the parent cell. In the other partitioning process, the genetic material must precisely halve so that fertilization will restore the diploid complement. This cellular process is **meiosis**.

Mitosis

Mitosis is the process that partitions newly replicated chromosomes equally into two daughter cells. The term mitosis (derived from the Greek word, meaning 'thread') was introduced by Walther Flemming in 1882. During mitosis, one round of DNA replication is followed by a single round of chromosome segregation and generating two genetically identical daughter cells.

Mitosis is a continuous process. They are conventionally divided into four substages: *prophase, metaphase, anaphase,* and *telophase* (Greek: *pro-,* before; *meta-,* mid; *ana-,* back; *telo-,* end).

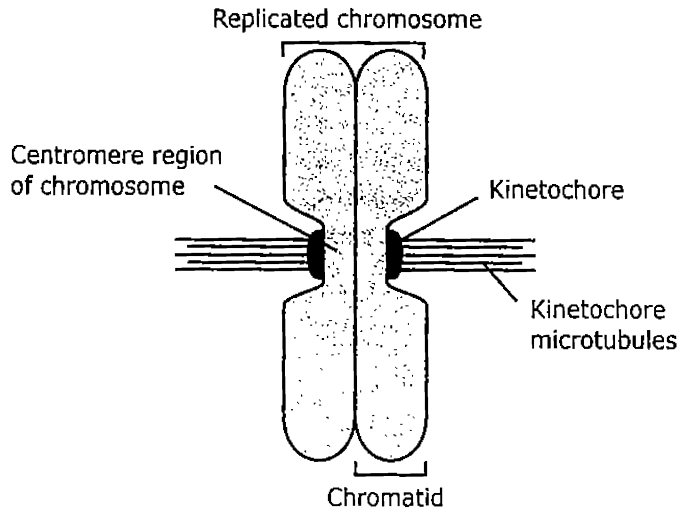
Prophase

The chromatin, which is diffuse in interphase, slowly condenses into well-defined chromosomes. Each chromosome has duplicated during the preceding S phase and consists of two sister chromatids; each of these contains a specific DNA sequence known as a *centromere*, which is required for proper segregation. At this point, the sister chromatids are kept together by a complex, called **cohesin**, made up of at least four different proteins.

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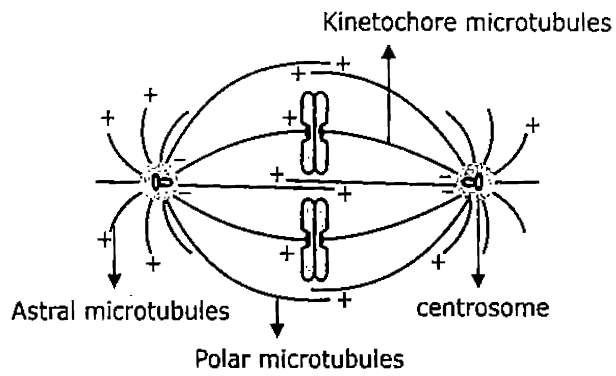
Prophase ends abruptly with disruption of the nuclear envelope, which breaks into membrane vesicles that are indistinguishable from bits of endoplasmic reticulum. These vesicles remain visible around the spindle during mitosis. At this time also the *nucleolus* disappears.

Toward the end of prophase, the cytoplasmic microtubules that are part of the interphase cytoskeleton disassemble and the main component of the mitotic apparatus, the mitotic spindle, begins to form. This is a bipolar structure composed of microtubules and associated proteins. The spindle initially assembles outside the nucleus between separating centrosomes.



Mitotic spindle

The mitotic spindle at metaphase is of three types : **Kinetochore microtubules**, which attach with chromosomes at specialized attachment sites on the chromosomes called *kinetochores*. The kinetochore is a specialized attachment site at the chromosome centromere. The sister chromatids of a metaphase chromosome are transported to each pole along the kinetochore microtubules. **Astral microtubules**, which forms the aster; they radiate outward from the centrosome toward the cortex of the cell. **Polar microtubules** do not interact with chromosomes but instead interdigitate with polar microtubules from the opposite pole.



Metaphase

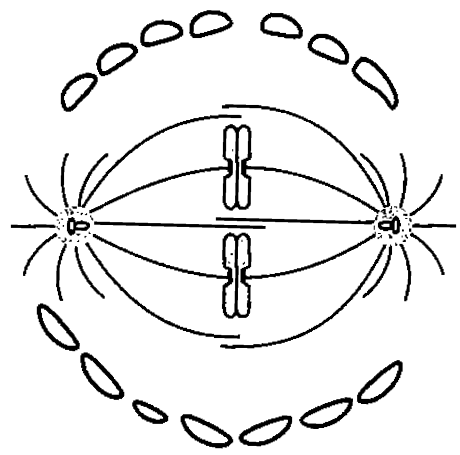
In the beginining of metaphase, specialized protein complexes called **kinetochores** mature on each centromere and attach to some of the spindle microtubules, which are then called kinetochore microtubules. The remaining microtubules in the spindle are called polar microtubules, while those outside the spindle are called astral microtubules.

The kinetochore microtubules align the chromosomes in one plane halfway between the spindle poles. Each chromosome is held in tension at this *metaphase plate* by the paired kinetochores and their associated microtubules, which are attached to opposite poles of the spindle.

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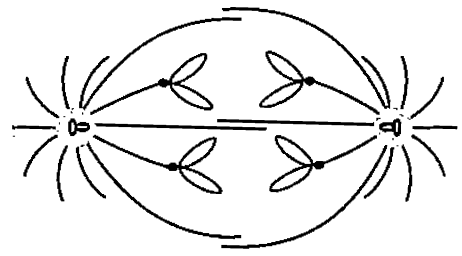
Assembly of the metaphase spindle requires two types of events : Attachment of spindle microtubules to the poles and capture of chromosomes by kinetochore microtubules. A kinetochore contacts the side of a microtubule and then slides along the microtubule to the (+) end in a process that may involve kinesins on the kinetochore. The kinetochore *caps* the (+) end of the microtubule. A combination of microtubule motor proteins at the kinetochore and microtubule dynamics at the (+) end of kinetochore microtubules is thought to position the chromosomes equally between the two spindle poles.



Metaphase

Anaphase

As mentioned, each sister chromatid of a metaphase chromosome is attached to microtubules via its kinetochore, a complex of proteins assembled at the centromere. The opposite ends of these kinetochore microtubules associate with one of the spindle poles. At anaphase, the kinetochore spindle fibers pull the two kinetochores toward the opposite poles.



Anaphase

Sister chromatids do not separate because they are held together at their centromeres by multiprotein complexes called **cohesins.** Cohesins associate with chromatin at S phase. During anaphase, **separase**, a ubiquitous *cysteine protease*, degrades cohesins. A **securin** protein keeps separase in an inactive state. The destruction of securin releases separase, which is then free to cleave cohesin complex. Destruction of securin is mediated by **anaphase-promoting complex** (APC), a *ubiquitin ligase*. Each chromatid (now called a chromosome) to be pulled slowly toward the pole it faces.

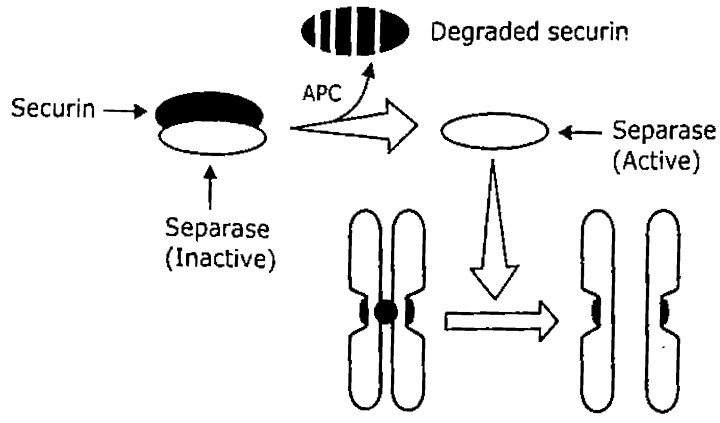


Figure : 10.5
Separation of sister-chromatid as a result of proteolysis of cohesin. The destruction of securin allows separase to cleave a subunit of cohesin.

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All of the newly separated chromosomes move at the same speed, typically about 1 μm per minute. The chromosomes move by two independent and overlapping processes. Hence anaphase can be divided into two distinct stages, *anaphase A* and *anaphase B* (or early and late anaphase). Anaphase A is characterized by the shortening of kinetochore microtubules, which pulls the chromosomes toward the poles. Microtubule shortening during anaphase A is due to depolymerization of kinetochore preferentially to the (+) ends of the microtubules. During anaphase B, the two poles move farther apart. It happens as a result of spindle elongation which occurs due to sliding of polar microtubules past one another and pulling forces exerted by astral microtubules.

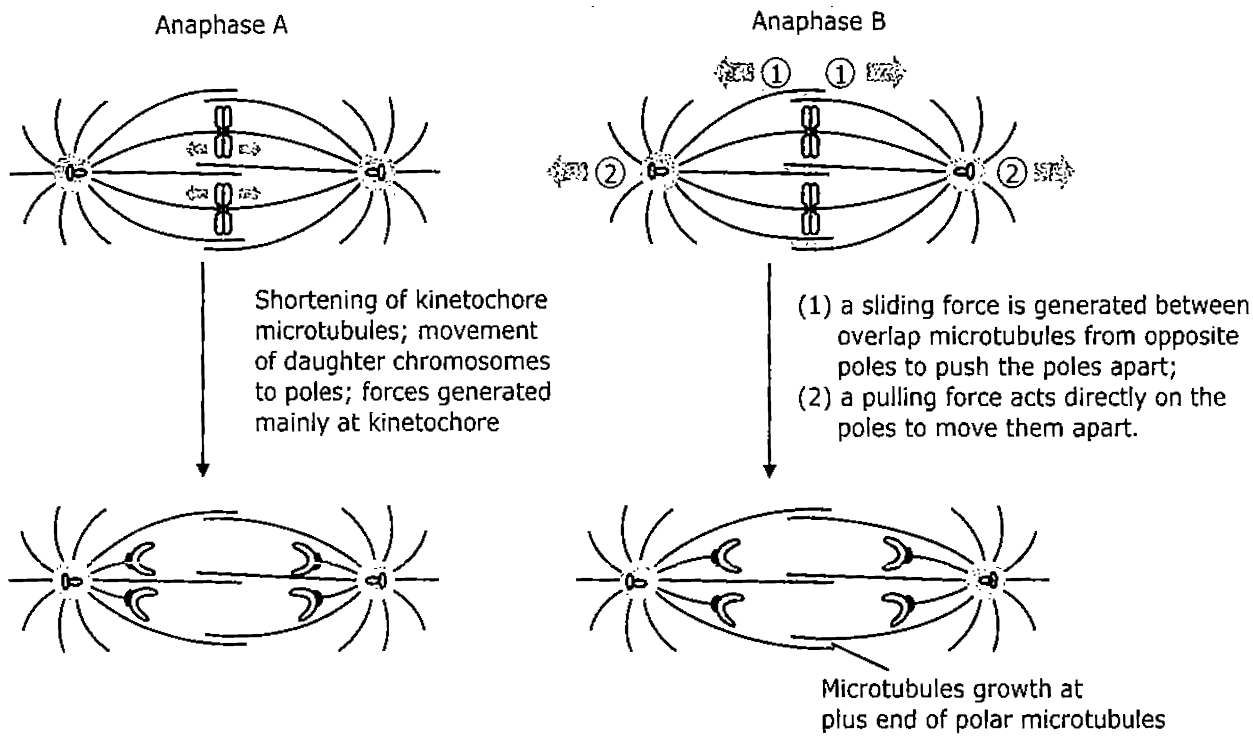


Figure 10.6 : Separation of daughter chromosomes at anaphase A and B in animal cells.
Adapted from Alberts et al., The cell, fourth edition, Garland Science.

Telophase

In telophase (telos- end) the separated daughter chromosomes arrive at the poles and the kinetochore microtubules disappear. The polar microtubules elongate still more, and a new nuclear envelope re-forms around each group of daughter chromosomes. The condensed chromatin expands once more, the nucleoli- which had disappeared at prophase-begin to reappear, and mitosis is at an end.

CYTOKINESIS

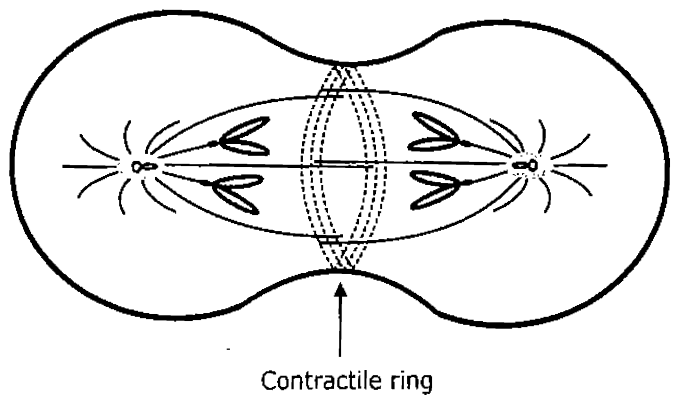
The cell cycle culminates in the division of the cytoplasm by *cytokinesis*. In a typical cell, cytokinesis accompanies every mitosis, although some cells, such as *Drosophila* embryos, undergo mitosis without cytokinesis and become multinucleate. Cytokinesis begins in anaphase and ends in telophase, reaching completion as the next interphase begins.

Cytokinesis in animal cell

Cytokinesis appears to be guided by organized bundles of actin filaments in animal cells. During cytokinesis the cytoplasm divides by a process called *cleavage*. Cleavage is accomplished by contraction of circumferentially oriented filaments *actin filament* and *myosin II* bound to the cytoplasmic face of the plasma membrane. This contractile ring defines the cleavage *furrow*. The muscle like sliding of actin and myosin II filaments in the contractile ring generates the force required for cleavage.

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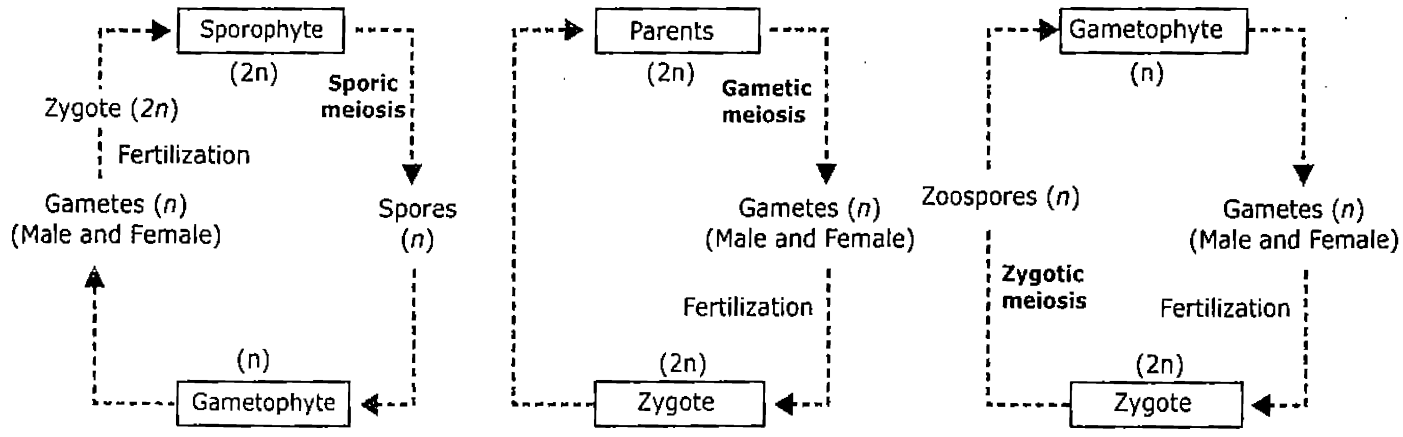


Cytokinesis in plant cell

Cytokinesis occurs by a special mechanism in higher plant cells, where the cytoplasm is partitioned by the construction of a new cell wall, the *cell plate*, inside the cell. A cytoskeleton framework determines the plane of plant cell division. Array of microtubules appears in G2 phase and forms a ring around the entire cell just beneath plasma membrane. It is called the **preprophase band**. It also contains numerous actin filaments in addition to microtubule. The microtubule disappears before metaphase, but the actin filaments remain and provide a memory of the predetermined division plane. The polar spindle microtubules assemble in this plane and forms a cylinder like structure called phragmoplast. Small membrane bound vesicles derived from the golgi apparatus and filled with cell walled precursors associate with microtubule and form the early cell plate. The polysaccharide molecule delivered by these vesicles forms the matrix polysaccharide. Some time later cellulose microfibrils are laid down within the cell plate to complete the new cell wall.

MEIOSIS

Meiosis (termed coined by Farmer and Moore) is a specialized form of cell division in which number of chromosomes is reduced to half. The reduction in chromosome number is achieved by one round of DNA replication being followed by two rounds of chromosome segregation with no intervening round of DNA replication. Meiosis occurs during sexual life cycle of all eukaryotes. Meiosis is of three types–*zygotic*, *sporogenic* and *gametic*–depending on the stage where it occurs during sexual cycle.



Meiosis is divided into two parts: *meiosis I* and *meiosis II*. At the end of the meiotic process, there are four daughter cells rather than the two produced at the end of the mitotic process. Each of the resulting daughter cells has one half of the number of chromosomes as the parent cell.

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MEIOSIS I

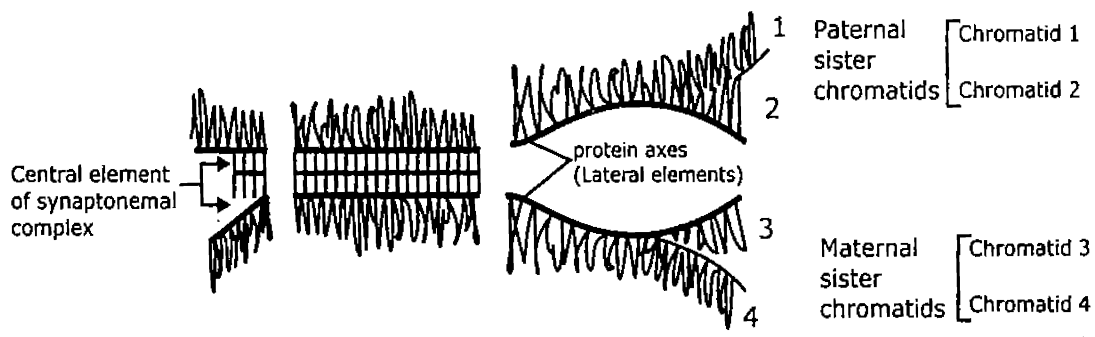
Prophase I of meiosis I is divided into five sub stages- Leptotene, Zygotene, Pachytene, Diplotene and Diakinesis.

Leptotene

Prophase I begin at the leptotene stage. When each chromosome is first seen to have condensed from its interphase conformation to produce a long thin thread with a proteinaceous central axis. Each chromosome is attached at both of the ends with the nuclear envelope via a specialized structure called an *attachment plaque*. Although each chromosome has replicated and consists of two sister chromatids, these chromatids are unusually closely apposed, and each chromosome therefore appears to be single (separate chromatids will not become visible until late in prophase, at either the diplotene stage or in diakinesis). In the leptotene to zygotene transition, the tips of the chromosomes move until most end up in a limited region near each other. This forms an arrangement called a *bouquet stage*.

Zygotene

Leptotene is considered to end and the zygotene stage of prophase to begin as soon as **synapsis**, or intimate pairing, between the two homologs is initiated. The initial recognition requires that the homologs recognize each other from a distance. *How the maternal and the paternal copy of each chromosome recognize each other is still unknown*? Synapsis often starts when the homologous ends of the two chromosomes are brought together on the nuclear envelope and continues inward in a zipperlike manner from both ends, aligning the two homologous chromosomes side by side, In other cases, synapsis may begin in internal regions of the chromosomes and proceed toward the ends, producing the same type of alignment. Each gene is thus thought to be brought into juxtaposition with its homologous gene on the opposite chromosome. As the homologs pair, their ropelike proteinaceous axes are brought together to form the two lateral elements, or sides, of the long ladderlike structure called the **synaptonemal complex**. Proteins such as Zip1 (Yeast) or Scp1 (Mammals) form the center of the synaptonemal complex. In mammalian cells, two other proteins Scp2 and Scp3, create a bipartite structure along the bivalent's axes. Each resulting chromosome pair in meiotic prophase I is usually called a **bivalent**, but since each homologous chromosome in the pair is a **tetrad**, it is another commonly used term.



Pachytene

As soon as synapsis is complete all along the chromosomes, the cells are said to have entered the pachytene stage of prophase, where they may remain for days. At this stage, large **recombination nodules** (large protein complexes, contain Rad51; eukaryotic version of RecA protein) appear at intervals on the synaptonemal complexes and are thought to mediate crossing over. These exchanges result in crossovers between two nonsister chromatids, that is, one from each of the two paired homologous chromosomes. Although invisible at pachytene each such crossover will appear later as a chiasma.

Crossing over : It is a physical exchange between non-sister chromatids in a pair of homologous chromosome. The exchange process consists of breaking and rejoining of two chromatids. It is the reciprocal exchange of

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equal and corresponding segment between them. Direct cytological evidence that homologous chromosome exchange part during crossing over was obtained in 1931 by Stern (working with *Drosophilla*) and by H.B.Creighton and B.McClintock (working on maize). Crossing occurs more or less random along the length of a chromosome pair. The crossing over occurs in the tetrad stage (bivalent) of pachytene stage of meiosis I. The crossing over may occur also in somatic cells as reported by Stern in *Drosophilla*. Male *Drosophilla* and female silkworm, *Bombyx mori* are example of organisms in which crossing over doesn't occur in meiotic cell.

Diplotene

Desynapsis begins the diplotene stage of meiotic prophase I. The synaptonemal complex dissolves, allowing the two homologous chromosomes in a bivalent to pull away from each other to some extent. However, each bivalent remains joined by one or more **chiasmata** representing the sites where crossing over has occurred. In oocytes (developing eggs), diplotene can last for months or years, since it is at this stage that the chromosomes decondense and engage in RNA synthesis to provide storage materials for the egg. In the extreme, the diplotene chromosomes can become highly active in RNA synthesis and expand to an enormous extent, producing the *lampbrush chromosomes* found in amphibians and some other organisms.

Diakinesis

Diplotene merges imperceptibly into diakinesis, the stage of transition to metaphase, as RNA synthesis ceases and the chromosomes condense, thicken, and become detached from the nuclear envelope. Each bivalent contains 4 separate chromatids, with each pair of sister chromatids linked at their centromeres. After prophase, cell enters into metaphase I and followed by anaphase I.

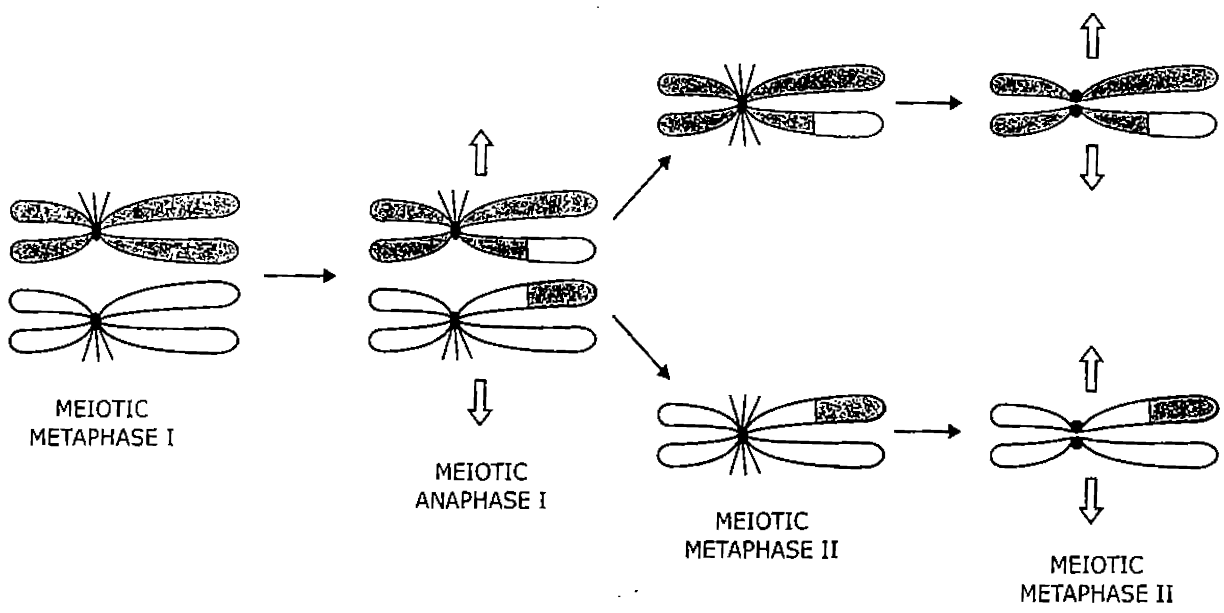


Figure 10.7 : Comparison of the mechanisms of chromosome alignment (at metaphase) and separation (at anaphase) in meiotic division I and meiotic division II.

Metaphase I, Anaphase I and **Telophase I**

Metaphase I is marked by the breakdown of the nuclear membrane and the attachment of kinetochore microtubules to the bivalent. During *metaphase I*, the homologous chromosomes move to the equatorial plane, called the *metaphase plate*. Unlike in mitosis, in which sister chromatids are pulled apart; in *anaphase I* homologous chromosomes are separated. Homologous chromosomes separate and move to opposite poles. This meiotic division is therefore called a reductional division because it reduces the number of chromosomes to half the diploid number in each daughter cell.

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MEIOSIS II

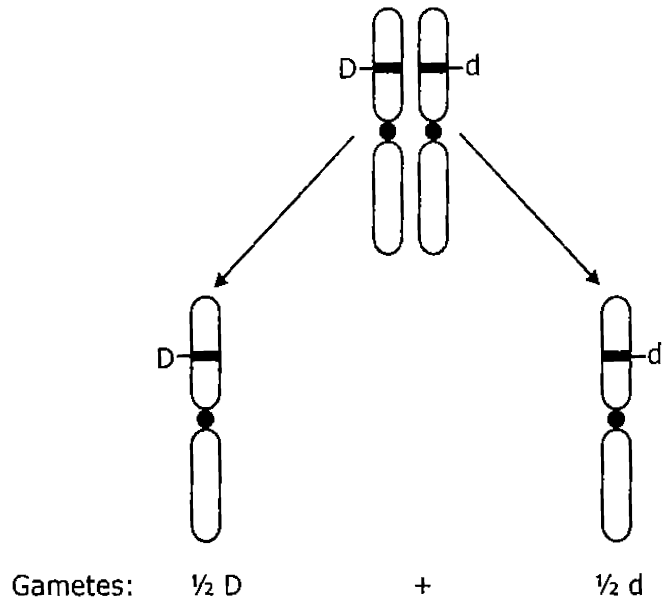
Meiosis II is similar to mitosis. However, there is no "S" phase. The chromatids of each chromosome are no longer identical because of recombination during meiosis I. Meiosis II separates the sister chromatids producing two daughter cells each with haploid chromosomes number.

Segregation and independent assortment during meiotic division

Segregation of homologous chromosomes:

In an organism that is heterozygous for two different alleles of a gene, such as tall (D) and dwarf (d) in Mendel's peas, the two alleles segregate from each other during the formation of gametes. The biological basis of this segregation is the pairing and subsequent separation of homologous chromosomes during anaphase I of meiosis I.

Segregation of homologous chromosomes
during the first division of meiosis



Independent assortment:

New combinations of genes on nonhomologous chromosomes are produced by the *independent assortment* of chromosomes during the first division of meiosis. Consider a cross between two double homozygotes, for example, AA BB and aa bb, where genes A and B are on different chromosomes. The F_1 progeny (F_1 for first filial generation) will be double heterozygotes (Aa Bb). Half of the gametes produced by F_1 progeny will have the same combination of alleles as the parents (1/4 AB + 1/4 ab). The other half will have new (recombinant) combinations of the alleles (1/4 Ab and 1/4 aB). The recombinations of pairs of alleles occur through independent assortment of the homologous chromosomes during the first division of meiosis. Each pair of homologous chromosomes lines up at the metaphase plate independently of every other pair. Both maternal chromosomes may be on the same side of the metaphase plate, with the two paternal chromosomes on the other side, or one maternal chromosome may be on the same side of the metaphase plate as the paternal member of the other chromosome pair, and vice versa. The result is that the segregation of the alleles of a gene on one chromosome occurs independently of the segregation of the alleles of any gene on a nonhomologous chromosome.

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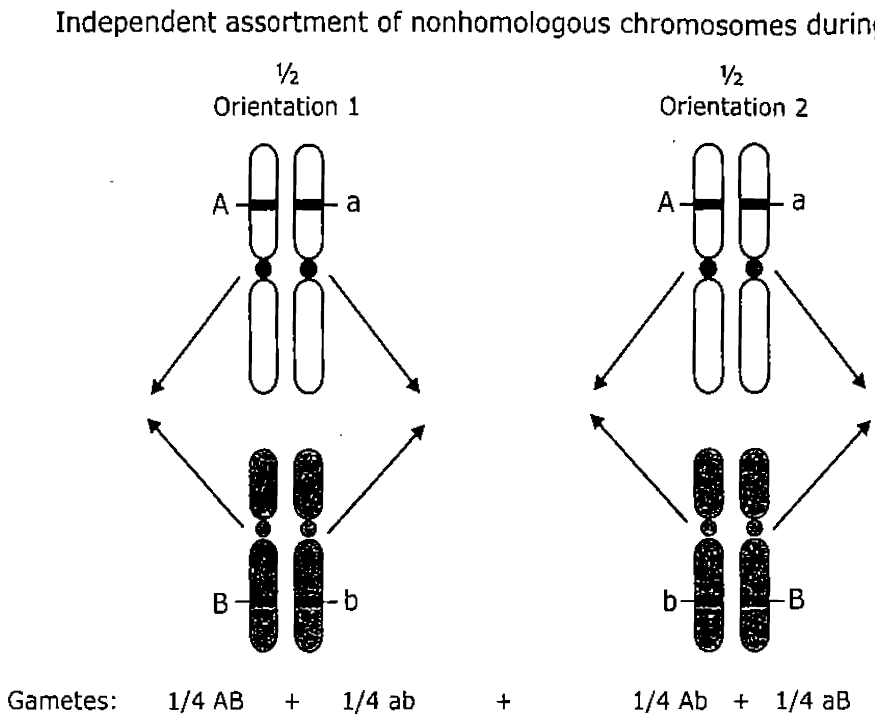


Table 10.2 : Comparison between mitosis and meiosis in animals

	Mitosis	**Meiosis**
Location	All tissues	Only in testis and ovary
Products	Diploid cells	Haploid gametes
DNA replication	One round of replication per cell division	One round of replication but two cell divisions
Duration of prophase	Short	Long
Pairing of homologs	Absent	Present
Recombination	Rare	Common
Relationship between daughter cells	Genetically identical	Genetically different

Nondisjunction and aneuploidy

During mitosis, each chromosome duplicates and the two daughter chromosomes separate, with one going to each progeny cell. Similarly, during meiosis, the two homologous chromosomes separate during the first division, and then the two daughter chromosomes (previously chromatids) move to separate cells during the second division. The result of a normal mitosis or meiosis is that all the progeny cells receive complete sets of chromosomes. Occasionally, however, homologous chromosomes and daughter chromosomes fail to separate or disjoin properly during mitosis or meiosis. The failure of chromosomes to separate properly during mitosis or meiosis is called *nondisjunction*. Nondisjunction produces cells or gametes with extra or missing chromosomes; it is the primary cause of aneuploidy.

10.3 APOPTOSIS

Apoptosis (from the Greek words *apo* = from and *ptosis* = falling,) is an energy dependent biochemical mechanism of programmed cell death. It is a genetically programmed process of deliberate suicide by an unwanted cell in a multicellular organism during embryogenesis, metamorphosis, and tissue turnover. It is the capacity of a cell to respond to a stimulus by initiating a pathway that leads to its death by a characteristic set of reactions. In contrast to **necrosis**, which is a form of an energy independent cell death that results from acute tissue injury, apoptosis is carried out in an ordered process that generally confers advantages

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during an organism's life cycle. For example, the differentiation of human fingers in a developing embryo requires the cells between the fingers to initiate apoptosis so that the fingers can separate. The demise of cells by apoptosis is marked by a well-defined sequence of morphological changes. Apoptotic cells become more compact, blebbing occur at the membranes, chromatin become condensed, and DNA is fragmented. Dying cells shrink and condense and then fragment, releasing small membrane-bound apoptotic bodies, which generally are phagocytosed by surrounding cells. Importantly, the intracellular constituents are not released into the extracellular milieu where they might have deleterious effects on neighboring cells. The highly stereotyped changes accompanying apoptosis suggest that this type of cell death is under the control of a strict cellular program. Apoptosis can be triggered by a variety of stimuli, including withdrawal of essential growth factors, treatment with glucocorticoids, gamma irradiation, and activation of certain receptors. Another means of initiating apoptosis is used in the immune system, where cytotoxic T lymphocytes attack target cells.

Mechanisms of apoptosis

The mechanisms of apoptosis are highly complex and regulated, involving an energy-dependent cascade of molecular events. There are multiple apoptotic pathways. These pathways are both caspase-dependent as well as caspase-independent. There are three main *caspase-dependent* apoptotic pathways:

- The *extrinsic* or death receptor pathway
- The *intrinsic* or mitochondrial pathway
- The *perforin/granzyme* pathway (induce apoptosis via granzyme B).

Caspases have proteolytic activity. Caspases are so named because they contain a key cysteine residue in the catalytic site and selectively cleave proteins at sites just C-terminal to aspartate residues). Once caspases are initially activated, there seems to be an irreversible commitment towards cell death. To date, more then 12 caspases have been identified and broadly categorized into *initiators* (caspase-2,-8,-9,-10) and *effectors* or executioners (caspase-3,-6,-7). All caspases are synthesized in the form of inactive procaspases. Each caspase when activated, cleaves certain target proteins at specific aspartate residues in the target polypeptide chains. There are multiple apoptotic pathways. While caspases are critical in many apoptosis events, caspase-independent apoptosis also occurs. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells.

Caspase-dependent extrinsic apoptotic pathway

Killer lymphocytes can induce apoptosis by extracellular activation. They induce apoptosis by producing a protein called **Fas ligand**, which binds to the death receptor protein **Fas** on the surface of the target cell. Fas is a cell surface receptor. The Fas ligand is a transmembrane protein.

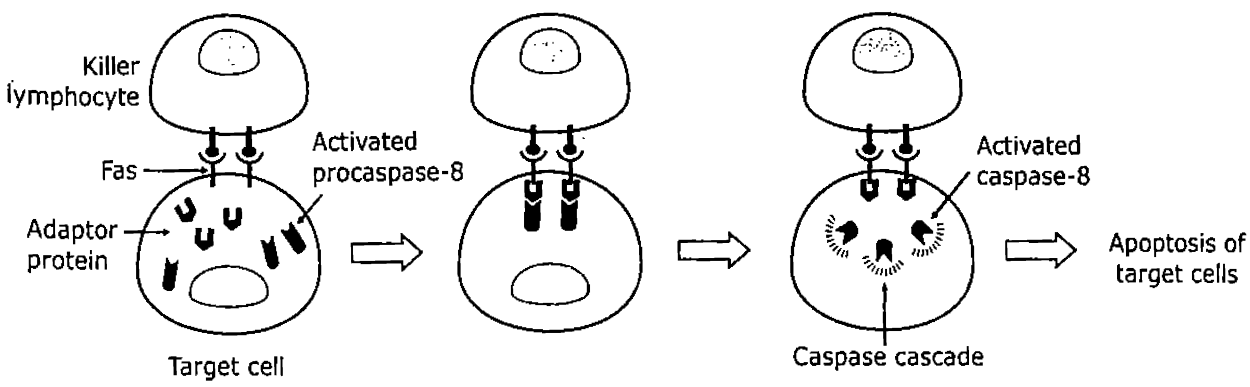


Figure 10.8 : A killer lymphocyte carrying the Fas ligand binds and activates Fas proteins on the surface of the target cell. Adaptor proteins bind to the intracellular region of aggregated Fas protein, causing the aggregation of procaspase-8 molecules. These then cleave one another to initiate the caspase cascade.

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The Fas receptor on a target cell is activated by interaction with the Fas ligand on an activating cell plasma membrane. Fas has an cytoplasmic domain called the *death domain* which is involved in protein-protein interactions. Fas proteins then recruit intracellular adaptor proteins that bind and aggregate procaspase-8 molecules, which cleave and activate one another. The activated caspase-8 molecules then activate downstream procaspases to induce apoptosis.

Caspase-dependent intrinsic apoptotic pathway

The mitochondrion plays a central role in apoptosis by releasing cytochrome *c*. The basic role of cytochrome *c* is to trigger the activation of caspase-9. Cytochrome *c* triggers the interaction of the cytosolic adaptor protein Apaf-1 with procaspase-9 in a complex called the **apoptosome.**

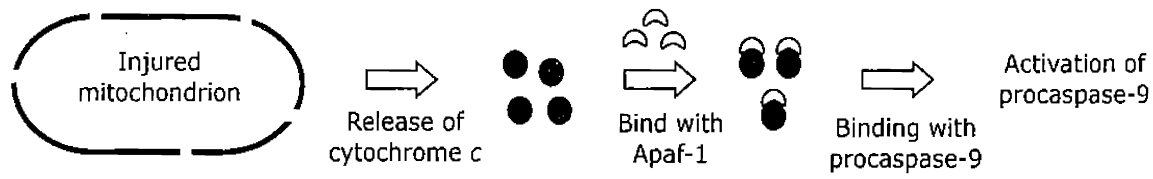


Figure 10.9 : Schematic representation of the role of mitochondria in apoptosis. Cytosolic cytochrome *c* interacts with Apaf-1, allowing subsequent caspase activation.

The incorporation of procaspase-9 into the apoptosome triggers the auto-activating cleavage. Caspase-9 in turn cleaves procaspase-3 to generate caspase-3 which cleaves targets that cause apoptosis of the cell.

Overview of apoptotic pathway

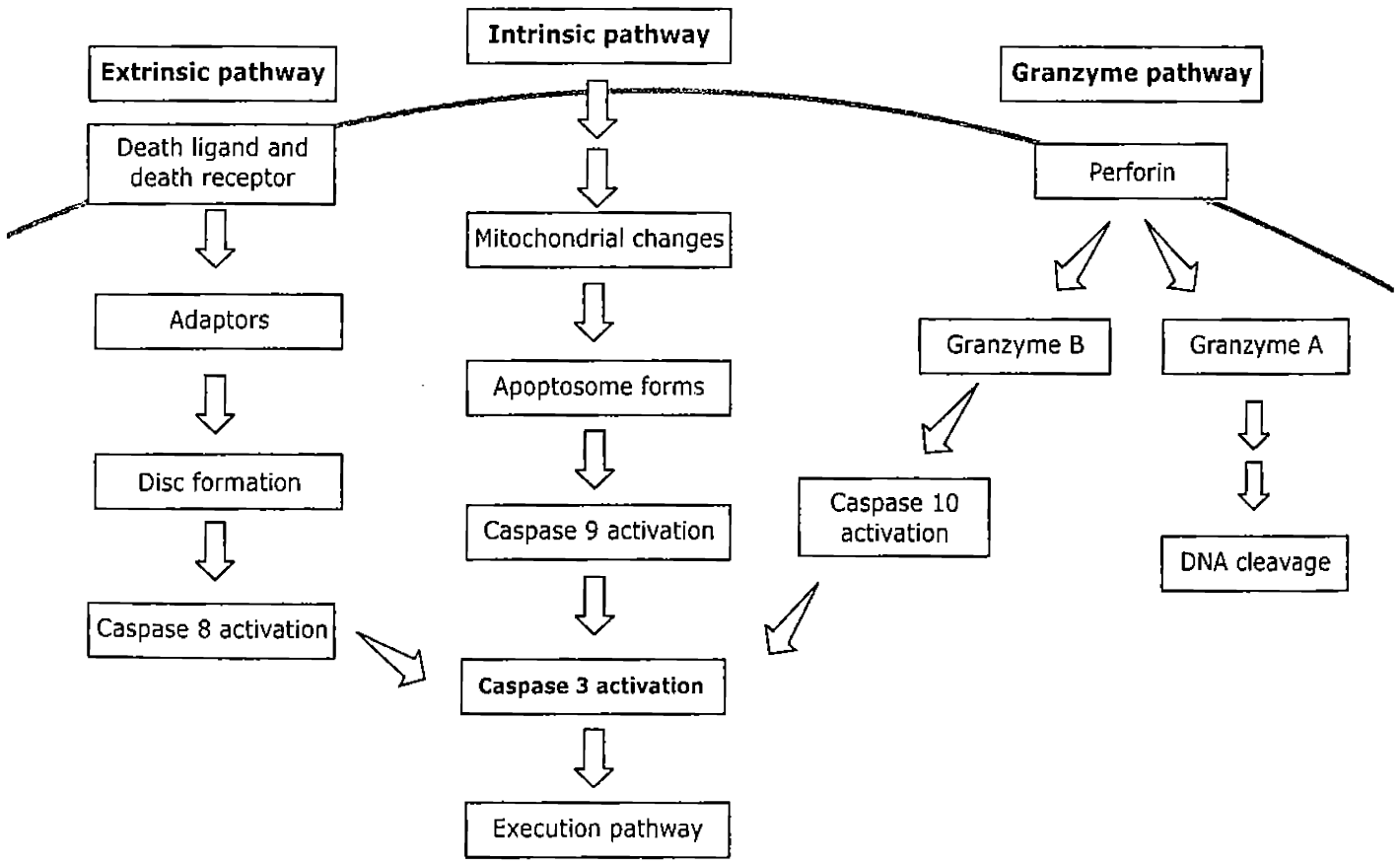


Figure 10.10 : Apoptotic pathways

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Apoptosis regulatory proteins

Regulatory proteins comprises both *anti-apoptotic* members and *pro-apoptotic* members. bcl-2 was the first oncogene shown to mediate its effects by an inhibition of apoptosis. It is the mammalian homologue of the CED-9 gene in *C. elegans*. Anti-apoptotic members like *Bcl-2*, Bcl-X_L inhibit apoptosis. The exact mechanism by which the anti-apoptotic members regulate apoptosis remain elusive. Similarly, Bax, Bad, Bik are proapoptotic members.

10.4 CANCER

A normal cell undergoes regulated division, differentiation and apoptosis (programmed cell death). When normal cells have lost the usual control over their division, differentiation and apoptosis they become tumor cells. So, tumor is the result of an abnormal proliferation of cells without differentiation and apoptosis. Tumor or neoplasm (any abnormal proliferation of cells) may be of two types: *Benign tumor* and *Malignant tumor*.

Benign tumor

Neoplastic cells remain clustered together in a single mass and cannot spread to other sites. It contains cells that closely resemble normal cells and that may function like normal cells.

Malignant tumor

Neoplastic cells that don't remain localized and encapsulated and becomes progressively invasive and malignant are described as *malignant tumors*. It invades surrounding normal tissues (called *invasiveness*) and spreading through out the body through circulatory or lymphatic systems (called *metastasis*). The term **cancer** refers specifically to a malignant tumors.

Table 10.3 : Comparison of benign and malignant tumours

Characteristics	*Benign*	*Malignant*
Differentiation	Well differentiated	Lack differentiation
Rate of growth	Slow	Rapid
Invasiveness	Absent	Present
Metastasis	Absent	Present

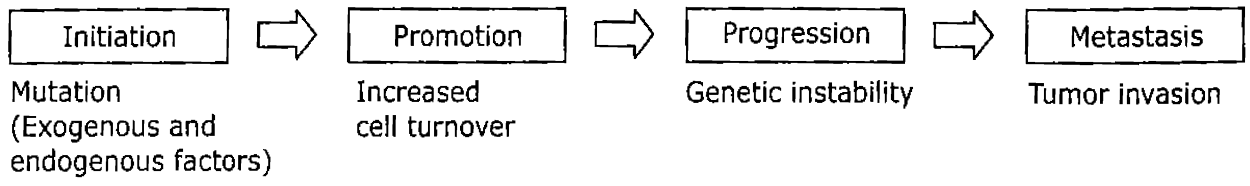
Both benign and malignant tumors are classified according to the type of cell from which they arise. Most cancer fall into three main groups- *Carcinomas* (tumors that arise from endodermal or ectodermal tissues), *Sarcomas* (malignancies of mesodermal connective tissues) and *Leukemia/Lymphomas* (from blood forming tissues and from cells of immune system).

Most cancers originate from single abnormal cell i.e., *monoclonal origin.* Cancers are probably initiated by change in the cell's DNA sequence (*genetic changes*) or change in pattern of gene expression without a change in DNA sequences (*epigenetic changes*). Most cancers are initiated by genetic change. Majority of cancer are caused by mutation in somatic cells and therefore are not transmitted to next generation. About 1% of all cancers is due to mutation in germinal cells and is therefore inherited. About 80% of these inherited cancers are dominant in nature.

The transition of a normal cell into a tumor cell is referred to as *transformation.* The transition from a normal to a transformed state is a multisteps process involving mutation and selection of cells with progressively increasing capacity for proliferation, invasion, and metastasis. The first step in the process is **tumor initiation.** It is a process in which normal cells are changed so that they are able to form tumors. Additional genetic changes occur within transformed cells. Additional changes followed by selection of

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more rapidly growing cells within the population then result in progression of the tumor to increasingly rapid growth and malignancy (**tumor progression**).



Genetic and phenotypic instability are hallmarks of cancer cells, but their cause is not clear. The leading hypothesis suggests that a poorly defined gene mutation generates genetic instability and that some of many subsequent mutations then cause cancer. The ensuing genetic instability drives tumour progression by generating mutations in cancer critical genes. These mutant genes provide cancer cells with a selective growth advantage, thereby leading to the clonal outgrowth of a tumor.

Properties of cancer cells

Cancer cells typically display several abnormal properties as compared to normal cells that provide a description of malignancy at the cellular level.

Density dependent inhibition

Normal cells in culture display *density dependent inhibition* of cell proliferation and proliferate until they reach a finite cell density, which is determined partly by availability of growth factors in culture medium. But the proliferation of cancer cells is not sensitive to density dependent inhibition.

Contact inhibition

Normal cells migrate across the surface of a culture dish until they make contact with a neighboring cell. Further cell migration is then inhibited and normal cells adhere to each other forming an orderly array of cells on the culture dish surface. This inhibition of growth after contact is called *contact inhibition*. Tumor cells in contrast continue moving after contact with their neighbouring cells migrating over adjacent cells and growing in disordered multilayered patterns.

Immortalization

Normal cell cultures do not survive indefinitely. For example human cell cultures die after about 50 generations, and chicken cell cultures have a much shorter life expectancy. On the other hand transformed cell cultures are immortal and can grow indefinitely.

Invasiveness and Metastasis

One of the most important characteristics of transformed cells is their *invasiveness.* Invasion refers to the direct migration and penetration by cancer cells into neighboring tissues. Metastasis refers to the ability of cancer cells to penetrate into lymphatic and blood vessels, circulate through the bloodstream, and then invade normal tissues elsewhere in the body.

There are two properties of cancer cells that play important roles in invasion and metastasis. *First*, malignant cells generally secrete proteases that digest extracellular matrix components, allowing the cancer cells to invade adjacent normal tissues. *Second*, cancer cells also secrete growth factors that promote the formation of new blood vessels (**angiogenesis**). Angiogenesis is needed to support the growth of a tumor by supplying oxygen and nutrients to the proliferating tumor cells.

Loss of anchorage dependence

Most normal cells must be attached to a rigid substratum (i.e, they must be anchored) in order to grow. Transformed cells can grow even when they are not attached to the substratum, as for example when they are suspended in a semisolid medium containing agar or methyl cellulose.

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Lower serum requirements

Growth of normal cells in a tissue culture medium requires a high concentration of serum. Some serum growth factors (somatomedins) resemble insulin in interacting with external receptors of the cell membrane to regulate biochemical activities within the cell. Transformed cells can grow in a culture medium containing much less serum than required by normal cells.

Fail to undergo apoptosis

Another general characteristic of most cancer cells is that they fail to undergo apoptosis and therefore exhibit increased life spans compared to their normal counterparts. This failure of cancer cells to undergo apoptosis contributes substantially to tumor development. Cancer cells are often characterized by increased resistance to apoptosis allowing their survival under abnormal growth stimulation. The failure to undergo apoptosis also contributes to the resistance of cancer cells to carcinogens, which act by damaging DNA.

Carcinogen

Agents those initiate or promote tumor formation are called *carcinogens*. Three classes of carcinogenic agents are known : Radiations (like UV radiation, γ ray, β ray), chemicals (like benzopyrene, benzene) and biological (oncovirus) agents. The ability of chemical and physical carcinogens to induce cancer can be accounted for by the DNA damage that they cause and by the errors introduced into DNA during the cells efforts to repair this damage.

Chemical carcinogen have a very broad range of structures that have little or no obvious structural or biochemical similarity to each other. There are two types of chemical carcinogens, one called *direct-acting* carcinogens that act directly without any metabolic activation and the other, called *indirect-acting* carcinogens, which require metabolic activation for them to act as carcinogens.

Aflatoxin is an example of indirect-acting carcinogen. It is activated into aflatoxin-2,3, epoxide by the action of intracellular enzymes which is associated with characteristic mutations of the p53 gene. Aflatoxins, mycotoxins produced by the fungi *Aspergillus flavus, Aspergillus parasiticus;* are among the most potent liver carcinogens known. Chemically, the aflatoxins consist of a difurofuran ring system fused to a substituted coumarin moiety, with a methoxy group attached at the corresponding benzene ring. After chemical modification by liver enzymes, aflatoxin becomes linked to G residues in DNA and induces G-to-T transversions.

Other common examples of chemical carcinogens which are associated with a number of human cancers are benzene (leukemia), arsenic (lung and skin), cadmium (prostrate), radon (lung), asbestos (lung and gastro-intestinal tract) and vinyl chloride (angiosarcoma, liver). Some carcinogens contribute to cancer development by stimulating cell proliferation, rather than by inducing mutations. Such compounds are referred to as **tumor promoters**. Phorbol esters that stimulate cell proliferation by activating protein kinase C are classic examples which stimulate proliferation of the mutated cells.

Oncovirus or **tumor virus**

The transforming activity of a tumor virus resides in a particular gene or genes carried in the viral genome. Oncogenes were given their name by virtue of their ability to convert normal cells to an oncogenic state. Oncovirus containing viral oncogen may be

- *DNA containing oncovirus* like hepatitis B viruses, Herpes virus, SV40 carry oncogenes without cellular counter parts; these oncogenes may work by inhibiting the activities of cellular tumour suppressor. DNA tumor viruses acts mainly by interfering with cell cycle controls. For example, papilloma virus (causes human warts and carcinomas of the uterine cervix) uses two viral proteins, E6 and E7, to sequester the host cell's p53 and Rb, respectively. Similarly, *E1A* and *E1B* proteins of adenoviruses inactivate the Rb and p53 tumor suppressor proteins, with E1A binding to Rb and E1B binding to p53.

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Name of *DNA containing oncovirus* that cause human cancer

- Hepatitis B virus (liver cancer),
- Papillomaviruses (cervical and other anogenital cancers),
- Epstein-Barr virus (Burkitt's lymphoma and nasopharyngeal carcinoma),
- Kaposi's sarcoma-associated herpesvirus (Kaposi's sarcoma)

- *RNA containing oncovirus* like transforming retrovirus contain oncogenes. Viral oncogenes were first reported in Rous Sarcoma virus by Peyton Rous in 1911, which transforms chicken embryo fibroblasts in culture and induces sarcoma. The first oncogene was originally found in the Rous sarcoma virus was designated as the *src* oncogene.

Table 10.4 : Examples of oncoviruses that cause human cancer

Virus	**Malignant disease**
Human T-cell lymphotropic virus (HTLV)-1	Adult T-cell leukemia/lymphoma
Epstein-Barr virus	B-cell lymphomas; Burkitt's lymphoma
Human herpesvirus	Kaposi's sarcoma
Hepatitis B virus	Hepatocellular carcinoma
Hepatitis C virus	Hepatocellular carcinoma
Human papilloma virus	Cancer of the uterine cervix; cancer of the penis
Simian virus 40 (SV40)	Non-Hodgkin's lymphoma

American microbiologist H. Varmous and M. Bishop in 1976 demonstrated that normal chicken cells contain genes that are closely related to the retroviral *src* oncogenes (codes for *Src* protein tyrosine kinase) of Rous Sarcoma virus. They published the remarkable conclusion that the oncogene in the virus did not represent a true viral gene but instead was a normal cellular gene, which the virus had acquired during replication in the host cell and thereafter carried along. *src* related sequences were also found in normal DNA of wide range of other vertebrates (including human). Until now more than 40 different oncogenes have been demonstrated. The normal cell genes from which the retroviral oncogenes originated are called **proto-oncogenes**. The proteins encoded by proto-oncogenes participate in various metabolic processes like *regulation of cell cycle, cell to cell signaling, transcription factor* and *intracellular signal transduction.*

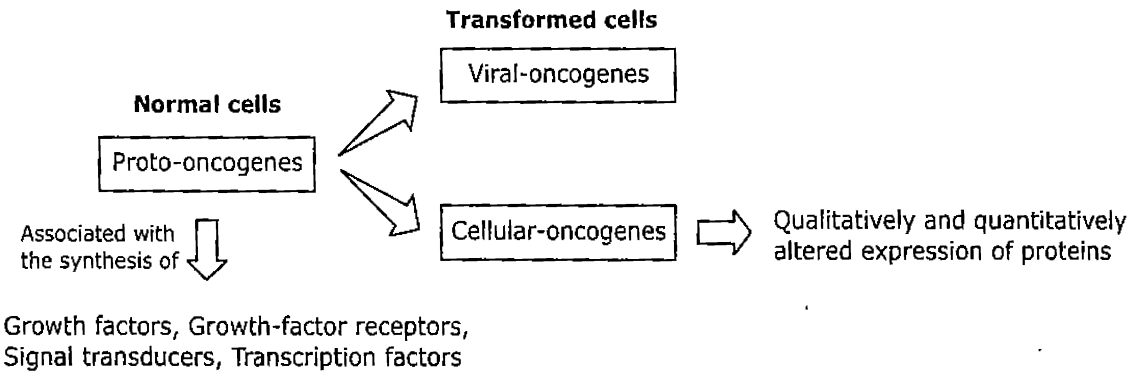
Table 10.5 : Example of proto-oncogenes and nature of their products

Name	*Nature of gene product*
sis	Synthesis of platelet-derived growth factor
erbB	Synthesis of epidermal growth factor receptor
src	Synthesis of tyrosine kinase
abl	Synthesis of tyrosine kinase
K-ras	Synthesis of GTPase
jun	Synthesis of transcription factors
fos	Synthesis of transcription factors
myc	Synthesis of transcription factors

In 1972, R.J. Huebner and G.J. Todaro suggested that mutations or genetic rearrangements of proto-oncogenes by carcinogens or viruses might alter the normally regulated function of these genes, converting them into potent cancer-causing oncogenes. The viral oncogenes and their cellular counterparts are described

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by using prefixes *v* for viral and *c* for cellular. So the oncogene carried by Rous sarcoma virus is called *v-src,* and the proto-oncogene related to it in cellular genomes is called *c-src.*



Proto-oncogenes in cell can change into an oncogene by many conceivable modes:

Point mutation

As for example the *ras* oncogenes are not present in normal cells, rather, they are generated in tumour cells as a consequence of point mutations that occur during tumour development. The *ras* oncogenes differ from their proto-oncogenes by point mutation resulting in single amino acid substitution at critical positions.

Chromosomal translocation

Chromosomal translocation may result in activation of proto-oncogene. Translocation induced over expression of a proto-oncogene is best exemplified by Burkitt's lymphoma. In Burkitt's lymphoma the c-*myc* containing segment of chromosome 8 (8q24) translocate to chromosome 14 (14q32). This translocation places c-*myc* close to the immunoglobulin heavy chain (Cm) gene. Similarly, in chronic mylogenous leukemia c-*abl*, a proto-oncogene present on chromosome 9 and exchange transfers the c-*abl* gene to chromosome 22 (known as Philadelphia chromosome). c-*abl* gene encodes a tyrosine kinase.

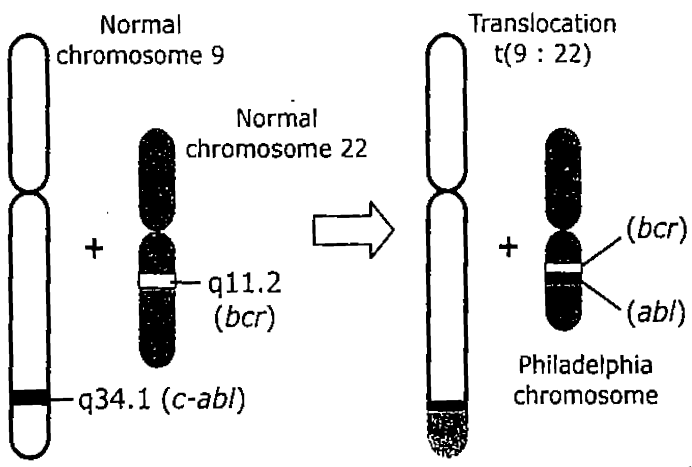


Figure : 10.11
Generation of the bcr-abl oncoprotein by chromosomal translocation. The abl gene in a normal cell, is located on chromosome 9 and encodes a tyrosine kinase. During malignant transformation of myeloid cells, a portion of chromosome 9 that contains the abl locus translocates to chromosomse 22 at the *breakpoint cluster region* (bcr) locus and generates the chimeric bcr-abl oncoprotein. Because the translocation results in deletion of the sequences that negatively regulate abl tyrosine kinase activity, the fusion protein has constitutive and increased levels of enzymatic activity.

Table 10.6 : Example of specific chromosome aberration and cancer

Cancer	*Chromosome alteration*
Chronic myelogenous leukemia	Translocation (9 ; 22)
Acute myelogenous leukemia	Translocation (8 ; 21)
Retinoblastoma	Deletion (13q)
Wilms tumor	Deletion (11p)

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Insertional activation

Insertion of a mobile genetic element such as retrovirus changes the expression of genes but leave their coding sequence unaltered. The best characterized is c-*myc*, whose expression is elevated after the insertion of a non-defective retrovirus in the vicinity of gene. Avian leukosis virus (ALV) is a retrovirus that does not carry any viral oncogenes and yet is able to transform B cells into lymphomas. This particular retrovirus has been shown to integrate within the *c-myc* proto-oncogene, which contains three exons. Exon 1 of *c-myc* has an unknown function; exons 2 and 3 encode the Myc protein. Insertion of AVL between exon 1 and exon 2 has been shown in some cases to allow the provirus promoter to increase transcription of exons 2 and 3, resulting in increased synthesis of c-Myc.

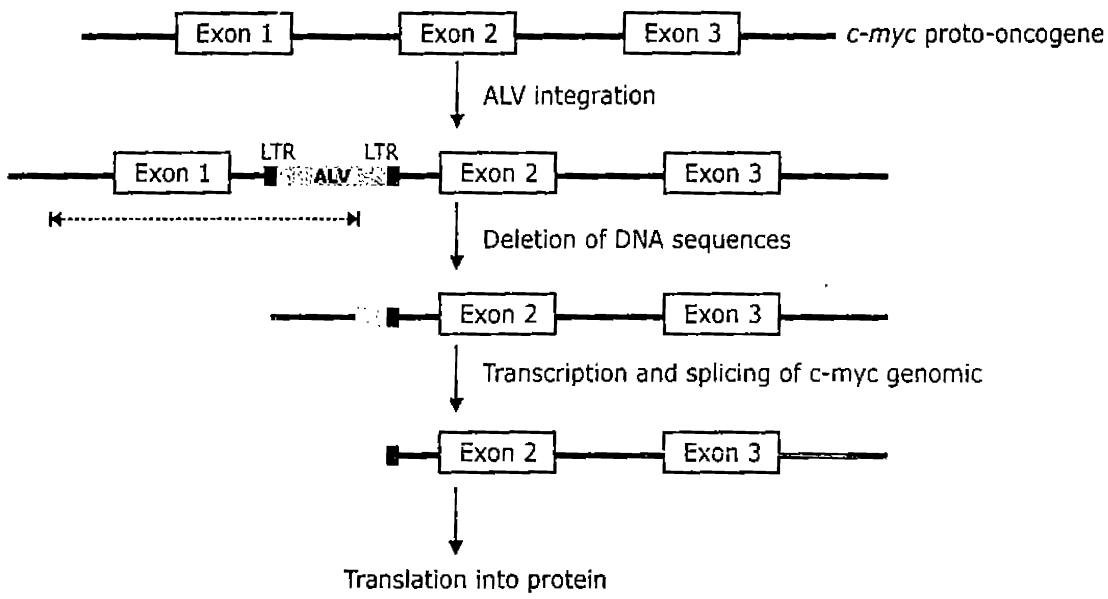


Figure 10.12 : Activation of the c-myc oncogene as a consequence of ALV integration. In a normal cell, the three exons of the c-myc gene are normally spliced into an mRNA that can be translated into the c-myc protein (only exons 2 and 3 actually code for the protein). When ALV integrates into the c-myc locus (usually between exons 1 and 2), the presence of the LTR sequences in the transcript results in increased expression of the c-myc protein, which is harmful to the cell. The integration of ALV is often followed by deletion of portions of viral sequences, leaving one of the LTRs intact.

Gene amplification

Activation of proto-oncogenes result from reduplication and amplification of their DNA sequences. The most interesting cases of amplification involve N-*myc* in neuroblastoma. Gene amplification is usually an unstable genetic condition that can only be maintained under strong selective pressure.

MOLECULAR BASIS OF CANCER

Cancer is a genetic disease that may be brought about by dysregulation of mainly five different pathways: Alteration or activation of proto-oncogenes; inactivation of tumour suppressor genes; decreased activity of differentiation pathways (e.g, *Hh*, *Apc*); decreased DNA repair (e.g, *Atm*, *Brca*); or decreased cell death (e.g, increased Bcl-2).

All genes whose mutation contributes to the causation of cancer is described as **cancer-critical genes**. Cancer-critical genes are grouped into two broad classes, according to whether the cancer risk arises from a *gain-of-function* mutation of a gene, or *loss-of-function* mutation. Genes for which a gain-of-function mutation drives a cell toward cancer, are called **proto-oncogenes**. Whereas genes for which a *loss-of-function* mutation creates the cancer are called **tumor suppressor genes**.

Mutation of a cancer-critical gene can be either dominant or recessive. Mutation of a proto-oncogene has a dominant, growth-promoting effect on a cell. On the other hand, the cancer-causing mutations of tumor

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suppressor genes are *generally* recessive. It means both copies of the normal gene must be removed or inactivated in the diploid somatic cell before an effect is seen. Tumor-suppressor genes encode proteins of diverse functions like intracellular proteins that regulate progression through a specific stage of the cell, receptors or signal transducers for secreted hormones, checkpoint-control proteins that arrest the cell cycle if DNA is damaged or chromosomes are abnormal, proteins that promote apoptosis and enzymes that participate in DNA repair.

Five broad classes of proteins are generally encoded by tumor-suppressor genes:

- Intracellular proteins that regulate or inhibit progression through a specific stage of the cell cycle (e.g., p16 and Rb).
- Receptors or signal transducers for secreted hormones or developmental signals that inhibit cell proliferation (e.g., the hedgehog receptor patched).
- Checkpoint-control proteins that arrest the cell cycle if DNA is damaged or chromosomes are abnormal (e.g., p53).
- Proteins that promote apoptosis.
- Enzymes that participate in DNA repair.

p53 is well-studied tumour suppressor gene and encode polypeptide chain of 375 amino acid residues. It acts as transcription factor that regulate the expression of cell cycle regulatory protein. p53 gene (named for its molecular size) located on chromosome number 17 of human was originally classified as an oncogene because missense mutations in it is oncogenic. p53 protein plays an important role in the maintenance of genomic stability during mitotic proliferation. These functions are carried out by composite regulation of key cellular responses to DNA damage. On the one hand, p53 mediates the arrest of cells at the G_1-S boundary and affects the G_2 checkpoints. This permits the cells to repair the DNA damage, prior to stages of its fixation and propagation, which may lead to carcinogenic transformation. Moreover, p53 was shown to facilitate general genomic repair of DNA damage and to bind proteins involved in DNA repair. On the other hand, in many cellular systems, p53 promotes *apoptosis* of cells harboring irreparable damaged DNA. Mutations in the *p53* tumor-suppressor gene occur in more than 50 percent of human cancers.

Alteration of genes involved in repair of damaged DNA are also responsible for number of human cancer. In view of the thousands of damage events that genomes suffer every day, coupled with the errors that occurs when the genome replicates, it is essential that cells possess efficient repair systems. Without these repair systems a genome would not be able to maintain its essential cellular functions for more than few hours before key genes became inactivated by DNA damage. Deficiency in DNA repair causes several human diseases. For example, Bloom's syndrome is caused by mutations in a helicase gene. The mutation results in an increased frequency of chromosomal breaks and sister chromatid exchanges. One of the best characterized of these is xeroderma pigmentosum, which results from a mutation in any one of several genes for proteins involved in nucleotide excision repair. A few diseases have been linked with defect in the transcription-coupled component of nucleotide excision repair. These include breast and ovarian cancers, the BRCA1 gene that confers susceptibility to these cancers coding for a protein that has been implicated with transcription-coupled repair.

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STUDY QUESTIONS

Q1. What are phorbol esters and how do they promote tumors?

Ans. Phorbol esters, found in croton oil, activate protein kinase C, an action that stimulates cell growth and division. However, unlike DAG, the molecule they mimic, phorbol esters continue to activate proteins kinase C for a prolonged time. This provides the affected cell with an advantage over unstimulated cells. Phorbol esters may transform a cell previously exposed to a carcinogenic initiating event into a cancerous cell whose unrestrained proliferation creates a tumor.

Q2. How does the effect of mutation in the p53 gene differ from the effect of mutation in the RB gene. What is the molecular basis for this difference?

Ans. Mutation of even one p53 allele is a dominant mutation in contrast to mutation in RB. Both RB alleles must be mutated for induction of retinoblastoma. This difference is caused by the fact that the p53 protein acts as an oligomer, and the presence of even one defective subunit in the complex abrogates its function. In contrast, the Rb protein acts as a monomer.

SELF TEST

Q1. Retinoblastoma is a tumor suppressor protein and is directly involved in

a. cell cycle progression
b. transmission of extra-cellular signals to the nucleus
c. cytoskeletal rearrangement
d. general inhibition of various oncoproteins **[JNU]**

Q2. A characteristic of homologous chromosomes is that

a. they carry alleles for the same genes in the same relative positions
b. they regularly exchange parts by crossing over at meiosis
c. they physically pair at meiosis
d. all of the above **[JNU]**

Q3. Which one of the following proteins is a major cell cycle regulator?

a. E2F
b. E47
c. EcoRI
d. Pit-I **[JNU]**

Q4. p53 protein is associated with all of the following, *except*

a. tumor suppression
b. programmed cell death
c. transcription
d. post-transcriptional modifications **[JNU]**

Q5. Diploid chromosome number in human is 46. However, if you count chromatids which are visible during mitotic or meiotic cell divisions, you would be able to count different sets of chromatid numbers depending on the stage and the type of the cell division in question. Match the chromatid numbers given on the left with cell division stage shown on the right

A. 46	1. Mitotic metaphase
B. 23	2. Aneuploid meiotic telophase II
C. 92	3. Meiotic telophase I
D. 24	4. Meiotic anaphase II

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a. A-3 ; B-4 ; C-1 ; D-2
b. A-2 ; B-4 ; C-1 ; D-3
c. A-1 ; B-4 ; C-2 ; D-3
d. A-1 ; B-2 ; C-3 ; D-4 **[GATE]**

Q6. FOS, JUN and MYC are
a. proteins expressed on the surface of cancerous cells
b. protein kinases that phosphorylate transcription factors in cancerous cells
c. proteins involved in regulation of expression of genes involved in growth promotion
d. proteins involved in ion transport in cancerous cells **[GATE]**

Q7. The proteins encoded by proto-oncogenes participate in various metabolic processes including
a. regulation of transcription
b. cell-to-cell signaling
c. intracellular signaling transduction
d. all of the above

Q8. Viral encoded Ras oncogene transforms normal mammalian cells into cancer cells. Viral Ras protein differs from its normal counterpart by
a. diminished GTPase activity
b. increased GTPase activity
c. diminished ATPase activity
d. increased ATPase activity **[GATE]**

Q9. In many tumors, there is a growth of blood vessels in the direction of tumors. The phenomenon is known as
a. necrosis
b. carcinogenesis
c. angiogenesis
d. morphogenesis **[CSIR-UGC]**

Q10. Malignant tumour is characterized by
a. slow simple expansion of cells
b. atypical tissue structure and uncontrolled growth
c. protooncogenes expression
d. no chromosomal abnormalities **[CSIR-UGC]**

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Chapter - 11

Prokaryotes and Virus

11.1 PHYLOGENETIC OVERVIEW AND GENERAL FEATURES OF PROKARYOTES

The basic physical unit of living organisms is the cell. All organisms more complex than viruses consist of cells, aqueous compartments bounded by membranes, which under restricted conditions are capable of existing independently. All cells are derived by cell division from other cells. All cellular organisms can be subdivided into two major classes, *prokaryotes* and *eukaryotes*, on the basis of the architecture of their cells. Prokaryotes are distinguished from eukaryotes by the absence of a unit membrane-bound nucleus and, usually, the lack of other cell organelles.

THREE DOMAINS SYSTEM

Historically, prokaryotes were classified on the basis of their phenotypic characteristics. Prokaryotic taxonomy therefore involved measuring a large number of characteristics, including morphology and biochemical characteristics (e.g., ability to grow on different substrates, cell wall structure, antibiotic sensitivities, and many others). This contrasts with classification of eukaryotic organisms, for which phylogenetic (evolution-based) classification was possible through the availability of fossil evidence.

A major revolution occurred with the realization that evolutionary relationships could be deduced on the basis of differences in gene sequence. The most important gene for prokaryote phylogeny is the 16S ribosomal RNA (rRNA) gene, which is present in all cells. The gene is approximately 1500 bases in length and possesses regions in which sequences are conserved, facilitating sequence alignment, and variable and hypervariable regions, which enable different organisms to be discriminated from one another.

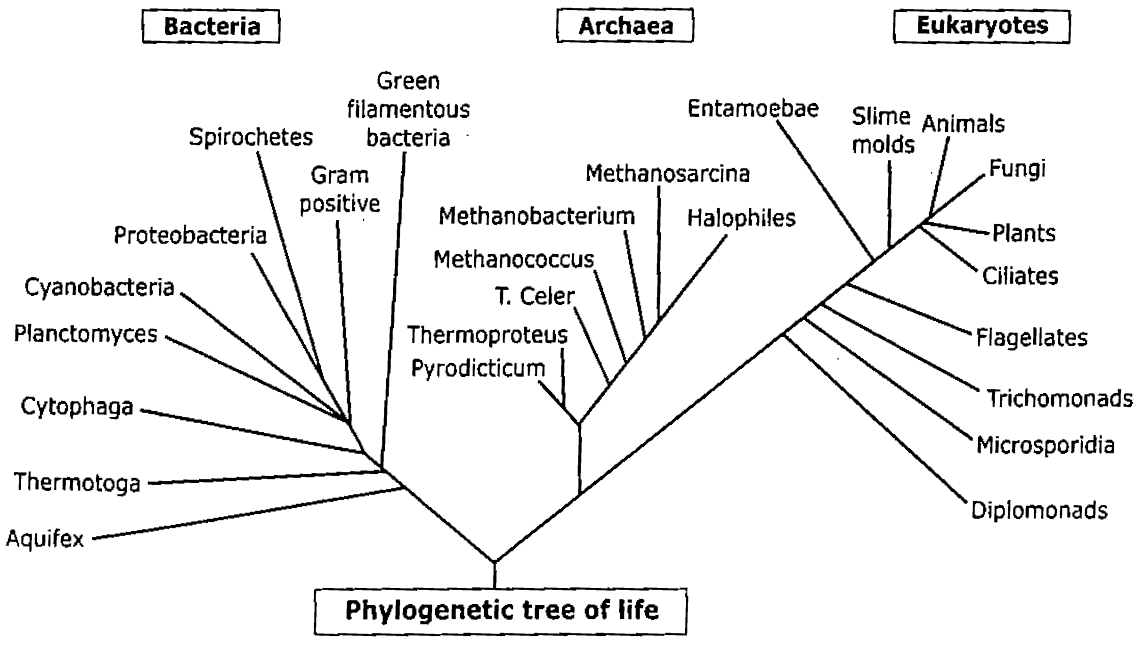


Figure 11.1 : A phylogenetic tree of living things, based on RNA data (proposed by Carl Woese), showing the separation of bacteria, archaea, and eukaryotes from a common ancestor.

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Based on ribosomal RNA data *Carl Woese* proposed a radical reorganization of the five kingdoms into three domains. In his classification system, Woese placed all four eukaryotic kingdoms (protista, fungi, plantae, animalia) into a single domain called *Eukarya*, also known as the eukaryotes. He then split the former kingdom of Monera into the *Eubacteria* (bacteria) and the *Archaea* (archaebacteria) domains. Unlike Whittaker's five kingdoms system, Woese's three domains system organizes biodiversity by evolutionary relationships.

General features of prokaryotic and eukaryotic cells

Features of prokaryotic organisms

True membrane bound nucleus	–	Absent
DNA complexed with histone	–	Absent
Number of chromosome	–	One (few contain more than one)
Mitosis and meiosis	–	Absent
Genetic recombination	–	Partial (unidirectional transfer of DNA)
Sterol in plasma membrane	–	Absent (but present in Mycoplasma)
Ribosome	–	70s
Unit membrane bound organelles	–	Absent
Cell wall	–	Made up of peptidoglycan in eubacteria (absent in Mycoplasma)

Features of eukaryotic organisms

True membrane bound nucleus	–	Present
DNA complexed with histone	–	Present
Number of chromosome	–	More than one
Mitosis and meiosis	–	Present
Genetic recombination	–	By crossing over
Sterol in plasma membrane	–	Present
Ribosome	–	80s (in cytosol) and 70s (in organelles)
Unit membrane bound organelles	–	Present
Cell wall	–	Made up of cellulose in plant and chitin in fungi (absent in animal cells)

Prokaryotic cells show similarities with eukaryotic organelles like mitochondria and chloroplast. The endosymbiotic theory (Margulis, 1993) proposes that the mitochondria and chloroplasts of eukaryotic cells originated as symbiotic prokaryotic cells. The presence of bacterial, circular, covalently closed DNA and 70S ribosomes in mitochondria supports this theory.

Table 11.1 : Comparison of prokaryotic cells and eukaryotic organelles

	Prokaryotic cells	**Eukaryotic organelles**
Nature of DNA	ds circular	ds circular
Histone protein	absent	absent
Ribosome type	70S	70S
Growth	binary fission	binary fission

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11.2 STRUCTURE OF BACTERIA CELL

Bacteria are microscopic, relatively simple, prokaryotic organisms whose cells lack a nucleus or nuclear membrane. Bacteria vary in size from less than 0.2 μm in diameter to more than 50 μm in diameter. The largest known bacterium in terms of total cells volume is *Thiomargarita namibiensis*, whereas smallest known example is *Mycoplasma pneumoniae*. The bacteria may appear as rod (bacilli), sphere (cocci), or spiral (spirilla or spirochetes) shaped. Bacteria reproduce by binary fission, have unique cell walls, and exist in most environments on earth. They live at temperatures ranging from 0°C to over 100°C and in conditions that are oxygen-rich or oxygen-free.

Prokaryotes can be distinguished from eukaryotes in terms of their cell structure and molecular make-up. Prokaryotic cells are morphologically simpler than eukaryotic cells. Although many structures are common to both cell types, some are unique to prokaryotes. Most prokaryotes lack extensive, complex, internal membrane systems. The major prokaryotic structures and their functions are summarized below.

Cell Wall

Prokaryotic cells almost always are bounded by a chemically complex cell wall. The cell wall of both Gram-positive and Gram-negative bacteria is chemically **peptidoglycans** (murein), which confer the characteristic cell shape. Peptidoglycans are unique to prokaryotic organisms. In gram-positive bacteria peptidoglycans cell wall consists of a single 20 to 80 nm thick homogeneous layer lying outside the plasma membrane. In contrast, the gram-negative cell wall consists of 2 to 7 nm thick peptidoglycan layer covered by a 7 to 8 nm thick *outer membrane*. The space that is present between the plasma membrane and the outer membrane in gram-negative bacteria is called the **peripiasmic space**. The substance that occupies the periplasmic space is called **periplasm**. Periplasmic space is also present in some gram-positive bacteria. However, the nature of the periplasmic space and periplasm differs in gram-positive and gram-negative bacteria.

Peptidoglycan is a polymer contains two sugar derivatives *N-acetylglucosamine* (NAG) and *N-acetylmuramic acid* (NAM) joined through β-1,4 glycosidic bond. A peptide chain of four alternating D- and L- amino acids called *tetrapeptide* is connected to the carboxyl group of the NAM. Amino acids present in tetrapeptide include L-alanine, D-alanine, D-glutamic acid, and either lysine or diaminopimilic acid (DAP). Often the carboxyl group of the terminal D-alanine is connected directly to the amino group of diaminopimelic acid. In some peptidoglycans a short peptide chain called **peptide interbridge** connects the tetrapeptide chains. The reaction that forms the peptide cross-links during peptidoglycan synthesis is called **transpeptidation**.

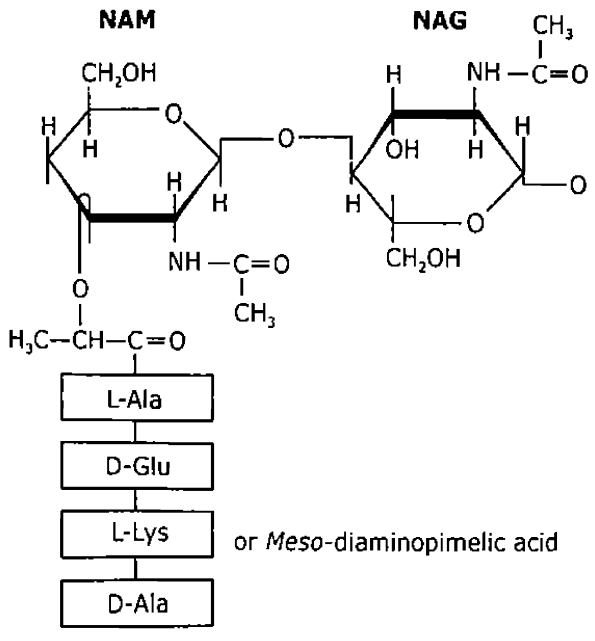


Figure 11.2
Structure of peptidoglycan. Heteropolymer chains of alternating N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM) are connected by glycosidic bond. The tetrapeptide side chain is composed of alternating D- and L- amino acids.

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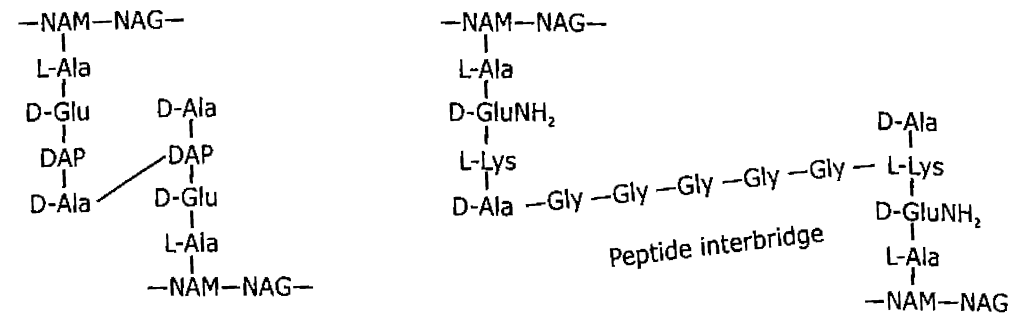


Figure (A) Figure (B)

Figure 11.3 : (A) Peptidoglycan with direct cross-linking, typical of many gram-negative bacteria. (B) The tetrapeptide side chains are connected by peptide interbridge (such as polyglycine) but the amino acids present in peptide interbridge vary in different species of bacteria.

Two groups of bacteria devoid of cell wall peptidoglycans are the **Mycoplasma** species, which possess a surface membrane structure, and the **L-forms** that arise from either Gram-positive or Gram-negative bacterial cells that have lost their ability to produce the peptidoglycan structures.

Many gram-positive bacteria have acidic substances called **teichoic acids**. Teichoic acids are polyol phosphate polymers bearing a strong negative charge. They are covalently linked to the peptidoglycan. They are strongly antigenic, but are generally absent in Gram-negative bacteria.

Biosynthesis of peptidoglycan

The biosynthesis of peptidoglycan can be divided into following stages:

The *first* stage occurs in the cytoplasm and leads to the synthesis of the nucleotide sugar-linked precursors UDP-NAM and UDP-NAG.

In the *second* stage, sequential addition of amino acids to UDP-NAM occurs which results into the formation NAM-pentapeptide.

In the *third* stage, which takes place at the cytoplasmic membrane, the UDP-NAM-pentapeptide make complex with bactoprenol and forms *lipid I*. Then, NAG from UDP-NAG is added to lipid I, yielding *lipid II*.

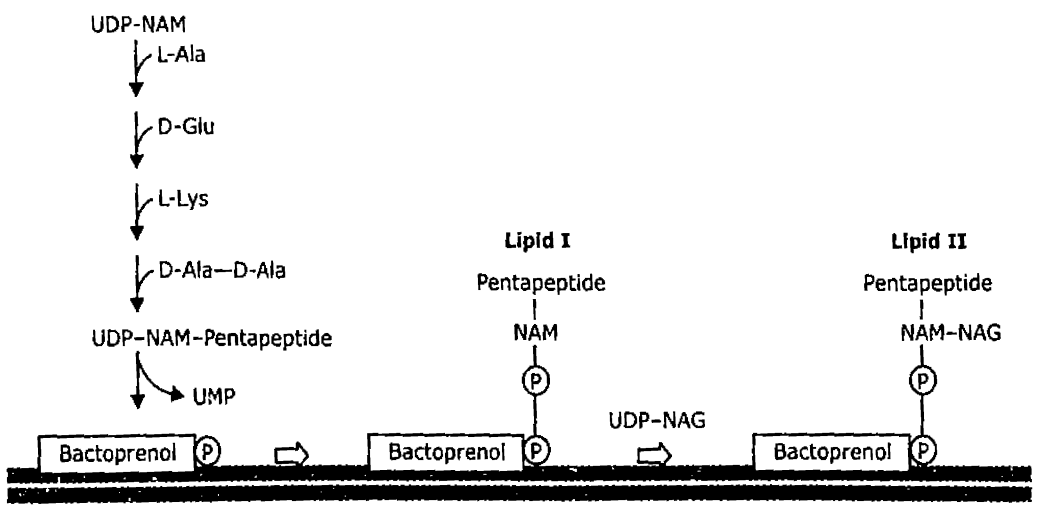


Figure 11.4 : Peptidoglycan synthesis

The *fourth* stage of peptidoglycan biosynthesis, which takes place at the outer side of the cytoplasmic membrane, involves the polymerization of the newly synthesized disaccharide-peptide units and incorporation into the growing peptidoglycan.

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Transpeptidation

During transpeptidation, the D-Ala-D-Ala bond of one stem peptide is first cleaved and an enzyme-substrate intermediate is formed, with the concomitant release of the terminal D-Ala. The cleavage reaction provides the energy necessary for the transpeptidation reaction, which occurs outside the cytoplasmic membrane. A second step involves the transfer of the peptidyl moiety to an acceptor. This acceptor is the non-alpha amino group of the dibasic amino acid in a second stem peptide (in bacteria with direct cross-linking) or the last amino acid of the peptide interbridge when it exists. The reaction results in the formation of a new peptide bond between the penultimate D-alanine of a donor peptide and an amino group of the cross bridge of an acceptor peptide.

Gram staining

Staining is a biochemical technique of adding a class-specific (DNA, proteins, lipids, carbohydrates) dye to a substrate to qualify or quantify the presence of a specific compound. Gram staining (or Gram's method) is an empirical method of differentiating bacterial species into two large groups based on the physical properties of their cell walls. The method is named after the inventor, the Danish scientist *Hans Christian Gram* (1853-1938), who developed the technique in 1884.

The gram staining procedure involves four basic steps:

1. The bacteria are first stained with the basic dye crystal violet. Both gram-positive and gram-negative bacteria become directly stained and appear purple-blue after this step.
2. The bacteria are then treated with gram's iodine solution. This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex. Both gram-positive and gram-negative bacteria remain purple-blue after this step.
3. Gram's decolorizer, a mixture of ethyl alcohol and acetone, is then added. This is the differential step. Gram-positive bacteria retain the crystal violet-iodine complex while gram-negative are decolorized.
4. Finally, the counterstain safranin (also a basic dye) is applied. Since the gram-positive bacteria are already stained purple, they are not affected by the counterstain. Gram-negative bacteria, that are now colorless, become directly stained by the safranin. Thus, gram-positive appear purple-blue, and gram-negative appear red or pink.

Acid-fast staining

The acid-fast stain is a differential stain used to identify acid-fast organisms such as members of the genus *Mycobacterium*. Acid-fast organisms are characterized by wax-like, nearly impermeable cell walls that contain large amount of **mycolic acid**.

The primary stain used in acid-fast staining, carbolfuchsin, is lipid-soluble and contains phenol, which helps the stain penetrate the cell wall. This is further assisted by the treatment with heat. The smear is then rinsed with a very strong decolorizer, which strips the stain from all non-acid-fast cells but does not permeate the cell wall of acid-fast organisms. The decolorized non-acid-fast cells then take up the counterstain.

CAPSULES

Some bacteria form a thick high-molecular-weight, viscous polysaccharide material on their outer surface. The terms **capsule** and **slime layer** are frequently used to describe these layers. The composition of these layers varies in different organisms but may be thick or thin, rigid or flexible, depending on their chemical nature. The rigid layers are organized in a tight matrix called *capsule*. If the layer is more easily deformed and washed off then it is called *slime layer*. Most capsules consist of polysaccharides. However, the capsules

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some bacilli (*Bacillus anthracis*) consist of polypeptides, mainly polyglutamic acid. The possession of capsules makes some pathogenic bacteria resistant to phagocytosis and enhances their virulence.

OUTER MEMBRANE

In addition to peptidoglycan cell wall, gram-negative bacteria contain an additional membrane, the **outer membrane**. This layer is not constructed solely of phospholipid and protein as is the plasma membrane. It also contains polysaccharide. The lipid and polysaccharide are linked in the outer membrane to from a *lipopolysaccharide complex*. Because of this, the outer membrane is often called the lipopolysaccharide layer (LPS). LPS consists of three components, the *core polysaccharide*, the *O-polysaccharide* and lipid portion referred to as *lipid A*. Lipid A is not a glycerol lipid, but instead the fatty acids are connected by amine ester linkage to a disaccharide composed of N-acetylglucosamine phosphate.

