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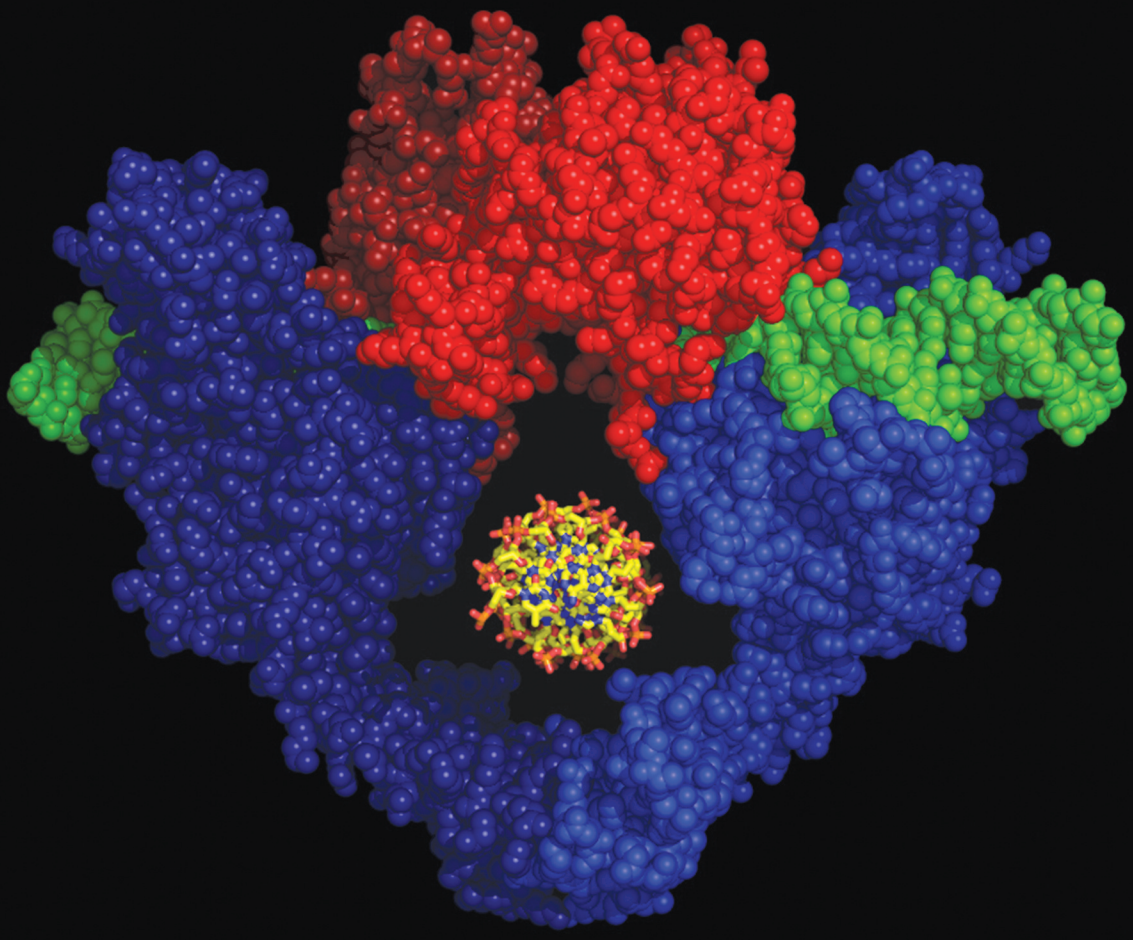
Life Sciences

Fundamentals and Practice - I

Fifth edition



Pranav Kumar | Usha Mina

Life Sciences

Fundamentals and Practice – I

Fifth edition

Pranav Kumar
Former faculty,
Department of Biotechnology
Jamia Millia Islamia,
New Delhi, India

Usha Mina
Senior Scientist,
CESCRA,
Indian Agricultural Research Institute (IARI),
New Delhi, India

Pathfinder Publication
New Delhi, India

Pranav Kumar
Former faculty,
Department of Biotechnology
Jamia Millia Islamia,
New Delhi, India

Usha Mina
Senior Scientist,
CESCRA,
Indian Agricultural Research Institute (IARI),
New Delhi, India

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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, interdisciplinary and up-to-date information and their understanding. This fifth edition of Life sciences, Fundamentals and practice includes extensive revisions of the previous edition. We have attempted to provide an extraordinarily large amount of information from the enormous and ever-growing field in an easily retrievable form. It is written in clear and concise language to enhance self-motivation and strategic learning skill of the students and empowering them with a mechanism to measure and analyze their abilities and the confidence of winning. We have given equal importance to text and illustrations. The fifth edition has a number of new figures to enhance understanding. At the same time, we avoid excess detail, which can obscure the main point of the figure. We have retained the design elements that have evolved through the previous editions to make the book easier to read. Sincere efforts have been made to support textual clarifications and explanations with the help of flow charts, figures and tables to make learning easy and convincing. The chapters have been supplemented with self-tests and questions so as to check one's own level of understanding. Although the chapters of this book can be read independently of one another, they are arranged in a logical sequence. Each page is carefully laid out to place related text, figures and tables near one another, minimizing the need for page turning while reading a topic. I have given equal importance to text and illustrations as well. We hope you will find this book interesting, relevant and challenging.

Acknowledgements

Our students were the original inspiration for the first edition of this book, and we remain continually grateful to them, because we learn from them how to think about the life sciences, and how to communicate knowledge in most meaningful way. We thank, Dr. Diwakar Kumar Singh and Mr. Ajay Kumar, reviewers of this book, whose comment and suggestions were invaluable in improving the text. Any book of this kind requires meticulous and painstaking efforts by all its contributors. Several diligent and hardworking minds have come together to bring out this book in this complete form. We are much beholden to each of them and especially to Dr. Neeraj Tiwari. This book is a team effort, and producing it would be impossible without the outstanding people of Pathfinder Publication. It was a pleasure to work with many other dedicated and creative people of Pathfinder Publication during the production of this book, especially Pradeep Verma.

Pranav Kumar

Usha Mina

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Chapter 01

Biomolecules and Catalysis

A *biomolecule* is an organic molecule that is produced by a living organism. Biomolecules act as building blocks of life and perform important functions in living organisms. More than 25 naturally occurring chemical elements are found in biomolecules. Most of the elements have relatively low atomic numbers. Biomolecules consist primarily of carbon, hydrogen, nitrogen, oxygen, phosphorus and sulfur. The four most abundant elements in living organisms, in terms of the percentage of the total number of atoms, are hydrogen, oxygen, nitrogen, and carbon, which together make up over 99% of the mass of most cells.

Nearly all of the biomolecules in a cell are carbon compounds, which account for more than one-half of the dry weight of the cells. Covalent bonding between carbon and other elements permit formation of a large number of compounds. Most biomolecules can be regarded as derivatives of hydrocarbons. The hydrogen atoms may be replaced by a variety of functional groups to yield different families of organic compounds. Typical families of organic compounds are the alcohols, which have one or more hydroxyl groups; amines, which have amino groups; aldehydes and ketones, which have carbonyl groups; and carboxylic acids, which have carboxyl groups. Many biomolecules are polyfunctional, containing two or more different kinds of functional groups. Functional groups determine chemical properties of biomolecules.

Sugars, fatty acids, amino acids and nucleotides constitute the four major families of biomolecules in cells. Many of the biomolecules found within cells are macromolecules and mostly are polymers (composed of small, covalently linked monomeric subunits). These macromolecules are proteins, carbohydrates, lipids and nucleic acids.

Small biomolecules	*Macromolecules*
Sugars	Polysaccharide
Fatty acids	Fats/Lipids
Amino acids	Proteins
Nucleotide	Nucleic acid

Nucleic acids and proteins are informational macromolecules. Proteins are polymers of amino acids and constitute the largest fraction (besides water) of cells. The nucleic acids, DNA and RNA, are polymers of nucleotides. They store, transmit, and translate genetic information. The polysaccharides, polymers of simple sugars, have two major functions. They serve as energy-yielding fuel stores and as extracellular structural elements.

1.1 Amino acids and Proteins

Amino acids are compounds containing carbon, hydrogen, oxygen and nitrogen. They serve as monomers (building blocks) of proteins and are composed of an amino group, a carboxyl group, a hydrogen atom, and a distinctive side chain, all bonded to a carbon atom, the α-carbon.

In an α-amino acid, the amino and carboxylate groups are attached to the same carbon atom, which is called the α-carbon. The various α-amino acids differ with respect to the side chain (R group) attached to their α-carbon. The general structure of an amino acid is:

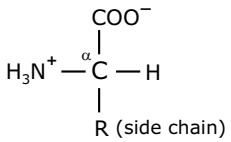

Figure 1.1 General structure of an amino acid.

This structure is common to all except one of the α-amino acids (*proline* 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.

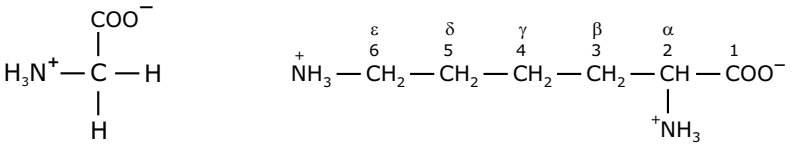

Figure 1.2 Structure of glycine and lysine.

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 depending upon the location of the C-atom to which amino group is attached.

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, an amino acid is an **amphoteric molecule**. At high concentrations of hydrogen ions (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*.

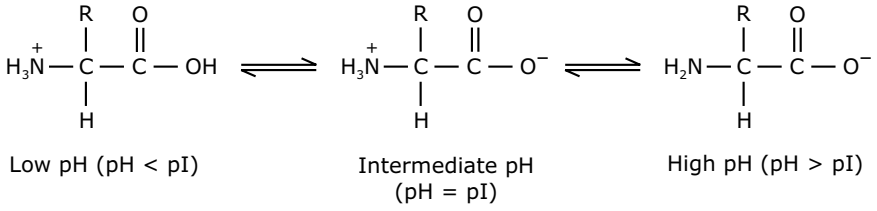

Figure 1.3 The acid-base behavior of an amino acid in solution. At low pH, the positively charged species predominates. As the pH increases, the electrically neutral zwitterion becomes predominant. At higher pH, the negatively charged species predominates.

1.1.1 Optical properties

All amino acids except glycine are optically active i.e. they rotate the plane of plane polarized light. Optically active molecules contain *chiral* carbon. A tetrahedral carbon atom with four different constituents are said to be chiral. All amino acids except glycine have chiral carbon and hence they are optically active.

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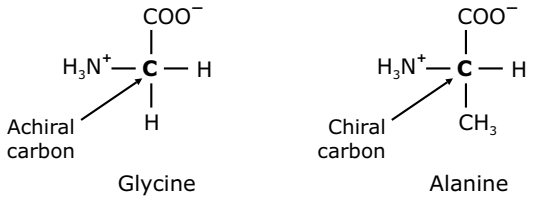


Figure 1.4 Amino acids showing achiral and chiral carbon.

An optically active compound can rotate the plane of polarized light either clockwise (to the right) or counterclockwise (to the left). Optically active compounds that rotate the plane of polarized light clockwise are said to be *dextrorotatory*. By convention, this direction is designated by a plus sign (+). Optically active compounds that rotate the plane of polarized light counterclockwise are said to be *levorotatory*. This is designated by a minus sign (–). The + and – forms have also been termed *d*- and *l*-, respectively.

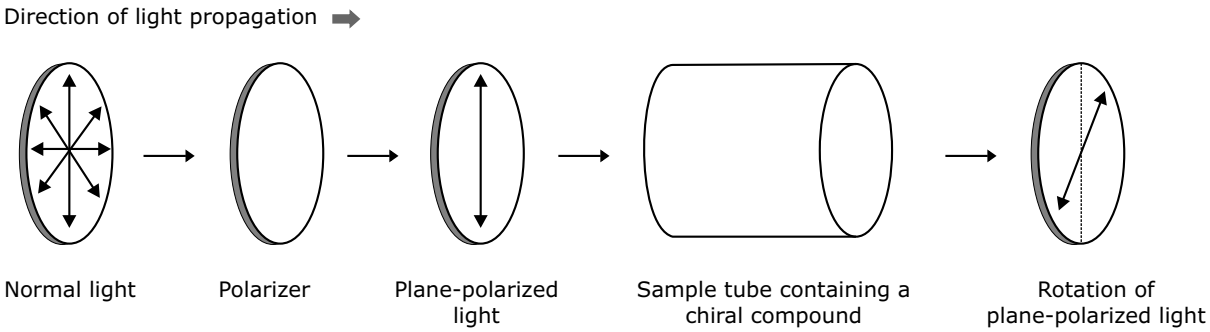


Figure 1.5 When plane polarized light is passed through a solution that contains an optically active compound, there is net rotation of the plane polarized light. The light is rotated either clockwise (dextrorotatory) or counterclockwise (levorotatory) by an angle that depends on the molecular structure and concentration of the compound, the path length and the wavelength of the light.

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 rotations of an optically active compound depend on the 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* $[\alpha]_{\lambda}^{T}$. The optical rotation of a solution at a given temperature and wavelength is given by

$$[\alpha]_{D}^{T} = \frac{\text{Å}}{l \times C}$$

Where, Å = observed rotation in degrees

$[\alpha]_{\lambda}^{T}$ = the specific rotation of compound at temperature, T (in degrees Celsius) and wavelength, λ (in nm). If the wavelength of the light used is 589 nm, the symbol 'D' is used, $[\alpha]_{D}^{T}$.

C = the concentration of solution in g/ml.

l = light path length in decimeter.

Problem

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.

Solution

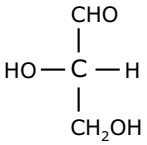
$$[\alpha]_D^T = \frac{\text{Å}}{l \times C}$$

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

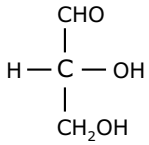
$[\alpha] = +15.1°$.

1.1.2 Absolute configuration

An amino acid with a chiral carbon can exist in two configurations that are non-superimposable mirror images of each other. These two configurations are called *enantiomers*. An enantiomer is identified by its absolute configuration. For example, 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.

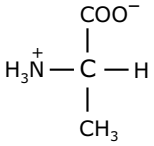


L-Glyceraldehyde

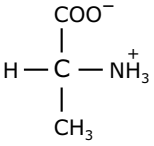


D-Glyceraldehyde

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 the amino acids ribosomically incorporated into proteins exhibit L-configuration. Therefore, they are all L-α-amino acids. The basis for preference for L-amino acids is not known. 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 eubacteria.

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 chiral carbon with four different substituents) are prioritized by decreasing the atomic number. Atoms of higher atomic number bonded to a chiral centre are ranked above those of lower atomic number. For example, the oxygen atom of a —OH group takes precedence over the carbon atom of the $—CH_3$ group that is bonded to the same chiral carbon atom. If any of the first substituent atoms are of the same element, the priority of these groups is established from the

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Table 1.3 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

Problem

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. Alanine obtained from a protein hydrolysate has the same absolute configuration as D-glyceraldehyde.

Solution

1. False; glycine has no asymmetric carbon atom.
2. True.
3. False; alanine obtained from a protein hydrolysis has the same absolute configuration as L-glyceraldehyde.

1.1.5 Peptide and polypeptide

A *peptide* is a compound consisting of two or more amino acids. When two amino acid molecules are linked, through peptide bonds then the product is called a *dipeptide*. Three amino acids can be joined by two peptide bonds to form a *tripeptide*, similarly amino acids can be linked to form tetrapeptides, pentapeptides and so forth. Peptide chains of more than 12 and less than about 20 amino acid residues are usually referred to as **oligopeptide**. Some peptides are cyclic in nature. Two cyclic decapeptides (peptides containing 10 amino acid residues) produced by the bacterium *Bacillus brevis* are common examples. Both of these peptides, gramicidin S and tyrocidine A, are antibiotics, and both contain D-amino acids as well as L-amino acids. In addition, both contain the amino acid ornithine, which does not occur in proteins. Small peptides play many roles in organisms. Some, such as *oxytocin* and *vasopressin*, are important hormones. Others, like *glutathione*, regulate oxidation–reduction reactions. Still others, such as *enkephalins*, are naturally occurring painkillers. *Aspartame* is a commercially synthesized dipeptide, L-aspartylphenylalanyl methylester, and is used as an artificial sweetener.

When many amino acid residues are joined, the product is called a **polypeptide**. Amino acids which have been incorporated into a peptide or polypeptide are termed *amino acid residues*. By convention, in a polypeptide the left end represented by the first amino acid while the right end represented by the last amino acid. The first amino acid is also called as N-terminal amino acid residue. The last amino acid is called the C-terminal amino acid residue.

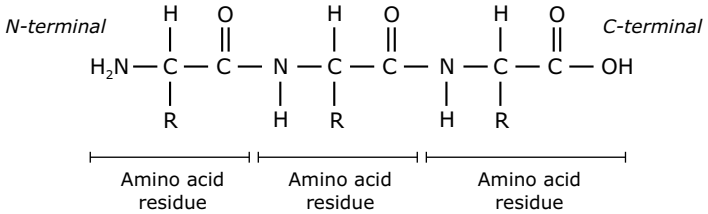

Figure 1.10 A series of amino acids joined by peptide bonds form a polypeptide chain, and each amino acid unit in a polypeptide is called a residue. A polypeptide chain has polarity because its ends are different, with an α-amino group at one end and an α-carboxyl group at the other.

The peptide bonds in proteins are formed 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.

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Concerted model

Monod, Wymann and Changeux in 1965, proposed a 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 an 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.

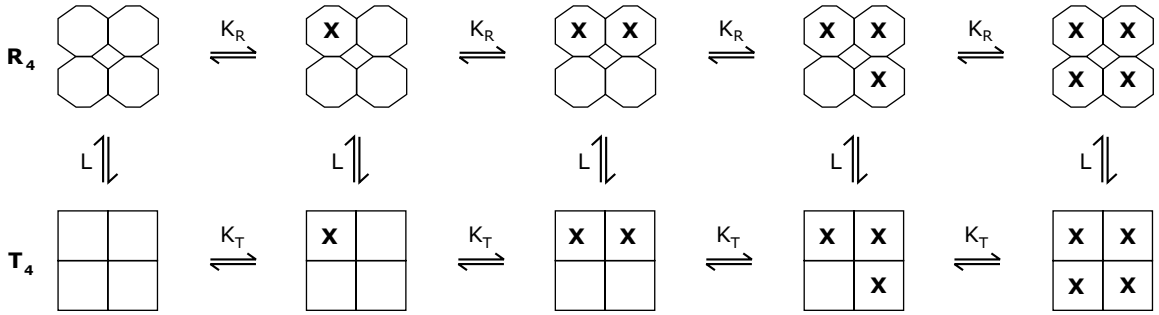

Figure 1.29 The concerted model. The squares represent the T state; the octagon represents the R state. In the absence of the ligand, the T state is favored. When ligand are bound to the oligomer (tetramer), the equilibrium gradually shifts to the R state, which has a higher affinity for the ligand.

As shown in the figure 1.29, the protein has two conformations, the R conformation, which binds substrate tightly and the T conformation, which binds substrate loosely. 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 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 an 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.

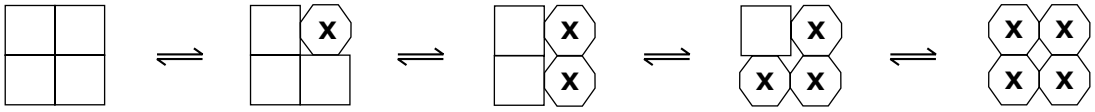

Figure 1.30 The sequential model. The squares represent the T state; octagon represents the R state. The binding of a ligand to a subunit changes the conformation of that subunit. This transition increases the affinity of the remaining subunits for the ligand.

1.3 Protein folding

Protein folding is the physical process by which a polypeptide folds into its characteristic and functional three-dimensional conformation. The correct three-dimensional structure is essential to protein function. Failure to fold into native structure produces inactive proteins. A protein molecule folds spontaneously during or after biosynthesis. However, the process also depends on the nature of solvent, the concentration of salts, the temperature, and the presence of molecular chaperones.

One of the most important experiment, which helped in understanding the process of protein folding was carried out by Christian Anfinsen and colleagues in the early 1960s. C. Anfinsen studied the refolding of protein ribonuclease A. Ribonuclease A isolated from bovine pancreas is an enzyme that has a molecular mass of 13,700 Da. It contains 124 amino acid residues and four disulfide linkages. In the presence of urea, a denaturant, and β-mercaptoethanol, a reducing agent, ribonuclease is denatured and the disulfide bonds are broken. When the protein is allowed to renature by removing the denaturant and the reductant, the protein regains its native conformation, including four correctly paired disulfide bonds. This finding provided the first evidence that the amino acid sequence of a polypeptide chain contains all the information required to fold the chain into its native three dimensional structure. However, when the reductant is removed while the denaturant is still present, the disulfide bonds are formed again in protein but most of the disulfide bonds are formed between incorrect partners. This indicates that weak interactions are required for correct positioning of disulfide bonds and assumption of the native conformation.

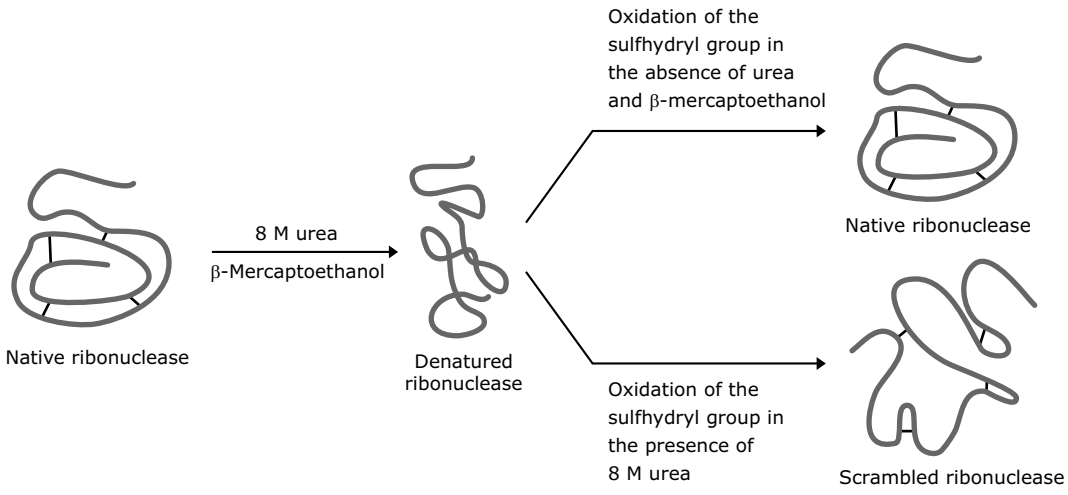

Figure 1.31 Denaturation and renaturation of ribonuclease. Depending on the conditions for renaturation, we obtain either native ribonuclease or scrambled ribonuclease.