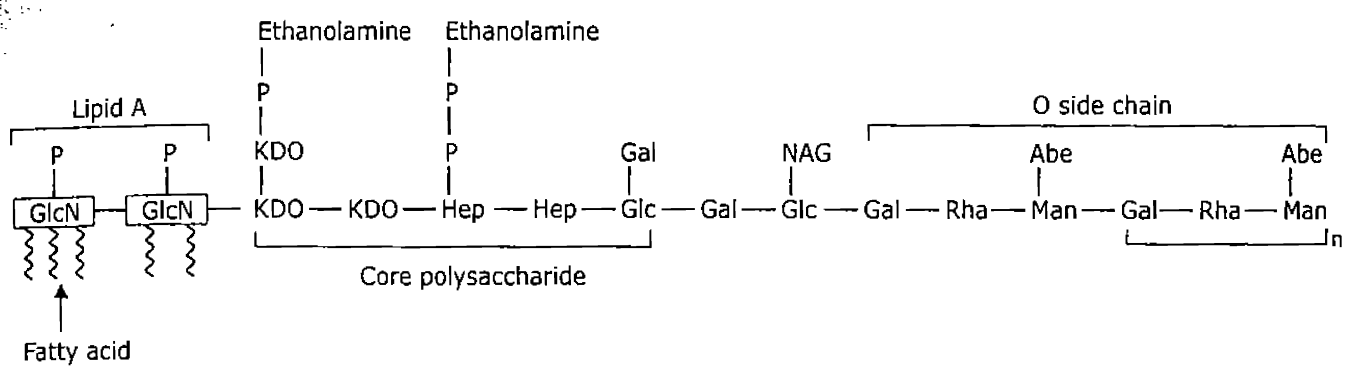


Figure 11.5 : Structure of lipopolysaccharide from *Salmonella* (Abe-abequose, Gal-galactose, Glc-glucose, GlcN-glucosamine, Hep-heptulose, KDO-keto deoxyoctonate, Man-mannose, NaG-N-acetylglucosamine, P-phosphate, Rha-L-rhamnose).

Outer membrane of gram-negative bacteria is relatively more permeable as compared to plasma membrane. This is because proteins called **porins** are present in the outer membrane that function as channels for the entrance and exit of hydrophilic low-molecular-weight substances.

PLASMA MEMBRANE

The bacterial plasma membrane is an unit membrane composed primarily of protein and phospholipid. One major difference in chemical composition of membranes between eukaryotic and prokaryotic cells is that eukaryotes have sterols in their membranes. Sterols are absent from the membranes of all bacteria except *Mycoplasma*. Molecules similar to sterols called **hopanoids**, are present in the membranes of many bacteria, and are likely to play a role similar to that of sterol in eukaryotic cells. Plasma membrane performs many functions, including transport, biosynthesis, and energy transduction. An invagination or infolding of the plasma membrane (called **mesosome**) is present in bacteria that may be involved in chromosome replication or cross wall formation in dividing bacteria.

CYTOPLASM

The bacterial cytoplasm is densely packed with 70S ribosomes. Bacterial cytoplasm also contains many organic and inorganic granules that may be *non-unit membrane* bound or membrane less. These granules may be *Glycogen*, *Poly-β-hydroxy butyrate* (PHB), *Cyanophycin* granules (composed of large polypeptides containing approximately equal amounts of amino acids arginine and aspartic acid; present in cyanobacteria), *Carboxysomes* (polyhedral inclusion bodies that contain the CO_2 fixation enzymes ribulose-1, 5-bisphosphate carboxylase; found in cyanobacteria, nitrifying bacteria, and thiobacilli) and *metachromatic granules* (granules of polyphosphate in the cytoplasm of some bacteria that show differences in colour when stained with a blue basic dye; acts as reserves of phosphate).

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SURFACE APPENDAGES

Two types of surface appendage can be recognized on certain bacterial species: the flagella, which are organs of locomotion, and pili (*Latin* hairs), meant for conjugation.

FLAGELLA

Flagella occur in both Gram-positive and Gram-negative bacteria. Structurally, bacterial flagella are long, filamentous surface appendages. A flagellum consists of three parts:

- The long filament, which lies external to the cell surface;
- The hook structure at the end of the filament; and
- The basal body, to which the hook is anchored and which imparts motion to the flagellum.

The protein subunits of a flagellum are assembled to form a cylindrical structure with a hollow core. Chemically, flagellar filament is constructed of a class of protein called **flagellins**. The hook and basal-body structures consist of numerous proteins.

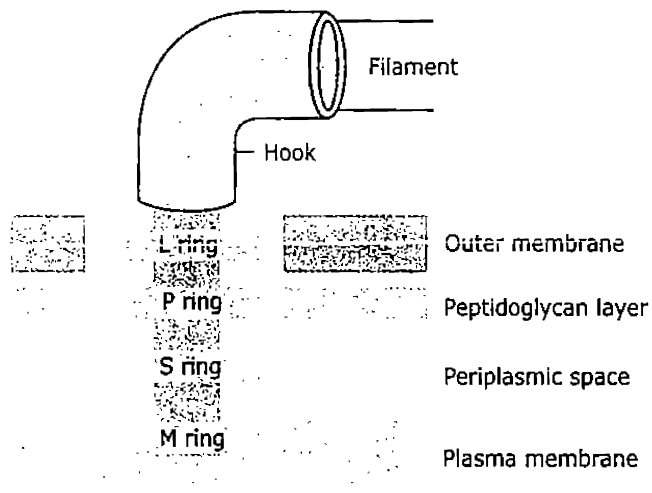


Figure 11.6 : Structure of a flagella (Gram negative bacteria)

The basal body traverses the outer wall and membrane structures. It consists of a rod and one or two pairs of discs (Gram positive - 2 rings in basal body and Gram negative - 4 rings in basal body).

The number and distribution of flagella on the bacterial surface are characteristic for a given species and hence are useful in identifying and classifying bacteria.

Monotrichous	–	Have one flagellum
Amphitrichous	–	One flagellum attached at each end
Lophotrichous	–	Cluster of flagella at one end or both end
Peritrichous	–	Flagella are spread fairly evenly over the whole surface
Atrichous	–	Without flagella

For example, *V. cholerae* has a single flagellum at one pole of the cell (i.e., it is monotrichous), whereas *Proteus vulgaris* and *E. coli* have many flagella distributed over the entire cell surface (i.e., they are peritrichous).

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Flagellar motion

Bacterial flagellar motion is rotatory in nature. The necessary free energy is derived from the proton gradient that exists across the plasma membrane. ATP is *not* directly required for flagellar motion. Membrane proteins MotA and MotB along with FliG create a proton channel that drives rotation of the flagellum. The rotation of flagella can be *clockwise* or *counterclockwise*. When the flagella rotate clockwise, forward motion ceases and the cells *tumble*. Whereas counterclockwise rotations of flagella impart forward motion (*run*).

PILI

Pili occur almost exclusively on Gram-negative bacteria. Only a few Gram-positive bacteria such as *Corynebacterium renale* bear pili. The terms *pili* and *fimbriae* are usually used interchangeably to describe the thin, hairlike appendages on the surface of many Gram-negative bacteria. Proteins that form pili are referred to as **pilins.** Pili are more rigid in appearance than flagella. In addition to the role of the pili in conjugation, they also function as receptors for donor-specific (male-specific) phages.

ENDOSPORES

Bacillus and *Clostridium* species can produce endospores: heat-resistant, dehydrated resting cells that are formed intracellularly and contain a genome and all essential metabolic machinery.

Endospore structure

Endospore structure is very complex and has many layers that are absent from the vegetative cell.

Exosporium : A thin delicate protein covering

Spore coat : Lies beneath the exosporium composed of several protein layers

Cortex : Present beneath the spore coat, made of a peptidoglycan

Spore cell wall or core wall : Inside the cortex and surrounds the protoplast or spore core.

Dipicholinic acids are present in large quantities in the bacterial endospore. It is thought to contribute to the endospore's heat resistance. Endospore morphology and location vary with species. Hence staining is use to determine the morphology and location. Staining of endospore through *Schaeffer-Fulton* procedure first involves heating of bacteria with malachite green that can penetrate endospores. It gives the endospore green colour. After malachite treatment cell is washed with water and is counterstained with safranin.

Endospore formation:

When a growing bacterium (the vegetative cell) is faced with starvation, the process of sporulation begins. The first morphological change is the transformation of the two identical chromosomes (the parent chromosome and the newly replicated daughter chromosome) into an axial filament of DNA. A septum then divides the cell, and the smaller prespore cell is engulfed by the spore mother cell. In this cell-within-a-cell, the plasma membranes separate the cytoplasm of one cell from the cytoplasm of the other and leave a space in between. In this space the spore wall material is deposited.

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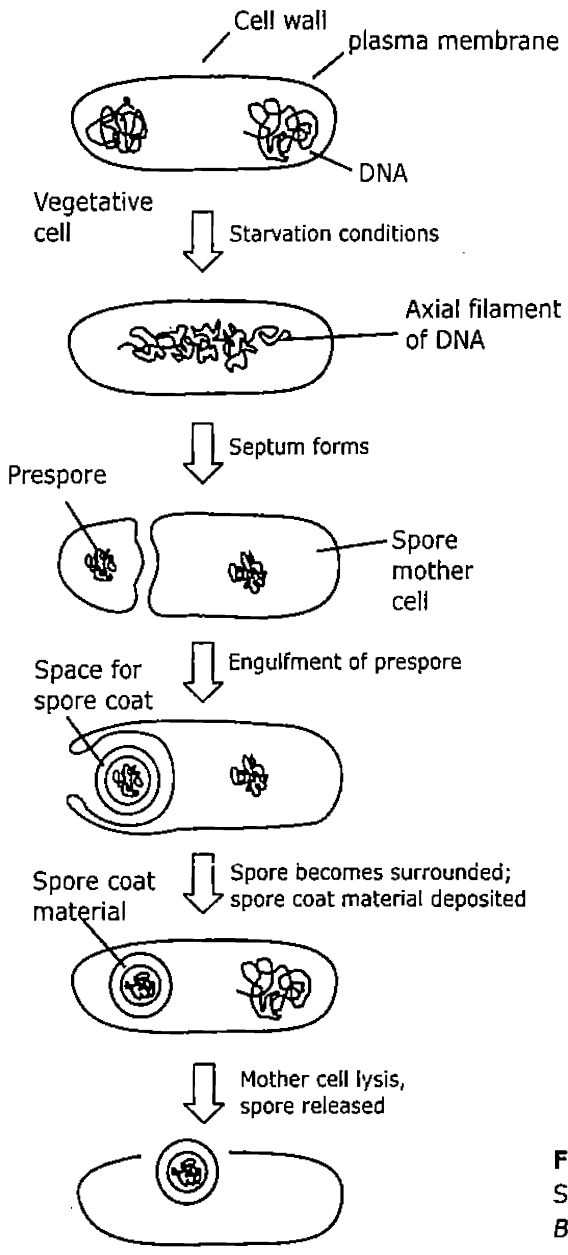


Figure : 11.7
Stages in the development of a *Bacillus subtilis* spore.

Table 11.2 : Differences between endospores and vegetative cells

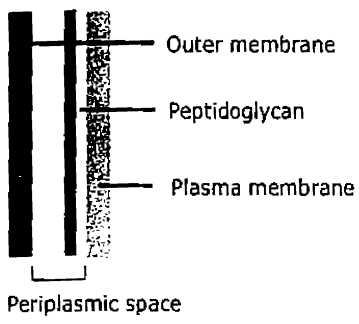
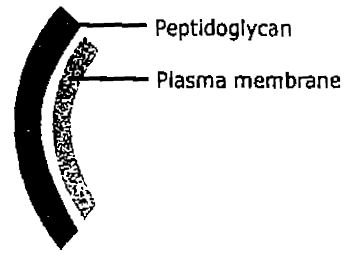
Characteristic	**Vegetative cell**	**Endospore**
Dipicolinic acid	Absent	Present
Enzymatic activity	High	Low
mRNA	Present	Low or absent
Heat resistance	Low	High
Radiation resistance	Low	High
Water content	High, 80-90%	Low, 10-25% in core
Cytoplasmic pH	About pH 7	About pH 5.5-6.0 (in core)
Calcium content	Low	High

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Difference between gram-negative and gram-positive bacteria

Table 11.3 : Some characteristic difference between gram-negative and gram-positive bacteria

Property	Gram-negative bacteria	Gram-positive bacteria
Cell wall	2 to 7 nm thick No teichoic acids	20 to 80 nm thick Teichoic acids present
	Gram-negative cell wall  	Gram-positive cell wall 
Cell Shape	Spheres, ovals, straight or curved rods, helices or filaments; some have sheaths or capsules.	Spheres, rods, or filaments; may show true branching
Reproduction	Binary fission, sometimes budding	Binary fission
Metabolism	Phototrophic, chemolithoautotrophic, or chemoorganoheterotrophic	Usually chemoorganoheterotrophic
Motility	Motile or nonmotile. Flagellation can be varied type - polar, lophotrichous, peritrichous.	Most often nonmotile; have peritrichous flagellation when motile.
Appendages	Can produce several types of appendages like pili, fimbriae	Usually lack appendages
Endospores	Cannot form endospores	Some groups can form endospores

11.3 BACTERIAL NUTRITION AND GROWTH

Bacterial growth is measured by an increase in cell number; whereas growth of multicellular organisms is measured by an increase in their size/mass. All bacteria require a source of carbon to synthesize components of cytoplasm as well as a source of energy and electron in order to grow.

NUTRITIONAL ASPECTS OF BACTERIAL GROWTH

In addition to the need for carbon, hydrogen and oxygen, all organisms require sources of energy and electrons for growth to take place. On the basis of sources of carbon, energy, and hydrogen/electrons bacteria can be categorized into following types-

Based on carbon sources

Autotrophs	CO_2 sole or principal biosynthetic carbon source
Heterotrophs	Reduced, preformed, organic molecules from other organisms

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Based on energy sources

Phototrophs	Light as their energy source
Chemotrophs	Oxidation of organic or inorganic compounds

Based on hydrogen/electron sources

Lithotrophs	Reduced inorganic molecules act as electron source
Organotrophs	Organic molecules act as an electron source

MAJOR NUTRITIONAL TYPES OF BACTERIA

Based on the primary source of carbon, energy and electrons, bacteria are placed in four major nutritional classes.

Photolithotrophic autotrophy (Photolithoautotrophy)

Source of Carbon	:	Carbon dioxide
Source of Energy	:	Light energy
Source of Electrons	:	Inorganic hydrogen/electron donor
Representative bacteria	:	Purple and green sulfur bacteria, Blue-green bacteria

Photoorganotrophic heterotrophy (Photoorganoheterotrophy)

Source of Carbon	:	Organic carbon source (CO_2 may also be used)
Source of Energy	:	Light energy
Source of Electrons	:	Organic hydrogen/electron donor
Representative bacteria	:	Purple nonsulfur bacteria, Green nonsulfur bacteria

Chemolithotrophic autotrophy (Chemolithoautotrophy)

Source of Carbon	:	Carbon dioxide
Source of Energy	:	Chemical energy source (inorganic)
Source of Electrons	:	Inorganic hydrogen/electron donor
Representative bacteria	:	Sulfur-oxidizing bacteria, Hydrogen bacteria, Nitrifying bacteria

Inorganic electron-donor	Groups	Representatives species
$NH_3 \xrightarrow{O_2} NO_2^-$	Ammonia oxidisers	*Nitrosomonas europaea*
$NO_2^- \xrightarrow{O_2} NO_3^-$	Nitrite oxidisers	*Nitrobacter winogradskyi*
$S^0, S_2O_3^{2-} \xrightarrow{O_2} SO_4^{2-}$	Sulphur oxidisers	*Thiobaillus thiooxidans*
$Fe^{2+} \xrightarrow{O_2} Fe^{3+}$	Iron bacteria	*Thiobacillus ferrooxidans*
$H_2 \xrightarrow{O_2} H_2O$	H_2 producers	*Alcaligenes eutrophus*
$CO \xrightarrow{O_2} CO_2$	Carboxidobacteria	*Pseudomanas carboxidovorans*

Chemoorganotrophic heterotrophy (Chemoorganoheterotrophy)

Source of Carbon	:	Organic carbon source
Source of Energy	:	Chemical energy source (organic)
Source of Electrons	:	Organic hydrogen/electron donor
Representative bacteria	:	Most nonphotosynthetic bacteria

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CULTURE MEDIA

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. A culture media is a solid or liquid medium that contain all the nutrients the microorganism requires for growth. It is used to grow, transport and store microorganisms. Depending upon the special needs of particular bacteria (as well as particular investigators) a large variety and types of culture media have been developed with different purposes and uses. Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical and physiological properties. Culture media can be classified on the basis of physical nature (liquid, semi-solid and solid), chemical composition (synthetic and complex) and functional type (supportive, enriched, selective and differential).

Synthetic media (Defined media)

A chemically-defined (synthetic) medium is one in which the exact chemical composition is known. A defined medium is a minimal medium if it provides only the exact nutrients (including any growth factors) needed by the organism for growth.

Complex media

A complex (undefined) medium is one in which the exact chemical constitution of the medium is not known. Example of some common complex media are nutrient broth, tryptic soy broth and MacConkey agar.

Enriched media

An enriched medium contains some component that permits the growth of specific types or species of bacteria, usually because they alone can utilize the component from their environment. However, an enrichment medium may have selective features. Blood agar is a example of enriched media because it encourage the growth of many fastidious microbes.

Selective media

A selective medium is one which has a component(s) added to it which will inhibit or prevent the growth of certain types or species of bacteria and/or promote the growth of desired species. For example bile salts and crystal violet favor the growth of gram-negative bacteria by inhibiting the growth of gram-positive bacteria. Eosin methylene blue agar, MacConkey agar and Mannitol salt agar are commonly used as selective media.

Differential media

A culture medium is described as a differential medium if it allows the investigator to distinguish between different types of bacteria based on some observable trait in their pattern of growth on the medium. Blood agar is an example of differential media and is used to distinguish between hemolytic and non-hemolytic bacteria. Eosin methylene blue agar, MacConkey agar and Mannitol salt agar are also used as differential media.

Pure culture

A pure culture is one that contains only a single kind of microbial population grown from a single cell. A pure culture is usually derived from a mixed culture (containing many species) by methods that separate the individual cells so that, when they multiply, each will form an individually distinct colony, which may then be used to establish new cultures with the assurance that only one type of organism will be present. Pure cultures may be more easily isolated if the growth medium of the original mixed culture favours the growth of one organism to the exclusion of others.

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BACTERIAL GROWTH

Growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves increase in cell mass and cell divisions. The asexual process of cell division in bacteria is known as *binary fission*. Binary fission begins when the DNA of the cell is replicated. Each circular strand of DNA then attaches to the plasma membrane. The cell elongates, causing the two chromosomes to separate. The plasma membrane then invaginates (grows inward) and splits the cell into two daughter cells through a process called cytokinesis. The principles that underlie chromosome segregation in bacteria and eukaryotes share similarities, although bacteria segregate DNA as it replicates and lack a eukaryote-like mitotic apparatus for segregating chromosomes. This may be because the distances that newly replicated bacterial chromosomes move apart before cell division are small as compared to those in eukaryotes.

E. coli divides by forming a septum across the middle of the cell. The biochemical mechanism underlying this process is unknown. FtsZ is required for cell division. FtsZ is homologous to tubulin, the building block of the microtubule in eukaryotes. The cellular concentration of FtsZ regulates the frequency of division. At the time of onset of septal invagination, the FtsZ protein is recruited from the cytoplasm to the division site, where it assembles into a ring that remains associated with the leading edge of the invaginating septum until septation is completed.

Bacterial growth curve

Under favorable conditions, a growing bacterial population doubles at regular intervals. Growth occurs in geometric progression: 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3.....2^n (where n = the number of generations). This growth is called **exponential growth**. In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in nature.

When a fresh medium is inoculated with a given number of cells, and the population growth is monitored over a period of time, plotting the data will yield a typical bacterial growth curve. Four characteristic phases of the growth cycle are recognized.

Lag phase

Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass.

Exponential (log) phase

The exponential phase of growth is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission, and are growing by *geometric progression*. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as **generation time**, also the *doubling time* of the bacterial population. The *generation time* is the time interval required for the cells (or population) to divide.

Stationary phase

Exponential growth cannot be continued forever in a *batch culture* (e.g., a closed system such as a test tube or flask). Population growth is limited by one of three factors:

- Exhaustion of available nutrients;
- Accumulation of inhibitory metabolites or end products;
- Exhaustion of space, in this case called a lack of "biological space".

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If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. During the death phase, the number of viable cells decreases geometrically (exponentially), essentially reverse of the growth during the log phase.

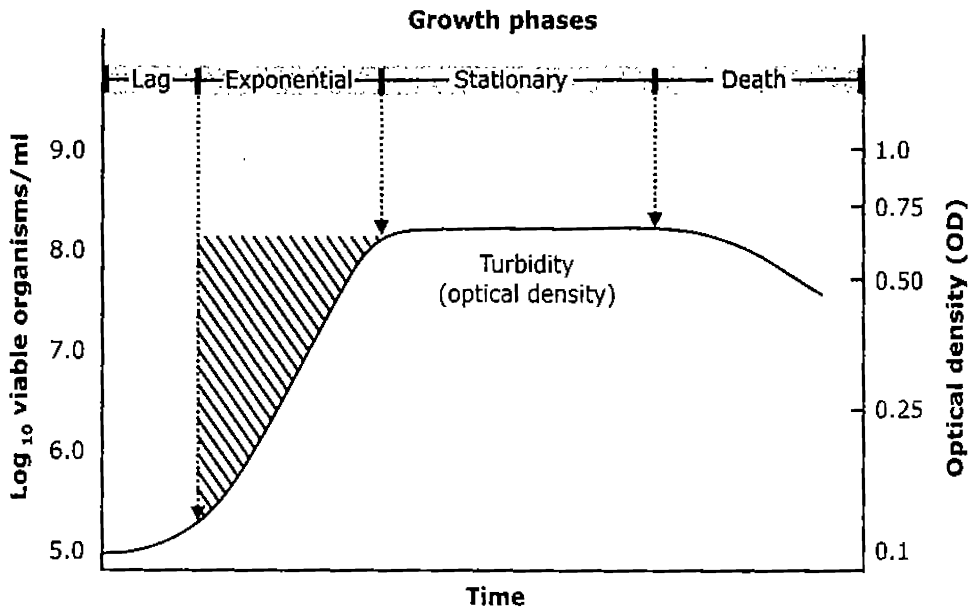


Figure 11.8 : Typical growth curve for a bacterial population.

Calculation of generation time

When growing exponentially by binary fission, the increase in a bacterial population is by geometric progression. If we start with one cell, when it divides, there are 2 cells in the first generation, 4 cells in the second generation, 8 cells in the third generation, and so on.

$$G \text{ (generation time)} = \frac{t \text{ (time, in minutes or hours)}}{n \text{ (number of generations)}}$$

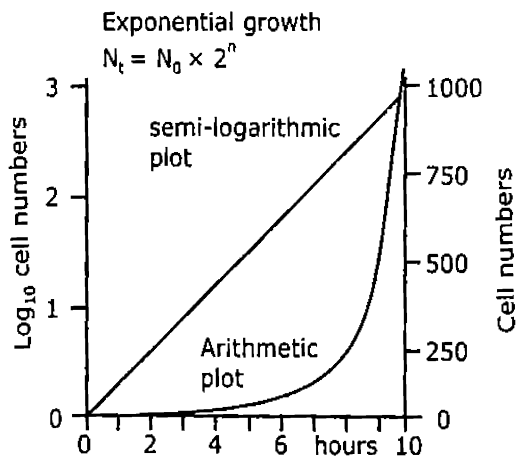


Figure : 11.9
Exponential growth of unicellular organisms. Arithmetic and semi-logarithmic plots of cell numbers versus time.

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A plot of the number of cells against the time gives an exponential curve. Such an arithmetic representation of a growth curve (as shown in the figure) is, however unsuitable for dealing with a large number of cell divisions. It is preferable, therefore, to use a semi-logarithmic plot. Such a logarithmic plot shows exponential growth as a straight line, the slope of the line corresponding to the division rate; the steeper the slope, the greater the division rate. Since exponential growth is characterised by a linear relationship between time and logarithm of cell number it is also referred to as **logarithmic growth.**

Calculation of number of generations

If N_0= number of bacteria at the beginning of a time interval; N = number of bacteria at the end of the time interval and n = number of generations (number of times the cell population doubles during the time interval) then $N = N_0 \times 2^n$ (This equation is an expression of growth by binary fission).

Applying logarithms, this becomes

$$\log N = \log N_0 + n \log 2$$

and for the number of cell divisions (n)

$$n = \frac{\log N - \log N_0}{\log 2} = \frac{\log N - \log N_0}{0.301} = 3.3 \log N / N_0$$

Calculation of bacterial density

Cell density is the number of cells per unit volume. The number of bacterial cells can be determined directly as well as indirectly. Direct measurement can be carried out by measuring colony number or by counting the number of cells in known volume with microscope. Whereas indirect method involves the measurement of optical absorbance through spectrophotometer.

Measurement of colony number

Bacterial cells form colonies on agar medium. As the bacteria divide, the progeny bacteria remain adjacent to the original bacterium. As the number of progeny increases to about 10^6 cells, a visible cluster of bacteria appears. This cluster is a population of bacterial cells called a *bacterial colony*. Because each viable cell can form a single colony, by counting the number of colonies formed when a known volume of diluted culture is plated one can determine the number of bacteria in an undiluted culture. Usually bacterial culture contains very high number of cells, hence it must be diluted before plating, and the dilution factor must be taken into account when calculating the cell density in undiluted culture. It can be calculated by using following formula :

$$\frac{\text{(Number of colonies formed)}}{\text{(ml plated)} \times \text{(dilution before plating)}} = \frac{\text{Number of viable cells}}{\text{ml of undiluted culture}}$$

Influence of environmental factors on growth

Growth of microbes are greatly affected by the chemical and physical conditions of their environments. Many environmental factors like temperature, pH, water availability, and oxygen play major roles in controlling growth of microorganisms. On the basis of their response to major environmental factors microbes can be divided into various class as given in the table.

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Table 11.4 : Effect of microbial responses to environmental factors

Term	Definition	Example
Halophile	Requires high levels of salt concentration	*Halobacterium*
Acidophile	Growth optimum between pH 0 and 5.5	*Sulfolobus*
Alkalophile	Growth optimum between pH 8.0 and 11.5	*Bacillus alcalophilus*
Psychrophile	Grows well at 0°C and has an optimum growth temperature of 15°C or lower	*Chlamydomonas nivalis*
Psychrotroph	Can grow at 0–7°C; has an optimum between 20 and 30°C	*Pseudomonas fluorescens*
Thermophile	Can grow at 55°C or higher; optimum often between 55 and 65°C	*Thermus aquaticus*
Hyperthermophile	Has an optimum between 80 and about 110°C	*Pyrococcus, Pyrodictium*
Obligate aerobe	Completely dependent on O_2 for growth	*Pseudomonas*
Facultative anaerobe	Does not require O_2 for growth, but grows better in its presence	*Escherichia, Enterococcus*
Aerotolerant anaerobe	Grows equally well in presence or absence of O_2	*Streptococcus pyogenes*
Obligate anaerobe	Does not tolerate O_2 and dies in its presence	*Clostridium*
Microaerophile	Requires O_2 levels below upto 10% for optimum growth	*Treponema pallidum*

11.4 BACTERIAL GENOME : BACTERIAL CHROMOSOME AND PLASMID

NUCLEOID

The nucleoid is the region in a prokaryotic cell that contains the genome. The bacterial nucleoid, which contains the dsDNA fibrils, lacks a limiting membrane. The bacterial chromosome is a single, continuous, circular DNA molecule. Bacterial chromosome does not contain basic histone proteins, but low-molecular-weight polyamines and magnesium ions may perform a function similar to that of eukaryotic histones. The bacterial chromosome is organized into independently supercoiled loops called *domains*. The domain organization enables the chromosomal DNA to undergo structural changes during different cellular processes (replication, transcription, and segregation) that take place simultaneously in a bacterial cell. Small nucleoid-associated proteins (HU, H-NS, IHF, Fis, Lrp, and Dps) and the high-molecular-weight protein SMC (structural maintenance of chromosomes) facilitate compaction of chromosomal DNA by bending, bridging, and wrapping. In addition, SMC protein is involved in chromosome segregation.

Variations in prokaryotic genome structure

Most prokaryotes, like *E. coli*, contain a single circular DNA molecule that makes up their entire genome, recent studies have indicated that some prokaryotes contain as many as four linear or circular chromosomes. For example, *Vibrio cholerae*, the bacteria that causes cholera, contains two circular chromosomes. One of these chromosomes contains the genes involved in metabolism and virulence, while the other contains the remaining essential genes. An even more extreme example is provided by *Borrelia burgdorferi*, the bacterium that causes Lyme disease. It contains up to 11 copies of a single linear chromosome. Unlike *E. coli*, *Borrelia* cannot supercoil its linear chromosomes into a tight ball within the nucleoid; rather, these strands are diffused throughout the cell. The DNA molecules of Archaea share many similarities with

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eukaryotes. Archaeans are the only group of prokaryotes that use eukaryote-like histones to condense their DNA molecules. DNA molecules of Archaea can also be negatively supercoiled, positively supercoiled, or not supercoiled at all.

Some example of bacterial chromosome

Bacteria	*Chromosome* (Number, shape and size)
Agrobacterium tumefaciens	One linear (2.1 Mb) + One circular (3.0 Mb)
Bacillus subtilis	One circular (4.2 Mb)
Brucella melitensis	Two circular (2.1 + 1.2 Mb)
Vibrio cholerae	Two circular (2.9 + 1.1 Mb)
Rhodobacter sphaeroides	Two circular (3.0 + 0.3 Mb)
E. coli K-12	One circular (4.6 Mb)

Prokaryotic versus Eukaryotic genome

Most prokaryotes reproduce asexually and are haploid, meaning that only a single copy of each gene is present. By contrast, eukaryotes that reproduce sexually generally contain multiple chromosomes and are said to be diploid, because two copies of each gene exist—with one copy coming from each of an organism's parents. Noncoding sequences account for an average of 12% of the prokaryotic genome, as opposed to upwards of 98% of the genetic material in eukaryotes. Another difference between prokaryotes and eukaryotes is that prokaryotic cells often contain one or more plasmids (i.e., extrachromosomal DNA molecules that are either linear or circular). Unlike eukaryotic chromosomes, most prokaryotic genomes are organized into polycistronic operons separated by only a few base pairs. The proteins encoded by each operon often collaborate on a single task, such as the metabolism of a sugar into by-products that can be used for energy. The organization of prokaryotic DNA thus differs from that of eukaryotes in several important ways. The most notable difference is the condensation process that prokaryotic DNA molecules undergo in order to fit inside relatively small cells.

Prokaryotic chromosomes

- Many prokaryotes contain a single circular chromosome.
- Prokaryotic chromosomes are condensed in the nucleoid via DNA supercoiling and the binding of various architectural proteins.
- Because prokaryotic DNA can interact with the cytoplasm, transcription and translation occur simultaneously. Most prokaryotes contain only one copy of each gene (i.e., they are haploid).
- Nonessential prokaryotic genes are commonly present on extrachromosomal plasmids.
- Prokaryotic genomes are efficient and compact, containing little repetitive DNA.

Eukaryotic chromosomes

- Eukaryotes contain multiple linear chromosomes.
- Eukaryotic chromosomes are condensed in a membrane-bound nucleus via histones.
- In eukaryotes, transcription occurs in the nucleus, and translation occurs in the cytoplasm.
- Most eukaryotes contain two copies of each gene (i.e., they are diploid).
- Some eukaryotic genomes are organized into operons, but most are not.
- Extrachromosomal plasmids are not commonly present in eukaryotes.
- Eukaryotes contain large amounts of noncoding and repetitive DNA.

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PLASMIDS

Plasmids (term coined by J.Lederberg) are replicons that are maintained as discrete, extrachromosomal genetic elements in bacteria (also present in some yeast and other fungi). They are usually much smaller than the bacterial chromosome, varying from less than 5 kbp to more than several hundred kbp, though plasmids as large as 2 Mbp also occur in some bacteria. Plasmids usually encode traits that are not essential for bacterial viability, and replicate independently of the chromosome. Most plasmids are supercoiled, circular, double-stranded DNA molecules, but linear plasmids have also been demonstrated in genera *Borrelia* and *Streptomyces*.

Plasmids can be classified into *conjugative* and *non-conjugative* type. Conjugative plasmids code for functions that promote transfer of the plasmid from the donor bacterium to other recipient bacteria, but non-conjugative plasmids do not. Conjugative plasmids have genes for the synthesis of pili that help in the process of conjugation. The best studied conjugative plasmid is the **F-factor** (or F-plasmid) of *E.coli*. The bacterial cell contain F-factor are described as F^+. F^+ cell acts as donor, where as F^- cell (a cell lacking an F-plasmid) acts as recipient.

The average number of molecules of a given plasmid per bacterial chromosome is called its *copy number*. Some plasmids are present in the cell in only 1-3 copies, whereas others may be present in over 100 copies. Large plasmids (>40 kilobase pairs) are often *conjugative*, have small copy numbers, code for all functions required for their replication, and partition themselves among daughter cells during cell division in a manner similar to the bacterial chromosome. Plasmids smaller than 7.5 kilobase pairs usually are *non-conjugative*, have high copy numbers (typically 1020 per chromosome), rely on their bacterial host to provide some functions required for replication, and are distributed randomly between daughter cells at division.

Many plasmids control medically important properties of pathogenic bacteria, including resistance to one or several antibiotics or production of toxins. Many plasmids confer antibiotic resistance on the cells that contain them and are described as **R-plasmid** (resistance plasmid). Some R-plasmids have only a single resistance gene, whereas others have many. Many R-plasmids are conjugative plasmids.

Col plasmids are able to produce *bacteriocin* such as colicin, proteins that prevent growth of susceptible bacterial strains that do not contain a Col plasmid. Bacteriocins are proteins that can interact with sensitive bacteria and inhibit one or more essential processes, such as DNA replication, transcription, translation or energy metabolism. Bacteriocins produced by *E.coli* are termed *colicins*. There are many types of colicins, each designated by a letter (for example, colicin B) and each having a particular mode of inhibition of sensitive cells.

Type of plasmid	Gene functions
Resistance	Antibiotic resistance
Fertillity	Conjugation and DNA transfer between bacteria
Killer	Synthesis of toxins that kill other bacteria
Degradative	Enzymes for metabolism of unusual molecules
Virulence	Pathogenicity

Host range

Most plasmids can exist in only a limited number of closely related bacteria; these are called *narrow host range* plasmids. Some plasmid like IncP of *E.coli* can be transferred to and maintained in bacteria of a large number of species. These are called *broad host range* plasmids. Why some plasmids have a broad host range in unknown.

Functions encoded by plasmids

Depending on their size, plasmids can encode a few or hundreds of different proteins. However, plasmids rarely encode gene products that are essential for growth, such as RNA polymerase, ribosomal subunits,

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or enzymes of the tricarboxylic acid cycle. Instead, plasmid genes usually give bacteria a selective advantage under only some conditions.

Gene products encoded by plasmids include enzymes for the utilization of unusual carbon sources such as toluene, resistance to substances such as heavy metals and antibiotics, synthesis of antibiotics, and synthesis of toxins and proteins that allow the successful infection of higher organisms. Table given below lists a few traits that are encoded by naturally occurring plasmids.

Table 11.5 : Some naturally occurring plasmids and the traits they carry

Plasmid	**Trait**
ColE1	Bacteriocin which kills *E.coli*
Tol	Degradation of toluene and benzoic acid
Ti	Tumor initiation in plants
pJP4	2,4-D degradation
pSym	Nodulation on roots of legume plants
RK2	Resistance to ampicillin, tetracycline, and kanamycin

Table 11.6 : List of some plasmid-coded traits

Trait	**Organisms in which trait is found**
Antibiotic resistance	*E. coli, Salmonella sp., Staphylococcus sp.*
Pilus synthesis	*E. coli*, Pseudomonas sp.
Tumor formation in plants	*Agrobacterium tumefaciens*
Nitrogen fixation (in plants)	*Rhizobium sp.*
Oil degradation	*Pseudomonas sp.*
Gas vacuole production	*Halobacterium sp.*
Insect toxin synthesis	*Bacillus thuringiensis*
Plant hormone synthesis	*Pseudomonas sp.*
Antibiotic synthesis	*Streptomyces sp.*
Increased virulence	*Yersinia enterocolitica*

Hfr formation

The F factor is an example of episome. An episome is a plasmid that can exist autonomously in a cell or can integrate into the bacterial chromosome. A bacterial cell that contains an integrated F factor is referred to as an **Hfr cell**. The frequency of insertion occurs at about 10^{-5}–10^{-7} per generation. Integration involves homologous recombination between two covalently closed circular DNA molecules forming one circular molecule containing both of the original DNA structures. It is thought that the insertion sequences (IS sequence) present in the F genome and those in the host chromosome serve as regions of homology for the insertional event.

Plasmids in eukaryotic microbes:

Plasmids are not limited to bacteria. For example, plasmids also found in eukaryotic microbes like yeast. One yeast plasmid is called the 2μ circle. The 2μ circle is a 6.3 kb circular, extrachromosomal element found in the nucleus of most *Saccharomyces cerevisiae* strains. It is stably maintained at about 50 to 100 copies per haploid genome of the yeast cell. Like the host chromosomes, the 2μ circle is coated with nucleosomes and replication is initiated by host replication enzymes once per cell cycle.

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11.5 HORIZONTAL GENE TRANSFER AND GENETIC RECOMBINATION

Gene transfer refers to the movement of genetic information between organisms. Gene transfer can be *horizontal* or *vertical*. Transfer of genes from parents to offsprings is termed *vertical* gene transfer whereas transfer of genes between two independent organisms is called *horizontal* or *lateral* gene transfer. Sexual reproduction in eukaryotes is an example of vertical gene transfer. Prokaryotes do vertical gene transfer when they reproduce by binary fission. Prokaryotes can also do horizontal gene transfer when they pass gene to other prokaryotic cells. Three mechanisms of horizontal gene transfer in prokaryotes have been discovered. These are *transformation, transduction* and *conjugation*.

Because donor DNA cannot persist in the recipient bacterium unless it is part of a replicon, recombination between donor and recipient genomes is often required to produce stable, hybrid progeny. Transfer of genes from one bacterial cell to other bacterial cell of same species or closely related species may result in substitution of donor alleles for recipient alleles. In molecular biology, the process of replacement of one allele by different alleles from the same genes by preserving the structure of genes is termed genetic *recombination*.

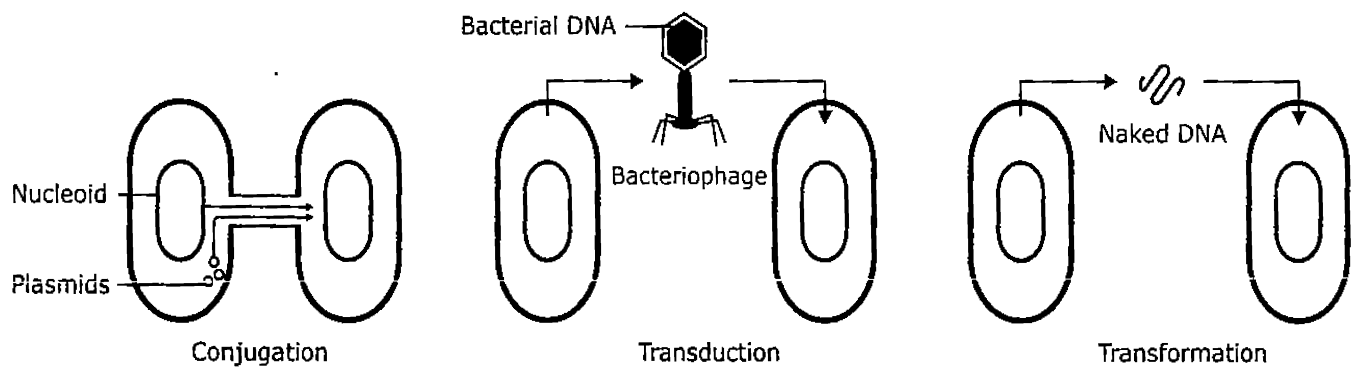


Figure 11.10 : Three ways of achieving DNA transfer between bacteria.

TRANSFORMATION

Transformation was the first mechanism of gene exchange in bacteria to be discovered. In 1928, Fred Griffith found that one form of the pathogenic pneumococci (now called *Streptococcus pneumoniae*) could be mysteriously "transformed" into another form. Griffith's experiments were based on the fact that *S.pneumoniae* makes two different types of colonies, one pathogenic and the other nonpathogenic. Because pathogenic strains excrete a polysaccharide capsule, they make colonies that appear smooth on agar plates. Apparently because the capsule allows them to survive in a vertebrate host, these bacteria can infect and kill mice. However, rough-colony-forming mutants that cannot make the capsule and are nonpathogenic in mice sometimes arise from the smooth-colony formers.

In his experiment, Griffith mixed dead *S. pneumoniae* cells that made smooth colonies with live nonpathogenic cells that made rough colonies and injected the mixture into mice. Mice given injections of only the rough-colony-forming bacteria survived, but mice that received a mixture of dead smooth-colony formers and live rough-colony formers died. Furthermore, Griffith isolated live smooth-colony-forming bacteria from the blood of the dead mice.

	Bacterial type	*Effect in mouse*	*Bacteria recovered*
A	Live type R	Nonpathogenic	None
B	Live type S	Pathogenic	Live type S
C	Heat-killed type S	Nonpathogenic	None
D	Mixture of live type R and heat-killed type S	Pathogenic	Live type S

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Concluding that the dead pathogenic bacteria gave off a "transforming principle" that changed the live nonpathogenic rough-colony-forming bacteria into the pathogenic smooth-colony form, he speculated that this transforming principle was the polysaccharide itself. About 16 years after Griffith did his experiments with mice, Oswald Avery and his collaborators used the *in vitro* system to purify the *transforming principle* and showed that it is DNA. The work of *Avery* and colleagues helped show that DNA, not proteins or other factors in the cell, is the hereditary material.

Competence

The ability of a recipient bacterium to take up free DNA and become transformed is known as *competence*. Competence is an inheritable characteristic of certain strains of bacteria. Some species of bacteria are *naturally* competent; transformation can occur at a high frequency since most cells in such a population can take up environmental DNA at any time. Competent bacteria that can take up DNA encode proteins called *competence factors*. These proteins facilitate the binding of DNA fragments to cell surface and uptake of DNA into cytoplasm. In competent bacteria, which include *Streptococcus pneumoniae*, *Bacillus subtilis*, and *Haemophilus influenzae*, linear, double-stranded DNA (ds DNA) enters the cell, but one strand is degraded while the other is integrated into the chromosome by homologous recombination mediated by RecA protein.

Process of transformation

Transformation is a mechanism of genetic transfer between bacteria in which the donor DNA is cell-free DNA in the recipient bacterium's immediate environment. DNA can be naturally released into the environment when cells die and subsequently lyse. Once the free DNA is brought into the cell, it may become integrated into the recipient's genome by homologous recombination. Recipient cells that undergo this process and acquire a new phenotype as a result are said to be **transformed**. This phenomenon allows only the uptake of DNA from the same or related species. Transformation was discovered in *Streptococcus pneumoniae* and occurs in other bacterial genera including *Haemophilus, Neisseria, Bacillus*, and *Staphylococcus*.

Mechanism of DNA transfer in bacteria by transformation

a. dsDNA attaches to membrane-bound dsDNA-binding protein.
b. One of the two strands of the transforming DNA passes into the cell while the other strand is degraded by a nuclease.
c. The ssDNA within the cell is bound by ssDNA-binding proteins. During this process, homologous recombination takes place and the transforming DNA is incorporated into the bacterial chromosome.
d. The transformed cell contains the segment of transforming DNA incorporated into its genome.

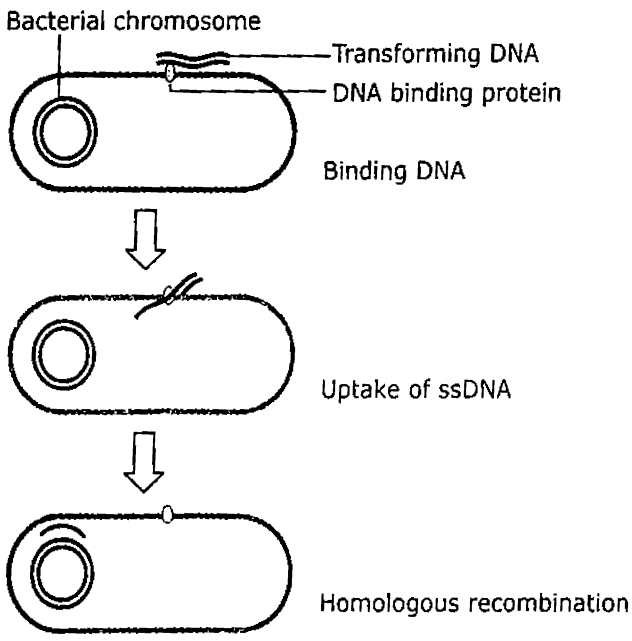


Figure : 11.11
Mechanism of transformation in a bacterium

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The fragment of DNA that has been transferred from a donor cell to a recipient cell is referred to as an **exogenote.** The recipient bacterial cell's own genetic material into which the donor DNA can integrate is called **endogenote.** A bacterial cell that has received an exogenote is initially diploid for part of its genome, and is said to be a **merozygote**. However, single-stranded exogenotes are unstable and will usually be degraded unless they are integrated into the endogenote. If the exogenote contains an allele of the endogenote, the resulting recombinant double helix would contain one or more mismatched base pairs, and is referred to as a **heteroduplex**. If progeny cells are to receive the new allele, mismatch repair must occur by excising a segment of the endogenote strand and using the exogenote strand as a template for its replacement.

TRANSDUCTION

In transduction, bacteriophages function as vectors to introduce DNA from donor bacteria into recipient bacteria by infection. The transfer of bacterial genes by phage was discovered by Lederberg and Zinder in 1951. There are two kinds of transduction: *generalized* and *specialized*. In **generalized transduction**, transducing phages produced during lytic growth are aberrant and contain a random fragment of the bacterial genome instead of phage DNA. Each individual transducing phage carries a different set of closely linked genes, representing a small segment of the bacterial genome.

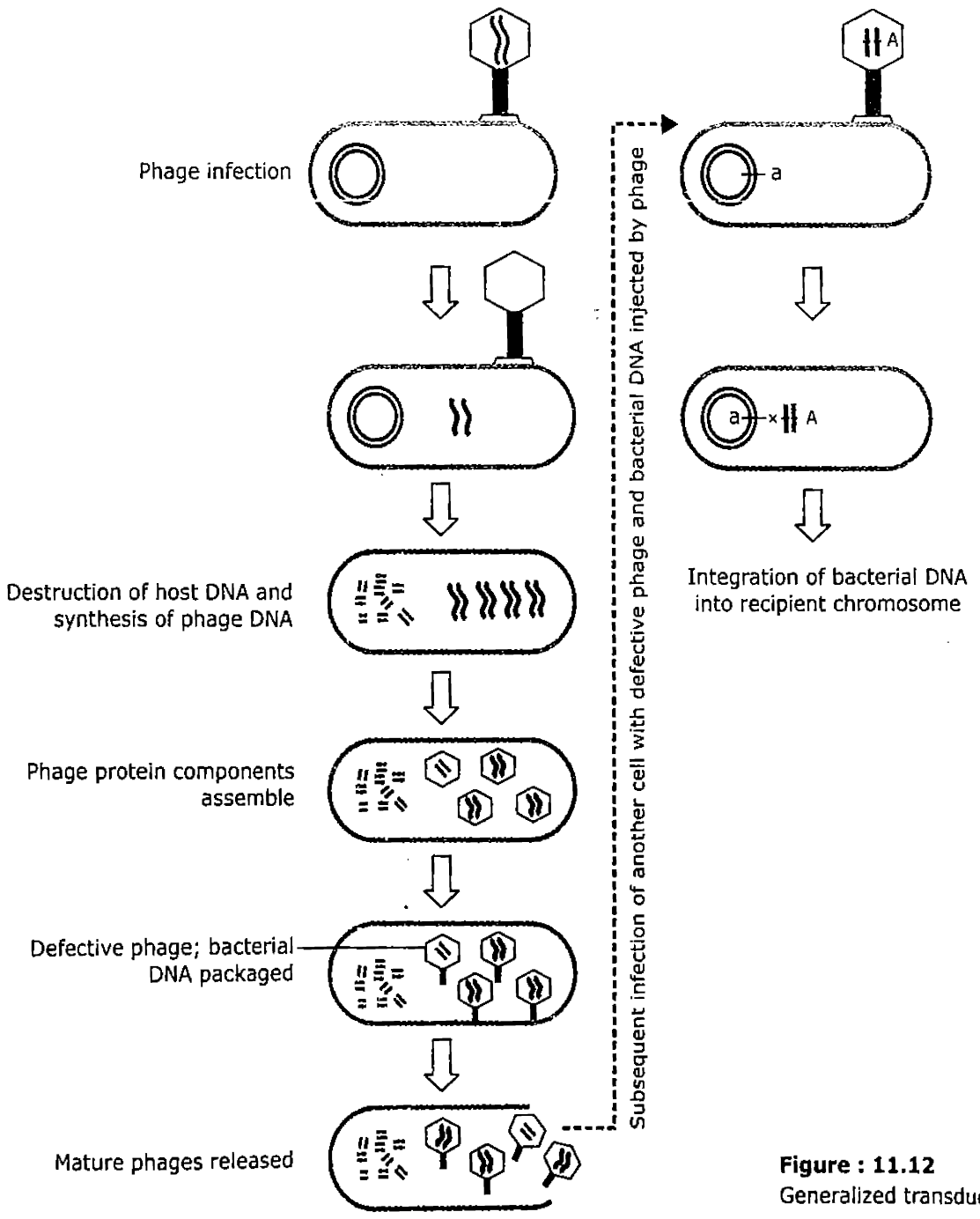


Figure : 11.12
Generalized transduction

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So in generalized transduction, each part of the bacterial genome has approximately the same probability of being transferred from donor to recipient bacteria. When a generalized transducing phage infects a recipient cell, expression of the transferred donor genes occurs. Typical phage that can mediate generalized transduction include P1 (*E. coli*) and P22 (*Salmonella*).

Generalized transduction may be **abortive** or **complete**. *Abortive transduction* refers to the transient expression of one or more donor genes without formation of recombinant progeny, whereas *complete transduction* is characterized by production of stable recombinants that inherit donor genes and retain the ability to express them. In abortive transduction, the donor DNA fragment does not replicate, and among the progeny of the original transductant only one bacterium contains the donor DNA fragment. The frequency of abortive transduction is typically one to two orders of magnitude greater than the frequency of complete transduction, indicating that most cells infected by generalized transducing phages do not produce recombinant progeny.

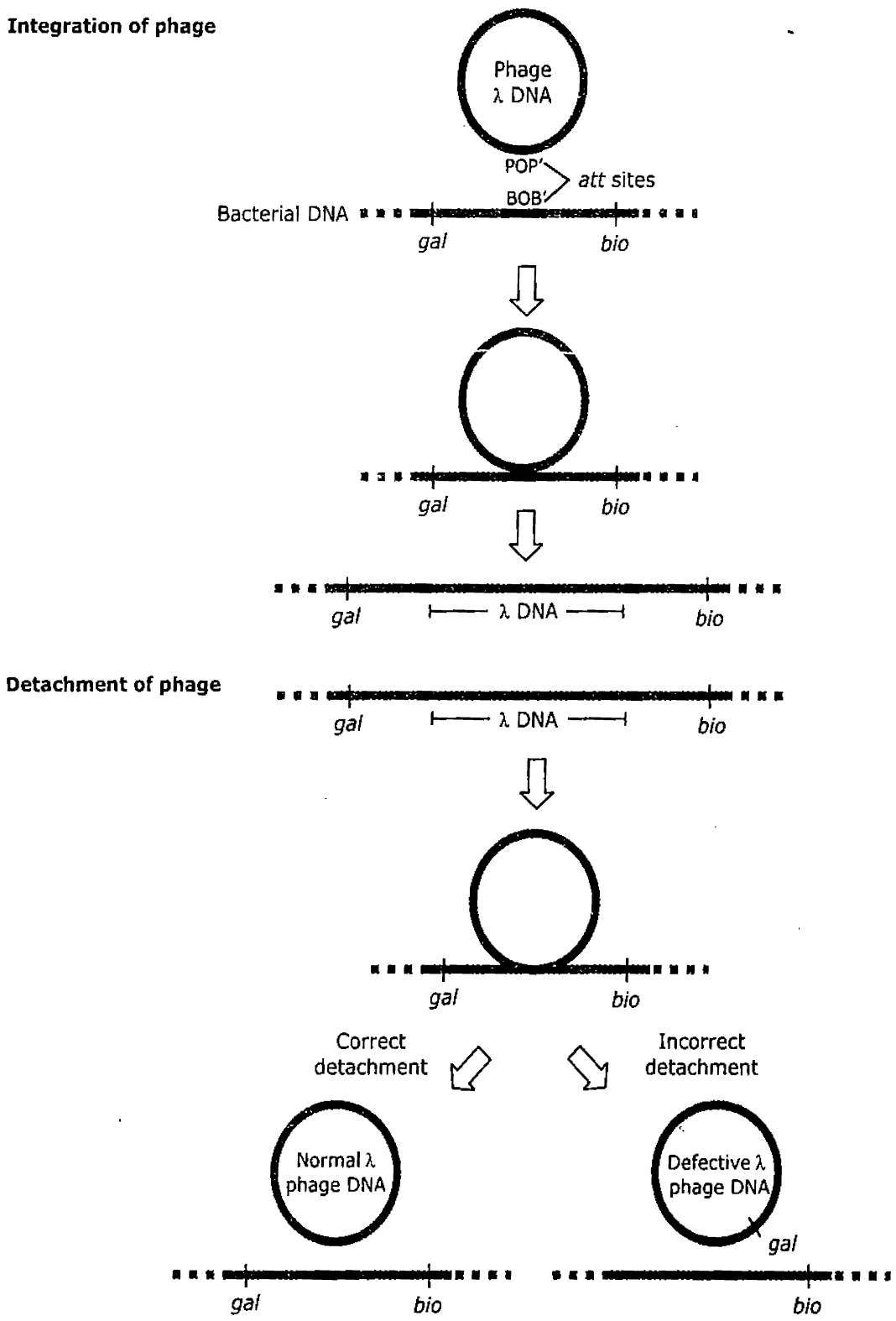


Figure 11.13 : The production of defective lambda phage.

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Specialized transduction differs from generalized transduction in several ways. It is mediated only by specific temperate phages, and only a few specific donor genes can be transferred to recipient bacteria. Specialized transducing phages are formed only when lysogenic donor bacteria enter the lytic cycle and release phage progeny. There are two distinguishing characteristics of specialized transduction.

1. The only bacterial genes that can be transduced are those very near the site at which the prophage is integrated. For example the only site at which lambda phage integrates into the host chromosome is between the genes for galactose fermentation (*gal*) and biotin synthesis (*bio*). So, if the prophage deintegrates abnormally from the host chromosome, only the *gal* or *bio* genes could be transduced.
2. It results from defective excision of the prophage from the host chromosome.

The specialized transducing phages contain both host and phage DNA linked in one single DNA molecule. They are rare recombinants which lack part of the normal phage genome and contain part of the bacterial chromosome located adjacent to the prophage attachment site.

Formation of the λ*gal* and λ*bio* transducing particles causes loss of some λ genes. λ*gal* particles lack the tail genes and sometimes head genes, both of which are located at the right end of the prophage; the λ*bio* particle lacks genes from the left end of the prophage (*int, xis*). The number of missing phage genes depends on the position of the cuts that generated the particle. The head and the tail genes are essential, so λ*gal* transducing particles are unable to perform lytic cycles and thus plaques. They are defective, and this is denoted λd*gal*. The genes missing in λ*bio* transducing particles are not essential for lytic growth, so λ*bio* phage are usually plaque forming and are called λp*bio*, in which the 'p' stands for *p*laque forming.

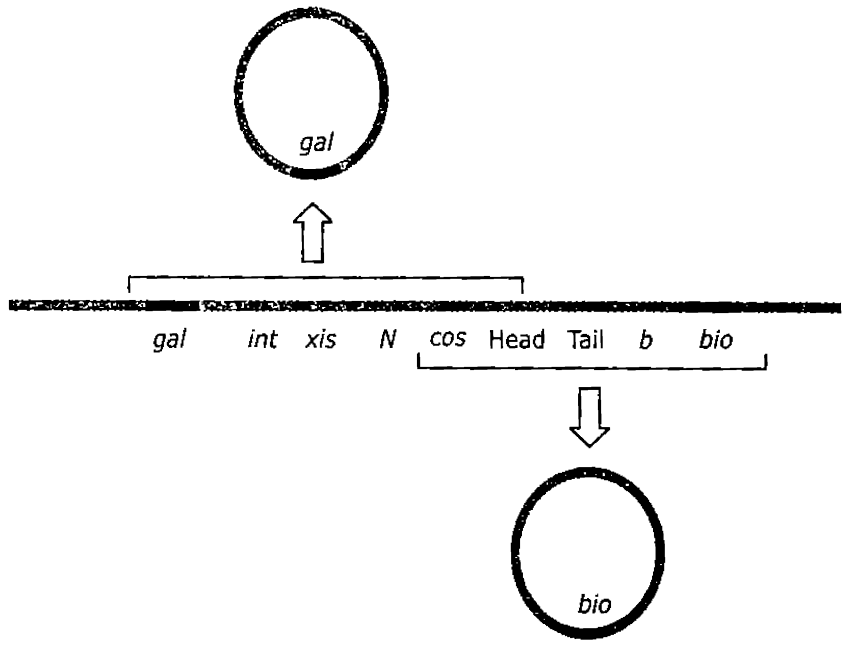


Figure 11.14 : Aberrant excision leading to production of λd*gal* and λp*bio* phages.

If λd*gal* transducing particles lack phage genes required to grow lytically, how are they formed? The transducing particle is produced by aberrant excision from a normal prophage. The prophage contains all of the essential genes, and hence the necessary gene products (head and tail proteins) are still present in the chromosome. Initially producing the defective transducing particles is not a problem, but there is a deficiency of essential gene products when the transducing particle infects a new cell. If a bacterial cell is double infected with a wild type lambda page and a λd*gal* phage, the wild-type phage can supply the functions missing in the defective phage, and the progeny will contain about equal numbers of both types.

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pecialized transducing phage are formed by abbarent excision of a lambda lysogen. This is a very rare vent compared to the normal excision via recombination between *attL* and *attR*. When the abbarent xcision initially occurs, it will yield less than one specialized transducing phage per 1,000,000 wild-type hage. Thus, this lysate is called a *Low frequency transducing lysate* or LFT. If this specialized transducing hage co-infects a new host together with a wild-type phage, a dilysogen can result. Induction of the ilysogen yields a lysate containing 50% specialized transducing phage and 50% wild-type phage. Thus, his lysate is called a *High frequency transducing lysate* or HFT.

able 11.7 : Comparison between generalized and specialized transduction

Generalized transduction	Specialized transduction
1. Occurs during the lytic cycle of virulent and temperate phage	1. Occurs during the lysogenic cycle of temperate phage
2. Phages penetrate bacterial cell and enter lytic cycle.	2. Phages penetrate bacterial cell and enter lysogenic cycle.
3. Viral DNA begins to replicate immediately in the bacterial cytoplasm.	3. Viral DNA integrates into the DNA of the bacterium. Replication begins at a later time.
5. Any bacterial genes are randomly packaged into new viruses.	5. Bacterial genes adjacent to previously incorporated virus are packaged into new viruses.
6. Some new viruses have no viral DNA.	6. Some new viruses have both viral DNA and bacterial DNA.
7. Donor bacterial genes are incorporated into chromosome of new bacterium.	7. Donor bacterial genes are incorporated into chromosome of new bacterium together with transducing viruses.

Cotransduction

During transduction, there is a maximum size of the DNA segment that can be packaged by bacteriophages. If two genes are close together along the chromosome, a bacteriophage may package a single piece of the chromosome that carries both genes and transfer that piece to another bacterium. This phenomenon is called *co-transduction*. The likelihood that two genes will be co-transduced depends on how close together they lie. If two genes are far apart along the bacterial chromosome, they will never be cotransduced, because the bacteriophage cannot physically package a DNA fragment that is larger. Co-transduction can be used to map genes present in bacterial chromosome. Mapping of genes by the process of co-transduction is based on the fact that the closer the genes to each other, the greater the probability they will be cotransduced. That is, the frequency of co-transduction is inversely proportional to the distance between two genes. As an example, consider three genes a, b and c for which the frequencies of co-transduction determined by two factor crosses are : a-b, 90%; a-c, 33%; and b-c, 42%. The values indicate the b is closer to a than to c. A mathematical formula (proposed by Wu) is used to relate co-transduction frequencies to map distance:

$$C = (1-d/L)^3$$

Where C is the co-transduction frequency, d is the distance in minutes between two genes and L is the size of the transducing fragment in minutes.

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CONJUGATION

In 1946, Joshua Lederberg and Edward Tatum showed that bacteria undergo conjugation, a process in which the genetic information from one bacteria is transferred to, and recombined with, that of other bacteria. In conjugation, direct contact between the donor and recipient bacteria leads to establishment of a cytoplasmic bridge between them and transfer of part or all of the donor genome to the recipient. Donor ability is determined by specific conjugative plasmids called fertility plasmids or sex plasmids.

$F^+ - F^-$ conjugation

The F plasmid (also called F factor) of *E.coli* is the prototype for fertility plasmids in Gram-negative bacteria. Strains of *E.coli* with an extrachromosomal F plasmid are called F^+ and function as *donors*, whereas strains that lack the F plasmid are F^- and behave as *recipients*. The conjugative functions of the F plasmid are specified by a cluster of at least 25 transfer (*tra*) genes which determine expression of *sex pili*, synthesis and transfer of DNA during mating, interference with the ability of F^+ bacteria to serve as recipients, and other functions. Each F^+ bacterium has 1 to 3 sex pili that bind to a specific outer membrane protein on recipient bacteria to initiate mating. An intercellular cytoplasmic bridge is formed, and one strand of the F plasmid DNA is transferred from donor to recipient, beginning at a unique origin and progressing in the 5′ to 3′ direction. The transferred strand is converted to circular double-stranded F plasmid DNA in the recipient bacterium. Both of the exconjugant bacteria are F^+, and the F plasmid can therefore spread by infection among genetically compatible populations of bacteria.

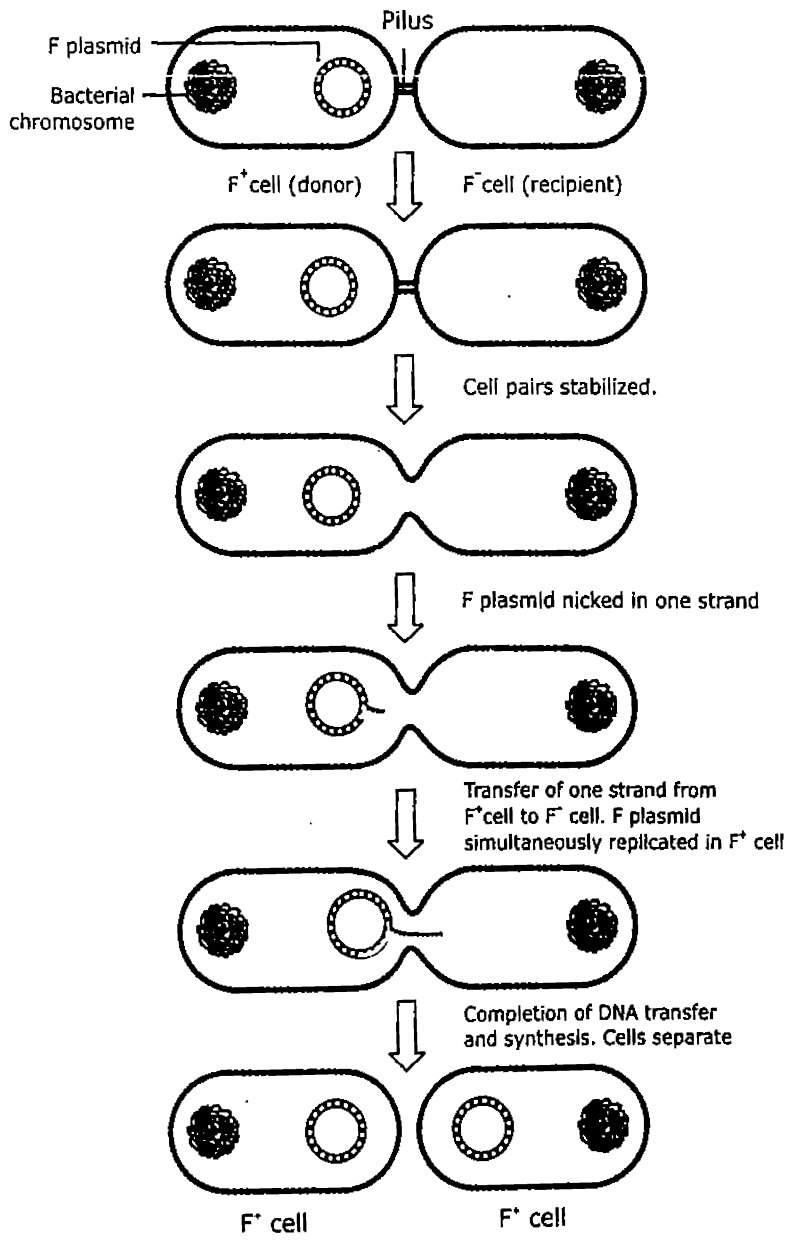


Figure : 11.15
Transfer of plasmid DNA by conjugation.

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Hfr – F^- conjugation

The F plasmid in *E.coli* can exist as an extrachromosomal genetic element or be integrated into the bacterial chromosome. An *E.coli* strain with an integrated F plasmid retains its ability to function as a donor in conjugal matings. Because donor strains with integrated F factors can transfer chromosomal genes to recipients with high efficiency, they are called **Hfr** (*High frequency recombination*) strains. Transfer of single-stranded DNA from an Hfr donor to a recipient begins from the origin within the F plasmid and proceeds as described above, except that the transferred DNA is the hybrid replicon consisting of F plasmid integrated into the bacterial chromosome.

In matings between F^+ and F^- bacteria, only the F plasmid is transferred with high efficiency to recipients. In case of matings between Hfr and F^- strains, the segment of the F plasmid containing the *tra* region is transferred last, after the entire bacterial chromosome has been transferred. Most recombinants from matings between Hfr and F^- cells fail to inherit the entire set of F plasmid genes and are phenotypically F^-.

F′ – F^- conjugation

Integrated F plasmids in Hfr strains can sometimes be excised from the bacterial chromosome. If excision precisely reverses the integration process, F^+ cells are produced. On rare occasions, however, excision occurs by recombinations involving insertion sequences or other genes on the bacterial chromosome that are located at some distance from the original integration site. In such cases segments of the bacterial chromosome can become incorporated into hybrid F plasmids that are called **F′ plasmids**. By similar processes, segments of the bacterial chromosome can sometimes become incorporated into R plasmids to produce hybrid R′ plasmids. Conjugative R′ plasmids can function as fertility plasmids because they can integrate into the bacterial chromosome by homologous recombination and mediate transfer of chromosomal genes during matings with recipient bacteria.

Conjugation also occurs in Gram-positive bacteria. Gram-positive donor bacteria produce adhesins that cause them to aggregate with recipient cells, but sex pili are not involved.

Gene mapping

Conjugation between different Hfr and F^- strains can be used to map the relative positions of genes in the bacterial chromosome. The mapping procedure is based on the fact that genes closer to the origin of transfer will be transferred at a higher frequency than genes farther away from the origin.

In an **interrupted mating experiment,** E. Wollman and F. Jacob mixed Hfr and F^- strains with suitable marker gene and assessed the recombination of specific genes at different time. To accomplish this, a culture containing a mixture of Hfr and F^- strains was incubated, samples were removed at various intervals and each was placed in agitating kitchen blender. Agitation in blender separates the conjugating bacteria and thus transfer of DNA. To assay the cells for genetic recombination following the blender treatment, they were grown on medium containing antibiotic in order to ensure the recovery of only recipient cells.

The rationale behind interrupted mating mapping strategy is that the time it takes for genes to enter a donor cell is directly related to their order along the bacterial chromosome. In this mapping strategy it was hypothesized that the chromosome of the donor strain in an Hfr mating is transferred in a linear manner to the recipient strain. The order of genes along the chromosome can be deduced by determining the time it takes various genes to enter the recipient strain. Thus, the interruptions of mating between Hfr and F^- at different times would lead to various lengths of the Hfr chromosome being transferred to the F^- recipient cell. If two bacterial cells had mated for a short period of time, only a small segment of the Hfr chromosome would be transferred to the recipient bacterium. However, if the bacterial cells were allowed to mate for a longer period of time before being interrupted, a longer segment of the Hfr chromosome could be transferred. Hence by determining which genes were transferred during short mating and which required longer mating times, the order of genes can be deduced.

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The following example can illustrate how three genes can be mapped by Hfr interrupted mating. The genetic markers are *leu*, *lac*, and *gal*. The Hfr donor is wild type for each (leu^+, lac^+ and gal^+), whereas the recipient is leu^-, lac^- and gal^- meaning it requires leucine and cannot grow on lactose or galactose as a carbon source. In addition, the Hfr is streptomycin sensitive (Str^S) while the recipient is resistant to this antibiotic (Str^R). After mixing donor and recipient cells (time=0), aliquots of the mating mixture are removed at different time intervals and mating pairs disrupted in a Waring blender. The mixture is then plated on three types of media:

1. Minimal glucose to select for leu^+ recombinants
2. Minimal lactose + leucine to select for lac^+ recombinants
3. Minimal galactose + leucine to select for gal^+ recombinants.

All three media should also contain streptomycin to prevent growth of the donor cells. Colonies that arise on any of these media must be recipient cells into which the donor wild-type gene was transferred.

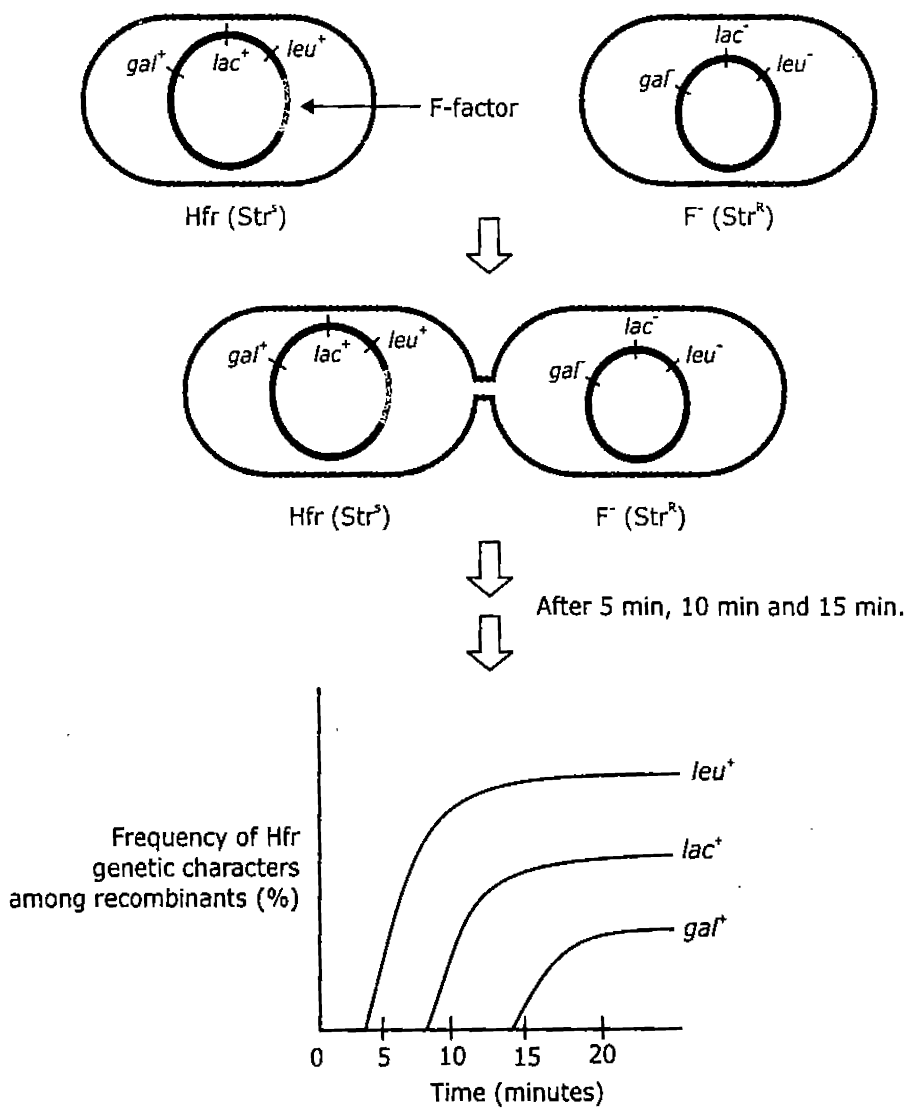


Figure 11.16 : Mapping by interrupted mating. The linear transfer of genes is stopped by breaking the conjugation bridge to study the sequence of gene entry into the recipient cell. The entering strand recombines with the resident chromosome, replacing the mutant alleles (leu^-, lac^-, gal^-) with wild-type counter-parts, and recombinants are selected by plating on medium with streptomycin (to stop growth of donors).

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Table 11.8 : Comparison of mechanism of DNA transfer

Mechanism	*Main Feature*	*Size of DNA transferred*	*Polarity*	*Sensitivity to DNase*
Transformation	Naked DNA transferred	About 20 genes	No	Yes
Transduction	DNA enclosed in a bacteriophage coat	Usually part of the chromosome	No	No
Conjugation				
Chromosomal DNA	Cell-to-cell contact required	Small fraction of chromosome	Yes	No
F plasmid	Cell-to-cell contact required	Entire F plasmid	Yes	No

11.6 GENERAL FEATURES OF IMPORTANT BACTERIAL GROUPS

RICKETTSIAE

The rickettsiae are a diverse collection of obligately intracellular, non motile, Gram-negative bacteria found in ticks, lice, fleas, mites, chiggers, and mammals. They include the genera *Rickettsiae, Ehrlichia, Orientia*, and *Coxiella*.

Some organisms in the family *Rickettsiaceae* are closely related genetically (e.g., *Rickettsia rickettsii*, *R. akari*, *R. prowazekii*, and *R. typhi*); others are related less closely to *Rickettsia* species (e.g., *Ehrlichia* and *Bartonella*); and others not related to *Rickettsia species* (e.g., *C. burnetii*). Rickettsioses are zoonoses that, except for Q fever, are usually transmitted to humans by arthropods (tick, mite, flea, louse, or chigger). Rickettsiae are important causes of human diseases like Rocky Mountain spotted fever, Q fever, murine typhus.

CHLAMYDIAE

Chlamydiae are obligate intracellular nonmotile coccoid bacteria. They lack several metabolic and biosynthetic pathways and depend on the host cell for intermediates, including ATP. Chlamydiae exist as two stages: (1) infectious particles called **elementary bodies** and (2) intracytoplasmic, reproductive forms called **reticulate bodies**. The chlamydiae consist of three species, *C. trachomatis, C. psittaci*, and *C. pneumoniae.*

Chlamydial cells are unable to carry out energy metabolism and lack many biosynthetic pathways; therefore they are entirely dependent on the host cell to supply them with ATP and other intermediates. Because of their dependence on host biosynthetic machinery, the chlamydiae were originally thought to be viruses; however, they have a cell wall and contain DNA, RNA, and ribosomes and therefore are now classified as bacteria. The group consists of a single genus, *Chlamydia* (order Chlamydiales, class Chlamydiaceae).

Table 11.9 : Comparison of typical bacterial, rickettsias/chlamydias and virus

	Typical bacteria	**Rickettsias/Chlamydias**	**Virus**
Intracellular parasite	No	Yes	Yes
Binary fission	Yes	Yes	No
DNA and RNA	both	both	DNA or RNA
Ribosome	Yes	Yes	No
Sensitive to antibiotics	Yes	Yes	No
Sensitive to interferon	No	No	Yes

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NOMYCETES

nomycetes are aerobic, gram-positive bacteria that form branching, usually nonfragmenting, hyphae asexual spores. If the asexual spores borne on aerial mycelia (are located at the tip of hyphae) then led conidiospores or conidia and sporangiospores when they are within sporangia.

rgey's Manual divides the actinomycetes into eight groups on the basis of properties like conidia angement, the presence of a sporangium, cell wall type.

reptomyces, the largest genus of actinomycetes, has chains of nonmotile conidia within a thin sheath. ost actinomycetes are not motile. When motility is present, it is confined to flagellated spores only. ctinomycetes cell wall composition varies greatly among different groups. Members of the genus are trict aerobes, and form chains of nonmotile conidia. Streptomycetes are best known for their synthesis of vast array of antibiotics like amphotericin B, chloremphenicol, erythromycin, neomycin, nystatin, streptomycin and tetracycline.

SPIROCHETES

Spirochetes are a group of gram-negative, chemoheterotrophic bacteria. They are slender, long organisms with a flexible, helical shape. Spirochetes lack external rotating flagella. They exhibit creeping or crawling movements. Their unique pattern of motility is due to an unusual morphological structure called the axial filament. The central **protoplasmic cylinder** contains cytoplasm and the nucleoid, is bounded by a plasma membrane and gram-negative type cell wall. Two or more than a hundred prokaryotic flagella, called **axial fibrils** or *periplasmic flagella*, extend from both ends of the cylinder often overlap. The whole complex of periplasmic flagella, the **axial filament,** lies inside a flexible outer membrane. The outer membrane contains lipid, protein, and carbohydrate. *Treponema pallidum* (causes syphilis) and *Borelia burgdorferi* (responsible for Lyme disease) are example of spirochetes.

MYCOPLASMAS

Mycoplasmas are the smallest and simplest self-replicating bacteria. Mycoplasmas are spherical to filamentous cells with no cell walls and they are consequently placed in a separate class *Mollicutes* (*mollis*, soft; *cutis,* skin). Formerly, Mycoplasmas were called "pleuropneumonia-like organisms" (PPLO) because it was first isolated from cattle suffering from pleuropneumonia. The trivial term mollicutes is frequently used as a general term to describe any member of the class, replacing in this respect the older term mycoplasmas. Characteristically, mycoplasmas growing on solid media produce "fried egg" colonies with a central dense region surrounded by a lighter peripheral zone.

11.7 ARCHAEBACTERIA

Archaebacteria are highly diverse with respect to morphology, reproduction, physiology and ecology. Archaebacteria can be gram positive or gram negative and may be spherical, rod shaped, spiral and other. They can be aerobic or anaerobic (facultative or obligate). Archaebacteria are usually present in extreme aquatic and terrestrial habitats. They are often present in anaerobic, hypersaline or high temperature environments.

Cell wall

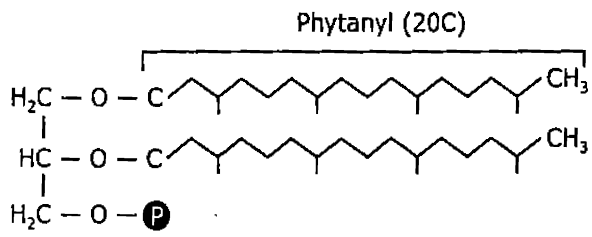
The cell walls of archaebacteria are distinctive from those of eubacteria. Archaebacterial cell walls are composed of different polysaccharides, glycoproteins or proteins, with no peptidoglycan. Many archaebacteria have cell walls made of the polysaccharide **pseudomurein** (a modified peptidoglycan lacking D-amino acids and containing N-acetyltalosaminuronic acid instead of N-acetylmuramic acid; found in methanogenic bacteria). All archaebacteria are resistant to lysozyme and beta-lactam antibiotics such as penicillin.

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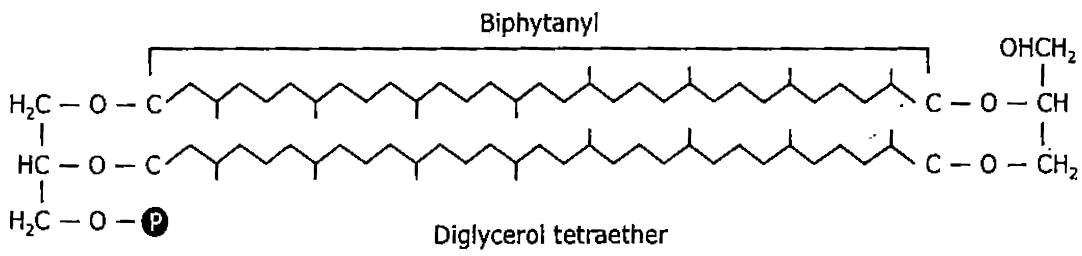
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Plasma membrane

Nature of archaebacterial membrane lipids differs from both eubacteria and eukaryotes. Archaebacterial membrane lipids contains branched chain hydrocarbons attached to glycerol by *ether links.* Sometimes two glycerols are linked to form a long tetraether. Usually the diether side chains are 20 carbons in size and the tetraether chains are 40 carbons. Eubacterial and eukaryotic lipids have glycerol connected to fatty acids by ester bonds.



Glycerol diether



Diglycerol tetraether

There are three major known groups of archaebacteria: *methanogens, extreme halophiles,* and *thermophiles.*

Major archaeal group	**General features**
Methanogens	Strict anaerobic bacteria that produce methane, found in sewage treatment plants, bogs, and the intestinal tracts of ruminants, the largest group of archaebacteria. Example : *Methanobacterium*, *Methanococcus*.
Extreme halophiles	Aerobic, chemoorganoheterotrophs, thrive in high salt concentrations, gram-negative or gram positive, neutrophilic or alkalophilic. Example : *Halobacterium*, *Halococcus*.
Thermophiles	Heat-loving bacteria found near hydrothermal vents and hot springs, gram-negative, usually strict anaerobes, acidophilic or neutrophilic, autotrophic or heterotrophic. Example : *Deslfurococcus*; *Pyrodictium*.

Table 11.10 : Difference between bacteria and archaea

Property	**Bacteria** (Eubacteria)	**Archaea** (Archacebacteria)
Nucleus	Absent	Absent
Unit membrane bound organelles	Absent	Absent
Cell Wall	Peptidoglycan containing muramic acid	Variety of types, no muramic acid
Membrane Lipid	Have ester-linked, straight-chained fatty acid	Have ether-linked, branched aliphatic chains
Transfer RNA	Thymine present	No thymine in T or TψC arm
	N-formylmethionine carried by initiator tRNA	Methionine carried by initiator tRNA

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Property	**Bacteria** (Eubacteria)	**Archaea** (Archacebacteria)
Ribosomes		
Elongation factor 2	Does not react with diphtheria toxin	Reacts
Sensitivity to chloramphenicol	Sensitive	Insensitive
Sensitivity to anisomycin	Insensitive	Sensitive
DNA-Dependent RNA Polymerase		
Number of enzymes	One	Several
Rifampicin sensitivity	Sensitive	Insensitive
Polymerase II Type Promoters	Absent	Present
Metabolism		
Methanogenesis	Absent	Present
Chlorophyll-based photosynthesis	Present	Absent

11.8 BACTERIAL TOXINS

A **toxin** (Latin *toxicum,* poison) is a specific substance, often a metabolic product of the organism that damages the host. Toxins can even induce disease in the absence of the organism that produced them. Diseases that result from the entrance of a specific toxin into the body of a host is called **intoxication.** The term **toxemia** refers to the condition caused by toxins that have entered the blood of the host. Toxins produced by organisms can be divided into two main categories: **exotoxins** and **endotoxins.**

Table 11.11 : Main features of exotoxins and endotoxins

Property	**Exotoxin**	**Endotoxin**
Source	Some species of gram-positive and gram-negative bacteria	Lipopolysaccharide of most gram negative bacteria
Secreted from cell	Yes	No
Chemical nature	Polypeptide	Lipopolysaccharide
Location of genes	Plasmid or prophage or chromosome	Bacterial chromosome
Toxicity	High (fatal dose on the order of 1µg)	Low (fatal dose on the order of hundreds of micrograms)
Antigenicity	Induces high-titer antibodies called antitoxins	Poorly antigenic
Vaccines	Toxoids used as vaccines	No toxoids formed and no vaccine available
Heat stability	Heat labile, inactivated rapidly at 60 to 80°C	Heat stable

TYPES OF EXOTOXIN

Exotoxins can be categorized by their mode of action on target cells into following three categories:

Toxins that act from the cell surface

Toxins that act from the cell surface bind to a receptor on the cell surface and stimulate intracellular signaling pathways. Two examples are described below.

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Superantigens

Superantigens are produced by several bacteria. The best characterized superantigens are those produced by the strains of *Staphylococcus aureus* and *Streptococcus pyogenes* that cause toxic shock syndrome. Superantigens bridge the MHC class II protein on antigen presenting cells with the T cell receptor on the surface of T cells. Consequently, up to 30% of all T cells are activated, leading to massive secretion of cytokines, which produce the symptoms of toxic shock.

Heat-stable enterotoxins

Some bacteria produce heat-stable enterotoxins. Different heat-stable enterotoxins recognize distinct receptors on the cell surface and thereby affect different intracellular signaling pathways. For example, STa enterotoxins bind and activate membrane-bound guanylate cyclase, which leads to the intracellular accumulation of cyclic GMP and downstream effects on several signaling pathways. These events lead to the loss of electrolytes and water from intestinal cells.

Membrane damaging toxins

Membrane damaging toxins disrupt the itegrity of the plasma membrane. There are two subtypes of membrane damaging toxins - the channel-forming toxins and toxins that function as enzymes that act on the membrane.

Channel-forming toxins

Channel-forming toxins inserts itself into the host cell membrane and make an open channel (pore). Alpha toxin of *Staphylococcus aureus* is an example of channel-forming toxins.

Toxins that enzymatically damage the membrane

One common example is *alpha toxin* of *Clostridium perfringens,* which causes gas gangrene. Alpha toxin has *phospholipase* activity which enzymatically damage the membrane by removing the charged polar head groups from the phospholipids.

AB toxins

AB toxins are intracellular toxins. The *B-subunit* attaches to target regions on cell membranes, the *A-subunit* enters through the membrane and possesses enzymatic function that affects internal cellular bio-mechanisms. Toxins of this type include cholera toxin, pertussis toxin, Shiga toxin, tetanus toxin, botulinum toxin, anthrax toxin and heat-labile enterotoxin from *E. coli.*

Table 11.12 : Important bacterial exotoxins and their mode of action

Bacterium	**Disease**	**Mode of action**
Corynebacterium diphtheriae	Diphtheria	Inactivates EF-2 by ADP-ribosylation
Clostridium tetane	Tetanus	Blocks release of the inhibitory neurotransmitter glycine.
Clostridium botulinum	Botulism	Blocks release of acetylcholine
Clostridium perfringens	Gas gangrene	Alpha toxin is a lecithinase
Bacillus anthracis	Anthrax	One of the toxins is an adenylate cyclase
Staphylococcus aureus	Toxic shock	Binds to class II MHC protein; induces IL-1 and IL-2
Vibrio cholerae	Cholera	Stimulates adenylate cyclase by ADP-ribosylation of Gsα
Bordetella pertussis	Whooping cough	Stimulates adenylate cyclase by ADP-ribosylation of Giα

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11.9 CONTROL OF MICROBIAL GROWTH

Control of microbial growth means to inhibit or prevent growth of microorganisms. This control is affected in two basic ways:

1. By killing microorganisms or
2. By inhibiting the growth of microorganisms.

Control of growth usually involves the use of physical or chemical agents which either kill or prevent the growth of microorganisms. Agents which kill cells are called **cidal** agents and agents which inhibit the growth of cells are referred to as **static** agents. Thus, the term **bactericidal** refers to killing bacteria, and **bacteriostatic** refers to inhibiting the growth of bacterial cells.

Terms commonly used to describe antimicrobial agents and processes:

Antiseptics

A disinfectant that is nontoxic enough to be used on skin. Examples include alcohols (ethanol, 50-70% - denatures proteins and solubilizes lipids), silver nitrate (precipitates proteins), iodine solution (inactivates proteins), detergents (disrupts cell membranes).

Disinfectants

A chemical used to destroy many microorganisms and viruses. Examples include, hypochlorites, chlorine compounds, copper sulfate, formaldehyde (reacts with NH_2, SH and COOH groups), phenolic compounds (denature proteins and disrupt cell membranes), and mercuric chloride (Inactivates proteins by reacting with sulfide groups).

Sterilization

Sterilization refers to the complete and total destruction or elimination of all viable organisms, including microbial endospore, in or on a substance being sterilized. Sterilization procedures involve the use of heat, radiation or chemicals, or physical removal of cells.

Pasteurization

Pasteurization is a brief heat treatment used to reduce the number of spoilage organisms and to kill disease-causing microbes. It uses precisely controlled heat to reduce the microbial populations in milk and other heat-sensitive liquids. The process is named after Louis Pasteur, who first used heat for controlling the spoilage of wine. Pasteurization does not kill all organisms and is therefore not synonymous with sterilization. Milk is usually pasteurized by heating, typically at 63°C for 30 minutes (batch method) or at 71°C for 15 seconds (flash method), to kill bacteria and extend the milk's usable life.

MICROBIAL CONTROL METHODS

Physical agents

Heat, radiation and filtration are most common physical agents normally used to control microbial growth. **Heat** is the most rapid and efficient physical agent used to control a population of microbes. Both *dry* and *moist* heat is used to kill microbes. Moist heat generally coagulates and causes denaturation in microoganisms. In dry heat, by contrast, the primary effect on microbes is due to oxidation of large molecules.

Similarly there are many different kinds of **radiations** having antimicrobial effects. Ionizing radiations which include x-rays and gamma rays, form free radicals in cytoplasm and the free radicals damage microbial protein and DNA. Another important form of radiation is ultraviolet radiations. These radiation damage nucleic acids.

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Filtration involves the physical removal of all cells in a liquid or gas. It is especially important for sterilization of solutions which would be denatured by heat (e.g. antibiotics, amino acids, vitamins, etc.). In this process solutions or gases are passed through a filter of sufficient pore diameter (generally 0.22 micron) to remove the smallest known bacterial cells.

Chemical agents

Large number of chemicals such as alcohol, aldehyde, heavy metals, halogens and dyes are commonly used as antimicrobial agents. *Alcohols* like ethyl alcohol and isopropyl alcohol cause denaturation of proteins and used as an antiseptic. Two *heavy metals*, mercury and silver, are used an antimicrobial agents. Mercury is used as mercuric chloride and as a component of Mercurochrome. Silver is commonly used as silver nitrate. Two important halogens, *chlorine* and *iodine*, act as oxidizing agents that react with the amino acids in proteins and change the nature of the protein. Similarly, gases like ethylene oxide, formaldehyde, and beta propiolactone are used to achieve sterilization.

ANTIBIOTICS

Antibiotics are low molecular-weight antimicrobial agents produced as secondary metabolites by microorganisms that kill or inhibit other microorganisms. The term, coined by Selman Waksman, originally described only those formulations derived from living organisms. A more broadened definition of an antibiotic includes any chemical of natural origin which can kill or inhibit the growth of other types cells. Since most clinically-useful antibiotics are produced by microorganisms and are used to kill or inhibit infectious bacteria, we will follow the classic definition.

Antibiotics may have a *cidal* effect or a *static* effect on a range of microbes. The range of bacteria or other microorganisms that are affected by a certain antibiotic is expressed as its **spectrum of action**. Antibiotics effective against a wide range of microoganisms such as both Gram-positive and Gram-negative bacteria are said to be **broad spectrum antibiotics**. If effective mainly against Gram-positive or Gram-negative bacteria but not both, they are **narrow spectrum**. If effective against a single organism or disease, they are referred to as **limited spectrum**. Antibiotic molecules produced by a microbe that are subsequently modified by an organic chemist to enhance their antimicrobial properties or to render them unique for a pharmaceutical patent are described as *semisynthetic antibiotics*.

Table 11.13 : Microbial sources of some antibiotics

Microorganism	**Antibiotic**
Bacteria	
Streptomyces spp.	Amphotericin B, Chloramphenicol (also synthetic), Erythromycin, Kanamycin, Neomycin, Nystatin, Rifampin, Streptomycin, Tetracyclines
Micromonospora spp.	Gentamicin
Bacillus spp.	Bacitracin, Polymyxins
Fungi	
Penicillium spp.	Griseofulvin, Penicillin
Cephalosporium spp.	Cephalosporins

Mode of action of antibiotics

Antibiotics inhibit microbial populations by any of five major methods. They disrupt cell wall synthesis; they interfere with cell membrane function; they inhibit protein synthesis or interfere with its completion; they disrupt the functioning of nucleic acids; and they interrupt selected metabolic pathways.

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ble 11.14 : Mechanism of action/target of some antibiotics

ntibiotics	**Mechanism of action**
ell wall synthesis inhibition	
enicillin	Inhibit transpeptidation enzymes involved in the cross-linking.
ancomycin	Binds directly to the D–Ala-terminus and inhibits transpeptidation.
acitracin	Inhibits cell wall synthesis
rotein synthesis inhibition	
ntibiotic	*Target*
uromycin	A site of ribosome
anamycin	16S rRNA
eomycin	16S rRNA
treptomycin	30S ribosome
hiostrepton	23S rRNA
Gentamicin	16S rRNA
Tetracycline	A site of ribosome
Chloramphenicol	Peptidyltransferase
Erythromycin	23S rRNA
Fusidic acid	Translation elongation factor G
Kirromycin	Translation elongation factor Tu

Nucleic acid synthesis inhibition

Antibiotics that block replication

Antibiotic	*Target*
Ciprofloxacin	Inhibit bacterial DNA gyrase
Hydroxyurea	Ribonucleotide reductase
Nalidixic acid	*gyr*A subunits of gyrase
Novobiocin	*gyr*B subunits of gyrase
Mitomycin C	Cross-links DNA

Antibiotics that block RNA synthesis

Antibiotic	*Target of action*
Streptolydigin	Beta subunit of RNA polymerase
Actinomycin D	Binds DNA
Rifampin	Beta subunit of RNA polymerase
Bleomycin	Cuts DNA

Cell membrane disruption

Polymyxine B	Disrupts the structure and permeability of plasma membrane

Metabolic antagonism

Sulfonamides	Inhibits folic acid synthesis by competition with p-aminobenzoic acid
Trimethoprim	Blocks tetrahydrofolate synthesis
Dapsone	Interferes with folic acid synthesis
Isoniazid	May disrupt pyridoxal or NAD metabolism and functioning

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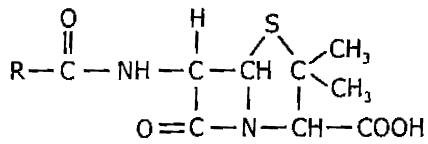
Example of some commonly used antibiotics :

Sulfonamides or **Sulfa drugs**

Sulfonamides or sulfa drugs are structurally related to sulfanilamide, an analogue of *p-aminobenzoic acid*. The latter substance is used in the synthesis of the cofactor folic acid. When sulfanilamide or another sulfonamide enters a bacterial cell, it competes with p-aminobenzoic acid for the active site. It results in decrease in folate concentration. The decline in folic acid is detrimental to the bacterium because folic acid is essential for the synthesis of purines and pyrimidines.

Penicillins

Penicillin was first discovered by Alexander Fleming as a metabolic by-product of the fungus *Penicillium notatum*. All penicillins share a common bicyclic nucleus that is composed of fused β-lactam and thiazolidine rings.The most crucial feature of the molecule is the β-lactam ring, which appears to be essential for activity. The mechanism of action of penicillins is still not completely known. It has been proposed that penicillins inhibit the enzyme catalyzing the transpeptidation reaction because of their structural similarity, which would block the synthesis of a complete, fully cross-linked peptidoglycan and lead to osmotic lysis.



Structure of penicillin nucleus

Cephalosporins

Cephalosporins originally isolated from the fungus *Cephalosporium*, and their β-lactam structure is very similar to that of the penicillins. Members of the cephalosporin family of antibiotics are effective in preventing the synthesis of bacterial cell walls and mostly useful against Gram-positive bacteria.

Tetracyclines

The tetracyclines are a family of antibiotics with a common four-ring structure to which a variety of side chains are attached. Tetracyclines are broad-spectrum antibiotics effective against gram-negative bacteria, gram-positive bacteria, rickettsias, chlamydiae, and mycoplasmas.

Aminoglycoside antibiotics

The aminoglycoside antibiotics are inhibitors of protein synthesis in bacteria. Members of this family of antibiotics all have amino groups and carbohydrate molecules associated with the main molecules. The members include gentamicin, kanamycin, tobramycin, and streptomycin. The aminoglycosides are most active against gram-negative pathogens.

Erythromycin

Erythromycin, a macrolide antibiotic, is synthesized by *Streptomyces erythraeus*. The macrolides contain a 12- to 22-carbon lactone ring linked to one or more sugars. Erythromycin is useful against Gram-positive bacteria and acts as inhibitor of protein synthesis.

Chloramphenicol

Chloramphenicol was first produced from cultures of *Streptomyces venezuelae*, it is now made through chemical synthesis. Antibiotic has a very broad spectrum of activity.

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11.10 VIRUS

Viruses are simple, noncellular entities consisting of one or more molecules of either DNA or RNA enclosed in a coat of protein. They can reproduce only within living cells and are *obligate intracellular parasites*. Viruses are smaller than prokaryotic cells ranging in size from 0.02 to 0.3 μm (small pox virus is largest virus about 200 nm in diameter and polio virus is the smallest virus about 28 nm in diameter).

A fully assembled infectious virus 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. Each viral species has a very limited *host range;* i.e., it can reproduce in only a small group of closely related species.

Viral structure

The structure of virions are very diverse, varying widely in size, shape and chemical composition. All viruses have a nucleocapsid composed of nucleic acid surrounded by a protein capsid.

- 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. Capsids are formed as single or double protein shells and consist of only one or a few structural protein species. The proteins used to build the capsid are called **capsomeres**. The nucleic acid together with the genome forms the nucleocapsid. Some viruses have a membranous envelope that lies outside the nucleocapsid. Those virions having an envelope are called **enveloped viruses**; where as those lacking an envelope are called **naked viruses**. In enveloped viruses, the nucleocapsid is surrounded by a lipid bilayer and glycoprotein derived from the modified host cell membrane. 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 membrane or another intracellular cell membrane, the lipid composition of the viral envelope closely reflects that of the particular host membrane.
- **Viral genomes** are smaller in size. The largest known viral genome, that of bacteriophage G, is 670 kbs. The genome of a virus may consist of DNA or RNA, which may be single stranded (ss) or double stranded (ds), linear or circular. The entire genome may occupy either one nucleic acid molecule (monopartite genome) or several nucleic acid segments (multipartite genome). The genomic RNA strand of single-stranded RNA viruses is called **sense** (positive sense, *plus* sense) in orientation if it can serve as mRNA, and **antisense** (negative sense, *minus* sense) if a complementary strand synthesized by a viral RNA transcriptase serves as mRNA.

Table 11.15 : Types of viral nucleic acids

Nucleic acid type	Nucleic acid structure
DNA	
Single stranded	Linear, single-stranded DNA
	Circular, single-stranded DNA
Double stranded	Linear, double-stranded DNA
RNA	
Single stranded	Linear, single-stranded, positive-strand RNA
	Linear, single-stranded, negative-strand RNA
	Linear, single-stranded, segmented, positive-strand RNA
	Linear, single-stranded, two identical single strands, positive-strand RNA
	Linear, single-stranded, segmented negative-strand RNA
Double stranded	Linear, double-stranded, segmented RNA

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Shape/symmetry

All viruses have a nucleocapsid (nucleic acid and protein) structure. The symmetry (refers to the way in which the capsomeres are arranged in the virus capsid) may be icosahedral (spherical shape) or helical (rod shape).

Helical symmetry is seen in nucleocapsids of many filamentous and pleomorphic viruses. Helical nucleocapsids consist of a helical array of capsomeres wrapped around a helical filament of nucleic acid. A typical virus with helical symmetry is TMV.

Icosahedral morphology is characteristic of the nucleocapsids of many spherical viruses. An icosahedron is a regular polyhedron with 20 equilateral triangular faces and 12 vertices. Complex structures have capsid symmetry that is neither purely icosachedral nor helical. As for example T4 virus of *E.coli.*

Classification of viruses

Viruses are classified on the basis of their nucleic acid's characteristics, capsid symmetry, the presence or absence of an envelope, their host, dimensions of the virion and capsid and other properties.

Table 11.16 : Some common virus groups and their characteristics

	Picornaviridae	*Orthomyxoviridae*	*Parvoviridae*	*Adenoviridae*	*Poxviridae*
Genome size	7–8 kb	10–15 kb	4–6 kb	28–45 kb	130–375 kb
Nucleic acid	RNA	RNA	DNA	DNA	DNA
Strandedness	Single	Single	Single	Double	Double
Capsid symmetry	Icosahedral	Helical	Icosahedral	Icosahedral	Complex
Envelope	–	+	–	–	+
Host range	Animal	Animal	Animal	Animal	Animal

The International Committee for Taxonomy of Viruses (ICTV) has described almost 2000 virus species and placed them in 3 orders, 73 families, 9 subfamilies, and 287 genera. Virus order names end in *virales*; virus family names in *viridae*; subfamily names, in *virinae*; and genus (and species) names, in *virus*.

The Baltimore system of virus classification

The Baltimore system of virus classification is used by many virologists to organize viruses based on their genome type and the mechanisms they use to synthesize mRNA and replicate their genomes.

Group I	Double-stranded DNA genome	*Replication* : dsDNA → dsDNA
Group II	Single-stranded DNA genome	*Replication* : ssDNA → dsDNA → ssDNA
Group III	Double-stranded RNA genome	*Replication* : dsRNA → ssRNA → dsRNA
Group IV	Single-stranded RNA genome of plus sense	*Replication* : +RNA → –RNA → + RNA
Group V	Single-stranded RNA genome of minus sense	*Replication* : – RNA → + RNA → – RNA
Group VI	Single-stranded RNA genome that replicates with DNA intermediate	*Replication* : ssRNA → dsDNA → ssRNA
Group VII	Double-stranded DNA genome that replicates with RNA intermediate	*Replication* : dsDNA → + RNA → – DNA

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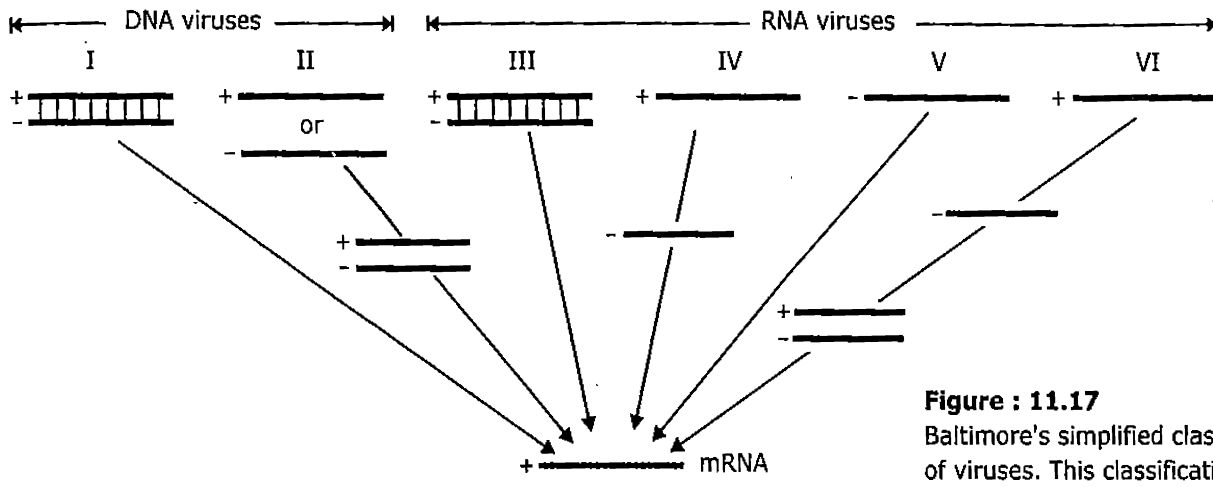


Figure : 11.17
Baltimore's simplified classification of viruses. This classification is based on the type of nucleic acid in the genome and the mode of production of mRNA.

BACTERIOPHAGE (Bacterial Virus)

Phages were first observed in 1915 by *F. Twort* in England and in 1917 by *F. d'Herelle* in France. D' Herelle named them bacteriophage (eaters of bacteria). Several morphologically distinct types of phage have been described, including polyhedral, filamentous, and complex. Complex phages have polyhedral heads to which tails and sometimes other appendages (tail plates, tail fibers, etc.) are attached.

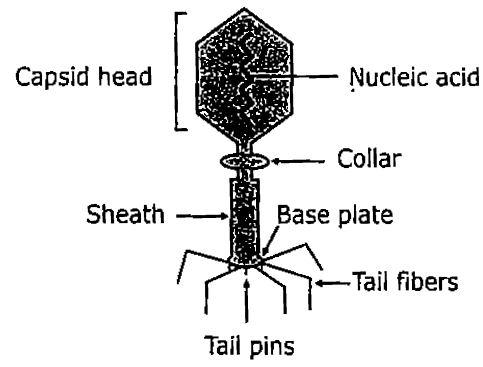


Figure 11.18 : T-even Coliphages

PHAGE GENOME

Most of bacterial viruses (phages) are double-stranded DNA viruses. However, some phages contain single-stranded DNA and single and double-stranded RNA genome. Few **examples of** phages and their nature of genome are given below.

Example

Single stranded DNA phages	ϕX174, fd
Double stranded DNA phages	T-phages, λ-phages
Single-stranded RNA genome of plus sense	MS2
Double-stranded RNA genome	ϕ6

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Life cycle of Bacteriophage

For a virus to replicate it must induce a living host cell to synthesize all the essential components needed to make more virions. These components must then be assembled into new virions and escape from the cell in order to infect other cells. There are two different types of viral life cycles : *Lytic* and *Lysogenic cycle*

Lytic cycle

Lytic or vegetative life cycle culminates in the lysis (rupture) of the host cell and the release of numerous viral progeny. Bacterial viruses exhibiting a lytic life cycle only are also known as **virulent** bacteriophages (or lytic phages) because they inevitably cause the death and destruction of the host bacterium. Examples of virulent phages are the T-even phages. The lytic life cycle consists of five steps. These are attachment, penetration, synthesis, assembly, and release.

The cycle begins by the **attachment** of the bacteriophage to specific host cell molecules (receptors) on the bacterial cell wall. After the virus attaches to its host, it introduces its genetic material into the cell (**penetration**). Once the bacteriophage genome enters the cytoplasm, it subverts the host's nucleic acid and protein synthesis apparatus and initiates the synthesis of viral proteins and DNA. As the virus proteins are synthesized, they self-assemble into viral components such as the head (containing the phage DNA), tail, and tail fibers. The **assembly** process results in the formation of numerous intact phage particles within the cell. After the assembly step is completed, viral proteins cause the lysis of the host cell, and all the viral progeny are **released** into the environment. The average number of phage particles produced by each infected cell, called the **burst size**, is characteristic for each virus and often ranges between 50 and several hundred.

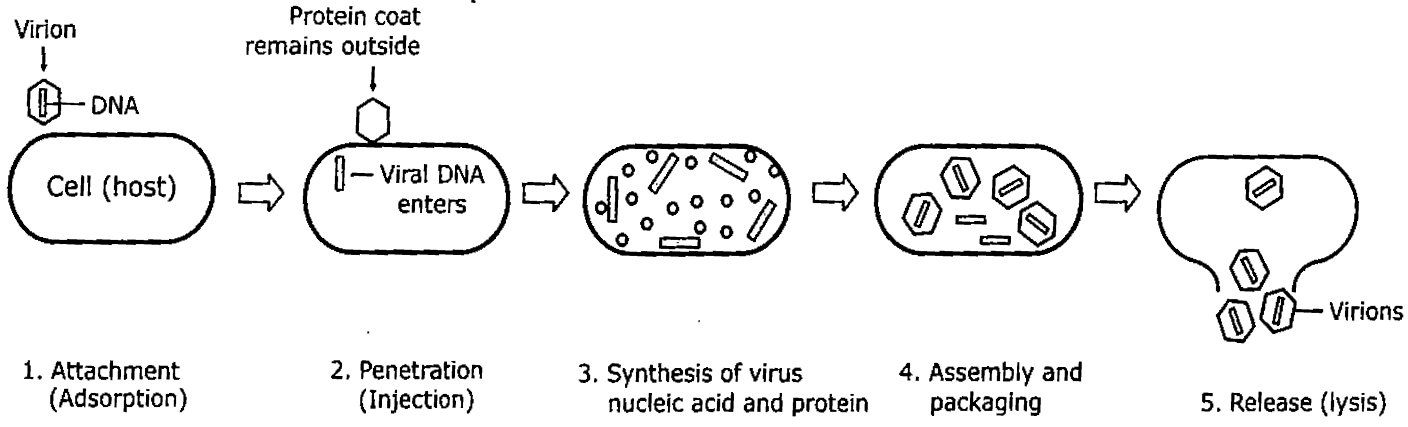


Figure 11.19 : The lytic cycle of a bacterial virus

Because the release of virions is a more or less simultaneous process, the virions are said to undergo a *one step growth curve*.

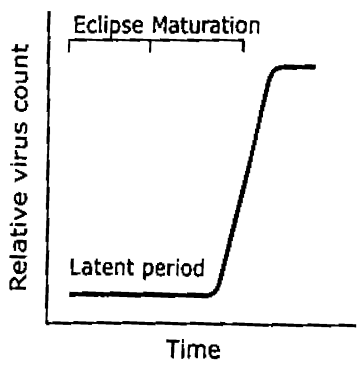


Figure : 11.20
The one-step growth curve of virus replication. This graph displays the results of a single round of viral multiplication in a population of cells. Following adsorption, the infectivity of the virus particles disappears, a phenomenon called *eclipse*. This is due to the uncoating of the virus particles. During the *latent period*, replication of viral nucleic acid and protein occurs.

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Lysogenic cycle

In the *lysogenic cycle*, the viral DNA is inserted into the host's DNA and reproduces as the host's DNA replicates. A lysogenic virus (**temperate phage**) can remain in this state for numerous replications of host cell DNA until it excises itself from the host DNA and undergoes a lytic life cycle. When the genetic material of these phages is inserted into the DNA of host cells, it is said to be in the **prophage** state. A cell that contains a prophage is known as a **lysogen.**

The physical state of the prophage is not identical for all temperate viruses. For example, the prophage of bacteriophage λ in *E coli* is integrated into the bacterial chromosome at a specific site and replicates as part of the bacterial chromosome, whereas the prophage of bacteriophage P1 in *E.coli* replicates as an extrachromosomal plasmid.

An example of a lysogenic virus is the bacteriophage of *E.coli* called lambda (λ). The lysogenic bacteriophage λ, like phage T4, also uses its tail to inject the viral DNA into the host. Once the viral DNA is inside the host, it can either undergo a lytic cycle similar to that of T4 or incorporate itself into the host DNA.

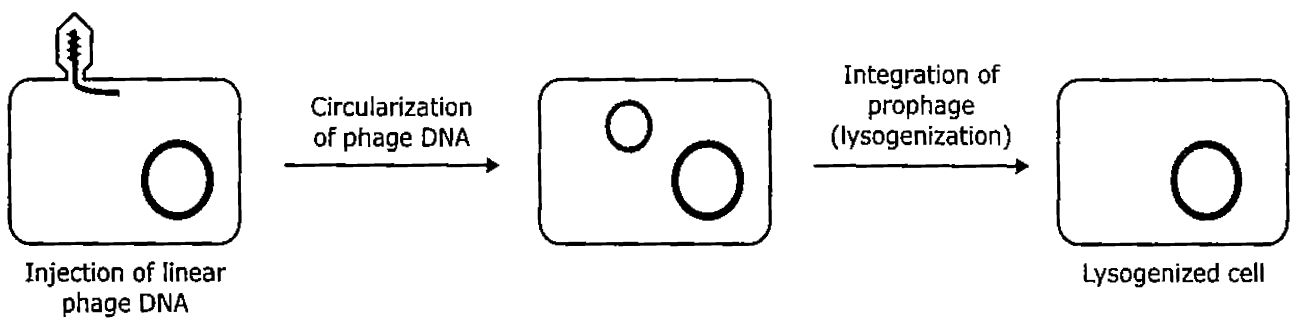


Figure 11.21 : Lambda-type lysogenic cycle.

The integration of the circular λ genome involves the presence of **attachment sites** in the genome of both bacteriophage and bacterium. The bacterial attachment site, called *att*B, consists of a specific DNA sequence composed of three domains: a B domain, an O domain and a B′ domain. The attachment site of the bacteriophage, called *att*P, is also made up of three domains: a P domain, an O domain and a P′ domain. The O domain has a sequence common to both *att*B and *att*P. The O domain is also known as the **core sequence**.

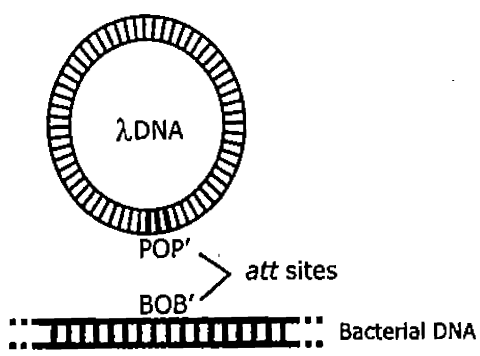


Figure : 11.22
Integration of the bacteriophage lambda genome into *E.coli* chromosomal DNA.

Lysogeny is maintained by a **repressor** protein encoded by the DNA of the λ prophage. The λ repressor blocks the expression of all the other λ genes, including those responsible for phage replication and synthesis of phage proteins. An intriguing property of lysogens is that they cannot be reinfected by phages of the type with which they are lysogenized. They are immune to superinfection. Under some conditions the prophage initiates synthesis of phage proteins that leads to the destruction of infected cells and release of new phages. This process is called **induction.** If ultraviolet radiation has damaged the host DNA, the ensuing

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prophage induction is termed **UV induction**. When a nonlysogenic F^- bacterial cell receives, by conjugation, a prophage from a lysogenic Hfr donor, the recipient cell dies by induction of the lytic phage cycle. This form of prophage induction is termed **zygotic induction**.

Immunity

The lysogens are immune to superinfecting phage. The resistance of a λ lysogen to superinfection by λ is called **immunity**. Immunity is due to expression of a repressor in the lysogens. Phage λ contains gene *cI* that codes protein λ-repressor. In a lysogen, the *cI* repressor is synthesized continuously. When a phage infects a lysogen, however, excess repressor molecules already present in the cytoplasm of the cell bind rapidly to the two operators in the infecting DNA molecule before RNA polymerase can bind to pL and pR.

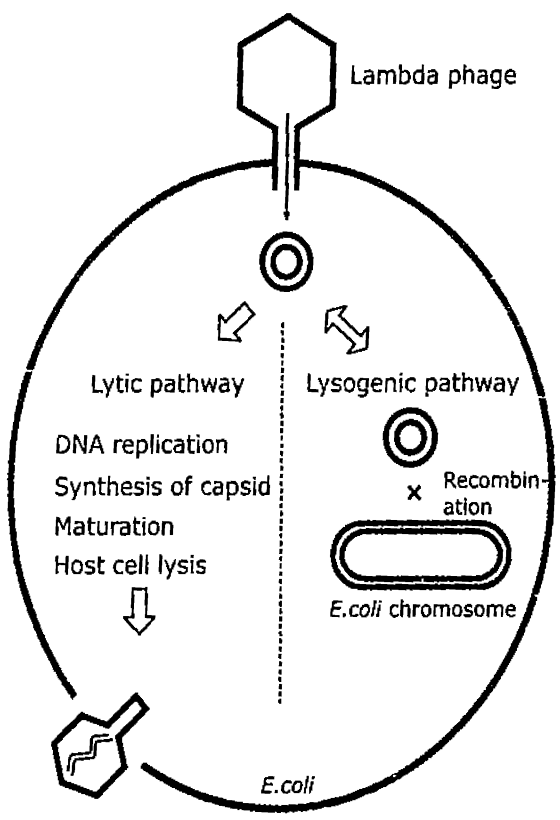


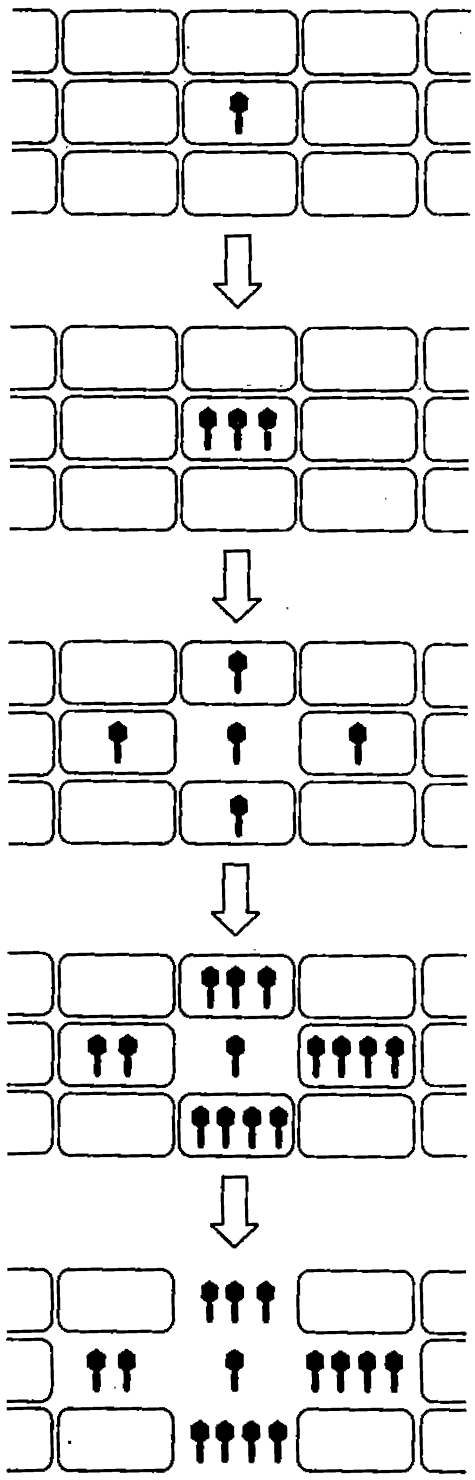
Figure : 11.23
Phage can enter both lytic and lysogenic pathways.

Plaque assay

When plated with a lawn of susceptible cells, a bacteriophage will form a circular area of reduced turbidity called a **plaque**. Depending on the bacteriophage, a plaque may be clear (such as those formed by phage T1) or a plaque can be an area containing slow-growing cells.

The number of infectious viral particles in a sample can be quantified by a plaque assay. This assay is performed by culturing a dilute sample of viral particles on a plate covered with host cells and then counting the number of local lesions, called *plaques,* that develop. A plaque develops on the plate wherever a single virion initially infects a single cell. The virus replicates in this initial host cell and then lyses the cell, releasing many progeny virions that infect the neighboring cells on the plate. After a few such cycles of infection, enough cells are lysed to produce a visible plaque in the layer of remaining uninfected cells. A bacteriophage capable of productively infecting a cell is called a **Plaque-forming unit** (PFU).

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A phage infects a single cell in a lawn of sensitive, growing bacteria

Phage reproduces in these cell typically yielding about 50 phage particles per infected cell

Lysis of the cell releases phage into the medium. The phage diffuses through the medium and infects adjacent cells

Phage reproduces in these cell releasing 50 additional progeny phage per infected cell

These cells lyse, releasing more phage which can then diffuse outward and infect the surrounding cells. Lysis of the cells results in a circular clearing in the lawn of bacteria. This region of visible lysis is called a plaque

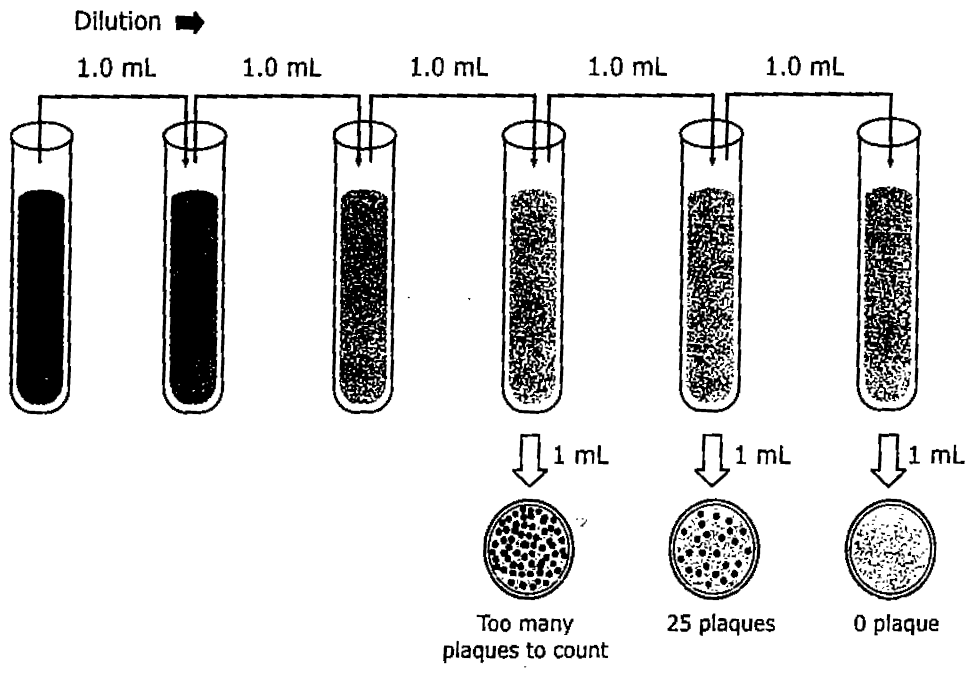
To obtain the PFU in a particular sample, the sample must be diluted. To make 10-fold (10^{-1}) dilution, one can mix 1.0 ml sample with 9.0 ml diluent. If 100 fold (10^{-2}) dilution is needed, 0.1 ml can mixed with 9.9 ml diluent. Dilutions (10^{-2}, 10^{-3}, 10^{-5}...) are analyzed using the plaque assay technique as mentioned in figure 11.24. In each case 1 ml of diluted culture is used. Each dilution can now be analyzed by plaques. Each plaque represents the initial infection of one bacterial cell by one bacteriophage. In the 10^{-3} dilution, too many plaques are present to count as shown in the figure. Whereas in the 10^{-4} dilution, countable numbers of plaques (25) are produced. In the 10^{-5} dilution, the dilution factor is so great that no phages are present in the 1 ml sample, and thus no plaques form. From the 1 ml sample of the 10^{-4} dilution, the original bacteriophage density can be calculated by using following formula:

$$\text{(Plaque number/ml)} \times \text{(Dilution factor)}$$

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Using the results shown in figure, it is observed that there are 25 phage plaques derived from the 1 ml aliquot of the 10^{-4} dilution. Therefore, we can estimate that there are 250 phages per milliliter at this dilution. This initial viral density in the undiluted sample, in which 25 plaques are observed from 1 ml of the 10^{-4} dilution, is calculated as

$$(250/ml) \times (10^4) = 230 \times 10^4/ml$$



Total volume	10 mL	10 mL	10 mL	10 mL	10 mL
Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Dilution factor	10	10^2	10^3	10^4	10^5

Figure 11.24 : The plaque assay for bacteriophage analysis.

ANIMAL VIRUSES

Animal viruses have a variety of shapes, sizes, and nature of genome. The genome of animal viruses may be DNA or RNA, ss or ds, linear or circular, segmented or non-segmented. The great diversity of animal viruses has been classified into seven groups based on characteristics such as genome type and mRNA synthesis pathway. Six classes of animal viruses are recognized.

Classification of animal virus

According to the Baltimore system of virus classification, which is based on various mechanisms of viral genome replication, animal viruses are classified into seven classes. The central theme in this system is that all viruses must generate positive strand mRNAs from their genomes, in order to produce proteins and replicate themselves. The precise mechanisms whereby this is achieved differ for each virus family.

CLASS I (Genome - dsDNA)

It is the most common type of animal virus. Viral DNA enters the cell nucleus, where cellular enzymes transcribe the DNA and process the resulting RNA into viral mRNA.

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Class Ia DNA replicates in host-cell nucleus; use host enzymes for viral mRNA synthesis. *Example : Adenoviruses, Herpesviruses, Papovaviruses* (SV40)

Class Ib DNA replicates in host-cell cytoplasm using viral enzymes. *Example* : *Pox virus* (Vaccinia)

CLASS II (Genome - ssDNA)

Example : *Parvoviruses* (from Latin *parvo,* poor), are simple viruses that contain one molecule of single-stranded DNA (ssDNA). Some parvoviruses encapsidate (enclose) both plus and minus strands of DNA, but in separate virions; others encapsidate only the minus strand. In both cases, the ssDNA is copied inside the cell into dsDNA, which is then itself copied into mRNA.

CLASS III (Genome - dsRNA)

Example : *Reoviruses*

CLASS IV (Genome - Plus sense ssRNA)

Example : *Picornaviruses* (Polio virus), *Coronaviruses, Togaviruses*

CLASS V (Genome - Minus sense ssRNA)

Contain a single negative strand of genomic RNA, whose sequence is complementary to that of the viral mRNA. The genomic RNA in the virion acts as a template for synthesis of mRNA but does not itself encode proteins. Typified by *influenza virus*, have segmented genomes; each segment acts as a template for the synthesis of a different mRNA species.

CLASS VI (Genome - Plus sense ssRNA that replicates with DNA intermediate)

Example : *Retroviruses*

CLASS VII (Genome - dsDNA that replicates with RNA intermediate)

Example : *Hepadnaviruse* such as human hepatitis B virus

Like the retroviruses, hepadnaviruses use reverse transcriptase in their replication cycle. However, unlike retroviruses, the DNA genome of hepadnaviruses is replicated through an RNA intermediate, the opposite of what occurs in retroviruses.

Table 11.17 : Examples of animal virus and their nature of nucleic acid

Herpesvirus	(Herpes simplex virus)	dsDNA
	(Epstein-Barr virus)	dsDNA
	(Varicella-zoster)	dsDNA
Pox virus		dsDNA
Papovavirus	(Papillomavirus)	dsDNA
	(Polyomavirus)	dsDNA
Adenovirus		dsDNA
Parvovirus		ssDNA
Reoviridae	(Rotavirus)	dsRNA
Togavirus		(+) ssRNA
Coronavirus		(+) ssRNA
Picornavirus		(+) ssRNA
Retrovirus		(+) ssRNA
Orthomyxovirus	(Influenza virus)	(–) ssRNA
Paramyxovirus		(–) ssRNA

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Effect of animal virus on animal cells:

Animal viruses can have several different effects on animal cells:

Lytic infection : Lytic infection results in the host cell lysis.

Persistent infection : In persistent infections, the infected cell remain alive and continue to produce virus indefinitely.

Latent infection : In a latent infection, there is a delay between infection by the virus and lytic events. Cold sores caused by the herpes simplex virus are a typical example of latent viral infection.

Transformation : Certain animal viruses can change the normal cell into a tumor cell, a process called transformation.

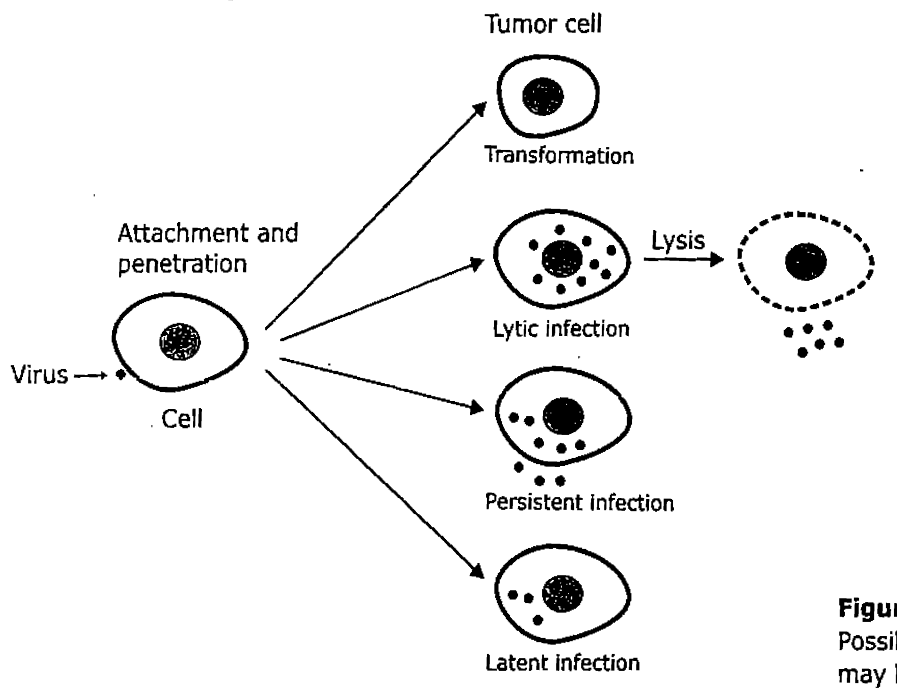


Figure : 11.25
Possible effects that animal viruses may have on cells they infect.

Life cycle of animal viruses

A generalized productive life cycle for animal viruses consisting of the following steps:

- Adsorption
- Viral entry
- Replication of the viral genome
- Viral assembly and
- Viral release

Viral adsorption/attachment to the host cell

Viruses typically attach to cells via specific cell surface receptors. Animal viruses have specialized attachment sites distributed over the surface of the virion. Adsorption involves the binding of attachment sites on the viral surface with receptor sites on the host cell cytoplasmic membrane.

A well studied example of this phenomenon is the interaction between the HIV-1 envelope glycoprotein, gp120, and the cell surface CD4 receptor as well as chemokine co-receptors present on T4-lymphocytes and macrophages.

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mple of some virus and their receptor

	Receptor
...no-associated virus	Heparan sulfate
...stein-Barr virus	CD21
...rpes simplex virus	Heparan sulfate
...V-1	CD4
...luenza virus	Sialic acid
...bies virus	Acetylcholine receptor

...ral entry into the host cell

...llowing attachment, viruses must enter cells. This requires that the virus must traverse the lipid bilayer ...rrounding the cell, without killing the cell. For many viruses, detailed mechanisms of penetration are ...nclear. One of following three different modes of entry is employed by most viruses:

Direct penetration of viral genome:

In this case, interaction between the viral capsid and the host cell cytoplasmic membrane causes a rearrangement of capsid proteins allowing the viral nucleic acid to pass through the membrane into the cytoplasm.

Direct fusion with plasma membrane (enveloped virus only):

The viral envelope fuse with the host cell cytoplasmic membrane and the nucleocapsid is released into the cytoplasm.

Receptor mediated endocytosis (naked or enveloped virus):

Most naked viruses and some enveloped viruses enter by receptor-mediated endocytosis whereby the host cell cytoplasmic membrane invaginates and pinches off, placing the virus in an endocytic vesicle.

Endocytic vesicle fuses with endosome; depending on the virus, escape of the nucleocapsid or its genome may occur either before or after vesicle fusion. Endosomal enzymes and low pHs often trigger the uncoating process.

Replication of the viral genome within the host cell

DNA viruses

Soon after penetration, the viral nucleic acid is prepared for expression and replication. Genome replication and transcription in DNA viruses follow a similar pattern. In DNA viruses, early viral mRNA and proteins are involved in taking over the host cell and the synthesis of viral DNA and RNA. DNA replication usually occurs in the host cell nucleus; *poxviruses* are exceptions since their genomes are replicated in the cytoplasm.

Hepadnaviruses (hepatitis B virus) are unique since they contain a partially dsDNA genome that must be converted into an RNA form by the virion enzyme reverse transcriptase during the virus life cycle. After infecting the cell, the virus DNA is released into the nucleus. Transcription *of* viral genes occurs in the nucleus using host RNA polymerase and yields mRNAs. The RNA moves to the cytoplasm where it is reverse transcribed into minus-strand DNA. After RNA has been degraded by RNaseH, the remaining RNA fragment serves as a primer for synthesis of the gapped dsDNA genome, using the minus-strand DNA as template.

RNA viruses

The RNA viruses are much more diverse in their reproductive strategies than are the DNA viruses. Most RNA viruses can be placed in one *of* four general groups based on their modes of genome replication and transcription, and their relationship to the host cell genome.

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1. The viral RNA genome can act as mRNA (positive strand viruses) or the complementary strand can be the mRNA (negative strand viruses).
2. All RNA viruses except retroviruses encode an *RNA-dependent RNA polymerase*. In the negative strand RNA viruses this polymerase is part of the virion, and it must enter the cytosol along with the viral genome. This is necessary in order for the virus to generate mRNAs from its genome.
3. All RNA viruses *replicate in the cytoplasm* except orthomyxoviruses (influenza A and B), hepatitis delta virus and retrovirses.
4. Retroviruses are unique. These viruses have a positive sense RNA genome which must be converted into a dsDNA form by the virion enzyme *reverse transcriptase* (an RNA-dependent DNA polymerase). This double-stranded DNA is then integrated (at random sites) into the host cell chromosome by the viral integrase enzyme. Upon integration into the host chromosome, the viral DNA can then be transcribed by cellular RNA polymerase II, to produce new genomic RNA molecules.

Viral assembly

Some *viral genes* direct the synthesis of capsid proteins, and these spontaneously self-assemble to form the capsid. The nucleic acid is then inserted into the capsid. The assembly of enveloped virus capsids is generally similar to that of naked virions.

Viral release from the host cell

Mechanisms of virion release differ between naked and enveloped viruses. Naked viruses are predominantly released by *host cell lysis*. *Enveloped viruses* released from host cell by *budding*. All viral envelopes are derived from host cell membranes by a multi-step process. Most envelopes arise from the plasma membrane. However, in herpes viruses, envelope formation usually involve the nuclear membrane. Prior to budding, viral proteins and glycoproteins are incorporated into the host cell's membranes. During budding the host cell membrane with incorporated viral proteins and glycoproteins pinches off to form the viral envelope.

Differences between bacteriophages and eukaryotic viruses

The differences between prokaryotic and eukaryotic cells also determine the features of the viruses that infect them. For example, in prokaryotes transcription and translation is coupled processes. In eukaryotes, by contrast, transcription and DNA replication occur in the nucleus while translation occurs in the cytoplasm. In addition, mRNAs in eukaryotes are capped and contain poly-A-tails. Some of the major differences between bacteriophage and eukaryotic viruses are mentioned in the table below.

Table 11.18 : Some differences between bacteriophages (virulent) and eukaryotic viruses

Bacteriophages	*Eukaryotic viruses*
1. Viral genome typically injected without capsid into the cell	1. Viral genome is not injected into host cells; virion is typically taken up inside cell
2. No segmented genomes	2. Some ssRNA viruses have segmented genomes
3. No 5′ capping	3. 5′ end of ssRNA (+) strand or viral mRNA is capped
4. No poly-A tails	4. 3′ end of ssRNA viruses have poly-A tail
5. Phage proteins rarely penetrate the host cell (phage M13 is an exception)	5. Viral proteins commonly enter the cell

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...iruses

...iruses are ssRNA (plus sense) containing animal virus that replicates through a DNA intermediates. ...iruses are enveloped viruses. The virion RNA is 7 to 12 kb long, and it is linear, single-stranded, ...gmented, and of positive polarity. The genome of the retrovirus is unique. It consists of two indentical ...stranded RNA molecules. The enzymes found in the virus particle are *reverse transcriptase*, *integrase* ...*protease*. The virion also contains specific cellular tRNA molecules. All retroviruses, contain the following ... and are arranged in the same order:

- ...*ag*, encoding structural proteins;
- ...*ol*, encoding reverse transcriptase and integrase; and
- ...*env*, encoding envelope proteins

...e complex retroviruses, such as HIV-I contains additional regulatory genes. The retroviral life cycle ...ceeds by reverse transcribing the RNA genome into duplex DNA, which is inserted into the host genome. ... enzyme, reverse transcriptase, converts the RNA into a linear duplex of DNA in the cytoplasm of the ...ected cell. Reverse transcriptase also has a DNA polymerase activity, which enables it to synthesize a ...plex DNA from the single-stranded reverse transcript of the RNA. The enzyme has an RNAase H activity, ...ich can degrade the RNA part of the RNA-DNA hybrid. Like other DNA polymerases, reverse transcriptase ...requires a primer. The native primer is tRNA. The linear DNA enters into the nucleus and become integrated into the host genome. A single enzyme, called *integrase*, is responsible for integration. Integrated retroviral ...DNA remains in the cellular genome as an endogenous **provirus**. The provirus is transcribed by the host ...machinery to produce viral RNAs. Retroviral mRNA has a conventional structure; it is capped at the 5′ end ...and polyadenylated at the 3′ end.

...Overall process of replication of a retrovirus

- Entrance into the cell via fusion with cell membrane.
- Uncoating of the virion
- Reverse transcription of one of the two RNA genomes into a single-stranded DNA that is subsequently converted to a linear double-stranded DNA by reverse transcriptase, which then enters the nucleus.
- Integration of retroviral DNA into the host genome.
- Transcription of retroviral DNA, leading to the formation of viral mRNAs and viral genomic RNA.
- Assembly and encapsidation of the genomic RNA into nucleocapsids in the cytoplasm.
- Budding of enveloped virions at the cytoplasmic membrane and release from the cell.

HIV AND AIDS

The acquired immunodeficiency syndrome (AIDS) was first recognized in the United States in 1981 in the Los Angeles, New York, and San Francisco when a group of patients displayed *Pneumocystis carinii* pneumonia (PCP), caused by opportunistic fungal pathogen *Pneumocystis carinii* and *Kaposi sarcoma*, an extremely rare skin tumor. A more complete evaluation of the patients showed that they had in common a mark deficiency in *cellular immune* responses and a significant decrease in the sub population of T-cells that carry the CD4 marker (the CD4 molecule is 55KD protein found predominantly on a subset of T-lymphocytes that are responsible for helper or inducer function in the immune system. It is also expressed on the surface of Monocytes/Macrophages).

Etiologic agent of AIDS is Human Immunodeficiency Virus (HIV). It was discovered and characterized by *Luc Montagnier* (in Paris) and *Robert Gallo* (in Bethesda) in year 1983 from a patient suffering from *lymphadenopathy*. HIV belongs to the family of human retrovirus and the sub family of lentiviruses.

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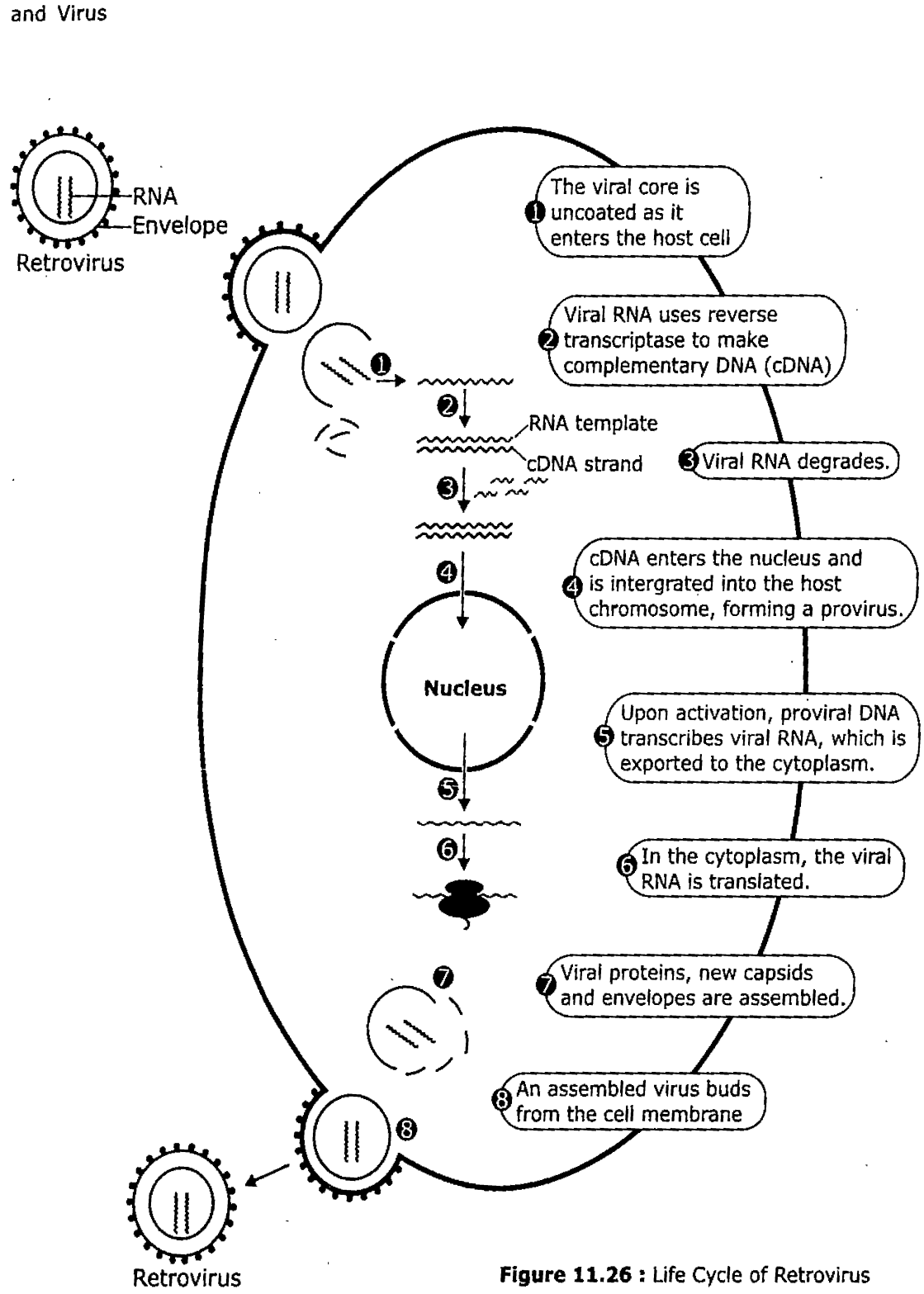


Figure 11.26 : Life Cycle of Retrovirus

AIDS is caused primarily by the HIV-1 virus (some cases result from an HIV-2 infection). This virus is closely related to HTLV-I, the cause of adult T-cell leukemia, or disabling progressive neurologic disorder called HTLV-I-associated myelopathy and HTLV-II, which has been isolated from individuals with hairy-cell leukemia.

Recently, a virus Simian immunodeficiency virus (SIV) related to HIV-1 and HIV-2, the strains primarily responsible for AIDS, has been isolated from African green monkeys and is believed to be the ancestor of the AIDS virus. Somehow, this virus entered in humans and mutated to the two current African human viruses SBL and IAV-2, which are intermediate viruses; these intermediate viruses may have evolved into the highly virulent HIV-1. HIV formerly called Lymphadenopathy associated virus (LAV), Human T Lymphotropic viruses (HTLV-III) and AIDS Associated Retrovirus (ARV).

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Structure of HIV-1

HIV-1 has an icosahedral structure. It is an enveloped *lentivirus* within the family *retroviridae*. The envelope derives from the host cell. Envelope contains 72 external spikes, made up of glycoproteins gp 120 (external) and gp 41 (transmembrane). Gp stands for glycoprotein and the number refers to the mass of protein, in thousands of Daltons. These glycoprotein serves as the viral receptor for CD4 on the host cells. Within the envelope is the viral core, or nucleocapsid, which includes two layers of a protein - an outer layer called p17 and an inner layer called p24.

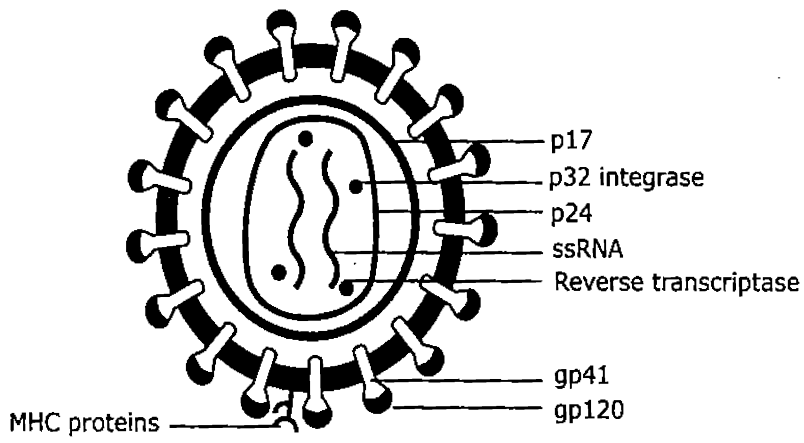


Figure 11.27 : HIV

HIV genome

HIV-I is a retrovirus. It contains two copies of *plus* single standard RNA genome. Most retroviruses that are capable of replication contain only three genes namely *gag*, *pol* and *env.* HIV-I contains nine different genes in its 9Kb RNA genome (*gag, pol, env, vif, vpu, vpr, tat, rev* and *nef*). Flanking these genes are the Long Terminal Repeats (LTR), which contain regulatory element involved in gene expression. The major difference between the genome of HIV-1 and HIV-2 is the that HIV-lacks the *vpu* gene and has a *vpx* gene not contained in HIV-1.

Gene	Funcion of encoded protein
gag	Nucleocapsid proteins
pol	Enzymes like Reverse transcriptase, Protease, Integrase, Ribonuclease
env	Envelope glycoprotein
vif	Promotes infectivity of viral particle
vpu	Required for efficient viral assembly and budding
vpr	Weakly activates transcription of proviral DNA
tat	Strongly activates transcription of proviral DNA
rev	Allows export of unspliced and singly spliced mRNAs from nucleus
nef	Increases viral replication down-regulates host-cell CD4

Life cycle of HIV

HIV-1 infects T-cells that carry the CD4 antigen on their surface; in addition certain HIV strains will infect monocytes and other cells that have CD4 on their surface. Binding to CD4 on cell surface and viral entry is also mediated by Chemokine co-receptors CXCR4 or fusin and CCR5 present on T-cell and monocytes, respectively. After the virus has entered the cell, the RNA genome of the virus is reverse transcribed and a

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cDNA (provirus) integrates into the host genome. The integrated provirus is transcribed and translated into proteins, which along with a complete new copy of the RNA genome are used to form new viral particles.

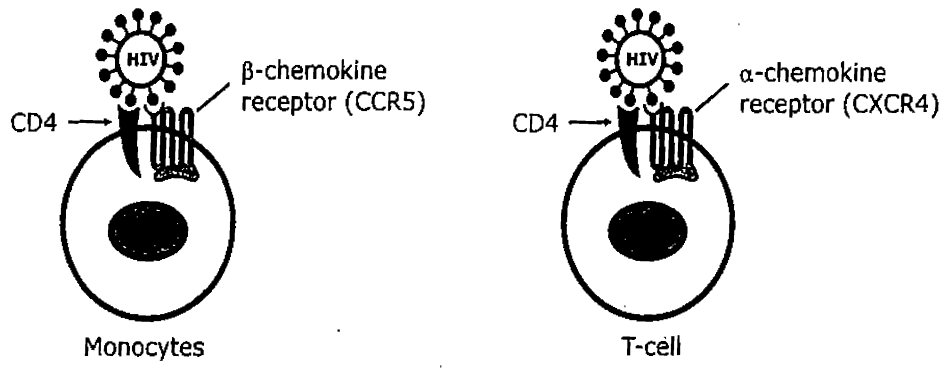


Figure 11.28 : Receptor and co-receptors for HIV

Infection of target cell

- HIV gp120 binds to CD4 on target cell
- Fusogenic domain in gp41 and CXCR4, a G-protein-linked receptor in the target-cell membrane, mediate fusion.
- Nucleocapsid containing viral genome and enzymes enters cells.
- Viral genome and enzymes are released following removal of core proteins.
- Viral *reverse transcriptase* catalyzes reverse transcription of ssRNA, forming RNA-DNA hybrid.
- Original RNA template is partially degraded by ribonucleases H, followed by *synthesis* of second DNA strand to yield HIV dsDNA.
- The viral dsDNA is then translocated to the nucleus and integrated *randomly* into the host chromosomal DNA by the viral *integrase* enzyme.

Activation of provirus

- Transcription factors stimulate transcription of proviral DNA into genomic ssRNA and, after processing, several mRNAs.
- Viral RNA is exported to cytoplasm.
- Host-cell ribosomes catalyze synthesis of viral precursor proteins.
- Viral proteases cleaves precursors into viral protein.
- HIV ssRNA and proteins assemble, into which gp41 and gp120 are inserted.
- The membrane buds out, forming the viral envelope.
- Released viral particles complete maturation; incorporated precursor proteins are cleaved by viral *protease* present in viral particles.

Therapeutic agents

At present there is no complete cure for AIDS. Primary treatment is directed at reducing the viral load and disease symptoms, and at treating opportunistic infections and malignancies. The antivirals currently approved for use in HIV disease are of two types.

1. **Reverse transcriptase inhibitors** are nucleoside analogues that inhibit the enzyme *reverse transcriptase* as it synthesizes DNA from RNA. Examples include AZT or zidovudine (Retrovir), didanosine (Videx), zalcitabine (HIVID), stavudine, lamivudine, delavirdine and nevirapine (Viramune).

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Protease inhibitors work by blocking the activity of the *HIV protease* and thus interfere with virion assembly. Examples include indinavir (Crixivan), ritonavir (Norvir), nelfinavir (Viracept), and saquinavir (Invirase).

However, the most successful treatment approach is to use drug combinations. An effective combination is a cocktail of AZT, lamivudine, and a protease inhibitor such as ritonavir.

HEPATITIS

Hepatitis is a liver inflammation commonly caused by an infectious agent. Hepatitis sometimes results in destruction of functional liver anatomy and cells, a condition known as **cirrhosis**. Some forms of hepatitis may lead to liver cancer. Although many viruses and a few bacteria can cause hepatitis, a restricted group of viruses is often associated with liver disease termed hepatitis viruses. Hepatitis viruses are diverse, and none of these viruses are genetically related, but all infect cells in the liver, causing hepatitis.

Characteristics of hepatitis viruses

	Features	*Incubation period*
Hepatitis A	SS RNA; No envelope	2-6 week
Hepatitis B	dS DNA; enveloped	4-26 week
Hepatitis C	SS RNA; enveloped	2-22 week
Hepatitis D	SS RNA; enveloped	6-26 week
Hepatitis E	SS RNA; No envelope	2-6 week

The genome of Hepatitis B virus (hepadnavirus) is among the smallest known of any viruses, 3-4 kb. Like retroviruses, Hepatitis B virus uses reverse transcriptase during replication cycle. However, unlike retroviruses the DNA genome of Hepatitis B virus is replicated through an RNA intermediate, the opposite of what occurs in retroviruses.

PLANT VIRUSES

Plant viruses exist in rod and polyhedral shape. Most plant viruses have genomes consisting of a single RNA strand of the (+) type. The best-known plant virus is the rod-shaped tobacco mosaic virus (TMV).

Relatively few plant viruses have DNA genomes. There are only two classes of DNA containing plant viruses. The cauliflower mosaic virus belongs to the first class, which contains a double-stranded DNA genome in a polyhedral capsule. The second class of DNA containing plant viruses are the geminiviruses (gemini = twins), characterized by a connected pair of capsids, each containing a circular, single-stranded DNA molecule of about 2500 nucleotides.

TOBACCO MOSAIC VIRUS

TMV causes leaf mottling and discoloration in tobacco and many other plants. It was the first virus to be discovered (by Dmitri Iwanowasky) and first virus to be crystallized (by W. Stanley). TMV is a rod shaped particle with ~2130 capsomere arranged in a hollow right handed helix. TMV's single RNA strand (~6400nt) is coaxially wound within the turns of the coat protein helix such that 3 nucleotides are bound to each protein subunit.

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11.11 PRIONS AND VIROID

Prions are proteinaceous infectious agents that are responsible for neurodegenerative diseases in animals including human. Prions are unprecedented infectious pathogens that are devoid of nucleic acid and seem to be composed exclusively of a modified isoform of PrP designated PrP^{Sc}. The normal, cellular PrP, denoted PrP^{C}, is converted into PrP^{Sc} through a process whereby a portion of its α-helical and coil structure is refolded into a β-sheet. This structural transition is accompanied by profound changes in the physicochemical properties of the PrP. PrP^{C} is sensitive to proteases whereas PrP^{SC} is protease resistant. High content of β-sheet in PrP^{SC} result in the formation of amyloid fibrillous structure that is absent from the PrP^{C} form. Both proteins are glycosylated and linked to the membrane by a GPI-linkage. The PrP^{SC} form can perpetuate itself by causing the newly sythesized PrP protein to take up the PrP^{SC} form instead of the PrP^{C} form.

Prions are novel transmissible pathogens causing a group of invariably fatal neurodegenerative diseases that can be perpetuated by inoculating animal with tissue extract from infected one. Prion diseases of humans are referred to as Creutzfeldt-Jakob disease, fatal familial insomnia, fatal sporadic insomnia and kuru. **Kuru** was the first naturally occurring subacute spongiform encephalopathy of humans shown to be caused by prions. It was first described by *Gajdusek* and *Zigas* in 1957. Kuru is characterized by cerebellar ataxia and a shivering-like tremor that produces complete motor incoordination.

Collectively, prion diseases are also described as **spongiform encephalopathies**. No prion diseases of plants are known. In 1997 the American scientist Stanley B. Pruisner won the Nobel Prize for this pioneering work with these diseases and with the prion proteins.

Table 11.19 : Prion disease of human/animals

S.No.	Disease	Organism
1.	Creutzfeldt-Jakob	Human
2.	Kuru	Human
3.	Bovine spongiform encephalopathy (Also known as Mad cow disease)	Cow
4.	Scrapie	Sheep
5.	Chronic wasting disease	Mule deer

Viroid and virusoid

Viroid is an infectious agent of plants that is a single-stranded, covalently closed circular RNA (about 250 to 400 nucleotides long) not associated with any protein. Viroid RNA does not code for any proteins. Viroids have so far been shown to infect plants only. A few well-studied viroids include *coconut cadang-cadang viroid* and *Potato Spindle-Tuber Viroid* (PSTV). No viroid diseases of animals are known, and the precise mechanisms by which viroids cause plant diseases remain unclear.

Virusoid are satellite nucleic acids. Satellite nucleic acids may be single stranded RNA, single-stranded DNA, or double-stranded RNA. Most of the characterized satellites are associated with plant viruses, and most are single-stranded RNA. Satellite nucleic acids are always functionally dependent on specific helper viruses and are encapsidated by the coat protein of these helper viruses. Nature of nucleic acid in virusoid is circular RNA which posesses **ribozyme** domain. Virusoids replicate via rolling circles. The strand of RNA that is packaged into the helper virus is called the plus strand. Similar to viroid it form a hammerhead structure that has a self-cleaving activity.

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Table 11.20 : Some important human disease and their causative agent

Bacterial Disease

Disease	**Causative agent**
Anthrax	*Bacillus anthracis*
Bacillary dysentery	*Shigella dysenteriae*
Botulism	*Clostridium botulinum*
Cholera	*Vibrio cholerae*
Legionnaire's disease	*Legionella pneumophila*
Typhoid fever	*Salmonella typhi*
Diphtheria	*Corynebacterium diphtheriae*
Meningococcal meningitis	*Neisseria meningitidis*
Pneumococcal pneumonia	*Streptococcus pneumoniae*
Tuberculosis	*Mycobacterium tuberculosis*
Whooping cough	*Bordetella pertussis*
Chlamydia	*Chlamydia trachomatis*
Gonorrhea	*Neisseria gonorrhoeae*
Syphilis	*Treponema pallidum*
Tetanus	*Clostridium tetani*

Viral Disease

Disease	**Causative agent**
Hepatitis	Hepatitis A, B, C, D, E
Influenza	Influenza virus
Measles	Measles virus
AIDS	Human immunodeficiency (HIV) virus
Rabies	Rabies virus

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SELF TEST

Q1. Which is the most modern method of classification of organisms?
- a. Morphology
- b. Gram staining
- c. Biochemical characterization
- d. 16S rRNA sequence

Q2. Which statement is *true* of both prokaryotic and eukaryotic cells?
- a. prokaryotic cells are generally much larger than eukaryotic cells
- b. eukaryotic cells have ribosomes and prokaryotic cells do not
- c. both have DNA as their primary genetic material
- d. eukaryotic cells have plasma membranes and prokaryotic cells do not

Q3. A bacterial culture contained 32 $\times 10^6$ cells after 2.5 hours of exponential growth. If the doubling time was 30 min, what was the initial population number in this culture?
- a. 20 $\times 10^4$ cells
- b. 10 $\times 10^5$ cells
- c. 40 $\times 10^5$ cells
- d. 16 $\times 10^6$ cells

Q4. Which of the following is a fact that argues against a symbiotic origin of eukaryotic flagella from bacterial flagella?
- a. bacterial flagella typically contain flagellin; eukaryotic flagella contain tubulin
- b. bacterial flagella rotate; eukaryotic flagella do not rotate
- c. bacterial flagella are directly energized by electrons and protons from the respiratory system of the plasma membrane; eukaryotes are not
- d. more than one of the above argue against the symbiotic theory

Q5. Which of the following is *not* true about bacterial flagella?
- a. most of their length consists of a hollow, rigid protein tube
- b. they are constructed largely of a single protein called flagellin
- c. they spin like wheels, either clockwise or counterclockwise
- d. they use cytoplasmic ATP as their primary energy source

Q6. Actin filaments are found in all of the following *except* the
- a. flagella of bacteria
- b. sarcomeres of skeletal muscle cells
- c. microvilli of the intestinal brush border
- d. contractile rings of dividing animal cells

Q7. In the exponential phase of growth of a bacterial culture, 100 cfu/ml cells increased to 3200 cfu/ml cells in 2 hours. What is the generation time for this bacterium?
- a. 12 minutes
- b. 15 minutes
- c. 24 minutes
- d. 30 minutes **[JNU]**

Q8. All of the following components of a retrovirus are encoded by the viral genome, *except*
- a. reverse transcriptase
- b. viral RNAs
- c. capsid proteins
- d. envelope lipids

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Q9. Potato spindle-tuber disease causes potatoes to become gnarled and cracked. This disease is caused by an infectious agent consisting of

a. circular DNA molecules without a protein coat
b. circular RNA molecule with a protein coat
c. DNA molecule with a protein coat
d. circular RNA molecule lacking a protein coat **[JNU]**

Q10. Match the correct combination of plasmid DNA to their properties

Plasmid DNA	*Property*
P. conjugative plasmid	1. can integrate into the chromosome and replicate when the chromosome is copied
Q. cryptic plasmid	2. capable of transferring itself between prokaryotes
R. episome	3. does not appear to have any function

a. P–1, Q–3, R–2
b. P–2, Q–3, R–1
c. P–2, Q–1, R–3
d. P–3, Q–2, R–1

Q11. An Hfr bacterium is one that contains

a. many unusual plasmids
b. chromosomal material acquired from a recipient cell
c. the ability to undergo transduction
d. a plasmid integrated into its chromosome

Q12. Evidence indicating the chloroplasts were originally free-living prokaryotes that subsequently evolved a symbiotic relationship with a eukaryotic host includes all of the following *except* the

a. similarities of rRNA sequences between chloroplasts and free-living prokaryotes
b. similarities of structures between chloroplasts and some contemporary free-living prokaryotes
c. presence of circular DNA in chloroplasts and in free-living prokaryotes
d. ability of chloroplasts to synthesize all their own protein

Q13. When a population of bacteria capable of conjugation transfers a specific chromosomal gene (say a gene coding for galactose metabolism) but no other genes, regardless of how long the bacteria are allowed to mate, these bacteria are said to be

a. F^+
b. F^-
c. Hfr
d. F'

Q14. Interrupted mating process is used to determine order in which genes are present on chromosome. It is based on the kinetics of transfer of individual makes. In case of interrupted mating conjugation is mediated between

P. Hfr donor and F^- recipient
Q. antibiotic resistant strain of Hfr and F^-
R. F^+ and F^-
S. prototropic Hfr donor and auxotropic F^- recipient

a. P and S
b. P and Q
c. R and S
d. Q and S

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Q15. Match the following

Column A	*Column B*
P. retroviridae	1. linear double stranded DNA
Q. herpesviridae	2. minus strand RNA
R. rhabdoviridae	3. circular double stranded DNA
S. baculoviridae	4. plus strand RNA

a. P-1, Q-2, R-3, S-4
b. P-2, Q-3, R-4, S-1
c. P-3, Q-4, R-1, S-2
d. P-4, Q-1, R-2, S-3

Q16. Which one of the following statement is *not* correct

a. almost all of the prokaryotes have a single circular chromosome
b. almost all of the prokaryotes have cell wall
c. all prokaryotes have plasmids
d. the genetic material in prokaryote is not enclosed in any structure surrounded by membranes

Q17. A prophage is

a. an auxotropic mutant
b. a gene
c. a phage DNA incorporated into the host genome
d. the DNA of lytic phage

Q18. Which of the following enzyme has both DNA polymerase and Rnase H activities

a. pancreatic ribonuclease A
b. reverse transcriptase
c. DNAzyme
d. RnaseP

Q19. HIV replicates its genome using unique mechanisms. Which of the following statements about HIV is not *correct*

a. HIV is an enveloped RNA virus
b. the virion contains an RNA dependent DNA polymerase
c. a DNA copy of the HIV genome intergrates into host cell DNA
d. virion contains an RNA dependent RNA polymerase **[IISc]**

Q20. Which of the following statement is *incorrect?*

a. enzyme activity associated with reverse transcriptase that digests the RNA template after DNA has been synthesized is RNase-H
b. viral envelope attaches the HIV to host cell is gp120
c. genomes of retroviruses contain two copies of plus ssRNA
d. HIV contains single copy of ssRNA

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Chapter - 12

Immunology

Immunology is the science that is concerned with immune response to foreign challenges. **Immunity** (derived from Latin term *immunis*, meaning *exempt*), is the ability of an organism to resists infection by pathogens or state of protection against foreign organisms or substances. The array of cells, tissues and organs which carry out this activity constitute the **immune system**. Immunity may be *active* or *passive* and *innate* or *adaptive*.

Active and Passive immunity

Active immunity is the adaptive immunity that is induced by natural exposure to a pathogen (natural) or by vaccination (artificial). In this kind of immunity a person develops his own immune response to a microbe. Immunity resulting from transfer of antibodies or immune cells from an immune to a non-immune individual is known as **passive immunity**. It may be *naturally* (involves the transfer of antibodies from one host to another) or *artificially* (when antibodies or lymphocytes that have been produced outside the host are introduced into a host) acquired.

Active Immunity	Passive Immunity
Exposure to antigen	No exposure of antigen
Immunity achieved by injecting antigens	Immunity achieved by injecting Ab or Ag reactive T-cells
Activation of immune system	No immune system activation
Immune state develops over a period of weeks	Immunity develops immediately
Immunological memory develops	No immunological memory develops

12.1 INNATE IMMUNITY

Innate (non-specific/natural) immunity is present from birth and consists of many factors that are relatively nonspecific—that is, it operates against almost any foreign molecules and pathogens. It provides *first line of defense* against pathogens. It is not specific to any one pathogen but rather acts against all foreign molecules and pathogens. It also does not rely on previous exposure to a pathogen and response is functional since birth and has no memory.

Elements of Innate Immunity

Physical barriers

Physical barriers are the *first line of defense* against microorganisms. It includes skin and mucous membrane. Most organisms and foreign substances cannot penetrate intact skin but can enter the body if the skin is damaged. Secondly the acidic pH of sweat and sebaceous secretions and the presence of various fatty acids and hydrolytic enzyme like lysozyme inhibits the growth of most microorganisms. Similarly respiratory and gastrointestinal tracts are lined by mucous membranes. Mucus membranes entraps foreign microorganisms. Respiratory tract is also covered by cilia, hair like projections of the epithelial-cell membranes. The synchronous movement of cilia propels mucus-entrapped microorganisms from these tracts. Similarly, the conjunctiva is a

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specialized, mucus-secreting epithelial membrane that lines the interior surface of each eyelid. It is kept moist by the continuous flushing action of tears (lacrimal fluid) from the lacrimal glands. Tears contain *lysozyme, lactoferrin, IgA* and thus provide chemical as well as physical protection.

Microorganisms do occasionally breach the epithelial barricades. It is then up to the innate and adaptive immune systems to recognize and destroy them, without harming the host. In case of innate immune response several antimicrobial chemicals and phagocytic cells provide protection against pathogens.

CHEMICAL MEDIATOR

A variety of chemicals mediate protection against microbes during the period before adaptive immunity develops. The molecules of the innate immune system include complement, cytokines, pattern recognition molecules, acute-phase proteins, cationic peptides, enzyme like lysozyme and many others.

Complement

Complement is a heat labile component of blood plasma that augments phagocytosis. This activity was said to *complement* the antibacterial activity of antibody; hence, the name complement. It also bridges innate and adaptive immunity and removes immune complexes. The complement system is composed of over 30 serum proteins. Activation of complements in response to certain microorganisms results in a controlled enzymatic cascade, which targets the membrane of pathogenic organisms and leads to their destruction.

Cytokines

The term **cytokine** is a generic term for any low molecular weight soluble protein or glycoprotein released by one cell population which acts as an intercellular mediator. It includes *Monokines, Lymphokines, Interleukins, Interferons* and others. Cytokines are required for immunoregulation of both innate as well as adaptive immune responses.

Interferons are cytokines made by cells in response to virus infection, which essentially induce a generalized antiviral state in surrounding cells.

Chemokines are small, positively charged secreted proteins that have a central role in guiding the migrations of various types of white blood cells. They bind to the surface of endothelial cells, and to negatively charged proteoglycans of the extracellular matrix in organs. By binding to G-protein-linked receptors on the surface of specific blood cells, chemokines attract these cells from the bloodstream into an organ, guide them to specific locations within the organ, and then help stop migration.

Pattern recognition molecule

Many molecules involved in innate immunity have the ability to recognize a given class of molecules i.e., recognize pattern. Patterns are conserved structures and invariant among microorganisms of a given class. Pattern recognition molecules that recognize *P*athogen-*A*ssociated *M*olecular *P*attern (PAMP) may be soluble circulating proteins or cell surface receptors. Many PAMPs are recognized by pattern recognition molecules present on the surface of phagocytic cells. Mannose-binding lectin (MBL) and C-reactive protein (CRP) are soluble pattern recognition molecules that bind to microbial surfaces and promote their opsonization. **Toll-like receptor** (TLRs) are a class of pattern recognition molecules that function exclusively as signaling receptors. There are at least 10 distinct proteins in this family of mammalian receptors. For example, TLR-4 signals the presence of bacterial lipopolysaccharide (LPS) and heat-shock proteins. TLR-2 signals the presence of bacterial lipoproteins and peptidoglycans.

Acute phase proteins are a heterogeneous group of plasma proteins mainly produced in the liver as the result of a microbial stimulus. They include C-reactive protein (CRP), serum amyloid protein A (SAA) and

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mannose binding protein (MBP). Cytokines (IL-1, IL-6, IL-8, etc) released by macrophages upon activation by bacteria stimulate the liver to rapidly produce acute-phase proteins. These proteins maximize activation of the complement system and opsonization of invading microbes.

CELLULAR DEFENSES

The cells responsible for both non-specific and specific immunity are the **leukocytes**. Many specialized cell types like *neutrophils, macrophages, monocytes, NK cells* participate in innate host defense mechanisms. Once a pathogen evades the physical and chemical barriers, these specialized cells play important role in protection. Phagocytosis is a fundamental protective mechanism carried out by these cell types, neutrophils, macrophages, monocytes and dendritic cells. **Phagocytosis** is the ingestion of invading foreign particles, such as bacteria by individual cell. Phagocytosis may be enhanced by a variety of factors collectively referred to as **opsonins** (Greek word meaning 'prepared food for'), consist of antibodies and various serum components of complement. The process by which particulate antigens are rendered more susceptible to phagocytosis is called **opsonization.** After ingestion, the foreign particle is entrapped in a phagocytic vacuole (phagosome), which fuses with lysosomes forming the *phagolysosome*. The antimicrobial and cytotoxic substances present within the lysosome destroy the phagocytosed microorganisms in following ways.

Oxygen dependent killing mechanisms

During phagocytosis, a metabolic process known as the **respiratory burst** occurs in activated phagocytes. It results in transient increase in oxygen consumption by cell. Activated phagocytes generate a number of toxic products like reactive oxygen intermediates (such as hydroxyl radicals, hypochlorite anion, superoxide anions, hydrogen peroxide) and reactive nitrogen intermediates (like NO, NO_2, $HNO_2^{\bullet}$) that have potent antimicrobial activity.

Oxygen independent killing mechanisms

Activated macrophages also synthesize **lysozyme**, **defensins** (cysteine rich cationic peptides containing 29-35 amino acid residues) and various hydrolytic enzymes/cytotoxic peptides whose degradative activities do not require oxygen.

INFLAMMATORY BARRIERS

Inflammation is an important nonspecific defense reaction to cell injury. The hallmark signs of inflammation are pain, redness (erythema), swelling (edema) and heat. Each of these is the result of specific changes in the local blood vessels. Pain is caused by *increased vascular diameter,* which leads to *increased blood flow*, thereby causing heat and redness in the area. The blood vessels become permeable to fluid and proteins, leading to local swelling and an accumulation of blood proteins that aid in defense. At the same time, the endothelial cells lining the local blood vessels are stimulated to express cell adhesion proteins that facilitate the attachment and *extravasion* (movement of blood cells through the vessel wall into the surrounding tissue) of white blood cells, including neutrophils, lymphocytes, and monocytes.

The inflammatory response is mediated by a variety of signaling molecules. Activated macrophages produce chemoattractants (known as **chemokines**). Some of these attract neutrophils, which are the *first* cells recruited in large numbers to the site of the new infection. Others later attract monocytes and dendritic cells. The dendritic cells pick up antigens from the invading pathogens and carry them to nearby lymph nodes, where they present the antigens to lymphocytes to marshal the forces of the adaptive immune system. Two principal mediators of inflammatory response are **histamine** (released by a variety of cells in response to tissue injury) and **kinins** (present in blood plasma in an inactive form). Both cause vasodilation and increased permeability of capillaries. Kinins are also very potent nerve stimulators and are the molecules most responsible for pain associated with inflammation.

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12.2 ADAPTIVE (SPECIFIC) IMMUNITY

Adaptive immunity, also known as *acquired* immunity, is capable of recognizing and selectively eliminating specific foreign antigens. It does not come into play until there is an antigenic challenge to the organism. Adaptive immunity display four characteristic features:

1. Antigenic specificity

   It is the ability to discriminate among different epitopes/antigens.

2. Immunologic memory

   It is the ability to recall previous contact with a foreign molecule and respond to it in a learned manner-that is, with a more rapid and larger response.

3. Diversity

   The ability to respond to many different epitopes even if the individual has not previously encountered them.

4. Self/non-self recognition

   It is the ability to recognize and respond to molecules that are foreign and to avoid making a response to those molecules that are self.

Table 12.1 : Difference between the Innate and Adaptive immune response

Innate	**Adaptive**
Antigen nonspecific	Antigen specific
Rapid response (hours)	Slow response (days)
No memory	Memory, response to repeated infection is identical to primary response.

Immune response of adaptive immunity

There are two arms (branches) of acquired immunity, which have different sets of participants and different purposes but one common aim: to eliminate the antigen. These two arms interact with each other and collaborate to achieve the final goal of eliminating the antigen. Of these two arms of the acquired immune response, one is mediated mainly by **B cells** and circulating **antibodies**, a form of immunity is referred to as **humoral** (the word *humors* was formerly used to define body fluids). The other is mediated by T cells, which, do not synthesize antibodies but instead synthesize and release various cytokines that affect other cells. Hence, this arm of the acquired immune response is termed **cellular** or **cell-mediated immunity.**

In humoral immunity, B lymphocytes synthesize and secrete antibodies with specificity against the foreign substance. Whereas the T lymphocytes, which also exhibit specificity against the foreign substance by virtue of their *T-cell receptors* (TCRs), do not make antibodies but perform various effector functions when antigen-presenting cells (APCs) bring antigens into the secondary lymphoid organs. T lymphocytes also interact with B cells and help the latter make antibodies; they activate macrophages and have a central role in the development and regulation of acquired immunity. Acquired immunity mediated by T lymphocytes is termed cell-mediated immunity.

Innate and adaptive immunity are not independent

The innate and adaptive immunity operate in cooperative and interdependent ways. The activation of innate immune responses produces signals that stimulates and direct subsequent adaptive immune responses. They cooperate in many ways.

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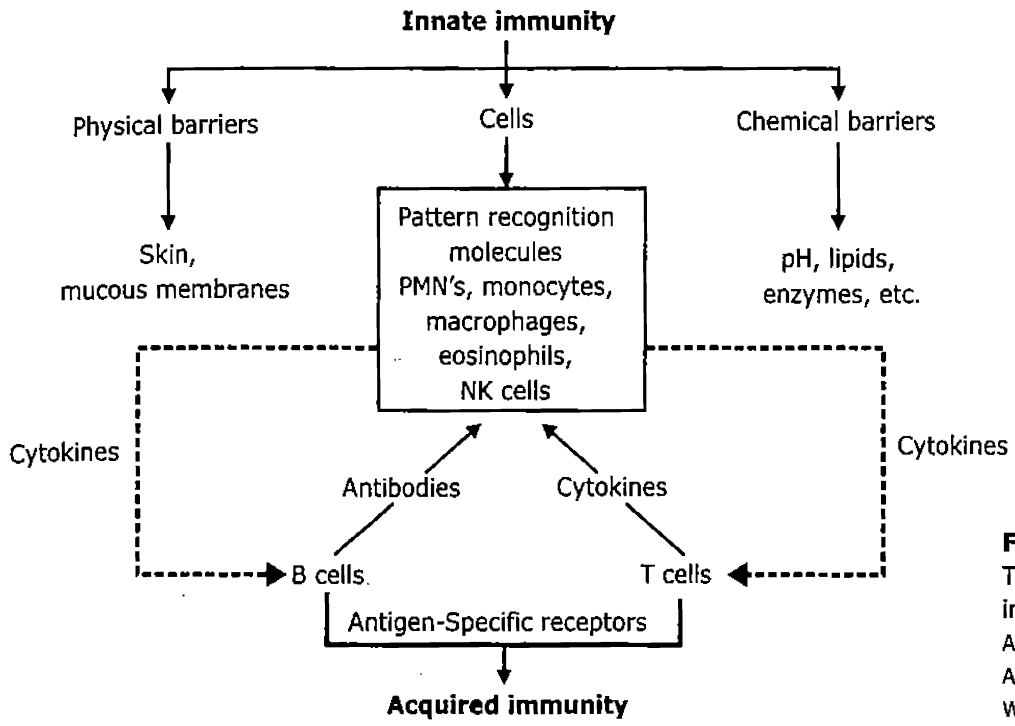


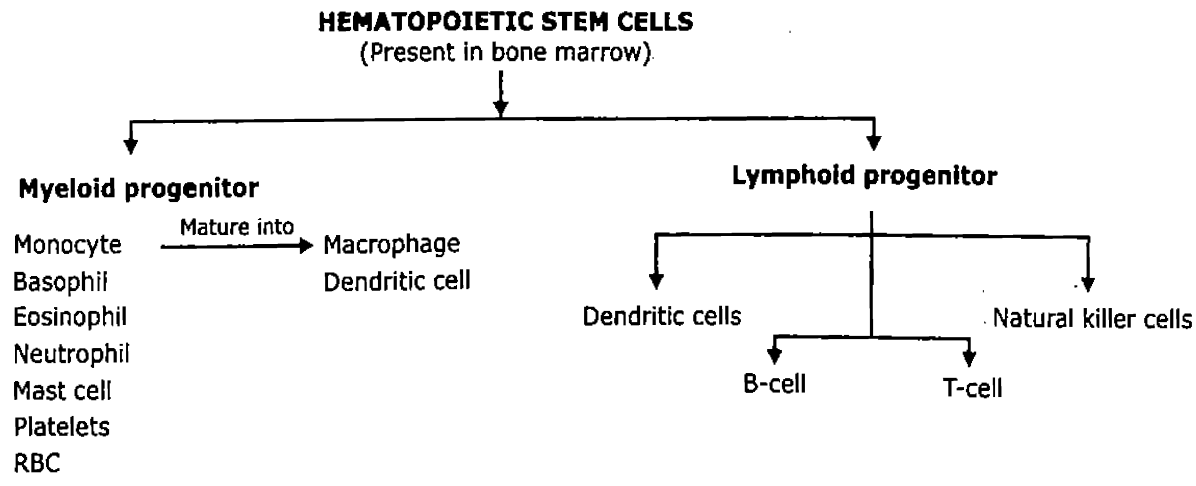
Figure : 12.1
The interrelationship between innate and acquired immunity.
Adapted and modified from Immunology A short Course, R. Coico et. al, Wiley-Liss Publication.