The amino acid sequence of a protein determines its native conformation. But, if this is true, how do proteins find the right conformation out of the simply endless number of potential three-dimensional forms that it could randomly fold into? After all, the folding of a protein is not a chemical reaction. The folding pathway of a polypeptide is very complicated, and not all the principles that guide the process have been worked out. However, there are several models to explain folding. According to one model, folding is initiated by a spontaneous collapse of the unfolded polypeptide chain into a partly organized globular state, mediated by hydrophobic interactions among nonpolar residues (*hydrophobic collapse*). The collapsed state is referred to as a **molten globule**. This state is clearly different from the native and the denatured state. The molten globule has most of the secondary structure of the native state but it is less compact and the proper packing interactions in the interior of the protein have not been formed. This event is very fast, usually completes within a few milliseconds. We therefore know almost nothing about the process that leads to the molten globule. However we know some of the properties of this state. As mentioned above the molten globule has most of the secondary structure of the native state. It is less compact than

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Chaperonins are of two types – Group I chaperonins and Group II chaperonins. Group I chaperonins (includes Hsp60s in mitochondria and chloroplasts of eukaryotes and GroEL in eubacteria) have seven-membered rings and functionally cooperate with Hsp10 proteins in eukaryotes (GroES in eubacteria), which form the lid of the folding cage. The group II chaperonins in archaea and the eukaryotic cytosol usually have eight-membered rings. They are independent of Hsp10 factors.

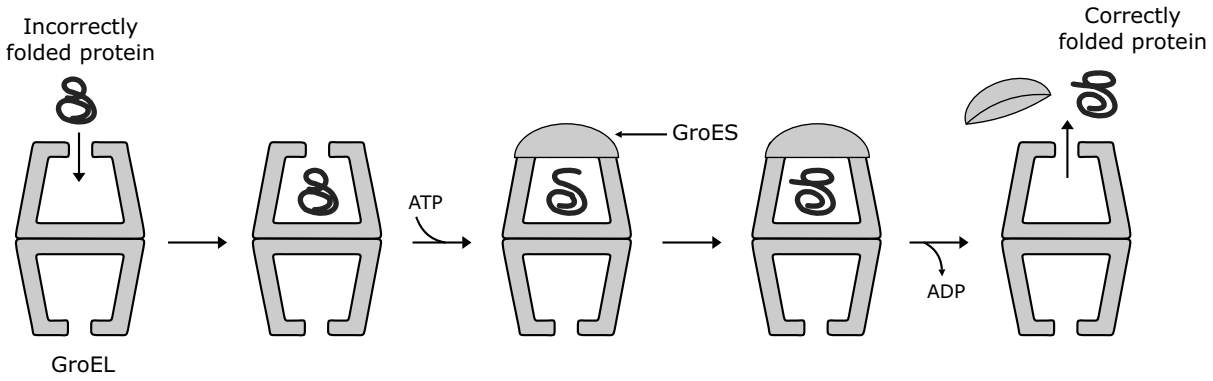

Figure 1.33 The structure and function of the chaperonin, GroEL. A misfolded protein molecule binds to one end of the GroEL. Subsequently ATP and protein cap (GroES) binds. ATP hydrolysis occurs as the protein is folding or un-folding inside the central cavity. ATP binding and hydrolysis are required for release of GroES and the protein molecule. A new un-folded protein molecule can now bind to GroEL.

Problem

The enzyme RNase A requires the formation of four disulphide bonds amongst its eight cysteine residues. 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?

Solution

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 $\frac{1}{7}\times\frac{1}{5}\times\frac{1}{3}=\frac{1}{105}$.

1.3.2 Amyloid

Amyloid refers to the abnormal fibrous, extra- as well as intracellular, proteinaceous deposits found in organs and tissues. It is insoluble, very resistant to degradation and is structurally dominated by β-sheet structure. Unlike other fibrous proteins, it does not commonly have a structural role but is associated with the pathology seen in a range of diseases known as the **amyloidoses**. These diseases include Alzheimer's, the spongiform encephalopathies and type II diabetes, all of which are progressive disorders.

Amyloid fibrils are insoluble and heterogeneous. Amino acid composition and sequence analysis of amyloid fibrils revealed that each amyloid disorder was associated with a particular protein or peptide. More than 20 plasma proteins have been identified that form amyloid. Interestingly, despite of the differences in amino acid sequences and native structure, these amyloidogenic peptides all appear to share a common β-sheet conformation of their polypeptide backbone. It is likely that this characteristic feature confers the proteolytic resistant and insoluble nature to all forms of amyloid.

Many proteins can take on the amyloid fibril structure as an alternative to their normal folded conformations. The proteins are secreted in an incompletely folded conformation. The core β-sheet forms before the rest of the protein folds correctly. The β-sheets from two or more incompletely folded protein molecules associate to begin forming an amyloid fibril. Additional protein molecules slowly associate with the amyloid and extend it to form a fibril.

1.3.3 Ubiquitin mediated protein degradation

The ability to degrade proteins is an essential function of all eukaryotic cells. The two main proteolytic systems within eukaryotic cells are *lysosomes* and *proteasomes*. Lysosomes are membrane-bound organelles that contain an assortment of acid hydrolases, including many proteolytic enzymes.

Proteasomes are cylindrical, multisubunit proteases found in both prokaryotes and eukaryotes. In eukaryotes, they are present in both cytosol and the nucleus. Eukaryotic proteasomes are of two types: 20S proteasome and 26S proteasome. The *20S proteasome* is a cylindrical barrel-shaped structure consisting of 28 subunits arranged as four rings of heptamers stacked upon one another. All subunits are members of the same superfamily of proteins, which group into two families, designated alpha and beta. In eukaryotic cells, the 20S proteasome assembles with two 19S regulatory complexes (RC) in an ATP dependent manner to form the *26S proteasome*.

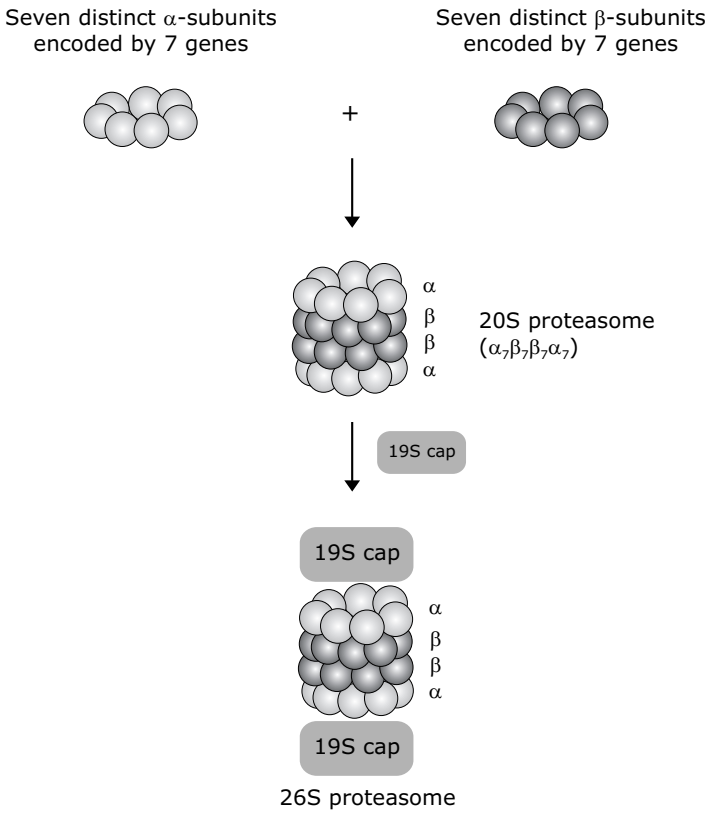

Figure 1.34 The 26S proteasome is a protease that is present in the cytosol, perinuclear regions and nucleus of eukaryotic cells. It consists of a 28-subunit catalytic core – 20S proteasome – which is an assembly of two outer and two inner heptameric rings stacked axially to form a hollow cylindrical structure in which proteolysis occurs. The 19S particle consists of ~19 individual proteins. The 19S regulatory unit has three functions. First, the 19S unit binds specifically to polyubiquitin chains, thereby ensuring that only ubiquitinated proteins are degraded. Second, an isopeptidase in the 19S unit cleaves off intact ubiquitin molecules from the proteins so that they can be reused. Finally, the protein is unfolded and directed into the catalytic core.

The 26S proteasome degrades proteins by an energy dependent mechanism. Proteasomes do not digest cellular proteins indiscriminately, but rather participate in the regulated breakdown of proteins that have been altered so as to be susceptible to degradation. It catalyses the ATP-dependent degradation of polyubiquitinated proteins.

Ubiquitination or ubiquitylation of substrates

The most well-established means of targeting proteins to proteasomes is by addition of ubiquitin. **Ubiquitin** is a highly conserved eukaryotic protein of 76 amino acid residues. The process by which ubiquitin is covalently attached

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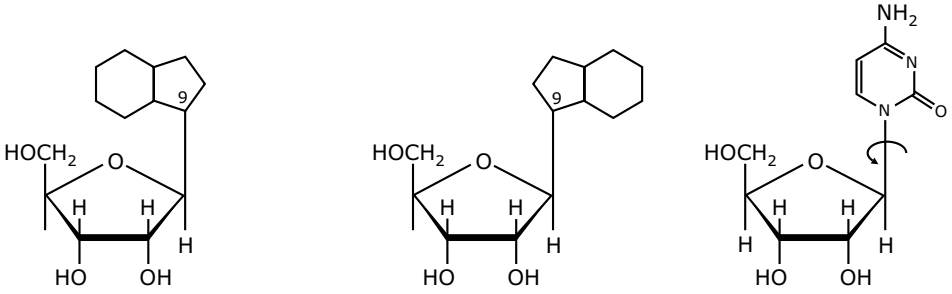
The base is free to rotate around the glycosidic bond. Due to rotation of the glycosidic bond, two different conformations are possible. The two standard conformations of the base around the glycosidic bond are *syn* and *anti.* Pyrimidines tend to adopt the *anti* conformation almost exclusively, because of steric interference between O2 and C5' in the *syn*-conformation, whereas purines are able to assume both forms (*syn* as well as *anti*).



Syn (purine) *Anti* (purine) *Anti* (pyrimidine)

Nucleotides

The nucleotides are phosphoric acid esters of nucleosides, with phosphate at position C5′. The nucleotide can have one, two, or three phosphate groups designated as α, β and γ for the first, second and third, respectively.

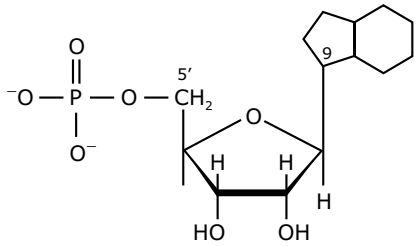


Figure 1.44 Structure of nucleotide.

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.
- Act as a precursor for several important coenzymes such as NAD^+, $NADP^+$, FAD and coenzyme A.
- Serving as a precursor for secondary messengers like cyclic AMP (cAMP), cGMP.

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. 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 a high energy bond. The active form of ATP is usually a complex of ATP with Mg^{2+} or Mn^{2+}.

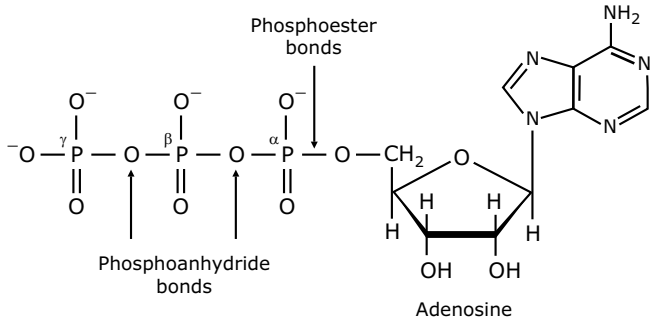


Figure 1.45 Structure of ATP indicating phosphoester and phosphoanhydride bonds.

Table 1.10 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'

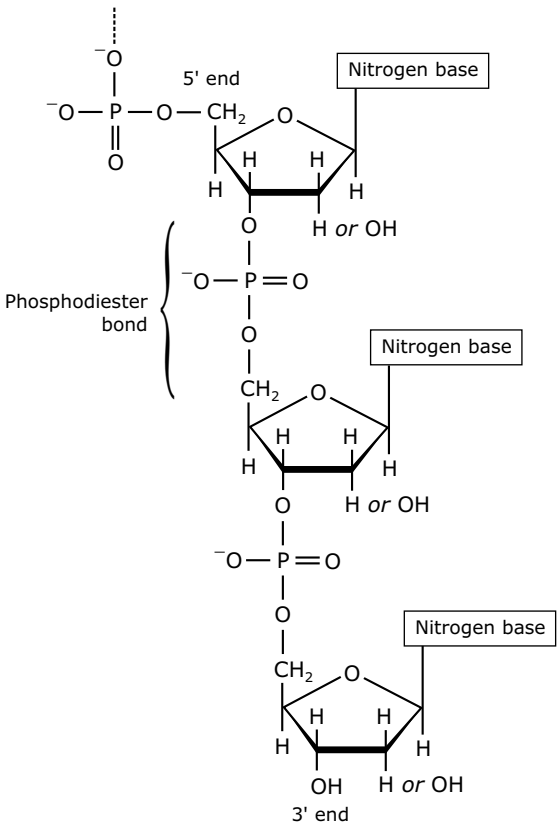


Figure 1.46 The polynucleotide has a 5' end, which is usually attached to a phosphate, and a 3' end, which is usually a free hydroxyl group. The backbones of these polynucleotide are formed by 3' to 5' phosphodiester linkages.

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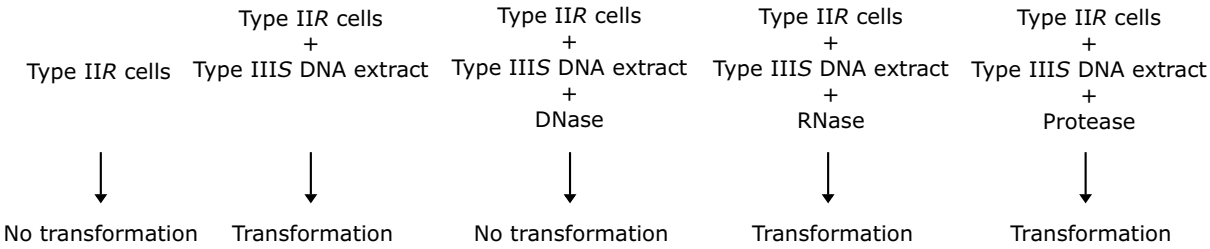

Figure 1.56 Avery, MacLeod, and McCarty had shown that only DNA extracts from S cells caused transformation of R cells to S cells. To demonstrate that contaminating molecules in the DNA extract were not responsible for transformation, the DNA extract from S cells was treated with RNase, DNase, and protease and then mixed with R cells.

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 virus that infects bacteria. It consists of a protein coat that encloses the dsDNA. Since phages consist only of nucleic acid surrounded by protein, they lend themselves nicely to the determination of whether the protein or the nucleic acid is the genetic material.

Hershey and Chase designed an experiment using radioactive isotopes of sulfur and phosphorus to keep separate track of the viral proteins and nucleic acids during the infection process. They used the T2 bacteriophage and the bacterium *Escherichia coli.* The phages were labeled by having them infect bacteria growing in culture medium containing the radioactive isotopes ^{35}S or ^{32}P. Hershey and Chase then proceeded to identify the material injected into the cell by phages attached to the bacterial wall. When ^{32}P-labeled phages were mixed with unlabeled *E. coli* cells, Hershey and Chase found that the ^{32}P label entered the bacterial cells and that the next generation of phages that burst from the infected cells carried a significant amount of the ^{32}P label. When ^{35}S-labeled phages were mixed with unlabeled *E. coli,* the researchers found that the ^{35}S label stayed outside the bacteria for the most part. Hershey and Chase thus demonstrated that the outer protein coat of a phage does not enter the bacterium it infects, whereas the phage's inner material, consisting of DNA, does enter the bacterial cell. Since the DNA is responsible for the production of the new phages during the infection process, the DNA, not the protein, must be the genetic material. Hershey shared the 1969 Nobel Prize in Physiology or Medicine for his *discoveries concerning the genetic structure of viruses*.

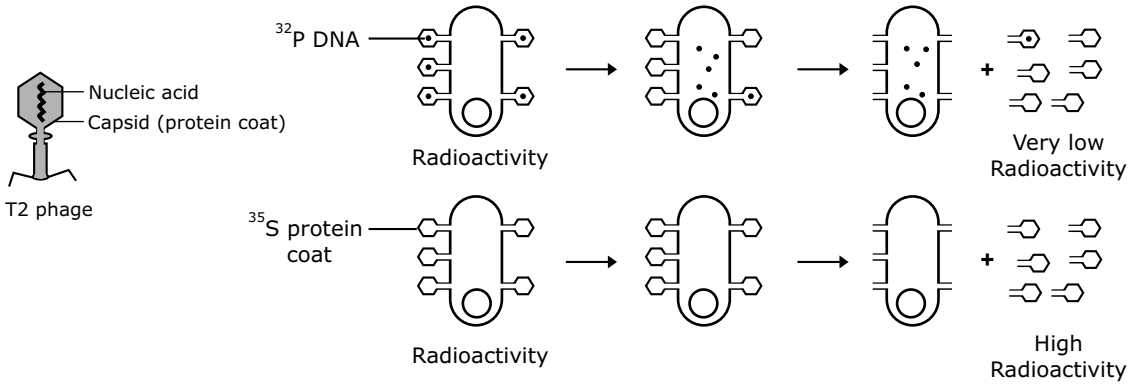

Figure 1.57 The Hershey-Chase experiment.

1.7 RNA

DNA contains all the information needed to maintain a cell's processes, but these precious blueprints never leave the protected nucleus. How, then, all these data are transmitted to the body of the cell itself where they are 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, RNA may be genetic or non genetic, catalytic or non-catalytic and coding (mRNA) or noncoding (like tRNA, rRNA). Within a given cell, RNA molecules are found in multiple copies and in multiple forms. Major RNA classes are mRNA, rRNA, tRNA, snRNA, SnoRNA, miRNA, XIST, scRNA, siRNA, tmRNA and telomerase RNA.

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 bases 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's 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.

RNA duplexes are more stable than DNA duplexes. At physiological pH, denaturation of a double stranded helical RNA often requires higher temperatures than those required for denaturation of a DNA molecule with a comparable sequence. However, the physical basis for these differences in thermal stability is not known.

1.7.1 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.

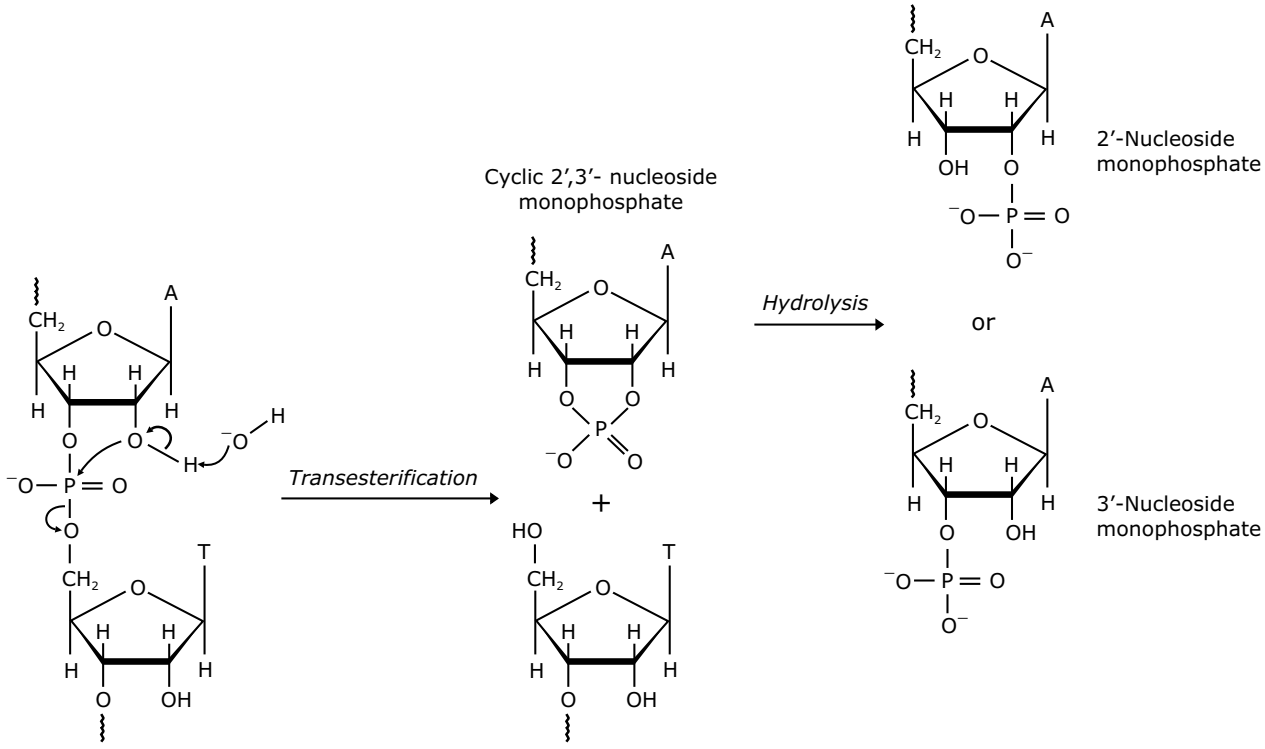

Figure 1.58 Alkali-catalyzed cleavage of RNA.

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Solution

1. The genetic code is a triplet code. That is, it takes a sequence of three nucleotides on the coding strand of DNA to specify one amino acid. The DNA of T4 contains:

   $\frac{1.3\times10^8}{650} = 2\times10^5$ nucloeotide pairs $= 2\times10^5$ nucleotides in the coding strand.

   $\frac{2\times10^5}{3} = \sim 6.7\times10^4$ codons.

2. The average MW of an amino acid residue is 110. A protein of MW 55000 contains:

   $\frac{55000}{110} = 500$ amino acids.

   6.7×10^4 codons can yield: $\frac{6.7\times10^4}{500} = 134$.

Nucleic acid conversion factors

Average MW of a DNA base pair = 650 Da

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

To calculate the concentration of plasmid DNA in solution using absorbance at 260 nm:

(Observed A_{260}) × (dilution factor) × (0.050) = DNA concentration in µg/µl

1.8 Carbohydrates

Carbohydrates are polyhydroxy aldehydes or polyhydroxy ketones, or compounds that can be hydrolyzed to them. In the majority of carbohydrates, H and O are present in the same ratio as in water, hence also called as *hydrates of carbon*. Carbohydrates are the most abundant biomolecules on Earth. Carbohydrates are classified into following classes depending upon whether these undergo hydrolysis and if so on the number of products form:

Monosaccharides are simple carbohydrates that cannot be hydrolyzed further into polyhydroxy aldehyde or ketone unit.