The encounter between macrophages and microbes can generate 'danger' signals that stimulate and direct adaptive responses. It may increase the ability of macrophages to display antigen for recognition by antigen specific T-cells. Macrophage stimulated by encounters with microbes also secrete immunoregulatory molecules, called **cytokines**. These cytokines guide adaptive immune response. Vice-versa, adaptive immune system also produces signals and components which stimulate and increase the efficacy of innate response. The cells of adaptive system (e.g., T-cells) secrete cytokines and increase the ability of macrophage to kill the ingested microbes. By binding to the pathogens, antibodies mark it as a target for attack by complement.

12.3 CELLS OF THE IMMUNE SYSTEM

The immune system is a defensive system in a host consisting of widely distributed cells, tissues and organs that recognize foreign substances and microorganisms and acts to neutralize or destroy them. The cell responsible for both nonspecific and specific immunity are the leukocytes or white blood cells. All of the leukocytes originate from pluripotent stem cells in the fetal liver and in the bone marrow of the animal.

Pluripotent stem cells in the bone marrow divide to form two blood cell lineages: the **lymphoid stem cell** gives rise to B cells, T cells and natural killer cells. The common **myeloid progenitor cell** gives rise to the granulocytes (neutrophils, eosinophils, basophils), monocytes that give rise to macrophages and dendritic cells, an unknown precursor that gives rise to mast cells, megakaryocytes that produce platelets, and the erythroblast that produces RBCs.



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LYMPHOCYTES

Lymphocytes (responsible for adaptive immune response) are mononuclear leukocyte which constitutes 20 to 40% of WBC. They occur in large numbers in blood and lymph and in lymphoid organs such as the thymus, lymph nodes, spleen and appendix. Upto 99% of lymphocytic cells are found in lymph. Lymphocytes are of three main types :

1. B Lymphocytes or B-cells
2. T Lymphocytes or T cells
3. Natural killer (NK) cells

B-Lymphocytes

A lymphocyte that matures in the bone marrow (in birds is Bursa of Fabricius) and expresses membrane-bound antibody. After interacting with antigen, it differentiates into antibody-secreting *plasma cells* and *memory cells*. They are the only cell type capable of producing antibody molecules and therefore the central cellular component of humoral immune responses. B-cells also serve as **antigen presenting cells** (APC).

T lymphocytes

T lymphocytes 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 the membrane. T cells do not make antibodies but perform various effector functions when APC bring antigens into the secondary lymphoid organ. T-cells help in eliminating APCs, cancer cells, virus-infected cells or grafts which have altered self-cells.

T-cells express distinct membrane molecules. On the basis of presence of one or the other of two membrane molecules, $CD4^+$ and $CD8^+$, there are two sub-population of T-cells - **T helper cells**, T_H (carries $CD4^+$ membrane glycoprotein on their surfaces) and **T cytotoxic cells**, T_C (carries $CD8^+$ membrane glycoprotein on their surfaces). In addition, all T- cell subpopulations express the T-cell receptor, that includes CD3. The ratio of $CD4^+$ and $CD8^+$ T cells is approximately 2:1 in normal human peripheral blood, but it may be significantly altered by immunodeficiency and autoimmune diseases.

Cytotoxic T cells provide protection against intracellular pathogens such as viruses and some bacteria and parasites that multiply in the host-cell cytoplasm, where they are sheltered from attack by antibodies. They provide this protection by killing the infected cell before the microbes can proliferate and escape from the infected cell to infect neighboring cells. Helper T cells are crucial for defense against both extracellular and intracellular pathogens. They help stimulate B cells to make antibodies that inactivate or eliminate extracellular pathogens and their toxic products. They also activate macrophages to destroy any intracellular pathogen multiplying within the macrophage's phagosomes, and they help activate cytotoxic T cells to kill infected target cells.

Natural killer cells

Natural killer cells (NK cells) are a class of lymphocytes that are distinct from cytotoxic T cells. However, NK cells also play a role in destroying cells infected with intracellular pathogens. NK cells are neither T cells nor B cells. They constitute 5-10% of lymphocyte population. Their numbers are not enhanced, nor do they exhibit memory after stimulation. NK cells destroy the target cell not by phagocytosis but by releasing biologically potent molecules. They resemble T_C cells in their ability to destroy infected cells. For example, NK cells also use perforin and granzymes to kill their targets. However, NK cells differ from T_C cells in that they kill targets in the *absence* of a specific antigen. NK cells are capable of destroying malignant and virus-infected cells without previous exposure or contact with the foreign antigen. **Chediak-Higashi syndrome** – an autosomal recessive disorder- is associated with a lack of **NK cells**.

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NK cells recognize targets through two ways : In some, they employ NK cells receptors that can detect abnormalities such as low MHC I display and unusual markers on cancer cells and cells infected by some viruses. Another way in which NK cells recognize potential target cells depend by certain viruses upon the fact that some tumor cells and cells infect 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 membrane receptor CD16 for the IgG, they can bind to these antibodies and subsequently destroy the targeted cells. This is an example of a process known as **antibody-dependent cell-mediated cytotoxicity** (ADCC).

Granulocytes

Granulocytes have irregular-shaped nuclei with two to five lobes, and are often called *polymorphonuclear leukocytes*. Their cytoplasmic matrix has granules that contain reactive substances that kill microorganisms and enhance inflammation. Three types of granulocytes exist: basophils, eosinophils, and neutrophils.

1. **Neutrophils** (due to irregular-shaped nuclei, also called *polymorphonuclear neutrophils* or PMNs) : The neutrophils have a multilobed nucleus and a granulated cytoplasm that stain with both acidic and basic dyes. Neutrophils are produced by hematopoiesis in the bone marrow. They are released into the peripheral blood and circulate for 7-10 hour before migrating into the tissues, where they have a life span of only a few days. 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 walls, and finally penetrates the vascular basement membrane, moving out into the tissue spaces. Like macrophages, neutrophils are active phagocytic cells.
2. **Eosinophils** have bilobed nucleus and stain with acidic dye *eosin*. It comprises 2-5% of WBCs and are motile phagocytic cells that can migrate from the blood into the tissue space. Their role is important in the defense against protozoan and helminth parasites, mainly by releasing cationic peptides and reactive oxygen intermediates into the extracellular fluid.
3. **Basophils** have lobed nucleus and stain with basic dye *methylene blue*. It comprises less than 1% of total WBCs. They are non-phagocytic, release substance that cause an **allergic responses.** These molecules include histamine, prostaglandins, serotonin, and leukotrienes. Basophils (and mast cells) possess high affinity receptors for one type of antibody, known as IgE.
4. **Mast cells** : Mast-cell precursors are formed in bone-marrow and released into blood in an undifferentiated state, until they reach tissues. They have large number of cytoplasmic granules containing **histamine**. Mast cells and basophils play role in allergic responses.

Mononuclear phagocytes

The mononuclear phagocytes include monocytes circulating in the blood and macrophages in the tissues.

Monocytes

Monocytes are mononuclear phagocytic leukocyte that circulate briefly in the blood stream before migrating into the tissues where it become macrophages or into dendritic cells.

Macrophages

Macrophages are **phagocytes** derived from blood **monocytes.** The monocyte is a small, spherical cell with few projections, abundant cytoplasm, and many granules. Following migration of monocytes from the blood to various tissues, they undergo further differentiation into a variety of histologic forms, all of which play a role in phagocytosis, including the following-

- *Kupffer cells*, in the liver; large cells with many cytoplasmic projections.

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- *Alveolar macrophages*, in the lung.
- *Splenic macrophages*, in the white pulp.
- *Peritoneal macrophages,* free-floating in peritoneal fluid.
- *Osteoclasts,* in bone.
- *Mesangial cells,* in the kidney.
- *Microglial cells,* in the central nervous tissue.

Dendritic cells

Dendritic cells are bone marrow derived cells that descend through the myeloid and lymphoid lineages and are specialized for antigen presentation to helper T-cells. Dendritic cells are classified into four types:

1. Langerhans cells
2. Interstitial dendritic cells
3. Myeloid dendritic cells
4. Lymphoid dendritic cells

Despite their differences they all constitutively express high levels of class II MHC molecules. Dendritic cells acquire antigen by phagocytosis; the antigen is processed, and mature dendritic cells present it to T_H cells.

Follicular dendritic cell

A cell with extensive dendritic extensions that is found in the follicles of lymph nodes. It does not arise in bone marrow and has a different function from the antigen presenting dendritic cells. Although they do not express MHC class II molecules, they are richly endowed with receptors for antibody which allow the binding of Ag-Ab complexes. They are of a lineage that is distinct from MHC class II bearing dendritic cells.

12.4 ORGANS INVOLVED IN THE ADAPTIVE IMMUNE RESPONSE

Lymphatic organs

The lymphatic organs are those organs in which B and T-lymphocytes maturation, differentiation, and proliferation take place. They are generally divided into two categories - **Primary** and **Secondary lymphoid organs**.

The *primary (central) lymphoid organs* are those in which the maturation of T and B lymphocytes into antigen-recognizing lymphocytes occurs. **Bone marrow** and **thymus** are example of primary (central) lymphoid organs.

Thymus gland

Thymus is the site where T lymphocytes mature. Progenitor cells from the bone marrow migrate into the thymus gland, where they differentiate into T-lymphocytes. 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, and the inner compartment, or *medulla*.

T lymphocytes mature in the cortex and migrate to the medulla, where they encounter macrophages and dendritic cells. Here, they undergo thymic selection, which results in the development of mature, functional T cells, which then leave to enter the peripheral blood circulation, through which they are transported to the secondary lymphoid organs. It is in these secondary lymphoid organs where the T cells encounter and respond to foreign antigens.

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Bursa of fabricius and Bone marrow

A primary lymphoid organ was first discovered in birds. In birds, B cells undergo maturation in the bursa of fabricius. This organ, situated near the cloaca, consists of lymphoid centers that contain epithelial cells and lymphocytes. These lymphocytes consist solely of antibody-producing B cells.

Mammals do not have a bursa of fabricius. B cells differentiate from hematopoietic stem cells in the fetal liver. After birth and for the life of the individual, this function moves to the bone marrow, a structure that is considered to be a primary lymphoid organ with functions equivalent to that of the avian bursa of fabricius. Each mature B lymphocyte bears antigen-specific receptors that have a structure and specificity identical to the antibody later synthesized by the B cell. The mature B cells are transported by the circulating blood to the secondary lymphoid organs, where they encounter and respond to foreign antigens.

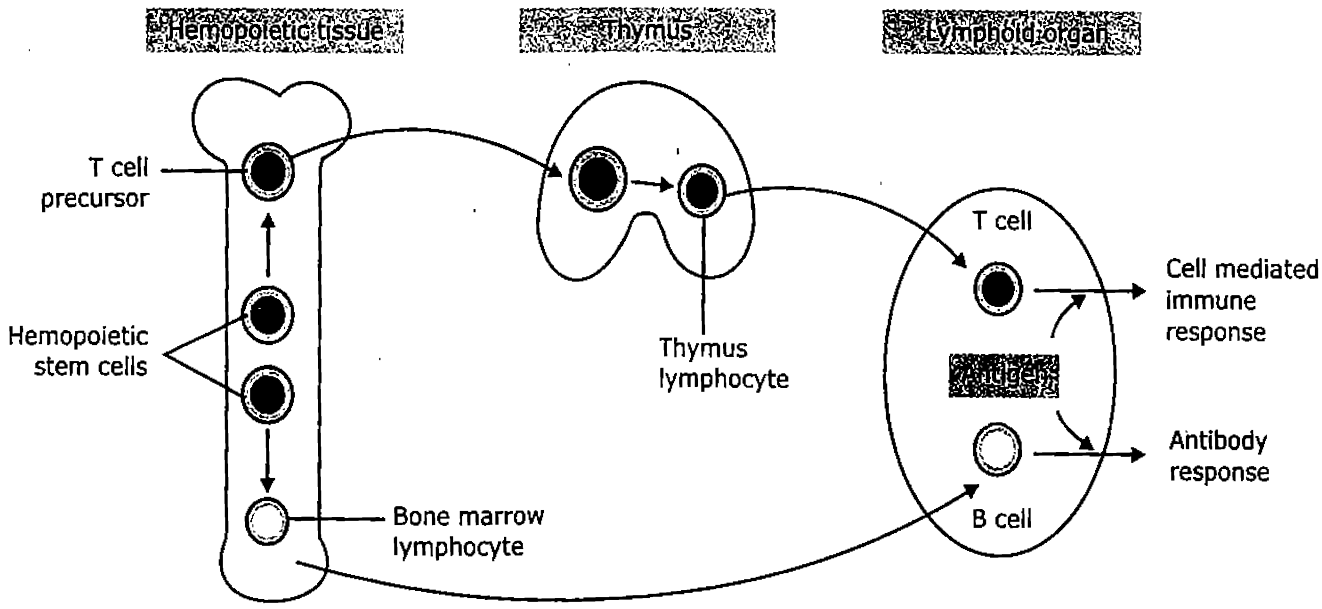


Figure 12.2 : The development and activation

STUDY QUESTIONS

Q1. What are the two primary characteristics that distinguish hematopoietic stem cells and progenitor cells?

Ans. Stem cells are capable of self-renewal and can give rise to more than one cell type, whereas progenitor cells have lost the capacity for self-renewal and are committed to a single cell lineage. Commitment of progenitor cells depends on their acquisition of responsiveness to particular growth factors.

Q2. What effect would removal of the bursa of fabricius (bursectomy) have on chickens?

Ans. The bursa of fabricius in birds is the primary site where B lymphocytes develop. Bursectomy would result in a lack of circulating B cells and humoral immunity, and it would probably be fatal.

Secondary lymphoid organs/tissues

Mature B and T lymphocytes migrate from bone marrow and thymus, respectively, through the bloodstream to the *secondary (peripheral) lymphoid organs*. These secondary (peripheral) lymphoid organs are those organs in which antigen-driven proliferation and differentiation take place.

The major secondary lymphoid organs are the **spleen**, the **lymph nodes** and **mucosa associated lymphoid tissue** (MALT). Spleen and lymph nodes are the highly organized secondary lymphoid organs. The secondary lymphoid organs have two major functions: They are highly efficient in trapping and concentrating foreign substances, and they are the main sites of production of antibodies and the induction of antigen-specific T lymphocytes.

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Spleen

The spleen is the *largest* of the secondary lymphoid organs. It is highly efficient in trapping and concentrating foreign substances carried in the blood. It is the major organ in the body in which antibodies are synthesized and from which they are released into the circulation.

The interior of spleen is a compartmentalized structure. The compartments are of two types - **Red pulp** and **White pulp**. *Red pulp is the site where old and defective RBCs are destroyed and removed*, whereas white pulp forms **PALS** (Periarteriolar lymphoid sheath) which are rich in T-cells. The marginal zone, located peripheral to the PALS, is rich in lymphocyte and macrophage. Approximately 50% of spleen cells are B lymphocytes; 30-40% are T lymphocytes.

Lymph nodes

Lymph nodes are small encapsulated bean shaped structures (normally <1cm in diameter) found in various regions throughout the body. The lymph nodes are composed of a *medulla* and a *cortex*, which is surrounded by a capsule of connective tissue. They are packed with lymphocytes, macrophages, and dendritic cells. The cortical region contains primary lymphoid follicles. After antigenic stimulation, these structures enlarge to form secondary lymphoid follicles with germinal centers containing dense populations of lymphocytes (mostly B cells). The deep cortical area or paracortical region contains T cells and dendritic cells. Antigens are brought into these areas by dendritic cells, which present antigen fragments to T cells. The medullary area of the lymph node contains antibody-secreting plasma cells that have travelled from the cortex to the medulla via lymphatic vessels.

Lymph nodes are highly efficient in trapping antigen that enters through the afferent lymphatic vessels. In the node, the antigen interacts with macrophages, T cells, and B cells, and that interaction brings about in immune response, manifested by the generation of antibodies and antigen-specific T cells.

Mucosa associated lymphoid tissue

The majority of secondary lymphoid tissue in the human body is located within the lining of respiratory, digestive and genitourinary tracts. These are collectively called as ***mucosa associated lymphoid tissue*** (MALT). There are several types of MALT. Two major MALT includes ***bronchial associated lymphoid tissue*** (BALT) and ***gut-associated lymphoid tissue*** (GALT). GALT includes the tonsils, adenoids, and specialized regions in the small intestine called **Peyer's patches**.

12.5 ANTIGENS

Adaptive immune responses arise as a result of exposure to foreign compounds. The compound that evokes the response is referred to as **antigen**, a term initially coined due to the ability of these compounds to cause *anti*body responses to be *gen*erated. An antigen is any agent capable of binding specifically to components of the immune response, such as the B cell receptor (BCR) on B lymphocytes and soluble antibodies. The ability of a compound to bound with antibodies and with cells of immune system is referred to as **antigenicity**. There is functional distinction between the term antigen and immunogens. An **immunogen** is any agent capable of inducing an immune response and is therefore **immunogenic**. The distinction between the terms is necessary because there are many compounds that are incapable of inducing an immune response, yet they are capable of binding with components of the immune system that have been induced specifically against them. Thus all immunogens are antigens, but not all antigens are immunogens.

The study of antigen-antibody reaction *in vitro* is called **serology**. Serological reactions are the basis for all diagnostic immunology tests.

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Requirements for immunogenicity

A substance must possess the following characteristics to be immunogenic:

1. Foreignness

The most important feature of an immunogen is that an effective immunogen must be *foreign* with respect to the host. The adaptive immune system recognizes and eliminates only foreign (nonself) antigens. Self antigens are not recognized and thus individuals are *tolerant* to their own self molecules, even though these same molecules have the capacity to act as immunogens in other individuals of the same species.

2. Size

The second requirement for being immunogenic is that the compound must have a certain minimal molecular weight. There is a relationship between the size of immunogen and its immunogenicity. In general, small compounds with a molecular weight <1000 Da (e.g., penicillin, aspirin) are not immunogenic; those of molecular weight between 1000 and 6000 Da (e.g., insulin, adrenocorticotropic hormone) may or may not be immunogenic; and those of molecular weight >6000 Da (e.g., albumin, tetanus toxin) are generally immunogenic. The most active immunogens tend to have a molecular mass of 100,000 Da or more. In short relatively small substances have decreased immunogenicity, whereas large substances have increased immunogenicity.

3. Chemical complexity

The third characteristic necessary for a compound to be immunogenic is a certain degree of chemical complexity. For example, homopolymers of amino acids or sugars are seldom good immunogens regardless of their size. Similarly, a homopolymer of poly-γ-D-glutamic acid (the capsular material of *Bacillus anthracis*) with a molecular weight of 50,000 Da is not immunogenic. The absence of immunogenicity is because these compounds, although of high molecular weight, are not sufficiently chemically complex.

Virtually all proteins are immunogenic. Thus the most common immune responses are those to proteins. Furthermore, the greater the degree of complexity of the protein, the more vigorous will be the immune response to that protein. Nucleic acids are poor immunogens by themselves, but they become immunogenic when they are conjugated to protein carriers. Lipids are rarely immunogenic, but an immune response to lipids may be induced if the lipids are conjugated to protein carriers.

4. Dosage and route of administration

Insufficient dose of antigen may not stimulate an immune response either because the amount administered fails to activate enough lymphocytes or because such a dose renders the responding cells unresponsive. Besides the need to administer a threshold amount of antigen to induce an immune response, the number of doses administered also affects the outcome of the immune response generated.

The route of administration also affect the outcome of the immunization because this determines which organs and cell populations will be involved in the response. Immunogens can be administered through a number of common routes: *Intravenous* (into a vein); *intradermal* (into the skin); *subcutaneous* (beneath the skin); *intramuscular* (into the muscle). Antigens administered via the most common route namely, *subcutaneously*, generally elicit the strongest immune responses. This is due to their uptake, processing, and presentation to effector cells by Langerhans cells present in the skin, which are among the most potent APCs. Antigens administered subcutaneously moves first to local lymph nodes. *Intravenously* administered antigens are carried first to the spleen, where they can either induce immune unresponsiveness or tolerance, or if presented by APCs, generate an immune response. Orally administered antigens (*gastrointestinal route*) elicit local antibody responses within the intestinal lamina propria.

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Haptens

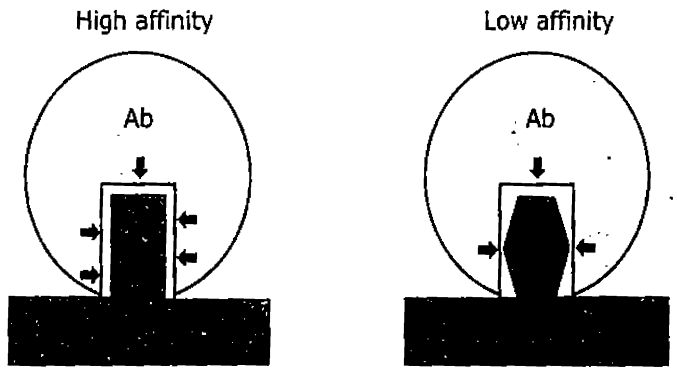
Substances called **haptens** (from the Greek *hapten*, which means *to grasp*) fail to induce immune responses in their native form because of their low molecular weight and their chemical simplicity. Haptens are antigenic but not immunogenic. These compounds become immunogenic when they are conjugated to high molecular weight, physiochemically complex **carriers**. Thus a hapten is a compound that, by itself, is incapable of inducing an immune response but against which an immune response can be induced by immunization with the hapten conjugated to a *carrier*.

Antigen-antibody interactions

Ag-Ab interaction is highly specific and occurs in similar way as bimolecular association of an enzyme-substrate. The binding between antigens and immune components involves weak non-covalent interactions. The binding forces are relatively weak and *reversible* and consist mainly of van der Waals forces, electrostatic forces, and hydrophobic forces, all of which require a very close proximity between the interacting moieties. The smallest unit of antigen that is capable of binding with antibodies is called an **antigenic determinant** or **epitope**. The corresponding area on the Ab molecule combining with the epitope is called **paratope**. The number of epitopes on the surface of an antigen is its **valence**. The valence determines the number of antibody molecules that can combine with the antigen at one time. If one epitope is present, the antigen is **monovalent**. Most antigens, however, have more than one copy of the same epitope and are termed **polyvalent**.

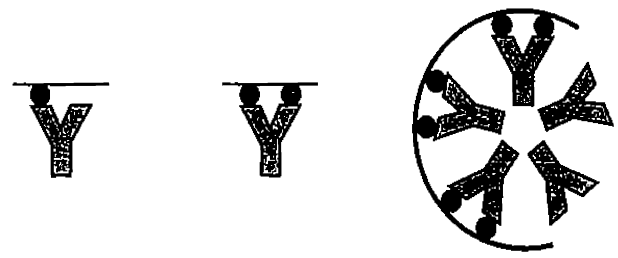
AFFINITY AND AVIDITY

The intrinsic association constant that characterizes the noncovalent interaction between single antigen binding site of an antibody with an epitope is termed **affinity**. Low-affinity antibodies bind antigen weakly and tend to dissociate readily, whereas high-affinity antibodies bind antigen more tightly and remain bound longer.



When the antigen consists of many repeating identical epitopes or when antigens are multivalent, the association between the entire antigen molecules and antibodies depends not only on the affinity between each epitope and its corresponding antibody but also on the sum of the affinities of all the epitopes involved. The term **avidity** is used to denote the overall binding between antibodies and a multivalent antigen. So, when complex Ag having multiple repeating epitopes, are mixed with Ab having multiple binding sites, the interaction of such type between multivalent Ab and Ag is called, the avidity. Thus, in general, IgM antibodies with 10 antigen binding sites are of higher avidity than IgG antibodies, although the binding of each Fab in the IgM antibody with ligand may be of the same affinity as that of the Fab from IgG.

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Affinity Avidity Avidity

Adjuvant

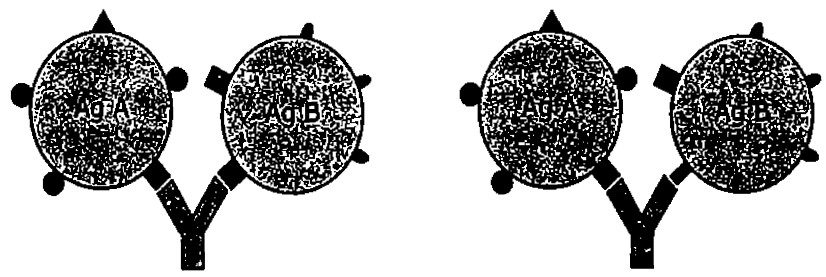
An adjuvant (from Latin word *adjuvare* means *to help*) is a substance that when mixed with an immunogen and injected with it, enhances the immune response against the immunogen. Adjuvants are often used to boost the immune response in case of weak immunogens or when antigens are available in small amount. A carrier for a hapten and an adjuvant is different. A hapten will become immunogenic when conjugated covalently to a carrier; it will not become immunogenic if mixed with an adjuvant. Thus an adjuvant enhances the immune response to immunogens but does not confer immunogenicity on haptens. How adjuvants augment the immune response has remained enigmatic.

While many adjuvants have been developed in animal models and tested experimentally in humans, only one has been used for routine vaccination. Currently, aluminum potassium sulfate (alum) is a common adjuvant used for human vaccines. Alum prolongs the persistence of antigen. When an antigen is mixed with alum, the salt precipitates the antigen.

Another commonly used adjuvant is *Freund's complete and incomplete adjuvant* . Freund's incomplete adjuvant contains antigen in aqueous solution, oil, and an emulsifying agent such as mannide monooleate. Freund's **complete adjuvant** contains heat-killed *Mycobacteria* as an additional ingredient.

Cross-reactivity

Although Ag-Ab reaction is very specific, sometimes antibody elicited by one antigen can cross-react with an unrelated antigen. An immunologic reaction in which a particular antibody or T-cell receptor react with two or more antigens that possess a common epitope is called a **cross-reaction**. Another form of cross-reactivity is seen when antibodies or cells with specificity to one epitope bind, usually more weakly, to another epitope that is not quite identical but has a structural resemblance to the first epitope.



Shared epitope Similar epitope

Comparison of antigen recognition by T cells and B cells

The recognition of antigens by T cells and B cells is fundamentally different. B cells recognize soluble antigens whereas most T cells recognize only peptides combined with MHC molecules.

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Table 12.2 : Antigen recognition by B and T cells

Characteristics	B cells	T cells
Antigen interaction	B cell receptor (membrane Ig) binds Ag	T cell receptor binds Ag and MHC
Nature of antigens	Protein, polysaccharide, glycolipid	Peptide
Binding soluble antigens	Yes	No
Epitopes recognized	Accessible areas of protein structure containing sequential amino acids and nonsequential amino acids	Antigens processed internally and presented as linear peptides bound to MHC molecules
MHC molecules	Not required	Required to display processed antigen

Adapted and modified from Immunology, Goldsby et. al, Freeman Publication.

Exogenous and endogenous antigens

Exogenous antigens are those antigens that originate outside the organism or cell. Exogenous antigens are internalized and degraded within the acidic endocytic compartments and subsequently pair with MHC class II molecules. *Endogenous antigens* are those antigens that originate within the organism or cell. Endogenous antigens are degraded into peptides within the cytosol by proteasomes and assemble with MHC class I molecules in the RER.

T-independent and T-dependent antigens

T-dependent (TD) *antigens* are those that requires direct contact with T_H cells, not simply exposure to T_H -derived cytokines to stimulate B-cells for the production of antibody. Proteins are T-dependent antigens. *T-independent* (TI) *antigens* are antigens that can activate B cells in the absence of any direct contact with T_H cells. Examples of TI antigens are lipopolysaccharide (LPS) of bacterial outer membrane, bacterial flagellin and cell-wall and capsular polysaccharides.

Unlike the T-dependent antigens, the T-independent antigens:

- do not produce isotype switching (IgM is almost exclusively produced).
- do not demonstrate affinity maturation (in which antibodies of progressively higher affinity are produced).
- do not show immunological memory hence secondary responses.

Superantigens

Superantigens are viral or bacterial proteins that bind simultaneously to the variable domain of β of a T-cell receptor and to the α chain of a class II MHC molecule. Because of their unique binding ability, superantigens can activate large numbers of T cells irrespective of their antigenic specificity. Superantigens canbe exogenous and endogenous. *Exogenous* superantigens are soluble proteins secreted by bacteria whereas *endogenous* superantigens are cell-membrane proteins encoded by certain viruses that infect mammalian cells.

REVIEW THE CONCEPT

Q1. a. What is the minimal molecular mass range of a protein essential for inducing an antibody response.

b. Distinguish between antigenicity and immunogenecity. **[CSIR-UGC]**

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12.6 MAJOR-HISTOCOMPATIBILITY COMPLEX (MHC)

MHC is a tightly linked cluster of genes present in every vertebrate species, the products of which play a role in intercellular recognition and in discrimination between self and non-self.

MHC is located on chromosome 6 in humans and on chromosome 17 in mice and referred to as the HLA (human leukocyte antigen) complex and H-2 (histocompatibility-2) complex, respectively. MHC are highly **polymorphic** i.e., many alternative form of the gene, or alleles, exist at each locus. Different individuals within a species have different alleles of MHC gene– that is, at a single MHC locus, different individuals have different types of a prototypical gene. The phenomenon of having multiple stable forms of one gene in the population is known as **genetic polymorphism**. MHC genes are *codominantly expressed* – that is, every cell that expresses MHC molecules expresses proteins transcribed from both the maternal and the paternal chromosome.

The genes of the MHC loci lie close together. A group of linked MHC genes is generally inherited as a unit from parents; these linked groups are called **haplotypes**. An individual inherits one haplotype from the mother and one haplotype from the father. Each individual expresses a distinct array of MHC molecules. This diversity comes about because different individuals within a species have a range of slightly different forms (alleles) of MHC genes (genetic polymorphism). Because of the extensive polymorphism of MHC genes, every individual has an almost unique array of inherited MHC genes.

Two major sets of MHC genes, known as **class I MHC** and **class II MHC**, and their cell-surface expressed products (MHC molecules, sometimes referred to as MHC antigens) are present.

1. **Class I MHC genes** express glycoproteins called MHC molecules, sometimes referred to as MHC antigen on nearly all nucleated cells. Each MHC class I gene codes for a transmembrane glycoprotein of approximate molecular weight 43 kDa, which is referred to as the α, or heavy chain. It comprises three extracellular domains: α_1, α_2 and α_3. The α_3 domain is highly conserved and interacts with the CD8 membrane molecule present on T_C cells. Every MHC class I molecule is expressed at the surface of a cell in *noncovalent* association with a small invariant polypeptide called β_2-**microglobulin** (β_2m; molecular weight 12 kDa), which is coded from another chromosome (human chromosome 15). In the absence of β_2m; the class I MHC chain is not expressed on the cell membrane.

   In general, class I MHC molecules are expressed on most nucleated cells, but the level of expression differs among different cell types. The highest levels of class I molecules are expressed by lymphocytes whereas fibroblasts, liver hepatocytes, and neural cells express very low levels of class I MHC molecules. A few cell types (e.g., neurons and sperm cells at certain stages of differentiation) appear to lack class I MHC molecules altogether.

2. **Class II MHC genes** code for α and β chains of approximate molecular weight 35,000 and 28,000Da, respectively. MHC class II molecules, like MHC class I molecules, are transmembrane glycoprotein molecules with cytoplasmic tails and extracellular Ig-like domains; the domains are referred to as α_1, α_2, β_1 and β_2. MHC class II molecules are also members of the Ig superfamily. The T cell molecule CD4 binds to the invariant portion of all MHC class II molecules.

   Class II MHC gene express MHC molecule *constitutively* (i.e., under all conditions) only on antigen presenting cells (APCs). APCs are classified as *professional* and *nonprofessional* antigen-presenting cells. Professional antigen-presenting cells are dendritic cells, macrophages, and B lymphocytes. Nonprofessional antigen-presenting cells like fibroblasts, thymic epithelial cells, glial cells can be induced to express class II MHC molecules or a co-stimulatory signal.

   In general, in the absence of inducing factors, most cells express MHC class I molecules without expressing MHC class II molecules. Certain cells, such as B cells, constitutively express both MHC class I and Class II molecules. *By convention*, cells that display peptides associated with class I MHC molecules to T_C cells are referred to as *target cells* and cells that display peptides associated with class II MHC to T_H cells are called *antigen presenting cells* (but sometimes we use APC for both).

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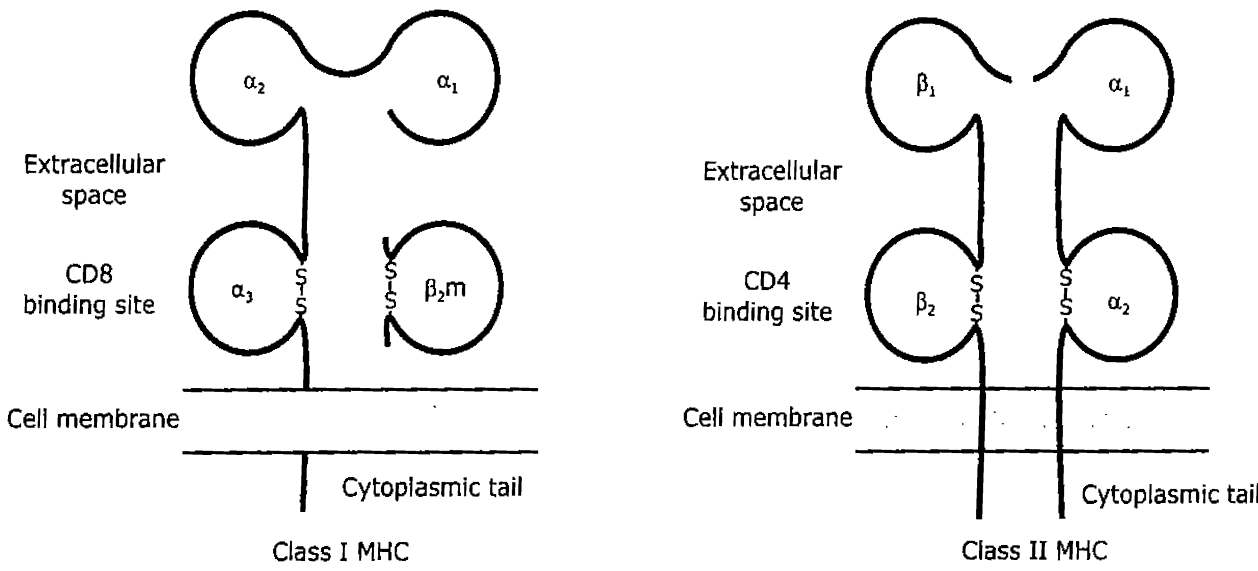


Figure 12.3 : MHC class I molecule are composed of a polymorphic α chain noncovalently attached to the nonpolymorphic β_2 microglobulin (β_2m). The α chain is glycosylated. MHC class II molecules are composed of a polymorphic α chain non-covalently attached to a polymorphic β chain. Both chains are glycosylated..

The three independent genes that code for the human MHC class I molecules; the genes and molecules are known as **HLA-A, HLA-B**, and **HLA-C**. The human MHC class II region is made up of three sets of genes, known as **HLA-DP, HLA-DQ**, and **HLA-DR**. Each MHC class II subregion contains an A and a B gene that codes for one chain, α or β, respectively, of a two-chain MHC class II molecule. Diversity of MHC molecules results not only due to multiple alleles of each gene but also from the presence of duplicated genes with similar functions. Because it includes genes with similar but not identical structure, and function (for example, HLA-A, -B, and -C), the MHC may be said to be **polygenic.**

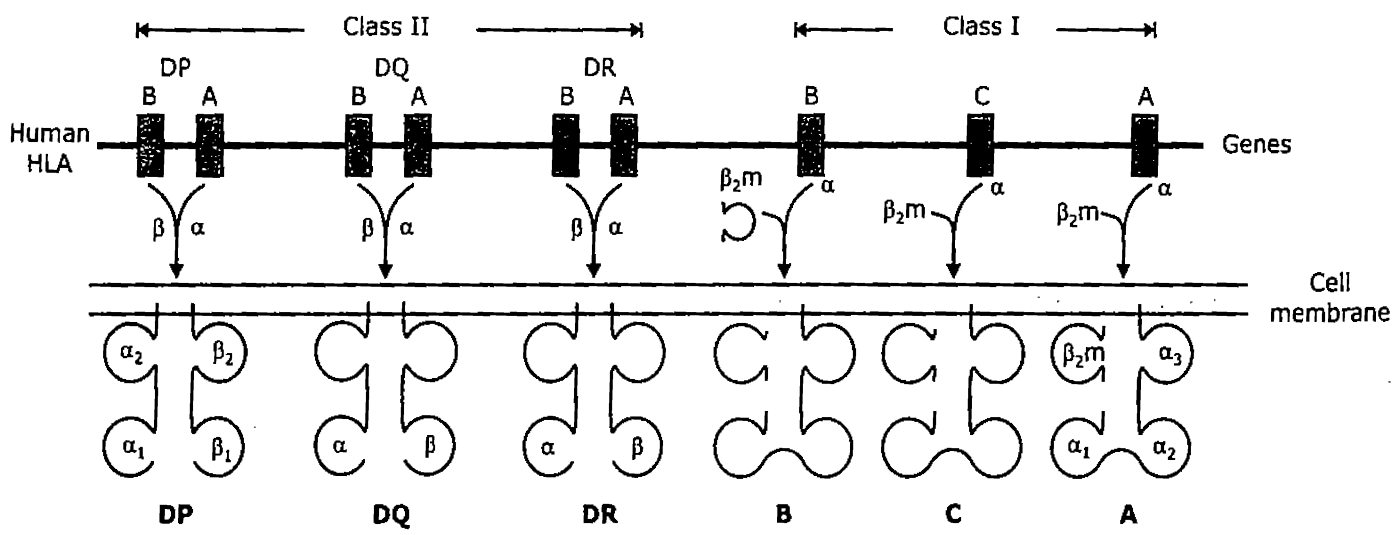


Figure 12.4 : Simplified depiction of the human MHC showing regions and genes coding for polymorphic MHC class I and II molecules. β_2m encoded outside the MHC. Adapted and modified from Immunology A short Course, R. Colco *et al.*, Wiley-Liss Publication.

MHC molecules and antigen presentation

Both types of membrane glycoproteins (class I and class II MHC) function as highly specialized antigen-presenting molecules. Both class I and class II MHC molecules present antigen to T-cells. T cells recognize only peptides combined with MHC molecules. Class I molecules present processed endogenous antigen to CD8 T-cells. Class II molecules present processed exogenous antigen to CD4 T-cells. The class I MHC

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molecule presents a peptide of 8-10 amino acids, whereas the class II molecules bind and present slightly longer peptides of 13-18 amino acids. Peptide binding by class I and II molecules does not exhibit high specificity. A given MHC molecule can bind numerous different peptides, and some peptides can bind to several different MHC molecules.

Table 12.3 : Feature of class I and class II MHC molecules

Feature	**Class I MHC**	**Class II MHC**
Structure	α chain and β_2m	α and β chain
Polymorphic domain	α_1 and α_2 domains	α_1 and β_1 domains
Binding site for T cell coreceptor	α_3 region binds CD8	β_2 region binds CD4
Constitutive cellular expression	Nearly all nucleated cells	APCs
Peptides derived from	Endogenous antigens	Exogenous antigens
Peptide presented to	$CD8^+$ T cells	$CD4^+$ T cells
Nomenclature		
Human	HLA-A, HLA-B, HLA-C	HLA-DR, HLA-DQ, HLA-DP
Mouse	H-2K, H-2D, H-2L	I-A, I-E

Important aspects of MHC

- Although there is a high degree of polymorphism for a species, an individual has maximum of six different class I MHC products and only slightly more class II MHC products (considering only the major loci).
- Each MHC molecule has only *one* binding site. The different peptides can bind to a given MHC molecules to the same site, but only one at a time.
- Because each MHC molecule can bind many different peptides, binding is termed degenerate.
- MHC polymorphism is determined only in the germline. There are *no* recombinational mechanisms for generating diversity.
- MHC molecules (class I and class II) are membrane-bound; recognition by T cells requires cell-cell contact.
- Alleles for MHC genes are co-dominant. Each MHC gene product is expressed on the cell surface of an individual nucleated cell.
- Cytokines (especially interferon-γ) increase level of expression of MHC.
- Endogenous peptides associate with class I MHC and are recognized by Tc cells. Exogenous peptides associate with class II MHC and are recognized by T_H cells.

ANTIGEN PROCESSING AND PRESENTATION

T-cell receptor does not recognize free antigens. T-cell receptor recognize only antigen that is bound to cell membrane protein called *MHC molecules*. Both T_H and T_C cells can recognize antigen only when it is presented by a self-MHC molecule, an attribute called *MHC restriction*. CD4 T_H cells recognize antigen with class II MHC molecules on antigen-processing cells. Thus, antigen recognition by the CD4 T_H cell is *class II MHC restricted.* Whereas CD8 T_C cells recognize antigen with class I MHC molecules on target cells. Thus, antigen recognition by CD8 T_C cells is *class*

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The events involved in the generation of peptide antigens from proteins inside cells, the binding of peptides to MHC molecules, and the display of peptide-MHC complexes at the cell surface for T-cell recognition are known collectively as **antigen processing** and **presentation.**

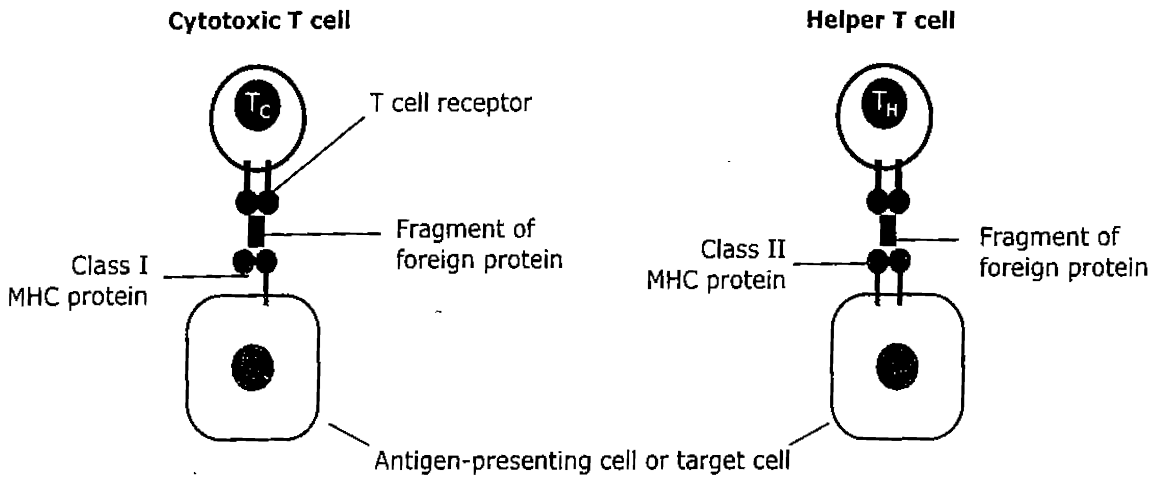


Figure 12.5 : Recognition by T cells of foreign peptides bound to MHC proteins. Cytotoxic T cell recognize foreign peptides in association with class I MHC proteins, whereas helper T cells recognize foreign peptides in association with class II MHC proteins. In both cases, the peptide MHC complexes are recognized on the surface of an antigen-presenting cell or a target cell.

How do peptides derived from protein antigens associated with MHC molecules? Protein antigens presented by APC belongs to two categories: exogenous and endogenous antigens. Endogenous and exogenous protein antigens use different pathways for processing and presentation to cytotoxic T cells and helper T cells, respectively.

STUDY QUESTIONS

Q3. Human red blood cells are not nucleated and do not express any MHC molecules. Why is this property fortuitous for blood transfusion?

Ans. If RBCs expressed MHC molecules, then extensive tissue typing would be required before a blood transfusion, and only a few individuals would be acceptable donors for a given individual.

Q4. Explain the difference between the terms antigen-presenting cell and target cell, as they are commonly used in immunology.

Ans. By convention, antigen-presenting cells are defined as those cells that can display antigenic peptides associated with class II MHC molecules and can deliver a co-stimulatory signal to CD_4^+ T_H cells. Target cells display peptides associated with class I MHC molecules to CD_8^+ T_C cells.

Processing and presentation of endogenous antigens : Cytosolic pathway

Endogenous antigens are synthesized inside a cell; typically they are derived from pathogens (e.g., viruses, bacteria, and parasites) that have *infected* the cell. MHC I proteins present peptide antigens derived from endogenous protein antigen. Processing of endogenous antigens occurs in the cytoplasm rather than in the acid vesicles. The major mechanisms for generating peptide fragments in the cytoplasm is via a giant protein complex known as the **proteasome**. This cleaves proteins into peptides about 15 amino acids in length. Cytosolic enzymes (amino peptidases) remove even more amino acids from the peptides. Peptide antigens are transported via an energy-dependent reaction into the endoplasmic reticulum (ER) through a pore formed by proteins, called the *transporters associated with antigen processing* (TAP). Once the peptides

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have entered the ER, they are bound by the MHC I protein, held in place near the TAP site by a group of *chaperone* proteins such as calnexin, calreticulin, tapasin and ERp57. The MHC I-peptide complex is then released from the chaperone and moves to the cell surface where it integrates into the membrane and can be recognized by T_c cells.

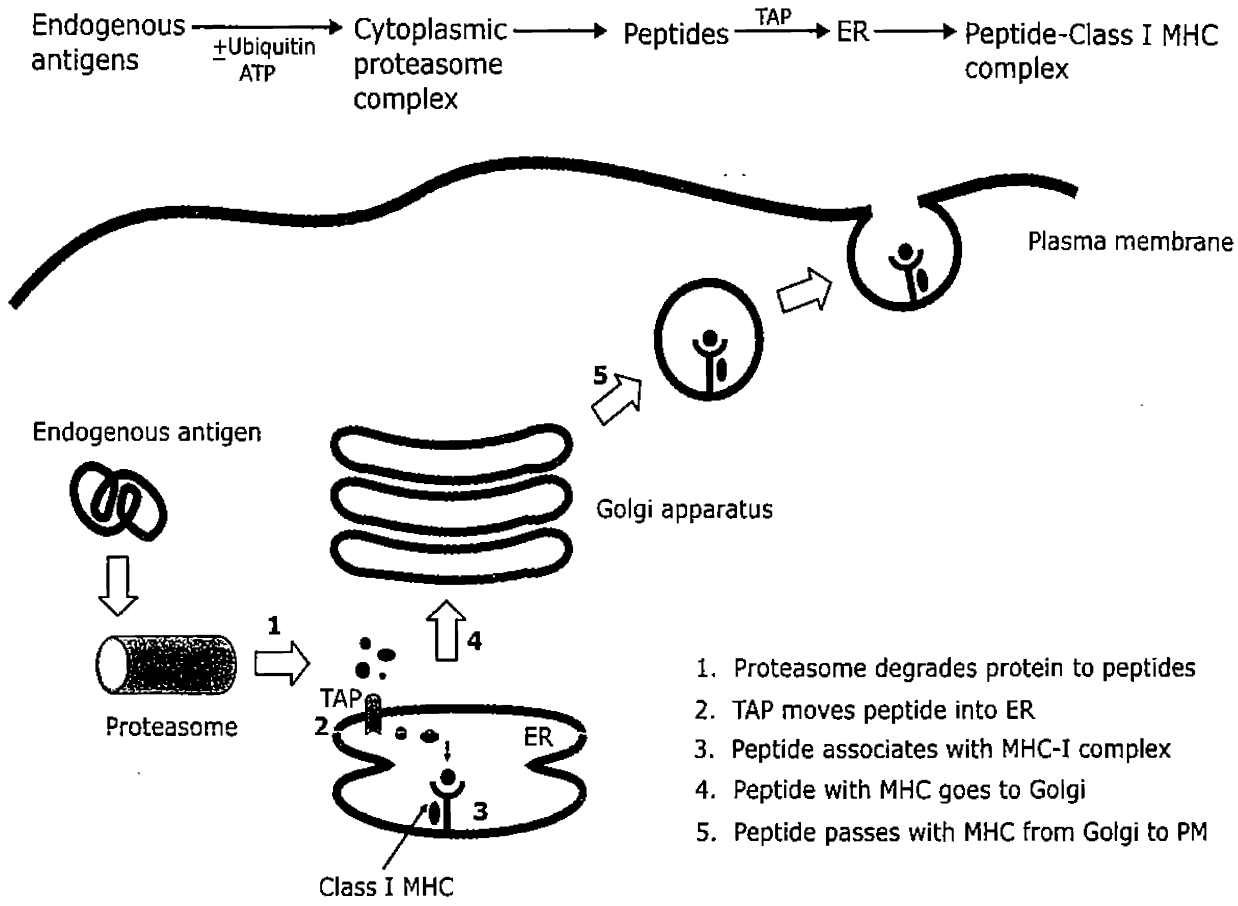


Figure 12.6 : The processing of an endogenous protein antigen for presentation to a cytotoxic T cell.

Processing and presentation of exogenous antigens : Endocytic pathway

Like the endogenous antigen presented to cytotoxic T cells, the proteins presented to helper T cells on APC are degraded fragments of foreign proteins. Rather than being derived from foreign protein synthesized in the cytosol of a cell, the foreign peptides presented to helper T cells are derived from endosomes. These peptides come from extracellular microbes or their products that the APC has endocytosed and degraded in the acidic environment of its endosomes. Processed antigens not enter the lumen of the ER, where the class II MHC proteins are synthesized and assembled, but instead bind to preassembled class II heterodimers in a special endosomal compartment. Once the peptide has bound, the class II MHC protein alters its conformation, trapping the peptides in the binding groove for presentation at the cell surface to helper T cells.

A newly synthesized class II MHC protein must avoid clogging its binding groove prematurely in the ER lumen with peptide derived from endogenously synthesized proteins. A special trimeric protein, called the **invariant chain**, ensures this by associating with newly synthesized class II MHC in the ER. Part of its polypeptide chain lies within the peptide-binding groove of the MHC protein, thereby blocking the groove from binding other peptides in the lumen of the ER. The invariant chain is cleaved by proteases in the late endosome. However, a short fragment of the invariant chain termed CLIP (for class II-associated invariant chain peptide) remain bound to the class II MHC, preventing any premature binding of antigenic peptide. This fragment is then released (mediated by a non-classical class II-MHC like protein called **HLA-DM**), freeing the MHC protein to bind peptides derived from endocytosed proteins. The peptide antigen-presenting MHC molecules are known as *classical* MHC molecules. Structurally related molecules that do not function in

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the presentation of peptide antigens to T cells are known as *non-classical* MHC molecules. Another non-classical class II MHC HLA-DO may act as negative regulator of class II antigen processing by binding to HLA–DM and inhibiting its role in the dissociation of CLIP from MHC.

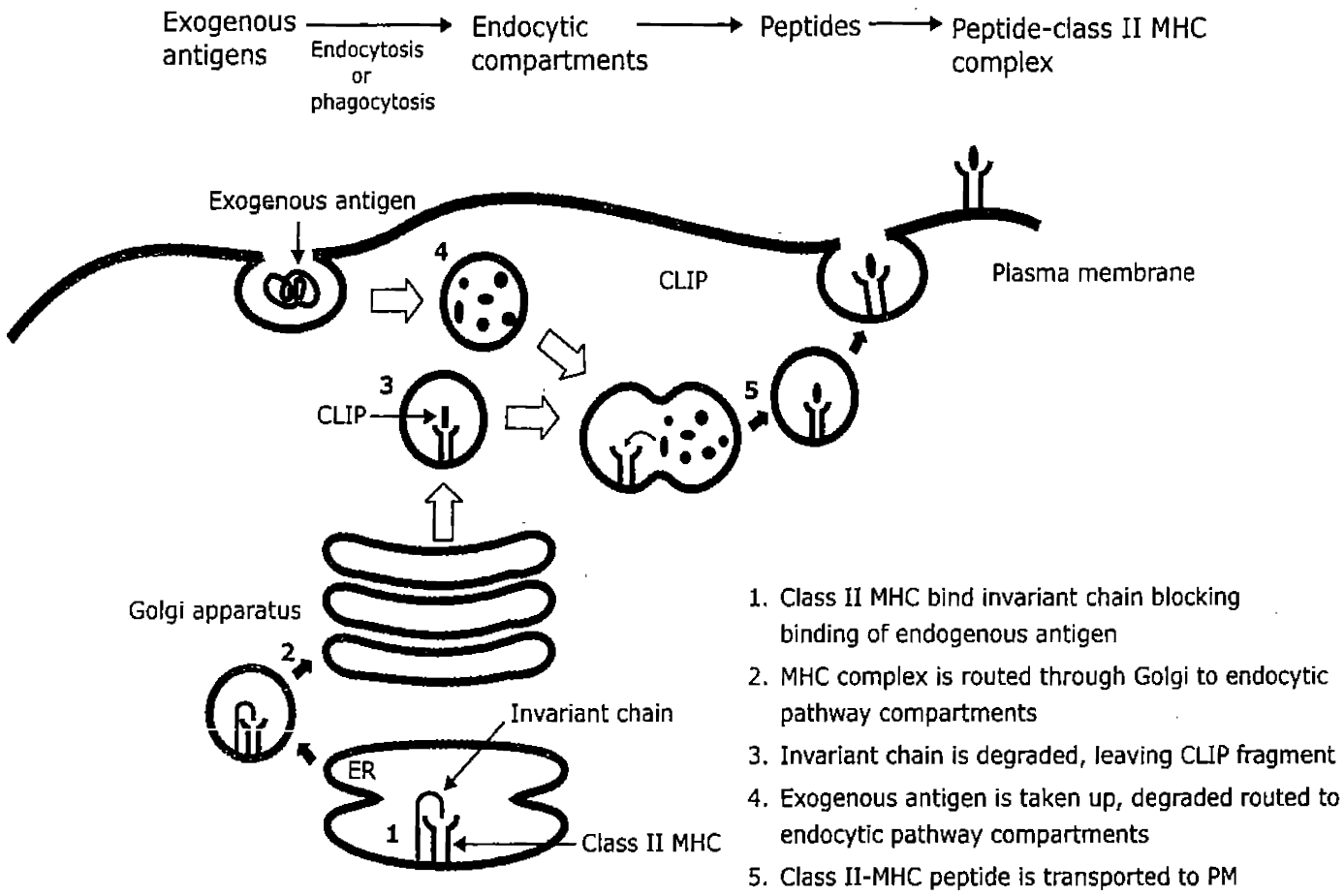


Figure 12.7 : The processing of an exogenous protein antigen for presentation to a helper T cell.

Presentation of non-peptide antigens

Non-peptide antigens (such as glycolipid, phospholipid) are presented by members of the CDI family belongs to non-classical type I MHC. It is presented to T-cells that express the γδ TCR.

12.7 IMMUNOGLOBULINS : STRUCTURE AND FUNCTION

Antibodies, the antigen-binding *glycoproteins* are synthesized exclusively by B cells and in billions of forms, each with different amino acid sequence and a different antigen binding site. Collectively called **immunoglobulins (Ig)**, they are among the most abundant protein components in the blood, constituting about 20% of the total protein components in the blood plasma. Antibodies (Ab) are present on the B-cell membrane and also are secreted by plasma cells.

Basic structure of antibody molecule

The simplest antibodies are Y-shaped molecules with two identical antigen-binding sites, one at the tip of each arm of the Y. Because of their two antigen-binding sites, they are described as *bivalent*.

Antibody has a common structure of 4 polypeptide chains. It is a heterodimer, consists of two **light (L) chains** (each containing about 220 amino acids residues, about 25000 MW) and 2 identical **heavy (H) chains** (each usually containing about 440 amino acids residues, about 50,000 MW). Each light chain is bound to heavy chain by disulfide bridges and other non-covalent linkages. Thus, antibody is a dimer of H–L chain.

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All species studied have two major classes of light chains: κ and λ. Any one individual of a species produces both types of light chain. However, in any one immunoglobulin molecule, the light chains are always either both κ both and λ, never one of each. While there are two types of light chains, the immunoglobulins of virtually all species have been shown to consist of five different types of heavy chains- α, γ, δ, ε and μ. These five different types of heavy chains are called *isotypes*. 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. Any individual of a species makes all heavy chains, but in any one antibody molecule, both heavy chains are identical. Thus an antibody molecule of the IgG class could have the structure κ2γ2 with two identical κ light chains and two identical γ heavy chains. Alternatively, it could have the structure λ2γ2 with two identical λ light chain and two identical γ heavy chains.

Immunoglobulin heavy chain isotypes

Isotype	*Heavy chain*
IgM	μ
IgD	δ
IgG	γ
IgA	α
IgE	ε

Minor differences in the amino-acid sequences of the α and the γ heavy chains led to further classification of the heavy chains into subclasses. In humans, there are two subclasses of a heavy chains (α_1 and α_2) and four subclasses of γ heavy chains (γ_1, γ_2, γ_3 and γ_4).

Both light and heavy chains have a variable sequence at their N-terminal ends but a constant sequence at their C-terminal ends. Light chains have a **constant region** (C_L) about 110 amino acids long and a **variable region** (V_L) of the same size. The variable region (V_H) of the heavy chains (at their N-terminus) is also about 110 amino acids long, but the heavy-chain constant region (C_H) is about three of four times longer (330 or 440 amino acids), depending on the class. It is the N-terminal ends of the light and heavy chains that come together to form the antigen-binding site.

The diversity in the variable regions of both light and heavy chains is for the most part restricted to three small *hypervariable regions* in each chain called **complementarity determining regions** (CDR); the remaining parts of the variable region, known as **framework regions**, are relatively constant. Only the 5-10 amino acids in each hypervariable region form the antigen-binding site.

Deduction of Ab structure

Porter and Edelman elucidated the basic structure of the Ig molecule but their approaches were different. Porter found that proteolytic treatment with the enzyme **papain** split the Ig molecule (molecular weight 150,000Da) into three fragments of about equal size. Two of these fragment were found of equal size, each of MW 45,000 called **Fab fragments** (fragment antigen binding) because they had Ag binding activity. Fab are considered to be univalent, possessing one binding site each and being in every way identical to each other. The third fragment of molecular weight 50000 called the **F_c fragment** (fragment crystallizable). It can not bind antigen.

At about the same time, Edelman discovered that when γ-globulin was extensively reduced by treatment with mercaptoethanol, the molecule fell apart into four chains: two identical light chains with a molecular weight of about 53,000Da each and two others of about 22,000 Da each. The large molecules were designated **heavy** (H) **chains** and the smaller ones, **light** (L) **chains**. On the basis of these results, the structure of immunoglobulin molecules, was proposed. This model was subsequently shown to be essentially correct, and Porter and Edelman shared the Nobel Prize for the elucidation of antibody structure.

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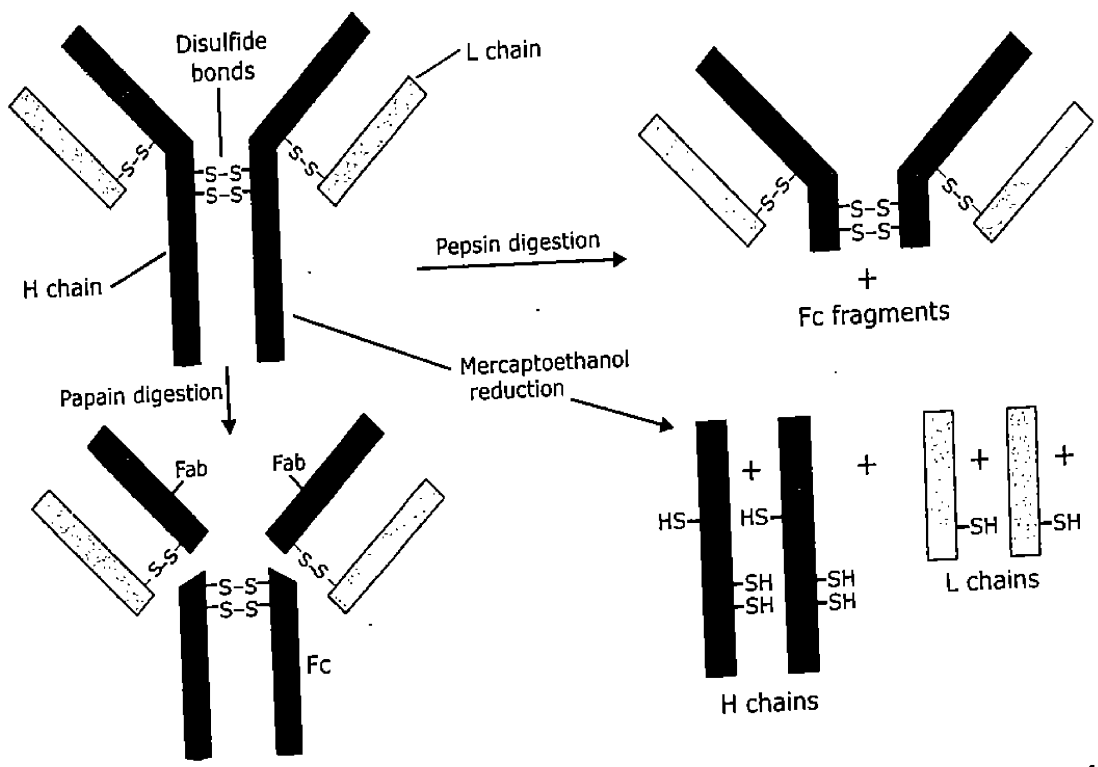


Figure 12.8 : Prototype structure of IgG, showing chain structure and interchain disulfide bonds. The fragments produced by various treatments are also indicated. Light (L) chains are depicted in gray and heavy (H) chains in black.

Another researcher, Nisonoff used **pepsin** to digest the Ig. It generated a single 100,000 MW fragment composed of two Fab-like fragments and designated $F(ab')_2$. Unlike Fab-fragments, $F(ab')_2$ fragment was able to precipitate antigens. The F_c fragment was not recovered from pepsin digestion, because it had been digested into multiple fragments.

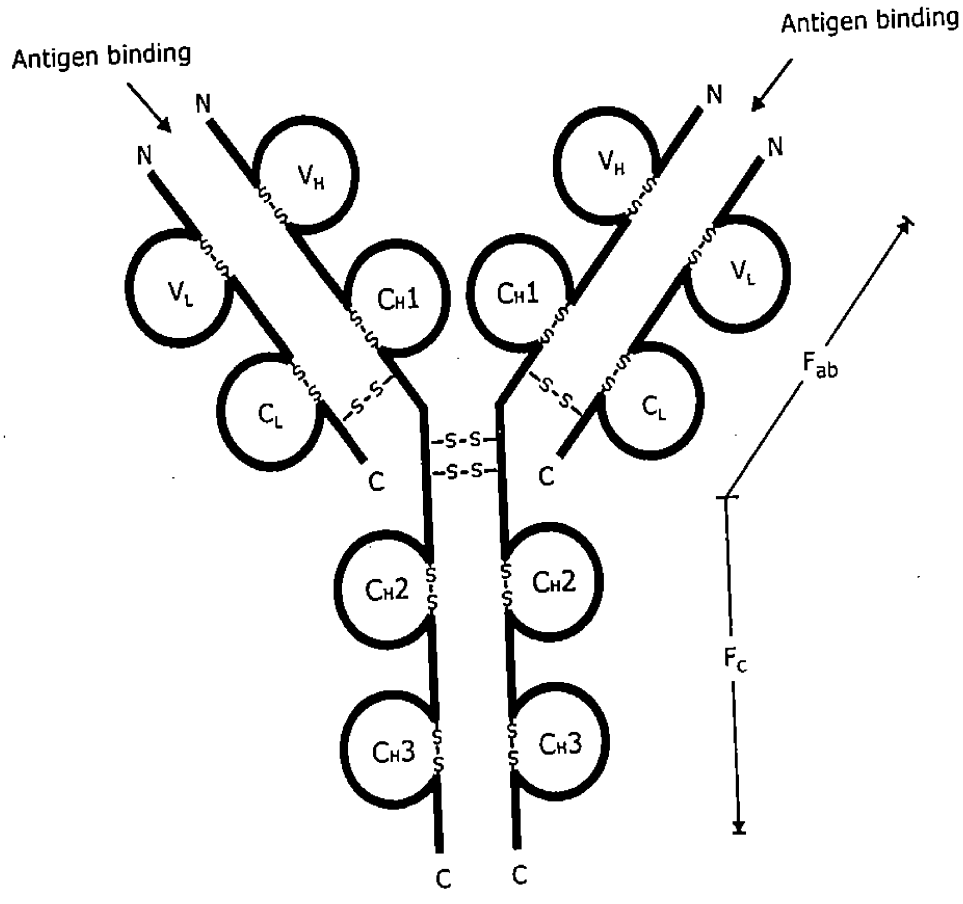


Figure 12.9 : Schematic diagram of structure of immunoglobulins

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Immunoglobulins domains

The structure of the immunoglobulin molecule is determined by the primary, secondary, tertiary and quaternary organization of the protein. The *primary structure* i.e., the amino-acid sequence, accounts for the variable and constant regions of the heavy and light chains. The *secondary structure* is formed by folding of the extended polypeptide chain back and forth upon itself into an *antiparallel β-pleated sheet*. The chains are then folded into a tertiary structure of compact globular domains, which are connected to neighboring domains by continuations of the polypeptide chain that lie outside the β-pleated sheets. Finally, the globular domains of adjacent heavy and light polypeptide chains interact in the *quaternary structure* forming functional domains that enable the molecule to specifically bind antigen and at the same time, perform a number of other biological effector functions.

Both light and heavy chains are made up of repeating segments—each about 110 amino acids long and each containing one intrachain disulfide bond. These repeating segments fold independently to form compact functional units called immunoglobulin (Ig) domains. A light chain consists of one variable (V_L) and one constant (C_L) domain. Similarly heavy chain consists of one variable (V_H) and three or four constant (C_H) domains. Most heavy chains have three constant domains (C_H1, C_H2, and C_H3), but those of IgM and IgE antibodies have four (C_H1, C_H2, C_H3 and C_H4). V_L and C_L domains of light chain pair with the variable (V_H) and first constant (C_H1) domain of the heavy chain to form the antigen-binding region.

The γ, δ and α-heavy chains contain an extended peptide sequence between the C_H1 and C_H2 domains that has no homology with the other domains. This region, called the **hinge region** is rich in proline and cysteine residues and is flexible, giving IgG, IgD and IgA flexibility between the two Fab arms of the Y-shaped antibody molecule.

Immunoglobulin can be expressed either as *secreted* immunoglobulin or as *membrane bound* immunoglobulin. The carboxyl-terminal domain in secreted immunoglobulin differs from membrane-bound immunoglobulin. Secreted immunoglobulin has a hydrophilic amino acid sequence at the carboxyl-terminal end. In membrane-bound immunoglobulin, the carboxyl terminal domain contains three regions: An extracellular hydrophilic spacer sequence, a hydrophobic transmembrane sequence and a short cytoplasmic tail.

DIFFERENT CLASSES OF IMMUNOGLOBULIN

In mammals, there are five *classes* of antibodies, IgA, IgD, IgE, IgG, and IgM, each with its own class of heavy chain–α, δ, ε, γ, and μ respectively. In addition, there are a number of subclasses of IgG and IgA immunoglobulins; for example, there are four human IgG subclasses (IgG1, IgG2, IgG3, and IgG4), having γ_1, γ_2, γ_3, and γ_4 heavy chains, respectively. Each class (and subclass) has characteristic properties of its own. Except for their variable regions, all the immunoglobulins within a class have about 90% homology in their amino acid sequences, but only 60% homology exists between classes (e.g., IgG and IgA).

IgG

IgG is the most abundant Ig in serum constituting about 80% of the total serum Ig. 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. Except for the IgG3 subclass, which has a rapid turnover, with a half-life of 7 days, the half-life of IgG is approximately 23 days, which is the longest half-life of all immunoglobulin isotypes.

The IgG isotype (except for subclass IgG2) is the only class of immunoglobulin that can pass through the placenta, enabling the mother to transfer her immunity to the fetus.

IgM

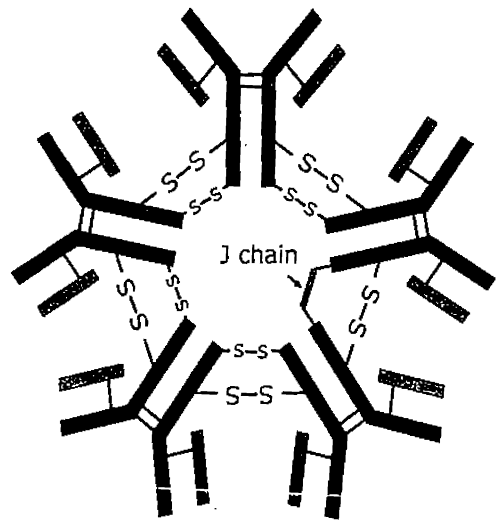
IgM is the first class of antibody made by a developing B cell. It accounts of 5%-10% of the total serum Ig. IgM is also found on the surface of mature B cells together with IgD where it serves as an antigen-specific

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BCR. 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 pentameric molecule, composed of five such units, each of which consists of two light and two heavy chains, all joined together by additional disulfide bonds between their F_c portions and by a polypeptide chain termed the **J chain**. The J chain is synthesized in the B cell or plasma cell. The half-life of the IgM molecule is approximately 5 days.

IgM is the first immunoglobulin class produced in a primary response to an antigen, and it is also the first Ig to be synthesized by the neonate. IgM is more efficient than IgG in activating complement. Complement activation requires two F_c regions in close proximity, and the pentameric structure of a single molecule of IgM fulfills this requirement.

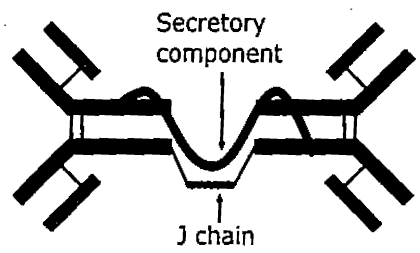


IgM (Pentamer)

IgA

IgA constitutes 10-15% of the total Ig in serum. It is the predominant Ig class in external secretions such as breast milk, saliva, tears and mucus of the bronchial, genitourinary and digestive tracts. The IgA class of immunoglobulins contains two subclasses: IgA_1 (93%) and IgA_2 (7%). Serum IgA has a half-life of 5.5 days. The IgA present in serum is predominantly monomeric. The IgA of external secretions, called *secretory IgA*, consists of a dimer or tetramer with a *J-chain* polypeptide and a polypeptide chain called *secretory component*. The daily production of secretory IgA is the maximum among other Ig.

It is the major immunoglobulin found in the **colostrum** of milk in nursing mothers, and it may provide the neonate with a major source of protection against pathogens during the first few weeks after birth.



IgA (dimer)

IgE

It mediates the immediate hypersensitivity reactions that are responsible for the symptoms of hay fever, asthma, hives and anaphylactic shock. IgE binds to F_c receptors on the membranes of blood basophils and tissue mast cells. Cross-linkage of receptor-bound IgE molecules by antigen (allergen) induces degranulation of basophils and mast cells; as a result a variety of pharmacologically active mediators giving rise to allergic manifestations. IgE mediated degranulation is necessary for anti-parasitic defense.

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IgD

It constitutes 0.2% of the total Ig in serum. IgD together with IgM, is the major membrane bound Ig expressed by mature B-cells. It is thought to function in the activation of B cells by Ag. No biological effector function has been identified for IgD.

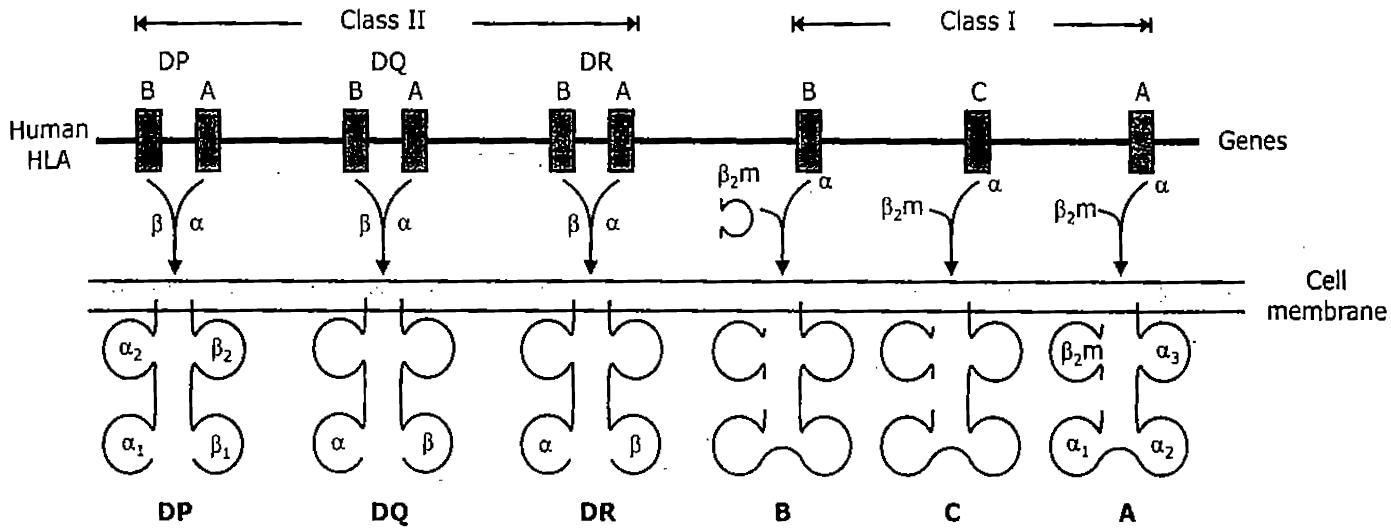


Figure 12.4 : Simplified depiction of the human MHC showing regions and genes coding for polymorphic MHC class I and II molecules. β_2m encoded outside the MHC. Adapted and modified from Immunology A short Course, R. Coico *et al.*, Wiley-Liss Publication.

STUDY QUESTION

Q5. IgG, which contains γ heavy chains, developed much more recently during evolution than IgM, which contains μ heavy chains. Describe two advantages and two disadvantages that IgG has in comparison with IgM.

Ans. Advantages of IgG compared with IgM are

1. its ability to cross the placenta and protect the developing fetus;
2. its higher serum concentration, which results in IgG antibodies binding to and neutralizing more antigen molecules and being more effective in antigen clearance;
3. its smaller size, which enables IgG to diffuse more readily into intercellular fluids.

The disadvantages of IgG compared with IgM are its lower capacity to (1) agglutinate antigens and (2) activate the complement system, both of which are due to the lower valency of IgG.

ACTION OF ANTIBODY

In addition to binding antigen, antibodies participate in a broad range of effector functions—that will result in removal of the antigens and death of the pathogens. Because these effector functions result from interactions between heavy-chain constant regions and other serum proteins or cell-membrane receptors, not all classes of antibodies have the similar functional properties. Some major antibody mediated effector functions are given below.

Opsonization

Opsonization is the process by which microorganisms or other foreign particles are coated with antibody and/or complement, and thus prepared for *recognition* and *ingestion* by phagocytic cells. Opsonizing antibodies, IgG, bind to Fc receptors on the surface of macrophages and neutrophils. This binding provides the phagocyte a method to capture antigens.

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Toxin/viral neutralization

Some Gram positive and negative bacteria produce extracellular toxins that contribute to their pathogenic effects. Immunity against such toxin depends on the production of specific antibodies that inactivate the toxins. This process is called **toxin neutralization**. Once neutralized, the toxin-antibody complex is either unable to attach to receptor sites on host target cells and is unable to enter the cell, or it is ingested by macrophages. An antibody capable of neutralizing a toxin is called **antitoxin**. Similarly, antibody like IgG, IgM, and IgA can bind to some viruses during their extracellular phase and inactivate them. This antibody mediated viral inactivation is called **viral neutralization**. Viral neutralization prevents a viral infection due to the inability of the virus to bind to and enter its target cell.

Activation of complement

Complement is a set of plasma proteins that can be activated either by binding to certain pathogens or by binding to antibody. Complement activation is often described as a series of cascading enzymatic events, leading to the generation of specific complement components that cause *opsonization* and *phagocytosis* of infectious agents as well as direct lysis of the invading organisms. IgM and most IgG subclasses can activate the complement system.

Immune complex formation

The interaction of antigen with antibodies may result in a variety of consequences, including **precipitation** and **agglutination**. Precipitation results from the interaction of a *soluble* antibody with a *soluble* antigen to form an *insoluble* complex. Antibodies that thus aggregate soluble antigens are called **precipitins**.

The reactions of antibody with a multivalent antigen that is **particulate** (i.e., an insoluble particle) results in the cross-linking of the various antigen particles by the antibodies. This cross-linking eventually results in the clumping or **agglutination** of the antigen particles by the antibodies.

Antibody dependent cell-mediated cytotoxicity (ADCC)

The NK function is to destroy malignant cells and cells infected with microorganisms. They recognize their targets with the help of binding with antibodies. They can bind to antibodies that coat infected or malignant cells; thus the antibody bridges the two cell types. This process is called *Antibody-Dependent Cell-mediated Cytotoxicity* (ADCC), and can result in the death of the target cell.

ANTIGENIC DETERMINANTS ON IMMUNOGLOBULINS

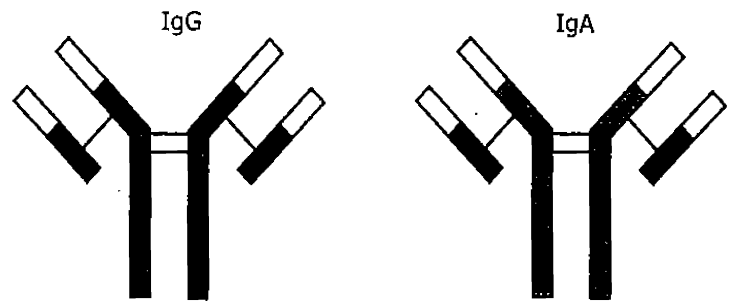
Since antibodies are glycoproteins, they can themselves function as potent immunogens to induce an antibody response. The antigenic determinants, or epitopes on Ig molecules fall into three major categories which are located in characteristic portions of the molecule.

Isotype

An antibody class is determined by the constant region sequence of the heavy chain. The five human isotypes, designated IgA, IgD, IgE, IgG, and IgM, exhibit structural and functional differences. Isotypic determinants are constant-region determinants that collectively define each heavy-chain class and sub-class within a species. Each isotype is encoded by a separate constant-region gene, and all members of a species carry the same constant-region genes. Different species inherit different constant-region genes and therefore express different isotypes. Therefore, when an antibody from one species is injected into another species, the isotype determinants will be recognized as foreign, inducing an antibody response to the isotypic determinants on the foreign antibody.

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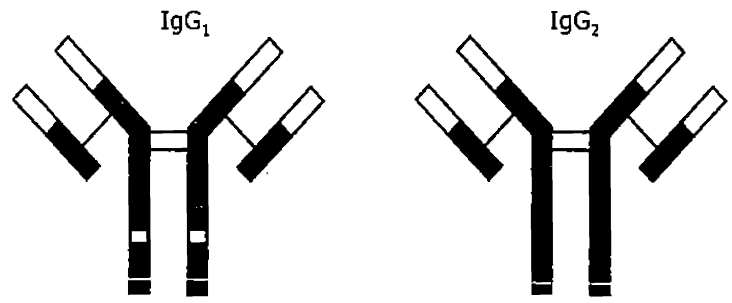
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Isotypic differences

Allotype

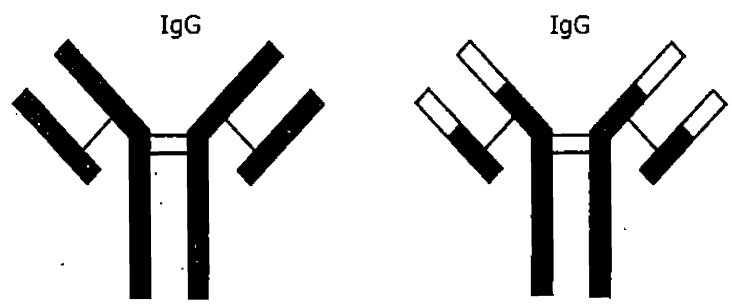
It is based on *genetic differences among individuals*. It depends on the existence of **allelic forms** of the same gene. These alleles encode minor amino-acid differences, called allotypic determinants, that occur in some but not all, members of a species. As a result of allotypy, a heavy or light chain constituent of any immunoglobulin can be present in some members of a species and absent in others. This situation contrasts with that of immunoglobulin classes or subclasses, which are present in all members of a species.



Allotypic differences

Idiotype

The unique amino-acid sequence of the variable domains of a given antibody can function not only as an antigen-binding site but also as a set of antigenic determinants. The idiotypic determinants are generated by the conformation of the heavy- and light-chain variable regions. Each individual antigenic determinant of the variable region is referred to as an *idiotype*.



Idiotypic differences

STUDY QUESTION

Q6. According to the clonal selection theory, all the immunoglobulin molecules on a single B cell have the same antigenic specificity. Explain why the presence of both IgM and IgD on the same B cell does not violate the unispecificity implied by clonal selection.

Ans. IgM and IgD differ in their constant-region domains, whereas antigenic specificity is determined by the variable-region domains. Molecules of IgM and IgD that have different C domains but identical V_H and V_L domains are found on a given B cell; thus, the cell is unispecific although it bears two isotypes.

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B-CELL MATURATION AND ACTIVATION

B-Cell maturation

B cells synthesis and maturation occur in bone marrow. Before birth, the yolk sac, fetal liver, and fetal bone marrow are the major sites of B-cell maturation; after birth, generation of mature B cells occurs in the bone marrow. B-cell development begins with the differentiation of lymphoid stem cells into *progenitor B cell* (**pro-B cell**). In the pro-B cell stage, heavy-chain gene rearrangement occurs. Upon completion of heavy-chain rearrangement, the cell is classified as a *precursor B cells* or pre-B cell. During the small pre-B cell stage, productive light-chain gene rearrangement occurs. If rearrangement is productive, the cell becomes an **immature B cell** expressing membrane IgM.

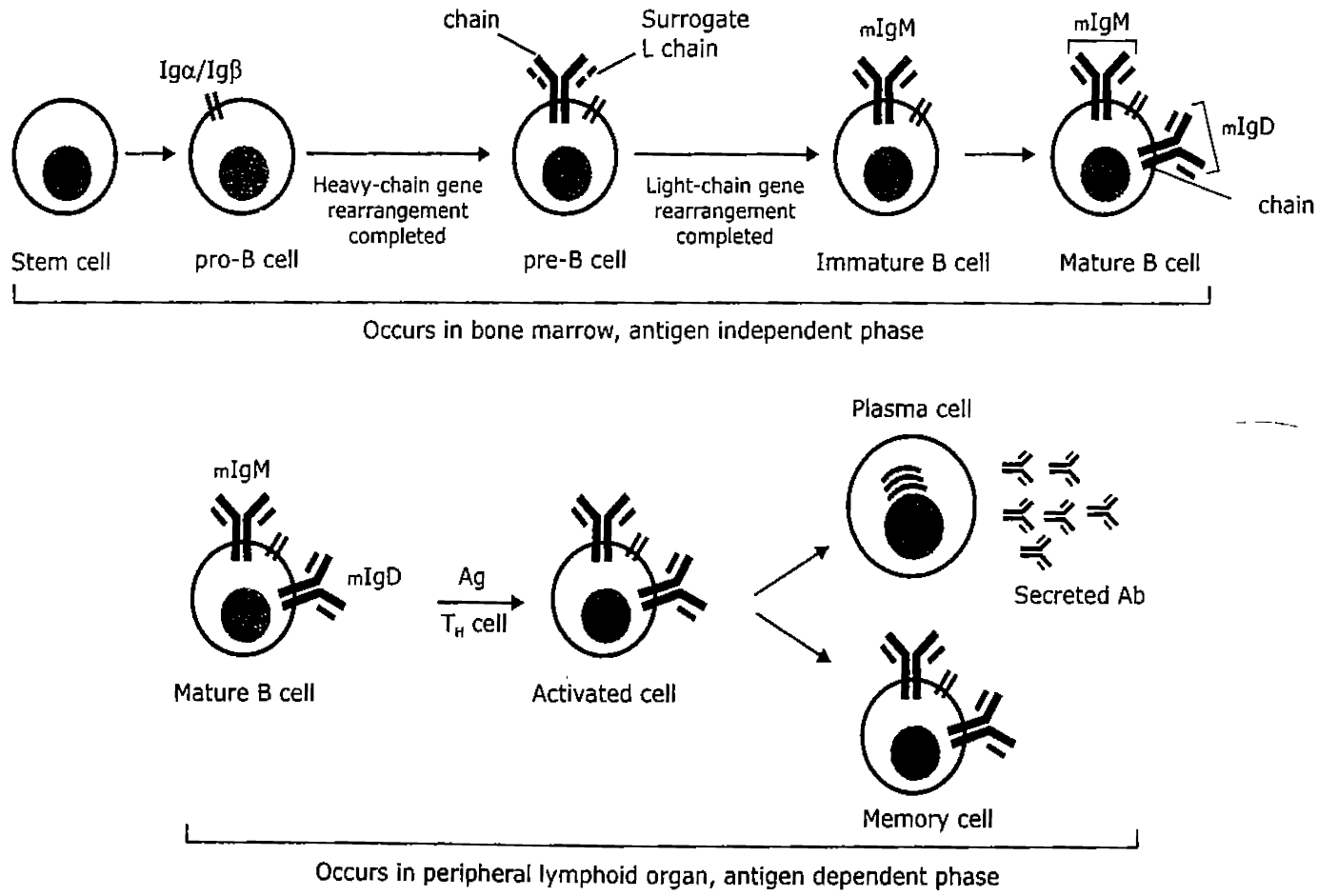


Figure 12.10 : The main stages in B cell development.

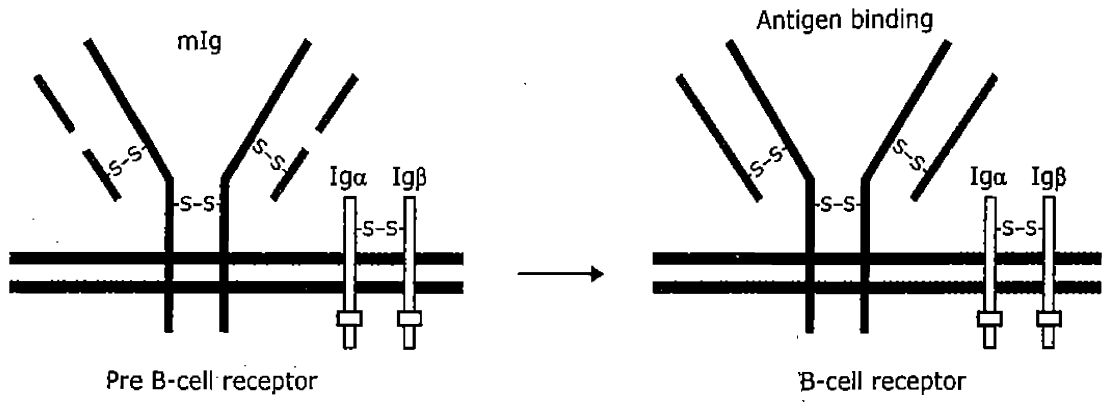
IgM on the cell surface with two closely associated transmembrane molecules Igα and Igβ, which are disulfide-linked to each other, form the B cell receptor. Further development yields mature B cells expressing both mIgM and mIgD. A mature B cell leaves the bone marrow expressing membrane- bound immunoglobulin (mIgM and mIgD) with a single antigenic specificity. These mature B cell circulate in the blood and lymph and are carried to the secondary lymphoid organs, most notably the spleen and lymph nodes. The mature B cells are activated by antigen. On contact with its appropriate antigen, the mature B cell undergoes *clonal proliferation*. Some activated B cells become long lived **memory cells**. The majority of activated B cells are transformed into **plasma cells**. Plasma cell is the antibodies secreting cell which continuously secretes antibodies at the astonishing rate of about 2000 molecules per second. A plasma cell makes an antibody of a single epitope specificity of a single Ig class and allotype and of a single light chain type only.

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B-CELL RECEPTOR

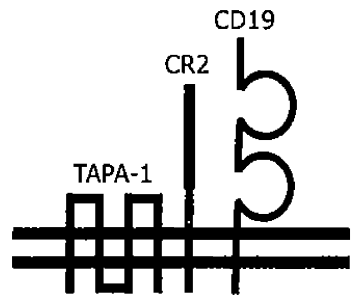
Igα and Igβ are associated with membrane Ig molecules on all cells in the B cell lineage, from the pre-B cell to the memory B cell. The complex of Igα and Igβ associated with membrane Ig molecules of more mature cells in the B lymphocyte lineage is known as the **B-cell receptor** (BCR). Igα and Igβ do not bind antigen. Their function is to transmit signals to the cell nucleus, leading to a change in the pattern of genes expressed; for this reason, Igα and Igβ are referred to as *signal transduction* molecules.



Pre B-cell receptor

B-cell receptor

Other molecules on the B cell membrane like **CD19, CD81** (also known as TAPA-1) and **CD21** (CR2) are associated in a complex, known as the **B cell co-receptor**, also affect the signal that is transmitted through the BCR.



B-cell coreceptor

CDs (Cluster of *D*ifferentiation molecules) are cell surface proteins present on lymphocytes (both B and T-cell) and other cells of immune system. To date, over 300 CDs have been characterized. CDs have both biological and diagnostic significance. The presence of various CDs on cell surface can be used to determine the cells identity.

B cells also express surface proteins known as **MHC class II** molecules. Unlike the expression pattern of many other cells types, B cell expression of MHC class II molecules is **constitutive**–that is, the molecules are always expressed. Activated B cells also express high levels of a family of molecules known as **B7** (**CD80/CD86**), which are referred to as **co-stimulatory molecules**, because they are required in addition to antigen to activate naïve T cells. Resting mature B cells express low levels of B7 and are poor APCs, whereas activated B cells are very efficient APCs.

B-CELL ACTIVATION

B cells activation require antigen. Depending on the nature of the antigen, B-cell activation proceeds by two different pathways – T_H cells dependent and T_H cells independent.

In *T_H cells dependent pathway*, T_H cell and B-cell cooperate in presence of **thymus-dependent antigens**. Thus the B-cell response to thymus-dependent antigens requires direct contact with T_H cells, not simply exposure to T_H-derived cytokines. In *T_H cells independent pathway*, antigens called **thymus-independent antigens**,

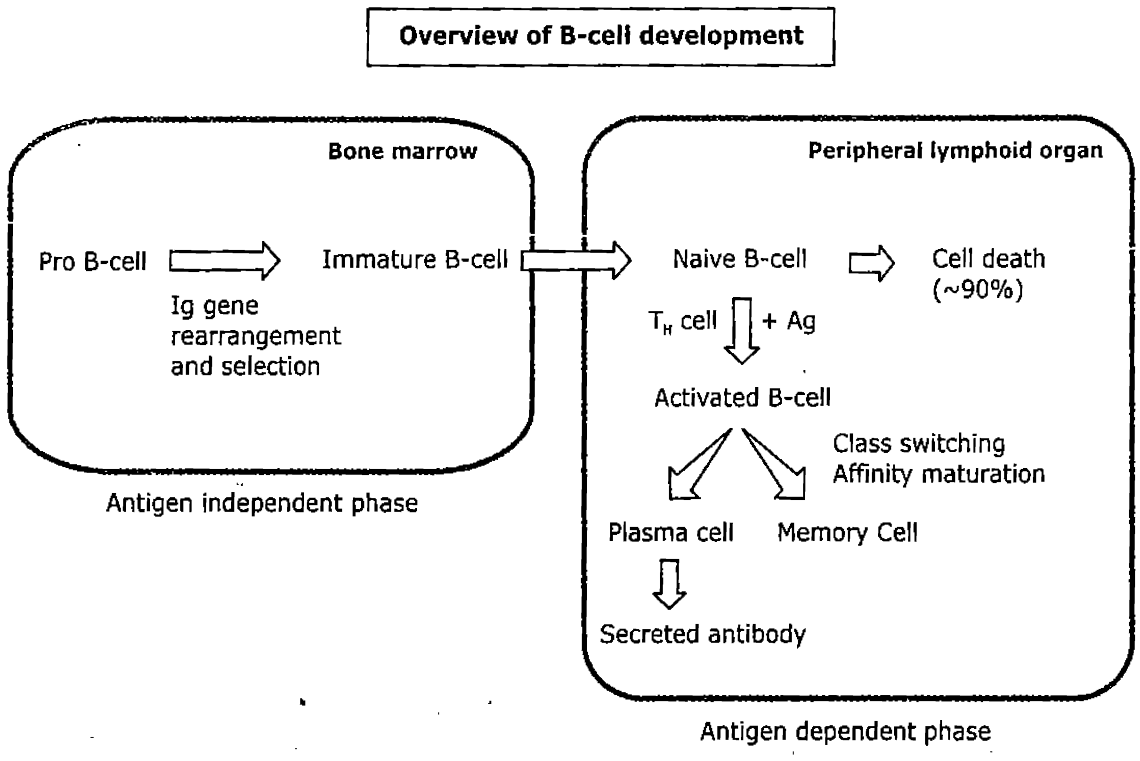
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activate B-cells without direct participation of T_H cells. Thymus-independent antigens are generally large polymeric molecules with multiple, repeating, antigenic determinants; for example, the components of bacterial cell membrane such as lipopolysaccharides and the capsule polysaccharide components of *Haemophilus influenzae*. The humoral response to thymus-independent antigens is different from the response to thymus-dependent antigens. The response to thymus-dependent antigens is stronger and involves synthesis of memory B cells, affinity maturation and class switching to other isotypes. Responses to thymus-independent antigens generate primarily IgM and do not give rise to memory.

Table 12.5 : Properties of thymus *dependent* and thymus *independent* antigen

Property	**Thymus dependent antigen**	**Thymus independent antigen**
Chemical nature	Soluble protein	LPS, polymeric protein, capsular polysaccharide
Isotype switching	Yes	No/Limited
Affinity maturation	Yes	No
Immunogenic memory	Yes	No



Summary of properties of B-cells

Origin	—	Bone marrow
Maturation	—	Bone marrow (Bursa of Fabricious in bird)
Expression of Ag receptor	—	Bone marrow
Differentiation	—	In lymphoid tissue
Surface immunoglobulin	—	Present
Immunity	—	Humoral
Distribution	—	Spleen, Lymph nodes, Bone marrow and other lymphoid tissue
Secretory product	—	Ab
Complement receptors	—	Present

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12.8 KINETICS OF THE ANTIBODY RESPONSE

Humoral immunity is mediated by serum antibodies which are the proteins secreted by the B cell compartment of the immune response. B cells are initially activated to secrete antibodies after the binding of antigens to specific membrane **immunoglobulin** molecules (B cells receptors), which are expressed by these cells. Once bind, the B cell receives signals to begin making the secreted form of this immunoglobulin, a process that initiates the full-blown antibody response whose purpose is to eliminate the antigen from the host. Antibodies are a heterogeneous mixture of serum globulins, all of which share the ability to bind individually to specific antigens.

Primary and secondary responses

The first exposure of an individual to an immunogen is referred to as the primary immunization, which generates a **primary response**. The primary antibody response may be divided into several phases, as follows:

1. *Lag or latent phase* : It is the immediate stage following antigenic stimulus during which no antibody is detectable in circulation. The length of this period is generally one to two weeks.
2. *Log or exponential phase* : In this phase there is steady rise in the titer of antibody and the concentration of antibody in the serum increases exponentially.
3. *Plateau or steady state* : During this phase there is an equilibrium between antibody synthesis and degradation.
4. *Declining phase :* The concentration of antibody in serum declines rapidly.

A second exposure to the same immunogen results in a **secondary response**. This second exposure may occur after the response to the first immune event has leveled off or has totally subsided. The secondary response is also called the *memory* or *anamnestic* response and the B and T lymphocytes that participate the memory response are termed **memory cells**.

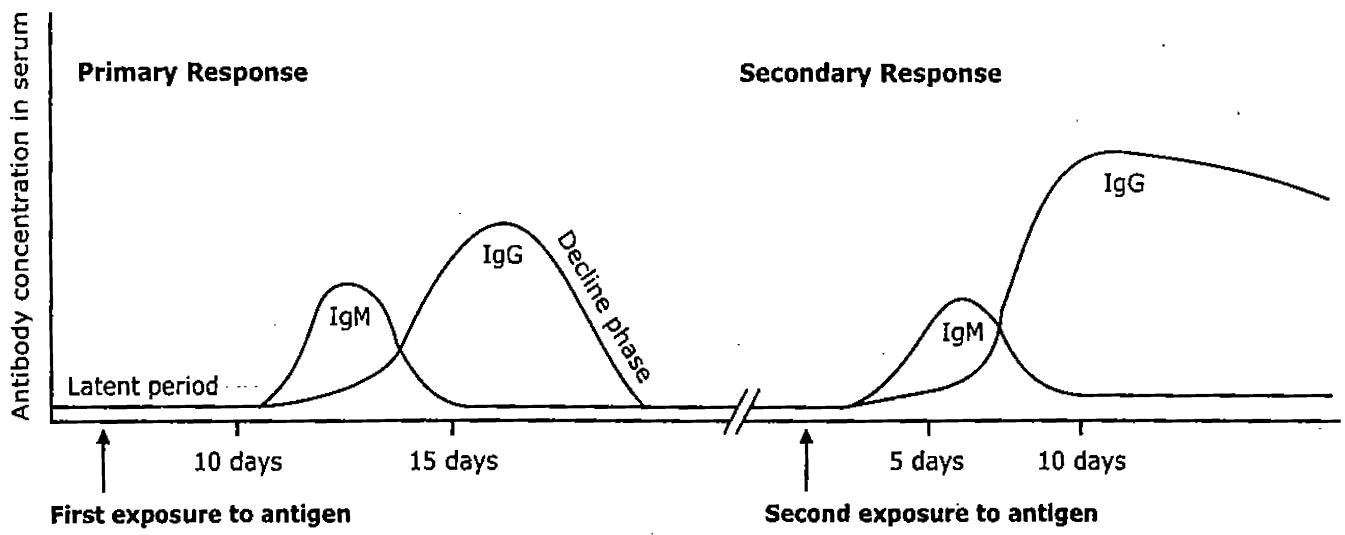


Figure 12.11 : Antibody production and kinetics

The primary response is slow, sluggish and short lived with a long lag phase and low titre of antibodies that does not persist for long. However the secondary response is prompt, powerful and prolonged, with a short or negligible lag phase and a much higher level of Ab that lasts for long periods.

In the primary response, the first class of antibody detected is generally IgM, then IgG, or another antibody class. There is a marked change in the type and quality of antibody produced in the secondary response. There is a shift in class response, known as **class switching**, with IgG antibodies appearing at higher concentrations and with greater persistence than IgM, which may be greatly reduced or disappear altogether. This may be also accompanied by the appearance of IgA and IgE.

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With the passage of time after immunization, there is usually a progressive increase in the affinity of the antibodies produced against the immunizing antigen. This phenomenon, known as **affinity maturation,** is due to the accumulation of point mutations specifically in both heavy-chain and light-chain V-region coding sequences.

Table 12.6 : Features of primary and secondary antibody responses

Feature	**Primary response**	**Secondary response**
Time lag after immunization	Usually 5–10 days	Usually 1–3 days
Peak response	Smaller	Larger
Antibody isotype	Usually IgM > IgG	Relative increase in IgG
Antibody affinity	Lower	Higher
Induced by	All immunogens	Only protein antigens
Required immunization	Relatively high doses of antigens, optimally with adjuvants	Low doses of antigens; adjuvants may not be necessary
Responding B-cell	Naive B-cell	Memory B-cell
Antigens	Thymus-dependent and thymus-independent	Thymus-dependent

MONOCLONAL ANTIBODIES (MAB)

A single antibody forming cell or clone produces antibody specifically directed against a single Ag or antigenic determinant only. However, antibodies produced ordinarily by infection or immunization are **polyclonal** because natural Ag have multiple epitopes, each of which generates clones of lymphocytes. This results in antisera containing immunoglobins of different classes with specificities against different epitopes of the antigens. On the other hand, when a clone of lymphocytes or plasma cells undergoes selective proliferation, as in multiple myeloma, antibodies with a single Ag specificity accumulate. Such antibodies produced by a single clone and directed against a single Ag determinant are called **monoclonal** antibodies.

Difference between *polyclonal* and *monoclonal* antibodies:

Polyclonal

- Contains many antibodies recognizing many determinants on an antigen.
- Various classes of antibodies are present (IgG, IgM and so on).

Monoclonal

- Contains a single antibody recognizing only a single determinant.
- Single class of antibody produced.

Monoclonal antibodies are very useful tools for diagnostic and research techniques. This techniques developed by Kohler and Milstein in 1975. This is called **Hybridoma technology**. Hybridomas are somatic cell hybrids produced by fusing antibodies forming spleen cells with myeloma cells. Antibody-producing B lymphocytes normally die after several weeks in cell culture *in vitro*. Therefore, antibody-producing B lymphocytes are fused with B-cell tumors called *myelomas*. These myelomas are capable of dividing indefinitely and are therefore often called immortal cell lines. The immortal cell lines that result from the B cell-myeloma fusion are hybrid cell lines called *hybridomas*. The hybridoma cell lines share the properties of both fusion partners. They grow indefinitely *in vitro* and produce antibodies. To produce a monoclonal antibody, a mouse is immunized with the antigen of interest. During the next several weeks, antigen-specific B cells proliferate and begin producing antibodies in the mouse. Spleen tissue, rich in B-lymphocytes, is then removed from the mouse, and the B cells are fused with myeloma cells.

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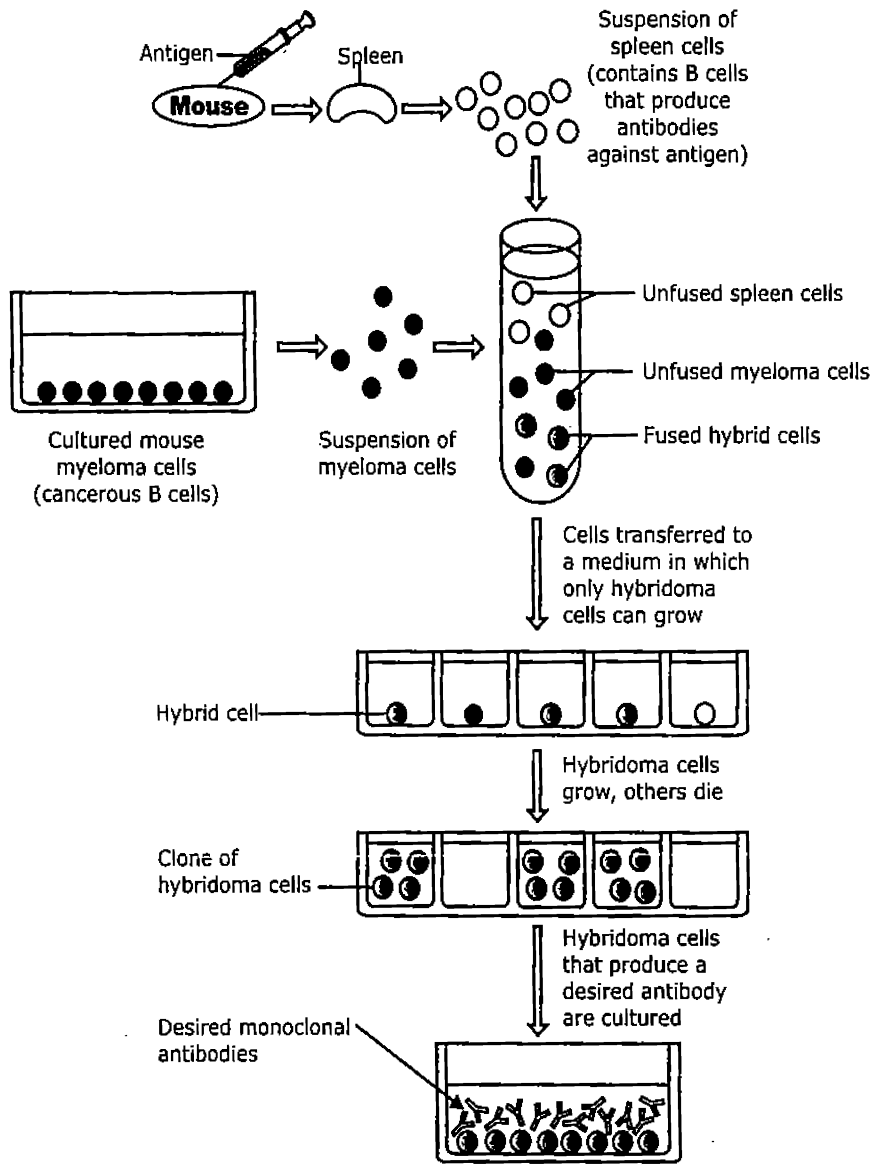


Figure 12.12 : Production of monoclonal antibodies

Hybridomas are selected by use of a selective medium in which the myeloma cells die, but hybridomas survive. The most widely used selective system involves the inclusion of the antibiotic aminopterin in growth medium. This inhibits the *de novo* nucleotide synthesis pathway. However, normal cells survive in this medium as they are able to use the *salvage pathway* for nucleic acid synthesis. But if cells are unable to produce the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), they are unable to utilize the salvage pathway and therefore die in aminopterin-containing medium.

In the procedure, myeloma cells are engineered to be deficient enzyme HGPRT. After fusion of lymphocytes with HGPRT-negative myeloma cells, aminopterin-containing medium, supplemented with hypoxanthine and thymidine to ensure and adequate supply of substrates for the salvage pathway (HAT medium) is added, which kills myeloma cells but allows hybridomas to survive as they inherit HGPRT from the lymphocyte parent. Unfused lymphocytes die after a short period of culture, which results in a pure preparation of hybridomas.

REVIEW THE CONCEPT

Q2. a. Explain the rationale for inducing aminoprotein in selective media during establishment of hybrids in hybridoma technology.

b. Briefly describe the screening method for selection of hybrids. **[CSIR-UGC]**

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CLONAL SELECTION THEORY

The clonal selection theory is a central paradigm of adaptive immunity. The most remarkable feature of the adaptive immune system is that it can respond to millions of different foreign antigens in a highly specific way. B cells, for example, make antibodies that react specifically with the antigen that induced their production. The clonal selection theory (formulated by Sir Macfarlane Burnet) explain how the adaptive immune system can respond to millions of different antigens in a highly specific way.

According to this theory, an animal first randomly generates a vast diversity of lymphocytes, and then those lymphocytes that can react against the foreign antigens that the animal actually encounters are specifically selected for action. As each lymphocyte develops in a central lymphoid organ, it becomes committed to react with a particular antigen before ever being exposed to the antigen. It expresses this commitment in the form of cell-surface receptor proteins that specifically fit the antigen.

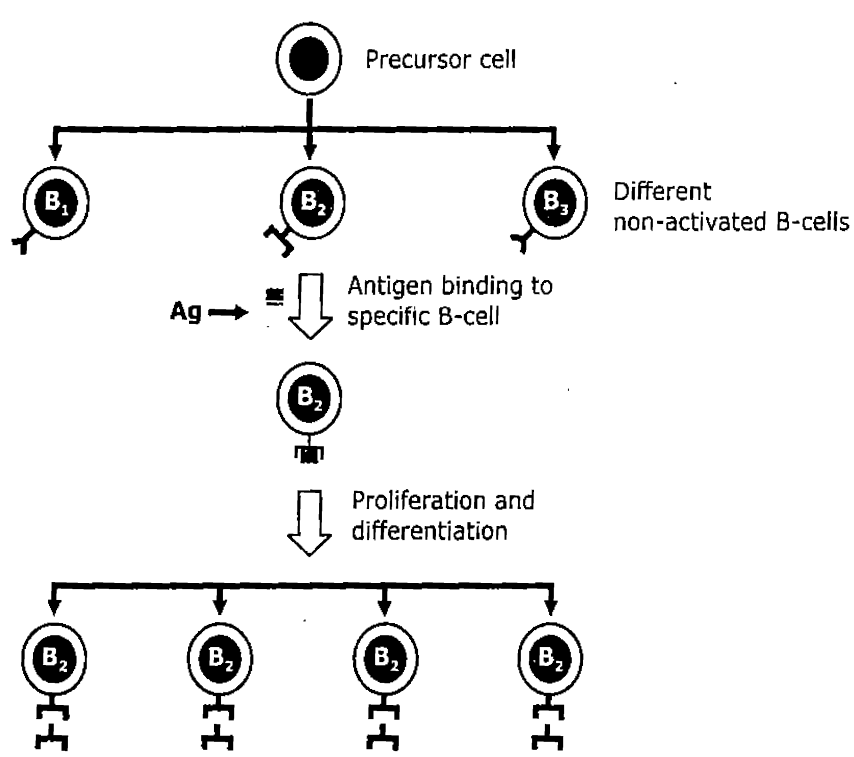


Figure 12.13 : The clonal selection theory of B-cells leading to antibody production.
Adapted from Molecular Biology of the cell,Albert et al., Garland Science.

When a lymphocyte encounters its antigen in a peripheral lymphoid organ, the binding of the antigen to the receptors activates the lymphocyte, causing it both to proliferate and to differentiate into an effector cell. An antigen therefore selectively stimulates those cells that express complementary antigen-specific receptors and are thus already committed to respond to it. This arrangement is what makes adaptive immune responses antigen-specific. According to the clonal selection theory, then, the immune system functions on the *ready-made* principle rather than the *made-to-order* one.

The term "clonal" in clonal selection theory derives from the postulate that the adaptive immune system is composed of millions of different families, or clones, of lymphocytes, each consisting to T or B cells descended from a common ancestor. Each ancestral cell was already committed to make one particular antigen-specific receptor protein, and so all cells in a clone have the same antigen specificity.

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12.9 ORGANIZATION AND EXPRESSION OF IMMUNOGLOBULIN GENES

One of the most remarkable features of the vertebrate immune system is its ability to respond to an apparently limitless array of foreign antigens. The genetic basis of such wide variation coupled with constancy in a single protein molecule lies in the organization of the Ig genes. In germ-line DNA, multiple gene segments encode portions of a single Ig heavy or light chain. These gene segments are carried in the germ cells but cannot be transcribed and translated into heavy and light chains until they are rearranged into functional genes. During B-cell maturation in the bone marrow, some of these gene segments are randomly shuffled by a dynamic genetic system capable of generating more than 10^{10} combinations.

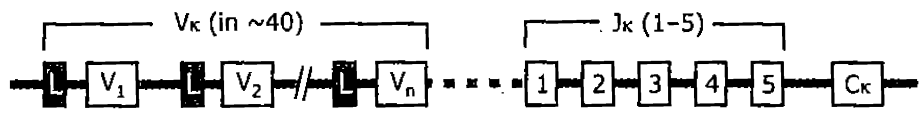
An Ig is a tetramer of two light chains and two heavy chains. Each chain has an N-terminal variable region (V) and a C-terminal constant region (C). V domains and C domains are separately coded by V gene segments and C gene segments. But V and C gene segment is not expressed as an independent unit. To construct a unit that can be expressed in the form of a light or heavy chain, a V gene segment must be joined physically to a C gene segment.

Germ line gene organization of light chain genes

Each κ and λ L-chain polypeptide consists of two major domains, a variable region and a constant region (V_L and C_L). V_L is coded for by two separate gene segments: a **variable (V) segment**, which codes for the amino-terminal residues, and a small **joining (J) segment**, coding for about 13 residues at the carboxy-terminal end of the variable region.

Kappa (κ) light chain gene

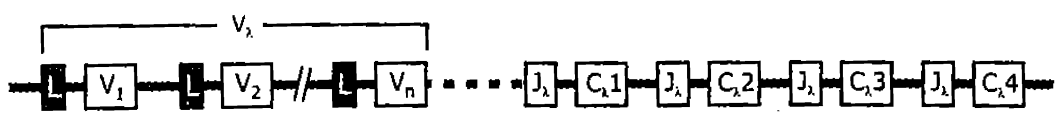
The κ locus is found on chromosome 2 in human. There are approximately 40 different V_κ genes, each of which can code for the N-terminal amino acids sequence of a κ variable region. These V_κ genes are arranged linearly, each with its own L (leader) sequence and all separated by introns. A series of 5 J_κ gene segments present between V_κ genes and C_κ gene. Each J_κ gene segment can encode the remaining amino acid residues of the κ variable region. A long intron separates the C_κ gene segment—the gene coding for the single constant region of the κ chain.



Kappa light chain genes

Lambda (λ) light chain gene

The λ locus is found on chromosome 22 in human. It is composed of 4 C region genes, one for each subtype of lambda chain, and approximately 30 V region genes. These Vκ genes are arranged linearly, each with its own L (leader) sequence and all separated by introns.



Lambda light chain genes

V-J rearrangements in κ chain synthesis

One V gene and one J gene are brought together in the genome to create a gene unit that, together with the C region gene, codes for an entire Ig L chain. This unique gene rearrangement mechanism—referred to as **V(D)J recombination** (D gene segments are discussed with H chain genes).

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To make a κ chain, an early cell in the B lymphocyte lineage selects one of the V_κ genes from its DNA and physically joins it to one of the J_κ segment. *How this selection of V and J genes is made is not known but is probably a random process.* Variable-region gene arrangements occur in an ordered sequence during B-cell maturation in the bone marrow. Rearranged κ genes contain the following regions in order from the 5′ to 3′ end : L exon, intron, VJ gene segment, intron and the constant region.

Rearrangement is mediated by an enzyme complex called the **V(D)J recombinase**. This complex contains two proteins that are specific to developing lymphocytes, as well as enzymes that help repair damaged DNA in all our cells. The lymphocyte-specific proteins of the V(D)J recombinase are encoded by two closely linked genes called *rag-1* and *rag-2* (rag = recombination activating genes). The products of these two genes, RAG-1 and RAG-2 express exclusively in lymphocytes.

After a cell in the B cell lineage rearranges its DNA, it makes a primary RNA transcript. This transcript is then spliced to remove all intervening noncoding sequences, bringing the V_κ, J_κ and C_κ exons together in a mature mRNA. The mRNA is translated into the κ polypeptide chain and at the cell's rough endoplasmic reticulum, the leader sequence is cleaved off. In the lumen of the endoplasmic reticulum, it joins with a newly synthesized H chain to form an Ig molecule. *The synthesis of λ chains is similar in principle to the synthesis of κ chains.*

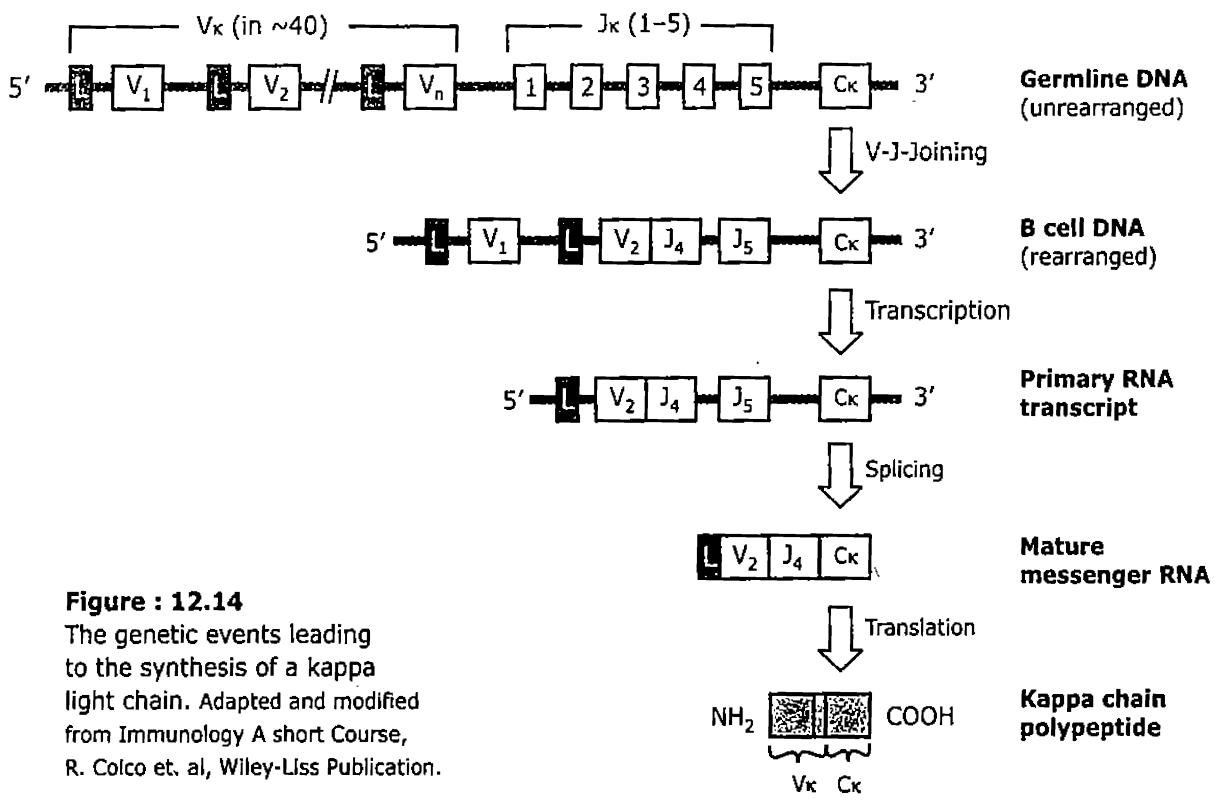


Figure : 12.14
The genetic events leading to the synthesis of a kappa light chain. Adapted and modified from Immunology A short Course, R. Colco et. al, Wiley-Liss Publication.

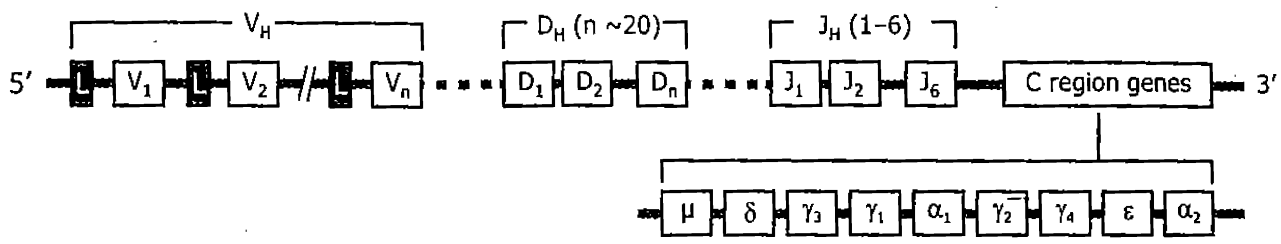
Germ line gene organization of the heavy chain genes

In contrast to the variable region of a light chain that is constructed from two gene segments, the variable region of a heavy chain is constructed from three gene segments V_H, D_H and J_H. Thus, in addition to V and J segments, genes coding for the variable region of a H chain also use a **diversity** (D) **segment**. The human H chain locus present on chromosome 14 includes approximately 50 V_H genes, about 20 D_H gene segments, and 6 J_H gene segments.

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The second key feature of the H chain genes is the presence in the germ line of multiple genes coding for the C region of the Ig, one for each class and subclass of Ig. The C region determines the class and hence nature of the particular antibody.



V-D-J rearrangement in heavy-chain DNA

Heavy chain synthesis uses the same mechanisms of rearrangement described for light chains. In the early stages of the life of a particular B cell, two rearrangements of germ line DNA must occur.

The first brings one D segment alongside one J segment. The second brings one V segment next to the DJ unit fixing the nature of the H chain. Rearranged heavy chain genes consist of the following sequences, starting from the 5′ end : L exon, intron, VDJ segment, intron and a series of C gene segment. As shown in the figure 12.15, the rearranged DNA is then transcribed along with the closest C region genes μ and δ. This primary transcript can be spliced in two different ways (**alternative splicing**) to yield either a VDJ-μ or a VDJ-δ mRNA.

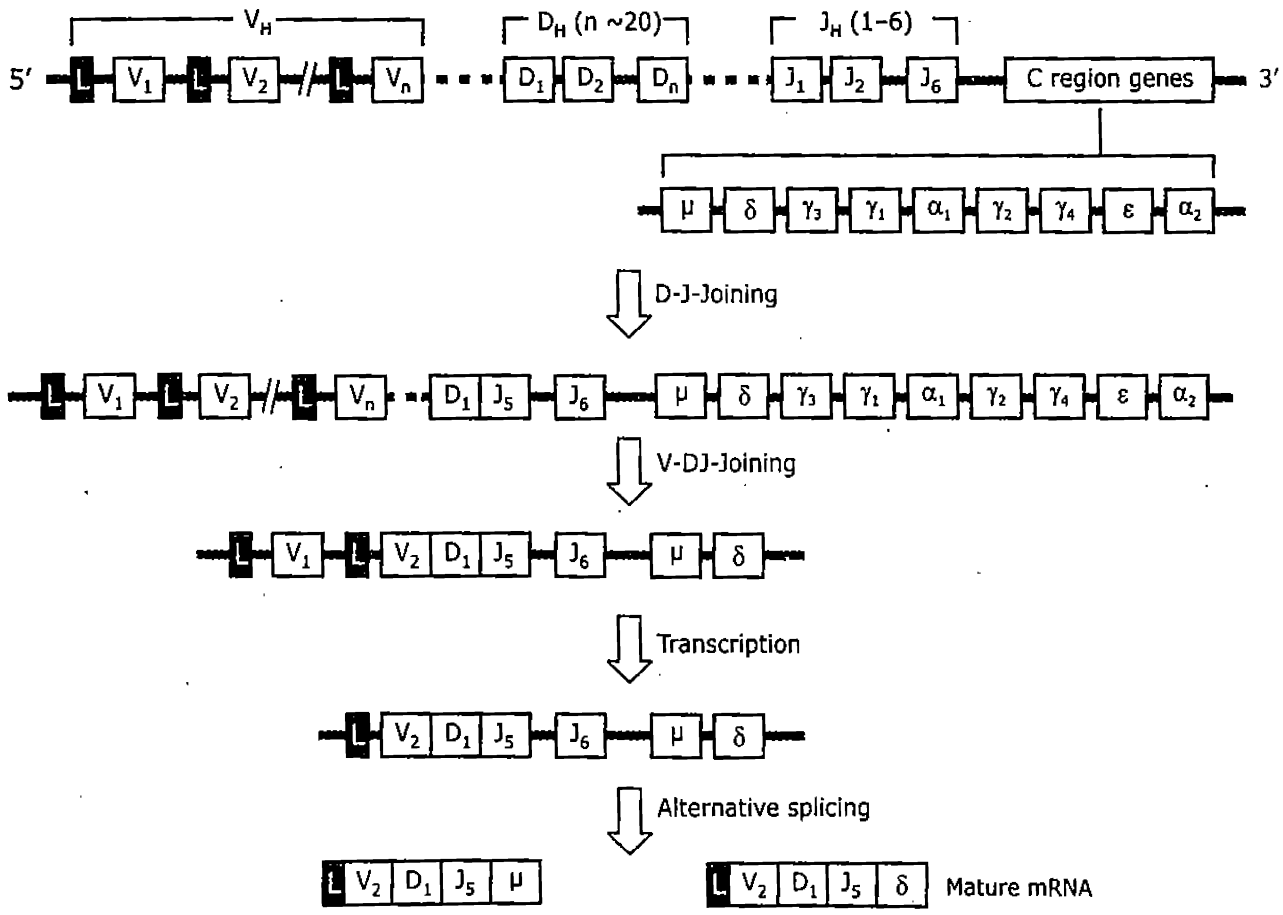


Figure 12.15 : The genetic events leading to the synthesis of a human heavy chain.
Adapted and modified from Immunology A short Course, R. Coico et. al, Wiley-Liss Publication.

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Mechanism of DNA rearrangements

Flanking the V, J and D exons there are unique sequences referred to as *recombination signal sequences* (RSS), which function in recombination. Each RSS consists of a conserved nonamer and a conserved heptamer that are separated by either 12 or 23 base pairs as illustrated in figure 12.16. The 12bp and 23 bp spaces correspond to one or two turns of the DNA helix.

Recombination only occurs between a 1 turn and a 2 turn signal. In the case of the lambda light chain there is a 1 turn signal upstream of the J exon and a 2 turn signal downstream of V_λ. In the case of the kappa light chain there is a 1 turn signal downstream of the V_κ gene and a 2 turn signal upstream of the J exon. In case of the heavy chains there are 1 turn signals on each side of the D exon and a 2 turn signal downstream of the V gene and a 2 turn signal upstream of the J exon. Thus, this ensures that the correct recombination events will occur.

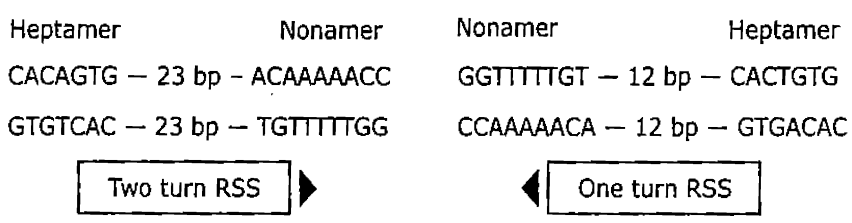
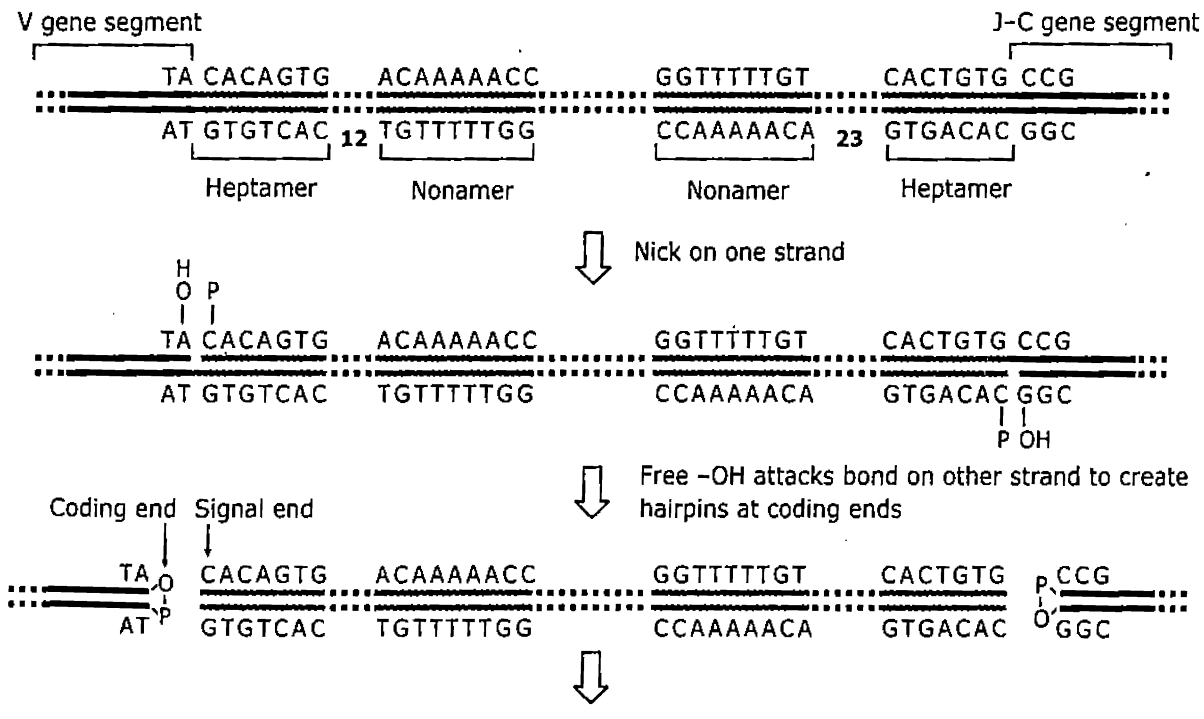


Figure 12.16 : Two conserved sequences in light-chain and heavy-chain DNA function as recombination signal sequences (RSSs). Both signal sequences consist of a conserved palindromic heptamer and conserved AT-rich nonamer. These are separated by nonconserved spacers of 12 or 23 bp.

Recombination occurs by double-strand breaks at the heptamers of two consensus sequences that lie at the ends of the coding units. In case of light chain gene this releases the entire fragment between the V gene segment and J-C gene segment; the cleaved termini of this fragment are called *signal ends*. The cleaved termini of the V and J-C loci are called *coding ends*. The two coding ends are covalently linked to form a *coding joint*. The RAG proteins catalyze the cleavage reaction. RAG1 recognizes the nonamer consensus sequences for recombination. RAG2 binds to RAG1 and cleaves at the heptamer. The reactions involved in recombination are shown in figure 12.17.



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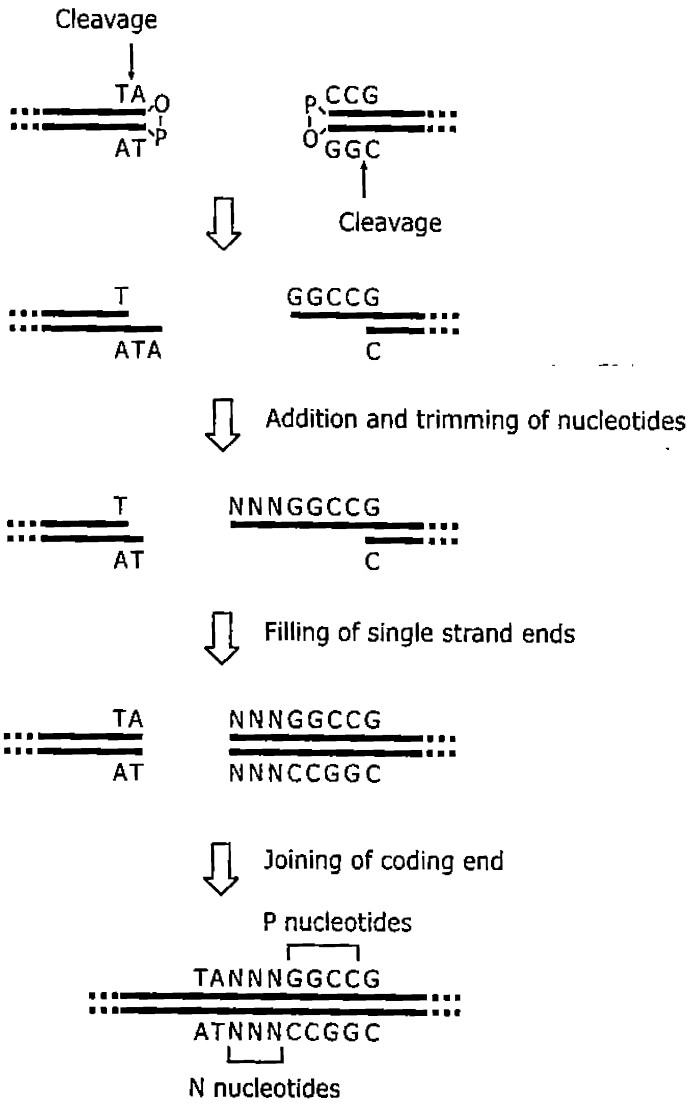


Figure 12.17 : Processing of coding ends introduces variability at the junction. The complex nicks one strand at each junction. The nick has 3′–OH and 5′–P ends. The free 3′–OH end then attacks the phosphate bond at the corresponding position in the complementary strand of the duplex. This creates a hairpin at the coding end, in which the 3′ end of one strand is covalently linked to the 5′ end of the other strand; it leaves a blunt double-strand break at the signal end. This second cleavage is a transesterification reaction. Next a single-strand break is introduced into one strand close to the hairpin. This generates a single-stranded protrusion. Incorporation of complementary nucleotides to the exposed single strand then converts the coding end to an extended duplex. This reaction explains the introduction of *P nucleotides* at coding ends. Some nucleotides are added randomly further. They are called *N nucleotides*. Their insertion is catalyzed by the enzyme deoxynucleoside transferase. Finally through the mechanism of non-homologous end-joining (NHEJ) the coding ends joins together. *Adapted from genes IX by Benjamin Lewin.*

STUDY QUESTION

Q7. Explain why V_H segment cannot join directly with a J_H segment in heavy-chain gene rearrangement.

Ans. V_H and J_H gene segments cannot join because both are flanked by recombination single sequences (RSSs) containing a 23-bp (2-turn) spacer. According to the one-turn/two-turn joining rule, signal sequences having a two-turn spacer can join only with signal sequences having a one-turn (12-bp) spacer.

Productive or Nonproductive rearrangements of Ig gene

The recombination of V-(D)-J gene segments results in a *productive rearrangement* if all the rearranged gene segments are in the correct reading frame and leads to expression of an active protein. A *nonproductive rearrangement* occurs when the rearranged gene segments are not in the correct reading frame and fails to generate a gene that codes for a functional protein. A productive rearrangement prevents any further rearrangement from occurring, but a nonproductive rearrangement does not.

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12.10 GENERATION OF ANTIBODY DIVERSITY

One unique property of antibodies is their remarkable diversity. How can be adaptive immune system produce lymphocytes that collectively display such an enormous diversity of receptors, including ones that recognize synthetic molecules that never occur in nature? There are several means of antibody diversification identified in mice and humans.

Combinatorial V(D)J joining

Random association of any V gene segment with any J gene segment can occur to form an L chain variable region, and similarly, any V can associate with any D or J gene segments in H chain gene rearrangement. As there are about 40 V_κ and 5J_κ genes coding for the κ chain variable region, assuming random association then 40×5 or 200κ chains can be formed.

Junctional and insertional diversification

Diversity generated by means of combinatorial V(D)J joining it further augmented by a phenomenon called *junctional diversification*. In most cases of site-specific recombination, DNA joining is precise. But during the joining of antibody (and T cell receptor) gene segments, a variable number of nucleotides are often lost from the ends of the recombining gene segments, and one or more randomly chosen nucleotides may also be inserted. This random loss and gain of nucleotides at joining sites is called **junctional diversification**, and it enormously increases the diversity of V-region coding sequences created by recombination.

Somatic Hypermutation

The V regions of germ-line DNA are susceptible to a high rate of somatic mutation during B-cell development in response to an antigen challenge. This phenomenon is referred to as **somatic hypermutation** because it occurs at the rate of at least 10,000-fold higher than the normal rate of mutation. The molecular mechanism of this process is still uncertain. However, these mutations allow B-cell clones to produce antibodies with somewhat different polypeptide sequences.

Combinatorial joining of H and L chains

In addition to V(D)J combinatorial association, any H chain may associate with any L chain.

ALLELIC EXCLUSION

B cell have two sets of chromosomes, one set from each parent, so theoretically, Ig genes located on both chromosomes could synthesize Ig molecules. This does not occur. In contrast to almost all other gene products, which are derived from genes from both parental chromosomes a B-cell expresses the rearranged genes for H and L chain from only one chromosome i.e., either from the maternal or the paternal chromosome. For example, the H chain may be coded for by genes on the paternal chromosome and the L chain (either κ or λ) by genes on the maternal chromosome. This phenomenon of using genes from only one parental chromosome is known as **allelic exclusion**.

The steps in rearrangement, allelic exclusion, and hence the synthesis of a complete Ig molecule are very tightly controlled, although all the controlling mechanisms are not yet completely clear. This mechanism of allelic exclusion ensures that every B cell and the antibody it synthesizes are **monospecific**–that is, are specific for only one epitope. It ensures that functional B cells never contain more than one $V_HD_HJ_H$ and one V_LJ_L unit. In this way, an individual B cell is prevented from forming and expressing Ig molecules with different antigenic specificities on its cell surface.

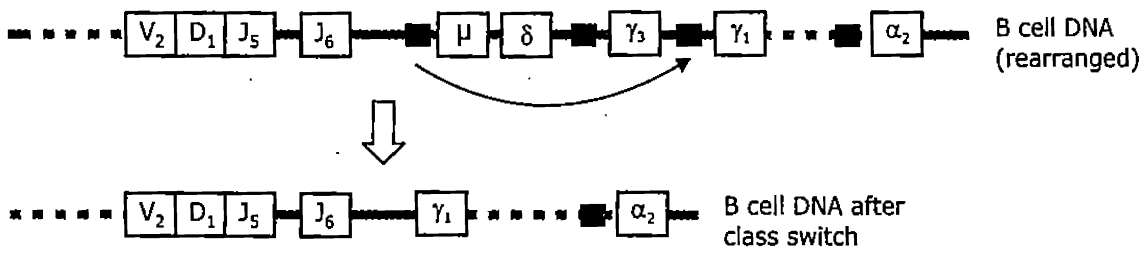
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CLASS SWITCHING

All lymphocytes start productive life as immature cells engaged in synthesis of IgM. A lymphocyte generally produce only a single class of immunoglobulin at any one time, but the class may change during the cell lineage. A change in expression is called **class switching** or **isotype switching**. Switching involves only the C_H gene segment; the same V_H gene segment continues to be expressed. Thus a given V_H gene segment may be expressed successively in combination with more than one C_H gene segment.

Class switching occurs in antigen-stimulated mature B cells synthesizing IgM and IgD and involves further DNA rearrangement. In addition to antigen, class switching is dependent on the presence of factors known as cytokines released by T cells. There is little or no class switching by B cells in the absence of such T cell-derived cytokines. Class switching involves DNA recombination events. But this system is different from the V(D)J joining. Switching involves sequence lie upstream of the C_H gene called **switch (S) regions**. An S region is an intron sequence. This S region permits any of the C_H genes to associate with the VDJ unit.

Recombination between switch regions (S) results in deletion of the material between the recombining S sites. In the following figure due to switching expression of C_μ is succeeded by expression of Cγ1. The Cγ1 gene segment is brought into the expressed position by recombination between the sites S_μ and Sγ1. The S_μ site lies between V-D-J and the C_μ gene segment. The Sγ1 site lies upstream of the Cγ1 gene segment. The DNA sequence between the two switch sites is excised as a circular molecule. *Once a class switch has been made, it becomes impossible to express any C_H gene segment that used to reside between C_μ and the new C_H gene segment.* In this example cells expressing Cγ1 will be unable to give rise to cells expressing Cγ3, which has been deleted.



12.11 T CELLS AND CMI (CELL MEDIATED IMMUNITY)

Ig molecules secreted from B cells, play a critical role in interacting with antigens when they are present *outside* cells; for example, when viruses are encountered in blood plasma or at mucosal surfaces. Once an antigen gets into a cell however, antibodies do not generally have access to it, and so antibodies are ineffective in dealing with antigens *Inside* cells. T cells deal with pathogens – such as viruses, bacteria, and parasites – that takes place inside cells of the host. T cells responses differ from B cell responses in at least two crucial ways.

First, T cells are activated by foreign antigen to proliferate and differentiate into effector cells only when the antigen is displayed on the surface of antigen-presenting cells/target cells in peripheral lymphoid organs.

The *second* difference is that, once activated, effector T cells act only at short range, either within a secondary lymphoid organ or after they have migrated into a site of infection. They interact directly with another cell in the body, which they either kill or signal in some way.

T-cell receptor

T-cells, like B cells, express on antigen specific receptor. The T-cell receptor (TCR) is a heterodimer and composed of two transmembrane glycoprotein chains, α and β. The extracellular portion of each chain

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consists of two domains, resembling immunoglobulin variable (V) and constant (C) domains, respectively. Both chains are glycosylated and connected with each other with the help of interchain disulfide bond. The trans-membrane helices of both chains are unusual in containing positively charged (basic) amino acid residues within the hydrophobic transmembrane segment. The α-chains carry two such residues; the β-chains have one.

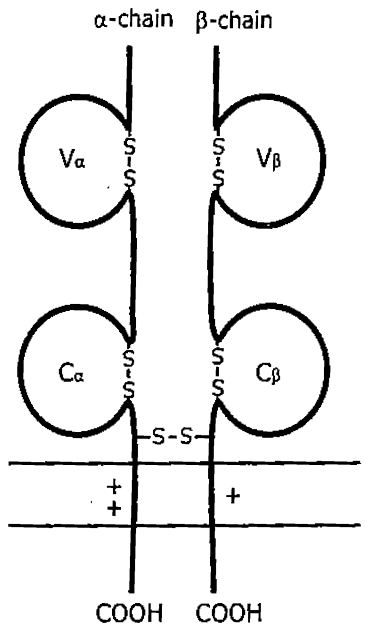


Figure : 12.18
The predominant form of the antigen-binding chains of TCR.

γδ T cells

Some T cells express a TCR distinct from αβ. This alternative TCR is known as γδ, and the cells expressing this receptor are referred to as γδ T-cells. In normal adult humans, γδ T-cells are found at much lower numbers than αβ cells, but their numbers are increases as a result of infections. αβ-expressing T-cells interact with the peptide antigens processed and presented on the surface of APCs/target cells. Whereas γδ T-cells react with antigen that is neither processed nor presented by a MHC molecules.

Table 12.7 : Comparison of αβ and γδ T-cells

Feature	**αβ T-cells**	**γδ T-cells**
$CD4^+$	Present	Absent
$CD8^+$	Present	Present
MHC restriction	$CD4^+$: MHC class II	No MHC restriction
	$CD8^+$: MHC class I	
Antigen	Peptide	Phospholipid/glycolipid

Comparison between TCR and mIg

The features of the TCR and Ig differ in several important ways :

- The TCR is a two-chain structure that forms a single binding site for antigen that is, the TCR is *monovalent*. By contrast, Ig is a four chain molecule with two antigen-binding sites (bivalent).
- Ig binds to many different types of antigens (carbohydrates, lipids, and proteins) that it encounters in fluids, such as serum. The TCR interacts with small fragments of proteins (peptides) that are presented on the surface of a APC/target cell. These peptides, generated by enzymatic degradation of the protein inside cells and presented by antigen presenting proteins, MHC. Thus the TCR interacts with a peptide bound to an MHC molecule on the surface of a APC/target cell.

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- In contrast to the variety of structures and shapes recognized by Ig molecules, the antigen recognized by the TCR is a combination of MHC molecule and a small linear sequence of peptide.
- Unlike Ig, the TCR does not exist in a specifically secreted form and is not secreted as a consequence of T cell activation. T cell activation results in cytokines secretion and/or the killing of infected host cells. By contrast after antigen binds to membrane Ig and activates the B cell, the B cell differentiates into a plasma cell that secretes Ig with the same antigenic specificity expressed by the B cell that initially bound antigen.

Co-receptor molecules

The TCR is expressed on the T cell surface in association with other transmembrane molecule, referred to as a **co-receptor**. This coreceptor can be one of two molecules on the mature T cell: either **CD4** or the two chain molecule **CD8** (both members of the Ig superfamily).

The extracellular portions of CD4 and CD8 bind to MHC molecules on the surface of a cell that presents antigen to T cells. CD4 binds selectively to the subset of MHC molecules known as *MHC class II* and CD8 binds selectively to the subset of MHC molecules known as *MHC class I*.

CD4 and CD8 are involved in signal transduction after antigen binding to the TCR. Specifically, the intracellular portions of CD4 and CD8 are linked to enzymes, known as protein tyrosine kinases, which are important components of the T cell activation pathways.

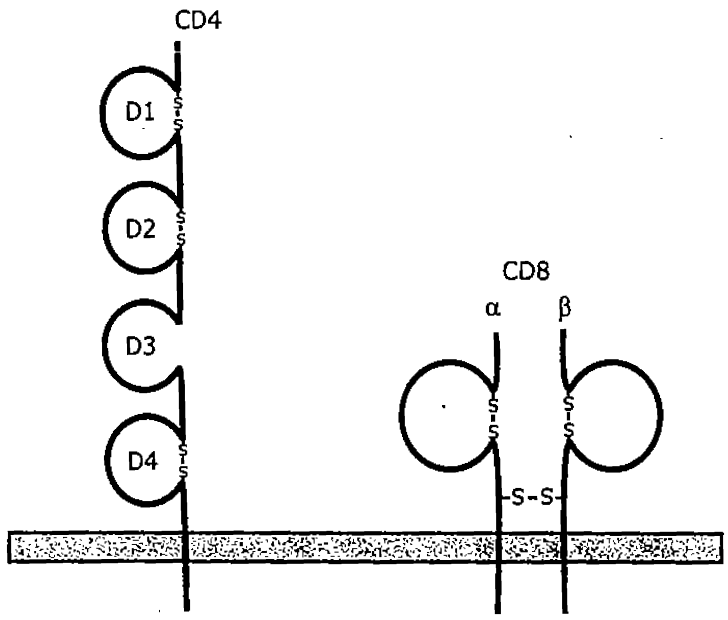


Figure : 12.19
General structure of the CD4 and CD8 co-receptors. CD8 may take the form of an αβ heterodimer, or an αα homodimer.

T cell receptor complex : TCR-CD3

The antigen-recognizing α and β chains of the TCR are also expressed on the surface of T-cells in tight, noncovalent association with CD3 molecule. The combination of the α and β chains of the TCR and CD3 is referred to as the **T cell receptor complex**, analogous to the B cell receptor complex.

CD3 is a complex of five polypeptide chains that associate to form three dimers : heterodimers of γε, δε and a homodimer ξξ or heterodimer ξη. CD3 acts as a *chaperone* and helps in transporting the newly synthesized TCR molecule through the cell to the cell surface. CD3 is invariant–that is, it is the same on all T cells–and because it is expressed exclusively on T cells it can be used as a marker to distinguish T cells from all other cells. CD3 do not bind antigen. They are signal transduction molecules activated after antigen binding to the TCR.

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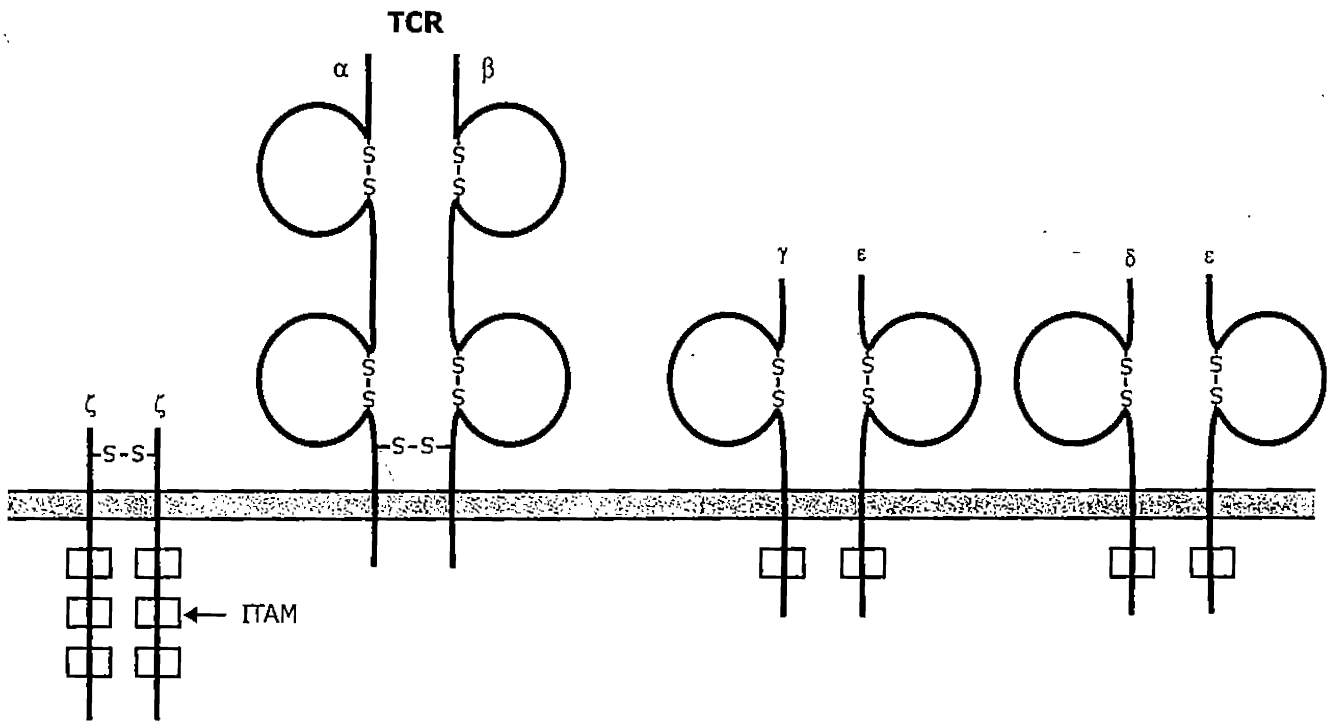


Figure 12.20 : Schematic diagram of the TCR-CD3 complex, which constitutes the T-cell antigen-binding receptor. The CD3 complex consists of the ξξ homodimer (or ξη heterodimer) plus γε and δε heterodimers. The long cytoplasmic tails of the CD3 chains contain a common sequence, the immunoreceptor tyrosine-based activation motif (ITAM), which functions is signal transduction. Adapted and modified from Immunology A short Course, R. Coico et. al, Wiley-Liss Publication.

Generation of T cell receptor diversity

The mechanisms for generating diversity in T cell receptors are very similar to the mechanisms of generating diversity in B cell receptors. The same fundamental principal of gene rearrangement, as described for Ig, apply in synthesizing the V and C regions of each chain of the T cell receptor α, β, γ and δ. Thus, TCR diversity is generated by *combinatorial joining* of chains and *junctional and insertional diversification*. However, there is one important difference between diversity generation in TCRs and Ig molecules. Ig but not the TCR undergoes somatic hypermutation following antigenic stimulation.

T-CELL MATURATION AND ACTIVATION

Progenitor T cells from the early sites of hematopoiesis begin to migrate of the thymus. In a manner similar to B-cell maturation in the bone marrow, T-cell maturation occurs in the thymus. In the thymus, developing T cells, known as *thymocytes*, proliferate and differentiate along developmental pathways that generate functionally distinct subpopulations of mature T cells.

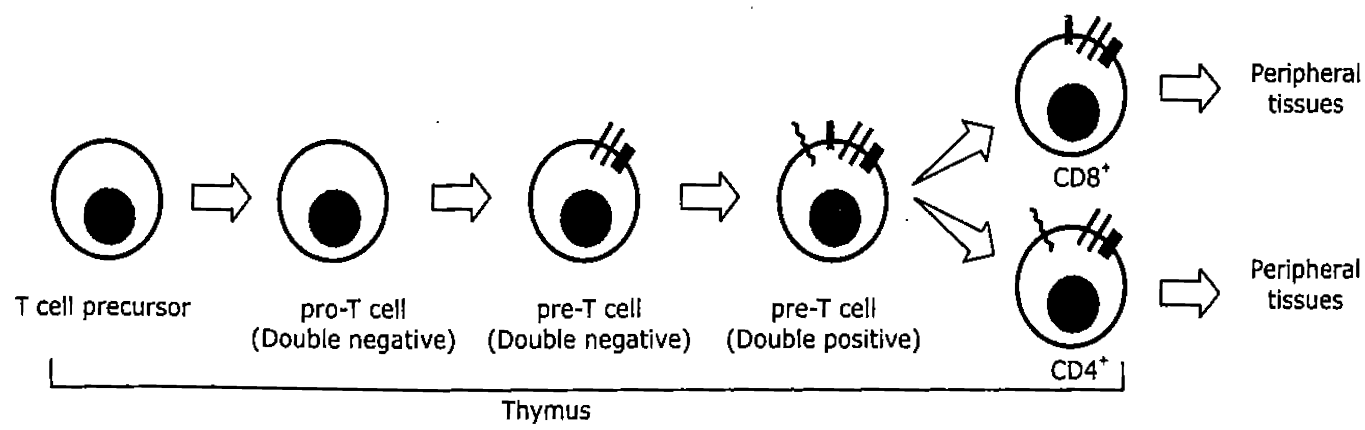


Figure 12.21 : Development of T cells. T cell precursors arrive at the thymus from bone marrow via the bloodstream, undergo development to mature T cells, and are exported to the periphery where they can undergo antigen-induced activation and differentiation into effector cells and memory cells.

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Thus thymus is absolutely required for the differentiation of immature precursor cells into cells with the characteristics of T cells. The adverse effects of lacking a thymus and consequently not having mature T cells can be seen in children born without a thymus (**DiGeorge syndrome**) or in mice genetically lacking a thymus (known as **nude mice** because they also lack hair). T cell differentiation in the thymus is a complex multistep process.

Thymic selection

In the thymus, Pro T-cell in the early state of development lack detectable CD4 and CD8 co-receptors. Because these cells are $CD4^-$ $CD8^-$ they are referred to as *double negative cells*. During the developmental process most double negative Pro T-cells develop into $CD4^+$ $CD8^+$ pre T-cells, referred to as *double positive cells*. The double positive pre T-cell undergoes a multistep process known as **thymic selection**. Thymocytes pre T cells undergo two selection processes in the thymus - **positive** and **negative selection.**

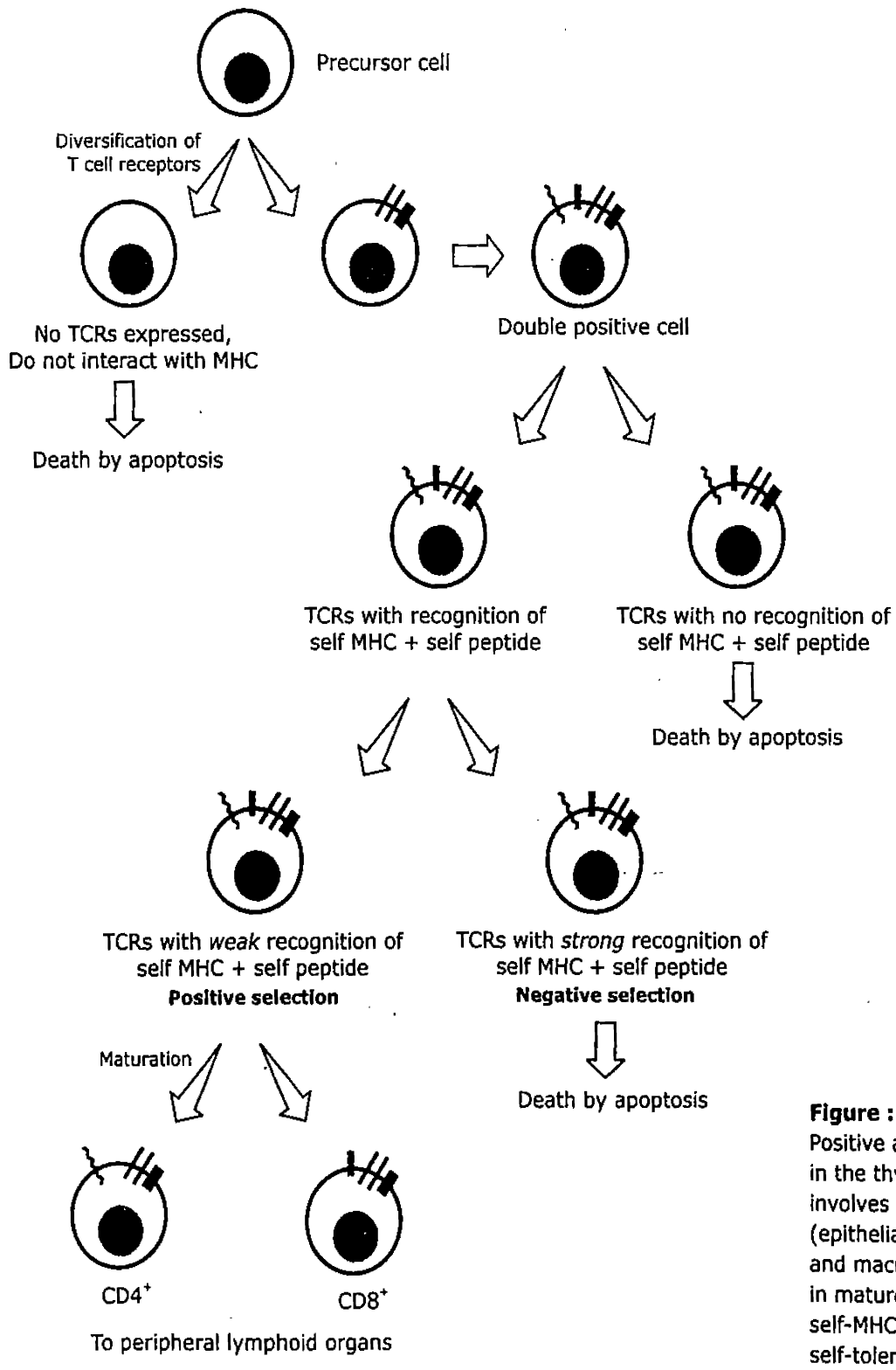


Figure : 12.22
Positive and negative selection in the thymus. Thymic selection involves thymic stromal cells (epithelial cells, dendritic cells, and macrophages), and results in mature T cells that are both self-MHC-restricted and self-tolerant.

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In case of **positive selection**, the TCR of the double positive thymocyte interacts with MHC molecules expressed on epithelial cells in the thymic cortex. This interaction results in the survival and differentiation of the double positive cells; double positive cells that do not make this critical interaction, and are thus not selected, die by apoptosis.

Thus, in positive selection developing αβ T cell becomes *educated* to the MHC molecules expressed by the thymic cortical epithelial cells. This means that for the rest of the life of the T cell, even as a mature cell when it leaves the thymus, it will respond to antigen only when the antigen is bound to the MHC molecules that the developing T cell encountered in the thymus. For this reason, the MHC molecules expressed in a person's thymus and that educate his or her developing T-cells are referred to as **self-MHC;** for that person, all other types of MHC molecules are **non-self**. This is the origin of the phenomenon known as **MHC restriction**, or more specifically, **self-MHC restriction**.

T cells expressing TCRs specific for both foreign and self antigens can develop in the thymus and survive positive selection. Allowing T-cells with strong reactivity to self components to leave the thymus and interact with these antigens in tissues could result in undesirable autoimmune response. To prevent this from occurring, the double positive cell undergoes a second selection step, known as **negative selection**.

Negative selection taking place when double positive cells interact with dendritic cells. Because dendritic cells have peptides associated with MHC molecules, the double positive cells likely interact with MHC and peptide expressed on the dendritic cell surface. A T-cell expressing a TCR that reacts with too high an affinity to the combination of MHC and peptide is deleted by apoptosis. Thus negative selection removes T-cells expressing TCRs with high reactivity to self components and result in **self-tolerance**.

Current evidence indicates that peptides expressed by cortical epithelial cells play a critical role in the positive selection step. These peptides are derived from self antigens either expressed in the thymus or brought in the thymus. It is not currently clear, however, how these peptides derived from self antigens select T cells with TCR specificities for non-self as well as self antigens. In addition, it is not clear whether the peptides expressed by the cortical epithelial cells in positive selection differ from those expressed by the dendritic cell in negative selection.

Double positive cells that survive negative selection downregulate expression of either CD4 or CD8 by a mechanism that is currently not well understood. This results in the development of either $CD4^+CD8^-$ or $CD4^-CD8^+$ (single positive) T cells.

Function of T cells

If a naïve T cell (T cells that have not yet encountered antigen) recognizes an antigen-MHC complex on APC or target cell, it will be activated, initiating a primary response. APC provides two kinds of signals for activation.

Signal 1 is provided by a foreign peptide bound to an MHC protein on the surface of the presenting cell. This peptide-MHC complex signals through the T cell receptor and its associated proteins.

Signal 2 is provided by costimulatory proteins, especially the **B7 proteins** (CD80 and CD86), which are recognized by the co-receptor protein **CD28** on the surface of the T cell. The expression of B7 proteins on an antigen-presenting cell is induced by pathogens during the innate response to an infection.

The naïve T cell enlarges into a *blast cell* and undergo repeated rounds of cell division. It generates a large clone of progeny cells, which differentiate into memory and effector T-cell populations. The effector T cells carry out specialized functions such as cytokine secretion, B-cell activation and cytotoxic killing activity.

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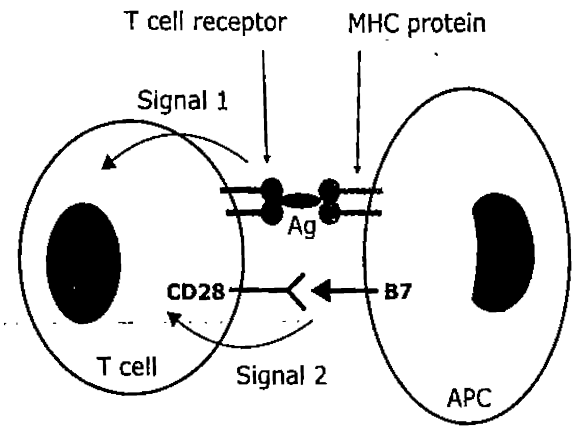


Figure 12.23 : Two signals that activate a helper T cell.

Function of T helper cells

Effector T helper cells can be divided into two subsets (T_H1 and T_H2) based on the different cytokines they produce. T_H1 cells synthesize and secrete IL-2, IFNγ, and TNFβ which is responsible for delayed-type hypersensitivity and the activation of cytotoxic T lymphocytes, NK cells and macrophages. T_H2 cells synthesize IL-4, IL-5, IL-10, and IL-13 which functions more effectively as a helper for B-cell activation..

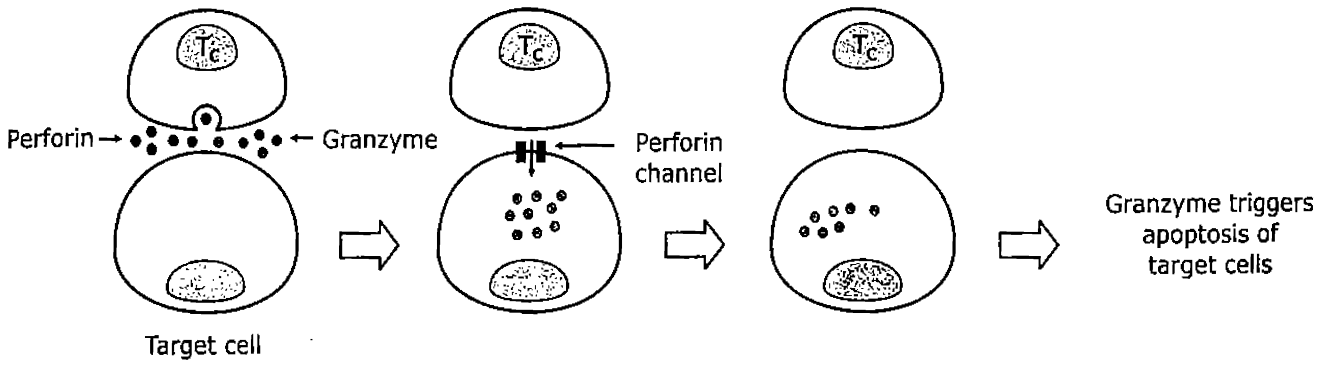
Function of cytotoxic T lymphocytes

The principal function of cytotoxic T lymphocytes (CTLs) is to kill cells that have been infected by bacteria and viruses. Cytotoxic T lymphocytes are also involved in killing of tumor cells and transplanted foreign cells during graft rejection. For this reason, cytotoxic T lymphocytes are frequently referred to as **killer T cell** . The cell killed by a CTL is known as a **target**, which can be a specialized antigen-presenting cell such as a dendritic cell, or any other cell in the body. The TCR of a CTL recognizes a combination of peptide in association with an MHC class I molecule on the surface of a cell. This interaction, in the presence of appropriate second signals results in the death of the cell presenting the peptide. CTL also synthesize cytokines; like IFNγ, which regulates certain viral and bacterial infections, as well as TNFβ, which plays a role in target cell killing.

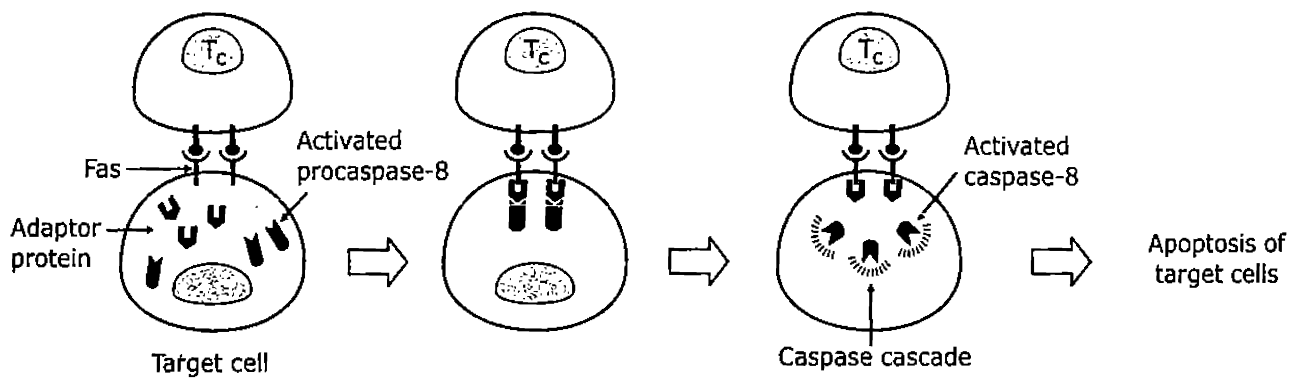
CTL killing of target cells

Once activated mature CTL initiates killing by attaching to the target cell. Killing by CTL is thought to occur by two pathways. The *first* predominant pathway involves the action of the cytotoxic substances contained in granules inside the T cell. Antigen-specific CTLs interact with appropriate target cells and undergo conjugate formation. Formation of a CTL–target cell conjugate is a Ca^{2+}-dependent, energy-requiring step. After attaching to the target cell, the CTL mobilizes its granules directionally toward the target and releases the contents of these granules onto the target cell. The major constituents of the granules involved in target-cell killing are **perforin** and **granzymes**. Perforin is a molecule that polymerizes to form transmembrane channels or pores in the target-cell membrane. The action of perforin on cell membranes is similar to the complement membrane attack complex, described later. CTL killing via this pathway also involves granzymes, a set of *serine proteases*. Granzymes pass into the target cell through the pores and interact with intracellular components of the target cell to induce apoptosis.

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A *second* pathway of target cell killing occurs via the interaction of the molecule **Fas ligand** on the CTL with **Fas**, a surface molecule expressed on many host cells. This interaction activates the apoptosis of the target cell. The cytosolic tail of Fas contains a death domain, which, when activated, binds to an adaptor protein, which in turn recruits a specific procaspase (procaspase-8). Clustered procaspase-8 molecules then cleave one another to produce active caspase-8 molecules that initiate the proteolytic caspase cascade leading to apoptosis.



Summary of properties of T-cells

Origin	—	Bone Marrow
Maturation	—	Thymus
Expression of Ag receptor	—	Thymus
Differentiation	—	Lymphoid tissue
Surface Ig	—	Absent
Immunity	—	Cell mediated as well as humoral
Secretory Product	—	Cytokines
Receptor	—	TCR on membrane

Immunological tolerance

Adaptive immune system *learns* not to respond to self antigen. The learning process that leads to self-tolerance involves **clonal deletion** (killing the self-reactive lymphocytes), **clonal anergy** (functional inactivation), and **receptor editing** (stimulating the cells to produce modified receptors that no longer recognize the self antigen. The process begins in the central lymphoid organs when newly formed self-reactive lymphocytes first encounter their self antigen. Instead of being activated by binding antigen, the immature lymphocytes are induced to either alter their receptors or die by apoptosis.

Now there is a question. Why does the binding of self antigen lead to tolerance rather than activation? A lymphocyte to be activated in a peripheral lymphoid organ, it must not only bind its antigen but also require a *costimulatory signal* provided by a helper T cell. Without a costimulatory signal, an antigen tends to kill or inactivate a lymphocyte rather than activate.

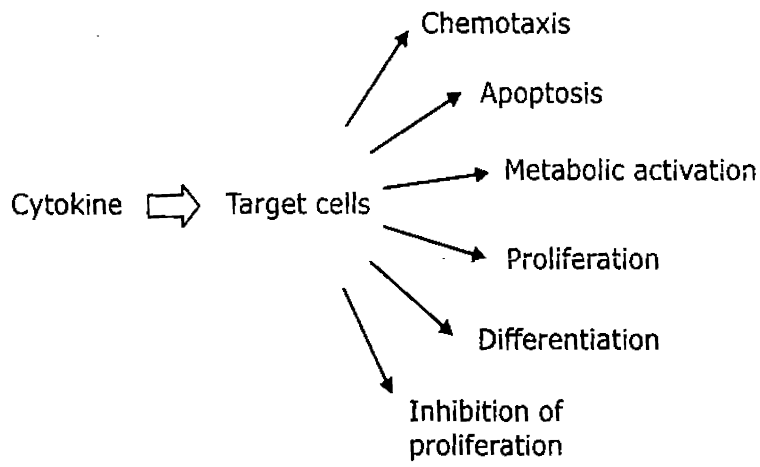
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12.12 CYTOKINES

Cytokines are low-molecular-weight proteins that are produced and secreted by a variety of cell types. They play major roles in the induction and regulation of the cellular interactions involving cells of the immune, inflammatory and hematopoietic system. There are over 200 different cytokines, most of which fall into one of the following families: hematopoietins, interferons, chemokines, and tumor necrosis factors.

Cytokines released by activated T lymphocytes are called **lymphokines**. Similar substances produced by monocyte or macrophage are called **monokines**. The term **interleukin** is used for those products of leukocytes which exert a regulatory influence on other leukocytes. Cytokines can affect the same cell responsible for their production (an autocrine function) or nearby cells (a paracrine function), or they can be distributed by the circulatory system to distant target cells (an endocrine function). They are highly potent hormone-like substances, active even at femto molar concentration. However differ from endocrine hormones as being not produced by glands but by widely distributed cells. Cytokines have range of effects on eukaryotic cells. Cytokines produce biological actions only when they bind to specific, high-affinity receptors on the surface of target cells.



The biological activities of cytokines exhibit **pleiotropy** (a given cytokines that has different biological effect on different target cells), **redundancy** (two or more cytokines that mediates similar functions), **synergy** (combined effect of two cytokines on cellular activity is greater than the additive effect of the individual cytokines) and **antagonism** (effect of one cytokines inhibit the effect of another cytokines).

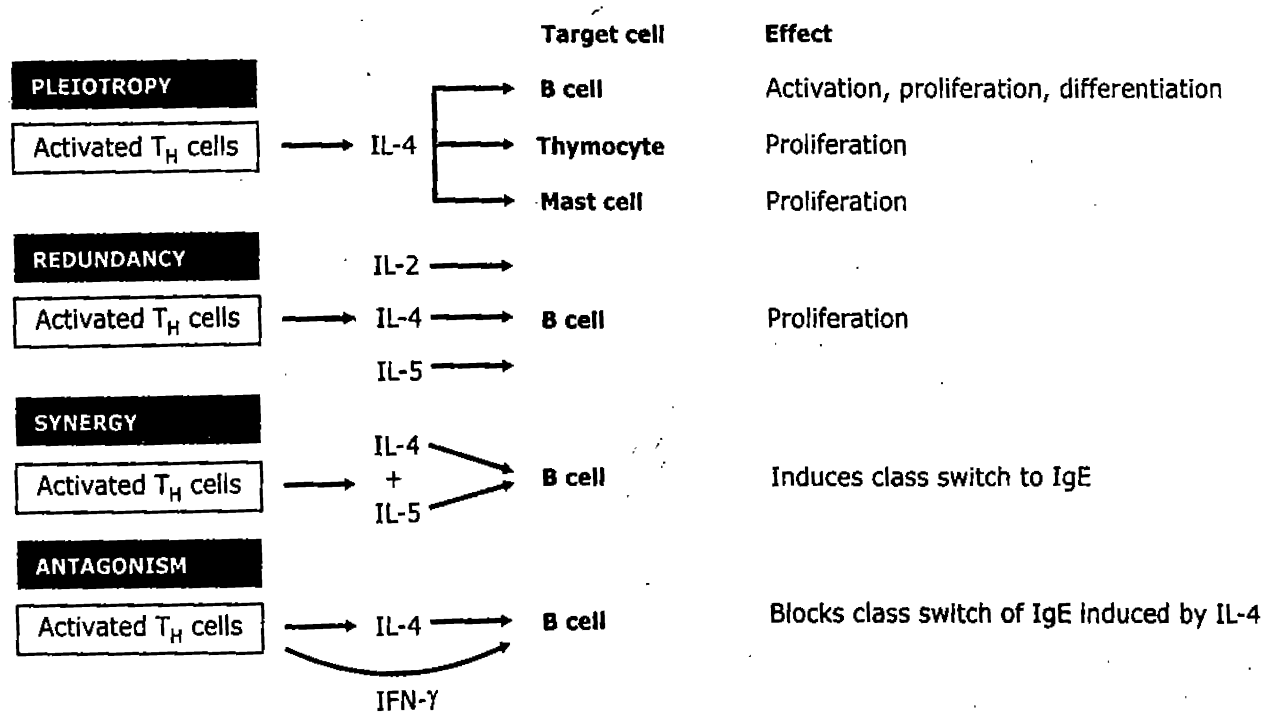


Figure 12.24 : Cytokine attributes of pleiotropy, redundancy, synergy (synergism), antagonism.

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Role of cytokines in immune responses

- Cytokines produce a variety of effects on a number of cell types and help to regulate immune response.
- Cytokines play an important role in alerting and activating the innate immune response to an infection.
- Cytokine plays an important role in the initial response to a bacterial infection. Macrophage activation by exposure to bacterial products results in TNF and IL-1 secretion. TNF and IL-1 activate nearby vascular endothelial cells to permit recruitment of leukocytes to the infection site. Activated macrophages also produce IL-12 leading to increased IFN-γ production from NK and T-cells. IFN-γ feeds back on the macrophage to increase the phagocytic potential and microbicidal properties of the macrophage.

Cytokine Receptors and Signaling

Cytokines mediate their biological effect through the binding of specific receptors on target cell surfaces. These receptors then convert an external signal (binding of the cytokine to the receptor) to an intracellular biochemical signal, usually resulting in new gene transcription. Cytokine receptors are transmembrane proteins where binding of the cytokine occurs in the extracellular region and interaction with signaling proteins occurs in the cytoplasm.

Cytokine receptor classes

Classification of cytokine receptors is based on structural homologies in the cytokine-binding regions. There are five families of cytokine receptors:

- Type I cytokine receptors (IL-2 and IL-4),
- Type II cytokine receptors (type I IFNs and IL-10),
- Ig superfamily receptors (IL-1),
- TNF receptors, and
- Serpentine receptors (chemokines).

JAK/STAT Signaling

The best-defined signaling pathway utilized by type I and type II cytokine receptors involves the activation of Janus kinases (JAKs) and transcription factors called *signal transducers and activators of transcription* (STATs). The JAK/STAT signaling pathway provides a direct link between cytokine binding at the cell surface to changes in gene expression at the level of new gene transcription. The general scheme consists of inactive JAK kinases bound specifically to the cytokine receptor cytoplasmic tail. Upon cytokine binding, the receptor chains are brought together and induce the activation of the JAKs through transphosphorylation. The JAKs also phosphorylate specific tyrosine residues on the cytoplasmic portion of the cytokine receptor, serving as a docking site for specific STAT proteins. The STAT proteins are also then phosphorylated, dimerize, and migrate to the nucleus. The dimerized STAT proteins are able to bind to specific DNA sequences in the promoters of cytokine inducible genes and activate the transcription of those genes directly. Different cytokine receptors bind to unique JAK and STAT combinations resulting in specific outcomes to cytokine exposure.

Negative regulation of cytokine signaling

Unopposed cytokine signaling can lead to a number of immune disorders so several regulatory mechanisms are in place to turn off JAK/STAT signaling after cytokine exposure. First, a family of cytoplasmic proteins called *suppressor of cytokine signaling* can directly interfere with JAK activity or with STAT binding to the cytokine receptor. The expression of the suppressor of cytokine signaling genes themselves is directly activated by the cytokine-induced STATs, consequently forming a classic negative feedback loop. JAK and

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STAT activity can also be negatively regulated by protein tyrosine phosphatases, which return the JAK and STAT proteins to their unphosphorylated, inactive states.