Oligosaccharides are polymers made up of two to ten monosaccharide units joined together by glycosidic linkages. Oligosaccharides can be classified as di-, tri-, tetra- depending upon the number of monosaccharides present. Amongst these the most abundant are the disaccharides, with two monosaccharide units.

Polysaccharides are polymers with hundreds or thousands of monosaccharide units. Polysaccharides are not sweet in taste hence they are also called *non-sugars*.

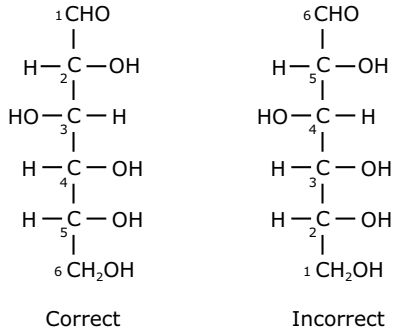
1.8.1 Monosaccharide

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 $C_nH_{2n}O_n$. Monosaccharides are colorless, crystalline solids that are freely soluble in water but insoluble in nonpolar solvents. 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.



Presence of aldehydes or ketones groups

Aldoses are monosaccharides with an aldehyde group.

Ketoses are monosaccharides containing a ketone group.

The monosaccharide *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-).

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.

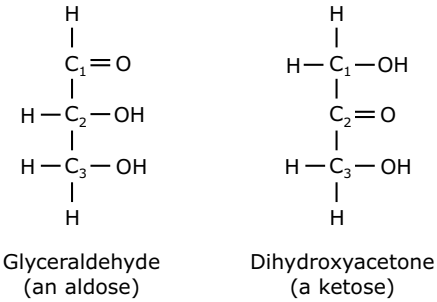


Figure 1.60 Trioses, the simplest monosaccharides.

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 non-superimposable mirror images of each other. These two forms are called *enantiomers*. An enantiomer 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.

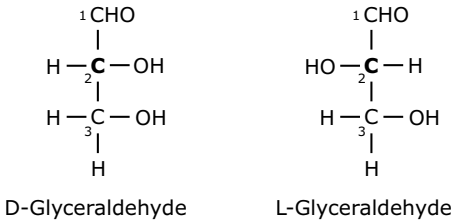


Figure 1.61 The enantiomers of glyceraldehyde. The configuration of groups around the chiral carbon 2 (shown in bold) distinguishes D-glyceraldehyde from L-glyceraldehyde. The two molecules are mirror images and cannot be superimposed on one another.

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1.8.5 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 a 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. Similarly, in *sucrose* anomeric carbon atoms of a glucose unit and a fructose unit are joined through $\alpha 1 \leftrightarrow 2\beta$ -glycosidic linkage and the configuration of this glycosidic linkage is α for glucose and β for fructose. The abbreviated name of sucrose is either Glc ($\alpha 1 \leftrightarrow 2\beta$) Fru or Fru ($2\beta \leftrightarrow \alpha 1$) Glc.

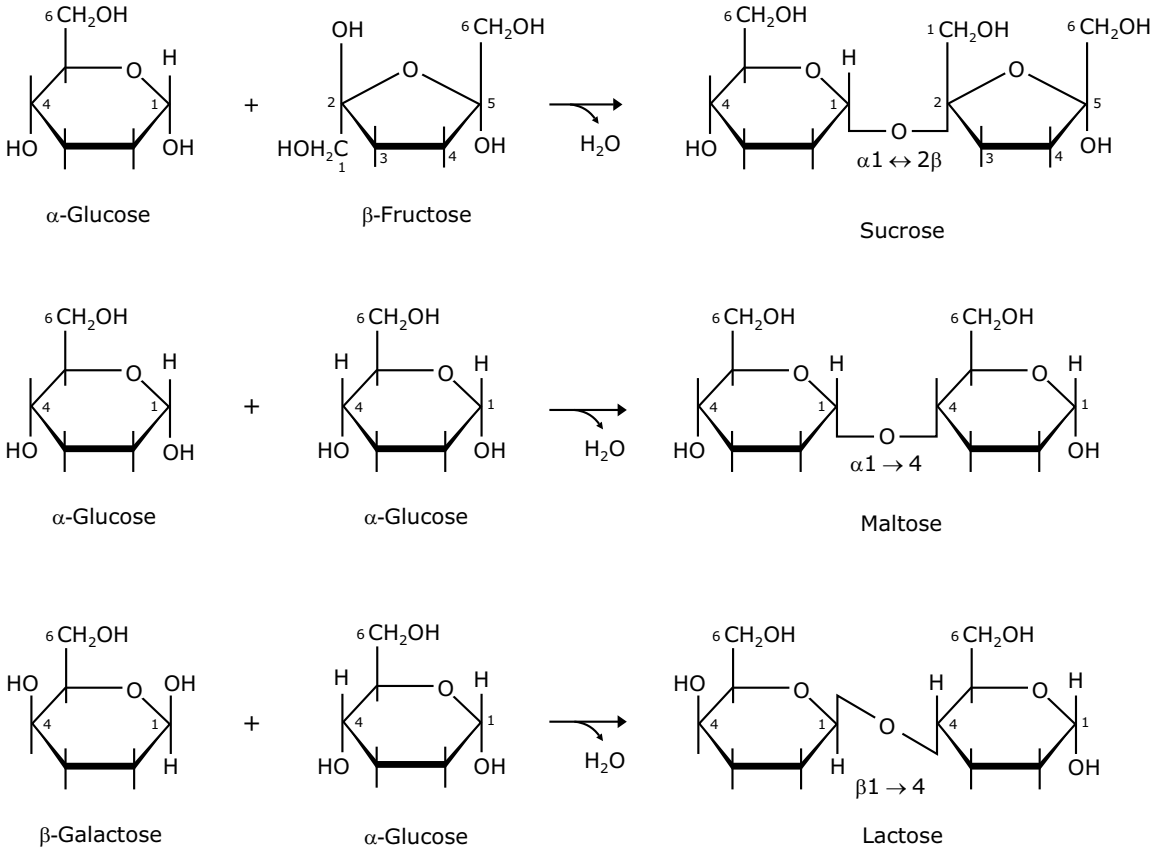

Figure 1.64 *Sucrose* is a disaccharide of glucose and fructose residues joined by a glycosidic linkage between C-1 (the anomeric carbon) of glucose residue and C-2 (the anomeric carbon) of the fructose residue. The anomeric carbons of both monosaccharide units are involved in the glycosidic bond. The disaccharide *maltose* contains two D-glucose residues joined by a glycosidic linkage between C-1 (the anomeric carbon) of one glucose residue and C-4 of the other. The configuration of the anomeric carbon atom in the glycosidic linkage between the two D-glucose residues is α. Lactose, the disaccharide of milk, consists of galactose joined to glucose by a β-1,4-glycosidic linkage.

Table 1.13 Occurrence and biochemical roles of some representative disaccharides

Disaccharides	*Structure*	*Physiological role*
Sucrose	Glucose ($\alpha 1 \leftrightarrow 2\beta$) Fructose	A product of photosynthesis.
Lactose	Galactose ($\beta 1 \rightarrow 4$) Glucose	A major animal energy source.
Trehalose	Glucose ($\alpha 1 \leftrightarrow 1\alpha$) Glucose	A major circulatory sugar in insects; used for energy.
Maltose	Glucose ($\alpha 1 \rightarrow 4$) Glucose	The dimer derived from the starch and glycogen.
Cellobiose	Glucose ($\beta 1 \rightarrow 4$) Glucose	The dimer of the cellulose polymer.
Gentiobiose	Glucose ($\beta 1 \rightarrow 6$) Glucose	Constituent of plant glycosides and some polysaccharides.

Hydrolysis of sucrose

Sucrose (invert sugar) 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, $[\alpha]_D$ = +52.7°, 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.

Problem

How many different disaccharides containing D-galactopyranose plus D-glucopyranose are possible?

Solution

There are 20 possible disaccharides containing galactose plus glucose in the pyranose ring forms:

Galactosides : 1 – 2, 1 – 3, 1 – 4, and 1 – 6 = 4
Linked α or β $\therefore \times 2$
= 8

Glucosides : 1 – 2, 1 – 3, 1 – 4, and 1 – 6 = 4
linked α or β $\therefore \times 2$
= 8

Nonreducing disaccharides : $\alpha - \alpha$, $\alpha - \beta$, $\beta - \alpha$ and $\beta - \beta$ = 4 (1 – 1 linked) Total : 20

1.8.6 Polysaccharides

Polysaccharides are ubiquitous in nature. They 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 *homo-polysaccharides* (contain only a single type of monomeric unit) or *heteropolysaccharides* (contain two or more different kinds of monomeric units).

Homopolysaccharides

Starch is a branched chain of D-glucose units. It is the storage form of glucose in plants. It contains a mixture of amylose and amylopectin. *Amylopectin* is a branched polymer of α-D-glucose with $\alpha 1 \rightarrow 4$-glycosidic linkages and with $\alpha 1 \rightarrow 6$ 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 $\alpha 1 \rightarrow 4$-glycosidic linkages.

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1.11 Reactive oxygen species and antioxidant

Reactive Oxygen Species (ROS) is a phrase used to describe a number of reactive chemical species and free radicals derived from molecular oxygen. *Free radicals* are atoms or molecules that have unpaired valence electrons, which explain their instability and high reactivity. Radicals can have positive, negative or neutral charge. They are formed as necessary intermediates in a variety of normal biochemical reactions. There are many types of radicals, but those of most concern in biological systems are derived from oxygen. Ground state oxygen may be converted to ROS either by *energy transfer* or by *electron transfer* reactions. The former leads to the formation of *singlet oxygen*, whereas the latter results in the sequential reduction to *superoxide radical*, *peroxide radical* and *hydroxyl radical*.

Oxygen	Superoxide radical	Peroxide radical	Hydroxyl radical
O_2	$O_2^{\bullet -}$	$O_2^{\bullet -2}$	$^{\bullet}OH$

When the level of ROS exceeds the normal level, a cell is said to be in a state of 'oxidative stress.' The enhanced level of ROS causes peroxidation of lipids (oxidations of unsaturated fatty acids), oxidation of proteins, damage to nucleic acids, enzyme inhibition and activation of programmed cell death pathway.

An *antioxidant* is a molecule that inhibits the oxidation of other molecules. Antioxidants remove free radical intermediates and inhibit oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols. Cells contain multiple types of antioxidants. Enzymatic antioxidant defences include superoxide dismutase, glutathione peroxidase and catalase. Non-enzymatic antioxidants are represented by vitamin C, vitamin E, glutathione, carotenoids, flavonoids and other antioxidants.

1.12 Enzymes

General features

An enzyme is a biocatalyst that increases the rate of chemical reaction without itself being changed in the overall process. It is a remarkable molecular device that determines the pattern of chemical transformations. Virtually all cellular reactions or processes are mediated by enzymes. Enzymes have several properties that make them unique.

- Most enzymes are proteins. With the exception of a small group of catalytic RNA molecules, all enzymes are proteins. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into its subunits, catalytic activity is usually lost.
- Enzymes are highly specific. They are highly specialized proteins and have a high degree of specificity for their substrates.
- 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 cofactor from a conjugated enzyme leaves only protein component, 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 1.21 Vitamin B-complex and their coenzyme forms

Vitamin	*Coenzyme form*	*Reaction or process promoted*
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 (B_5)	Coenzyme A	Acyl group transfer
Biotin	Biocytin	Carboxylation
Folic acid	Tetrahydrofolic acid	One-carbon group transfer
Vitamin B_{12}	Deoxyadenosylcobalamin	Intramolecular rearrangements

The vitamins in the human diet that are coenzyme precursors are all water soluble vitamins.

Table 1.22 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
Mg^{2+}	Hexokinase, Enolase, Glucose-6-phosphatase
Mn^{2+}	Arginase, Enolase, Pyruvate carboxylase
K^+	Pyruvate kinase
Ni^{2+}	Urease
Mo	Dinitrogenase, Xanthine oxidase
Se	Glutathione peroxidase

1.12.1 Naming and classification of enzyme

Many enzymes have common names. For example, trypsin, a proteolytic enzyme, is 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 helps in breaking down ATP, whereas ATP synthase is an enzyme that helps in synthesis of 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.

The 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-subclass, 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 the class of enzymes. There are six classes to which different enzymes belong. These classes are:

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EC 1 Oxidoreductase

Oxidoreductase catalyzes oxidation-reduction reactions.

$A_{red} + B_{ox} \longrightarrow A_{ox} + B_{red}$

Example

Oxidases	Use oxygen as an electron acceptor but do not incorporate it into the substrate.
Dehydrogenases	Use molecules other than oxygen (e.g. NAD^+) as an electron acceptor.
Oxygenases	Directly incorporate oxygen into the substrate.
Peroxidases	Use H_2O_2 as an electron acceptor.

EC 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*.

A–B + C ⟶ A + B–C

Examples

Transcarboxylases	Transfers a carboxylate group to a substrate.
Transaminases	Transfer amino group from amino acids to keto acids.
Kinases	Transfer phosphate from ATP to a substrate.
Phosphorylases	Transfer inorganic phosphate to a substrate.

EC 3 Hydrolases

Hydrolases catalyze reactions in which the cleavage of bonds is accomplished by adding water.

$A–B + H_2O \longrightarrow A–H + B–OH$

Example

Phosphodiesterases	Cleave phosphodiester bonds.
Phosphatases	Remove phosphate from a substrate.
Peptidases	Cleave amide bonds such as those in proteins.

EC 4 Lyases

Lyases are enzymes that catalyzes the breaking of C—C, C—O, C—N, C—S and other bonds by means other than hydrolysis or oxidation. These bonds are cleaved by the process of elimination and the result in the formation of a double bond or a new ring, or conversely adding groups to double bonds.

A = B + HX ⟶ A – X + B–H

Example

Aldolases	Removal of aldehydes *via* elimination reactions.
Synthases	Link two molecules without involvement of ATP.
Dehydratases	Removal of H_2O *via* elimination reactions.
Decarboxylases	Removal of CO_2 *via* elimination reactions.

EC 5 Isomerases

Isomerases catalyze several types of intramolecular rearrangements and yield isomeric forms.

A B ⟶ B A

Example

Mutases	Catalyze the intramolecular transfer of functional groups.
Cis-trans isomerase	Catalyzes the isomerization of geometric isomers.
Epimerases	Catalyze the inversion of asymmetric carbon atoms.
Racemases	Interconvert L and D stereoisomers.

EC 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. Other common names for ligases include *synthetases*, because they are used to synthesize new molecules.

$A + B + ATP \longrightarrow A - B + ADP$

Example

Carboxylases Use CO_2 as a substrate.

Biochemical nomenclature distinguishes synthetases from synthases. *Synthases* catalyze condensation reactions in which no nucleoside triphosphate (ATP and GTP) is required as an energy source. *Synthetases* catalyze condensations that *do* use nucleoside triphosphate as a source of energy for the synthetic reaction. A synthase is a lyase and does not require any energy, whereas a synthetase is a ligase and thus requires energy.

1.12.2 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 molecules interacting have the minimum amount of kinetic energy that 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. 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 only increases the rate of a reaction by lowering the activation energy.

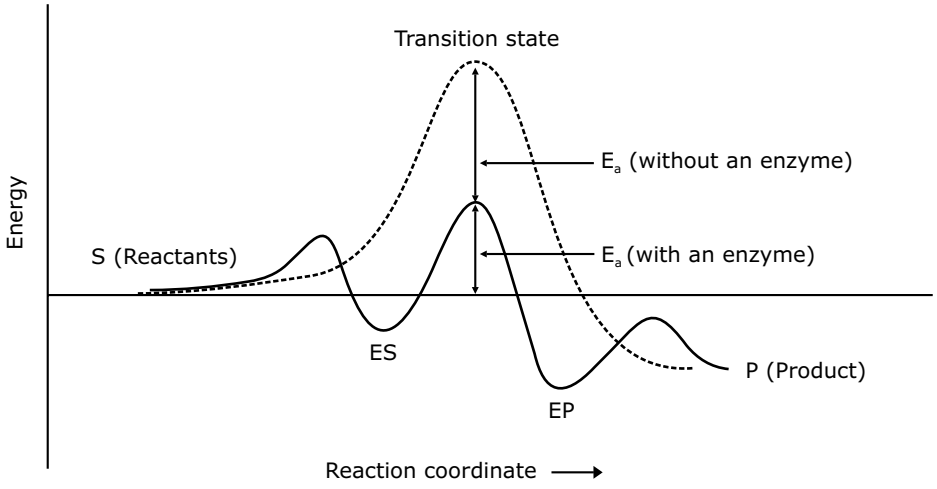

Figure 1.88 Energy profile of a simple enzyme-catalyzed reaction. The non-enzyme catalyzed reaction proceeds via a higher energy transition state and hence the reaction has a higher activation energy than the enzyme catalyzed reaction.

1.12.3 How enzymes operate?

In the first step of 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*. *This binding energy is used to lower the activation energy*. Only the correct substrate can participate in most or all of the interactions with the enzyme and thus maximize binding energy. The full complement of such interactions is formed only when the substrate is converted into the transition state.

The binding of the substrate to the enzyme may be represented by:

$$E + S \rightleftharpoons E - S \rightleftharpoons E - P \rightleftharpoons E + P$$

Where E is the enzyme, S is the substrate, the product E – S is the enzyme-substrate complex and E – P is a complex of the enzyme and the product. The binding of enzyme and substrate is highly specific. A given enzyme usually binds to only one kind of 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. However, the transition state is too unstable to exist for long. It collapses to either substrate or product, but which of the two accumulates is determined only by the free energy difference between the substrate 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.

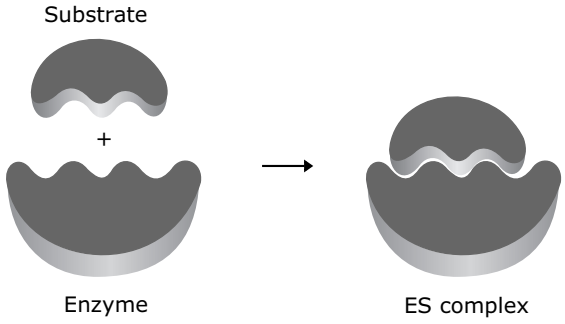

Figure 1.89 Lock-and-key model. In this model, the active site of the unbound enzyme is complementary in shape to the substrate.

The 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 a product. The product is released from the enzyme.

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In a double-reciprocal form the equation will be

$$\frac{1}{V} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$

In competitive inhibition, V_{max} stays same and K_m increases, but the inhibitor does not affect the turnover 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.

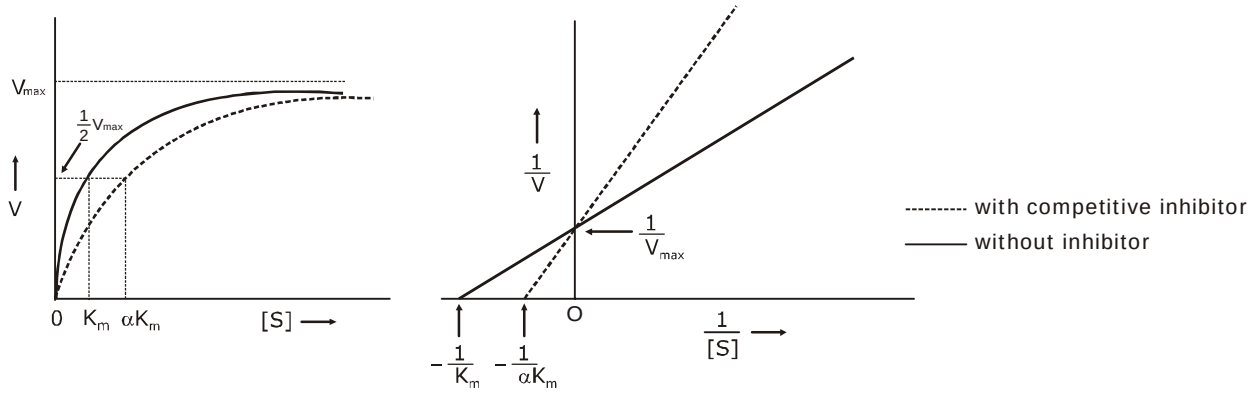
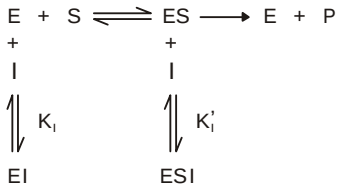


Figure 1.93 Michaelis-Menten and Lineweaver-Burk plot of uninhibited enzyme activity versus competitive inhibition.

Non-competitive inhibition

In non-competitive 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. There are two types of non-competitive inhibition— pure and mixed. In *pure non-competitive inhibition*, substrate and inhibitor bind at different sites on enzyme and binding of inhibitor does not affect binding of substrate. The inhibitor binds to *either* free enzyme or the ES complex. Hence, inhibition is not reversed by increasing the concentration of substrate.



$$K_I = \frac{[E]\,[I]}{[EI]} \quad \text{and} \quad K_I' = \frac{[ES]\,[I]}{[ES\,I]}$$

Pure non-competitive inhibition occurs if $K_I = K_I'$. In this type of inhibition, *V_{max} decreases and K_m stays constant.* In the presence of pure non-competitive inhibitor, the Michaelis-Menten equation becomes

$$V = \frac{V_{max}\,[S]}{\alpha K_m + \alpha'\,[S]} \qquad \text{Where, } \alpha' = 1 + \frac{[I]}{K_I'} \text{ and } \alpha = 1 + \frac{[I]}{K_I}$$

$$V = \frac{V_{max}\,[S]}{\alpha K_m + \alpha\,[S]} \qquad \text{Since, } \alpha' = \alpha$$

In a double-reciprocal form the equation will be

$$\frac{1}{V} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha}{V_{max}}$$

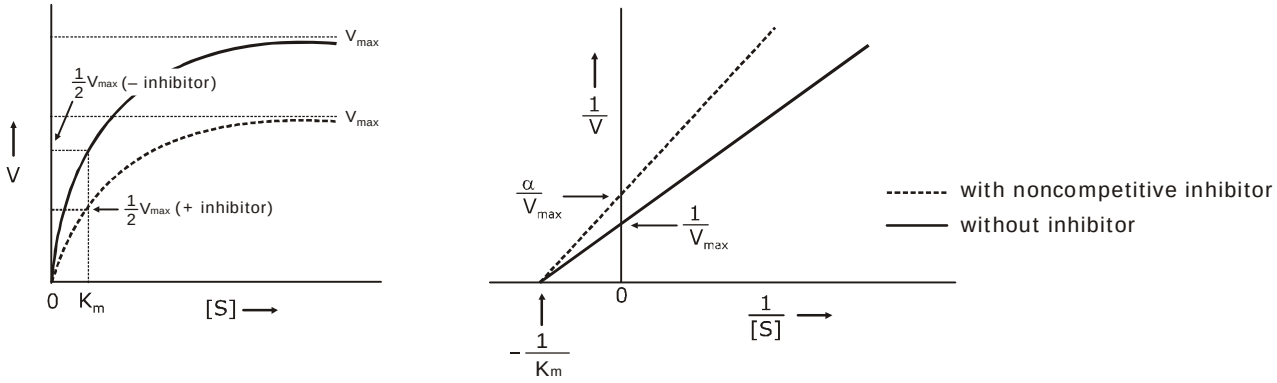


Figure 1.94 Michaelis-Menten and Lineweaver-Burk plot of uninhibited enzyme activity versus pure non-competitive inhibition.