STUDY QUESTION

Q8. Briefly describe the similarities and differences among cytokines, growth factors, and hormones.

Ans. Cytokines, growth factors, and hormones are all secreted proteins that bind to receptors on target cells, eliciting various biological effects. Cytokines tend to be produced by a variety of cells, although their production is carefully regulated, and they exert their effects on several cell types; most cytokines, are often produced constitutively. Hormones, unlike cytokines, generally act over long distances (endocrine effect) on one or a few types of target cells.

12.13 THE COMPLEMENT SYSTEM

The complement (term coined by Paul Ehrlich) system, made up of approximately 30 circulating and membrane-expressed proteins/glycoproteins, is an important effector arm of both the innate and antibody-mediated acquired immune responses. The most important functions of the complement system are *lysis of cells*, *opsonization*, *inflammatory responses* and *immune clearance* which removes the immune complexes from the circulation and deposit them in spleen and liver.

Complement components are designated by numerals (C1–C9), by letter symbols (e.g., factor D), or by trivial names (e.g., homologous restriction factor). Designation C1, C2,...C9 denotes the order in which the components were discovered, rather than their position in the activation sequence.

Complement components are synthesized mainly by liver hepatocytes. Monocytes, tissue macrophages, and epithelial cells of the gastrointestinal tract also produce significant amounts of complement components. In the circulation, the concentration of all complement proteins in about 3 mg/mL. Some complement components are found at high concentrations (e.g., C3 at about 1 mg/mL), while others (such as factor D and C2) are found in only trace amounts.

Complement activation pathways

Most complement components present in the serum in functionally inactive forms as proenzymes or zymogens. Complement activation pathways involves the activation of one component that triggers the activation of the next component in the sequence.

Upon activation, individual components are split into fragments, designated by lower-case letters. The smaller of the cleaved fragments is generally designated with a lower-case *a*, and the large fragment with a lower-case *b*; for historical reasons, however, the large cleavage fragment of C2 is usually referred to as C2a and the smaller fragment C2b. The larger fragments bind to the target near the site of activation, and the smaller fragments diffuse from the site and can initiate localized inflammatory responses.

The complement fragments interact with one another to form functional complexes. Those complexes that have enzymatic activity are designated by a bar over the number or symbol (e.g., $\overline{C3bBb}$). There are three pathways of complement activation. These are the **classical, lectin**, and **alternative pathways.**

The classical pathways

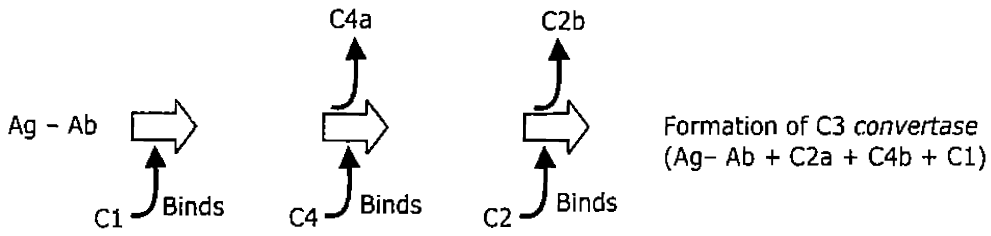
The classical pathway was so named because it was the first complement pathway to be worked out. The classical pathway commonly begins with the formation of antigen-antibody complexes. Thus this pathway is a major effector pathway of the humoral adaptive immune response. Other activators include some viruses, necrotic cells and subcellular membranes (e.g., from mitochondria), aggregated immunoglobulins, and beta-amyloid, found in Alzheimer disease plaques.

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The classical pathway is initiated when C1 binds to the antibody in an antigen-antibody complex. C1 is a complex of three different proteins: C1q (made up of six identical subunits) combined with two molecules each of C1r and C1s. IgM and certain subclasses of IgG (human IgG1, IgG2, and IgG3) are efficient complement-activating antibodies. Among the human immunoglobulins, the ability to bind and activate C1 is, in decreasing order, IgM > IgG3 > IgG1 >> IgG2. IgG4, IgD, IgA, and IgE do not have C1q receptors; these antibodies do not bind or activate C1 and thus do not activate the classical complement pathway.

Classical Pathway (Ab dependent)

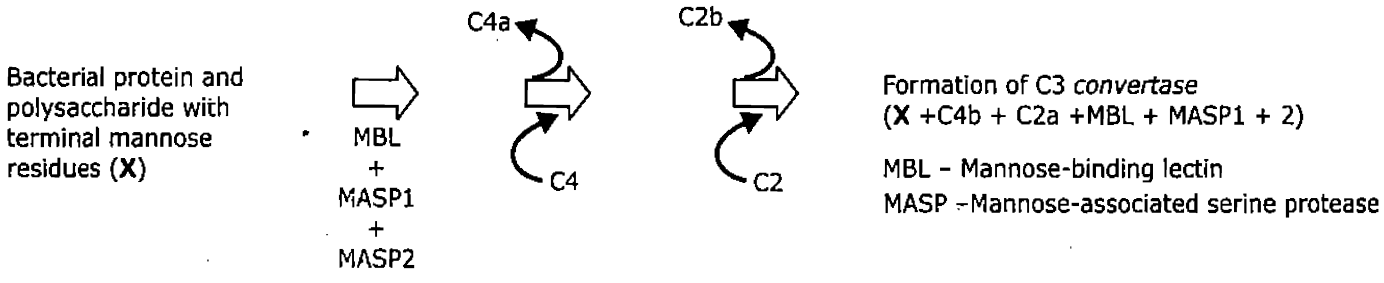


C1q of C1 binds to the Fc region of antibodies. This activates C1r, a *serine proteinase* that initiates the cascade of the classic pathway. First, C4 is proteolytically activated into C4b, which in turn cleaves C2 into C2a and C2b. C4B and C2a together form *C3 convertase*, which finally catalyzes the cleavage of C3 into C3a and C3b. Small amounts of C3b also arise from non-enzymatic hydrolysis of C3.

The lectin pathway

Lectins are proteins that recognize and bind to specific carbohydrate target. The lectin pathway is activated by terminal mannose residues of glycoprotein or polysaccharides found on the surface of bacteria. This pathway does not depend on Ab for activation. It is activated by binding of *mannose binding lectin* (an acute phase protein) to mannose residues. These terminal mannose residues are not found on the surface of mammalian cells, and so the lectin pathway of complement activation may be responsible for discrimination between self and non-self. Because this pathway is activated in the absence of antibody, it is part of the innate immune defenses.

Lectin Pathway (Ab independent)



The alternative or **properdin pathway**

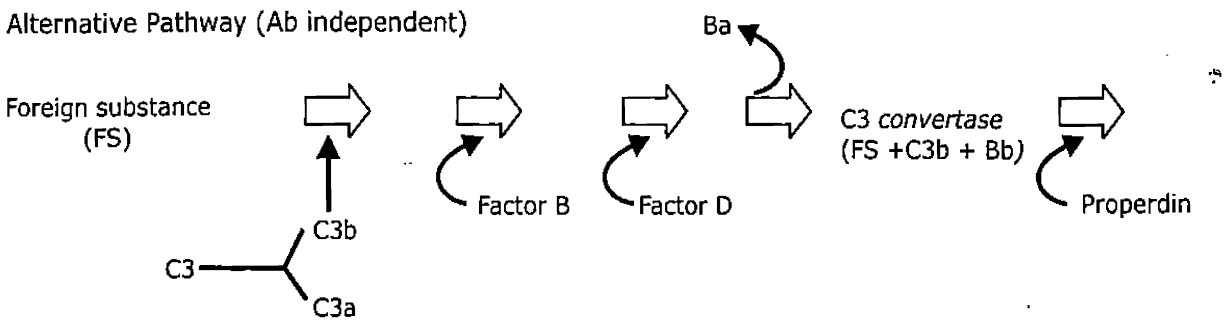
The alternative pathway is antibody-independent. This pathway of complement activation is triggered by almost any foreign substance. The most widely studied include lipopolysaccharide from the outer membrane of gram-negative bacteria, the cell walls of some yeasts, and a protein present in cobra venom, known as cobra venom factor. Some agents that activate the classical pathway–viruses, aggregated immunoglobulins, and necrotic cells– also trigger the alternative pathway. Activation of the alternative pathway occurs in the absence of specific antibody. Thus the alternative pathway of complement activation is an effector arm of the innate immune defenses. This major pathway of complement activation involves four serum proteins: C3, factor B, factor D, and **properdin**, also known as **factor P**.

In the alternative pathway, serum C3 is subject to spontaneous hydrolysis to yield C3a and C3b. The C3b component can bind to foreign surface antigens. The C3b present on the surface of the foreign cells can

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bind another serum protein called factor B to form a complex. Binding to C3b serves as the substrate for an enzymatically active serum protein called factor D. Factor D cleaves the C3b-bound factor B, releasing a small fragment (Ba) that diffuses away and generating C3 convertase. Properdin binds with C3 convertase and stabilize it.



Activation of C3 and C5

C3 cleavage is a critical step in all three complement pathways, the C3 convertases of the classical and alternate pathways cleave C3 into two fragments: the smaller, C3a, is a fluid-phase **anaphylatoxin**. The large fragment, C3b, continues the complement activation cascade by binding to the cells surface around the site of complement activation. Anaphylatoxin bind to receptors on mast cells and blood basophils and induce degranulation, with release of histamine and other active mediators. Anaphylatoxin also induce smooth muscle contraction and increased vascular permeability.

C3b binding to either the classical or alternative pathway allows the next component in the sequence, C5, to bind and be cleaved. For this reason, the C3 convertases with bound C3b are referred to as C5 convertases. The cleavage of C5 produces two fragments: C5a is released into the fluid phase and is potent anaphylatoxin. C5b binds to the cell surface and forms the nucleus for this binding of the terminal complement components.

The terminal pathway

The terminal components of the complement cascades are C5b, C6, C7, C8, and C9. These components bind to each other sequentially to form a macromolecular structure called the **Membrane Attack Complex** (MAC) that results in cell lysis. The three complement pathways converge at the membrane-attack complex. This complex forms a large channel through the membrane of the target cell, enabling ions and small molecules to diffuse freely across the membrane. The first step in MAC formation is C6 binding to C5b on a cell surface. C7 then binds to C5b and C6 and inserts into the outer membrane of the cell. The subsequent binding of C8 to C5b67 results in the complex penetrating deeper into the cell's membrane. The final step in formation of the MAC is the binding and polymerization of C9, a perforin-like molecule, to the C5b678 complex. C5b678 on the cell membrane acts as a receptor for C9 that binds to C8. Additional C9 molecules interact with the C9 molecule in the complex to form polymerized C9 (poly-C9). This poly-C9 forms a transmembrane channel that disturbs the cell's osmotic equilibrium: Ions pass through the channel, and water enters the cell. The cell swells, and the membrane becomes permeable to macromolecules, which then escape from the cell. The result is cell lysis.

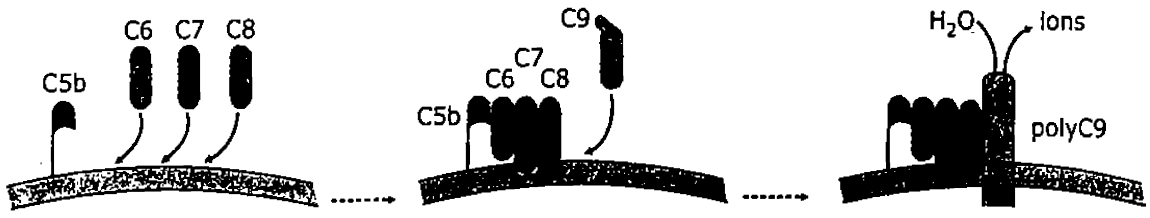


Figure 12.25 : Formation of the membrane attack complex. Late stage complement components C5b-C9 bind sequentially to form a complex on the cell surface. Multiple C9 component bind to this complex and polymerize to form poly-C9, creating a channel that disrupts the cell membrane.

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Biologically active products of complement activation

Activation of complement results in the production of several biologically active molecules which contribute to resistance, anaphylaxis and inflammation.

Kinin production

C2b generated during the classical pathway of C activation is a prokinin which becomes biologically active following enzymatic alteration by plasmin. Excess C2b production is prevented by limiting C2 activation by C1 inhibitor (C1-INH) also known as serpin which displaces C1rs from the C1qrs complex.

Anaphylotoxins

C4a, C3a and C5a (in increasing order of activity) are all anaphylotoxins which cause basophil/mast cell degranulation and smooth muscle contraction.

Chemotactic factors

C5a and MAC (C5b67) are both chemotactic. C5a is also a potent activator of neutrophils, basophils and macrophages and causes induction of adhesion molecules on vascular endothelial cells.

Opsonins

C3b and C4b in the surface of microorganisms attach to C-receptor on phagocytic cells and promote phagocytosis.

STUDY QUESTION

Q9. Explain why serum IgM cannot activate complement by itself?

Ans. Serum IgM is in a planar form in which the complement-binding sites in the Fc region are not accessible. Only after binding to antigen does IgM assume a conformation in which the complement-binding sites are accessible.

12.14 HYPERSENSITIVITY

Hypersensitivity is an exaggerated immune response that results in tissue damage and is manifested in the individual on a second or subsequent contact with an antigen.

Hypersensitivity has been traditionally classified into *immediate* and *delayed* types based on the time required for a sensitized host to develop clinical reactions on re-exposure to the antigen. Later, Gell and Coombs proposed a classification scheme which defined 4 types of hypersensitivity reactions.

Type I Hypersensitivity

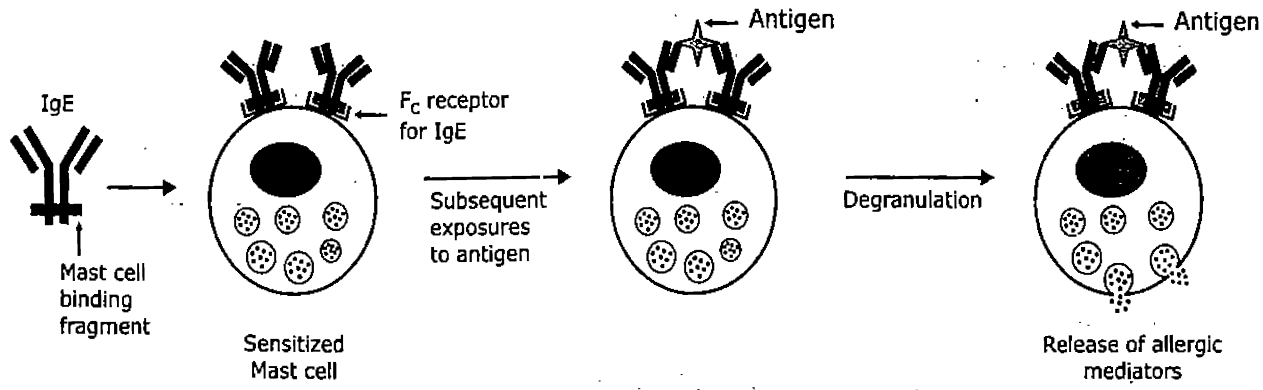
Type I hypersensitivity (also known as *allergic reaction*) is induced by antigens referred to as *allergens*. The term allergen refers specifically to nonparasitic antigens capable of stimulating type I hypersensitive responses. Type I hypersensitive reactions are IgE-mediated humoral antibody responses. These IgE-mediated reactions are stimulated by the binding of IgE (via its Fc region) to high-affinity IgE-specific Fc receptors expressed on mast cells and basophils. When cross linked by antigens, the IgE antibodies trigger the mast cells and basophils to release *primary mediators,* vasoactive amines, stored in the granules (degranulation). The most significant primary mediators are histamine, proteases, eosinophil chemotactic factor, neutrophil chemotactic factor, and heparin.

These mediators cause all the normal consequences of an acute inflammatory reaction - increased vascular permeability, smooth muscle contraction, granulocyte chaemotaxis and extravasation etc. Mast cell activation

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via Fc also leads to the production of two other type of mediators. These *secondary mediators*, unlike the stored granule contents, must be synthesized *de novo* and comprise arachadonic acid derivatives (prostaglandins and leukotrienes), platelet-activating factor, bradykinins, and various cytokines.

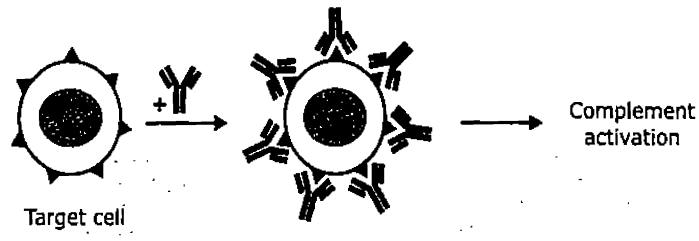


Type I hypersensitivity can be *anaphylaxis* or *atopy*. Anaphylaxis is a very rapid, life-threatening, severe whole body allergic reaction. It is caused by re-exposure to a previously encountered antigen. Atopy (atopic allergy) is a hereditary tendency to develop allergic reaction to substances such as pollen, food, insect venome etc.

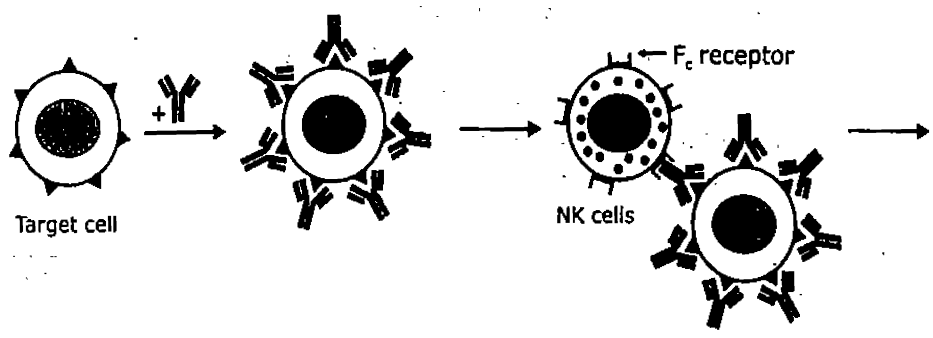
Type II Hypersensitivity

Type II hypersensitivity is generally called a *cytolytic* or *cytotoxic reaction* because it results in the destruction of host cells, either by lysis or toxic mediators. Type II Hypersensitivity is caused by antibodies binding to cells or tissue antigens. The antibodies are of the IgM or IgG classes and cause cell destruction by Fc dependent mechanisms either directly or by recruiting complement via the classical pathway. Classical examples of type II hypersensitivity reactions are the response exhibited by a person who receives a transfusion with blood from a donor with a different blood group and erythroblastosis fetalis.

Two different antibody-mediated mechanisms are involved in these cytotoxic reactions. In **complement-mediated hypersensitivity** reactions, antibodies react with a cell membrane component, leading to complement fixation. This activates the complement cascade and leads either to lysis of the cell or opsonization. Blood cells are most commonly affected by this mechanism.



Antibody-dependent cell mediated cytotoxicity (ADCC) used Fc receptors expressed on many cell types (e.g., natural killer cells, macrophages, neutrophils, eosinophils) as a means of bringing these cells into contact with antibody-coated target cells. Lysis of these target cells requires contact but does not involve phagocytosis or complement fixation. Instead, ADCC lysis of target cells is analogous to that of cytotoxic T cells and involves the release of cytoplasmic granules containing **perforin** and **granzymes** that activate events leading to apoptosis. ADCC reactions involve IgG and IgG Fc receptors.



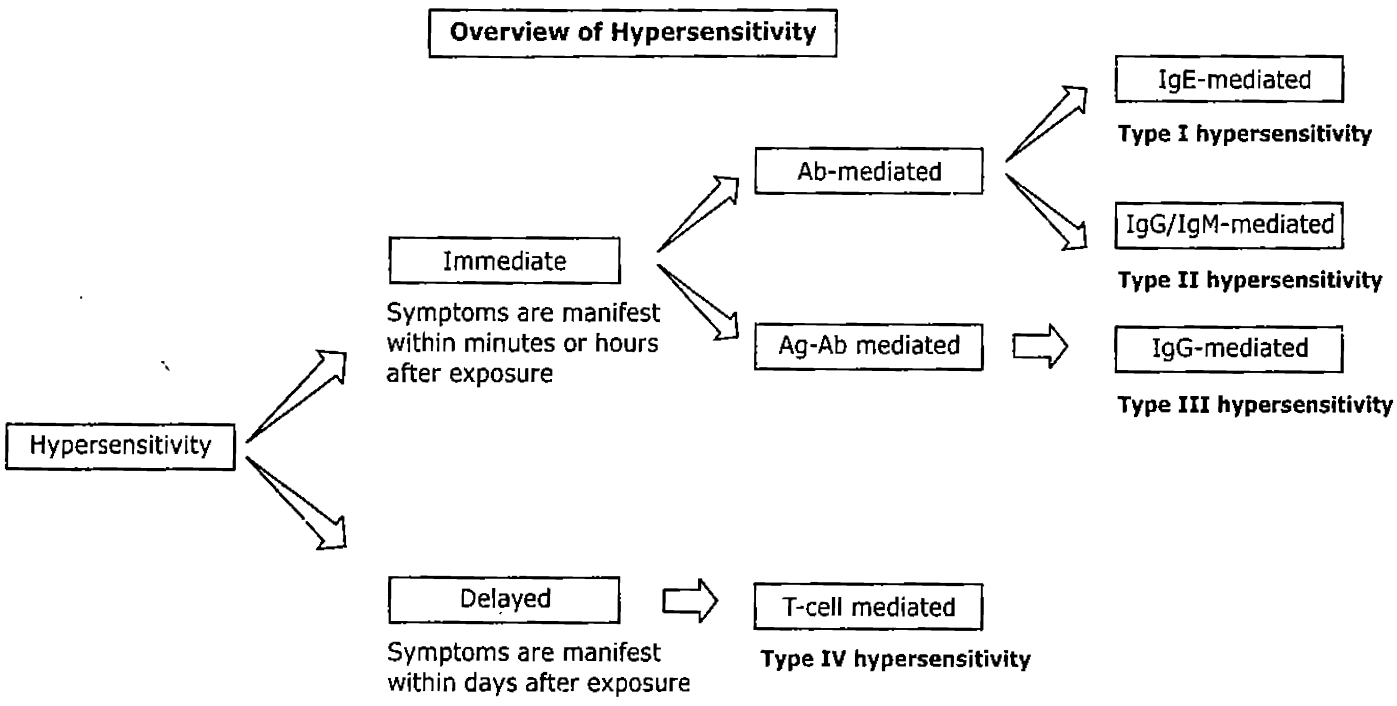
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Type III Hypersensitivity

Type III hypersensitivity is mediated by *immune complexes* essentially of IgG antibodies with soluble antigens. Circulating immune complexes may lodge at various tissue sites where they activate complement and subsequently cause tissue cell lysis. Normally these complexes are phagocytosed effectively by the fixed monocytes and macrophages of the monocyte-macrophage system. In the presence of excess amounts of some soluble antigens, the antigen-antibody complexes may not be efficiently removed. Their accumulation can lead to a hypersensitivity reaction from complement that triggers a variety of inflammatory process. This form of hypersensitivity has a lot in common with type I except that the antibody involved is IgG and therefore not prebound to mast cells.

Type IV Hypersensitivity

Type IV hypersensitivity, commonly called **delayed type hypersensitivity**, is the only class of hypersensitive reactions to be triggered by antigen-specific T cells (cell-mediated immunity reactions). This is mediated by T-cell-dependent effector mechanisms involving T_H cells, primarily of the T_H1 subtype, but in a few cases $CD8^+$ cells. Antibodies do not play a role in type IV hypersensitivity reactions. On activation, the T_H1 cells release cytokines that cause accumulation and activation of macrophages, which, in turn, cause local damage. The *tuberculin skin test* is an example of a type IV hypersensitivity.



12.15 AUTOIMMUNITY

The body is normally able to distinguish its own self-antigens from foreign nonself antigens and does not mount an immunologic attack against itself. This phenomenon is called *immune tolerance*. **Autoimmunity** is a condition in which structural or functional damage is produced by the action of immunologically competent cells or Ab against self antigen. Auto immunity literally means *protection against self*, but actually it implies *injury to self*, and therefore sometimes the term is also under criticism.

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Autoimmune disease results from the activation of self-reactive T and B cells that, following stimulation by genetic or environmental triggers, cause actual tissue damage. Four factors influence the development of autoimmune disease. These factors are genetic, viral, hormonal and psycho-neuro-immunological (the influence of stress and neurochemicals). All four of these factors can affect gene expression, which directly or indirectly interferes with important immunoregulatory actions. Based on the site of involvement and nature of lesions autoimmune diseases may be classified as *hemocytolytic*, *localized* (or organ specific), *systemic* (or non-specific) and *transitory* diseases. Important examples of autoimmune diseases in human and their respective autoantigen are given below in the table.

Table 12.8 : Some autoimmune diseases in humans

Disease	*Autoantigen*
Autoimmune hemolytic anemia	Rh blood group
Graves disease	Thyroid-stimulating hormone receptor
Multiple sclerosis	Myelin basic protein
Myasthenia gravis	Acetylcholine receptor
Rheumatoid arthritis	Unknown synovial joint antigen
Systemic lupus erythematosus	DNA, histones, SnRNP
Type 1 diabetes mellitus	Pancreatic beta cell antigen

TRANSPLANTATION

The immune system has evolved as a way of discriminating between self and non-self. This discriminating power of the immune system between self and non-self is undesirable in case of tissue transplant from one individual to another for therapeutic purposes. Indeed, result of transplants culminates in the phenomenon of **graft rejection**. Before the discussion about the immunological mechanisms associated with graft rejection, it is important to understand the various gradations in relationship from donor to recipient.

Isograft : Graft between genetically identical individuals (syngeneic). In humans, an isograft (or *syngraft*) can be performed between monozygotic twins.

Allograft : Transplants between genetically different individuals within a species.

Xenograft : A graft between individuals from different species.

Autograft : A graft or transplant from one area to another on the same individual.

Transplanting tissue that is not immunologically privileged generates the possibility that the recipient's cells will recognize the donor's tissue as foreign. This triggers the recipient's immune mechanisms, which may destroy the donor tissue. Such a response is called a *graft rejection reaction*. Some transplanted tissues do not stimulate an immune response. For example, a transplanted cornea is rarely rejected because lymphocytes do not circulate into the anterior chamber of the eye. This site is considered an immunologically privileged site. Another example of a privileged tissue is the heart valve.

A tissue rejection reaction can occur by two different mechanisms. *First*, foreign class II MHC molecules on transplanted tissue, or the graft are recognized by host T-helper cells, which aid cytotoxic T cells in graft destruction. Cytotoxic T cells then recognize the graft through the foreign class I MHC molecules. This response is much like the activation of CTLs by virally infected host cells. A *second* mechanism involves the T-helper cells reacting to the graft and releasing cytokines. The cytokines stimulate macrophages to enter, accumulate within the graft, and destroy it. The MHC molecules play a dominant role is tissue rejection reaction because of their unique association with the recognition system of T cells.

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12.16 IMMUNODEFICIENCY DISEASES

Immunodeficiencies occur when one or more components of the immune system is defective. Most of immunodeficiency diseases are inherited.

Acquired immunodeficiencies are caused due to malnutrition or by agents, such as drugs or irradiation that damage lymphocytes, or infection with measles or HIV. Immunodeficiencies may be *primary* and *secondary*. *Primary immuno deficiency* diseases are disorders caused by a defect in one or more genes involved in the development or function of the immune system. By contrast, a *secondary immune deficiency* disease is one in which the immune system is compromised by factors from the outside, such as viruses, radiation treatments or chemotherapy.

Example of primary immuno deficiency diseases:

Severe combined immunodeficiency (SCID)

SCID is a genetic disorder which is characterized by a very low number of circulating lymphocytes. Both arms (B cells and T cells) of the adaptive immune system become non-functional. As such patients make neither specific T-cell dependent antibody responses nor cell-mediated immune responses, and thus cannot develop immunological memory. Several different defects can lead to the SCID phenotype. In X-linked SCID, which is the commonest form of SCID, T cells fail to develop because of defect in the genes code for several cytokine receptors, including those for the interleukins IL-2, IL-4, IL-7, IL-9, and IL-15. The autosomally inherited SCID occurs due to *adenosine deaminase deficiency*. Adenosine deaminase catalyzes conversion of adenosine to inosine, and its deficiency results in accumulation of adenosine, which interferes with purine metabolism which result in an accumulation of nucleotide metabolites that are particularly toxic to developing T cells.

Chediak-Higashi syndrome

Chediak-Higashi syndrome is an autosomal recessive disease. It is characterized by recurrent bacterial infections, lack of skin and eye pigment. Phagocytes from patients with this immune defect contain giant granules but do not have the ability to kill bacteria. With time, patients develop massive infiltrates of lymphocytes and macrophages in the liver, spleen, and lymph nodes. The molecular basis of the defect is a mutation in a protein involved in the regulation of intracellular trafficking. The mutation impairs the targeting of proteins to lysosomes.

DiGeorge syndrome

DiGeorge syndrome, or *congenital thymic aplasia*, is not hereditary but occurs sporadically and is result of a deletion in chromosome 22. The syndrome is caused by defective migration of fetal neural crest cells into the third and fourth *pharyngeal pouch*. DiGeorge syndrome in its most severe form is the complete absence of a thymus. This developmental defect causes immunodeficiency along with hypoparathyroidism, and congenital heart disease. The immune defect includes a profound depression of T-cell numbers and absence of T-cell responses. Although B cells are present in normal numbers, affected individuals do not produce antibody in response to immunization with specific antigens.

12.17 FAILURES OF HOST DEFENSE MECHANISMS

The propagation of a pathogen depends on its ability to multiply in a host. Hence pathogens must therefore grow without activating an immune response. The most successful pathogens persist either because they do not elicit an immune response, or because they evade the response once it has occurred. Pathogens

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have developed various strategies for avoiding destruction by the immune system. Some of strategies are mentioned below:

- One way in which a pathogenic agent can evade immune response is by altering its antigens. There are three ways in which antigenic variation can occur. First, many pathogenic agents exist in a wide variety of antigenic types. For example, there are many types of *Streptococcus pneumoniae*. Each type differs from the others in the structure of its polysaccharide capsule. The different types are distinguished by serological tests and so are often known as *serotypes*. Infection with one serotype of *Streptococcus pneumoniae* can lead to type-specific immunity, which protects against reinfection with that type but not with a different serotype.
- A second, more dynamic mechanism of antigenic variation arises due to **antigenic drift** and **antigenic shift**. *Antigenic drift* is caused by point mutations in the genes encoding epitopes. For example in influenza virus new variant arises with mutations in genes encoding the major surface proteins *hemagglutinin* and *neuraminidase*. Thus individuals who were previously infected with, and hence are immune to, the old variant are thus susceptible to the new variant. Periodically influenza viruses also show an *antigenic shift* through reassortment of their segmented genome with another influenza virus, changing their surface antigens radically. Such antigenic shift variants are not recognized by individuals immune to influenza.
- The third mechanism of antigenic variation involves programmed rearrangements in the DNA of the pathogen. For example, African trypanosomes which cause sleeping sickness in humans changes the major surface antigen repeatedly within a single infected host. The trypanosome is coated with a single type of glycoprotein, the *variant-specific glycoprotein* (VSG), which elicits an antibody response. The trypanosome genome, however, contains about 1000 VSG genes, each encoding a protein with distinct antigenic properties. Only one of these is expressed at any one time. The VSG gene expressed can be changed by gene rearrangement. So, by having their own system of gene rearrangement that can change the VSG protein produced, trypanosomes keep one step ahead of an immune system capable of generating many distinct antibodies by gene rearrangement.
- Some pathogens have evolved specialized mechanisms to resist destruction by host defense mechanisms. For instance, some bacteria that are engulfed in the normal way by macrophages have evolved means of avoiding destruction by these phagocytes. For example, *Mycobacterium tuberculosis* is taken up by macrophages but prevents the fusion of the phagosome with the lysosome, protecting itself from the bactericidal actions of the lysosomal contents.

12.18 IMMUNOTECHNIQUES

Precipitation reactions

Precipitation results from the interaction of a *soluble* antibody with a *soluble* antigen to form an *insoluble* complex. Antibodies that thus aggregate soluble antigens are called **precipitins**. Formations of an Ag-Ab lattice depends on the valency of both antibody and antigen. The antibody must be bivalent for precipitation reaction. Monovalent Fab fragments can not form precipitate with antigen. Similarly, 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.

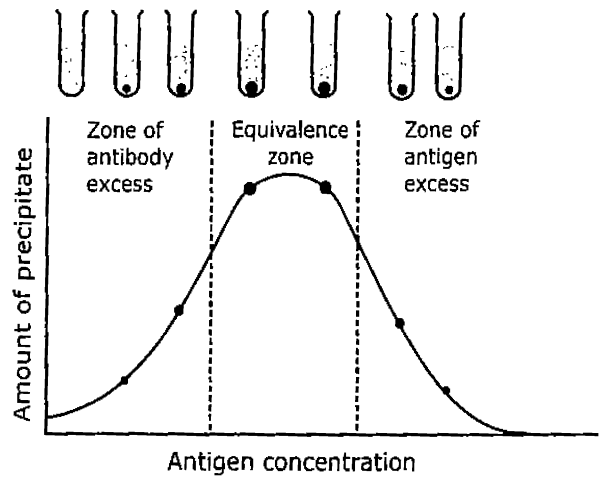
Precipitation reaction in fluids

Precipitation occurs maximally only when there are optimal proportions of the two reacting substances-antigen and antibody. Hence an insoluble antibody-antigen complex formation occurs within a narrow

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concentration range known as the **zone of equivalence**. This represents the conditions under which macromolecular antigen/antibody complexes are formed that are sufficiently large to be precipitated. Outside the equivalence concentration, conditions known as *antigen* or *antibody excess* occur, which result in the formation of small, soluble complexes.



The precipitin reaction

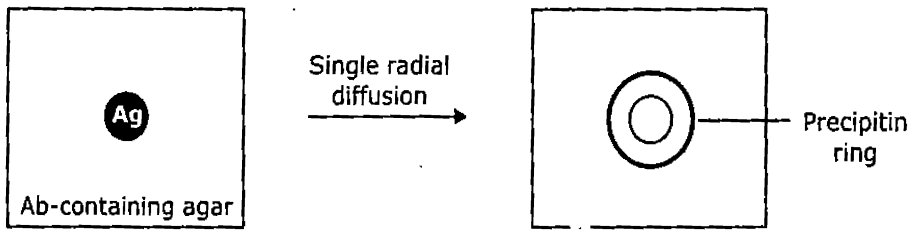
When increasing concentrations of antigen are added to a series of tubes that contain a constant concentration of antibodies, variable amounts of precipitate form. If the amount of the precipitate is plotted against the amount of antigen added, a **precipitin curve**, as shown in the figure above, is obtained.

Precipitation reactions in gels

Precipitation reactions carried out in agar gels are referred to as *immunodiffusion reactions*. When Ag and Ab diffuse toward one another in agar, or when Ab is incorporated into the agar and Ag diffuses into the Ab-containing matrix, a visible line of precipitation will form. Visible precipitation occurs in the region of equivalence. No visible precipitate forms in regions of antibody excess or Ag excess. Two types of immunodiffusion reactions can be used to determine relative concentrations of antibodies or antigens as well as the identity of antigens. These immunodiffusion techniques are *radial immunodiffusion* and *double immunodiffusion*.

Radial immunodiffusion (Mancini method)

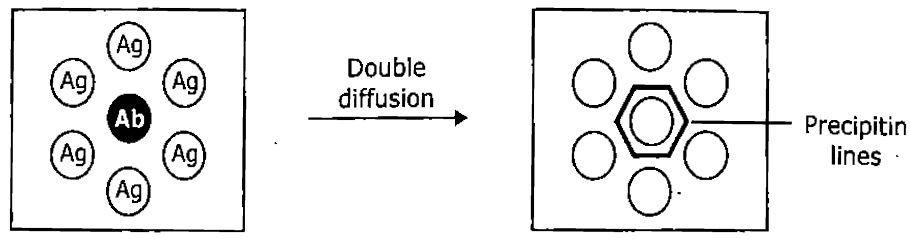
The relative concentration of an Ag can be determined by a simple quantitative assay in which an Ag sample is placed in a well and allowed to diffuse into agar containing a suitable dilution of an antiserum. At the region of equivalence, a precipitation ring form around the well, the area which, is proportional to the concentration of Ag. By comparing the area of the precipitation ring with a standard curve, the concentration of the antigen sample can be determined.



Double immunodiffusion (Ouchterlony method)

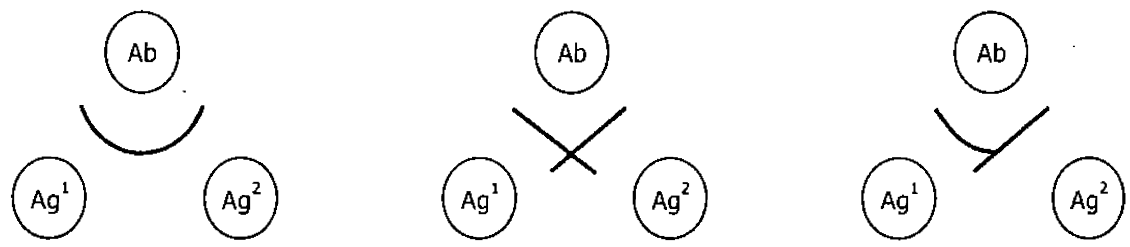
In the Ouchterlony method, both Ag and Ab diffuse radially from wells towards each other, thereby establishing a concentration gradient. It is an effective qualitative tool for determining the relationship between Ag and number of different Ag-Ab systems present.

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The pattern of the precipitin lines that form when two different Ag preparations are placed in adjacent wells indicates whether they share epitopes:

- **Identity** occurs when two antigens share identical epitopes (Figure A).
- **Non-identity** occurs when two antigens are unrelated i.e., share no common epitopes. The antisera forms an independent precipitin line with each Ag, and the two lines cross (Figure B).
- **Partial identity** occurs when two Ag's share some epitope but one of the other has a unique epitope. The antiserum forms a line of identity with the common epitope and a curved spur with unique epitope (Figure C).



(A) Identity (B) Non-identity (C) Partial identity

Agglutination reactions

Agglutination is the visible clumping of a particulate antigen when mixed with antibodies specific for the particulate antigens. Antibodies that produce such reactions are called **agglutinins**. The general term agglutinin is used to describe antibodies that agglutinate particulate antigens. When the antigen is an erythrocyte the term **hemagglutination** is used. All antibodies can theoretically agglutinate particulate antigens but IgM, due to its high valence, is particularly good agglutinin.

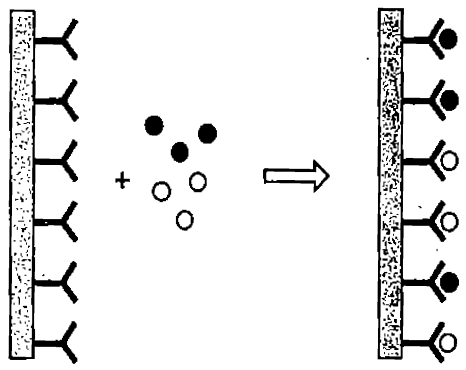
Agglutination reactions are similar in principle to precipitation reactions. Just as an excess of antibody inhibits precipitation reactions, such excess can also inhibit agglutination reactions; this inhibition is called the **prozone effect**. When the antigen is an integral part of the surface of a cell or other insoluble particle, the agglutination reaction is referred to as **direct agglutination**. When the agglutination reaction takes place between antibodies and soluble antigen that has been attached to an insoluble particle, the reaction is referred to as **passive agglutination**.

IMMUNOASSAYS

Immunoassays are based on the specific antibody-antigen reaction. All immunoassays depend on the measurement of fractional binding site occupancy of the antibody by analyte. *Noncompetitive* immunoassays directly measure occupied sites, while *competitive* assays are based on the detection of total and unoccupied sites by analyte. In competitive immunoassays, labeled and unlabeled analyte are simultaneously exposed to the antibody. In noncompetitive immunoassays the labeled antibody detects the bound analyte. Maximal sensitivity will be reached by decreasing the amount of antibody in competitive assays and increasing the antibody concentration in noncompetitive assays.

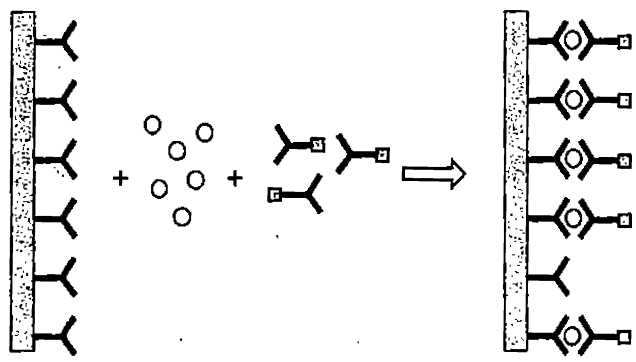
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O Unlabeled antigen
● Labeled antigen

Figure : 12.26
Competitive immunoassay



Labeled antibody

Figure : 12.27
Non-competitive immunoassay

RADIOIMMUNOASSAY

Radioimmunoassay (RIA) is a technique for determining the concentration of a particular molecule in a sample, based on competitive binding between non-labeled and isotope labeled antigen for its specific antibody. Berson and Yalow developed the RIA method, and first applied it to the assay of human and animal insulins in plasma. It is one of the most sensitive techniques for detecting Ag or Ab. The principle of RIA involves competitive binding of radiolabelled antigen and unlabeled antigen to a high-affinity antibody.Typically, a fixed amount of the antibody is attached to the bottom of a tube. Then a fixed amount of isotope labeled antigen is added to the tube. The amount of isotope labeled antigen is typically high enough to saturate all of the antibody molecules attached to the bottom of the tube. Because the interaction between the antibody and the antigen involves very strong non-covalent forces, the contents of the tube can be discarded without disturbing the isotope labeled antigen that are bound to the antibody molecules in the bottom of the tube. The tube can now be counted in a gamma counter to obtain a counts per minute (CPM). This value represents the total count, and represents a situation when all of the antibody binding sites are occupied by the isotope labeled antigen. This value alone, however, tells us nothing. In order to calibrate the measurements, known amounts of non-labeled antigen is added to tubes that contain the same fixed amount of antibody attached to the bottom, and in addition, the same fixed amount of isotope labeled antigen in the tube. Now in the tube, there is competition between the non-labeled and labeled antigen to bind to the antibody binding sites. The relative amount of non-labeled or labeled antigen that binds to the antibody is strictly a function of their relative amounts in the tube. Because the labeled and the non-labeled antigen bind to the antibody with the same affinity. The higher the concentration of the non-labeled antigen, the better it can compete for the binding sites. Therefore, less labeled antigen will bind, which will result in a smaller CPM. In this fashion, a standard curve can be obtained by measuring counts from tubes, which have increasing known concentrations of the non-labeled antigen.

Enzyme-linked immunosorbent assay

Enzyme immunoassays exploit an enzymatic reaction for detecting the immune reaction. In 1971, Engvall and Perlmann and van Weemen and Schuurs described independently the use of enzyme-labeled agents. The most common type of enzyme immunoassays in use is *enzyme-linked immunosorbent assays.*

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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 Ab reacts with a colorless substrate to generate a colored reaction product. Such a substrate is called chromogenic substrate. A number of enzymes have been employed for ELISA, including alkaline phosphatase, horse-radish peroxidase, urease and beta galactosidase.

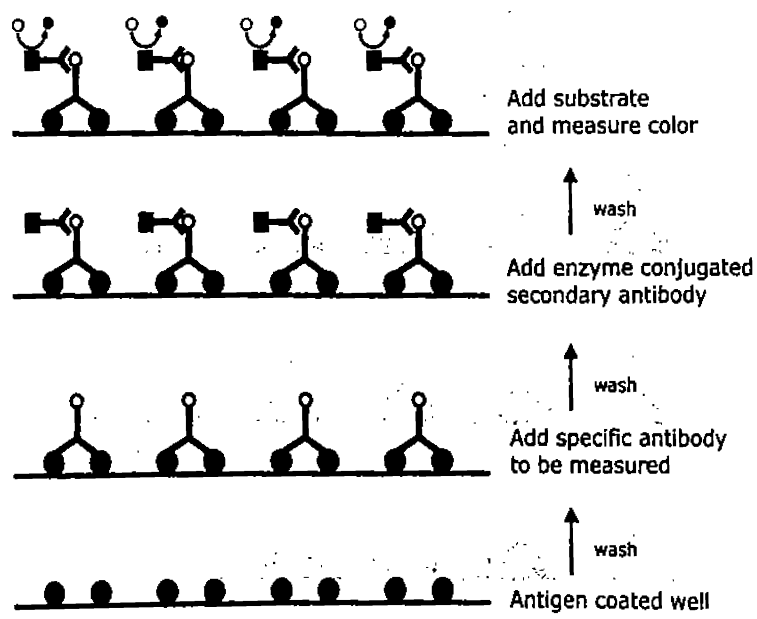
Table 12.9 : Enzymes used for conjugation of antibodies

Enzyme	**Source**	**Reaction catalyzed**
Peroxidase	Horseradish	H_2O_2 + oxidisable substrate → oxidized product + $2H_2O$
Alkaline phosphatase	Calf intestine	R-O-P + H_2O → R-OH + P_i
β-Galactosidase	*E.coli*	β-D-Galactoside + H_2O → galactose + alcohol
Urease	Jack bean	$(NH_2)_2CO$ + $3H_2O$ → CO_2 + $2NH_4OH$

Different variants of ELISA have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody. Each type of ELISA can be used qualitatively to detect the presence of Ab or Ag. Alternatively, a standard curve based on known concentrations of Ab or Ag is prepared from which the unknown concentrations of a sample can be determined.

Indirect ELISA

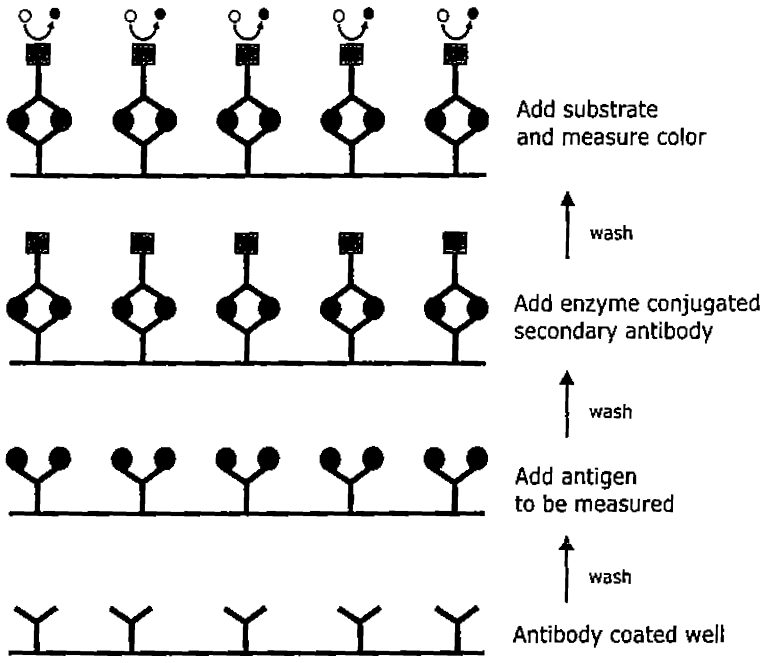
Ab can be detected or quantitatively determined with an indirect ELISA. Serum or some other sample containing primary Ab (Ab1) is added to an Ag-coated micro-titer well and allowed to react with the Ag attached to the well. After any free Ab, is washed away, the presence of Ab bound to the Ag is detected by adding an enzyme-conjugated secondary anti-isotype (Ab2), which binds to the primary Ab. Any free Ab2 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 less than a few minutes. Indirect ELISA is the method of choice to detect the presence of serum antibodies against HIV.



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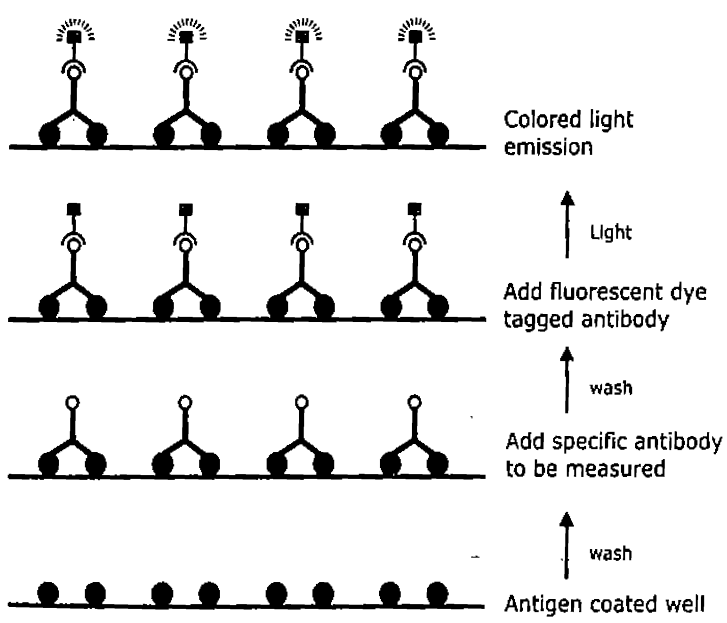
Sandwich ELISA

Antigen can be detected or measured by a sandwich ELISA. 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 Ab specific for a different epitope on the antigen is added and allowed to react with the bound Ag. Any free second Ab then is washed away and a substrate for the enzyme is added. Finally the amount of colored reaction product that forms is measured.



Immunofluorescence

Ab could be labeled with molecules that have the property of fluorescence. Fluorescent molecules absorb light of one wavelength (excitation) and emit light of another wavelength (emission). If Ab molecules are tagged with a fluorescent dye, or fluorochrome, immune complexes containing these fluorescently labeled antibodies can be detected by colored light emission when excited by light of the appropriate wavelength. Ab molecules bound to antigens in cells or tissue sections can similarly be visualized. This technique is known as *immunofluorescence*. The most commonly used fluorescent dyes are **fluorescein** and **rhodamine**: other dyes such as phycoerythrin and phycobiliproteins are also used now-a-days.



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Fluorescence, chemiluminescence and **bioluminescence**

In the case of *fluorescence*, the energy input is the absorption of light. The chemical characteristics of a molecule will determine which wavelengths (color) of light it will absorb. In the case of chemiluminescence, the input energy is derived from the making and breaking of bonds that occur during a chemical reaction.

Chemiluminescence and *bioluminescence* are terms that refer to the same physical process of light emission without heat. Bioluminescence is the process by which a living system or components isolated from a living system, such as a firefly tail, carries out a series of reactions that result in emission of light. Chemiluminescence is the same kind of process, but involving molecules that are not of biological origin. Bioluminescence reactions, unlike chemiluminescence, require an enzymatic catalyst for the reaction to occur.

Immunoelectrophoresis

Immunoelectrophoresis is a qualitative technique. It combines the process of electrophoresis as well as double immunodiffusion. Electrophoresis is used for separation of antigens on the basis of charge and double immunodiffusion for the purpose of their identification. Immunoelectrophoresis is used in clinical laboratories to detect the presence or absence of proteins in the serum.

In this technique antigen mixture is placed in a well cut in the center of a slide that is coated with agar gel. The antigen mixture is then subjected to electrophoresis, which separates the various components according to their charge in the electrical field. After electrophoresis, a trough is cut along the side of the slide, and antibodies to antigen mixture are placed in the trough. The antibodies diffuse in the agar, as do the separated antigen mixture. At an optimal antigen to antibody ratio for each antigen and its corresponding antibodies, precipitin lines form.

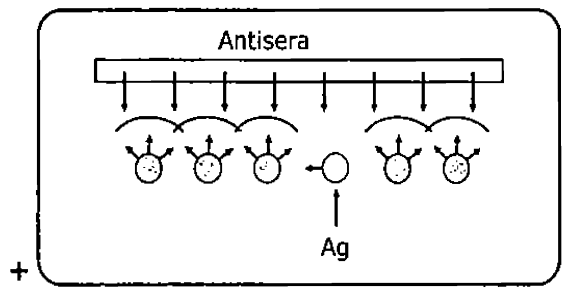


Figure : 12.28
Identification of antigens using gel electrophoresis. Ag (e.g. serum) is placed in a well cut in a gel and subjected to a voltage gradient which causes the various antigens to migrate different distances through the gel depending on their charge. After electrophoresis, a trough is cut in the gel into which antibodies (e.g. horse anti-human serum) are placed.
Adapted from Lydyard et al., 2003, Immunology, BIOS Scientific Pub Ltd.

Rocket electrophoresis

In rocket electrophoresis, a negatively charged antigen is electrophoresed in a gel containing antibody. During electrophoresis, as the protein antigen starts to leave the well and enter the gel, antigen molecules will start to interact, and bind with, antibody molecules. However, at this early stage, there is considerable antigen excess over antibody and no precipitation occurs. However, as the antigens sample electrophoreses further through the gel, more antibody molecules are encountered that interact with the antigen, until eventually there is sufficient antibody-antigen crosslinking such that "equivalence" is reached and the antigen-antibody complex precipitates. The precipitin lines appear like the shape of a rocket.. The majority of the antibody-antigen precipitate is indeed at the head of this rocket, but the fine precipitation lines up the side of the rockets are formed by a small amount of antigen diffusing sideways as the antigen passes through the gel. This small amount of antigen very quickly meets sufficient antibody to reach equivalence and precipitate.

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12.19 VACCINES

An individual may be exposed to an antigen to induce formation of antibodies, a type of immunity known as *artificial active immunity*. The material used to induce artificial active immunity, the antigen or mixture of antigens, is known as a **vaccine** or an **immunogen**, and the process of generating such an immune response is **immunization**. Immunization is commonly known as *vaccination*. The term vaccine has been derived from Latin word *vaccinus* meaning *from cows.* It can be defined as a non toxic or non virulent preparation of antigenic material that can be used to induce long term humoral as well as cell mediated immune response against pathogens.

Type of antigens used in vaccines

In principle anything from whole organisms to small subcellular fragment can be used as antigen in vaccines. Most current vaccines in use for humans consist of whole organisms, described as **whole organism vaccine**, which include live but attenuated organisms or killed organisms and **subunit vaccines** (toxoid, capsular polysaccharide and recombinant surface antigens).

Whole organism vaccines

It consists of live but alternated or inactivated bacterial cells or viral particles.

Live but attenuated vaccines

These vaccines are prepared by attenuating pathogenic organisms by growing them in unfavorable conditions which result in gene mutations due to which organism looses pathogenicity but retains their capacity for transient growth. Attenuated vaccines have advantages as well as disadvantages. Due to their capacity for transient growth these vaccines show prolonged immunogenicity and eliminate the need for repeated boosters. But a major disadvantage of these vaccines is the associated risk of reverting back to virulent form. However these days genetic engineering is also used to cause site directed mutation causing irreversible removal of virulence genes from attenuated organisms making them safe for use.

The Sabin polio vaccine is an example of *attenuated vaccine*, consisting of three attenuated strains of poliovirus. Sabin vaccine in the intestines induces production of secretory IgA, which serves as an important defense against naturally acquired poliovirus. The vaccine also induces IgM and IgG classes of antibody.

Inactivated (killed) vaccines

Inactivated vaccines are produced by inactivation of pathogenic organisms by heat or chemical treatment so that organisms are unable to multiply in the host. Inactivation is carried out in such a way so that structure of epitopes on surface antigens remain maintained. Hence heat treatment is generally not satisfactory since it can cause protein degradation and thus loss of some epitopes. Thus chemical inactivation using formaldehyde or other alkylating agent is more common. Inactivated vaccines are effective, but they are less immunogenic so often require several boosters and normally do not adequately stimulate cell-mediated immunity or secretory IgA production. In contrast, attenuated vaccines usually are given in a single dose and stimulate both humoral and cell-mediated immunity.

Table 12.10 : Comparison of attenuated and inactivated vaccine

	Attenuated vaccine	*Inactivated vaccine*
Booster dose	Generally requires single booster	Requires multiple boosters
Relative stability	Less stable	More stable
Immunity induced	Humoral and cell-mediated	Mainly humoral
Reversion to virulent form	May revert to virulent form	Cannot revert to virulent form

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Subunit vaccines

Three general forms of subunit vaccines are in current use: Toxoid, Capsular polysaccharide, Recombinant microbial antigens. Many exotoxins can be modified chemically so they retain their antigenicity but are no longer toxic. Such a modified exotoxin is called a **toxoid**. Toxoids are usually not as efficient as the original exotoxin for producing immunity, but they can be given safely and in high doses. In case of *recombinant antigen vaccine*, the gene encoding any immunogenic protein canbe cloned and expressed in bacterial, yeast or mammalian cells using recombinant DNA technology. The first recombinant antigen vaccine approved for human use is the hepatitis B vaccine.

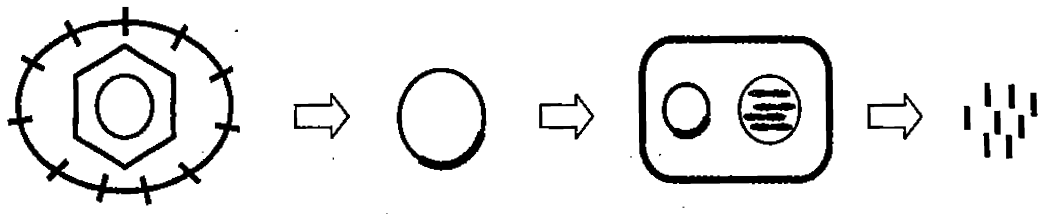


Figure 12.29 : Schematic representation of the development of a subunit vaccine.

DNA vaccines

DNA vaccines, also known as *genetic vaccines*, use the genetic material of the pathogen itself to immunize the individual. In this class of vaccine fragments of pathogen's genome encoding antigenic proteins are injected directly into the host cells where they can integrate into the chromosomal DNA or exist as episomes. Expression of genes within the host generate foreign proteins to which the host immune system responds. Hence in DNA vaccine, an immune response is made against the protein encoded by the vaccine DNA. The DNA itself is not immunogenic. DNA vaccines induce both humoral and cell mediated immunity.

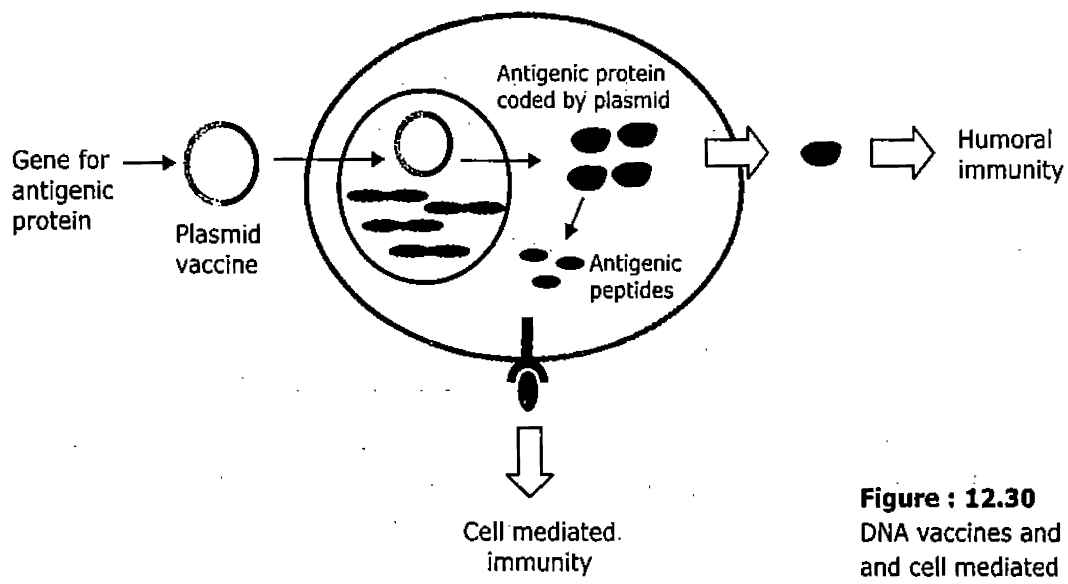


Figure : 12.30
DNA vaccines and humoral and cell mediated immunity.

Recombinant vector vaccines

In recombinant vector vaccines genes that encode antigens isolated from a pathogen can be inserted into nonvirulent viruses or bacteria. Such recombinant microorganisms serve as vectors, replicating within the host and expressing the gene product of the pathogen-encoded antigenic proteins. A number of organisms have been used for vector vaccines, including vaccinia virus, the canary pox virus, attenuated polio virus, adenoviruses and others. The antigens elicit humoral immunity as well as cellular immunity.

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Table 12.11 : Vaccines for infectious diseases in humans

Disease	Type of vaccine used
Bacterial diseases	
Anthrax	Toxoid
Diphtheria	Toxoid
Tetanus	Toxoid
Pertussis	Killed bacteria (*bordetella pertussis*) or acellular proteins
Typhoid fever	Killed bacteria (*Salmonella typhi*)
Paratyphoid fever	Killed bacteria (*Salmonella paratyphi*)
Cholera	Killed cells or cell extract (*Vibrio cholerae*)
Plague	Killed cells or cell extract (*Yersinia pestis*)
Tuberculosis	Attenuated strain of *Mycobacterium tuberculosis* (BCG)
Meningitis	Purified polysaccharide from *Neisseria meningitidis*
Bacterial pneumonia	Purified polysaccharide from *Streptococcus pneumoniae*
Typhus fever	Killed bacteria (*Rickettsia prowazekii*)
Viral diseases	
Measles	Attenuated virus
Mumps	Attenuated virus
Rubella	Attenuated virus
Polio	Attenuated virus (Sabin) or inactivated virus (Salk)
Influenza	Inactivated virus
Rabies	Inactivated virus
Smallpox	Cross-reacting virus (vaccinia)
Hepatitis B	Recombinant DNA vaccine or inactivated virus
Varicella (chickenpox)	Attenuated virus

Adapted from Biology of Microorganisms, Michael T. Madigan et. al, Pearson Prentice Hall

Herd immunity

If enough people in a community are immunized against certain diseases, then it is more difficult for that disease to get passed between those who are not immunized. A type of immunity that occurs when the vaccination of a portion of the population (or herd) provides protection to unprotected individuals is termed *herd immunity*. Herd immunity does not apply to all diseases.

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SELF TEST

Q1. Macrophages, which are also called monocytes, have the ability to
a. process and present antigens to T cells
b. produce antibodies
c. express IgM molecules on their cell surface
d. differentiate into dendritic cells when necessary **[JNU]**

Q2. Indicate which statement is *not* correct. T helper cells
a. stimulate migration of macrophages
b. help B cells to produce antibodies
c. are cytotoxic to virus infected cells
d. help in generation of cytotoxic T cells **[JNU]**

Q3. Identify the *wrong* statement about dendritic cells
a. they take up antigen by endocytosis
b. they express class-II molecules constitutively
c. they cannot activate naive T cells
d. they express B_7 molecules all the time on the membrane **[JNU]**

Q4. Identify the *wrong* statement about B cells
a. they can present both exogenous and endogenous antigens
b. they can present only antigens for which they have surface immunoglobulin
c. they can present only protein antigen
d. they can present both protein and non-protein antigens **[JNU]**

Q5. An IgG molecule after treatment with an enzyme generated a disulfide linked antigen binding fragment which could agglutinate cells. The enzyme is
a. trypsin
b. papain
c. pepsin
d. V_8 protease **[JNU]**

Q6. Monoclonal antibodies are secreted by hybridomas which are generated by
a. fusion of immune spleen cells with any type of cells capable of growing in tissue culture
b. fusion of immune spleen cells with plasmacytoma cells
c. growing immune spleen cells in the presence of HAT
d. growing immune spleen cells in the presence of B cell growth factors **[JNU]**

Q7. Identify the *correct* statements for the 'HAT medium'
P. includes drug aminopterin to block major pathway for synthesis of deoxyribonucleotides
Q. hypoxanthine is precursor for thymidine
R. includes drug aminopterin to block major pathway for synthesis of polypeptides
S. cells can grow in presence of aminopterin only if they have enzymes thymidine kinase and hypoxanthine-guanine phosphoribosyl transferase
a. P, Q
b. P, S
c. R, S
d. Q, S

Q8. Serum IgM cannot activate the complement by itself because
a. it does not have complement binding site
b. it is planar in which complement binding sites in the Fc region are not accessible

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c. it gets degraded and hence unable to activate the complement
d. it needs metal ions to activate complement

Q9. The following activates the alternative pathway of complement
a. lipopolysaccharides
b. some viruses and virus-infected cells
c. fungal and yeast cell walls
d. all of the above

Q10. Somatic mutation of immunoglobulin genes accounts for
a. allelic exclusion
b. class switching from IgM to IgG
c. affinity maturation
d. increased expression of the Ig gene **[IISc]**

Q11. An MHC class I molecule was run on an SDS-acrylamide gel. How many protein bands will be observed in the gel
a. 4 b. 2
c. 1 d. 3 **[IISc]**

Q12. Anti-idiotypic antibodies refer to
a. antibodies produced in vitro
b. antibodies that are highly cross-reactive
c. antibodies that are generated against the unique determinants in the variable regions of a specific antibody molecule
d. antibodies directed against the constant region of antibody only **[IISc]**

Q13. Nucleated cells tends to be more resistant to complement mediated lysis than RBC because
a. many nucleated cells can endocytose the membrane attack complex
b. membrane attack complex cannot get inserted in the nucleated cell membrane
c. membrane attack complex can get inactivated by the nucleated cells
d. membrane attack complex get inactivated hence cannot get inserted in the nucleated cell membrane

Q14. Choose the correct match from A, B, C and D

Group I	*Group II*
P. IgA	1. Basophils
Q. IgE	2. δ heavy chain
R. IgG	3. Secretory component
S. IgM	4. Pentamer
	5. Crosses placenta

a. P-3, Q-1, R-5, S-4
b. P-3, Q-5, R-2, S-1
c. P-2, Q-3, R-5, S-4
d. P-2, Q-1, R-3, S-5

Q15. Choose the correct combination of statements from the following for MHC II protein
P. has α_1, α_2, α_3 and β_2 domains
Q. α_1 and β_1 are the polymorphic domains
R. involved in presenting antigen to cytotoxic T cells
S. only α-chain is glycosylated
a. QR b. RS
c. PS d. PQ

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Chapter - 13

Classical Genetics

13.1 MENDELIAN GENETICS

All living organisms reproduce. Reproduction results in the formation of offspring of the same kind. However, the resulting offspring need not and most often do not totally resemble the parent. Several characteristics may differ between individuals belonging to the same species. These differences are termed *variations.* The mechanism of transmission of characters, resemblances as well as differences, from the parental generation to the offspring, is called as *heredity.* The scientific study of heredity, variations and the environmental factors responsible for these, is known as *genetics* (from the Greek word *genno* = give birth). The word *genetics* was first suggested to describe the study of inheritance and the science of variation by the prominent British scientist William Bateson.

Gregor Johann Mendel (1822-1884) known as *Father of Genetics* was an Austrian monk. In 1856 he published the results of *hybridization experiments* titled *Experiments on Plant Hybrids* in a journal "The proceding of the Brunn society of natural history" and postulated the principles of inheritance which are popularly known as **Mendel's laws.** But his work was largely ignored by scientists at that time. In 1900, the work of Mendel was independently rediscovered by three biologists- Hugo de Vries of Holland, Carl Correns of Germany and Erich Tschermak of Austria. Mendel did a statistical study (he had a mathematical background). He discovered that individual traits are inherited as discrete *factors* which retain their physical identity in a hybrid. Later these factors came to be known as *genes*. The term was coined by Danish botanist wilhelm Johannsen in 1909. A gene is defined as *unit of heredity* that may influence the outcome of an organism's traits.

Mendel's experiment

Mendel chose the garden pea, *Pisum sativum*, for his experiments since it had the following advantage.

1. Well defined discrete characters
2. Bisexual flowers
3. Predominantly self fertilization
4. Easy hybridization
5. Easy to cultivate and relatively short life cycle

Characters studied by Mendel

The characteristics of an organism are described as *characters* or *traits*. Mendel studied seven characters/ traits (all having two variants). These characters are

		Dominant	*Recessive*
1.	Stem length	Tall	Dwarf
2.	Flower position	Axial	Terminal
3.	Flower color	Violet	White
	Seed coat color	Grey	White
4.	Pod shape	Inflated	Constricted
5.	Pod color	Green	Yellow
6.	Cotyledon color	Yellow	Green
7.	Seed form	Round	Wrinkled

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Flower color is positively correlated with seed coat colors. Seeds with white seed coats were produced by plants that had white flowers and those with gray seed coats came from plants that had violet flower.

What is Alleles?

Alleles are alternative forms of a gene which is present at same locus. In other words, alleles mean a particular form of a gene. We may also define alleles as genes occupying corresponding positions on homologous chromosomes and controlling the same characteristic (e.g., height of plant) but producing different effects (tall or short).

Wild-type versus Mutant alleles

Prevalent alleles in a population are called **wild-type alleles**. These typically encode proteins that are made in the right amount and function normally. Alleles that are present at less than 1% in the population and have been altered by mutation are called **mutant alleles**. Such alleles *usually* result in a reduction in the amount or function of the wild-type protein and are most often inherited in a recessive fashion.

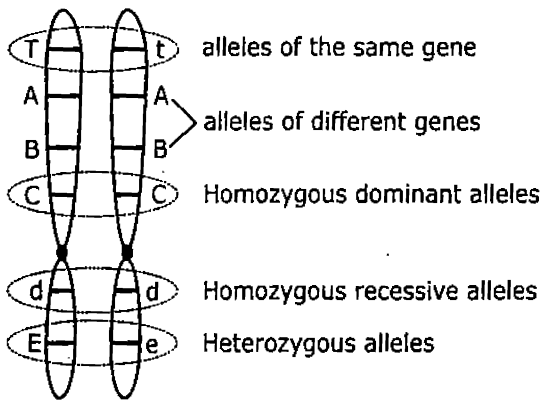
Dominant and Recessive alleles

A *dominant allele* masks or hides expression of a recessive allele and it is represented by an *uppercase letter.* A *recessive allele* is an allele that exerts its effect only in the *homozygous state* and in heterozygous condition its expression is masked by a dominant allele. It is represented by a *lowercase letter* (t).

Homozygous and Heterozygous

Each parent (diploid) has two alleles for a trait — they may be

1. **Homozygous**, indicating they possess two identical alleles for a trait.
   a. *Homozygous dominant* genotypes possess two dominant alleles for a trait (TT).
   b. *Homozygous recessive* genotypes possess two recessive alleles for a trait (tt).
2. **Heterozygous** genotypes possess one of each allele for a particular trait (Tt).



GENOTYPE AND PHENOTYPE

To distinguish physical appearance from genetic constitution two different terms are used in genetics i.e., *genotype* and *phenotype*. The genotype is defined as the genetic constitution of an individual for any particular character or trait. The genotype of an individual is usually expressed by a symbol e.g., tt, Tt or TT etc. The phenotype is defined as the physical appearance of an individual for any particular trait. The phenotype of an individual is dependent on its genetic constitution.

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MENDEL'S LAWS OF INHERITANCE

On the basis of hybridization experiment on *Pisum sativum,* Mendel proposed the principles of inheritance known as Mendel's laws:

Law of segregation

On the basis of *monohybrid cross* (a cross involving only one trait) Mendel formulated the *law of segregation*. This law states that each individual possesses two factors (later termed as *genes*) for a particular character. At the time of formation of gametes each member of the pair of genes separate from one another so that each gamete carries only one factor (gene) i.e., gametes are always pure (*law of purity of gametes*). It also explains that hereditary factors are discrete and don't blend when present together. Law of segregation applies only to diploid organisms that form haploid gamete to reproduce sexually.

Explanation

Let's use Mendel's cross of tall and dwarf pea plants as an example. The letters T and t are used to represent the alleles of the gene that determine plant height; by conventions the upper case letter represents the dominant allele and the recessive allele is represented by the same letter in lower case. For the P cross, both parents are true breeding plants, the tall plant is homozygous for the tall allele 'T', while the dwarf plant is homozygous for the dwarf allele 't'.

Mendel tracked each trait through two generations. *P generation* is the parental generation in a breeding experiment. When true breeding plants were crossed to each other, this is called a P cross and offspring comprise the *first filial* or *F1 generation*. When the members of the F1 generation were crossed, this produced the F2 generation or second filial generation. A cross between true breeding tall and dwarf plants of P generation yield phenotypically tall plants. Now understand the reason why all the plants were tall in F1 generation which were obtained by crossing of pure tall with pure short plants.

To determine the kind and frequencies of the various types of offspring expected we usually use *Punnett squares* (used to predict the outcome of simple genetic crosses, proposed by R. Punnett). The genetic constitution of gametes of one sex are kept on top of squares and those of other sex on one side. The genetic constitution of all possible zygote is then entered in squares of the grid.

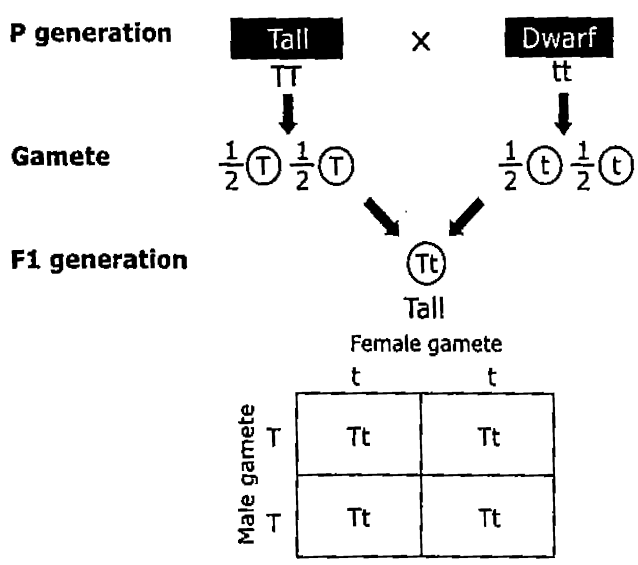


Figure : 13.1
The monohybrid cross between tall and dwarf pea plants. The symbols T and t designate the tall and dwarf unit factors, respectively, in the genotypes of mature plants.

At the time of formation of gametes members of the pair of genes separate from each other. The number of chromosomes reduces to half in gametes. Thus the pair of genes present at homologous loci separates from each other so that each gamete carries only one gene, never both as depicted in figure 13.1.

When plants with these genetic constitution are crossed (mated), the offspring get 'T' gene from one parent and 't' gene from the other. All the hybrid plants of F1 generation are genetically heterozygous with the genotype Tt.

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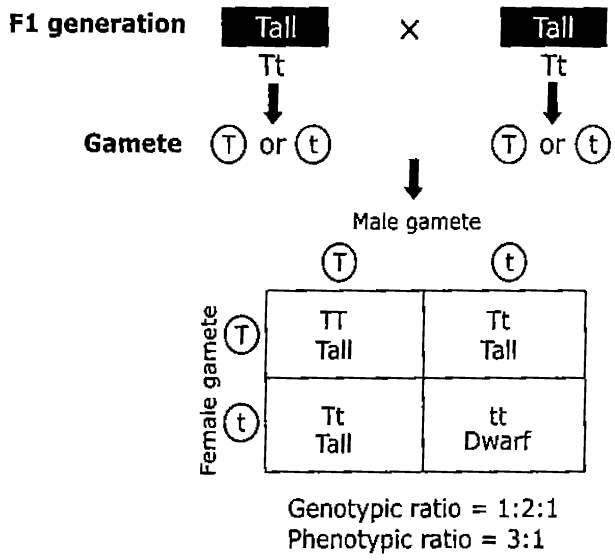


Figure : 13.2
The use of a Punnett square in generating the F2 ratio from the F1 × F1 cross.

Though all the hybrid plants of F1 generation are genetically Tt but all these plants were tall similar to the pure bred tall plants with genetic constitution TT. Thus we see that though their physical appearance is same (all tall) but their genetic constitution has different TT and Tt.

When plants of F1 generation were allowed to self pollinate, both tall and short plants appeared in the second generation (F2). From above figure 13.2 it is evident that all the plants of F1 generation (hybrid) were tall but in F2 generation both tall and short plants appeared. This indicates that the character of tallness dominates or conceals the character of shortness in first generation so that the character of shortness could not appear at all. Thus the character of tallness which expressed itself in heterozygous condition (Tt) in F1 generation is considered as **dominant** character and gene 'T' is considered as dominant gene. On the other hand the character for shortness, which could not express itself in F1 generation, is considered as **recessive** and gene 't' is considered as recessive gene. Thus a recessive gene 't' can not express itself when it is in single dose. However, the character of shortness reappeared in F2 generation where genes were in double dose (tt) and the dominant gene (T) was absent. Genotypic ratio 1:2:1 or phenotypic ratio of 3:1 (classical Mendelian ratio) provides the good test of Mendel's law of segregation.

Mendel's 3:1 phenotypic ratio of monohybrid cross states that

a. F1 hybrids contained two factors for each trait, one dominant and one recessive;

b. Factors separated when gametes were formed; a gamete carried one copy of each factor;

c. Random fusion of all possible gametes occurred upon fertilization.

Test cross

A **test cross** is between an individual with dominant phenotype and individual with recessive phenotype to see if the individual with dominant phenotype is homozygous or heterozygous.

For example, consider the case in which a test cross is performed with a dwarf pea plant and a tall pea plant of unknown genotype.

Scenario A: *The tall pea plant is homozygous.*

P	Tall plant (TT)	×	Dwarf plant (tt)
Gametes	only T		only t
F1		Tt (tall)	

Conclusion: The tall plant must be producing *only* one kind of gamete and therefore it is homozygous dominant (TT).

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Scenario B: *The tall pea plant is heterozygous.*

P	tall plant (Tt)	×	dwarf plant (tt)
Gametes	T and t		only t
F1	1Tt (tall) : 1tt (dwarf)		

Conclusion: The tall plant must be producing two kind of gamete and therefore it is heterozygous dominant (TT).

Law of independent assortment

The law of independent assortment was deduced from Mendel's experiment with dihybrid cross. A cross that involves the analysis of two independent traits is termed a *dihybrid cross*.

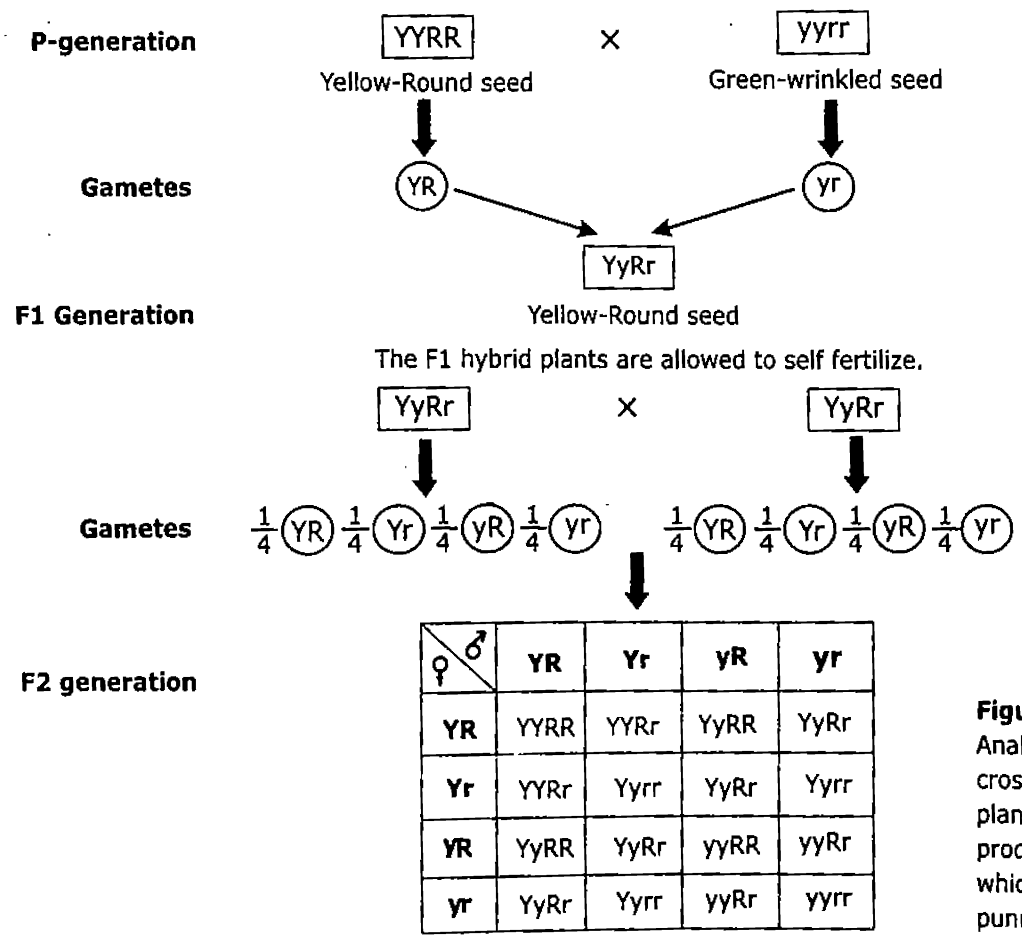
This law states that assortment of genes of one pair is independent of the other pair at the time of gametogenesis i.e., each pair of contrasting character behaves independently and bears no association with a particular character. Because of such independent assortment of characters, new combination of characters are produced in offspring.

Explanation

Suppose crosses are made between a pea plant with round and yellow seeds and one with wrinkled and green ones. The all F1 hybrids give yellow and round seeds. Since yellow colour is dominant over the green and the round shape is dominant over the wrinkled. When the F1 hybrid plants are crossed to each other or allowed to self fertilize, an F2 generation forms as represented in the following figure 13.3.

Now we have one question. Is the segregation of the seed colour characters independent of that of the seed shape or are the two characters somehow tied together?

Since the original varieties crossed had yellow round and green wrinkled seeds respectively, will the yellow color continue to be associated with the round shape and the green color with the wrinkled shape of the seeds? or will all combinations of the characters appears.



♀ \ ♂	YR	Yr	yR	yr
YR	YYRR	YYRr	YyRR	YyRr
Yr	YYRr	Yyrr	YyRr	Yyrr
yR	YyRR	YyRr	yyRR	yyRr
yr	YyRr	Yyrr	yyRr	yyrr

Figure : 13.3
Analysis of the dihybrid crosses. The F1 heterozygous plants are self-fertilized to produce an F2 generation, which is computed using a punnett square.

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Outcome of the dihybrid cross make it very clear that segregation of the seed colour is independent of the seed shape and both the parental and new combinations of the characters appear in the F2 offspring. i.e., assortment of genes of one pair is independent of the other pair.

In the above figure 13.3, F1 hybrid plants (YyRr) produce four kinds of gametes in equal frequencies (YR, yR, Yr, yr). It can be figured out in following way

Allele 1	Allele 2	Gametes	Frequency
$\frac{1}{2}$ Y	$\frac{1}{2}$ R	YR	$\frac{1}{4}$
	$\frac{1}{2}$ r	Yr	$\frac{1}{4}$
$\frac{1}{2}$ y	$\frac{1}{2}$ R	yR	$\frac{1}{4}$
	$\frac{1}{2}$ r	yr	$\frac{1}{4}$

These gametes then randomly combine in the F1 cross. The use of a punnett square helps to demonstrate all 16 possible combinations of these gametes.

Summary of F2 generation

Genotype	Phenotype	Number
RRYY	Round Yellow	1 (1/16)
RRYy	Round Yellow	2 (2/16)
RRyy	Round Yellow	1 (1/16)
RrYY	Round Yellow	2 (2/16)
RrYy	Round Yellow	4 (4/16)
Rryy	Round Green	2 (2/16)
rrYY	Wrinkled Yellow	1 (1/16)
rrYy	Wrinkled Yellow	2 (2/16)
rryy	Wrinkled Green	1 (1/16)

The phenotype in this conventional dihybrid cross give a 9:3:3:1 ratio

$\frac{9}{16}$ Round and yellow $\frac{3}{16}$ Wrinkled and yellow $\frac{3}{16}$ Round and green $\frac{1}{16}$ Wrinkled and green

From above results it is evident that each pair of contrasting characters behaves independently and bears no association with a particular character.

INCOMPLETE DOMINANCE

Incomplete dominance or partial dominance or semidominance is a condition when neither allele is dominant over the other. The condition is recognized by the heterozygotes expressing an intermediate phenotype relative to the parental phenotypes. For example in four O'clock plants (*Mirabilis jalapa*) when red flowered plant is crossed with white flowered one, pink flowered F1 plants obtained. If pink flowered F1 plants are crossed, the F2 plants appear in a phenotypic ratio of 1:2:1, having red, pink and white. In this case red colour producing gene is not completely dominant over allele producing white colour. So, genotypic and phenotypic ratio is same and equal to 1:2:1.

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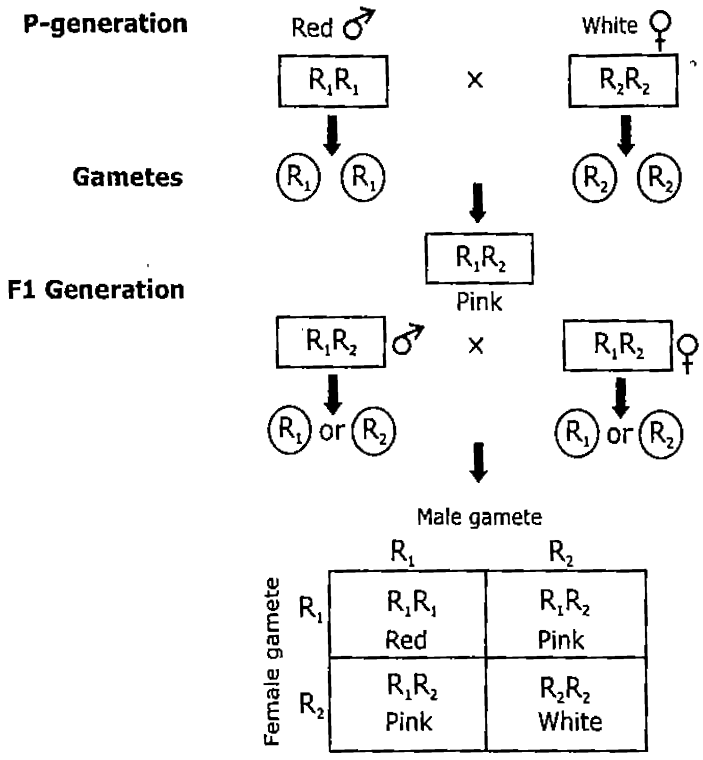


Figure : 13.4
Incomplete dominance in *Mirabilis jalapa* show outcome of pink colour

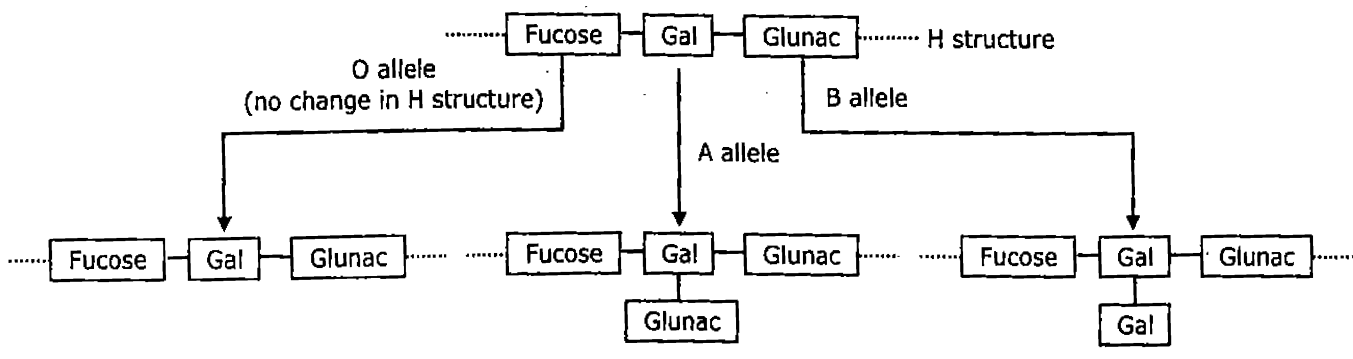
CODOMINANT ALLELES

Alleles that lack dominant and recessive relationship and are both observed phenotypically to same degree are called *codominant*. So, the phenotypic effect of each allele is observable in the heterozygous condition. The heterozygous genotype gives rise to a phenotype distinctly different from either of the homozygous genotype. The alleles governing the M-N blood group in human are example of codominant allele. In this case genotype and phenotype ratio remain same.

MULTIPLE ALLELES

In each of the seven pairs of characters studied by Mendel, there were only two alternative forms for each character. When more than two different forms of a given gene exists in a species, they are referred to as multiple alleles. The number of different genotypes possible among diploid organisms is a function of the number of alleles that exists for any given gene. If n is the number of alleles of a gene, the number of different genotypes possible is $n(n+1)/2$.

A classical example of multiple alleles is found in the ABO blood group system of human. The I^A and I^B alleles are responsible for the production of the A and B antigens found on the surface of the RBC. The I^A and I^B alleles coding for *glycosyl transferase* enzyme, each cause a different modification to the terminal sugar of a mucopolysaccharide (H-structure) found on the surface of red cells.



Gal = Galactose ; Galnac = N-Acetylgalactosamine ; Glunac = N-Acetylglucosamine

Figure 13.5 : Function of the A, B, and O alleles of the ABO gene.

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The allele I^A for the A antigen is codominant with the allele I^B for the B-antigen. Both I^A and I^B are completely dominant to the allele i, which fails to specify any detectable antigenic structure. The hierarchy of dominance relationship is symbolized as $(I^A=I^B)>$ i. I^A and I^B alleles produce slightly different glycoproteins (antigen) that are on the surface of each cell. Homozygous A individuals have only the A-antigen, homozygous B individuals have only the B-antigen, homozygous O individuals produce neither antigen, while a fourth phenotype (AB) produces both A and B antigens.

Genotype	Reaction with		Blood group (Phenotype)
	Anti A	*Anti B*	
I^AI^A, I^Ai	+	–	A
I^BI^B, I^Bi	–	+	B
I^AI^B	+	+	AB
i i	–	–	O

Rh blood group

The Rh blood group is so named because the antigen was discovered in the blood of the *Rhesus* monkey. The alleles of three genes (C, D and E) may code for the Rh antigen. People whose RBCs have Rh antigens are designated *Rh positive* and those who lack Rh antigens are designated *Rh negative*. Normally, blood plasma does not contain anti-Rh antibodies. If Rh negative person receives an Rh positive transfusion, however, the immune system starts to make anti-Rh antibodies that will remain in the blood. If a second transfusion of Rh positive blood is given later, the previously formed anti-Rh antibodies will cause agglutination and hemolysis of the RBCs in the donated blood, and a severe reaction may occur.

Table 13.1 : Multihybrid self-fertilization, where n equals number of gene segregating two alleles each

	Monohybrid	**Dihybrid**	**Trihybrid**	**General rule**
	n = 1	n = 2	n = 3	
Number of gametes	2	4	8	2^n
Proportion of recessive homozygotes among the F2 individuals	1/4	1/16	1/64	$1/(2^n)^2$
Number of different F2 Phenotypes (*given complete dominance*)	2	4	8	2^n
Number of different genotype (or *phenotypes if there is no dominance*)	3	9	27	3^n

Lethal alleles

Certain genes are absolutely essential for survival. The alleles created by mutations in these genes are called *lethal alleles*. The phenotypic manifestation of these genes is the death of the individual organism. Hence lethal alleles can be defined as any gene whose expression results in death of an organism at any stage of life usually during embryogenesis. Lethal genes may be *recessive* or *dominant* lethal alleles. Recessive lethal alleles are lethal when present in homozygous conditions whereas dominant lethal alleles show lethal effects even in heterozygous conditions. Dominant lethal alleles are very rare.

Lethal alleles fall into four categories:

- *Early onset* : Genes which result in early death of an organism, during embryogenesis.
- *Late onset* : Lethal genes which have delayed effect so that the organism can live for some time but eventually succumb to the disease.

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- *Conditional* : Such lethal genes kill organism under certain environmental conditions only. For example temperature sensitive lethal allele kills organism at 30°C but not at 22°C.
- *Semilethal* : These mutants kill only some individuals in the population but not all.

Penetrance and expressivity

The percentage of individuals that shown a particular phenotype among those capable of showing it is known as *penetrance*. Let us take example of polydactyly in human which is produced by a dominant gene. Homozygous recessive genotype not cause polydactyly. However, some heterozygous individuals are not polydactylous. If suppose 20% of heterozygous individuals do not show polydactyly, this means genes has a penetrance of 80%. Degree of expression of a trait controlled by a gene. A particular gene may produce different degrees of expression in different individuals. This is known as *expressivity*.

Phenocopy

A phenotype that is not genetically controlled but looks like a genetically controlled phenotype is called *phenocopy*. It is an environmentally induced phenotype that resembles the phenotype determined by the genotype. An example of a phenocopy is Vitamin-D-resistant rickets. A dietary deficiency of vitamin D, for example, produces rickets that is virtually indistinguishable from genetically caused rickets.

PROBABILITY

The chance that an event will occur in the future is called the event's **probability**. For example, if you flip a coin, the probability is 0.50, or 50%, that the head side will be showing when the coin lands. Probability depends on the number of possible outcomes. In this case, there are two possible outcomes (heads and tails), which are equally likely. This allows us to predict that there is a 50% chance that a coin flip will produce heads. The general formula for probability is

$$\text{Probability} = \frac{\text{Number of times an event occurs}}{\text{Total number of events}}$$

$$P_{heads} = 1 \text{ heads}/(1 \text{ heads} + 1 \text{ tails}) = 1/2 = 50\%$$

A probability calculation allows us to predict the likelihood that an event will occur in the future. The accuracy of this prediction, however, depends to a great extent on the size of the sample. For example, if we toss a coin 6 times, our probability prediction would suggest that 50% of the time we should get heads (i.e., 3 heads and 3 tails).

In genetic problems, we are often interested in the probability that a particular type of offspring will be produced. For example, when two heterozygous tall pea plants (Tt) are crossed, the phenotypic ratio of the offspring is 3 tall : 1 dwarf. This information can be used to calculate the probability for either type of offspring:

$$\text{Probability} = \frac{\text{Number of individuals with a given phenotype}}{\text{Total number of individuals}}$$

$$P_{tall} = 3 \text{ tall}/(3 \text{ tall} + 1 \text{ dwarf}) = 3/4 = 0.75 = 75\%$$

$$P_{dwarf} = 1 \text{ dwarf}/(3 \text{ tall} + 1 \text{ dwarf}) = 1/4 = 0.25 = 25\%$$

The probability of obtaining a tall plant is 75%, a dwarf plant 25%. When we add together the probabilities of all the possible outcomes (tall and dwarf), we should get a sum of 100% (here, 75% + 25% = 100%).

There are two laws of probability that are used for genetic analysis. The first law, the *Multiplicative law (product rule) of probability* states that the chance of two or more independent events occurring together is the product of the probability of the events occurring separately. For example, when a single coin is tossed repeatedly, the chance of heads occurring twice in succession would be $1/2 \times 1/2 = 1/4$. The chance of three such occurrences would be $1/2 \times 1/2 \times 1/2 = 1/8$.

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Similarly, when two coins are tossed together, the head-head combination is expected in one-fourth of the trial (1/2 × 1/2 =1/4) and the chance of occurrence of two such head-head combinations for two coins in succession would be 1/4 × 1/4 or 1/16.

The second law, the *Additive law* (*sum rule*) *of probability* calculates probability of an event that occurs in two or more independent ways; it is sum of individual probabilities of each way an event can occur.

We have already calculated that the probability of the head-head combination when two coins are tossed together is 1/4. Now we can calculate the probability of either two heads or two tails. Because these outcomes are mutually exclusive, the sum rule can be used to tell us that the answer is 1/2 + 1/2. This probability can be written as follows: p(two heads or two tails) = 1/2 + 1/2

Now we can consider a genetic example. Assume that we have two plants of genotypes *A/a*; *b/b*; *C/c*; *D/d* and *A/a*; *B/b*; *C/c*; *d/d* and that, from a cross between these plants, we want to recover a progeny plant of genotype *a/a*; *b/b*; *c/c*; *d/d*. If we assume that all the gene pairs assort independently, then we can do this calculation easily by using the product rule. The four different gene pairs are considered individually, as if four separate crosses, and then the appropriate probabilities are multiplied together to arrive at the answer. From *A/a* ×*A/a* , one-fourth of the progeny will be *a/a*; from *b/b* ×*B/b*, one-half of the progeny will be *b/b*; from *C/c* ×*C/c*, one-fourth of the progeny will be *c /c*; from *D/d* ×*d/d*, one-half of the progeny will be *d /d*. Therefore, the overall probability of progeny of genotype *a/a*; *b/b*; *c/c*; *d/d* will be 1/4 × 1/2 × 1/4 × 1/2 = 1/64.

STUDY QUESTION

Q1. An unaffected women (i.e., without disease symptoms) who is heterozygous for the X–linked trait Duchene's muscular dystrophy has children with a normal man. What are the probabilities of the following combinations of offspring?

a. an unaffected son

b. an unaffected son or daughter

c. a family of three children, all of whom are affected

Ans. a. 1/4

b. 3/4 (apply sum rule)

c. 1/4 X 1/4 X 1/4 =1/64 (apply product rule)

13.2 CHROMOSOMAL BASIS OF INHERITANCE

In 1902, *Walter S. Sutton* and *T. Boveri* proposed the *chromosomal theory of heredity*. The chromosomal theory of inheritance provides a way to explain how the cellular transmission or chromosomes passes genetic determinant (i.e., genes) from parent to offspring. According to this view

1. Chromosome contain the genetic material (genes) that is transmitted from parent to offspring.
2. Chromosomes are replicated and passed along generation after generation from parent to offspring.
3. The nuclei of most eukaryotic cells contain chromosomes that are found in homologous pairs (i.e., they are diploid). One member of each pair is inherited from the mother, the other from the father. At meiosis, one of the two members of each pair segregates into one daughter nucleus and the other segregates into different daughter nucleus. Therefore, gametes contain one set of chromosomes (i.e., they are haploid) as shown in figure 13.6.
4. During gamete formation, different types of chromosomes segregate independently of each other.

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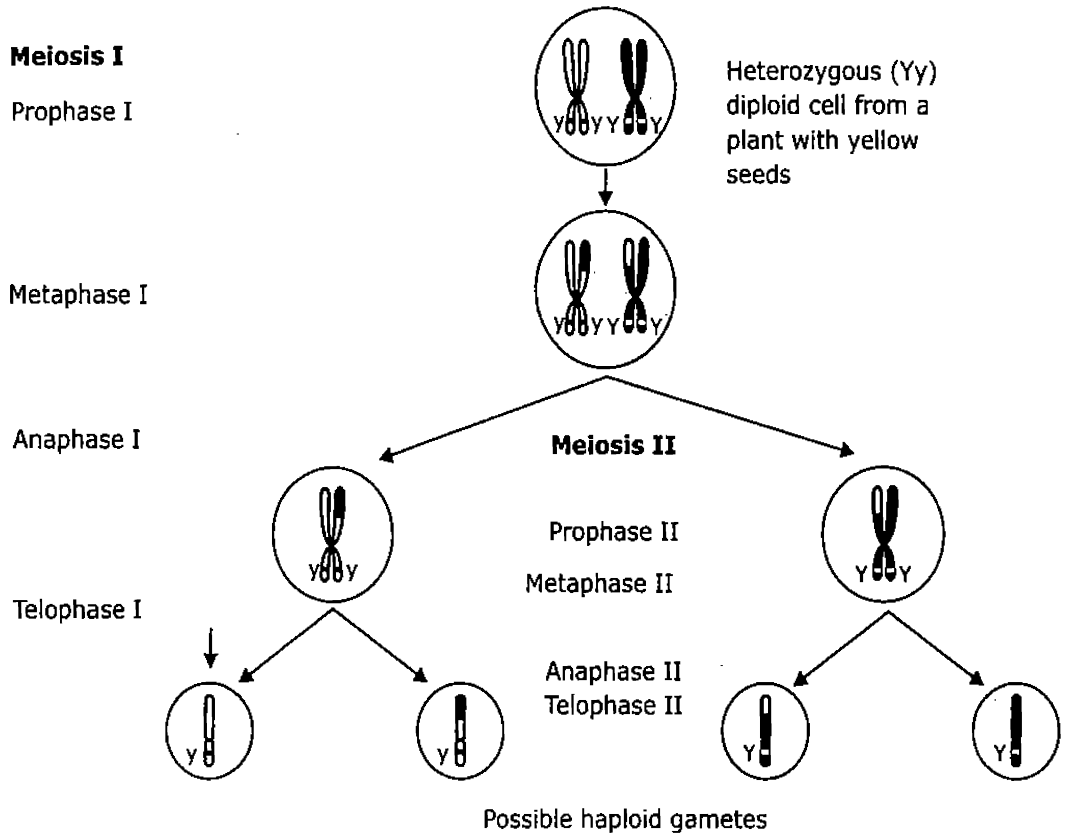


Figure 13.6 : Segregation of homologues during meiosis explains Mendel's law of segregation

5. Each parent contributes one set of chromosomes to its offspring.

   Hence, the chromosome theory of inheritance describes the relationship between Mendel's Law and chromosomal transmission.

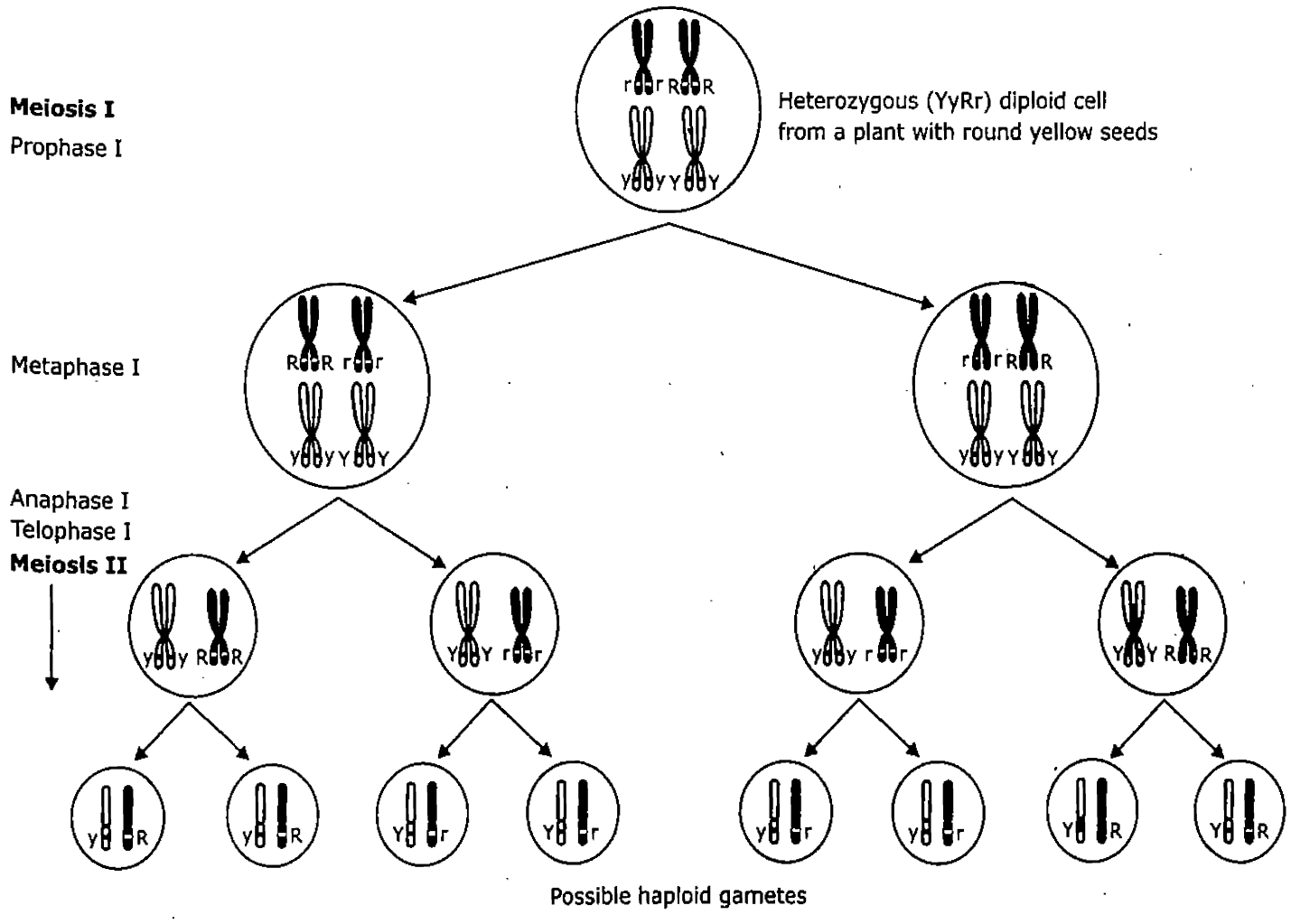


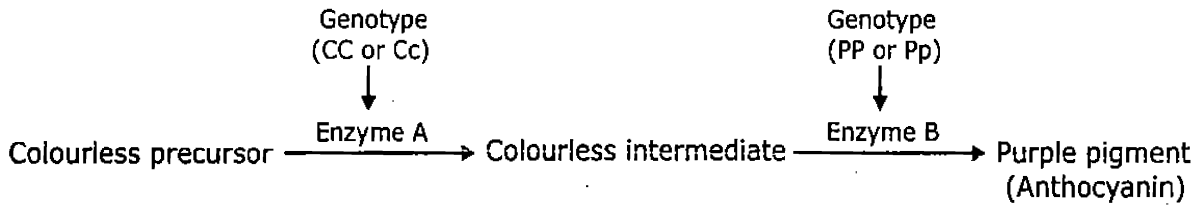
Figure 13.7 : Random alignment of tetrads during prophase of meiosis I explains Mendel's law of independent assortment

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13.3 GENE INTERACTION

According to Mendel, genes functioning independently of each other i.e., each of seven traits considered was controlled by a single gene. But many traits of an organism are determined by the complex contribution of many different genes. When two or more different genes (non-allelic) influence the outcome of single trait, this is known as a *gene interaction*.

The first case of two different genes interacting to affect a single trait was discovered by William Bateson and Reginald Punnett in 1906. Bateson and Punnett discovered an unexpected gene interaction when they studied crosses involving the sweet pea, *Lathyrus odoratus*. When they crossed true breeding purple flowered plant to a true breeding white flowered plant, the F1 generation was all purple flowered plants and the F2 generation (produced by self fertilization of the F1 generation) contained purple and white flowered plants in a 3:1 ratio. But when Bateson and Punnett crossed two different varieties of white flowered plant then all F1 generation plants had purple flowers when these purple flower plants were allowed to self fertilized, the F2 generation contained purple and white flowers in a ratio of 9 purple : 7 white. How can this unexpected result be explained? This surprising result was explained by Bateson and Punnett by considering the involvement of two different (non-allelic) genes because the F2 9:7 ratio is a variation of the 9:3:3:1 ratio. Let us consider the formation of the purple pigment in which products of two different genes are involved.



C (purple colour producing) allele is dominant to c (white)

P (purple colour producing) allele is dominant to p (white)

In the above pathway a colourless precursor molecule must be acted on by two different enzymes to produce the purple pigment. Gene C encodes a functional enzyme A, which converts the colourless precursor into a colourless intermediate and finally gene P encodes enzyme B, which gives purple colour by converting colourless intermediate. If any of these two genes will be in homozygous recessive condition (cc or pp) then purple colour will not appear. Thus the genotype cc can hide or mask the phenotype expression of genotype PP or Pp.

P generation White flowered plant (CCpp) × White flowered plant (ccPP)

F1 generation All purple (CcPp)

F1(CcPp) × F1(CcPp)

CCPP Purple	CCPp Purple	CcPP Purple	CcPp Purple
CCPp Purple	CCpp White	CcPp Purple	Ccpp White
CcPP Purple	CcPp Purple	ccPP White	ccPp White
CcPp Purple	Ccpp White	ccPp White	ccpp White

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The purple colour appears only when dominant alleles of both genes are present. When one or both genes have only recessive alleles, the colour will be white.

Epistasis

Epistasis (Greek for *standing upon*) is the term applied when one gene masks or inhibits the expression of another gene at distinct locus. Any gene that masks the expression of another nonallelelic gene is epistatic to that gene. The gene suppressed is hypostatic. In the pathway discussed for formation of purple colour, when either is homozygous recessive (cc or pp) that gene is epistatic to the other.

Epistasis is different from dominance. Epistasis is the interaction between different genes (non-alleles). Dominance is the interaction between different alleles of the same gene i.e., intraallelic.

Table 13.2 : Comparisons between dominance and epistasis

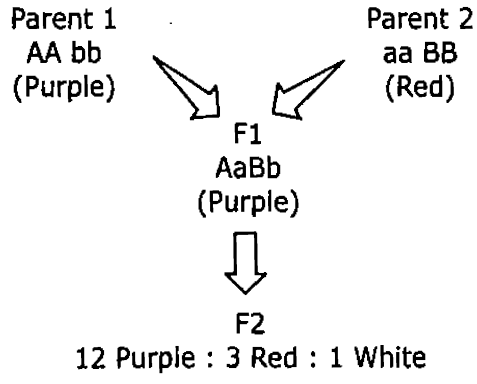
Dominance	**Epistasis**
1. Allelic suppression.	1. Non-allelic suppression
2. It involves single pair of alleles	2. It involves two pairs of alleles
3. A gene suppresses the expression of it's allele	3. A gene suppresses the expression of it's non-allele
4. The effect of only recessive allele is suppressed	4. Epistatic allele suppresses the effect of both dominant and recessive non-allele
5. The effect is only due to dominant allele	5. Epistasis may be due to dominant or recessive allele.

Now the term epistasis has come to be synonymous with almost any type of gene interaction that involves the masking of one of the gene effects. When epistasis is operative between two gene loci, the number of phenotype appears in the offspring will be less than four (normal F2 phenotypic classes in case of dihybrid crosses is four, 9:3:3:1). Such bigenic (two gene) epistatic interactions may be of several types.

Dominant epistasis (12:3:1)

When out of two genes, the dominant allele (e.g., A) of one gene masked the activity of alleles of another gene (e.g., B) and expressed itself phenotypically, then A gene locus is said to be epistatic to the B gene locus. The dominant allele A can express itself only in the presence of either B or b allele, such type of epistasis is termed as *dominant epistasis*. Example : Colour of squash (12 white: 3 yellow: 1 green)

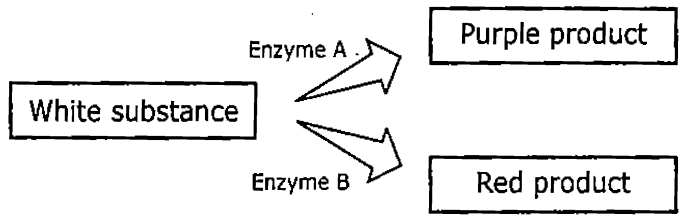
Explanation : Let us take the following case,



In this example two non-allelic genes are interactive because the F2 phenotypic class obtained is less than 4. This phenotypic ratio can be explained by following consideration

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In this case two enzymes A and B competes for the same substrate. Enzyme A which converts the substrate to a purple product has much higher affinity for substrate than enzyme B which converts the substrate to a red product. The difference in the affinity for the substrate is so marked that enzyme B can only work effectively if no enzyme A is present. So,

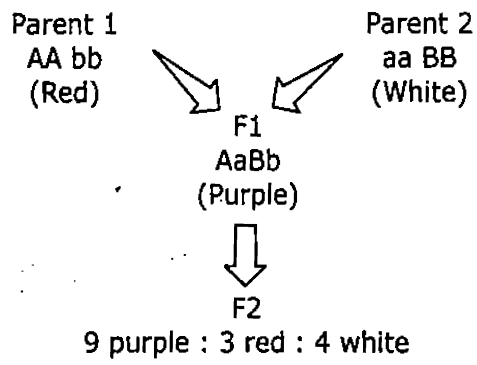
AABB(1), ABAb(2), AaBB(2) AaBb(4), AAbb(1), Aabb(2) *(Purple 12)*	These have at least one functional allele A and convert all the substrate to purple product.
aaBB(2), aaBb(1) *(Red 3)*	Lack any functional enzyme A, but have functional enzyme B which converts the substrate to a red product.
aabb(1) *(White 1)*	Have no functional enzymes and cannot synthesize any coloured pigment.

Recessive epistasis or **Supplementary genes** (9:3:4)

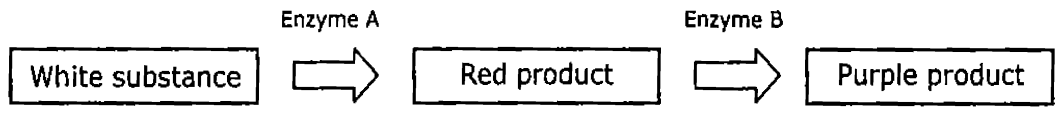
Supplementary genes are a pair of non allelic genes, of which one produces its phenotypic effect independently in dominant state, but another cannot produce phenotypic effect independently. However, later can produce its effect when they are together in dominant state. If there are A and B two non-allelic genes. A can produce phenotypic effect independently in dominant state but second gene B can produce its effect when they are present together (AB). Example : Coat colour of mouse (9 agouti: 3 black: 4 albino)

Explanation

Let us take the following case,



In this example the biochemical pathway would again be a simple chain, but in this case the product of enzyme A would be red in colour.



AABB(1), AaBB(2), AaBB(2), AaBb(4) *(Purple 9)*	have at least one functional copy of both A and B and therefore can synthesize the purple pigment.

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AAbb(2), Aabb(1) *(Red 3)*	have only functional enzyme A and produce red pigment but do not convert it to purple pigment.
aaBB(2), aaBb(1)	have no functional enzyme A and so cannot synthesize the red product that is the substrate for enzyme B and will remain white.
aabb(1) *(White 4)*	have no functional enzymes and cannot synthesize purple pigment.

Duplicate recessive interaction (9 : 7)

If both gene loci have homozygous recessive alleles and both of them produce identical phenotypes, the F2 ratio 9:3:3:1 would become 9:7. In such case, the genotype aaBB, aaBb, AAbb and aabb produce one phenotype. Both dominant alleles when present together, complement each other and are called **complementary genes** and produce a different phenotype.

Complementary versus supplementary gene

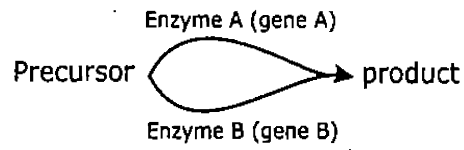
- In complementary gene, both the non-allelic pair independently produce similar phenotypic trait while in supplementary gene out of two non-allelic pair only one is able to express its effect independently.
- In complementary gene, both the non-allelic gene produce a completely new trait, while in supplementary gene interaction, character of the independently expressing gene is modified.

Duplicate dominant interaction (15:1)

If the alleles of both gene loci produce the same phenotype without cumulative effect, the 9:3:3:1 ratio is modified into a 15:1 ratio. This modified ratio was observed by G.H. Shull from his studies of seed capsule in shepherd's purse belonging to the genus *Capsella* (15 triangular : 1 ovoid).

Explanation

For example, if two different (non-allelic genes) code for enzymes that are able to catalyze the same reaction, such as by the following duplicate pathway one can easily see how a 15:1 F2 dihybrid phenotypic segregation ratio might result. If the recessive alleles a and b, of these two genes produce inactive forms of the two enzymes, a cross of aaBB×AAbb would yield a 15:1 F2 phenotypic segregation ratio.



Dominant and recessive interaction (13:3)

Sometimes, the dominant alleles of one gene locus (A) in homozygous (AA) and heterozygous (Aa) condition and the homozygous recessive alleles (bb) of another gene locus (B) produce the same phenotype, the F2 phenotypic ratio becomes 13:3 instead of 9:3:3:1. In such case, the genotype AA BB, AA Bb, Aa BB, Aa Bb, AA bb and Aa bb produce same phenotype and the genotype aa BB, aa Bb and aa bb produce another but same phenotype. Example : Colour of chicken (13 white : 3 coloured).

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Summary of different forms of gene interaction

Each gene pair affecting a different character

1. Complete dominance at both gene pairs

   Example : *Pisum sativum*

Phenotype classes	**Genotypes**
9 yellow round	AABB (1), AABb (2), AaBB (2), AaBb (4)
3 yellow wrinkled	AAbb (1), Aabb (2)
3 green round	aaBB (1), aaBb (2)
1 green wrinkled	aabb (1)
Epistasis	absent

Each gene pair affecting a same character

2. Complete dominance at both gene pairs, but one gene, when homozygous recessive, is epistatic to the other.

   Example : Mouse coat colour

   Gene pair A : Colour dominant over albino

   Gene pair B : Agouti colour dominant over black

Phenotype classes	**Genotypes**
9 agouti	AABB (1), AABb (2), AaBB (2), AaBb (4)
3 black	AAbb (1), Aabb (2)
4 albino	aaBB (1), aaBb (2), aabb (1)
Epistasis	aa Epistatic to B, b

3. Complete dominance at both gene pairs, but either recessive homozygote is epistatic to the effects of the other gene.

   Example : Sweet pea flower colour

   Gene pair A : Purple dominant over white

   Gene pair B : Colour dominant to white

Phenotype classes	Genotypes
9 purple	AABB (1), AABb (2), AaBB (2), AaBb (4)
7 white	AAbb (1), Aabb (2), aaBB (1), aaBb (2), aabb (1)
Epistasis	aa epistatic to B, b; bb epistatic to A, a

4. Complete dominance at both gene pairs, but one gene, when dominant, epistatic to the other gene.

   Example : Fruit colour in squash

   Gene pair A : White dominant over colour

   Gene pair B : Yellow dominant to green

Phenotype classes	**Genotypes**
12 white	AABB (1), AABb (2), AaBB (2), AaBb (4), AAbb (1), Aabb (2)
3 yellow	aaBB (1), aaBb (2)
1 green	aabb (1)
Epistasis	A epistatic to B, b

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5. Complete dominance at both gene pairs, but either gene, when dominant, epistatic to the other gene.

   Example : Seed capsules of Shepherd's purse

   Gene pair A : Triangular shape dominant to ovoid

   Gene pair B : Triangular dominant to ovoid

Phenotype classes	**Genotypes**
15 triangular	AABB (1), AABb (2), AaBB (2), AaBb (4), AAbb (1), Aabb (2), aaBB (1), aaBb (2)
1 ovoid	aabb (1)
Epistasis	A epistatic to B, b; B epistatic to A, a

6. Complete dominance at both gene pairs, but one gene, when dominant, epistatic to the second gene, and the second gene, when homozygous recessive, epistatic to the first.

   Example : Feather colur in fowl

   Gene pair A : Colour inhibition is dominant to colour appearance

   Gene pair B : Colour is dominant to white

Phenotype classes	**Genotypes**
13 white	AABB (1), AABb (2), AaBB (2), AaBb (4), AAbb (1), Aabb (2), aabb (1)
3 colour	aaBB (1), aaBb (2)
Epistasis	A epistatic to B, b; bb epistatic to A, a

PLEIOTROPY

Most of the biochemical pathways in the living organism are interconnected. Products and intermediates of one pathway may be used in several other metabolic processes. Hence the phenotypic expression of a gene usually effect more than one character/trait. The term *pleiotropy* refers to the effect of a single gene on more than one character/trait. Sometimes one trait will be very evident and other will be less evident. Numerous examples exist in which genes appear to have pleotropic effects. For example gene for white eyes in *Drosophila* also affect the shape of organs in female responsible for sperm storage as well as other structure. Other example is the *frizzle-trait* in chickens. The primary result of this gene is the production of defective feathers. Secondary results are both good and bad; good include increased adaptation to warm temperatures, bad include increased metabolic rate, decreased egg-laying, changes in heart, kidney and spleen. Similarly sickle-cell anemia is a human genetic disease. Sickle-celled individuals suffer from a number of problems, all of which are pleiotropic effects of the sickle-cell allele.

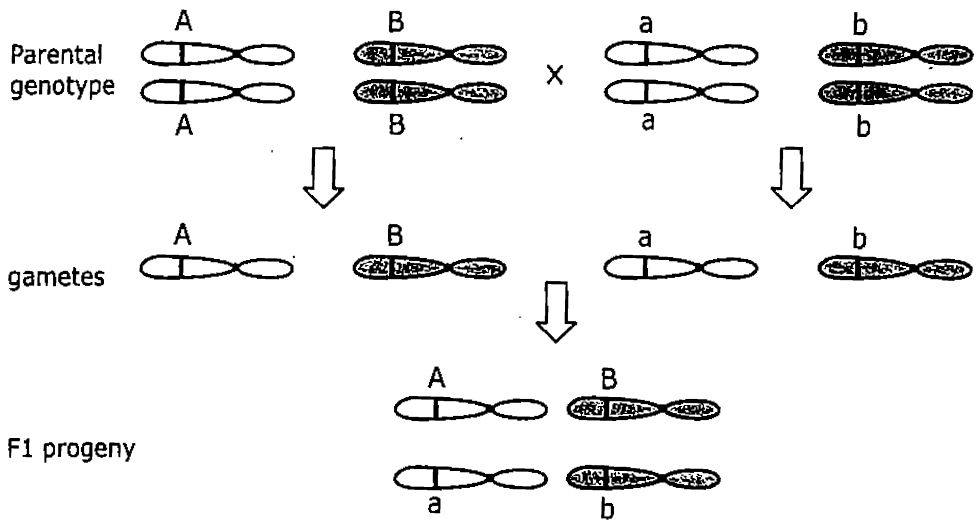
13.4 GENETIC LINKAGE AND GENE MAPPING

On the basis of his studies of garden peas in the 1850s, Gregor Mendel formulated some of the fundamental principles concerning the mechanisms of heredity.

First, he deduced that the units of inheritance are discrete, occur as pairs, and can exist in alternative forms. Later, by about 1905, the units of inheritance were called *genes* (by Johanson), and each variant of a gene was called an allele.

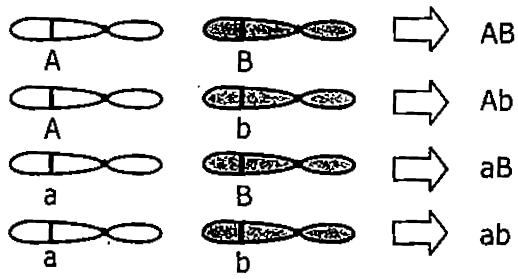
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Second, Mendel found that only one member of each pair of genes is distributed to a sex cell (gamete).



Third, he concluded that pairs of genes assort independently of one another, with the result that all possible genetic combinations will occur if enough offspring from a single genetically appropriate mating are sampled. If the two genes considered were located on different (nonhomologous) chromosomes then its crosses show independent assortment in above figure.

F1 progeny will produce gametes of following types in equal proportions (1:1:1:1).



When F1 progeny will be test crossed then the expected frequencies of test crossed (AaBb×aabb) progeny will be

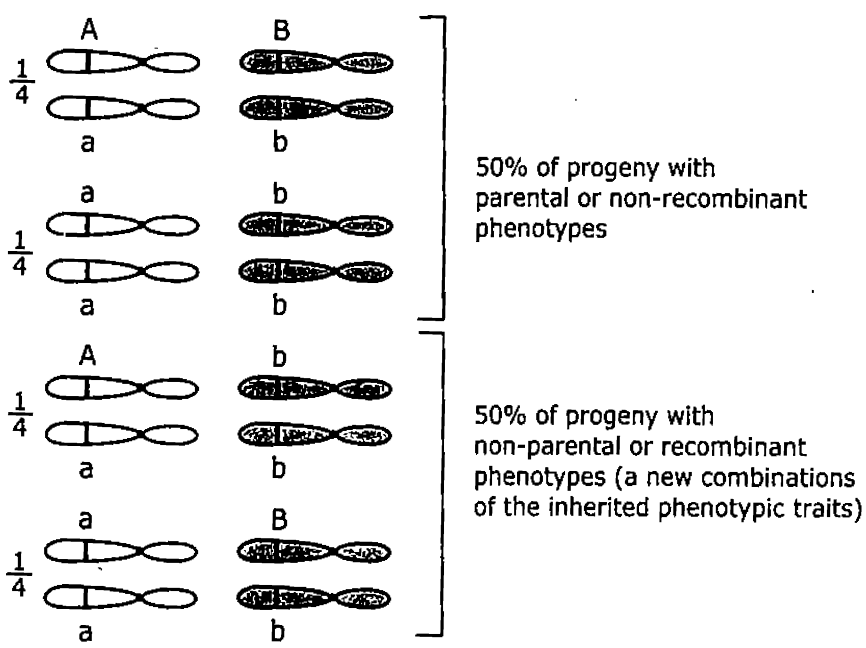
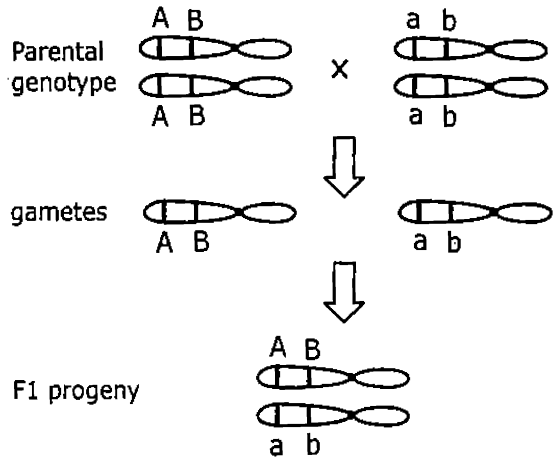


Figure 13.8 : Independent assortment of genes present on non-homologous chromosome

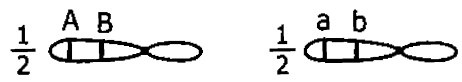
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When two or more genes reside on the same chromosome, they are said to be **linked** and their transmission pattern is called **linkage.** They may be linked together on one of the autosomes or connected together on the sex chromosome. Genes on different non-homologous chromosomes are distributed into gametes independently of one another (*Mendel's Law of Independent Assortment*). Gene on the same chromosome, however, tend to stay together during the formation of gametes. Thus, the results of testcrossing dihybrid individuals will yield different results, depending upon whether the genes are linked or on different chromosome.

Now, suppose that gene B is located on the same chromosome as gene A. So, in dihybrid cross the alleles of gene A and B will segregate in following way.



F1 progeny will form gametes of following two types and all are parental type i.e., AB, ab (no recombinant type i.e., Ab, aB gametes would be produced). A gamete that show the same configuration as the parent is referred to as *parental type* and where the configuration is altered, the gamete is referred to as a *recombinant type.*



A test cross (AaBb×aabb) of the F1 progeny would be

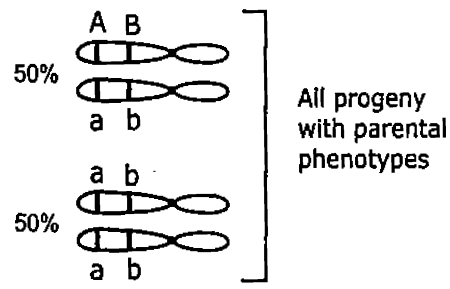


Figure 13.9 : Genes located on same chromosome shows complete linkage

So, genes located on the same chromosome thus tend to remain together and do not exhibit independent assortment. In the above illustrations (figure 13.9) genes a and b are completely linked and zero percent recombinant found. So linkage is considered as **complete linkage.**

Theoretically, if genetic linkage were complete (Complete linkage), then all genes along any chromosome would be passed on to the sex cells as an intact block, with no new genetic combinations on the chromosomes being formed during the meiotic process. However, complete linkage does not always prevent the formation of recombinant. In most instances test crosses involving two genes located on the same chromosome, gives not only parental phenotypes. Due to the process of crossing over even the genes present on same chromosome gives recombinant combination with recombination frequencies from 0 to 50% (two genes that

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undergo independent assortment indicated by a recombination frequency of 50%). 0% denotes complete linkage and no crossing over between two loci and 50% if far apart on the same chromosome.

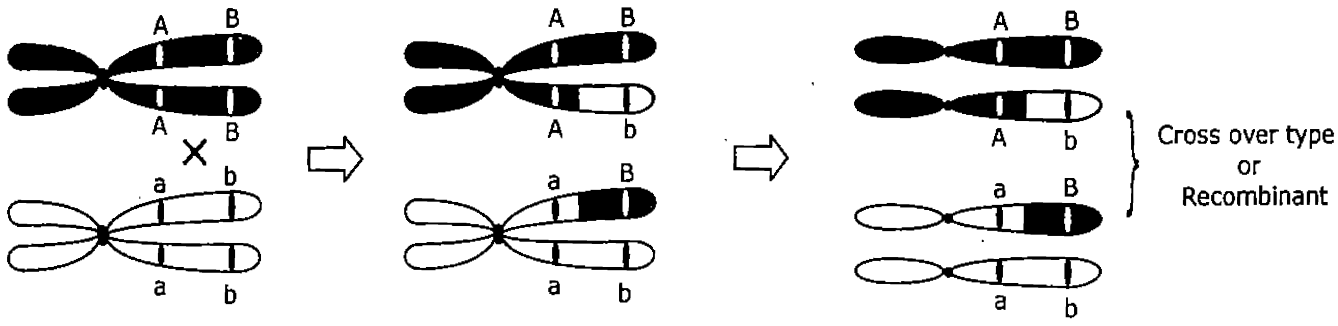


Figure 13.10 : Illustrating the occurrence of crossing over between two loci.

Now take one example

Two different traits affecting pod characteristics in garden pea plants are encoded by genes found on chromosome 5. Narrow pod is recessive to normal pod; yellow pod is recessive to green pod. A true-breeding plant with narrow, green pods was crossed to a true-breeding plant with normal, yellow pods. The F1 offspring were then test crossed to plants with narrow, yellow pods. The following results were obtained.

144 narrow green pods
150 normal yellow pods } 93.6% parental combination

11 narrow yellow pods
9 normal green pods } 6.36% recombinant

In this case, pea plants with narrow and green pods and normal and yellow pods show parental combinations. Pea plants with narrow and yellow pods and normal and green pods show recombinant combinations, because if genes for pod shape and colour are present on same (5^{th}) chromosome i.e., linked, recombinant will be $\leq 50\%$.

The effects of linkage were first evident in the result of a dihybrid cross in sweet pea by Bateson and Punnett in 1906. However, Bateson and Punnett did not interpret their results in terms of the behaviour of genes located on the same chromosome. When Bateson and Punnett conducted a cross in the sweet pea involving two different traits, flower color and pollen shape. Crossing was conducted between true-breeding strain with purple flowers (*PP*) and long pollen (*LL*) to a strain with red flowers (*pp*) and round pollen (*ll*). This yielded an F1 generation of plants that all had purple flowers and long pollen (*PpLl*). The unexpected result came from the F2 generation, the observed numbers of offspring did not confirm to a 9:3:3:1 ratio. Bateson and Punnett found that the F2 generation had a much greater proportion of the two parental types: purple flowers with long pollen, and red flowers with round pollen. Therefore, they suggested that the transmission of these two traits from the parental generation to the F2 generation was somehow coupled and not easily assorted in an independent manner. However, Bateson and Punnett did not realize that this coupling was due to the linkage of the flower color gene and the pollen shape gene on the same chromosome.

Morgan's work

Morgan was the first to discover the phenomenon of linkage. He crossed mutant *yellow* bodies and *white* eyes females with wild types male (*gray* bodies and *red* eyes). The F_1 females were wild type, whereas the F_1 males expressed both mutant traits. In the F_2 98.7% of the total offspring showed the parental phenotype – yellow bodied, white eyed flies and wild type flies (gray bodied, red eyed). The remaining 1.3% of

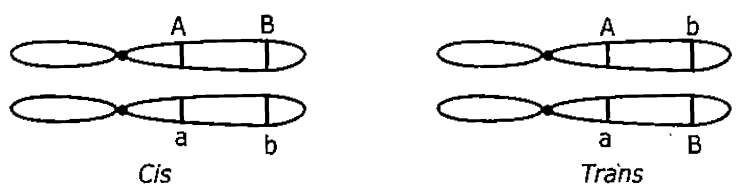
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the flies were either yellow bodied with red eyes or gray bodied with white eyes. Morgan proposed that 1.3% flies with recombinant feature develop as a result of separation of genes. Morgan postulated that separation of linked genes occur due to formation of **chiasmata** which represent points of genetic exchange. He used the term *crossing over* to describe the physical exchange leading to recombination. Morgan also proposed that two genes located relatively close to each other along a chromosome are less likely to have a chiasma form between them than if the two genes are farther apart on the chromosome.

Additional points about genetic linkage

First, to score the occurrence of new genetic combinations (recombinants), the genetic arrangement of one of the parents must be at least doubly heterozygous (AB/ab or Ab/aB).

Second, doubly heterozygous genotypes can be in two configurations. When two linked genes on each chromosome are of the same type (i.e., both dominant, AB, or both recessive, ab), this arrangement is called the **coupling** (*cis*) phase. Alternatively, when two linked genes on each chromosome are of different types (i.e., one dominant and one recessive allele, aB or Ab), the arrangement is the **repulsion** (*trans*) phase.



Third, recombination between two linked genes occur with the same frequency whether the alleles of the genes are in the *trans* or *cis* configuration.

Fourth, 0% recombination denotes complete linkage, and a value of 50% recombination between two loci means that the genes are either on different chromosomes or so far apart on the same chromosome that overall the numbers of even and odd crossovers between sites are the same.

Fifth, *Drosophila* is unusual in that crossing over (hence recombination) does not occurs in male. The result in that all allele located in a particular chromosomes show complete linkage in *Drosophila* males. Crossing over in also completely suppressed in female silkworms.

LIMITS OF RECOMBINATION

If two gene loci are so far apart in the chromosome that the probability of a chiasma forming between them is 100% then 50% of the gametes will be parental type (non-crossover) and 50% recombinant (crossover) type. When such dihybrid individuals are testcrossed, they are expected to produce progeny in a 1:1:1:1 ratio as would be expected for genes on different chromosomes. Recombination between two linked gene cannot exceed 50% even when multiple crossovers occur between them.

Calculation of recombinant frequency

Frequency of recombination can be calculated using following formula,

$$\text{Frequency of recombination (crossing over)} = \frac{\text{Number of Recombinant}}{\text{Total number of Progeny}} \times 100$$

LINKAGE GROUP

All the genes present on a particular chromosome form a **linkage group**. The number of linkage group of a species correspond to the total number of chromosomes of that species. For example,

In human male = 22 pairs autosomes + 1X chromosome + 1Y chromosome i.e., 24 linkage groups and in female = 22 pairs autosomes + 2X chromosomes i.e., 23 linkage groups.

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GENETIC MAPPING

The linkage of the genes in a chromosome can be represented in the form of a *genetic map* or *linkage map* or *chromosomal map*. The first individual to construct a genetic map was Alfred Sturtevant. He used the frequency of crossing over between two genes to prepare the first genetic map of X-chromosome of *Drosophila*.

There are two major aspects to genetic mapping:

1. The determination of the linear order with which the genetic units are arranged with respect to one another (gene order) and
2. The determination of the relative distance between the genetic units (gene distance).

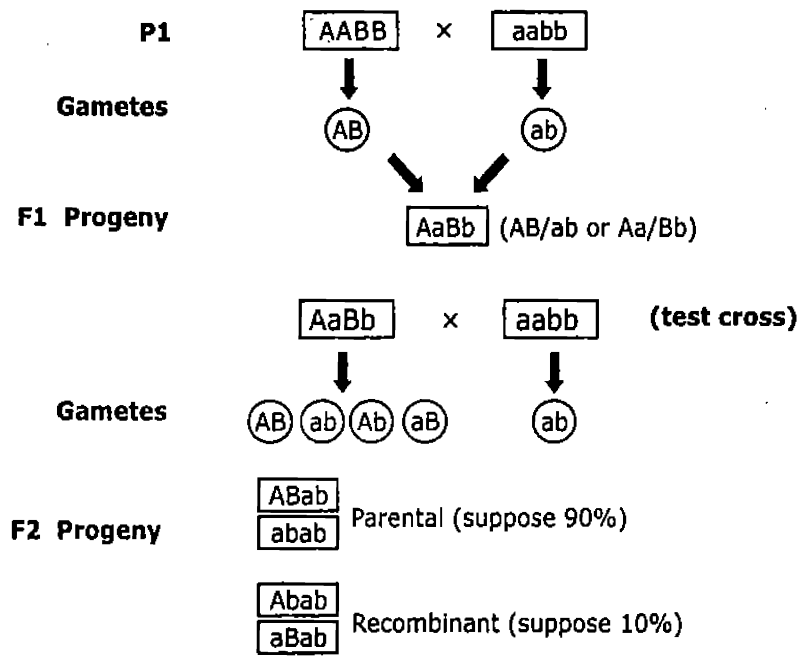
Because recombination tends to occur with increasing frequency as the distance between two specific gene loci increases, the percentage of recombination can be used to represent a measure of distance (map distance) between the two genes. The frequency of crossing over (% recombination) between two loci directly related to the physical distance between the loci. One unit of map distance is therefore equivalent to 1% crossing over or recombination. Map units are often referred to as **centimorgans** in honor of the work of Thomas Hunt Morgan, a famous *Drosophila* geneticist.

Thus, by analyzing recombination frequencies among the progeny of parents that are heterozygous for a number of linked genes, a genetic map that places the genes in a linear array can be constructed. The distance between loci represents the frequency of occurrence of recombination and is not equivalent to a precise physical distance. However approximations for the value of percentage recombination and number of base pairs of DNA have been deduced by corelating physical chromosome maps with genetic maps.

$$\text{So,} \quad \text{Map distance} = \frac{\text{Number of recombinant offspring}}{\text{Total number of offspring}} \times 100$$

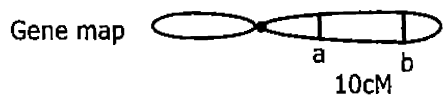
The unit of measurement for genetic linkage is known as a **map unit** or **centiMorgan** (cM). If two loci are 1 cM apart, then a cross-over occur between them during on average only one in every 100 meioses. cM is a measure of the genetic or linkage distance between two loci. One map unit is equal to 1 cM and 1 cM is equal to 1% recombination. Thus, two genes that recombine with a frequency of 3.5% are said to be located 3.5 map units apart. This is not the same as physical distance, which is measured in base pairs. In humans, 1 cM is roughly equivalent to 1×10^6 base pairs (bp).

Example,



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10% recombinant indicates that loci a (A) and b (B) are 10 map units apart,



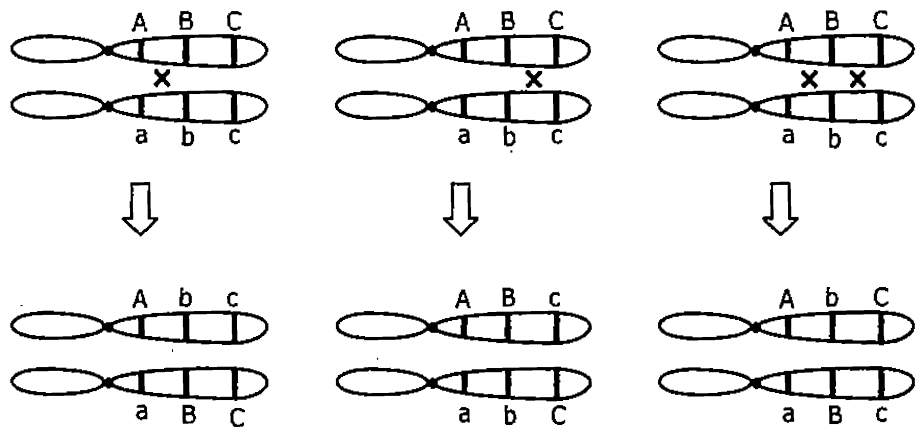
Genetic maps have limited accuracy because it is based on the assumption that crossing overs occur at random along chromosomes. This assumption is only partly correct because crossovers occurs with greater frequency at some points rather than at others.

GENE MAPPING FROM TWO-POINT CROSS

A cross involving two loci is usually referred to as a *two point cross*. The easiest way to detect crossover gametes in a dihybrid is through the testcross progeny. Suppose we testcross dihybrid individuals in coupling phase (AC/ac) and find in the progeny phenotypes 37% dominant at both loci, 37% recessive at both loci, 13% dominant at the first locus and recessive at the second, and 13% dominant at the second locus and recessive at the first. Obviously, the last two groups (genotypically Ac/ac and aC/ac) were produced by crossover gametes from the dihybrid parent. Thus, 26% of all gametes (13+13) were of crossover types and the distance between the loci A and C is estimated to be 26 map units, or 26 cM.

GENE MAPPING FROM THREE-POINT CROSS

By adding a third gene, we now have several different types of crossing over products that can be obtained. The following figure shows the different recombinant products that are possible.



Now if we were to perform a testcross with F_1, we would expect a 1:1:1:1:1:1:1:1 ratio. As with the two-point crosses, deviation from this expected ratio indicates that linkage is occurring.

Let us take an example of genes A, B, and C. We first make a cross between individuals that are AABBCC and aabbcc. Next the F_1 is test crossed to an individual that is aabbcc. We will use that following data to determine the gene order and linkage distances. As with the two-point data, we will consider the F_1 gamete composition.

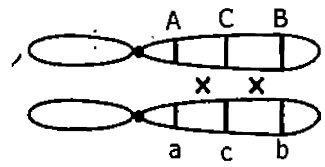
Genotype	**Observed**	**Type of Gamete**
ABC	390	Parental
abc	374	Parental
AbC	27	Single-crossover between genes C and B
aBc	30	Single-crossover between genes C and B
ABc	5	Double-crossover
abC	8	Double-crossover
Abc	81	Single-crossover between genes A and C
aBC	85	Single-crossover between genes A and C
Total	*1000*	

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The best way to solve these problems is to develop a systematic approach. First determine which of the genotypes are the parental genotypes. *The genotypes found most frequently are the parental genotypes.* From the above table it is clear that the ABC and abc genotypes were the parental genotypes.

Next we need to determine the order of the genes. Once we have determined the parental genotypes, we use that information along with the information obtained from the double-crossover. *The double-crossover gametes are always in the lowest frequency.* From the table the ABc and abC genotypes are in the lowest frequency. The next important point is that a double-crossover event moves the middle allele from one sister chromatid to the other. This effectively places the non-parental allele of the middle gene onto a chromosome with the parental alleles of the two flanking genes. We can see from the table that the C gene must be in the middle because the recessive c allele is now on the same chromosome as the A and B alleles, and the dominant C allele is on the same chromosome as the recessive a and b alleles.



Now we know the gene order i,e., ACB, we can go about determining the linkage distances between A and C, and C and B. The linkage distance is calculated by dividing the total number of recombinant gametes into the total number of gametes. This is the same approach we used with the two-point analyses that we performed earlier. What is different is the we must now also consider the double-crossover events.
So the distance between genes A and C is

$$\frac{(81+85+5+8)}{1000}\times 100 = 17.9\,\text{cM}$$

and the distance between C and B is

$$\frac{(27+30+5+8)}{1000}\times 100 = 7\,\text{cM}$$

Now we take other example. Suppose that we testcross trihybrid individuals of genotype ABC/abc and find in the progeny the following:

36% ABC/abc
36% abc/abc
72% Parental type

9% Abc/abc
9% aBC/abc
18% Single crossovers : between A and B (region I)

4% ABc/abc
4% abC/abc
8% Single crossovers : between B and C (region II)

1% AbC/abc
1% aBc / abc
2% Double crossovers

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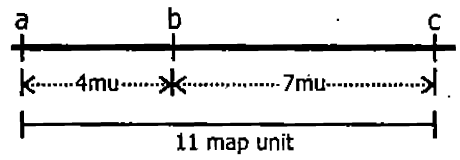
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To find the distance *A-B* we must count all crossovers (both singles and doubles) that occurred in region I = 18% + 2% = 20% or 20 map units between the loci *A* and *B*. To find the distance *B – C* we must count all crossovers (both singles and doubles) that occurred in region II = 8% + 2% = 10% or 10 map units between the loci *B* and *C*. The *A–C* distance is therefore 30 map units when double crossovers are detected in a three-point linkage experiment and 26 map units when double crossovers are undetected in the two-point linkage experiment above.

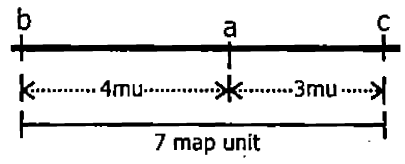
GENE ORDER

Map distances between two genes allows us to place genes in their proper linear order. Assume that the recombination frequency between genes a and b is 4% and frequency between b and c is 7%. The order of the gene can be either abc or bac.

Case I : If gene b is in between a and c



Case II : If gene a is in between b and c



Which of the above alternative orders is correct can be determined by a separate management of the recombination frequency between a and c; i.e., if a and c are found to recombine with a frequency of 11%, we can deduce that b is located between them (Case I). On the other hand, if a and c recombine with a frequency of 3%, we would conclude that the order of the gene is bac (Case II).

INTERFERENCE AND COINCIDENCE

Crossing over does not occur uniformly along a chromosome and also the formation of one chiasma reduces the probability of another chiasma formation in an immediately adjacent region of the chromosome. This lack of independence is known as **interference.** The net result of interference is the observation of fewer double cross over types than would be expected according to true map distances. This phenomenon was first time observed by H. J. Muller in 1916, also termed as **chiasma interference.** Interference varies in different sections of the chromosome and is measured by the **coefficient of coincidence.** Coefficient of coincidence is the ratio between the observed and the expected double crossovers. The expected double crossovers are equal to frequency of crossing over in the first region × frequency of crossing over in the second region × number of progeny.

So, Coincidence + Interference = 1.0

If coefficient of coincidence is

equal to 0; then interference is complete, no double crossovers are observed.

between 0 and 1 indicate partial interference.

equal to 1; then there is no interference, the expected double crossovers are observed

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STUDY QUESTION

Q2. An individual heterozygous for four genes. A/a . B/b . C/c is testcrossed to a/a . b/b . c/c and 1000 progeny are classified by the gametic contribution of the heterozygous parent as follows:

a . B . C	42
A . b . c	43
A . B . C	140
a . b . c	145
a . B . c	6
A . b . C	9
A . B . c	305
a . b . C	310

Draw a linkage map of the linked genes, showing the order and the distance in map units. Calculate an interference value, if appropriate.

Ans. According to data given in the question, A B c and a b C are parental types (most frequent) and a B c and A b C are double recombinants types (least frequent). The gene order can be determined either by the map distances or by comparing double recombinants with the parentals. The gene order is B A C. Now we can rewrite the progeny, this time putting the genes in the proper order, and classify the progeny

B a C	42	CO A–B
b A c	43	CO A–B
B A C	140	CO A–C
b a c	145	CO A–C
B a c	6	DCO
b A C	9	DCO
B A c	305	parental
b a C	310	parental

To construct the map of these genes, we can use the following formula:

$$\text{Distance between two genes} = \frac{(100\%)\ (\text{number of single CO} + \text{number of DCO})}{\text{total number of progeny}}$$

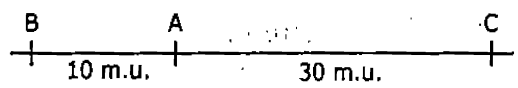
For the A to B distance:

$$= \frac{(42+43+6+9)}{1000} \times 100 = 10 \text{ m.u.a}$$

For the A to C distance:

$$= \frac{(140+145+6+9)}{1000} \times 100 = 30 \text{ m.u.}$$

The map is:



Interference = 1 – (observed DCO/expected DCO)

= 1 – (6 + 9)/[(0.10) (0.30) (1000)]

= 1 – 15/30 = 0.5

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GENETIC MAPPING IN HAPLOID ORGANISMS (TETRAD ANALYSIS)

Certain species of lower eukaryotes, particularly unicellular algae and fungi, which spend the greatest part of their life cycle in the haploid state, have also been used in mapping studies. The sac fungi (ascomycetes) have been particularly useful to geneticists because of their unique style of sexual reproduction.

Fungi may be unicellular or multicellular. Fungal cells are typically haploid (1n) and can reproduce asexually. In addition, fungi can also reproduce sexually by the fusion of two haploid cells to create a diploid zygote (2n). The diploid zygote can then proceed through meiosis to produce four haploid cells, which are called **spores.** This group of four spores is known as a **tetrad.** In some species, meiosis is followed by a mitotic division to produce eight cells, known as an **octad**. The cells of a tetrad or octad are contained within a sac known as an **ascus** (plural, asci).

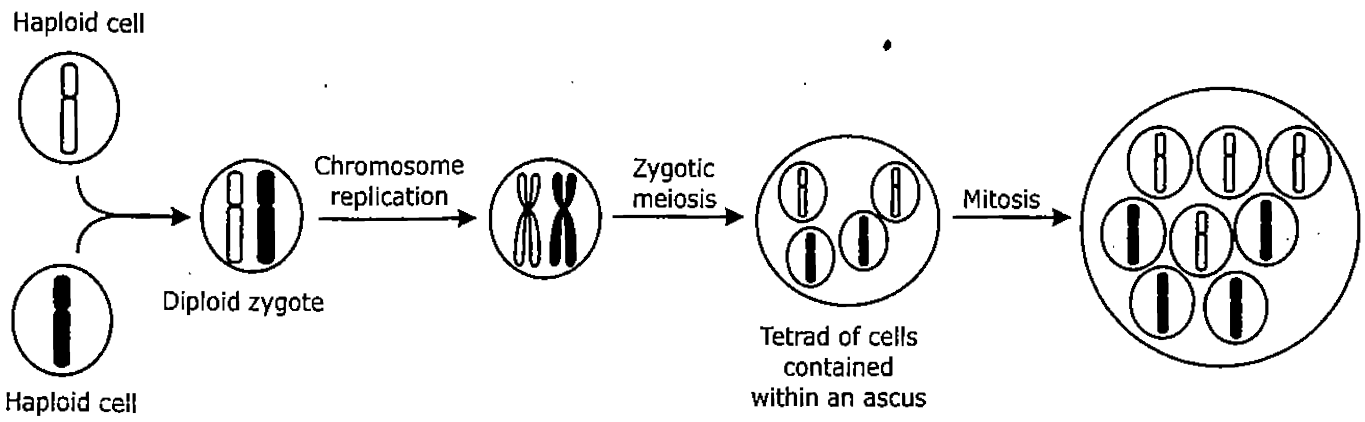


Figure 13.11 : Sexual reproduction in ascomycetes. For simplicity, this diagram shows each haploid cell as having only one chromosome per haploid set. However, fungal species actually contain several chromosomes per haploid set.

ORDERED *OR* UNORDERED TETRAD/OCTAD

The arrangement of spores within an ascus varies from species to species. In some cases, the ascus provides enough space for the tetrads or octads of spores to randomly mix together. This is known as an *unordered tetrad* or *octad*. These occur in fungal species such as *S. cerevisiae*. By comparison, other species of fungi produce a very tight ascus that prevents spores from randomly moving around. This can create a *linear tetrad or octad* found in *N.crassa.*

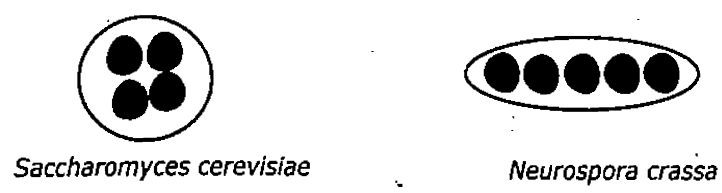


Figure 13.12 : Different arrangements of fungal spores

A key feature of linear tetrads or octads is that the position and order of spores within the ascus reflects their relationship to each other as they were produced by meiosis and mitosis. This idea is schematically shown in figure 13.13. After the original diploid cell has undergone chromosome replication, the first meiotic division produces two cells that are arranged next to each other within the sac. The second meiotic division then produces four cells that are also arranged in a straight row. Due to the tight enclosure of the sac around the cells, each pair of daughter cells is forced to lie next to each other in a linear fashion. Likewise, when each of these four cells divides by mitosis, each of daughter cells is located next to each other.

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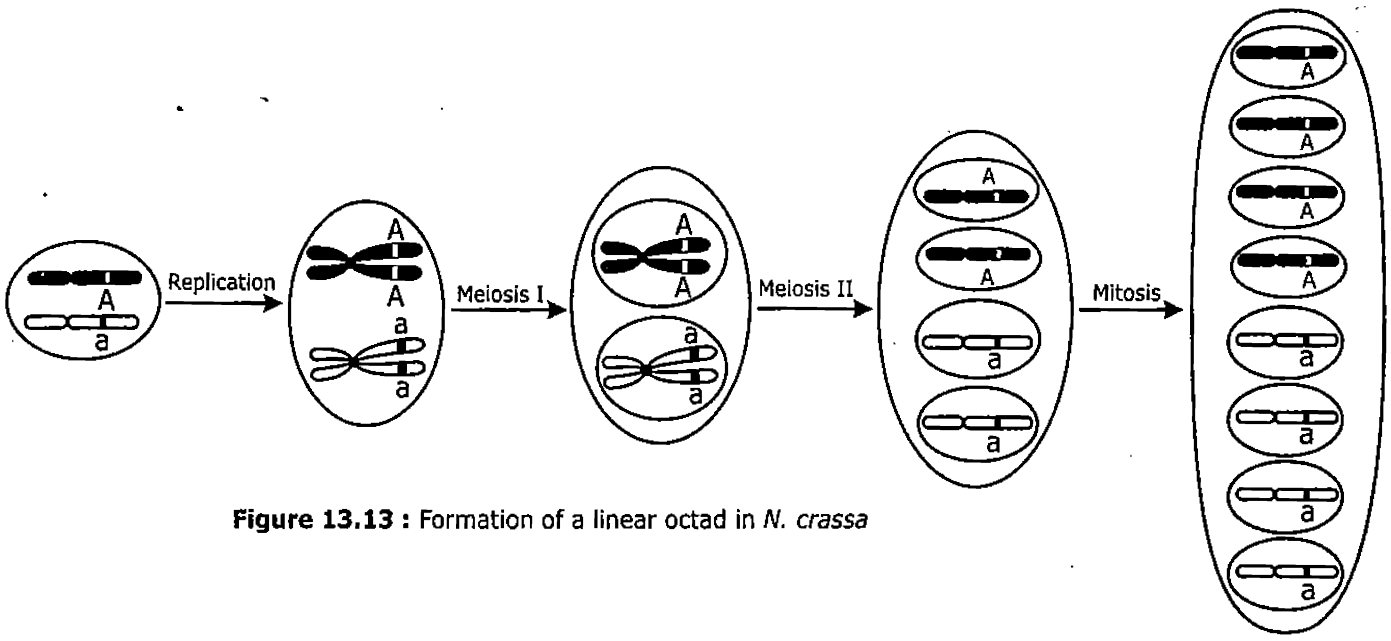


Figure 13.13 : Formation of a linear octad in *N. crassa*

ANALYSIS OF ORDERED TETRAD

Linear tetrad analysis can be used to map the distance between a gene and the centromere. This approach has been extensively exploited in *N. crassa*. In *N. crassa,* the products of meiosis are contained in an ordered arrey of spores. Each mature ascus contains eight ascospores in four pairs, each pair representing one of the products of meiosis. The ordered arrangement of meiotic product makes it possible to map each gene with respect to its centromere; i.e., to determine the recombination frequency between a gene and its centromere. Two cases are possible depending upon whether or not there is a crossover between the locus and its centromere.

First case

In the absence of crossing over between a gene and its centromere, the alleles of the gene (for example A and a) must separate in the first meiotic division, this separation is called **First Division Segregation** (FDS). Octad contains a linear arrangement of four haploid cells carrying the A allele, which are adjacent to four haploid cells that contain the a allele i.e., 4:4 arrangement of spores within the ascus (figure 13.14).

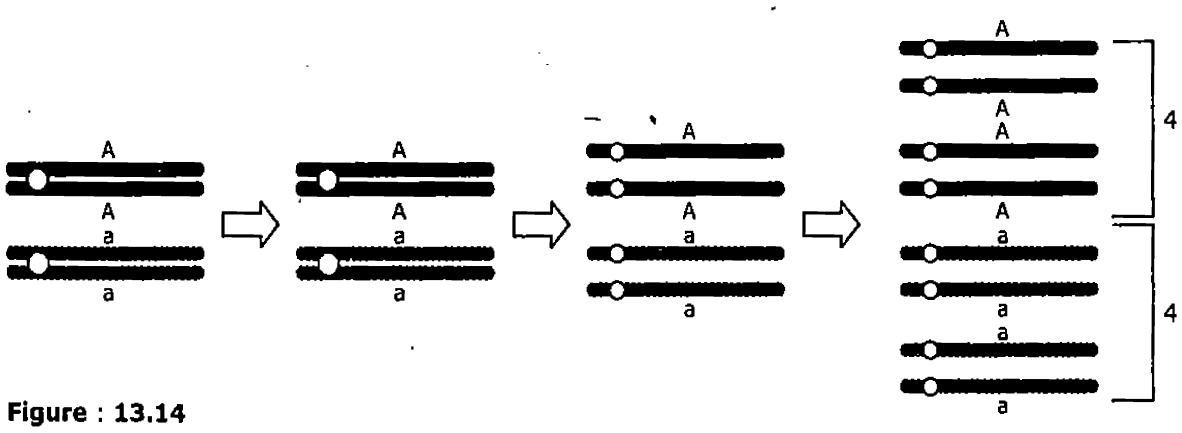


Figure : 13.14
First Division Segregation (FDS) : No crossing over produces a 4:4 arrangement

Second case

If a cross over does occur between the gene and its centromere, the A and a allele donot become separated until the second meiotic division, this separation is called **second division segregation**. So second division

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segregation deviate from the 4:4 pattern. Depending on the relative locations of the two chromatids that participated in the crossover, the ascus will contain a 2:2:2:2 or 2:4:2 pattern (figure 13.15 and 13.16). Since a pattern of SDS is a result of crossing over, the percentage of SDS asci can be used to calculate the map distance between the centromere and the gene of interest. As shown in the figure a cross over will only separate a gene from its original centromere if it begins in the region between the centromere and that gene. Therefore, the chances of getting a 2:2:2:2 or 2:4:2 pattern depend on the distance between the gene of interest and the centromere.

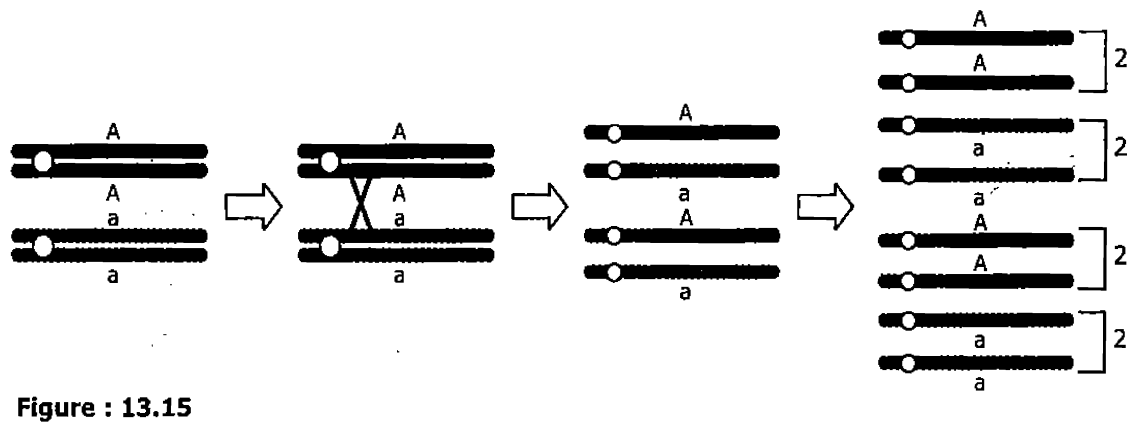


Figure : 13.15
Second Division Segregation (SDS) : A single crossing over produces a 2:2:2:2 arrangement

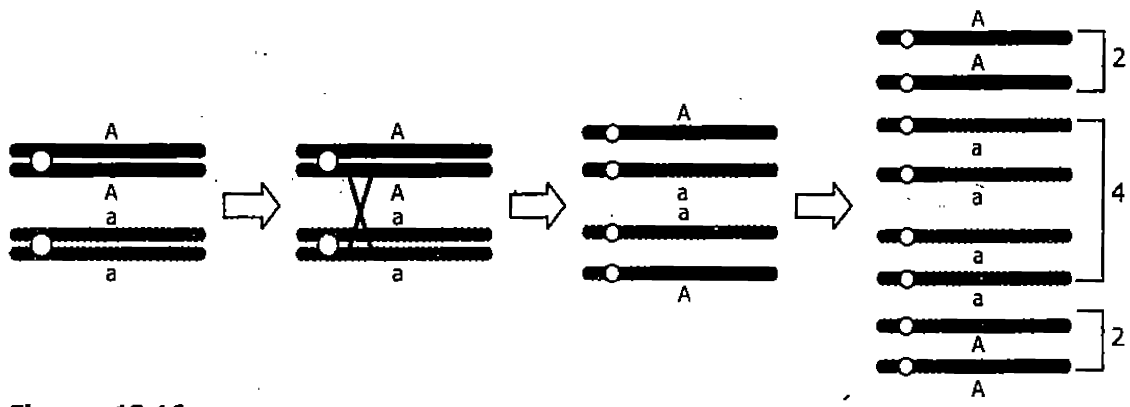


Figure : 13.16
Second Division Segregation (SDS) : A single crossing over produces a 2:4:2 arrangement

Calculation of map distance

The percentage of asci with second division segregation patterns for a gene can be used to map the genes with respect to its centromere. To determine the map distance between the centromere and a gene we first count the number of SDS asci and the total number of asci. In SDS asci, only half of the spores are actually the product of a crossover. Therefore, the map distance is calculated as

$$\text{Map distance} = \frac{\frac{1}{2} \times \text{Number of SDS asci}}{\text{Total number of asci}} \times 100$$

ANALYSIS OF UNORDERED TETRAD

Unordered tetrad contain a group of spores that are randomly arranged and the product of meiosis. Unordered tetrad analysis can be used to map genes in dihybrid crosses. This analysis can determine if two genes are linked or assort independently. If two genes are linked, a tetrad analysis can also be used to compute map distance.

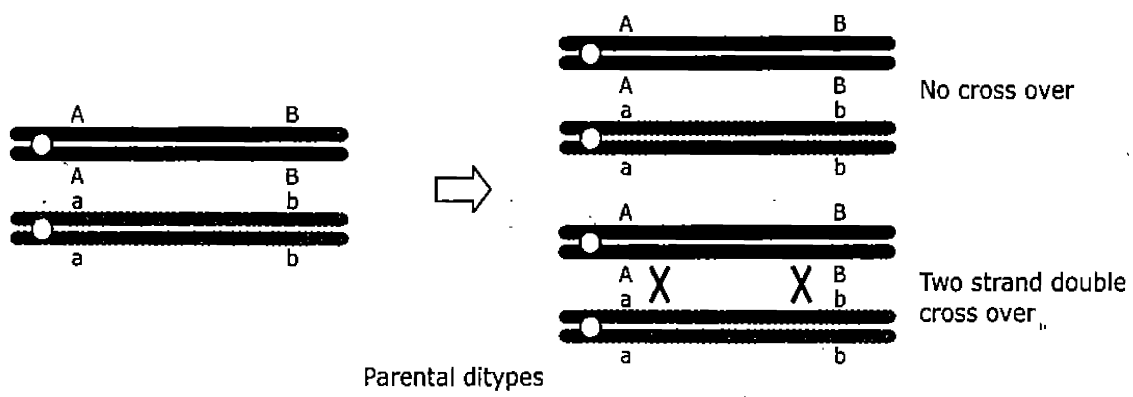
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Three patterns of segregation are possible in the tetrads when two pairs of alleles are segregating. For example, in a cross AB×ab, the three types of tetrad are

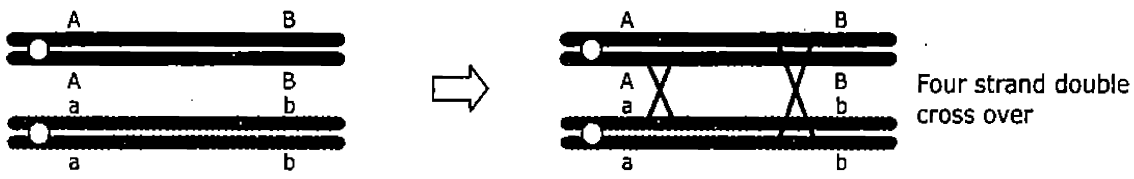
Type 1 : AB AB ab ab

If a crossover does not occur between these two loci or if a two-strand double crossover occurs between them, the resulting meiotic products will be of two kinds, resembling the parental combinations. Such a tetrad referred to as **parental ditype** or PD.



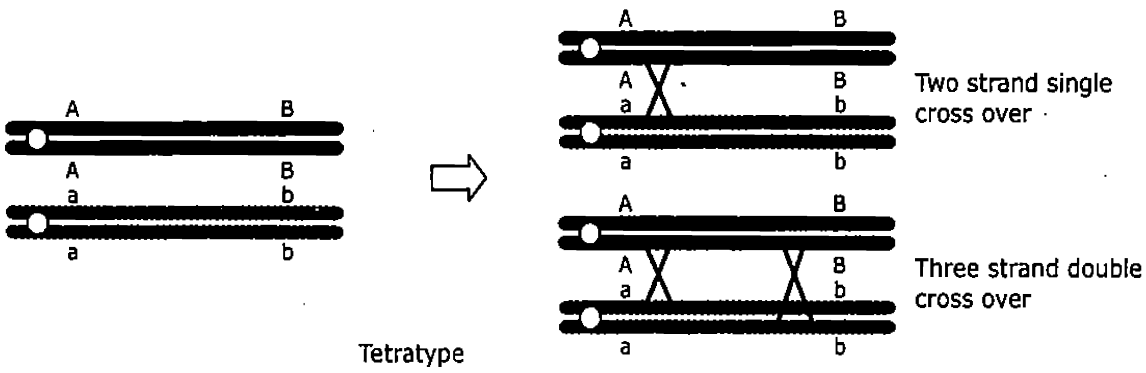
Type 2 : Ab Ab aB aB

A four-strand double crossover between the two genes results in two kinds of products, neither of which are parental combinations. This tetrad is referred to as **non-parental ditype** or NPD.



Type 3 : AB Ab aB ab

A two-strand single crossover or a three-strand double crossover (of two types) between the two genes results in formation of both parental and non-parental type combinations. This tetrad is referred to as **tetratype** or TT. All four of the possible genotypes are present (50% parental type and 50% non-parental type)



To estimate the amount of recombination between the two markers, we can use the formula:

$$\text{Recombination frequency} = \frac{\text{NPD} + 1/2\ \text{TT}}{\text{Total number of tetrads}}$$

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13.5 SEX CHROMOSOMES AND SEX DETERMINATION

Sex chromosome

The first sound indication for a connection between chromosomes and sex came from H. V. Henking (1891). He worked on spermatogenesis and its association with the egg development in the hemiptera *Pyrrhocoris apterus* and found out that half of the daughter cells contained one element more than the other during anaphase II. He was not sure whether it was an additional chromosome and thus called the structure X. Similar observations were made in other insects also. In 1902, C. E. McClung postulated that the X-element was connected to sex determination. The proof came in 1905 from E. B. Wilson (Columbia University, New York), who counted 13 (= 2n) chromosomes in the male *Protenor belfragei* and 14 in the female and could assign one X-chromosome to the male and two to the female. He coined the term **X-chromosome**.

Most animals and many plants show sexual dimorphism; in other words, an individual can be either male or female. In most of these cases, sex is determined by special sex chromosomes. In these organisms, there are two categories of chromosomes, **sex chromosomes** and **autosomes** (the chromosomes other than the sex chromosomes). The rules of inheritance considered so far, with the use of Mendel's analysis as an example, are the rules of autosomes. Most of the chromosomes in a genome are autosomes. The sex chromosomes are fewer in number, and, generally in diploid organisms, there is just one pair. Let us look at the human situation as an example.

Human body cells have 46 chromosomes: 22 homologous pairs of autosomes plus 2 sex chromosomes. In females, there is a pair of identical sex chromosomes called the **X chromosomes** (i.e., homomorphic). In males, there is a nonidentical pair, consisting of one X and one Y (i.e., heteromorphic). The **Y chromosome** is considerably shorter than the X. At meiosis in females, the two X chromosomes pair and segregate like autosomes so that each egg receives one X chromosome. Hence the female is said to be the **homogametic sex.**

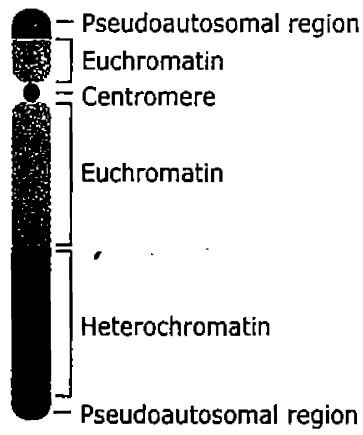


Figure 13.17 : Human Y chromosome

At meiosis in males, the X and the Y pair over a short region, which ensures that the X and Y separate so that half the sperm cells receive X and the other half receive Y. Therefore the male is called the **heterogametic sex.** The pairing of the X and Y chromosomes is end-to-end rather than along the whole length, and it is made possible by a 2.6 mega base pairs (Mb) region of homology between the X and Y chromosomes at the tips of their short arms, known as the major **pseudoautosomal region.** Genes in the pairing segment have some interesting properties : they are not subject to X-inactivation and because of the crossing over, alleles at these loci do not show the normal X-linked or Y-linked patterns of inheritance, but segregate like autosomal alleles. A second smaller pseudoautosomal region of 320 kb is located at the tips of the long arms of both chromosomes, but pairing and crossing over in this minor pseudoautosomal region is not an obligatory feature of male meiosis.

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Chromosomal basis of sex determination

Basically, four types of chromosomal sex-determining mechanisms exist: the XY, ZW, X0 and haplo-diploid system. In the XY system, the females are homomorphic with a pair of X chromosomes (XX) and males are heteromorphic with one X and Y chromosomes (XY). In the ZW case, as in birds; males are homomorphic (ZZ), and females are heteromorphic (ZW). In the X0 system males have only one X-chromosome (X0) and females have two X-chromosomes (XX). In haplo-diploid system, as in bees; males are haploid and females (both workers and queens) are diploid. In this case sex chromosomes are not involved in sex determination.

Different systems of sex determination

1.	X-Y system (Example-Human)	XX (female)	XY (male)
2.	X-O system (Example-Grasshopper)	XX (female)	XO (male)
3.	Z-W system (Example-Birds)	ZW (female)	ZZ (male)
4	Haplo-diploid system (Example-Bee)	Diploid (female)	Haploid (male)

STUDY QUESTION

Q3. Sex determination in the grasshopper is by the XO method. The somatic cells of a grasshopper are analyzed and found to contain 23 chromosomes.

a. What sex is this individual?

b. Determine the frequency with which different types of gametes (number of autosomes and sex chromosomes) can be formed in this individual.

c. What is the diploid number of the opposite sex?

Ans. a. Male

b. 1/2(11A + 1X) : 1/2(11A)

c. 24

Sex determination in humans

The sex of human is determined by its sex chromosomes. Females have two X chromosomes in all of their somatic cells, whereas males have one X and one Y. Individuals with a Y chromosome develop as males no matter how many X chromosomes they have, where as individuals without a Y chromosome develop as females.

Males develop functional testis. Testis are induced by the activity of the **Testis Determining Factor** (TDF) encoded by the **Sex Determining Region, Y** (SRY) located on the short arm of Y chromosome. Considerable evidence suggests that the SRY gene is the only gene on the Y chromosome that is both necessary and sufficient to initiate testis development. However recent advances have shown that mutations in X chromosome and autosomal loci are also associated with sex reversal, suggesting the presence of at least one other sex determining gene.

SRY is expressed only in a subset of the somatic cells of the developing gonad and it causes these cells to differentiate into **sertoli cells**, which are the main type of supporting cells found in the testis. The sertoli cells produce *anti-mullerian hormone*, which suppresses the development of female reproductive tract by causing the mullerian duct to regress. Sertoli cells also help to induce other somatic cells in the developing gonad to become **Leydig cells**, which secrete the male sex hormone *testosterone*; this hormone is responsible for inducing all male secondary sexual characters.

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In the absence of normal SRY expression, the cortex of the undifferentiated gonad develops into an ovary. The supporting cells become follicle cells instead of sertoli cells. Other somatic cells become *theca cells* instead of Leydig cells and, secrete the female sex hormone *estrogen* instead of *testosterone*. This normal process of female development is sometimes referred to as the 'default' pathway.

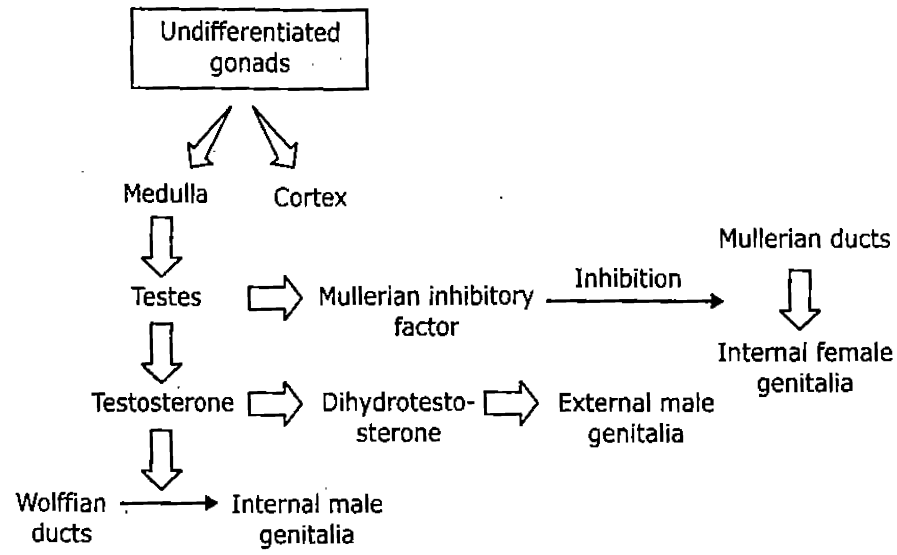


Figure 13.18 : Summary of the main events involved in sex determination.

Genic balance theory of sex determination in *Drosophila*

Fruit flies also have XX females and XY males. However, the mechanism of sex determination in *Drosophila* differs from that in mammals. Calvin Bridges suggested in 1921 that sex in *Drosophila* is determined by the balance (ratio) of autosomal alleles that favour maleness and alleles on the X-chromosomes that favour femaleness. He found that a ratio of X-chromosomes to autosomal sets determines the sex of *Drosophila*. A normal diploid male contains 2 sets of autosomes and XY chromosomes. Similarly a normal diploid female has 2 sets of autosomes and two X chromosomes.

Table 13.3 : Sex determination by genic balance in *Drosophila*

Number of X chromosome	Number of Autosomal sets (A)	Total number of chromosomes	X/A ratio	Sex
3	2	9	1.5	Meta female
2	2	8	1.00	Female
1	1	4	1	Female
2	3	11	0.67	Intersex
1	2	7	0.50	Male
1	3	10	0.33	Meta male

Bridges results show that in *Drosophila* factors that cause a fly to develop into a male are not localized on the sex chromosome, but are instead found on the autosomes. X-chromosomes contain some female-determining factor's genes. A sex switch gene directs the female development. This sex switch gene, sex-lethal (Sxl) is located on the X-chromosome. In the *on* state, it diverts female development and in the *off* state maleness. Other genes located on X-chromosome and the autosomes regulate this sex switch gene. Activation of the Sxl gene relies on a ratio of X-chromosomes to sets of autosomes. The presence of the Y chromosome in *Drosophila,* although it is essential for male fertility, has nothing to do with the determination of sex.