In *mixed non-competitive inhibition*, the binding of inhibitor with enzyme influences the binding of substrate with enzyme. Either the binding sites for inhibitor and substrate are near one another or conformational changes in enzyme caused by inhibitor affect substrate binding. In this case, K_I and K_I' are not equal and both K_m and V_{max} are altered by the presence of inhibitor.

Uncompetitive inhibition

In uncompetitive inhibition, 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 decrease.

$$E + S \rightleftharpoons ES \longrightarrow E + P$$
$$+$$
$$I$$
$$\Updownarrow K_I'$$
$$ESI$$

$$K_I' = \frac{[ES]\,[I]}{[ES\,I]}$$

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 a double-reciprocal form the equation will be

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{\alpha'}{V_{max}}$$

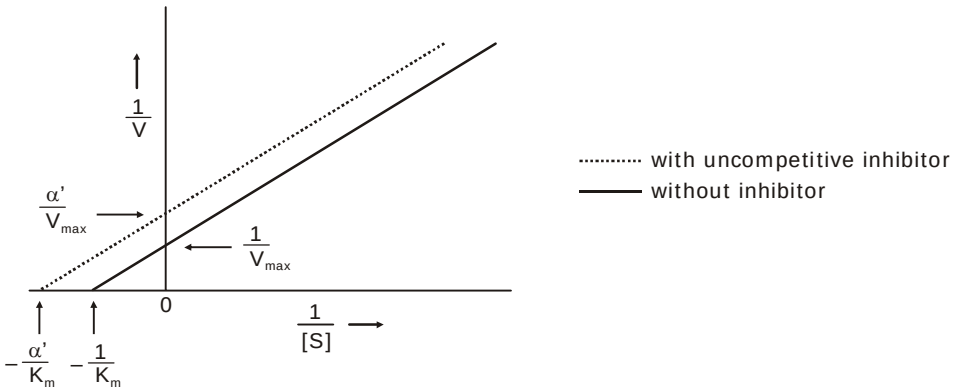


Figure 1.95 Lineweaver-Burk plot of uninhibited enzyme activity versus uncompetitive inhibition.

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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 figure 1.101 shows three central steps in this enzymatically catalyzed reaction.

Problem

The mechanism for lysozyme cleavage of its polysaccharide substrate requires Glu^{35} in its nonionized form, whereas the nearby Asp^{52} must be ionized. The pKs for the side chain carboxyl groups on the two amino acids in solution are virtually identical.

a. How can one carboxyl group be charged and the other uncharged in the active site of lysozyme?

b. The pH optimum for lysozyme is about 5? Why do you suppose that the activity decreases above and below this optimum?

Solution

a. Amino acid side chains in proteins often have quite different p*K*s than they do in solution. Glu^{35} is uncharged because its local environment is nonpolar, which makes ionization less favorable (raises its p*K*). The local environment of Asp^{52} is more polar, permitting ionization near its solution p*K*.

b. As the pH drops below 5, Asp^{52} picks up a proton and become nonionized, interfering with the mechanism. As the pH rises above 5, Glu^{35} begins to release its proton, also interfering with the mechanism.

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*, *acetyl-cholinesterase* 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 the 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 acts as a charge relay system. This grouping has been found in a whole group of enzymes called the serine proteases.

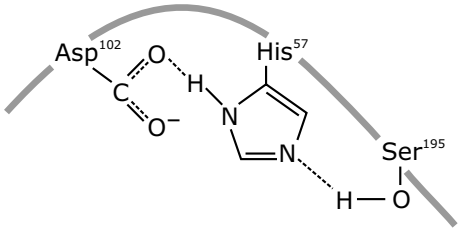

Figure 1.102 Catalytic triad acts as a charge relay system.

RNase A

Ribonucleases or RNases is a group of enzymes that catalyze the 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 the 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} acts as a general acid catalyst.

The 2′-3′ phosphodiester intermediate hydrolyzes in next steps. The 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}.

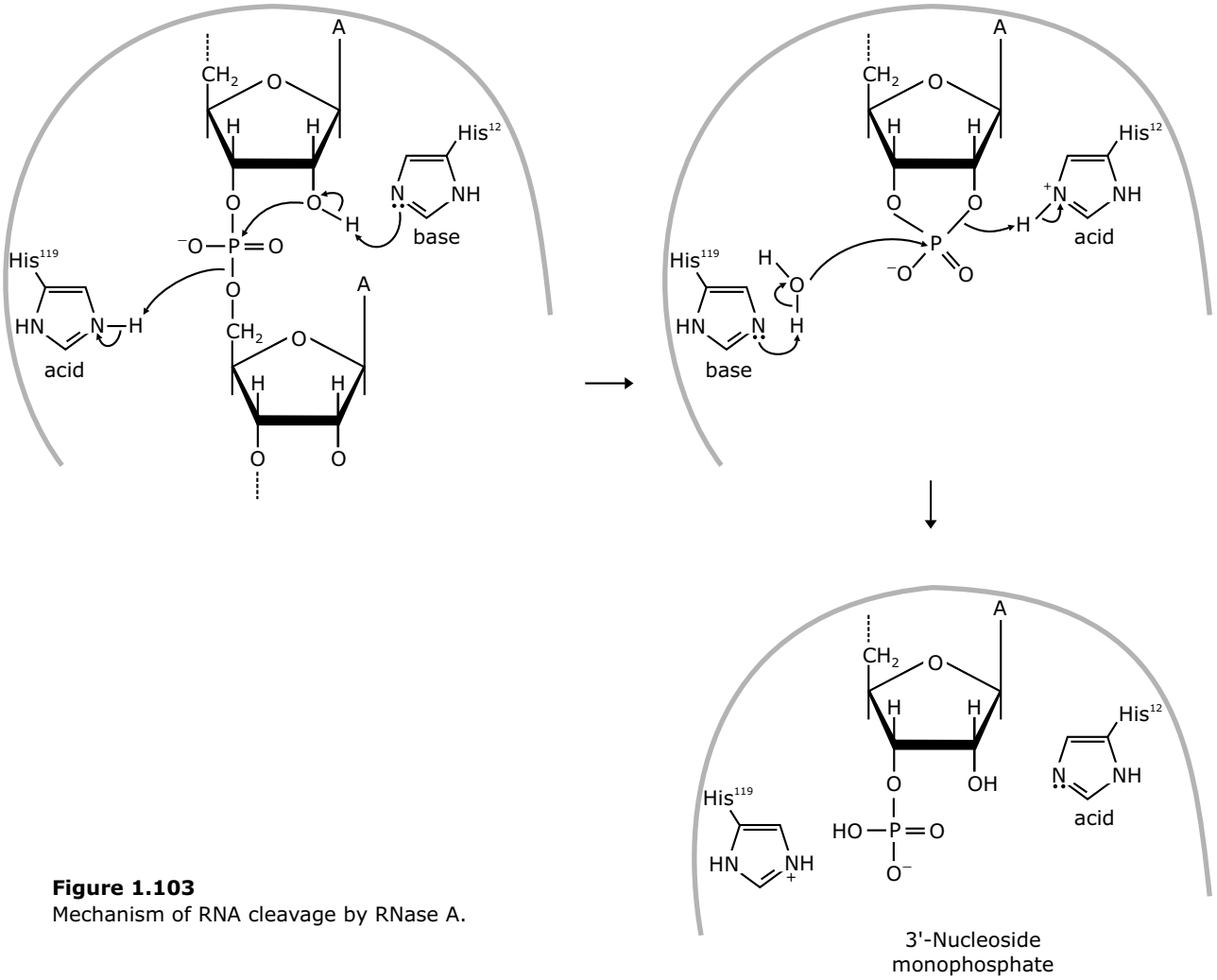

Figure 1.103
Mechanism of RNA cleavage by RNase A.

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Chapter 02

Bioenergetics and Metabolism

2.1 Bioenergetics

Bioenergetics is the quantitative study of the energy transductions that occur in living cells and of the nature and functions of the chemical processes underlying these transductions.

Thermodynamic 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, remains constant.

Mathematically, it can be expressed as:

$$\Delta U = \Delta q - \Delta w$$

ΔU is the change in internal energy,

Δq is the heat exchanged from the surroundings,

Δw is 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:

$$\Delta S \geq \frac{\Delta q}{T}$$ where, ΔS is the change in entropy of the system

Entropy is unavailable form of energy and it is very difficult to determine it, so a new thermodynamic term called *free energy* is defined.

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:

$$\Delta G = \Delta H - T\Delta S$$

Where, ΔH is the change in enthalpy and T is absolute temperature. ΔH is the measure of change in heat content of 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 the loss of free energy (**exergonic**),

ΔG is positive, the reaction proceeds only when 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 reactant and product. At constant temperature and pressure, the following relation can be derived:

$$\Delta G = \Delta G^0 + RT \ln \frac{[B]}{[A]}$$

Where, ΔG^0 is the standard free energy change;

R is the gas constant;

T is the absolute temperature;

[A] and [B] are the actual concentrations of reactant and product.

Standard free energy change

The actual change in free energy (ΔG) 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^0.

The chemical reaction has a characteristic standard free energy change and it is 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^0.

Relationship between ΔG^0 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, B is also being converted to A, as fast as A into B. 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 concentrations 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 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,

$$\Delta G^0 = -RT \ln \frac{[B]_{eq}}{[A]_{eq}}$$

So, $\Delta G^0 = -RT \ln K_{eq}$

This equation allows some simple predictions:

K_{eq}	ΔG^0	Reaction
> 1.0	Negative	proceeds forward
1.0	Zero	is at equilibrium
< 1.0	Positive	proceeds in reverse

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 is valid only at pH=7. Hence, under biochemistry convention, ΔG^0 is symbolized by $\Delta G^{0'}$ and likewise, the biochemical equilibrium constant is represented by K'_{eq}.

So, $\Delta G^{0'} = -RT \ln K'_{eq}$

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However, all reactions do not take place at standard conditions. A relationship has been worked out by which a distinction can be made between $\Delta G^{0'}$ and the actual free energy change, ΔG.

$$\Delta G = \Delta G^{0'} + RT \ln \frac{[\text{Product}]}{[\text{Reactant}]}$$

If concentration of products and reactants is unity, then $\Delta G = \Delta G^{0'}$. So, standard free energy change, $\Delta G^{0'}$, is the change in free energy when products and reactants are present in unit concentration.

Note : In all spontaneous biochemical reaction, the free energy of the reacting system ΔG is negative, but $\Delta G^{0'}$ may be *positive*, *negative* or *zero* depending upon the equilibrium constant of the reaction. As an example, let us 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:

$$\begin{aligned} \Delta G^{0'} &= -RT \ln K'_{eq} \\ &= -(8.315 \text{ JK}^{-1}\text{mol}^{-1})\ (298 \text{ K})\ (\ln 19) \\ &= -7.3 \text{ kJ/mol} \end{aligned}$$

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 characteristic standard free energy change, $\Delta G_1^{0'}$ and $\Delta G_2^{0'}$. 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, $\Delta G_{total}^{0'}$.

For the overall reaction A $\rightleftharpoons$ C, $\Delta G_{total}^{0'}$ is the sum of individual standard free-energy changes, $\Delta G_1^{0'}$ and $\Delta G_2^{0'}$, of the two reactions: $\Delta G_{total}^{0'} = \Delta G_1^{0'} + \Delta G_2^{0'}$.

Free energy change for ATP

ATP is the chemical link between catabolism and anabolism. It is the energy currency of living cells. It acts as a donor of high energy phosphate. Its central role in energy metabolism was first explained by Fritz Lipmann and Herman Kalckar. In aqueous condition, the phosphoanhydride bonds of ATP cleave with the incorporation of water.

$ATP + H_2O \rightleftharpoons ADP + P_i$ (Orthophosphate, PO_4^{3-})

$ATP + H_2O \rightleftharpoons AMP + PP_i$ (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	$\Delta G^{0'}$(kJ. mol^{-1})	$\Delta G^{0'}$(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 is *resonance stabilization, electrostatic repulsion* and *stabilization due to hydration*. ADP and, in particular, P_i, have greater resonance stabilization than ATP. Some compounds like phosphoenolpyruvate, 1,3-bisphosphoglycerate and creatine phosphate in biological systems have higher phosphoryl transfer potential than that of ATP.

Table 2.1 Standard Gibb's free energies of hydrolysis of some phosphate compounds at pH 7

Phosphates	*Type of phosphate bond*	*$\Delta G^{0'}$ 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 favourable if the standard free-energy change (ΔG^0) 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:

$$\Delta G = \Delta G^{0'} + RT \ln \frac{[ADP][P_i]}{[ATP]}$$

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 $\xrightleftharpoons[ADP \quad ATP]{\text{Creatine kinase}}$ Creatine $\quad \Delta G^{0'} = -10.3$ kcal mol^{-1}.

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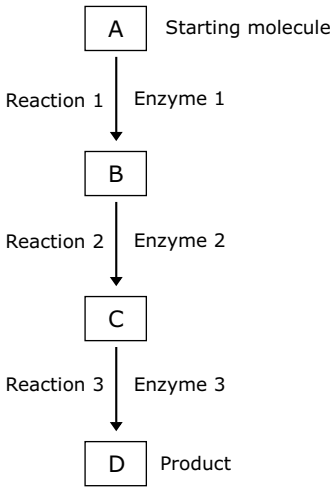
2.2 Metabolism

Metabolism (derives from the Greek word for *change*) is a series of interconnected chemical reactions occurring within a cell; the chemical compounds involved in this process are known as *metabolites*. It consists of hundreds of enzymatic reactions organized into discrete pathways. These pathways proceed in a stepwise manner, transforming substrates into end products through many specific chemical *intermediates*. Each step of metabolic pathways is catalyzed by a specific enzyme.



Metabolic pathways can be linear (such as glycolysis), cyclic (such as the citric acid cycle) or spiral (such as the biosynthesis of fatty acids). Metabolism serves two fundamentally different purposes: generation of energy to drive vital functions and the synthesis of biological molecules. To achieve these, metabolic pathways fall into two categories: anabolic and catabolic pathways. *Anabolic pathways* are involved in the synthesis of compounds and endergonic in nature. *Catabolic pathways* are involved in the oxidative breakdown of larger complex molecules and usually exergonic in nature. The basic strategy of catabolic metabolism is to form ATP and reducing power for biosyntheses. Some pathways can be either anabolic or catabolic, depending on the energy conditions in the cell. They are referred to as *amphibolic pathways*. Amphibolic pathways occur at the 'crossroads' of metabolism, acting as links between the anabolic and catabolic pathways, e.g. the citric acid cycle.

Characteristics of metabolic pathways are:

1. They are irreversible.
2. Each one has a first committed step.
3. Those in eukaryotic cells occur in specific cellular locations.
4. They are regulated. Regulation occurs in following different ways:
   I. Availability of substrate; the rate of reaction depends on substrate concentration.
   II. Allosteric regulation of enzymes by a metabolic intermediate or coenzyme.
   III. By extracellular signal such as growth factors and hormones that act from outside the cell in multicellular organisms; changes the cellular concentration of an enzyme by altering the rate of its synthesis or degradation.

A number of central metabolic pathways are common to most cells and organisms. These pathways, which serve for synthesis, degradation, interconversion of important metabolites, and energy conservation, are referred to as the *intermediary metabolism*.

Metabolic pathways involve several enzyme-catalyzed reactions. Most of the reactions in living cells fall into one of five general categories: oxidation-reductions; reactions that make or break carbon–carbon bonds; group transfers; internal rearrangements, isomerizations and eliminations; and free radical reactions.

2.3 Respiration

Living cells require an input of free energy. Energy is required for the maintenance of highly organized structures, synthesis of cellular components, movement, generation of electrical currents and for many other processes. Cells acquire free energy from the oxidation of organic compounds that are rich in potential energy.

Respiration is an oxidative process, in which free energy released from organic compounds is used in the formation of ATP. The compounds that are oxidized during the process of respiration are known as *respiratory substrates,* which may be carbohydrates, fats, proteins or organic acids. Carbohydrates are most commonly used as respiratory substrates.

During oxidation within a cell, all the energy contained in respiratory substrates is not released free in a single step. Free energy is released in multiple steps in a controlled manner and used to synthesise ATP, which is broken down whenever (and wherever) energy is needed. Hence, ATP acts as the energy currency of the cell.

During cellular respiration, respiratory substrates such as glucose may undergo *complete or incomplete* oxidation. The complete oxidation of substrates occurs in the presence of oxygen, which releases CO_2, water and a large amount of energy present in the substrate. A complete oxidation of respiratory substrates in the presence of oxygen is termed as *aerobic respiration*.

Although carbohydrates, fats and proteins can all be oxidized as fuel, but here processes have been described by taking glucose as a *respiratory substrate.* Oxidation of glucose is an exergonic process. An exergonic reaction proceeds with a net release of free energy. When one mole of glucose (180 g) is completely oxidized into CO_2 and water, approximately 2870 kJ or 686 kcal energy is liberated. Part of this energy is used for synthesis of ATP. For each molecule of glucose degraded to carbon dioxide and water by respiration, the cell makes up to about 30 or 32 ATP molecules, each with 7.3 kcal/mol of free energy.

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + \text{Energy (ATP + Heat)}$$

The incomplete oxidation of respiratory substrates occurs under anaerobic conditions i.e. in the absence of oxygen. As the substrate is never totally oxidized, the energy generated through this type of respiration is lesser than that during aerobic respiration.

2.3.1 Aerobic respiration

Enzyme catalyzed reactions during aerobic respiration can be grouped into three major processes: glycolysis, citric acid cycle and oxidative phosphorylation. Glycolysis takes place in the cytosol of cells in all living organisms. The citric acid cycle takes place within the mitochondrial matrix of eukaryotic cells and in the cytosol of prokaryotic cells. The oxidative phosphorylation takes place in the inner mitochondrial membrane. However, in prokaryotes, oxidative phosphorylation takes place in the plasma membrane.

Table 2.3 Intracellular location of major processes of aerobic respiration

In eukaryotes,		
Glycolysis	–	Cytosol
Citric acid cycle	–	Mitochondrial matrix
Oxidative phosphorylation	–	Inner mitochondrial membrane
In prokaryotes,		
Glycolysis	–	Cytosol
Citric acid cycle	–	Cytosol
Oxidative phosphorylation	–	Plasma membrane

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Solution

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.

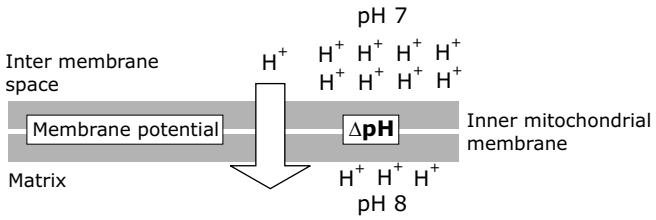
b. Antimycin A strongly inhibits the oxidation of Q in the respiratory chain, reducing the rate of electron transfer and leading to the consequences described in (a).

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 $FADH_2$.

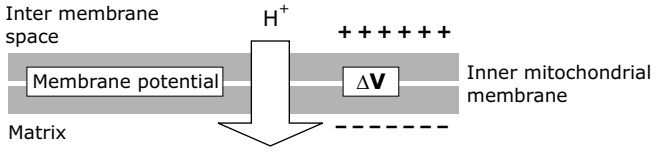
2.3.8 Electrochemical proton gradient

Transfer of electrons through the electron transport chain is accompanied by pumping of protons across inner mitochondrial membrane, from the mitochondrial matrix to inter membrane 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 inter membrane space).



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 160 mV (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 tenfold 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, 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

Proton motive force (pmf) is the electrochemical potential difference between protons in the aqueous phases on different sides of any membrane. The sum of the membrane potential and the pH gradient together constitute the pmf. This indicates the total potential energy stored in the transmembrane gradients, which is available to drive protons back into the matrix space, and provide the power for biologically useful processes. Proton motive force has two components - an electrical term and a concentration term. These are related by the equation:

$$pmf = \Delta\psi - z\Delta pH$$

where $\Delta\psi$ is the electrical potential difference or membrane potential (cytoplasm – matrix),

z is 2.303 RT/F, with values of 59 mV at 25°C.

Thus, $pmf \text{ (in millivolts)} = \Delta\psi - 59\ \Delta pH$

Measurements on respiring mitochondria have shown that the electrical potential difference 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 70 percent. In mitochondria, the electrical gradient (~160 mV, inside negative) makes a larger contribution than the pH gradient (~1 pH unit, inside alkaline). In chloroplasts, pH gradient makes a larger contribution than the transmembrane electric potential.

2.3.9 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 inter membrane 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.

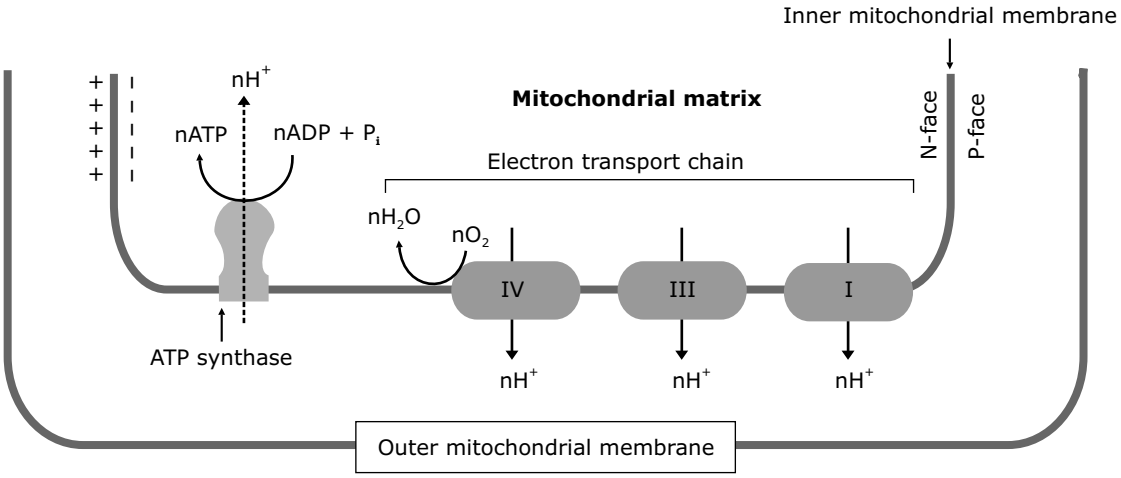


Figure 2.14 The general mechanism of oxidative phosphorylation. As a high-energy electron is passed along the electron-transport chain, some of the energy released is used to drive three respiratory enzyme complexes that pump H^+ out of the matrix space. The resulting electrochemical proton gradient 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.

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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, result in premature cell lysis. Peroxides are eliminated through the action of glutathione peroxidase, yielding glutathione disulfide (GSSG). So, G6PD deficiency results in hemolytic anemia caused by the inability to detoxify oxidizing agents.

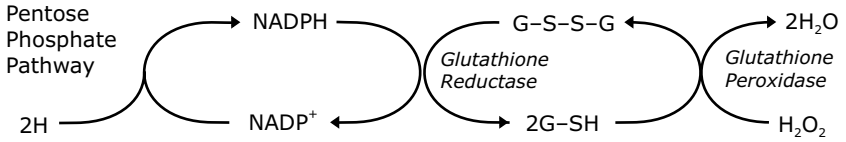

Figure 2.30 Role of the pentose phosphate pathway in the reduction of oxidized glutathione.

2.6 Entner–Doudoroff pathway

Entner-Doudoroff pathway is an alternative pathway that catabolizes glucose to pyruvate using a set of enzymes different from those used in either glycolysis or the pentose phosphate pathway. This pathway, first reported by Michael Doudoroff and Nathan Entner, occurs only in prokaryotes, mostly in gram-negative bacteria such as *Pseudomonas aeruginosa, Azotobacter, Rhizobium*.

In this pathway, glucose phosphate is oxidized to 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) which is cleaved by *2-keto-3-deoxyglucose-phosphate aldolase* to pyruvate and glyceraldehyde-3-phosphate. The latter is oxidized to pyruvate by glycolytic pathway where in two ATPs are produced by substrate level phosphorylations. This process yields one ATP as well as one NADH and one NADPH for every glucose molecule.

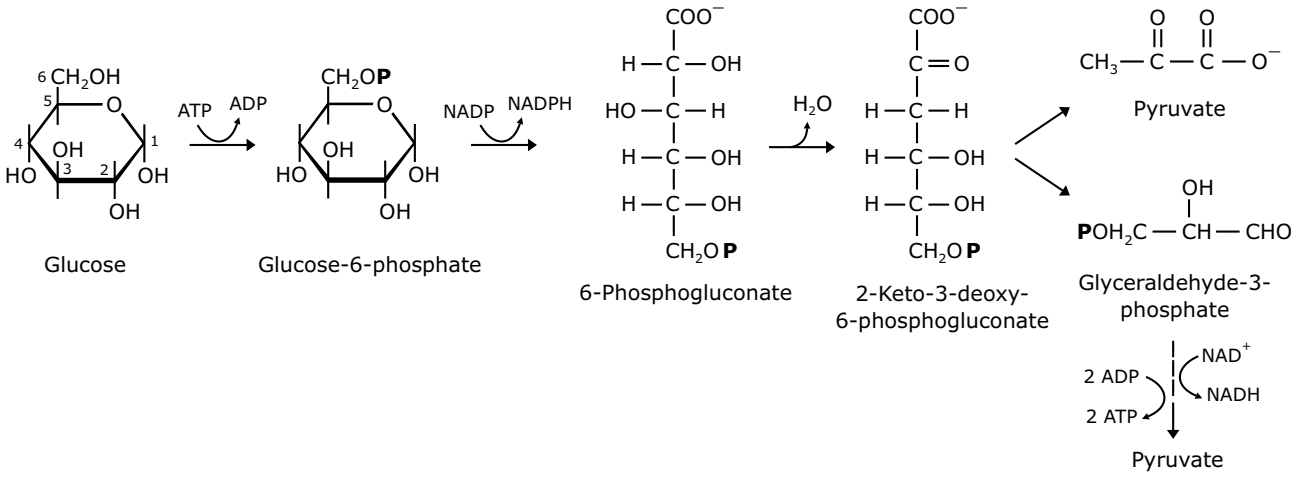

Figure 2.31 Entner-Doudoroff pathway.

2.7 Photosynthesis

Photosynthesis is a physiochemical 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.

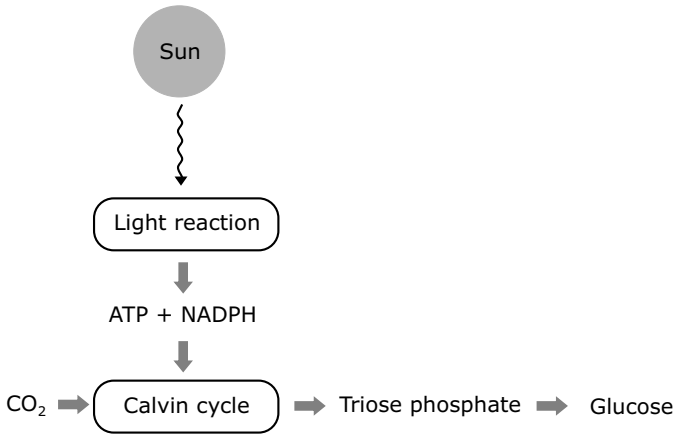

Figure 2.32 Photosynthesis is a two stage process. The first process is a light dependent one (light reactions) that requires the direct energy of light to make energy carrier molecules that are used in the second process. The Calvin cycle (light independent process) occurs when the products of the light reaction are used in the formation of carbohydrate.

On the basis of generation of oxygen during photosynthesis, the photosynthetic organisms may be **oxygenic** or **anoxygenic**. Oxygenic photosynthetic organisms include both eukaryotes as well as prokaryotes whereas anoxygenic photosynthetic organisms include only prokaryotes.

Oxygenic photosynthetic organisms

Eukaryotes – Plants and Photosynthetic protists

Prokaryotes – Cyanobacteria

Anoxygenic photosynthetic organisms

Prokaryotes – Green and purple photosynthetic bacteria

In oxygenic photosynthetic organisms, photosynthetic oxygen generation occurs via the light-dependent oxidation of water to molecular oxygen. This can be written as the following simplified chemical reaction:

$$nCO_2 + nH_2O \xrightarrow[Chl]{Light} (CH_2O)_n + nH_2O + nO_2$$

2.7.1 Photosynthetic pigment

The solar energy required for photosynthesis is captured by photosynthetic pigment molecules. Different types of pigments, described as photosynthetic pigment, participate in this process. The major photosynthetic pigment is the chlorophyll.

Chlorophylls

Chlorophyll, a light-absorbing green pigment, contains a polycyclic, planar tetrapyrrole ring structure. Chlorophyll is a *lipid soluble* pigment. It has the following important features:

1. The central metal ion in chlorophyll is Mg^{2+}.
2. Chlorophyll has a cyclopentanone ring (ring V) fused to pyrrole ring III.
3. The propionyl group on a ring IV of chlorophyll is esterified to a long-chain *tetraisoprenoid alcohol*. In chlorophyll a and b it is phytol.

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2.7.3 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:

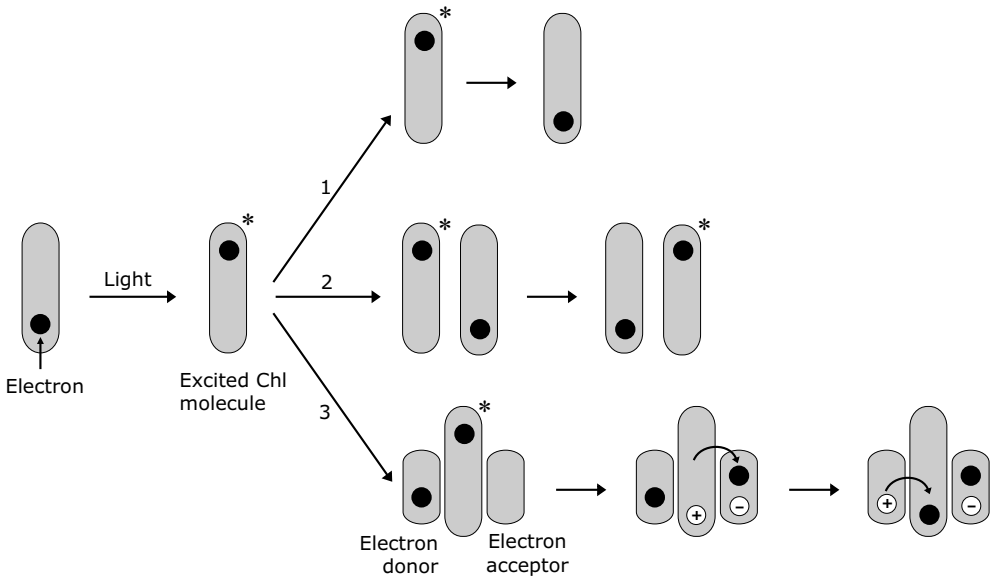

Figure 2.37 An excited molecule is unstable and will tend to return to its original, unexcited state in one of three ways: 1. by converting the extra energy into heat or to some combination of heat and light of a longer wavelength (fluorescence) 2. by transferring the energy directly to a neighboring chlorophyll molecule and 3. by transferring the high-energy electron to another nearby molecule (an electron acceptor) and then returning to its original state by taking up a low-energy electron from some other molecule (an electron donor). The last two mechanisms are exploited in the process of photosynthesis. Adapted and modified from Molecular biology of the cell, Alberts et. al, Garland Science.

1. By converting the extra energy into heat or to some combination of heat and light of a higher wavelength (**fluorescence**). An excited molecule in a singlet state usually has a maximum lifetime of ~10^{-8} sec due to an inherent tendency to fluoresce, but can undergo various modes of nonradiative as well as radiative decay, the latter generally called *luminescence*. The fate of the excited molecule depends on the pathway taken by the excitation energy. Depending on the mode of radiative decay, luminescence may also be called fluorescence, phosphorescence, etc. Once the excited molecule has returned to one of the lower vibrational sublevels of the first excited singlet state, it can return to one of the lower vibrational sublevels of the ground state by emitting a photon as *fluorescence*.

   Another important energy pathway is called *intersystem crossing*, which refers to the conversion of an excited 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 state transition is called *phosphorescence*.

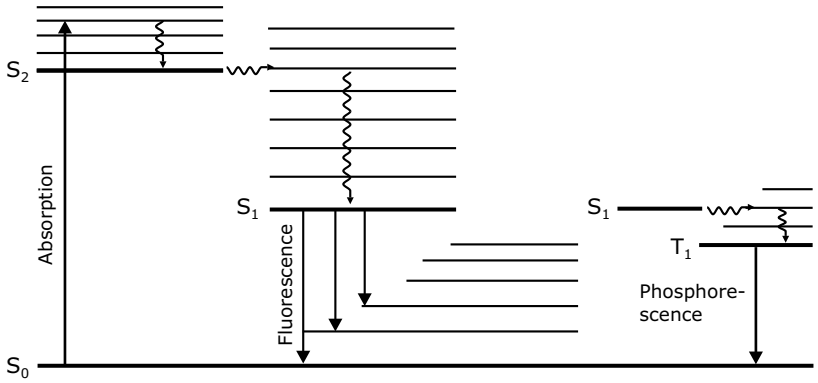

Figure 2.38 Excited states of Chl. S_0 represents the ground state while S_1 and S_2 represent the lowest vibrational energy level of the first and second excited singlet states, respectively. T_1 represents the lowest vibrational energy level of the triplet state. The other horizontal lines represent 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 chlorophyll has lower energy than that of the antenna molecules.

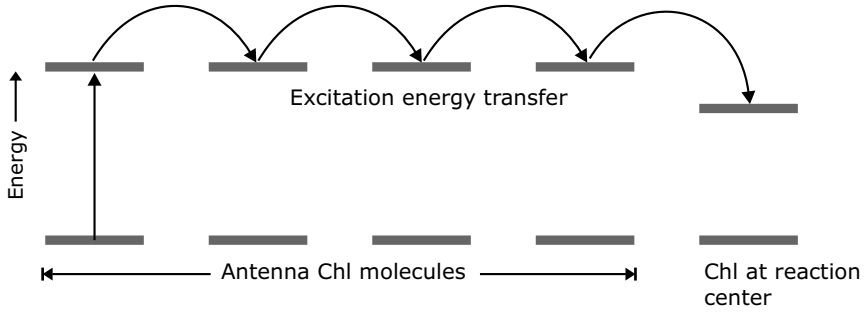

Figure 2.39 Energy level diagram illustrating excitation transfer. The excitation energy is trapped by the reaction center because the excited state of its Chl has a lower energy than that of the antenna molecules.

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., photochemical reaction. The photochemical reactions of photosynthesis are among the fastest known chemical reactions.

2.7.4 Concept of photosynthetic unit

In 1932, Robert Emerson and William Arnold provided the first evidence for the cooperation of many chlorophyll molecules in energy conversion during photosynthesis. They suggested that not all the chlorophyll molecules in a chloroplast were directly involved in the photochemical reaction. Using suspensions of the alga *Chlorella* and lights of saturating intensity for very short duration (~10μsec), 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 after absorption of 8 photons of light for about 2400 chlorophyll molecules present. Emerson and Arnold explained that about 2400 chlorophyll molecules act as a unit (called **photosynthetic unit**) for producing one molecule of oxygen per 8 photons absorbed. In a photosynthetic unit, most chlorophyll molecules (constitute light harvesting antenna complex) absorb light and

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proton-motive force of about 200 mV across the thylakoid membrane (nearly all of which is contributed by the pH gradient rather than by a membrane potential), which drives ATP synthesis by the ATP synthase embedded in this membrane. Like the stroma, the mitochondrial matrix has a pH of about 8, but this is created by pumping protons out of the mitochondrion into the cytosol (pH about 7) rather than into an interior space in the organelle. Thus the pH gradient is relatively small, and most of the pmf across the mitochondrial inner membrane, which is about the same as that across the chloroplast thylakoid membrane, is caused by the resulting membrane potential.

Cyclic electron flow

In certain cases, photoexcited electrons follow cyclic path, which involves PSI but not PSII. In cyclic electron flow, photoexcited electrons from P700 of PSI move through the b_6f complex and back to P700. This cyclic electron flow is coupled to proton pumping into the thylakoid lumen. When protons flow down their electrochemical gradient through ATP synthase complexes, ATP synthesis occurs. *The formation of ATP due to light-induced cyclic electron flow is called cyclic photophosphorylation.* There is no production of NADPH and no release of oxygen in cyclic electron flow. In plants, the cyclic flow of electrons is utilized only when the concentration of NADPH is sufficient, but still needs ATP to power other activities in the chloroplast. Cyclic photophosphorylation is somewhat more productive as compared to non-cyclic photophosphorylation with regard to ATP synthesis. The absorption of 4 photons by PSI results in the release of 8 protons into the lumen by the Cyt b_6f complex. These protons flow through ATP synthase to yield ~3 molecules of ATP (assuming the same ratio of ATP molecules generated per proton).

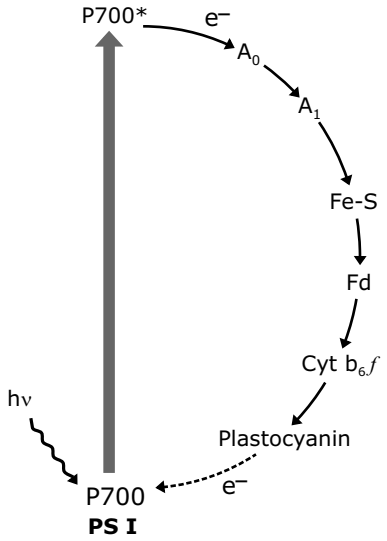

Figure 2.51 Cyclic electron flow. Photoexcited electrons from PSI are returned from ferredoxin to P700 via the cytochrome complex and plastocyanin. This electron flow generates electrochemical proton gradient and thus ATP (via chemiosmosis) but produces no NADPH.

Table 2.12 Differences between non-cyclic and cyclic electron flow

Non-cyclic electron flow	*Cyclic electron flow*
Both PSI and PSII	Only PSI
Photolysis of water	No photolysis of water
Formation of oxygen	No oxygen formation
ATP synthesis occurs	ATP synthesis occurs
NADPH synthesis occurs	No NADPH synthesis

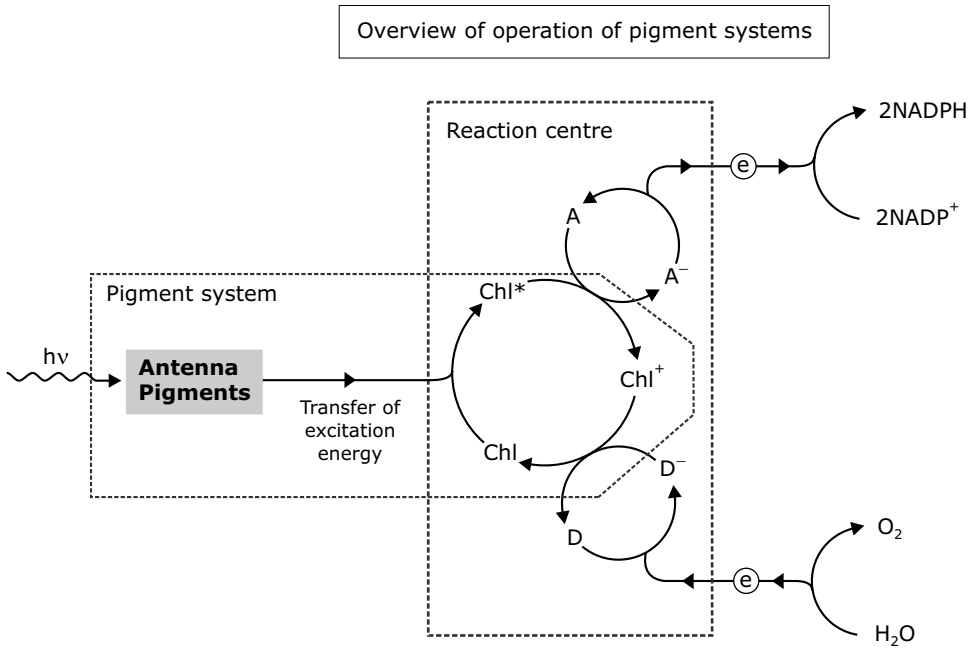

Figure 2.52 Simple concept of the operation of PSI and PSII. PSI and PSII are inserted at two different points in the chain of redox systems carrying electrons from H_2O to $NADP^+$. They each have a different acceptor (A/A^-) and donor (D/D^-) redox system.

Problem

The substance DCMU is a herbicide that inhibits photosynthesis by blocking electron transfer between plastoquinones in PSII.

a. Would you expect DCMU to interfere with cyclic photophosphorylation?

b. Normally, DCMU blocks O_2 evolution, but the addition of ferricyanide to chloroplasts allows O_2 evolution in the presence of DCMU. Explain.

Solution

a. No, because plastoquinones are not involved in this process.

b. The addition of ferricyanide as an electron acceptor allows a Hill reaction.

Problem

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 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.

Solution

a. Decrease

b. Increase

c. Increase

2.7.10 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 in 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 type of reaction center and perform anoxygenic photosynthesis (also called **bacterial photosynthesis**). In purple bacteria, the reaction center is similar to photosystem II and in green bacteria it is similar to photosystem I. However, neither of these two types of bacterial reaction center is capable of extracting electrons from water, so they do not evolve O_2. Because they are unable to use water as an electron source, they employ reduced molecules such as hydrogen sulfide, sulfur, hydrogen, and organic matter as their electron source for the generation of NADH and NADPH.

Reaction center in anoxygenic photosynthetic bacteria:

- Pheophytin-Quinone type reaction center (similar to PSII).
- Fe-S type reaction center (similar to PSI).

Despite these differences, the general principles of energy transduction are the same in anoxygenic and oxygenic photosynthesis. Anoxygenic photosynthetic bacteria depend on bacteriochlorophyll, a family of molecules that are similar to the chlorophyll, that absorb strongly in the infrared between 700 and 1000 nm. The electron carriers include quinone (e.g. ubiquinone, menaquinone) and the cytochrome bc complex. As in oxygenic photosynthesis, electron transfer is coupled to the generation of an electrochemical proton gradient that drives ATP synthesis.

Table 2.13 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

Purple photosynthetic bacteria

There are two divisions of photosynthetic purple bacteria, the non-sulfur purple bacteria (e.g. *Rhodobacter sphaeroides* and *Rhodospeudomonas viridis*) and the sulfur purple bacteria (e.g. *Chromatium vinosum*). The determination of the three-dimensional structures of the reaction center of the *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* has provided an unprecedented opportunity to understand the structure and function of photosynthetic reaction centers. In 1984, Michel, Deisenhofer and Huber first described the three-dimensional structure of the Pheophytin-Quinone reaction center of *Rhodopseudomonas viridis*. Pheophytin-Quinone reaction center of *R. viridis* consists of three transmembrane proteins, H, L, M, four molecules of Bchl b, two molecules of bacteriopheophytin (BPh) and two quinones (one ubiquinone and other menaquinone). A C-type cytochrome containing four heme also forms an integral component of reaction center of *R. viridis*. The reaction center of other purple photosynthetic bacteria like *Rhodobacter sphaeroides* (formerly called *Rhodopseudomonas sphaeroides*), and *Rhodospirillum rubrum* also have similar organization except that the C-subunit is not present.

Photochemical reaction in Rhodobacter sphaeroides

Light drives the *cyclic* flow of electrons. The reaction begins with light absorption by a *special pair* of BChl *a* molecules. This *special pair* absorbs light maximally at 870 nm. For this reason, the special pair is often referred to as *P870* (P stands for pigment). Components of the reaction center include one L subunit, one M subunit, one H subunit, four bacteriochlorophyll a, two bacteriopheophytin, two ubiquinone (designated as Q_A and Q_B), one non-heme-Fe-protein and one carotenoid. Excitation of the special pair leads to the ejection of an electron, which is

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Light compensation point

In the absence of light there is no photosynthetic carbon assimilation and CO_2 is given off by the plant because of respiration. In the presence of light, photosynthetic carbon assimilation starts. As the intensity of light increases, photosynthetic CO_2 assimilation increases until it equals CO_2 released by the process of respiration. The light intensity at which CO_2 uptake by photosynthesis equals CO_2 release by respiration (or O_2 uptake by respiration equals O_2 release by photosynthesis) is referred to as the *light compensation point*. There is no net CO_2 fixation at compensation point. The low compensation point is indicative of photosynthetic efficiency as the plant is, then, using the maximum amount of available CO_2.