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Sex determination in plants

Vascular plants show a variety of sexual arrangements. Dioecious species are the ones showing animal-like sexual dimorphism, with female plants bearing flowers containing only ovaries and male plants bearing flowers containing only anthers. Some, but not all, dioecious plants have a nonidentical pair of chromosomes associated with (and almost certainly determining) the sex of the plant. Of the species with nonidentical sex chromosomes, a large proportion have an XY system. For example, the dioecious plant *Melandrium album* has 22 chromosomes per cell: 20 autosomes plus 2 sex chromosomes, with XX females and XY males. Other dioecious plants have no visibly different pair of chromosomes; they may still have sex chromosomes but not visibly distinguishable types.

Mosaicism

Some individual is made up of several cell lines, each with different chromosome numbers. These individuals are referred to as **mosaics**, depending on the sources of the cell lines. Such conditions can be the result of nondisjunction or chromosomal lagging during mitosis in the zygote or in nuclei in the early embryo. If one of the X chromosome is lost in one of the dividing somatic cells, it result in an XX cell line and an X0 cell line. If this chromosomal lagging occurs early in development of *Drosophila*, an organism that is part male (X0) and part female (XX) develops. A special case in mosaic has a name **gynandromorph** in which chromosomal lagging has occurred at the one-cell stage i.e., during the first mitotic division, causing the fly to be half male and half female. In this case one of the two X chromosomes is lost at the first mitotic division following the fusion of the sperm and egg pronuclei in a newly fertilized XX embryo. This X instability occurs only at the first division. In all subsequent divisions, nuclei containing two X chromosomes give rise to daughter nuclei with two X chromosomes, while nuclei with just one X chromosome give rise to daughters containing a single X.

SEX- LINKED INHERITANCE

Sex linked genes

In an XY chromosomal system of sex determination both X and Y chromosomes are sex chromosomes. In general, genes on sex chromosomes are described as sex linked genes. However, the term *sex linked* usually refers to loci found only on the X chromosome; the term *Y linked* is used to refer to loci found only on the Y chromosome, which control holandric traits (traits found only in male).

Cytogeneticists have divided the X and Y chromosomes of some species into homologous and non-homologous regions. The latter are called *differential* regions. These differential regions contain genes that have no counterparts on the other sex chromosome. Genes in the differential regions are said to be **hemizygous** (half zygous). Genes in the differential region of the X show an inheritance pattern called **X linkage**; those in the differential region of the Y show **Y linkage**. Genes in the homologous region show what might be called *X-and-Y linkage*.

Another important feature of sex linked genes in XY chromosomal system of sex determination is that females have two X chromosomes, they can have normal homozygous and heterozygous allelic combinations. But males, with only one copy of the X chromosome can be neither homozygous nor heterozygous. Hence the term hemizygous is used for X-linked genes in males. Since only one allele is present, a single copy of a recessive allele can determines the phenotype, a phenomenon called **pseudodominance**. This is the same way that one copy of a dominant autosomal allele would determine the phenotype of a normal diploid organism. Hence the term pseudodominance.

The genes on the differential regions of the sex chromosomes show patterns of inheritance related to sex. The inheritance patterns of genes on the autosomes produce male and female progeny in the same

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phenotypic proportions, as typified by Mendel's data (for example, both sexes might show a 3:1 ratio). However, crosses following the inheritance of genes on the sex chromosomes often show male and female progeny with different phenotypic ratios. T.H.Morgan demonstrated the X-linked pattern of inheritance in *Drosophila* in 1910, when a white eyed male appeared in a culture of wild type (red-eyed) flies.

Let's look at an example from *Drosophila*. When white-eyed males are crossed with red-eyed females, all the F_1 progeny have red eyes, showing that the allele for white is recessive. Crossing the red-eyed F_1 males and females produces a 3:1 F_2 ratio of red-eyed to white-eyed flies, but all the white-eyed flies are males.

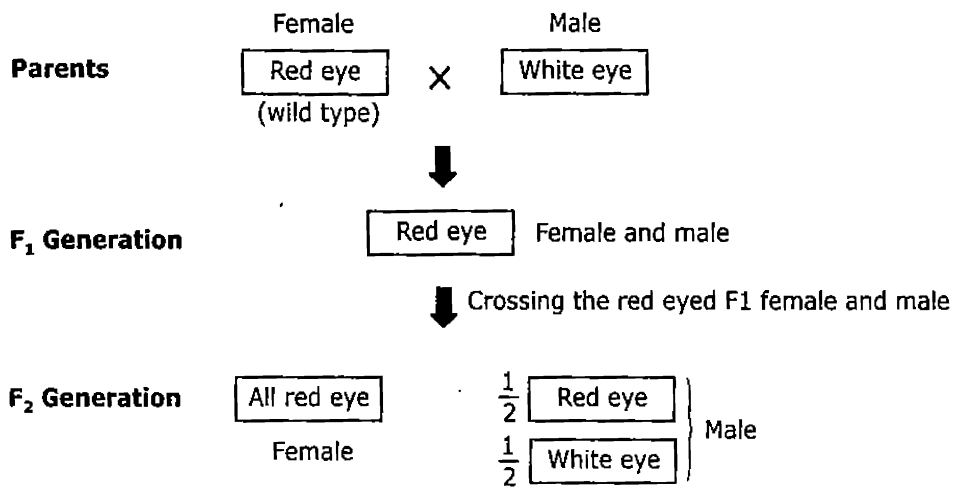


Figure 13.19 : Pattern of inheritance of the white eye trait in *Drosophila*.

This inheritance pattern is explained by the alleles being located on the differential region of the X chromosome; in other words, by X-linkage.

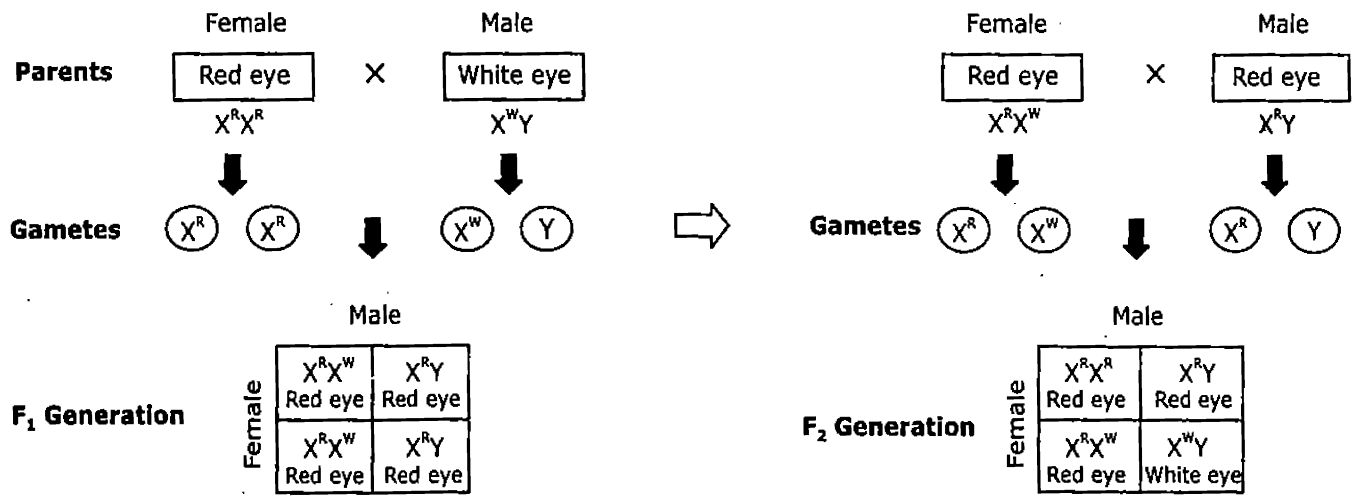


Figure 13.20 : Crosses by considering eye colour locous on X-chromosome.

In X-linked inheritance the *reciprocal cross* gives a different result. A reciprocal cross between white-eyed females and red-eyed males gives a F_1 in which all the females are red eyed, but all the males are white eyed. The F_2 consists of one-half red-eyed and one-half white-eyed flies of both sexes. Hence in sex linkage, we see examples not only of different ratios in different sexes, but also of differences between reciprocal crosses.

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In X-linked inheritance, the pattern of inheritance for loci on the heteromorphic sex chromosome differs from the pattern for loci on the homomorphic autosomal chromosomes, because sex chromosome alleles are inherited in association with the sex of offspring. Alleles on a male's X-chromosome go to his daughters but not to his sons, because the presence of his X chromosome normally determines that his offspring is a daughter. Since the father passes a trait to his daughters, who passes it to their sons. Hence, this pattern of inheritance is known as **criss-cross** pattern of inheritance.

In *Drosophila,* eye color has nothing to do with sex determination, so we see that genes on the sex chromosomes are not necessarily related to sexual function. The same is true in humans, for whom pedigree analysis has revealed many X-linked genes, of which few could be constructed as being connected to sexual function.

SEX LIMITED INHERITANCE

Sex hormones influence the action of certain genes. In some cases, a given genotype is so dependent on the presence of these hormones that its expression is limited exclusively to one sex. The result is a sex limited trait. Sex limited traits expressed in only one sex, although the genes are present in both sexes. Sex limited traits are usually determined by autosomal genes and primarily concerned with the secondary sexual characters.

In humans, for example, breast development is a trait that is normally limited to female, where as beard growth is limited to males.

SEX-INFLUENCEED OR **SEX-CONDITIONED INHERITANCE**

Sex limited trait is an extreme example of how the expression of gene can be controlled by hormones. In other less extreme cases of sex controlled characteristics, only the dominance relationship of the two alleles is affected. Characteristics of this type are known as sex influenced traits. In sex influenced inheritance an allele is dominant in one gender but recessive in the opposite gender. In human, pattern of baldness provides an example of sex influenced trait. Pattern baldness is characterized by the premature loss of hair from the front and top of the head. It is more common in males than in females. Women who have the genotype for pattern baldness typically show only thinning of hair rather than complete loss. The gene that causes pattern baldness is inherited as an autosomal trait. When a male is heterozygous for the baldness allele, he will become bald.

Genotype	**Phenotype**	
	Male	*Female*
BB	Bald	Bald
Bb	Bald	Non-bald
bb	Non-bald	Non-bald

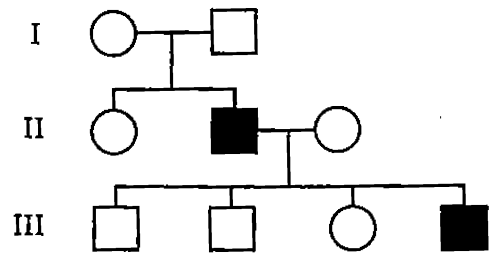
In contrast, a heterozygous female will not be bald. Women who are homozygous for the baldness allele will develop the trait. Sex influence nature of pattern baldness appears to be related to the levels of the male sex hormones.

PEDIGREE ANALYSIS

A pedigree is a family tree or chart made of symbols and lines that represent a patient's genetic family history. In pedigree symbols represent people and lines to represent genetic relationships.

The pedigree is a visual tool for documenting biological relationship in families and to determine the mode of inheritance (dominant, recessive etc.) of genetic diseases. Pedigrees are most often constructed by medical geneticists or genetic counselors. A sample pedigree is given below:

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In this pedigree, squares represent males and circles represent females. Horizontal lines connecting a male and female represent mating. Vertical lines extending downward from a couple represent their children. Subsequent generations are therefore written underneath the parental generations and the oldest individuals are found at the top of the pedigree. Each generation is given a Roman numeral designation and individuals within the same generation are numbered from left to right.

On the basis of the information in a pedigree geneticists attempt to determine the mode of inheritance of a trait. There are two types of questions the pedigree might be used to answer.

First, are there patterns within the pedigree that are consistent with a particular mode of inheritance?

Second, are there patterns within the pedigree that are inconsistent with a particular mode of inheritance?

Symbols used in the human pedigree analysis

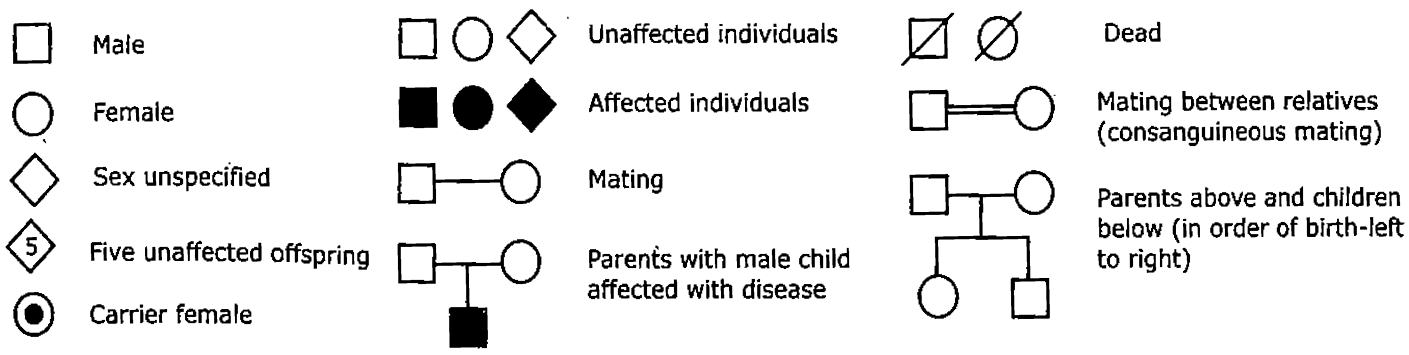


Figure 13.21 : The symbols used in the human pedigree analysis

Expected patterns for the various types of inheritance in pedigrees

Autosomal recessive inheritance

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

Autosomal dominant inheritance

1. Trait should not skip generations.
2. An affected person mating with an unaffected person should produce approximately 50% affected offspring (indicating also that the affected individual is heterozygous).
3. Distribution of the trait among sexes should be almost equal.

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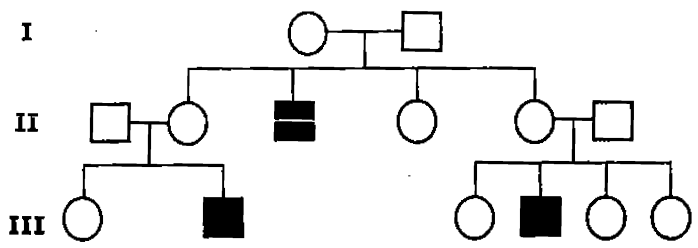
Sex-Linked recessive inheritance

1. Most affected individuals are male.
2. Affected males result from mothers who are affected or who are known to be carriers (heterozygotes).
3. Affected females come from affected father and affected or carrier mothers.
4. The sons of affected females should be affected.
5. Approximately half the sons of carrier should be affected.

Sex-Linked dominant inheritance

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

Example



This pedigree is of a common form of inherited colour blindness. What pattern of inheritance best accounts for this pedigree?

Explanation

In the above pedigree *X-linked recessive inheritance* is characterized by a predominance of affected males and an oblique pattern. It also indicates that transmission must be through females because there is no evidence of male-to-male transmission. The lack of affected females would make autosomal dominant inheritance less likely. In case of autosomal recessive Inheritance there should be an almost equal number of affected males and females but here only male in affected. So it eliminates the autosomal recessive inheritance.

Examples of autosomal and X-linked genetic disorders

Autosomal dominant

Familial hypercholesterolemia, Huntington chorea, Marfan syndrome, Retinoblastoma, Brachydactyly, Ehlers-Danlos syndrome.

Autosomal recessive

Sickle cell anemia, Thalassimias, Cystic fibrosis, Tay-Sachs disease, Phenylketonuria, Glycogen storage diseases (all types), Galactosemia, Wilson disease, Alkaptonuria, Homocystinuria, Lysosomal storage diseases.

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X-Linked recessive (*Sex linked recessive*)

Duchenne muscular dystrophy, Hemophilia A and B, Glucose-6-phosphate dehydrogenase deficiency, Fragile X-syndrome, Lesch-Nyhan syndrome, Color blindness

Sex linked dominant

Vit D resistant rickets

Table 13.4 : Some Important Genetic Disorders

Condition	**Characteristics**
Color blindness, deutan type	Insensitivity to green light
Color blindness, protan type	Insensitivity to red light
Fabry's disease	Deficiency of galactosidase A
G6PD deficiency	Deficiency of glucose 6-phosphate dehydrogenase
Hemophilia A	Classical form of clotting deficiency; lack of clotting factor VIII
Hemophilia B	Christmas disease; deficiency of clotting factor IX
Lesch-Nyhan syndrome	Deficiency of hypoxanthine-guanine phosphoribosyl transferase enzyme
Muscular dystrophy	Deficiency of the protein dystrophin
Cystic fibrosis	Failure of chloride ion transport mechanism
Sickle-cell anemia	Abnormal hemoglobin molecules
Tay-Sachs disease	Defective form of enzyme hexosaminidase A
Phenylketonuria	Defective form of enzyme phenylalanine hydroxylase
Huntigton's disease	Production of an inhibitor of brain cell metabolism
Hypercholesterolemia	Abnormal form of cholesterol cell-surface receptor

13.6 QUANTITATIVE INHERITANCE

The classical Mendelian traits are qualitative in nature; i.e., traits that are easily classified into distinct phenotypic categories. These discrete phenotypes are under the genetic control of only one or a very few genes with little or no environmental modification to obscure the gene effects.

In contrast to this, the variability exhibited by many traits fails to fit into separate phenotypic classes (*discontinuous variability*), but instead forms a spectrum of phenotypes that blend imperceptively from one type to another (*continuous variability*). Traits that exhibit continuous variation can usually be quantified by measuring, weighing, counting and so on. Traits such as body weight gains, mature plant heights, egg, or milk production records, and yield of grain per acre are **quantitative** or **complex traits** (historically referred to as **metric traits**) with continuous variability.

Let us compare the discontinuous and continuous variation by considering the example – height of pea plants and kernel colour of wheat. Height of pea plant is a Mendelian trait. So, in term of height pea plants can be either tall or dwarf; there is no overlap. All of the F1 generation plants have tall height, when these plants are self fertilized the ratio of tall and dwarf plant in the F2 is 3:1 respectively. This is inheritance involving one locus with two alleles.

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Kernel colour of wheat vary continuously from one phenotypic extreme i.e., dark red to the other i.e., white with no clear-cut breaks in between. When a particular strain of wheat having dark red kernels is crossed with another strain having white kernels, all the F1 plants have kernels that are intermediate in colour. When these plants are self-fertilized, the ratio of kernels in the F2 is 1 red : 2 intermediate : 1 white.

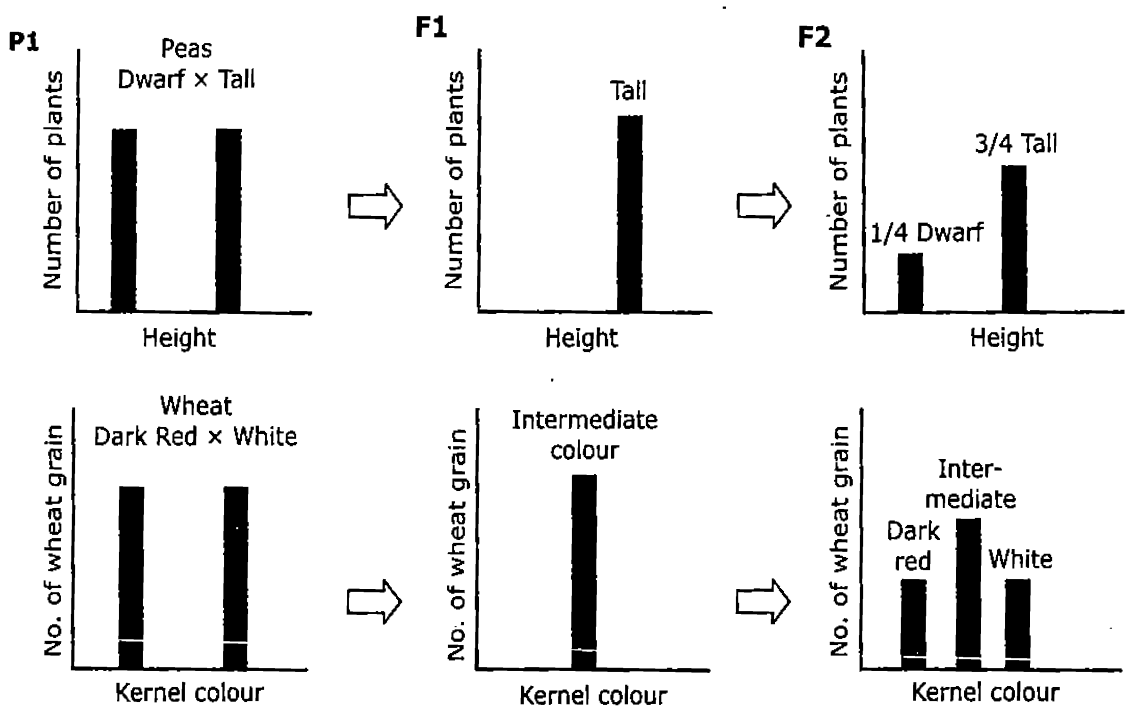


Figure 13.22 : Comparison of discontinuous variation in qualitative trait (height in pea plant) with continuous variation in quantitative trait (kernel colour in wheat)

The basic differences between qualitative and quantitative traits involve the number of genes contributing to the phenotypic variability and the degree to which the phenotype can be modified by environmental factors. Quantitative traits are governed by many genes at different loci and each contributing such a small amount to the phenotype that their individual effects cannot be detected by Mendelian methods. Each gene exerting a small additive effect i.e., effects of the genes are cumulative. Becuase more than one gene determines the expression of a given trait, hence quantitative traits are also referred to as **polygenic traits.** In quantitative traits, no allelic pairs exhibit dominance and there is no genetic interaction between alleles of different loci and no linkage between the loci is involved. Each gene locus may be occupied by either a *contributing allele*, which contributes a constant amount to the phenotype, or by a non-contributing allele, which does not contribute quantitatively to the phenotype.

The continuous variation in quantitative traits results from the combined effects of genetic and environmental variations. The contribution of genetic or environmental effects vary, depending upon the trait, but in most cases neither factor governs the variation exclusively.

To illustrate this, let us examine Nilsson-Ehle's experiments involving grain colour in wheat. In one set of experiments, wheat with red grain was crossed to wheat with white grain. The F1 generation demonstrated an intermediate colour. When these plants were self fertilized, at least seven colour classes, from red to white were distinguishable in a ratio of 1 : 6 : 15 : 20 : 15 : 6 : 1. This result is explained by assuming that three loci are assorting independently, each with two alleles, such that one allele produces a unit of red colour whereas the other allele does not. If three gene pairs were operating, each with one potential contributing allele and one potential non-contributing allele, we can envision the generation of such variations.

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AABBCC X **aabbcc**
Dark red White

AaBbCc
Intermediate red *Selfed*

Class	Phenotype	Ratio	Number of contributing allele
I	Dark red	1	6
II	Moderate red	6	5
III	Red	15	4
IV	Intermediate red	20	3
V	Light red	15	2
VI	Very light red	6	1
VII	White	1	0

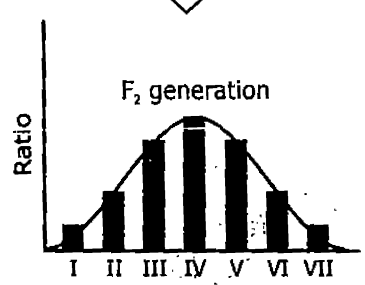


Figure : 13.23
Polygenic inheritance where three genes are involved in determining a quantitative trait (kernel colour in wheat)

Let us examine the inheritance by presuming that this trait is under the control of three gene pairs with two alleles each (AaBbCc). In this example, A, B and C are *contributing alleles* for the trait, and a, b and c are *non-contributing* alleles. The kernel colour will depend on the number of contributing alleles. For instance, a dark red kernel would have all the contributing alleles (AABBCC), a white kernel would have no contributing alleles (aabbcc) and a intermediate red kernel would have half the contributing alleles (AaBbCc).

As more gene pairs become involved, greater and greater numbers of classes would be expected to appear in more complex ratio. The study of a quantitative trait in a large population usually reveals that very few individuals possess the extreme phenotypes and that progressively more individuals are found nearer the average value for that population. This type of symmetrical distribution is characteristically bell-shaped and is called a *normal (Gaussian) distribution.* The spread of distribution about the mean is determined by the standard deviation.

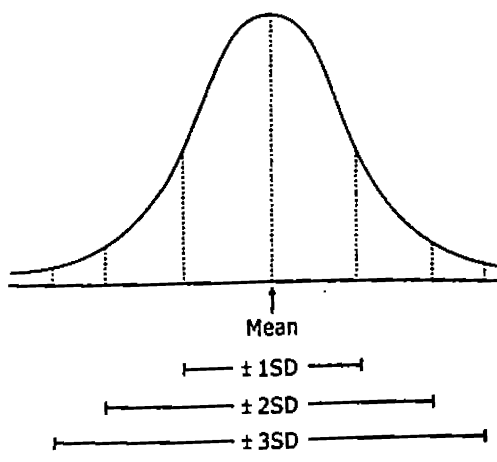


Figure : 13.24
The normal distribution

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13.7 EXTRANUCLEAR INHERITANCE AND MATERNAL EFFECT

Genes located on nuclear chromosomes obey Mendelian inheritance and are segregated in typical Mendelian ratios. Nevertheless, there are certain characters that are transmitted in a non-mendelian fashion. Such characters are either governed by non-nuclear genes like mitochondrial or chloroplast genes or endosymbiont (like Kappa particles, Sigma particles etc). This phenomenon is called as **extranuclear** or **cytoplasmic inheritance**. The characters that are controlled by cytoplasmic genes show certain features:

a. a lack of Mendelian segregation and typical Mendelian ratios

b. persistence of characters over many generations

c. different phenotypes of progeny in reciprocal crosses

In cytoplasmic inheritance transmission of a trait usually occurs through only one parent i.e., *uniparental inheritance*. Uniparental transmission through the mother constitutes **maternal inheritance** and through the father is **paternal inheritance**. Maternal inheritance is most common uniparental inheritance.

There is a difference between *maternal inheritance* and *maternal effect*. Maternal inheritance occurs when the hereditary determinants of a trait are extranuclear and genetic transmission is only through the maternal cytoplasm, where as maternal effect occurs when the nuclear genotype of the mother determines the phenotype of progeny. In maternal effect the hereditary determinants are nuclear genes transmitted by both sexes.

One of the first convincing examples of cytoplasmic inheritance is the inheritance of leaf variegation in four O'clock (*Mirabilis jalapa*) plant, which was reported by *Carl Correns* in 1909. Variegated plants have some branches which carry normal green leaves, some branches with variegated leaves (mosaic of green and white patches) and some branches which have all white leaves. Correns discovered that seed produced by flowers carried on the green branches gave progeny which were all normal green. It made no difference whether the phenotype of the branch which carried the flower used for pollen was green, white or variegated. Seed taken from white branches likewise gave all white progeny, regardless of the pollen donor phenotype. Seeds from flowers on variegated branches gave three kinds of progeny, green, white and variegated, in varying proportions; again regardless of the pollen donor phenotype.

Crosses and progeny phenotypes

Phenotype of branch bearing egg	**Phenotype of branch bearing pollen**	**Phenotype of progeny**
white	white	white
white	green	white
white	variegated	white
green	white	green
green	green	green
green	variegated	green
variegated	white	variegated, green or white
variegated	green	variegated, green or white
variegated	variegated	variegated, green or white

On the basis of above outcome Correns concluded that the phenotype of the progeny always resembles the female parent and the male makes no contribution at all to the character. The explanation for this unusual pattern of inheritance is that the genes concerned are located in the chloroplasts within the cytoplasm, not in the nucleus, and are therefore transmitted only through the female parent.

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The inheritance of streptomycin resistance in *Chlamydomonas reinhardii* and inheritance of striped leaves in maize are also governed by chloroplast genes and show uniparental maternal inheritance.

Respiration defective mitochondrial mutant

There are some characters that are controlled by mitochondrial genes, and the inheritance pattern of *petite mutants* of baker's yeast, *Saccharomyces cerevisiae* is one such example. Yeast cells normally form large colonies called as *grande* (French for 'big') on nutrient agar plates. Rarely, small or minute colonies called as *petite* (French for 'small'), also appear amongst them. Petite colonies were small not because the cells were small but because the growth rate of the mutant petite strain is significantly slower than that of the wild type. Thus there are fewer cells in the petite colonies. These petite colonies grow slowly because of low respiration due to defects in the mitochondrial electron transport proteins.

When petites are crossed with the wild-type, three different types of petites are observed (shown in the figure 13.25) : The **segregational, neutral** and **suppressive petites.**

The *segregational* (nuclear) petite, caused by mutation of a chromosomal gene, exhibits normal Mendelian segregation. In a cross with wild type, the diploid progeny are normal, and if the diploids are allowed to undergo meiosis half of the spores in an ascus produce petite colonies and half form wild type colonies i.e., segregation of ascospores into 2 : 2 ratio. Two other classes of petites, the *neutral* petites and *suppressive* petites exhibit the traits of extranuclear inheritance.

When a neutral petite is crossed with normal wild-type cells, the resulting diploids all produce grande colonies. The name neutral, then, refers to the fact that this class of petites does not affect the wild-type. The explanation for these result is that the majority of neutral petites lack most or all mitochondrial DNA, in which many of the genes determining oxidative respiration. When a neutral-petite cell mates with a wild type cell, the cytoplasm of the later is the source of the normal mitochondrial DNA in the resulting progeny spore. The third category of petites, *suppressive petites*, the cross between petite and wild type producing all petite colonies. The suppressive petites are different from the neutrals because they do have an effect on the wild type. Most petite mutants are of the suppressive type. Suppressive petites also have deletions of mtDNA, but they are not nearly as extensive as deletions in the neutral petites. Two major hypothesis have been given to explain suppressiveness. One hypothesis suggests that the mutant mitochondrial DNA replicates more rapidly, resulting in the mutant mitochondria dominating the phenotype by numbers alone. The second hypothesis consider the view that recombination occurs between the mutant and wild-type mitochondrial DNA, introducing errors into normal mitochondrial DNA.

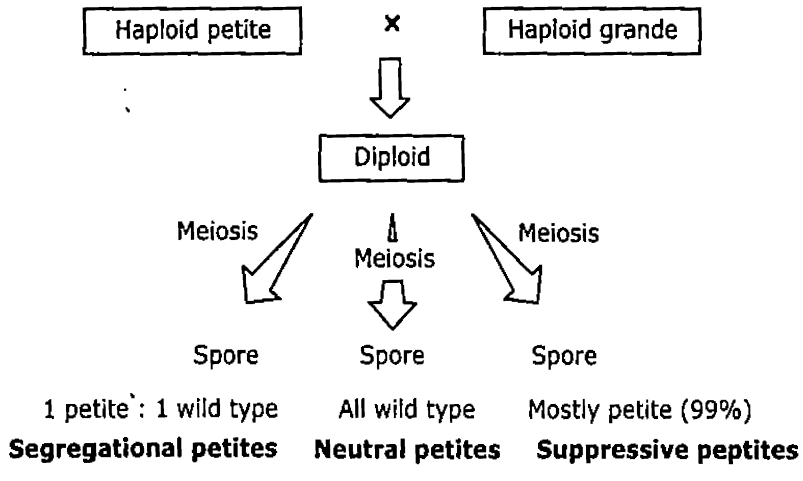


Figure 13.25 : The categories of petites yeasts based on segregation patterns

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Cytoplasmic male sterility (CMS)

CMS, first observed by M. Rhoades, is an example of cytoplasmic inheritance in plants. In this condition the plant does not produce active pollen but the female reproductive process and fertility are normal. This type of sterility is not controlled by nuclear genes but is transmitted through the egg cytoplasm from generation to generation. Cytoplasmic male steriility in maize results from the expression of an abnormal protein in the mitochondria of the anthers. Although the mutation is present in all the mitochondria of the plant, only the anthers show a phenotype. It is not yet understood how the abnormal proteins disrupt mitochondrial function. However, these result suggest that efficient mitochondrial function is particularly important during pollen development.

Endosymbiont

In eukaryotes, a variety of cytoplasmically transmitted traits result from the presence of bacteria living in the cytoplasm of certain cells. One of the best known is the killer phenomenon found in certain strains of the protozoan *Paramecium aurelia.* Killer strains of paramecium release toxin to the surrounding medium called *paramicin* that is lethal to many other strains of protzozoan called sensitive strain. The killer phenotype requires the presence of cytoplasmic bacteria referred to as **kappa particles**. The kappa particles are the cells of *Caedobacter taeniospiralis* which produce the killer substance. Kappa is not the only infective agent known in *Paramecium.* Another agent is the **mu particles**. Killer cells containing mu particles, termed mate-killers, do not release a toxin into the environment, but instead kill their mates during conjugation.

MATERNAL EFFECT

The embryo is formed when a female gamete unites with a male gamete. In the vast majority of species, the female gamete is physically larger than the male gamete and provides the cytoplasm for the developing embryo. Within this cytoplasm are factors that were released by the nuclear genes of the female. Those factors may have specific effects upon the developing embryo. Those phenotypes that are controlled by factor present in cytoplasm of female but encoded by nuclear genes are said to express a **maternal effect**. So, a **maternal effect,** is the phenomenon where the nuclear genotype of a mother is expressed in the phenotype of its offspring, unaltered by paternal genetic influence. However, the female cytoplasm also contributes the mitochondria for all species as well as the chloroplast for plant species. These two organelles contain DNA and control certain traits in the offspring. Those phenotypes controlled by organelle genes exhibit **maternal inheritance.**

The classic phenotype which exhibits maternal effects is coiling direction of shell in snail *Limnaea peregra*. Shell coiling in *Limnaea peregra* is of two types - left handed or sinistral and right handed or dextral. The coiling phenotype that is seen in the offspring is *controlled by the genotype of the mother*. The nuclear control over left handed or right handed coiling can be interpreted by analyzing the outcome upto F_3 generation. The following crosses were made between pure line snails, and the following results were seen.

These results at first glance appear to be at odds with Mendel's laws. First, the F1 phenotype is not the same for both crosses. With other experiments, the results of reciprocal crosses were equivalent, but with this experiment it appears that the female controls the phenotype. Yet, the F2 appears to contradict this hypothesis because the left- and right-coiled F1 individuals produced all right progeny. Furthermore, the 3:1 Mendelian ratio is not seen in the F2, but rather appears in the F3 generation.

How can this result be explained?

First, let's look for results that are familiar. The F3 ratio of 3 *right*:1 *left* for both crosses suggests that right-coiled shells are dominant to left-coiled shells. If this is the case, then we can assign the following genotypes to the pure lines:

- Right-coiled shell: DD
- Left-coiled shell: dd

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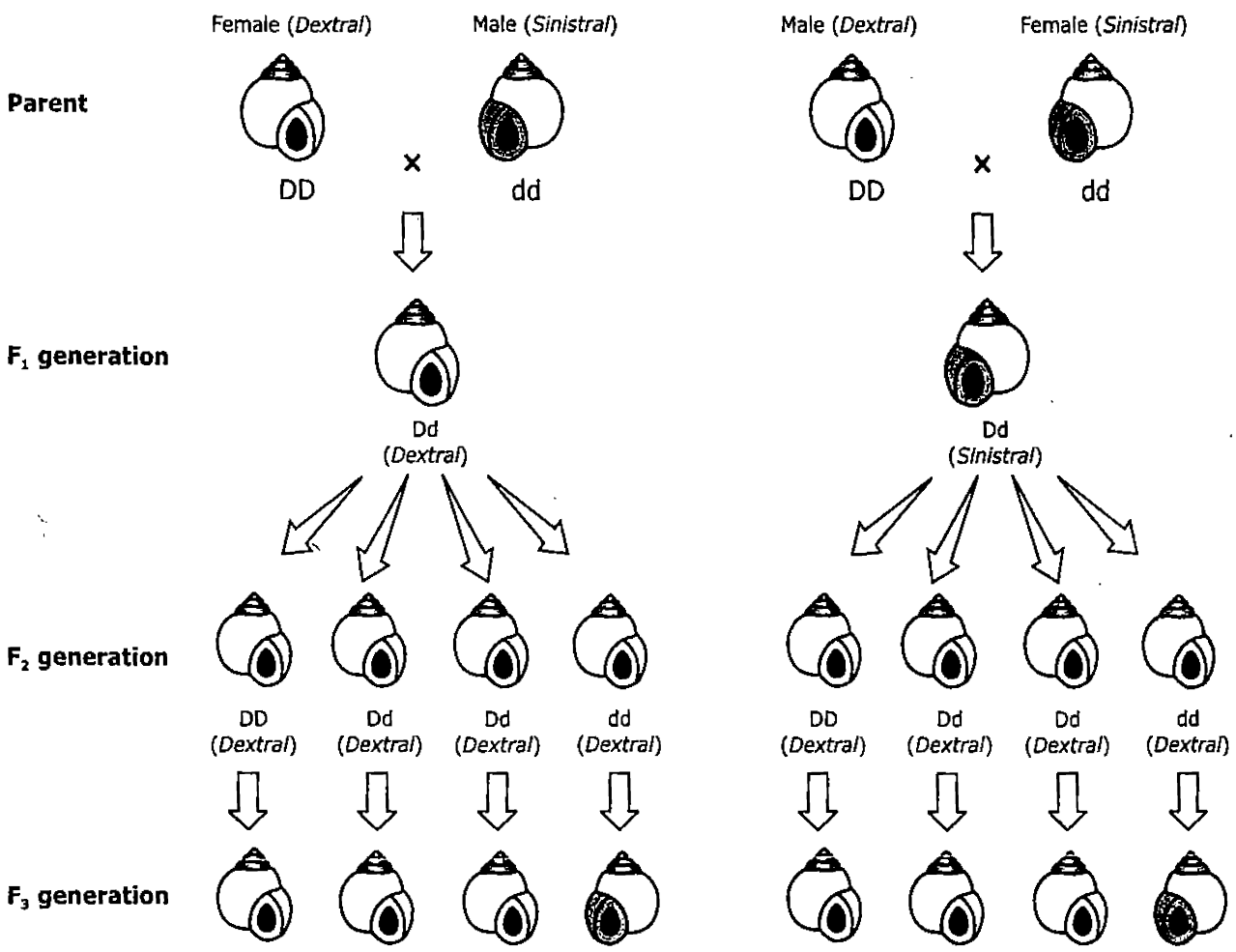


Figure 13.26 : Inheritance of the direction of shell coiling in the snail *Limnea*. Sinistral coiling is determined by recessive allele d, and dextral coiling by dominant allele D. The F_2 and F_3 generations are obtained by self-fertilization.

The next observation is that the phenotype of the F1 generation is always that of the female parent. One hypothesis would suggest that the genotype of the female controls the genotype of its offspring. *Can these results be confirmed in the subsequent generations*? If the genotypes we assigned to the parents are correct, then the genotype of F1 individuals from each cross are Dd (from DD×dd and dd×DD). If the female genotype does control the phenotype of its offspring, then we would predict that all the F2 snails would have right coils. This is the exact result that is seen. But what would the genotypes of the F2 snails be? If we intermate snails with the genotype Dd the genotypic ratio should be 3 D_ to 1 dd. These genotypes would not be expressed as a phenotype until the F3 generation. These are the results that were obtained. A general conclusion from all traits that express a *maternal effect* is that the normal Mendelian ratios are expressed one generation than expected.

Cytological analysis of developing eggs has provided the explanation of above mentioned result : the genotype of the mother determines the orientation of the mitotic spindle during the second cleavage (mitotic) division in the zygote, and this in turn controls the direction of shell coiling of the offspring.

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SELF TEST

Q1. Phenotypic expression of the heterozygote being greater than that of either homozygote is called:
a. dominance
b. midparent value
c. overdominance
d. pseudodominance

Q2. Independent assortment of genes occurs due to the orientation of chromosomes at
a. metaphase of mitosis
b. metaphase I of meiosis
c. metaphase II of meiosis
d. any phase of the cell division

Q3. When dominant epistasis is operative between two gene loci, the classical 9 : 3 : 3 : 1 ratio becomes modified into
a. 9 : 3 : 4 ratio
b. 9 : 6 : 4 ratio
c. 12 : 3 : 1 ratio
d. 15 : 1 ratio **[JNU]**

Q4. The most common cause of the pleiotropic effect of a gene is due to
a. the same product of the given gene being involved in different metabolic pathways
b. the gene making very different products in different cell types
c. the DNA sequence of the gene getting changed in cell specific manner
d. the gene not functioning in some cells

Q5. Recombination percentage in a diploid cannot exceed
a. 100
b. 50
c. 25
d. 75

Q6. Two linked genes a and b show 20% recombination. The individuals of a dihybrid cross between + + /+ + ab/ ab shall show gametes
a. + + 80 : ab : 20
b. + + 50 : ab : 50
c. + + 40 : ab 40 : + a 10 : + b : 10
d. + + 30 : ab 30 : + a 20 : + b : 20

Q7. In a diploid organism, the genes A, B and C are present on the same chromosome in that order. The AB interval is 10 map units and BC interval is 20 map units. In an ABC/abc heterozygous individual, what will be the proportion of gametes that carry the genotype AbC?
a. 1%
b. 10%
c. 20%
d. 30% **[IISc]**

Q8. In a three-factor cross among genetic markers A, B and C, the relative frequencies of recombinants were AB(1), AC(0.2) and BC(0.3). Which of the following is the most probable order of these markers on the genome?

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a. ABC
b. BCA
c. CAB
d. the data do not reflect the order of genes **[JNU]**

Q9. Pseudoautosomal gene is present on
a. both X- and Y- chromosomes
b. both X- and autosomes
c. both Y- and autosomes
d. only autosomes

Q10. What are the sexual phenotypes of the following genotypes in *Drosophila* : XXY, XO?
a. male, female
b. male, male
c. intersex, female
d. female, male **[JNU]**

Q11. In *Drosophila* Y-chromosome is:
a. essential for male sex determination
b. required for development of secondary sex-characters
c. required for dosage compensation
d. not required for male sex determination

Q12. One of the following statements is most appropriate about the Y chromosome in mammals
a. essential for the development of embryo
b. non-essential for the development of the embryo but important for male sex determination
c. non-essential for sex determination but important for male fertility
d. essential for male sex determination and fertility **[IISc]**

Q13. Why is the gene for pattern baldness in man exhibits dominance in man but acts recessively in woman? Because
a. baldness gene expression in regulated by hormones
b. baldness gene is autosomal
c. baldness gene is on X-chromosome
d. baldness gene is on Y-chromosome

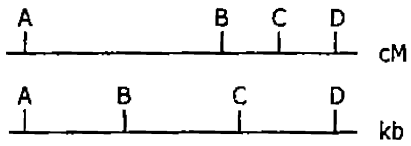
Q14. In mice, a mutation results in the production of yellow coat colour, as opposed to the normal gray coat. Crossing yellow mice with normal mice results in equal proportions of normal and yellow mice, whereas crossing yellow mice amongst themselves results in a ratio of 2 yellow : 1 normal progeny. The results suggest that
a. more than one gene is involved in conferring the yellow phenotype
b. the yellow allele is lethal under homozygous condition
c. the yellow allele exhibits cytoplasmic heredity
d. the results cannot be explained by any known genetic principles **[IISc]**

Q15. If a man who is colour-blind marries a woman who is homogyzygous for normal colour vision, the probability of their son being color-blind is
a. 0
b. 1
c. 0.5
d. 0.75

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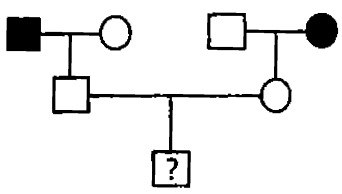
16. People who carry an allele for normal hemoglobin and an allele for sickle cell hemoglobin are resistant to malaria. This phenomenon is called
    a. heterozygote advantage
    b. polyploidy
    c. mutation selection balance
    d. recessive superiority

17. Hypophosphatemia is caused by an X-linked dominant gene in humans. If a hypophosphatemic man marries a normal woman, which of their children will have hypophosphatemia?
    a. all daughters
    b. half of their daughters
    c. all of the sons
    d. half of their sons

18. 

The figure above shows the locations of four genes on the genetic map of an organism; the lower panel shows the location of the same four genes on a physical map derived from the nucleotide sequence of the DNA of that organism. What is the reason for the maps to be *not* identical?
    a. there is no relationship between the position of genes in a genetic map and their positions on the DNA
    b. recombination frequencies per kb of DNA are not uniform throughout a chromosome
    c. the farther apart two genes are, the more likely they are to recombine
    d. the closer two genes are, the more likely they are to recombine **[IISc]**

19. The accompanying pedigree shows two individuals who are affected with tyrosinase-negative albinism (autosomal recessive). What is the risk that their grandchild will be affected with this disorder?



    a. 25%
    b. 50%
    c. 12.5%
    d. 0%

20. In a test cross of an individual heterozygous for each of three linked genes, the most frequent classes of progeny were ABc/abc and abC/abc, and the least frequent classes were ABC/abc and abc/abc. What Is the order of the genes.
    a. A – C – B
    b. B – A – C
    c. A – B – C
    d. none

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Chapter - 14

Cytogenetics and Population genetics

CYTOGENETICS

A chromosome is an organized structure of DNA and protein that is found in the nucleus of a cell. The study of the structure, function and abnormalities of chromosome is called *cytogenetics*, a discipline that combines cytology with genetics.

14.1 HUMAN KARYOTYPE

The number, sizes, and shapes of the metaphase chromosomes constitute the **karyotype** or karyogram which is distinctive for each species. Karyotype consisting of photograph or diagram of all the metaphasic chromosomes arranged in homologous pairs according to decreasing length, thickness, position of centromere, shapes etc is described as **idiogram.**

Table 14.1 : Symbol used in describing a karyotype

Symbol	*Meaning*
p	Short arm
q	Long arm
cen	Centromere
del	Deletion
dup	Duplication
inv	Inversion
t	Translocation
ter	Telomere
+ or –	Indicate gain or loss of part of chromosome

Tijo and *Levan* (1956) of Sweden found that human cells have 23 pairs or 46 chromosomes. Of the 23 pairs of human chromosome, 22 are perfectly matched in both males and females and are called autosomes. The remaining pair, the sex chromosomes, consists of two similar chromosomes in females and two dissimilar chromosomes in males. In human, females are designated XX and males XY.

Denver system

According to 'Denver system' of classification, the 22 pairs of human chromosomes are placed in seven groups as

	Group	**Position of centromere**	**Idiogram number**
I	(A)	Metacentric or submetacentric	1,2,3
II	(B)	Submetacentric	4,5

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III	(C)	Submetacentric	6,7,8,9,10,11,12 and X
IV	(D)	Acrocentric	13, 14 and 15
V	(E)	Metacentric or submetacentric	16, 17 and 18
VI	(F)	Metacentric	19 and 20
VII	(G)	Metacentric	21, 22 and Y

Chromosome banding

Chromosome banding is a cytological procedure of differential staining of mitotic chromosome along the longitudinal axis. The differential staining reactions reflect the heterogeneity and complexity of the chromosome along its length. The molecular mechanisms involved in producing the various banding patterns are not precisely defined. Bands are classified according to their relative location on the short arm (p) or the long arm (q) of specific chromosomes e.g., 12q1 means band 1 on the long arm of chromosome 12. One of the first chromosome-banding techniques was devised by Pardue and Gall. Features of commonly used banding technique are described in the table 14.2.

Table 14.2 : Chromosome banding techniques

Technique	*Procedure*	*Banding Pattern*
G-banding	Mild proteolysis followed by staining with Giemsa	Dark bands are AT-rich Pale bands are GC-rich
R-banding	Heat denature followed by staining with Giemsa	Dark bands are GC-rich Pale bands are AT-rich
Q-banding	Stain with quinacrine	Dark bands are AT-rich Pale bands are GC-rich
C-banding	Denature with barium hydroxide and then stain with Giemsa	Dark bands contain constitutive heterochromatin

14.2 PLOIDY

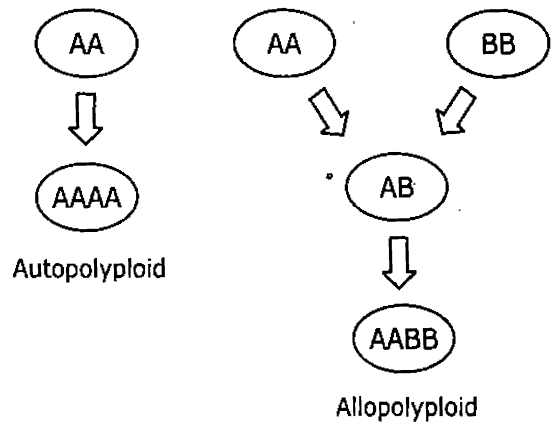
Numerical changes in chromosome number are referred to as changes in ploidy. Variation in chromosome number ranges from the addition or loss of one or more chromosomes to the addition of one or more haploid sets of chromosomes. **Euploidy** involves variations in the number of copies of the genome or complete sets of chromosomes in a cell or an organism. Whereas **aneuploidy** occurs when there are missing or extra copies of one or a few chromosomes.

Euploidy

The number of chromosomes in a basic set is called the *monoploid* (x) value. Diploid organisms contain two basic sets of chromosomes. However, not all organisms are diploids; some animals, for example, certain fish and salamanders, and many plants are polypoid, that is, contain more than two monoploid value or basic sets of chromosomes.

Polyploidy is of two different types: *autopolyploidy* and *allopolyploidy*. **Autopolyploidy** is the polyploidy condition resulting from multiplication of the same genome. In this case, each additional set of chromosome is identical to the parent species. **Allopolyploidy** is a polyploid conditon formed by the union of two or more distinct chromosome sets, with a subsequent doubling of chromosomes i.e., multiples of different genomes.

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Aneuploidy

Aneuploidy involves changes in chromosome number by additions or deletions of less than a whole set. In this case organism gains or loses one or more chromosomes but not a complete set.

Table 14.3 : List of terms to describe aneuploidy

Type	*Formula*
Normal	2*n*
Monosomic	2*n* - 1
Nullisomic	2*n* - 2
Double monosomic	2*n* - 1 - 1
Trisomic	2*n* + 1
Tetrasomic	2*n* + 2
Double trisomic	2*n* +1 + 1

Aneuploidy in human beings

Aneuploidy is common in human population. It includes both autosomal as well as sex chromosomal aneuploids. Following are the example of viable human aneuploids who survive long enough after birth to have a named syndrome.

Autosomal aneuploidy in humans

Down syndrome (47, +21) : It is usually caused by an extra copy of chromosome 21 (trisomy 21). Characteristics include decreased muscles tone, asymmetrical skull, slanting eyes and mild to moderate mental retardation.

Edward's syndrome (47, +18) : This is the second most common trisomy after Down syndrome. It is a trisomy of chromosome 18. Symptoms include mental and motor retardation as well as numerous congenital anomalies causing serious health problems. Ninety percent patients die in infancy; however those who live past their first birthday usually are quite healthy thereafter. They have a characteristic hand appearance with clenched hands and overlapping fingers.

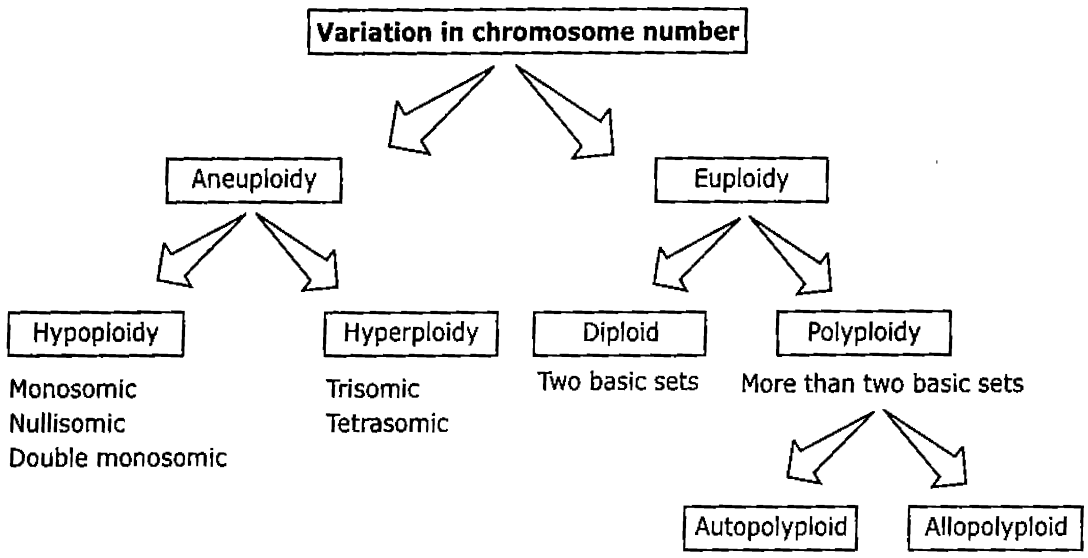
Patau's Syndrome (47, +13) : Patau's syndrome is also called D-syndrome or trisomy-13. Symptoms are somewhat similar to those of trisomy-18, but they do not have the characteristic hand shape.

Sex chromosomal aneuploidy in humans

Turner syndrome (45,X) : Female with retarded sexual development, usually sterile, short stature, webbing of skin in neck region, cardiovascular abnormalities, hearing impairment.

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Klinefelter syndrome (47,XXY) : Male, subfertile with small testes, developed breasts, feminine pitched voice, long limbs, knock knees, rambling talkativeness. Typically, male with the Klinefelter's syndromes have one extra copy of the X chromosome in the cell, for a total of two X chromosome and one Y chromosome. Less commonly, affected males may have two or three extra X chromosomes (48, XXXY or 49, XXXXY) or extra copies of both the X and Y chromosome (48, XXYY).



Nondisjunction

Aneuploidy arises as a random error during the production of gametes. Aneuploidy arises if, during one of the divisions of meiosis, one of the homologous chromosomes in meiosis I (or chromatids in meiosis II) does not migrate to the opposite pole. This result in an uneven distribution of that particular chromosome. This failure of chromosomes or chromatids to disjoin and move to opposite poles during division is known as **nondisjunction.** Depending upon where in meiosis this nondisjunction occurs, some gametes will end up with one or more extra chromosomes and other will end up with fewer chromosomes than a complete set.

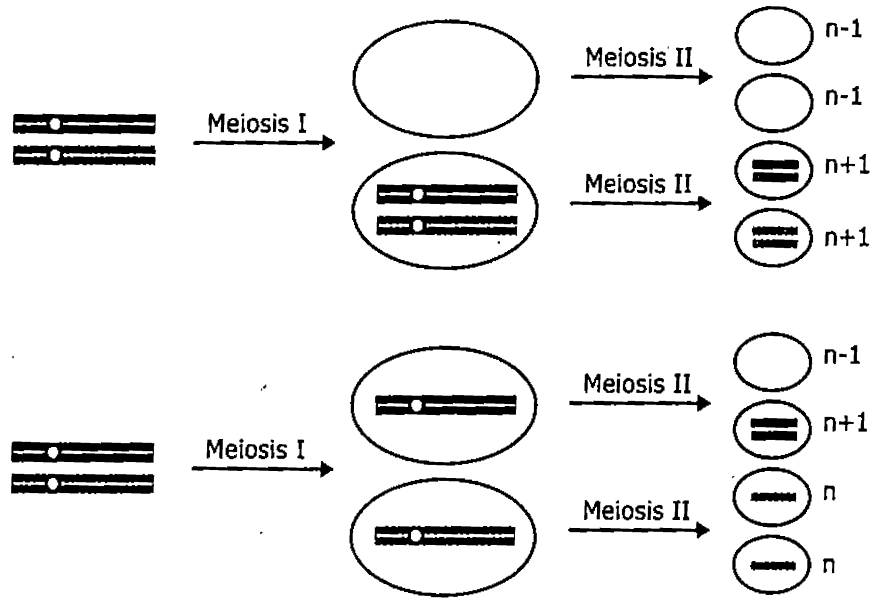


Figure 14.1 : Mechanisms of formation of aneuploid gametes during meiosis.

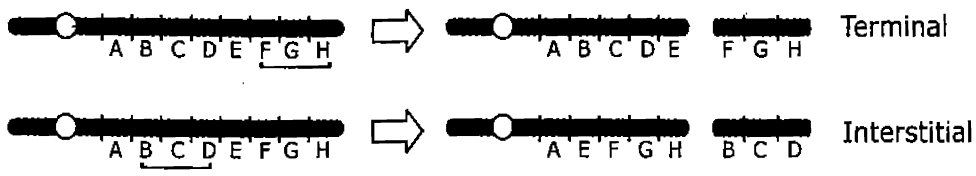
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14.3 CHROMOSOME ABERRATIONS

There are four types of *chromosome aberrations* : deletions, duplications, inversions, and translocations.

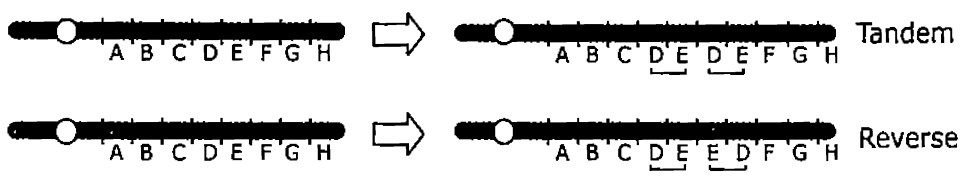
Deletion

A *deletion* is the loss of a segment of a chromosome. Deletion can be of two types - *terminal* and *interstitial.*

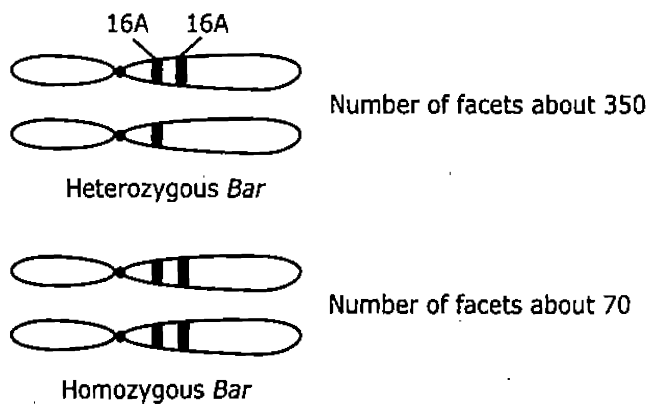


Duplication

A *duplication* is the occurrence of a segment of a chromosome in two or more copies per genome. Duplication can be *tandem* or *reverse*. The duplicate regions can be located adjacent to each other, or dispersed on the same chromosome.



Duplications of certain genetic regions may produce specific phenotypes. One interesting example is the *Bar* eye phenotype in *Drosophila.* The wild-type fruit fly has about 800 facets in each eye. *Bar* phenotype occurs as a result of duplication of the 16A locus of the X chromosome. The *Bar* heterozygote has about 350 facets whereas *Bar* homozygote has about 70.



Inversions

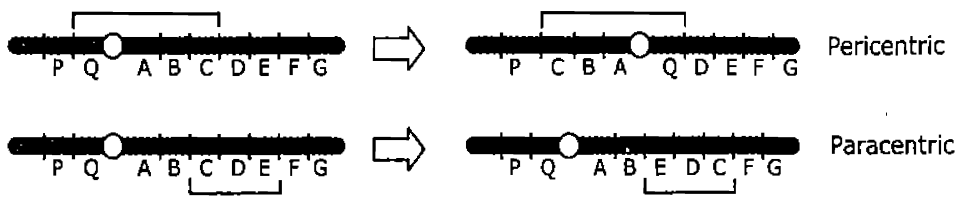
If two breaks occur in one chromosome, sometimes the region between the breaks rotates 180 degrees before rejoining with the two end fragments. Such an event creates a chromosomal mutation called an inversion. Unlike deletions and duplications, inversions do not change the overall amount of the genetic material, so inversions are generally viable and show no particular abnormalities at the phenotypic level. In some cases, one of the chromosome breaks is within a gene of essential function, and then that breakpoint acts as a lethal gene mutation linked to the inversion.



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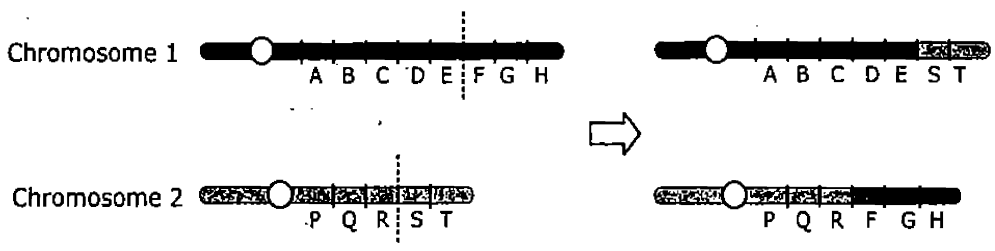
The location of the centromere relative to the inverted segment determines the genetic behavior of the chromosome. If the centromere is outside the inversion, then the inversion is said to be **paracentric**, whereas inversions spanning the centromere are **pericentric**.



How do inversions behave genetically? Crossing-over within the inversion loop of a paracentric inversion connects homologous centromeres in a **dicentric bridge** while also producing an **acentric fragment** - a fragment without a centromere. Then, as the chromosomes separate in anaphase I, the centromeres remain linked by the bridge, which orients the centromeres so that the noncrossover chromatids lie farthest apart. The acentric fragment cannot align itself or move and is, consequently, lost. In a pericentric inversion, because the centromeres are contained within the inverted region, the chromosomes that have crossed over disjoin in the normal fashion, without the creation of a bridge. However, the crossover produces chromatids that contain a duplication and a deficiency for different parts of the chromosome.

Translocations

When two non-homologous chromosomes exchange their parts, the resulting chromosomal rearrangements are translocations. Translocation may be *reciprocal* or *non-reciprocal*. Non-reciprocal translocation involves the transfer of a segment in one direction from one chromsomes to another. *Reciprocal translocations*, the most common type - a segment from one chromosome is exchanged with a segment from another non-homologous one, so two translocation chromosomes are generated simultaneously. The exchange of chromosome parts between nonhomologs establishes new linkage relations.



Reciprocal translocation between nonhomologous chromosomes

Meiosis in heterozygotes having two translocated chromosomes and their normal counterparts causes some important genetic and cytological effects. The pairing of homologous regions develop a characteristic configuration for chromosomes during meiosis. According to the law of independent assortment, there are two common patterns of disjunction. The segregation of each of the structurally normal chromosomes with one of the translocated ones ($T_1 \cdot N_2$ and $T_2 \cdot N_1$) is called **adjacent-1 segregation**. Both meiotic products are duplicated and deficient for different arms of the cross. These products are inviable. On the other hand, the two normal chromosomes may segregate together, as will the reciprocal parts of the translocated ones, to produce $N_1 \cdot N_2$ and $T_1 \cdot T_2$ products. This segregation is called **alternate segregation**. These products are viable.

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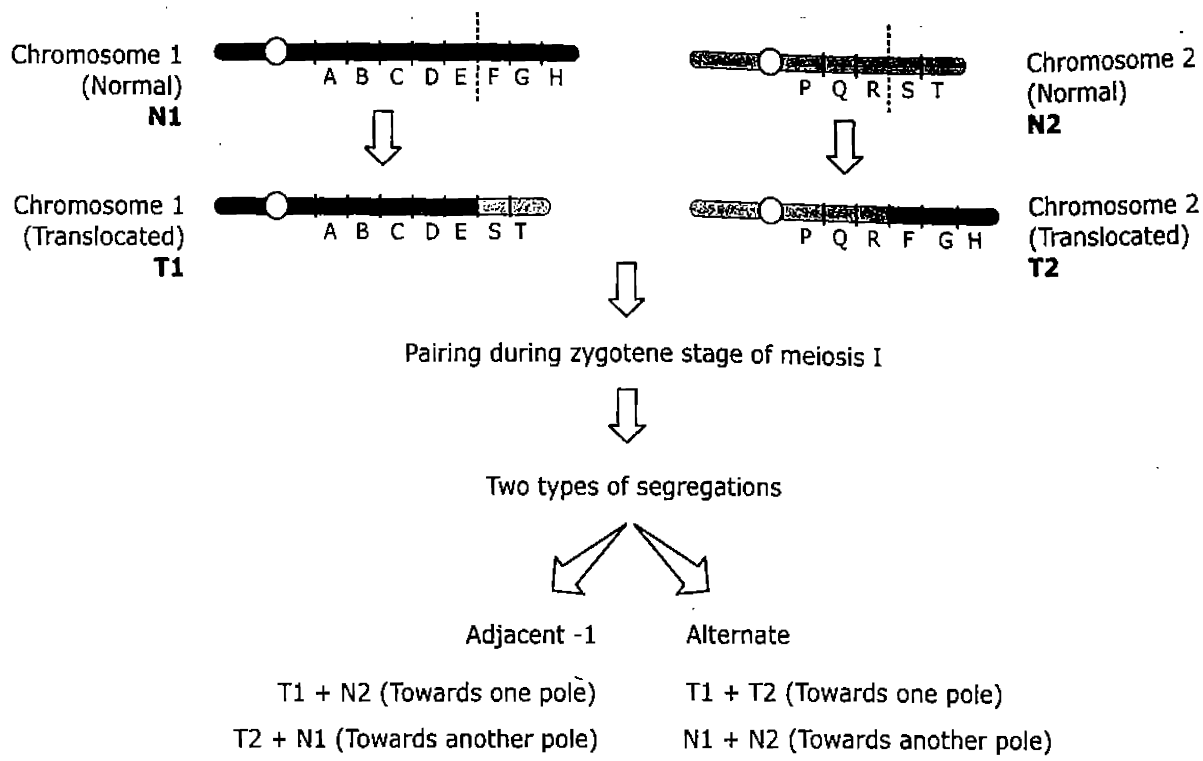


Figure 14.2 : The meiotic products resulting from the segregation of translocated chromosome.

A special pattern of translocation involving two acrocentric chromosomes is called **centric fusion-type**, or **Robertsonian translocation**. Typically, the breaks occur close to the centromere, affecting the short arms of both the chromosomes. Transfer of the segments leads to one very large chromosome and one extremely small one.

Ring chromosomes

A ring chromosome is formed when a break occurs on each arm of a chromosome leaving two sticky ends on the central portion that reunite as a ring.

Isochromosomes

An isochromosome shows loss of one arm with duplication of the other. The most probable explanation for the formation of an isochromosome is that the centomere has divided transversely rather than longitudinally.

Position effect

The change in the phenotypic expression of one or more genes as a result of a change in position in the genome is called **position effect.** Position effect may be exhibited if a gene located in euchromatin is brought near heterochromatin. Position effects are either *stable*, as in Bar eye of *Drosophila* or *variegated*, as with *Drosophila* eye colour. The locus for white eye colour in *Drosophila* is near the tip of the X-chromosome.

In case of *position effect variegation*, some of the regions in the eye lack colour, whereas others are red. Cells in which white is inactive give patches of white eye, whereas cells in which white is active give red patches. The white gene was inactivated by adjacent heterochromatin in some cells, but remain active in others.

Inactivation spreads from heterochromatin into the adjacent region for a variable distance. In some cells it goes far enough to inactivate a nearby gene, but in others it does not. This happens at a certain point in embryonic development, and after that point the state of the gene is inherited by all the progeny cells. Cells descended from an ancestor in which the gene was inactivated form patches corresponding to the phenotype of loss-of-function. The closer a gene lies to heterochromatin, the higher the probability that it will be inactivated.

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There is also a position effect in the Bar system. A homozygous Bar and a heterozygous double Bar both have four copies of the 16A regions. It would therefore be reasonable to expect that both genotypes would produce the same phenotype. However, the homozygous Bar has about 68 facets in each eye whereas the heterozygote has about 45.

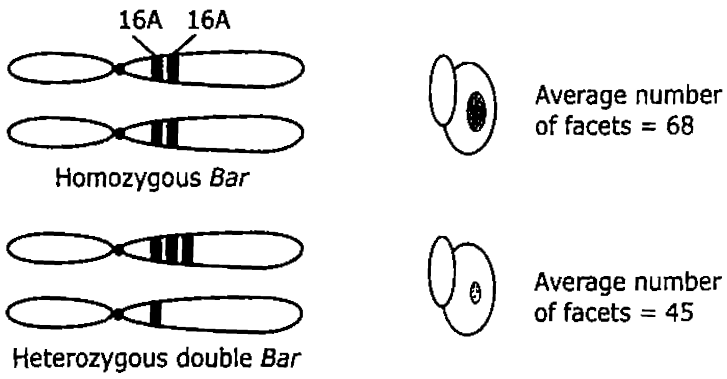


Figure : 14.3
Position effect and Bar eye in *Drosophila*. In Homozygous Bar females there are four copies of X chromosome segment 16A, two on each homolog; these flies have an average of 68 facets in their reduced eyes. In females heterozygous for double-Bar there are also four copies of segment 16A, three on the double-Bar chromosome and one on the normal chromosome; even though the number of segments is the same, these flies have smaller eyes, with an average number of 45 facets.

POPULATION GENETICS

Populations is a interbreeding groups of individuals of a particular species at a particular place. Populations are usually subdivided into partially isolated breeding groups called *demes*. The complete set of genetic information contained within the members in a population is called the *gene pool*. Gene pool includes all alleles present in the population. *Population genetics* is the study of changes in the frequencies of genes and genotypes within a population.

14.4 CALCULATING ALLELIC FREQUENCIES

If we consider an autosomal locus in a diploid, sexually reproducing species, allelic frequencies can be measured in either of two ways. The first way is simply by counting genes:

Consider, for example, the phenotypic distribution of MN blood types (controlled by the codominant *M* and *N* alleles) among two hundred persons.

Type M (*MM* genotype) = 114

Type MN (*MN* genotype) = 76

Type N (*NN* genotype) = 10

Since the homozygotes have two of a given allele and heterozygotes have only one, and since the total number of alleles is twice the number of individuals (each individual carries two alleles), we can calculate allelic frequencies in the following manner.

$$\text{Frequency of the } M \text{ allele} = \frac{\text{number of } M \text{ alleles}}{\text{total number of alleles}}$$

$$= \frac{(2 \times \text{number of MM homozygote}) + (\text{number of MN heterozygote})}{(2 \times \text{total no. of individual})}$$

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The expression 'frequency of' can be shortened to *f*(). For example, the frequency of the *M* allele will be written as *f*(M).

Then,

Similarly, $f(N) = q = \frac{2(10)+76}{2(200)} = \frac{96}{400} = 0.24$

Alternatively, because the frequencies of the two alleles, *M* and *N*, must add up to unity (p + q = 1), q = 1 – p (and p = 1 – q), if we know that p = 0.76, then q = 1 – 0.76 = 0.24.

Another way of calculating allelic frequencies is based on knowledge of the genotypic frequencies, which in this example are

$$f(MN) = \frac{114}{200} = 0.57$$

$$f(MN) = \frac{76}{200} = 0.38$$

$$f(NN) = \frac{10}{200} = 0.05$$

We derive an expression for calculating p and q based on genotypic frequencies as follows:

$$p = f(M) = \frac{2\times\text{number of MM}+\text{number of MN}}{2\times\text{total number}} = \frac{2\times\text{number of MM}}{2\times\text{total number}} + \frac{\text{number of MN}}{2\times\text{total number}}$$

$$p = f(MM) + (1/2)\, f(MN)$$

$$q = f(N) = \frac{2\times\text{number of NN}+\text{number of MN}}{2\times\text{total number}} = \frac{2\times\text{number of NN}}{2\times\text{total number}} + \frac{\text{number of MN}}{2\times\text{total number}}$$

$$q = f(NN) + (1/2)\, f(MN)$$

Thus, allelic frequencies can be calculated as the frequency of homozygotes plus half the frequency of heterozygotes as follows:

$$p = f(M) = f(MM) + (1/2)\, f(MN) = 0.57 + (1/2)0.38 = 0.76$$

$$q = f(N) = f(NN) + (1/2)\, f(MN) = 0.05 + (1/2)0.38 = 0.24$$

Note : *Allele and genotype frequencies must always be between 0 and 1.*

Calculation of allelic frequency with multiple alleles employ the same rule that we used with two alleles. Suppose we have three alleles – A1, A2, and A3 at a locus. Let p, q and r represent the frequencies of alleles A1, A2 and A3, respectively. In this case allelic frequency will be :

$$p = f(A1) = \frac{(2\times A1A1)+(A1A2)+(A1A3)}{(2\times\text{total number of individual})}$$

$$q = f(A2) = \frac{(2\times A2A2)+(A1A2)+(A2A3)}{(2\times\text{total number of individual})}$$

$$r = f(A3) = \frac{(2\times A3A3)+(A1A3)+(A2A3)}{(2\times\text{total number of individual})}$$

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14.5 HARDY-WEINBERG LAW

In 1908, *G.H. Hardy*, a British mathematician, and *W.Weinberg* a German physician, independently discovered a rule that relates allelic and genotypic frequencies in a population of diploid, sexually reproducing individuals. The rule has three aspects.

1. The allelic frequencies at an autosomal locus in a population will not change from one generation to the next (*allelic-frequency equilibrium*).
2. The genotypic frequencies of the population are determined in a predictable way by the allelic frequencies (*genotypic-frequency equilibrium*).
3. The equilibrium is neutral. That is, if it is perturbed, it will be reestablished within one generation of random mating at the new allelic frequencies (if all the other requirements are maintained).

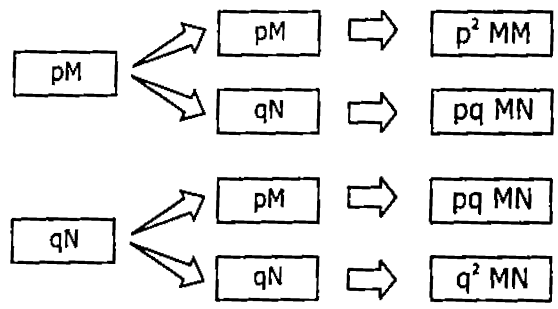
This principle based on certain assumptions. The following major assumptions are necessary for the Hardy Weinberg equilibrium to hold.

1. *Random mating* i.e., each individual of the population has equal opportunity of mating with any other individual of that population. If members of a population choose individuals of a particular phenotype as mates more or less often than at random, the population is engaged in **assortative mating**.
2. Allele frequencies are the same in males and females.
3. *No natural selection* i.e., all genotypes are equal in viability and fertility. No individual will have a reproductive advantage over another individual because of its genotype.
4. *No mutation*; If a particular locus shows a high mutation rate then there will be a steady increase in the proportion of mutant alleles in a population.
5. *No migration*; Allelic and genotypic frequencies may change through the loss or addition of alleles through migration (immigration or emigration) of individuals from or into a population.
6. The population is sufficiently large so that the frequencies of alleles do not change from generation to generation because of chance. When populations are small, changes in allelic frequencies take place due to chance alone. These changes are referred to as **genetic drift**.

In summary, the Hardy-Weinberg equilibrium holds for an infinitely large, randomly mating population in which mutation, migration, and natural selection do not occur.

Derivation of the Hardy-Weinberg Law

The Hardy-Weinberg law states that when a population is in equilibrium, the genotypic frequencies will be in the proportions P^2, 2pq and q^2. To understand the basis of these frequencies at equilibrium, consider the alleles M and N in a population whose allele frequencies are p and q, respectively (remember that p + q = 1). With random mating (each individual of the population has equal opportunity of mating with any other individual of that population), the relation between allele frequency and genotype frequency is particularly simple, because random mating of individuals is equivalent to random union of gametes. Conceptually, we may imagine all the gametes of a population to be present in a large container. To form zygote genotypes, pairs of gametes are withdrawn from the container at random. The genotype frequencies expected with random mating in this case can be deduced from the following diagram:



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In the above diagram, the gametes at the left represent the sperm and those in the middle the eggs. The genotypes that can be formed with two alleles are shown at the right, and with random mating the frequency of each genotype is calculated by multiplying the allele frequencies of the corresponding gametes. However, the genotype MN can be formed in two ways—the M allele could have come from the father (top part of diagram), or from the mother (bottom part of diagram). In each case, the frequency of the MN genotype is pq; considering both possibilities, the frequency of MN is pq + pq = 2pq. Consequently, the overall genotype frequencies expected with random mating are:

$$MM: p^2 \quad MN: 2pq \quad NN: q^2$$

The relationship between gene frequency and genotype frequency can be expressed as $p^2 + 2pq + q^2 = 1$ or $(p + q)^2 = 1$. It is known as the **Hardy-Weinberg** principle.

After random mating, the frequency of the M homozygote is p^2 and the frequency of the heterozygote is 2pq. Thus the frequency of the M allele, the frequency of its homozygote plus half the frequency of the heterozygotes, is

$$f(M) = f(MM) + (1/2)\, f(MN) = p^2 + (1/2)(2pq) = p^2 + pq = p(p + q)$$

The Hardy Weinberg law holds true for any frequencies of M and N, as long as the frequencies add to 1 and all the assumptions are evoked. A population in which the allele frequencies remain constant from generation to generation and in which genotype frequencies can be predicted from the allele frequencies is said to be in a state of Hardy Weinberg equilibrium for that locus.

Validity of Hardy-Weinberg equilibrium

It is possible to establish whether a population is in Hardy-Weinberg equilibrium for a particular trait if all possible genotypes can be identified. Consider a system with two alleles A and a, with three resulting genotypes AA, Aa/aA and aa. Amongst 1000 individuals selected at random, the following genotype distributions are observed.

AA	800
Aa/aA	185
Aa	15

From this data,

The frequency of the allele A (p) = [(2×800) + 185]/2000 = 0.8925

The frequency of the allele a (q) equals [185 + (2×15]/2000 = 0.1075.

Now consider what the expected genotype frequencies would be if the population is in Hardy-Weinberg equilibrium and compare these with the observed values.

Genotype	*Observed*	*Expected*
AA	800	796.5 (p^2 × 1000)
Aa/aA	185	192 (2pq × 1000)
aa	15	11.5 (q^2 × 1000)

These observed and expected values correspond closely and formal statistical analysis with a test would confirm that the observed values do not differ significantly from those expected if the population is in equilibrium.

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Now take a different system with two alleles B and b. Amongst 1000 randomly selected individuals the observed genotype distributions are:

BB	430
Bb/bB	540
bb	30

From this data,

The frequency of the allele B (p) = [(2×430)+540]/2000 = 0.7

The frequency of the allele b (q) = [540 + 2 (2×30)]/2000 = 0.3

Using these values for p and q the observed and expected genotype distributions can be compared.

Genotype	*Observed*	*Expected*
BB	430	490 (p^2 × 1000)
Bb/bB	540	420 (2pq × 1000)
bb	30	90 (q^2 × 1000)

These values differ considerably, with an increased number of heterozygotes at the expense of homozygotes. This deviation from Hardy-Weinberg equilibrium may be due to increased numbers of heterozygotes, such as heterozygote advantage or negative assortative mating, i.e. the attraction of opposites.

STUDY QUESTIONS

Q1. The diploid number of the garden pea is 2n = 14.

a. How many different trisomics could be formed?

b. How many different double trisomics could be formed?

Ans. a. 7

b. 21

Q2. Explain what type of abnormal sperm unites with a normal egg to produce an XYY offspring. Specifically, how does such an abnormal gamete arise?

Ans. A sperm bearing two Y chromosomes is produced by non-disjunction of the Y sister chromatids during the second meiotic division. When united with a normal egg, an XYY offspring would be expected.

Q3. How many *A* and a alleles are present in a sample of organisms consisting of 10 *AA*, 15 *Aa*, and 4 *aa* individuals? What are the allele frequencies in this sample?

Ans. The 10 *AA* individuals contain 20 *A* alleles, the 15 *Aa* contain 15 *A* and 15 *a*, and the 4 *aa* contain 8 *a*. Altogether there are 35 *A* and 23 *a* alleles, for a total of 58. The allele frequency of *A* is 35/58 = 0.603 and that of *a* is 23/58 = 0.397.

Q4. Given gene *A* at frequency 0.2 and gene *B* at frequency 0.6, find the equilibrium frequencies of the gametes *AB*, *Ab*, *aB* and *ab*.

Ans. *AB* = 0.12, *Ab* = 0.08, *aB* = 0.48, *ab* = 0.32

Q5. If the frequency of a homozygous dominant genotype in a randomly mating population is 0.09, what is the frequency of the dominant allele? What is the combined frequency of all the other alleles of this gene?

Ans. $p^2 = 0.09$, and so $p = (0.09)^{1/2} = 0.30$. All other alleles have a combined frequency of 1 – 0.30 = 0.70.

Q6. A particular recessive disorder is present in one in ten thousand individuals. If the population is in Hardy-Weinberg equilibrium, what are the frequencies of the two alleles?

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Ans. $p = 0.99$, $q = 0.01$. If the population is in equilibrium, there should be p^2 of AA + $2pq$ of Aa + q^2 of aa individuals. Since 1/10,000 shows the recessive trait, this is q^2. Therefore,

Since $p + q = 1$, $p = 1 - 0.01 = 0.99$.

Q7. Hartnup disease is an autosomal-recessive disorder of intestinal and renal transport of amino acids. The frequency of affected newborn infants is about 1 in 14,000. Assuming random mating, what is the frequency of heterozygotes?

Ans. The frequency of the recessive allele is $q = (1/14{,}000)^{1/2} = 0.0085$. Hence,
$p = 1 - 0.0085 = 0.9915$, and the frequency of heterozygotes is $2pq = 0.017$ (about 1 in 60).

Q8. White wool is dependent upon a dominant allele *B* and black wool upon its recessive allele *b*. Suppose that a sample of 900 sheep of the Rambouillet breed gave the following data: 891 white and 9 black. Estimate the allelic frequencies.

Ans. $q = \sqrt{q^2} = \sqrt{9/900} = 0.1$ = frequency of allele *b*,

Since $p + q = 1$, the frequency of allele *B* is 0.9.

SELF TEST

Q1. Certain types of cancers can be correlated with specific changes in chromosome structure. In patients suffering from myelogenous leukemia, the abnormal chromosome detected was termed Philadelphia chromosome. Which of the following chromosome is altered in this disease?

a. chromosome 10
b. chromosome 11
c. chromosome 20
d. chromosome 22

Q2. A dicentric chromosome is unstable because

a. it cannot resynthesize its telomeres during replication
b. it pairs with non-homologous chromosomes in meiosis
c. it pairs with non-homologous chromosomes in mitosis
d. it is often simultaneously drawn to opposing spindle poles in mitosis

Q3. The forces that can change the frequency of an allele in a population are

a. forward mutation, gene conversion, neutral evolution and recombination
b. selection, mutation, migration, inbreeding and random genetic drift
c. dominance, family selection, fitness and diversification
d. gene interaction, gene transfer, gene mutation and out breeding **[JNU]**

Q4. Which of the following statements are *correct*

P. Down's syndrome/mongolism is due to extra 21st chromosome
Q. Down's syndrome is due to non-disjunction of chromosomes
R. Trisomy has chromosome complement of $2n + 1$
S. Monosomics are $2n - 1$

a. P, Q
b. R, S
c. P, Q, R
d. P, Q, R, S

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Cytogenetics and population genetics

Q5. Which of the following statements are *correct*

P. Cri-du-chat syndrome is due to chromosomal change involving deletion

Q. Formation of multivalents in meiosis is due to reciprocal translocation

R. Klinefelter's syndrome has 44 + XXY

S. In Down's syndrome of a male child, the sex complement is XY

a. P, Q

b. Q, R

c. P, Q, S

d. P, Q, R, S

Q6. Consider two linked loci, labeled A and B. Each locus has two alleles, labeled 1 and 2. The frequencies of the alleles in a population are: A1 = 0.6, A2 = 0.4, B1 = 0.7, B2 = 0.3. If there is linkage equilibrium between these two loci in the population, what is the expected frequency of chromosomes carrying a combination of the A1 and B2 alleles?

a. 0.24

b. 0.12

c. 0.18

d. 1.0

Q7. Why are some autosomal dominant disorders (e.g., Marfan syndrome) seen more commonly in the offspring of older fathers?

a. replication errors accumulate as sperm-producing stem cells continue to divide

b. rate of nondisjunction increases in older males

c. recombination rates increase in older males

d. none of the above

Q8. Which of the following will *not* cause triploidy

a. Fusion of an egg and polar body with subsequent fertilization by sperm cell

b. Meiotic failure, producing diploid sperm or egg

c. Dispermy

d. Mitotic failure in the early embryo

Q9. Each of the following chromosome abnormalities involves a 20 megabase region of the long arm of chromosome 5 (5q). Which abnormality is *most* likely to cause severe disease?

a. Deletion of the region

b. Duplication of the region

c. A balanced translocation involving the region (i.e., in the translocation carrier)

d. Pericentric inversion

Q10. Which of the following most accurately explains the cause for the abnormal numbers of chromosomes during human reproduction that can result in Down syndrome, Turner's syndrome, or Klinefelter's syndrome?

a. the occurrence of non-disjunction of homologous chromosomes during meiosis

b. the duplicative production of extra chromosomes during DNA replication

c. the abnormal pairing of non-homologous chromosomes during prophase of meiosis I

d. the selective loss of particular chromosomes from the sex cells after formation of the mature gamete

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ANSWERS OF SELF TEST

Chapter - 01 : Amino acids and Proteins

01. c	02. b	03. c	04. d	05. d	06. b	07. b
08. b	09. a	10. d	11. b	12. b	13. a	14. a
15. c	16. d	17. a	18. d	19. d	20. b	

Chapter - 02 : Nucleic acids

01. c	02. c	03. c	04. c	05. b	06. c	07. d
08. c	09. b	10. c	11. d	12. c	13. d	14. a
15. a	16. b	17. b	18. c	19. c	20. b	

Chapter - 03 : Biophysical techniques

01. c	02. a	03. a	04. d	05. d	06. b	07. b
08. b	09. a	10. a	11. d	12. c	13. c	14. b
15. c						

Chapter - 04 : Carbohydrates

01. c	02. c	03. b	04. c	05. c	06. b	07. b
08. d	09. d	10. c				

Chapter - 05 : Lipids

01. b	02. a	03. d	04. a	05. b	06. c	07. d
08. b	09. c	10. a	11. d	12. d	13. b	14. c
15. d						

Chapter - 06 : Enzymes and Vitamins

01. d	02. d	03. a	04. c	05. c	06. d	07. d
08. c	09. b	10. b	11. b	12. b	13. d	14. a
15. c						

Chapter - 07 : Respiration

01. c	02. d	03. b	04. b	05. a	06. a	07. a
08. b	09. a	10. I.b, II.a	11. b	12. d	13. d	14. c
15. a						

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Answer

Chapter - 08 : Photosynthesis

01. d	02. b	03. d	04. a	05. c	06. d	07. c
08. b	09. d	10. c	11. a	12. b	13. b	14. a
15. c	16. b	17. c	18. d	19. d	20. d	

Chapter - 09 : Cell Structure and Functions

01. c	02. b	03. d	04. b	05. b	06. b	07. b
08. b	09. c	10. a	11. d	12. d	13. c	14. c
15. c	16. d	17. c	18. d	19. d	20. c	21. d
22. b	23. b	24. c	25. d	26. d	27. c	28. d
29. c	30. c					

Chapter - 10 : Cell Cycle and Cancer

01. a	02. d	03. a	04. d	05. a	06. c	07. d
08. b	09. c	10. b				

Chapter - 11 : Prokaryotes and Virus

01. d	02. c	03. b	04. d	05. d	06. a	07. c
08. d	09. d	10. b	11. d	12. d	13. d	14. a
15. d	16. c	17. c	18. b	19. d	20. d	

Chapter - 12 : Immunology

01. a	02. c	03. c	04. d	05. c	06. b	07. b
08. b	09. d	10. c	11. b	12. c	13. a	14. a
15. d						

Chapter - 13 : Classical genetics

01. c	02. b	03. c	04. a	05. b	06. c	07. a
08. d	09. a	10. d	11. d	12. d	13. a	14. b
15. a	16. a	17. a	18. b	19. a	20. a	

Chapter - 14 : Cytogenetics and Population genetics

01. d	02. d	03. b	04. d	05. d	06. c	07. a
08. d	09. a	10. a				

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