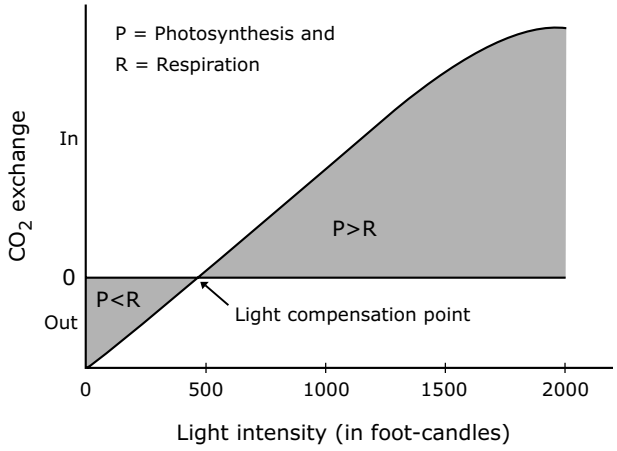
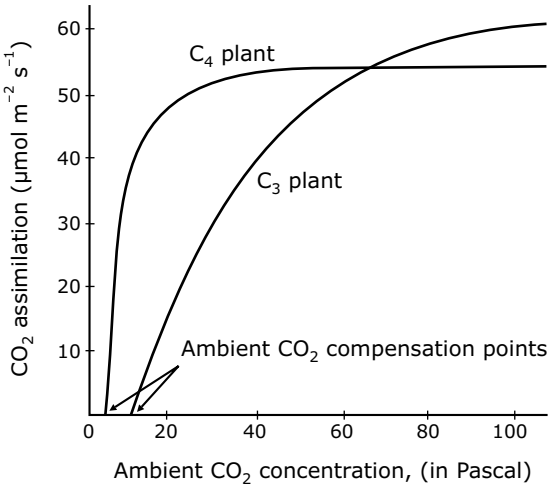


Figure 2.62 Relationship between photosynthesis and respiration.

Similarly, CO_2 concentrations at which CO_2 fixed by photosynthesis equals CO_2 release by respiration is termed as *CO_2 compensation point*. At this point, the net efflux of CO_2 from the plant is zero. This concept is similar to that of the light compensation point. The CO_2 compensation point reflects the balance between photosynthesis and respiration as a function of CO_2 concentration, and the light compensation point reflects that balance as a function of light intensity. For C_3 plants the CO_2 compensation point is 30-70 ppm at 25°C. The C_4 plants are characterized by a much lower CO_2 compensation point, often less than 10 ppm. Low compensation point in C_4 plants is due to the CO_2-concentrating mechanisms, CO_2 concentration at the carboxylation sites within C_4 chloroplasts is often saturating for rubisco activity.



Quantum yields and effect of temperature and CO_2 concentration

Quantum yield is the number of oxygen molecules produced per photon absorbed. Quantum yields vary from 0, where none of the light energy is used in photosynthesis, to 1, where all the absorbed light is used. In normal air, the quantum yield of C_3 plants is lower, typically 0.05. Quantum yield in C_3 and C_4 plants varies with temperature and CO_2 concentration because of their effect on the ratio of the carboxylase and oxygenase reactions of RuBisCo.

Effect of temperature: The quantum yield of photosynthetic carbon fixation in a C_3 plant and in a C_4 plant is a function of leaf temperature. In normal air, photorespiration increases with temperature in C_3 plants, and thus the energy cost of net CO_2 fixation increases accordingly. This higher energy cost results in decrease quantum yields. Hence in the C_3 plant, the quantum yield decreases with temperature. In C_4 plants, due to presence of the CO_2 concentrating mechanisms, photorespiration is low and the quantum yield does not show a temperature dependence. However, at lower temperatures (below 30°C), quantum yields of C_3 plants are generally higher than those of C_4 plants; above 30°C, the situation is usually reversed. This indicates that photosynthesis in C_3 plants is more efficient at lower temperatures.

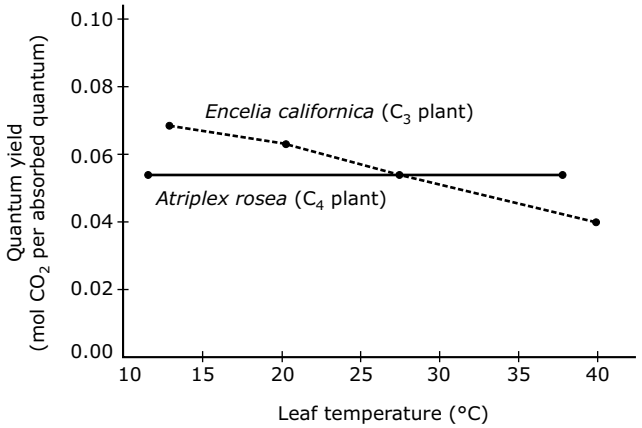


Figure 2.63 In the C_4 plant the quantum yield remains constant with temperature, reflecting typical low rates of photorespiration. In the C_3 plant, the quantum yield decreases with temperature, reflecting a stimulation of photorespiration by temperature and an ensuing higher energy demand per net CO_2 fixed.

Effect of CO_2 concentrations: In the presence of adequate amount of light, higher CO_2 concentrations support higher photosynthetic rates. In C_3 plants, increase in atmospheric CO_2 concentrations above the compensation point stimulates photosynthesis over a wide concentration range. Thus, C_3 plants may benefit more from ongoing increases in atmospheric CO_2 concentrations. In contrast, photosynthesis in C_4 plants is CO_2 saturated at low concentrations, and as a result C_4 plants do not benefit from increases in atmospheric CO_2 concentrations.

2.9 Carbohydrate metabolism

2.9.1 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 the liver and, to a smaller extent, in the 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 conditions, the amount contributed by the liver decreases considerably.

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Conversion of PEP to glucose

This pathway is opposite of glycolysis. However,

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.

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

$$2\ \text{Pyruvate} + 2\text{NADH} + 4\text{ATP} + 2\text{GTP} + 6H_2O \longrightarrow \text{Glucose} + 2\text{NAD}^+ + 2\text{GDP} + 4\text{ADP}$$

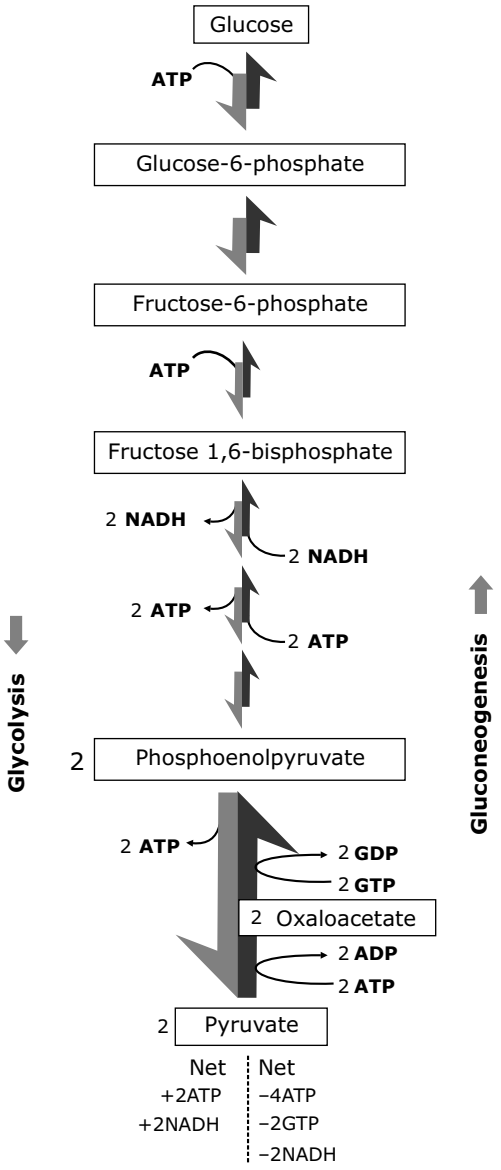
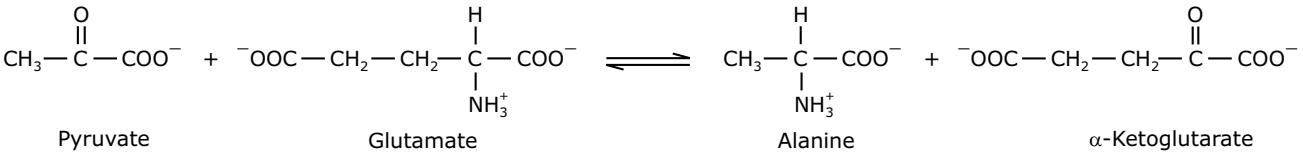


Figure 2.65 Reactions of glycolysis and gluconeogenesis.

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 gluconeogenetic pathway.



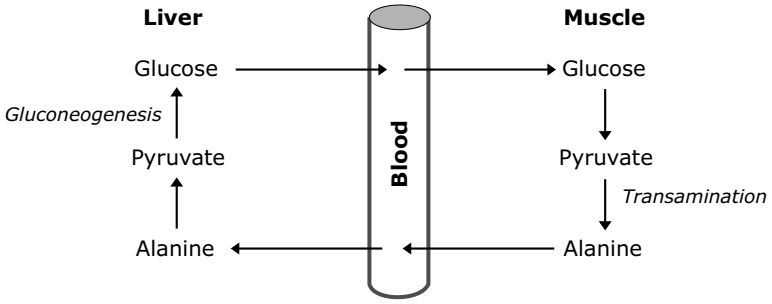


Figure 2.66
In glucose-alanine cycle, glucose is transported from liver to muscle with formation of pyruvate, followed by transamination to alanine, then transports alanine to the liver, followed by gluconeogenesis back to glucose.

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 the 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 sites of allosteric regulation and substrate level control are described in the following diagram.

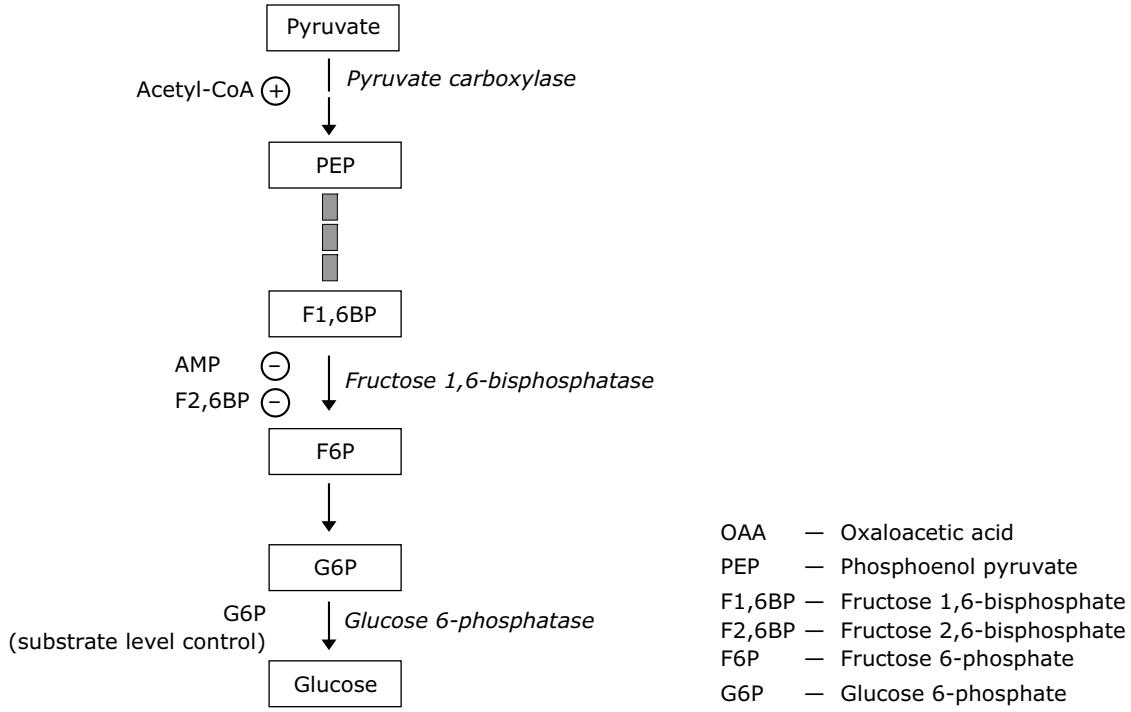


Figure 2.67 Regulation of gluconeogenesis. Activators are indicated by plus signs and inhibitors by minus signs.

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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.

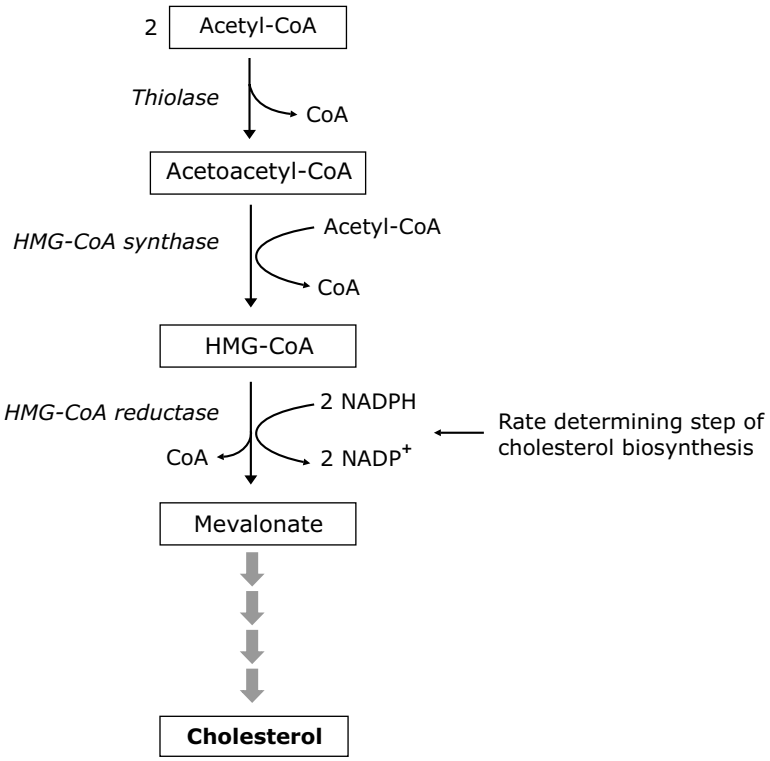

Figure 2.86 Formation of mevalonate from acetyl-CoA.

2.10.5 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 a 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.

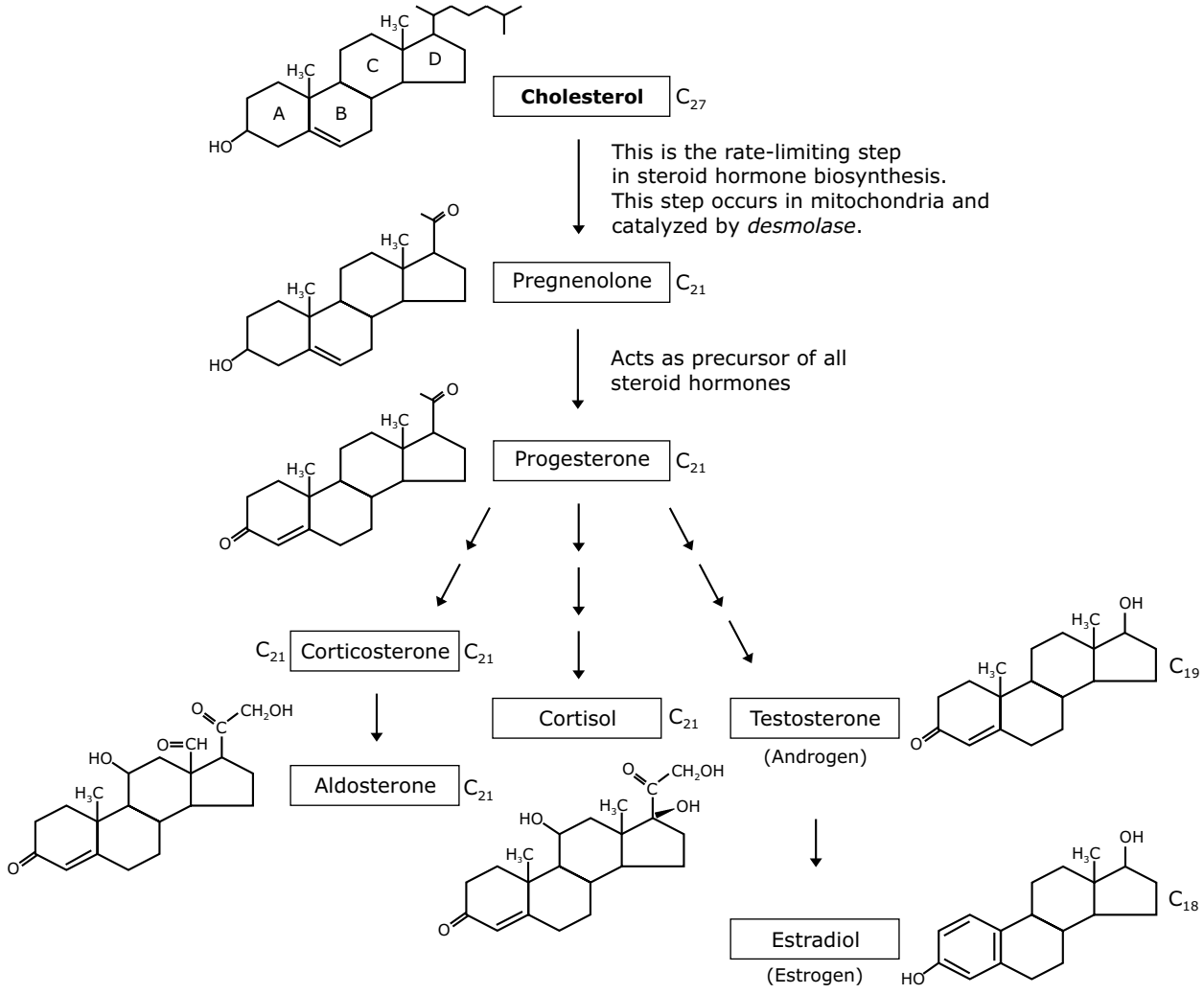

Figure 2.87 Biosynthetic routes from cholesterol to steroid hormones. Pregnenolone is an intermediate en route from cholesterol to steroid hormones.

3. The *mineralocorticoids* (aldosterone), which regulate ion balance by promoting reabsorption of potassium, sodium, chloride and bicarbonate ions 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, chenodeoxycholic acid and lithocholic acid–are present in human bile. The most abundant bile acids in humans are *cholic acid* and *chenodeoxycholic acid*.

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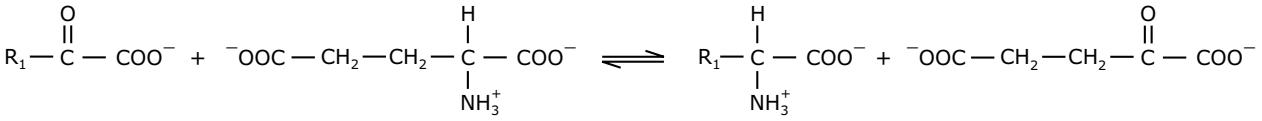
Transamination

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 the enzyme called *aminotransferases* (formerly called transaminases). Aminotransferases require the 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. 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 aminotransferases 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-keto acids by transamination reactions. Transamination reactions participate in the synthesis of most amino acids.



Acceptor α-keto acid | Glutamate | New amino acid | α-Ketoglutarate

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.

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 of histidine family–histidine–derive their carbon skeletons from ribose 5-phosphate.

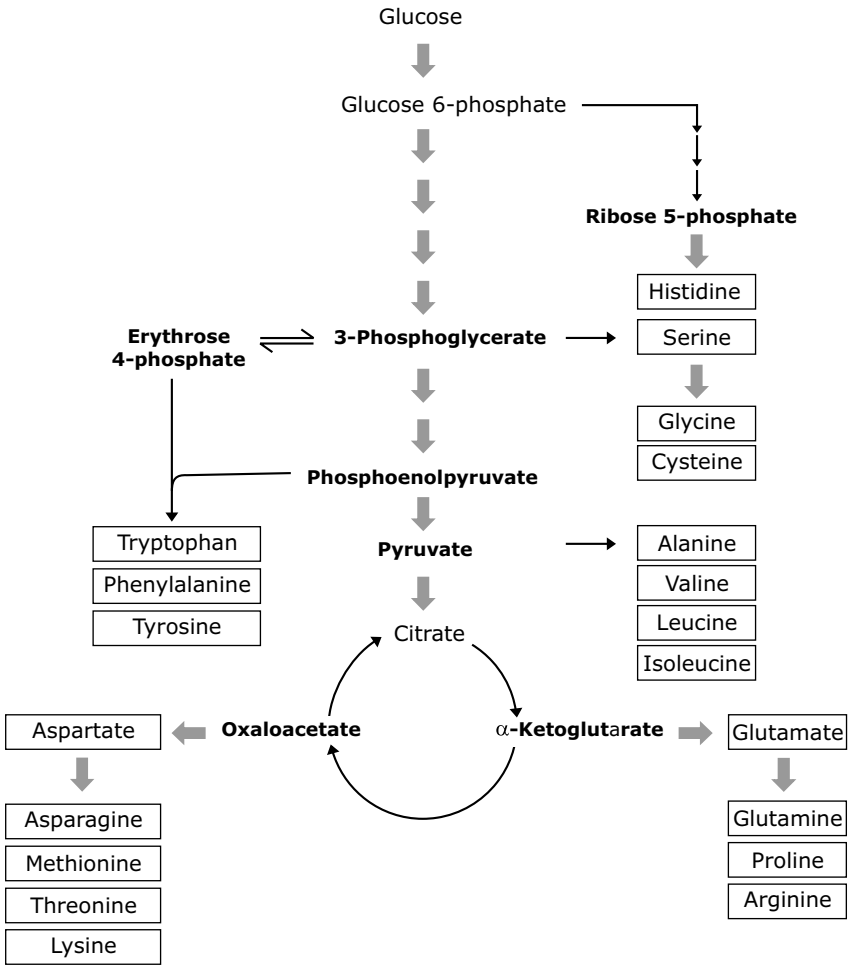


Figure 2.89 Overview of amino acid biosynthesis. The carbon skeleton precursors are derived from three sources—glycolysis, TCA cycle and pentose phosphate pathway.

2.11.2 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:

1. Removal of α-amino group from amino acids (*amino acid deamination*) and conversion of amino group to ammonia.
2. Incorporation of ammonia into urea.
3. Conversion of amino acid's carbon skeletons to common metabolic intermediate.

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Chapter 03

Cell Structure and Functions

3.1 What is a Cell?

The basic structural and functional unit of cellular organisms is the *cell*. It is an aqueous compartment bound by cell membrane, which is capable of independent existence and performing the essential functions of life. All organisms, more complex than viruses, consist of cells. Viruses are *noncellular* organisms because they lack cell or cell-like structure. In the year 1665, Robert Hooke first discovered cells in a piece of cork and also coined the word *cell*. The word cell is derived from the Latin word *cellula,* which means small compartment. Hooke published his findings in his famous work, *Micrographia*. Actually, Hooke only observed cell walls because cork cells are dead and without cytoplasmic contents. Anton van Leeuwenhoek was the first person who observed living cells under a microscope and named them animalcules, meaning *little animals.*

On the basis of the internal architecture, all cells can be subdivided into two major classes, *prokaryotic cells* and *eukaryotic cells*. Cells that have unit membrane bound nuclei are called eukaryotic, whereas cells that lack a membrane bound nucleus are prokaryotic. Eukaryotic cells have a much more complex intracellular organization with internal membranes as compared to prokaryotic cells. Besides the nucleus, the eukaryotic cells have other membrane bound **organelles** (*little organs*) like the endoplasmic reticulum, Golgi complex, lysosomes, mitochondria, microbodies and vacuoles. The region of the cell lying between the plasma membrane and the nucleus is the **cytoplasm**, comprising the cytosol (or cytoplasmic matrix) and the organelles. The prokaryotic cells lack such unit membrane bound organelles.

Cell theory

In 1839, Schleiden, a German botanist, and Schwann, a British zoologist, led to the development of the *cell theory* or *cell doctrine*. According to this theory *all living things are made up of cells* and *cell is the basic structural and functional unit of life.* In 1855, Rudolf Virchow proposed an important extension of cell theory that *all living cells arise from pre-existing cells* (*omnis cellula e cellula*). The cell theory holds true for all cellular organisms. Non-cellular organisms such as virus do not obey cell theory. Over the time, the theory has continued to evolve. The modern cell theory includes the following components:

- All known living things are made up of one or more cells.
- The cell is the structural and functional unit of life.
- All cells arise from pre-existing cells by division.
- Energy flow occurs within cells.
- Cells contain hereditary information (DNA) which is passed from cell to cell.
- All cells have basically the same chemical composition.

Evolution of the cell

The earliest cells probably arose about 3.5 billion years ago in the rich mixture of organic compounds, the *primordial soup*, of prebiotic times; they were almost certainly chemoheterotrophs. Primitive heterotrophs gradually acquired

the capability to derive energy from certain compounds in their environment and to use that energy to synthesize more and more of their own precursor molecules, thereby becoming less dependent on outside sources of these molecules-less extremely heterotrophic. A very significant evolutionary event was the development of photosynthetic ability to fix CO_2 into more complex organic compounds. The original electron (hydrogen) donor for these photosynthetic organisms was probably H_2S, yielding elemental sulfur as the byproduct, but at some point, cells developed the enzymatic capacity to use H_2O as the electron donor in photosynthetic reactions, producing O_2. The cyanobacteria are the modern descendants of these early photosynthetic O_2 producers.

One important landmark along this evolutionary road occurred when there was a transition from small cells with relatively simple internal structures - the so-called prokaryotic cells, which include various types of bacteria - to a flourishing of larger and radically more complex *eukaryotic* cells such as are found in higher animals and plants. The fossil record shows that earliest eukaryotic cells evolved about 1.5 billion years ago. Details of the evolutionary path from prokaryotes to eukaryotes cannot be deduced from the fossil record alone, but morphological and biochemical comparison of modern organisms has suggested a reasonable sequence of events consistent with the fossil evidence.

Three major changes must have occurred as prokaryotes gave rise to eukaryotes. *First*, as cells acquired more DNA, mechanisms evolved to fold it compactly into discrete complexes with specific proteins and to divide it equally between daughter cells at cell division. These DNA-protein complexes called chromosomes become especially compact at the time of cell division. *Second,* as cells became larger and intracellular membrane organelles developed. Eukaryotic cells have a *nucleus* which contains most of the cell's DNA, enclosed by a double layer of membrane. The DNA is, thereby, kept in a compartment separate from the rest of the contents of the cell, the cytoplasm, where most of the cell's metabolic reactions occur.

Finally, primitive eukaryotic cells, which were incapable of photosynthesis or of aerobic metabolism, pooled their assets with those of aerobic bacteria or photosynthetic bacteria to form symbiotic associations that became permanent. Some aerobic bacteria evolved into the mitochondria of modern eukaryotes, and some photosynthetic cyanobacteria became the chloroplasts of modern plant cells.

3.2 Structure of eukaryotic cells

3.2.1 Plasma membrane

Plasma membrane 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. The plasma membrane exhibits selective permeability; that is, it allows some solutes to cross it more easily than others. Different models were proposed to explain the structure and composition 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.

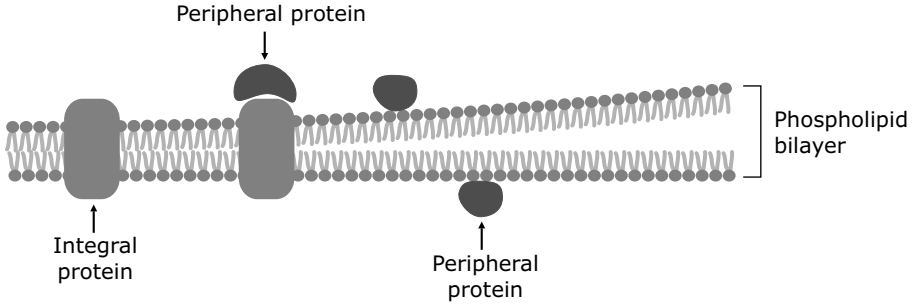

Figure 3.1 Fluid mosaic model for membrane structure. The fatty acyl chains in the lipid bilayer form a fluid, hydrophobic region. Integral proteins float in this lipid bilayer. Both proteins and lipids are free to move laterally in the plane of the bilayer, but movement of either from one face of the bilayer to the other is restricted.

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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 depends 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 comprise 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.

Lipid bilayer

The basic structure of the plasma membrane is the lipid bilayer. This bilayer is composed of two *leaflets* of amphipathic lipid 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. The primary physical forces for organizing lipid bilayer are *hydrophobic interactions*. Three classes of lipid molecules present in lipid bilayer - phospholipids, glycolipids and sterol.

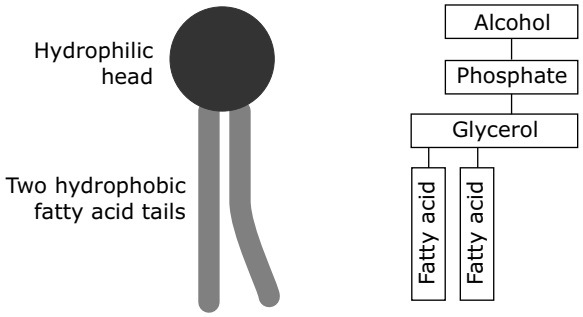

Figure 3.2 Phospholipid structure. The hydrophilic unit, also called the polar head group, is represented by a circle, whereas the hydrocarbon tails are depicted by straight lines.

Phospholipids

Phospholipids are made up of four components: an alcohol (glycerol or *sphingosine),* fatty acids, phosphate, and an alcohol attached to the phosphate. The fatty acid components are hydrophobic, whereas the remainder of the molecule has hydrophilic. There are two types of phospholipids: *glycerophospholipids* and *sphingophospholipids*.

Phospholipids derived from glycerol are called *glycerophospholipids.* Glycerophospholipids (or phosphoglycerides) contain glycerol, fatty acids, phosphate and an alcohol (e.g. choline). Phosphoglyceride molecules are classified according to the types of alcohol linked 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. Phosphoglycerides are the most numerous phospholipid molecules found in plasma membranes.

Sphingophospholipids contain an amino alcohol called **sphingosine** instead of glycerol, a fatty acid, phosphate and an alcohol attached to the phosphate. In sphingophospholipid, the amino group of the sphingosine backbone is linked to a fatty acid by an amide bond. *Sphingomyelin is the most abundant sphingophospholipid.*

The plasma membrane of animal cells contains 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 and phosphatidyl-ethanolamine) or it may have net negative charges (phosphatidylinositol and phosphatidylserine). Rarer phospholipids have a net positive charge.

Glycolipids

Glycolipids contain carbohydrate (either monosaccharide or oligosaccharide) covalently attached to the lipid. These can derive from either glycerol or sphingosine. 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

The 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 a rigid structure. *Cholesterol* is the major sterol present in the plasma membrane of animal cells. The plasma membrane of plant cells lacks cholesterol, but they contain other sterols like stigmasterol, sitosterol. With rare exceptions like *Mycoplasma*, bacterial plasma membrane also lacks cholesterol.

Table 3.1 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.

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. The rafts in cells appear to be heterogeneous both in terms of their protein and lipid content, and can be localized to different regions of the cell. There are two types of lipid rafts – planar (or non-caveolar) and caveolae. Caveolae are flask shaped invaginations of the plasma membrane that contain *caveolin* proteins. Caveolin, an intrinsic membrane protein, is a cholesterol binding protein. 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 contains only one fatty acid chain, the molecules form a small and spherical micellar structure (diameter usually <20 nm) in which the hydrophobic fatty acid chains 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 the third type of lipid aggregate, lipid bilayer forms a hollow sphere called a **liposome**. Liposomes are closed, self sealing, solvent filled vesicles that are bound by only a single bilayer.

Asymmetry of lipid bilayer

The phospholipids 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.

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Classes of proteins that mediate facilitated diffusion:

Carrier proteins mediated

Carrier proteins (called *transporters* or *permeases*) non-covalently bind 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, fuses with the plasma membrane, thereby increasing the rate of sugar uptake by several-fold.

The rate of transport (V) of glucose through GLUT can be well described by the following equation that is analogous to Michaelis-Menten equation:

$$V = \frac{V_{max}}{\left(1 + \frac{K_m}{C}\right)}$$

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 can be viewed as the affinity of the isoforms for glucose.

The *chloride-bicarbonate exchanger* (also called anion exchange protein) present on plasma membrane of RBC mediates the transport of two anions simultaneously. HCO_3^- ion moves in one direction and Cl^- moves in the opposite direction. The exchange is *electroneutral* because of no net transfer of charge (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).

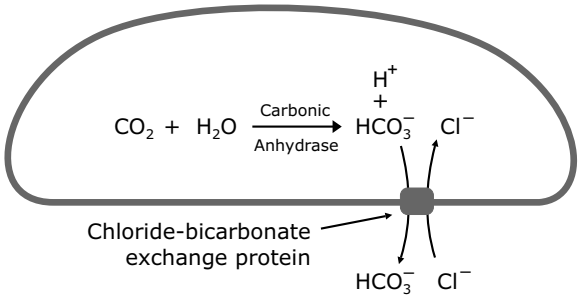

Figure 3.12 The anion exchange protein (chloride-bicarbonate exchange protein) of the RBC membrane allows the exit of HCO_3^-. For each HCO_3^- ion that moves in one direction, one Cl^- ion moves in the opposite direction. The result of this paired movement of two monovalent anions is no net change in the charge or electrical potential across the RBC 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**. 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 response to the binding of ligands.

Many animal and plant cells contain specialized *water channel* in their plasma membrane to facilitate the water flow called **aquaporins**. These are a family of transport proteins that allow water and a few other small uncharged molecules, such as glycerol, to cross membrane. Aquaporins (AQP) assemble as homotetramers in which each monomer, consists of six membrane-spanning α-helical domains with cytoplasmically oriented amino and carboxy termini. Each monomer functions as an independent pore. Two hydrophobic loops contain conserved, asparagine–proline–alanine (NPA) motif for selectivity of the channel.

The water permeability of epithelial cells lining the renal collecting duct in the kidney is due to the presence of an aquaporin (AQP-2). Antidiuretic hormone (ADH) regulates the retention of water by mobilizing AQP-2 molecules stored in vesicle membranes within the epithelial cells, much as insulin mobilizes GLUT4 in muscle and adipose tissue. When the vesicles fuse with the epithelial cell plasma membrane, water permeability greatly increases and more water is reabsorbed from the collecting duct and returned to the blood. When the ADH level drops, AQP-2 is resequestered within vesicles, reducing water retention.

The properties of ion channels can be studied by means of the **patch clamp technique**. In this technique, a glass pipette with a very small opening is used to make tight contact with a tiny area or patch of membrane. After the application of a small amount of suction to the back of the pipette, the seal between pipette and membrane becomes so tight that no ions can flow between the pipette and the membrane. Thus, all the ions that flow when a single ion channel opens must flow into the pipette. The resulting electrical current, though small, can be measured with an ultrasensitive electronic amplifier connected to the pipette. This technique has provided lots of valuable information about ion channels and its inventors, Erwin Neher and Bert Sakmann, were awarded the Nobel Prize in 1991.

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* (which move within in the bilayer) and *channel formers* (which span the lipid bilayer).

Valinomycin (isolated from *Streptomyces fulvissimus*) is an example of a mobile ion carrier. It transports K^+ down its electrochemical gradient. Similarly, ionophore *momensin* acts as a carrier for Na^+ ions. **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 a 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 3.3 Comparison of facilitated diffusion and active transport

Facilitated diffusion	*Active transport*
Selective	Selective
Passive	Active
Occurs along the concentration gradient	Occurs against the concentration gradient
Transport protein involved	Transport protein involved
Saturable	Saturable
Entropy increases	Entropy decreases

Active transport is of two types: Primary active transport and secondary active transport

Primary active transport

Primary (*direct*) active transport is coupled directly with a metabolic source of energy, such as ATP hydrolysis, or absorption of light by a carrier protein (in halobacteria). Transport of Na^+ and K^+ by carrier protein, Na^+-K^+

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Na^+-glucose symporters are a family of glucose transporter present on the apical surface (facing the lumen) of the epithelium cell of the small intestine and actively transports glucose molecules into the cell from the gut. The Na^+ flows down their concentration gradient while the glucose molecules are transported against their concentration gradient into the cell. Later, the Na^+ is pumped back out of the cell by the Na^+-K^+ ATPase and thus maintaining the inward Na^+ gradient. The GLUT confined to the basolateral (basal and lateral) surfaces of the cell allows the same molecules to leave the cell by facilitated diffusion into the extracellular fluid on the other side of the epithelium.

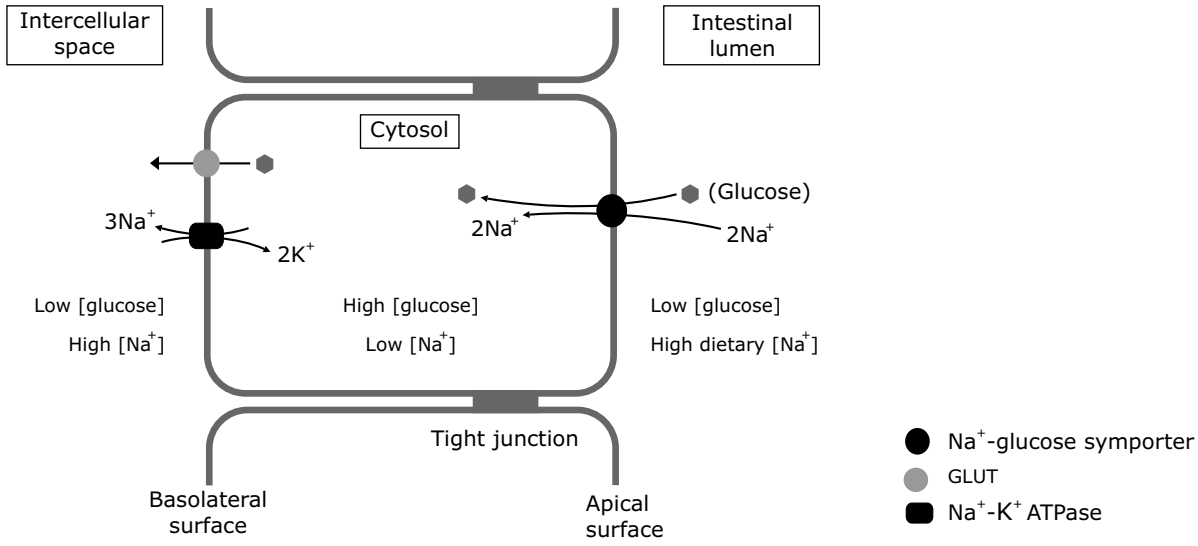

Figure 3.15 Glucose is actively transported into the cell by Na^+-driven glucose symporters at the apical surface, and it diffuses out of the cell by facilitated diffusion mediated by glucose carriers (GLUT) in the basolateral membrane. Tight junctions block the backflow of glucose from the basal side of the epithelium into the gut lumen.

Similarly, *lactose permease* (also known as galactoside permease) present in the plasma membrane of bacteria such as *E. coli*, utilizes the proton gradient across the membrane to co-transport proton and lactose (lactose/proton symport).

Thermodynamics of transport

The amount of energy needed for the transport of a solute against a concentration gradient can be calculated from the initial concentration gradient. When there is transport of one mole of a solute (uncharged) from a region in which its concentration is C_1 to a place where its concentration is C_2 and the standard free energy change (ΔG^0) is zero, then free energy change (ΔG) is given by

$$\Delta G = RT \ln \frac{C_2}{C_1} \qquad \ldots (1)$$

According to this equation, if C_2 is less than C_1, ΔG is negative, and the process is thermodynamically favourable. 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.

However, if the solute is an ion of charge Z, then the free energy change for transport across a cell membrane involves two contributors: 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. If we consider a process in which ions are transported from outside to inside of a cell, then ΔG is given by:

$$\Delta G = RT \ln \frac{C_{in}}{C_{out}} + Z.F.\Delta\psi$$

Here F is the *Faraday constant* (96.5 kJ $mole^{-1}$ V^{-1}) and $\Delta\psi$ is the trans-membrane electrical potential (in volts). Eukaryotic cells typically have electrical potentials across their plasma membranes of about 0.05 to 0.1 V (with the inside negative relative to the outside).

3.3 Membrane potential

All cells have an electrical potential difference, or *membrane potential*, across their plasma membrane. Electrical potential across plasma membranes is a function of the ions 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 electrochemical gradients, plasma membrane also contains channel protein that allows ions to move along their electrochemical gradients. Movement of ions occurs passively through ion channels. Ion channels may be either leaky channels or gated channels. Leaky channels, which are open all the time, permit unregulated leakage of specific ion across the membrane. Gated channels, in contrast, have gates that can be open or closed, permitting ion passage through the channels when open and preventing ion passage through the channels when closed. Ion concentration gradients across plasma membrane and selective movements of ions along gradient create a difference in electric potential or voltage across the plasma membrane. This is called *membrane potential*.

How membrane potentials arise?

To help explain how an electric potential across the plasma membrane can arise, we first consider a set of simplified experimental systems in which a membrane, which is only permeable for K^+ separates a 1 M KCl solution on the left from a 1 M KCl solution on the right. Because the concentrations of K^+ across the membrane are equal, there is no net flow of ions across the membrane and thus no electric potential is generated. If the concentration of K^+ ions across the membrane is different as shown in the figure, then K^+ ions tend to move down their concentration gradient from the left side to the right, leaving an excess of negative Cl^- ions compared with K^+ ions on the left side and generate an excess of positive K^+ ions compared with Cl^- ions on the right side. The resulting separation of charge across the membrane constitutes an electric potential, or voltage, with the left side of the membrane having excess negative charge with respect to the right. However, continued left-to-right movement of the K^+ ions eventually is inhibited by the mutual repulsion between the excess positive charges accumulated on the right side of the membrane and by the attraction of K^+ ions to the excess negative charges built up on the left side. The system soon reaches an equilibrium point at which the two opposing factors that determine the movement of K^+ ions—the membrane electric potential and the ion concentration gradient—balance each other out. At equilibrium, no net movement of K^+ ions occurs across the membrane.

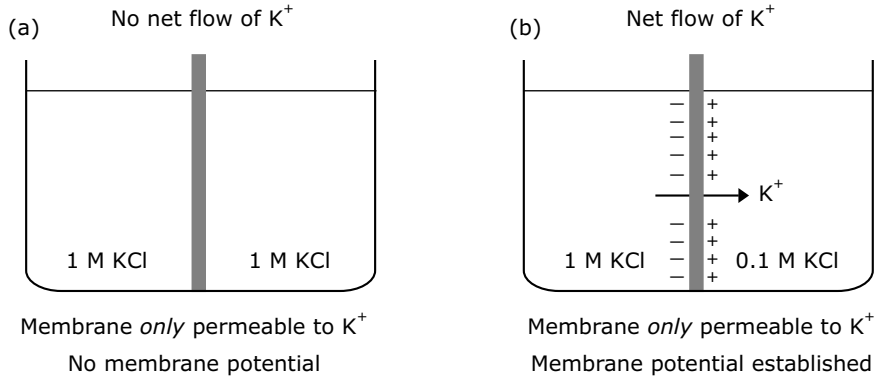

Figure 3.16 Two compartments are separated by a membrane permeable only to K^+ ions. (a) Because the concentrations in the two compartments are equal, there is no net flow of ions across the membrane and no electrical potential. (b) A difference in concentration causes K^+ ions to move from the left compartment to the right one. At equilibrium, an electrical potential is established across the membrane due to an accumulation of negative charges on the left side and positive charges on the right.

At equilibrium, the electric potential across the membrane equals the potassium equilibrium potential in volts, E_K. The magnitude of E_K is given by the *Nernst equation*,

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3.4 Transport of macromolecules across plasma membrane

The plasma membrane is a dynamic structure that functions to segregate the chemically distinct intracellular milieu (the cytoplasm) from the extracellular environment by regulating and coordinating the entry and exit of small and large molecules. Essential small molecules, such as amino acids, sugars and ions, can traverse the plasma membrane through the action of integral membrane protein pumps or channels. Macromolecules must be carried into the cell in membrane bound vesicles derived by the invagination and pinching-off of pieces of the plasma membrane in a process termed *endocytosis*.

3.4.1 Endocytosis

The term *endocytosis* was coined by Christian de Duve in the year 1963. Endocytosis is a 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 or *cell eating* (first reported by Metchnikoff) describes the internalization of large particles following particle binding to specific plasma membrane receptors and by the formation of large endocytic vesicles (generally >250 nm in diameter) called **phagosomes**. The phagosomes fuse with lysosomes to form **phagolysosomes**. In protozoa, phagocytosis is a form of feeding: large food particles taken up into phagosomes end up in lysosomes. In multicellular eukaryotes, few specialized cells – so called *professional phagocyte*s perform phagocytosis for non-nutritive purposes. In mammals, two classes of white blood cells act as professional phagocytes—*macrophage* and *neutrophils*. Phagocytosis is an active, actin mediated and highly regulated process involving specific cell-surface receptors and signalling cascades mediated by Rho-family GTPases.

Pinocytosis or *cell drinking* (also termed as *fluid-phase endocytosis*) involves the ingestion of fluid by the formation of small endocytic vesicles (termed **pinocytic vesicles**) of about 100 nm in diameter. Virtually all eukaryotic cells perform pinocytosis. Uptake of soluble material dissolved in extracellular fluid during pinocytosis occurs both selectively as well as non-selectively. Selective and efficient uptake occurs when solutes are captured by specific high-affinity receptors (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. Pinocytosis occurs in all cells by at least four basic mechanisms: *macropinocytosis*, *clathrin-mediated endocytosis*, *caveolae-mediated endocytosis* and *clathrin- and caveolae independent endocytosis*.

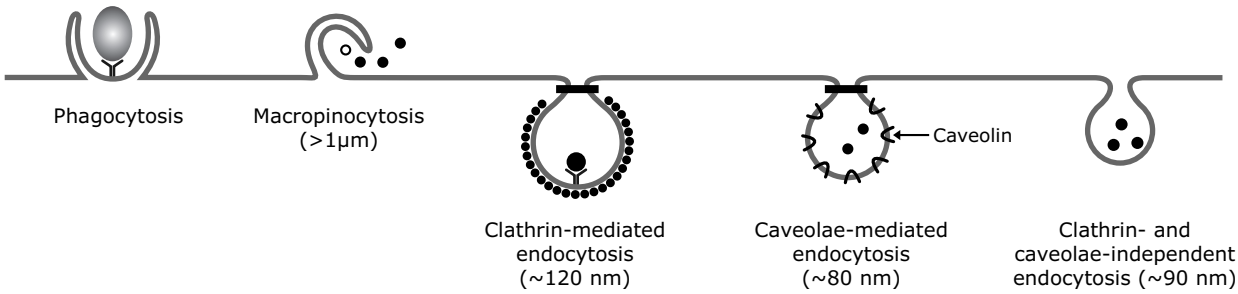

Figure 3.22 The endocytic pathways differ with regard to the size of the endocytic vesicle, the nature of the cargo (ligands, receptors and lipids) and the mechanism of vesicle formation.

Macropinocytosis

Macropinocytosis involves the membrane ruffling that is induced in many cell types upon stimulation by growth factors or other signals. Like phagocytosis, the signalling cascades that induce macropinocytosis involve Rho-family GTPases, which trigger the actin-driven formation of membrane protrusions. However, unlike phagocytosis, these protrusions do not 'zipper up' along a ligand coated particle, but they collapse onto and fuse with the plasma membrane to generate large endocytic vesicles, called **macropinosomes**.

Clathrin mediated endocytosis

Clathrin mediated endocytosis was previously referred to as *receptor mediated* endocytosis, but it is now clear that this is a misnomer, because most pinocytic pathways involve specific receptor and ligand interactions. In case of clathrin mediated endocytosis, macromolecules bind to cell-surface receptors, accumulate in clathrin coated pits, and enter the cell as a receptor and ligand complexes in clathrin-coated vesicles. Each *clathrin* 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 and assemble into a basket like convex framework of hexagons and pentagons to form coat. Clathrin assembles into coats on the cytoplasmic side of the plasma membrane by interacting with its *adaptor proteins.* There are four structurally related adaptor protein complexes (AP1, AP2, AP3 and AP4). Each of these four complexes are localized to different intracellular compartments and vary in their receptor specificity.

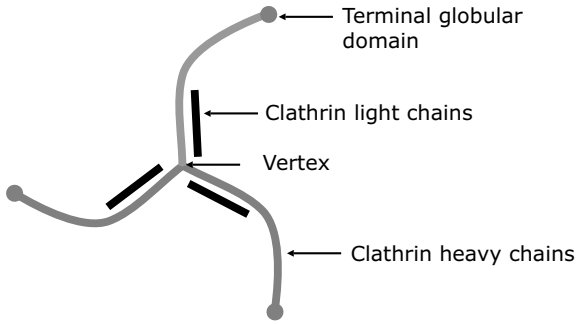

Figure 3.23 Structure of clathrin triskelion. Each triskelion consists of three clathrin heavy chains radiating from a central vertex, with a terminal globular domain at the tip of each triskelion leg and a clathrin light chain bound to the inner half of each leg.

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. AP2 is heterotetramer 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 clathrin-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 acid 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. This sequence binds to the AP2 complex. In most cell surface receptors such as transferrin receptor, the amino acid sequence present is *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.

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 ~120 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. The clathrin coat is rapidly lost shortly after the vesicle forms. An Hsp70 chaperone function as an uncoating ATPase to peel off the clathrin coat. **Auxillin** is believed to activate the ATPase.

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3.6.3 Transport of proteins from ER to *cis* Golgi

Proteins entering into the lumen of the ER are of two types – *resident* proteins such as BiP and *export* proteins such as secretory proteins and lysosomal proteins. Following the ER-specific folding, oligomerization and processing, export proteins are exported from the ER to the *cis* Golgi network, the first compartment of the Golgi apparatus. This transport occurs through the formation of transport vesicles followed by the targeting and fusion of these vesicles. Most of the protein components in transport vesicle are highly specific in order to maintain organelle distinction. To be transported from one compartment to another, protein products must be packaged into transport vesicles. Transport vesicles arise from specialized coated regions of membranes, which 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 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 proteins (APs). Clathrin that is associated with AP1 and AP3 forms vesicles for transport from the trans-Golgi network to the lysosome. Clathrin associated with AP2 forms vesicles from the plasma membrane during endocytosis that transport to the early endosomes.

COPI coated : COPI (Coat protein I) forms vesicles for both intra-Golgi transport and *retrograde* transport from the Golgi to the ER. ADP-ribosylation factor 1 (ARF1) is a small GTPase that regulates COPI vesicle formation by recruiting *coatomer* (for coat protomer). Like all small GTPase, activation of ARF1 is catalyzed by a guanine nucleotide exchange factor (GEF), while its deactivation is catalyzed by a GTPase-activating protein (GAP). A lactone antibiotic, **Brefeldin A,** prevents COPI coated vesicles formation. It targets the activity of GEF which catalyzes the activation of ARF1.

COPII coated : COPII forms vesicles for *anterograde* transport from the ER to the Golgi. COPII coat consists of the small GTPase Sar1p, the Sec23/24p complex, and the Sec13/31p complex that sequentially bind on the ER membrane. Budding from the ER involves activation of Sar1p-GDP to Sar1p-GTP by the ER-resident protein Sec12p, a guanine-nucleotide exchange factor (GEF).

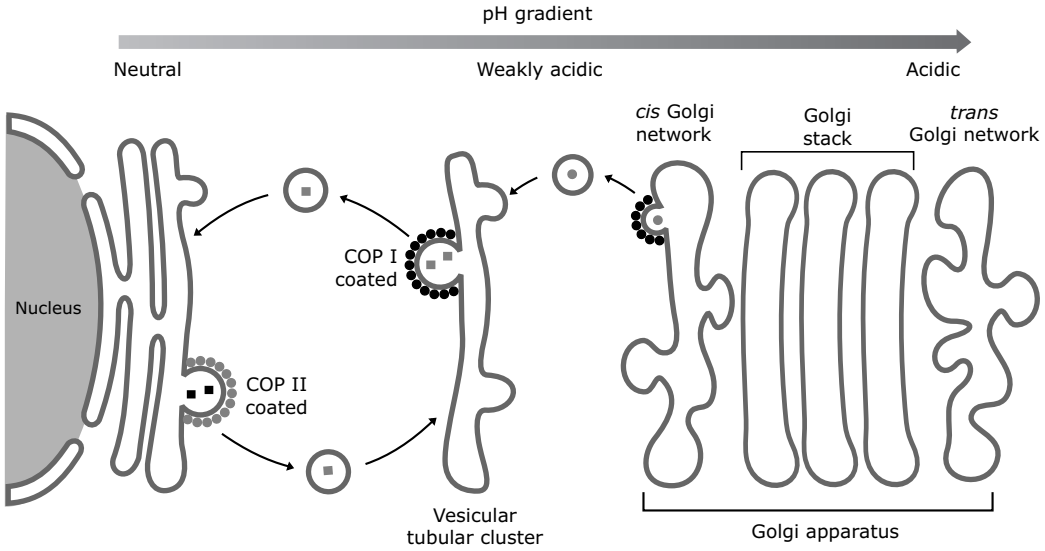

Figure 3.41 Coated transport vesicle-mediated protein trafficking between ER and cis Golgi.

Table 3.8 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 the ER or intra Golgi complex
COPII	COPII	ER to Golgi complex

The coat proteins surrounding transport vesicles that move from the late endosome to lysosomes and to the plasma membrane have not yet been identified.

ER-resident proteins often are retrieved from the *Cis*-Golgi

As we have mentioned in the previous section that proteins entering into the lumen of the ER are of two types- *resident* proteins and *export* 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. 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 the *KDEL sequence* which acts as sorting signal, is both necessary and sufficient for retention in the ER. If this *ER retention signal* is removed from BiP, for example, the protein is secreted from the cell; and if the signal is transferred to a protein that is normally secreted, the protein is now retained 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 sorting signal that have escaped to the *cis*-Golgi network and returns 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 also supports this concept. The KDEL receptor acts mainly to retrieve soluble proteins containing the KDEL sorting signal. The retention of transmembrane proteins in the ER is carried out by short C-terminal sequences that contain two lysine residues (KKXX sequences).

The affinity of the KDEL receptor for proteins with KDEL sorting signal changes in different compartments. How can the affinity of the KDEL receptor change depending on the compartment in which it resides? The answer may be related to the differences in pH. In the low-pH environment of *cis*-Golgi and transport vesicles, the KDEL receptor has greater binding affinity with the KDEL sorting signal whereas in the neutral-pH environment of the ER, the ER proteins dissociate from the KDEL receptor due to lesser affinity.

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-bound transport vesicles is an important feature of protein targeting as we will encounter them several times in our study of the subsequent stages in the maturation of secretory and membrane proteins.

3.7 Golgi complex

The Golgi complex was first discovered in 1897 by Italian physician Camillo Golgi. The Golgi complex, also termed as *Golgi body* or *Golgi apparatus,* is a single membrane bound organelle and part of endomembrane system. It consists of five to eight flattened membrane-bound sacs called the **cisternae**. Each stack of cisternae is termed as *Golgi stack* (or dictyosome). The cisternae in *Golgi stack* vary in number, shape and organization in different cell types. The typical diagrammatic representation of three major cisternae (cis, medial and trans) as shown in the figure 3.42 is actually a simplification. In some unicellular flagellates, however, as many as 60 cisternae may combine to make up the Golgi stack. The number of Golgi complexes in a cell varies according to its function. A mammalian cell typically contains 40 to 100 stacks. In mammalian cells, multiple Golgi stacks are linked together at their edges.

Each Golgi stack has two distinct faces: a ***cis* face** (or entry face or forming face) and a ***trans* face** (or maturing face). Both *cis* and *trans* faces are closely associated with special compartments: the ***cis* Golgi network** (CGN) and the ***trans* Golgi network** (TGN), respectively. TGN was earlier known as GERL (Golgi apparatus - ER - lysosome). 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,

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3.10 Vacuoles

Most plants and fungal cells contain one or several very large, fluid-filled vesicles called **vacuoles**. They are surrounded by single membrane called *tonoplast* and 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 contains water and dissolved inorganic ions, organic acids, sugars, enzymes and a variety of secondary metabolites. Solute accumulation causes osmotic water uptake by the vacuole, which is required for plant cell enlargement. This water uptake generates the turgor pressure.

The vacuole is different from contractile vacuole. A contractile vacuole is an organelle involved in osmoregulation. It pumps excess water out of the cell. It is found predominantly in protists (such as *Paramecium, Amoeba*) and in unicellular algae (*Chlamydomonas*). It was previously known as pulsatile or pulsating vacuole.

3.11 Mitochondria

Mitochondria (term coined by C. Benda) are *energy-converting organelles*, which are present in virtually all eukaryotic cells. They are the sites of aerobic respiration. They produce cellular energy in the form of ATP, hence they are called 'power houses' of the cell. Mitochondria are membrane-bound mobile as well as plastic organelle. Each mitochondrion is a double membrane-bound structure with outer and inner membranes. The outer membrane is fairly smooth. But the inner membrane is highly convoluted; forming folds called *cristae.* The inner membrane is also very impermeable to many solutes due to very high content of a phospholipid called *cardiolipin*. The cristae greatly increase the inner membrane's surface area. The two faces of this membrane are referred to as the *matrix side* (N-side) and the *cytosolic side* (P-side). Inner membrane contains enzyme complex called ATP synthase (or F_0-F_1 ATPase or oxysome) that makes ATP. The outer membrane protects the organelle, and contains specialized transport proteins such as *porin* which allows free passage for various molecules into the *intermembrane space* (the space between the inner and outer membranes) of the mitochondria. Mitochondrial porins, or voltage-dependent anion-selective channels (VDAC) allow the passage of small molecules across the mitochondrial outer membrane.

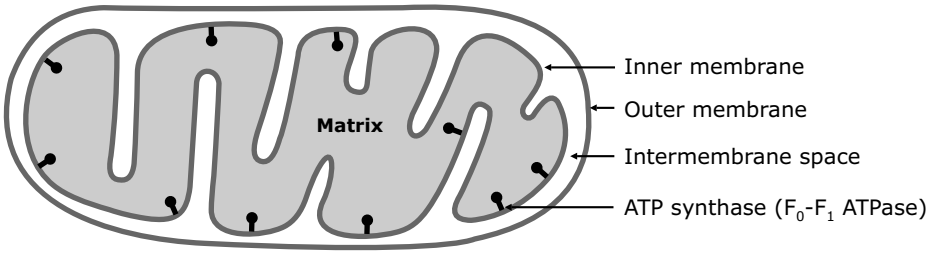

Figure 3.48 A mitochondrion has double-membraned organization and contains: the outer mitochondrial membrane, the intermembrane space (the space between the outer and inner membranes), the inner mitochondrial membrane, and the matrix (space within the inner membrane).

The matrix (large internal space) contains several identical copies of the dsDNA (as genetic material), mitochondrial ribosomes (ranging from 55S-75S), tRNAs and various proteins. Mitochondrial dsDNA is *mostly* circular. The size of mitochondrial DNA also 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

Kinetoplastids such as *Trypanosoma brucei, Trypanosoma cruzi,* possess a network of DNA, known as the *kinetoplast DNA* (kDNA), within mitochondria. It consists of two types of circular DNA molecules, maxicircles and minicircles, which are topologically interlocked into a single, massive network.

Mitochondria are *semi-autonomous* organelle and divide by *binary fission* just like bacteria. The similarities with the prokaryotic characters (especially with genetic systems), suggest that mitochondria evolved from bacteria. According to *endosymbiotic theory* (proposed by Lynn Margulis), mitochondria are supposed to have evolved in eukaryotes from endosymbiotic association of purple photosynthetic bacteria about 1.5×10^9 years ago. The captured cell (the endosymbiont) was then reduced to a functional organelle bound by two membranes, and was transmitted cytoplasmically to subsequent generations. Mitochondrial inheritance in higher eukaryotes is nearly *uniparental* (and more precisely *maternal inheritance* because female gametes mostly contribute mitochondria to the zygote). In lower eukaryotes such as yeasts, both parents contribute equal amounts of mitochondria to the zygote. Thus, mitochondrial inheritance in yeasts is therefore *biparental.*

Evidences to support endosymbiotic theory

- Mitochondria are self-replicating bodies like bacteria and divide in a manner resembling binary fission in bacteria.
- Mitochondria are surrounded by two membranes and the innermost of these membranes is very similar in composition to bacteria.
- Mitochondria have their own DNA, which is structurally similar to bacterial DNA.
- Mitochondrial ribosomes, enzymes and transport systems are all similar to those of bacteria.
- Mitochondria are of approximately the same size as bacteria.
- Protein synthesis in mitochondria is inhibited by a variety of antibiotics (e.g. chloramphenicol, tetracycline, erythromycin) that also inhibit protein synthesis by bacterial ribosomes, but have little effect on the cytosolic ribosome of eukaryotic cells.

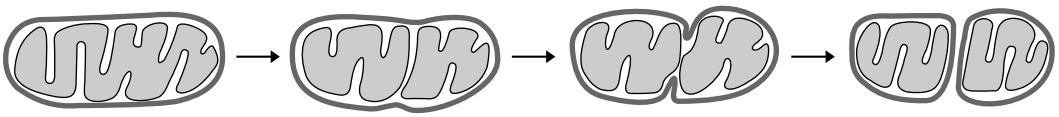

Figure 3.49 Mitochondrial division. It is mediated by a conserved, large dynamin-related GTPase called Dnm1 in yeasts (Drp1 in mammals). The dynamin-like Drp1/Dnm1 protein aggregates in ring- or spiral-like structures around the outer surface of mitochondria at regions where mitochondria will soon divide.

Hydrogenosome and mitosomes

Some protists (such as *Trichomonas vaginalis*) adapted to anoxic or microaerobic environments lack mitochondria. These protists contain a specialized organelle, involved in synthesis of ATP and hydrogen, called the **hydrogenosome**. Hydrogenosomes are the site of pyruvate fermentation, coupled with ATP production via substrate level phosphorylation. Pyruvate is broken down to acetate, CO_2 and molecular hydrogen. Like mitochondria, hydrogenosomes are surrounded by a double-membrane and produce ATP. In contrast to mitochondria, hydrogenosomes produce molecular hydrogen through fermentations, lack cytochromes and usually lack DNA.

Mitosomes, organelles of mitochondrial origin, are double-membrane bound organelles found in some unicellular eukaryotes, including *Entamoeba histolytica* and microsporidia. The name 'mitosome' was proposed to indicate that the organelles are highly reduced (cryptic) mitochondria. The mitosome has been detected only in anaerobic organisms that do not have mitochondria.

Targeting of mitochondrial proteins

Mitochondrial proteins are synthesized by 80S cytosolic as well as 70S matrix ribosomes. About 99% of mitochondrial proteins are encoded by nuclear genes and are synthesized as precursors on cytosolic 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.

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the *stroma*. 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 photosynthetic pigments. 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.

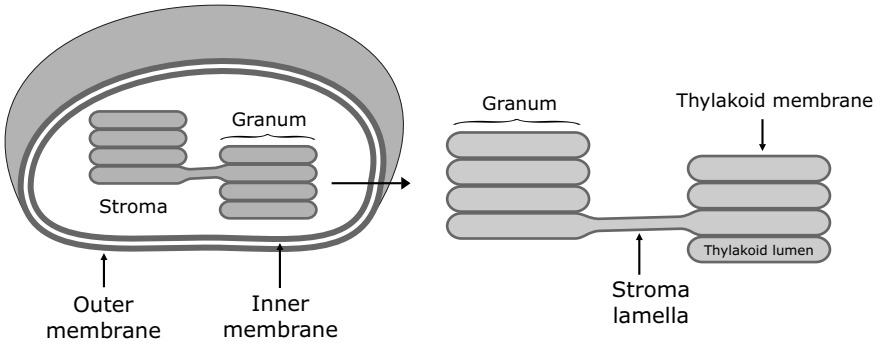

Figure 3.51 The two envelope membranes enclose the stroma. The stacks of the thylakoid termed grana are connected by tubes, forming a continuous thylakoid lumen.

In the dark grown plants, proplastids develop into **etioplasts**, which have a yellow chlorophyll precursor pigment protochlorophyll instead of chlorophyll. When exposed to light, the etioplasts rapidly change into chloroplasts by converting this precursor to chlorophyll.

Chromoplasts are plastids responsible for pigment synthesis and storage. They are rich in carotenoids and mainly responsible for the yellow, orange, or red colors of many fruits and flowers, as well as of autumn leaves. *Leucoplasts* are colorless (non-pigmented) plastids and act as storage organelles. Based on the kind of substance they store, they are further classified into *amyloplasts* (for starch storage), *elaioplasts* (for fat storage) and *proteinoplasts* or *aleuroplasts* (for storing and modifying proteins).

3.13 Peroxisome

Peroxisome (discovered by *Christian de Duve* in 1965) is a single membrane bound small organelle (approximately 0.5–1 μm in diameter) present in all eukaryotes. The term, peroxisome, was proposed by de Duve because it produced and consumed hydrogen peroxide. Peroxisomes lack DNA and ribosomes. Thus, all peroxisomal proteins (peroxisomal matrix and membrane proteins) are encoded by nuclear genes, synthesized on ribosomes present in the cytosol and then incorporated into pre-existing peroxisomes.

The ability of peroxisomes to divide themselves suggests that the peroxisome may have had an endosymbiotic origin similar to mitochondria. However, the localization of peroxisomal proteins to the endoplasmic reticulum and the similarity of some peroxisomal proteins to those localized in the ER suggest an alternative hypothesis: that the peroxisome was developed from the ER (*de novo* origin). Aspects of both views may be true. Most peroxisomal membrane proteins are made in the cytosol by membrane free ribosomes and insert into the membrane of preexisting ones. However, few others are first synthesized by membrane bound ribosomes of ER and then integrated into the ER membrane from where they may bud in specialized peroxisomal precursor vesicles. New peroxisome precursor vesicles may then fuse with one another and begin importing additional peroxisomal proteins synthesized by membrane free cytosolic ribosomes to grow into mature peroxisomes, which can enter into a cycle of growth and fission.

Like mitochondria, peroxisomes contain several *oxidative enzymes,* such as catalase, oxidases. Some peroxisomal enzymes such as oxidases transfer hydrogen atoms to molecular oxygen and form hydrogen peroxide. The enzyme

catalase (a member of the peroxidase family) present in the peroxisome uses the hydrogen peroxide to oxidize a variety of other substrates such as phenols, formic acid, formaldehyde and alcohol by the peroxidation reaction.

$$H_2O_2 + RH_2 \xrightarrow{\text{Catalase}} R + 2H_2O$$

When excess hydrogen peroxide accumulates in the cell, catalase converts it to H_2O through the reaction:

$$H_2O_2 + H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

A major oxidative reaction carried out in peroxisomes is the **β-oxidation**. β-oxidation in mammalian cells occur both in mitochondria and peroxisomes; in plant cells, however, this is exclusively found in peroxisomes. Peroxisomes also have two important roles in plants - *photorespiration* and *glyoxylate cycle.* In **photorespiration**, 2-phosphoglycolate 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.

Glyoxysome is a specialized form of peroxisome present in some plant cells, mainly the cells of germinating seeds. Glyoxysomes contain the enzymes of the **glyoxylate cycle** – which help to convert stored lipid into carbohydrates that can be translocated throughout the young plant to provide energy for growth. In the glyoxylate cycle, two molecules of acetyl-CoA produced by fatty acid breakdown are used to make succinic acid, which then leaves the glyoxysome and is converted into glucose in the cytosol. The glyoxylate cycle does not occur in animal cells, and animals are therefore unable to convert the fatty acids in fats into carbohydrates.

Targeting of peroxisomal proteins from cytosol to peroxisome synthesized by membrane free ribosomes

Transport of proteins from cytosol to peroxisomes occur *post-translationally*. Peroxisomal proteins synthesized on cytosolic ribosomes are generally fold into their mature conformation in the cytosol before import into the organelle. Proteins that are involved in peroxisome biogenesis, including peroxisome generation, division as well as matrix and membrane protein import are called **peroxins**. At least 23 distinct peroxins participate in the import process, which is driven by ATP hydrolysis. 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. The consensus sequence of PTS1 is (S/A/C)–(K/R/H)–(L/M). It was first characterized in catalase as a *Ser-Lys-Leu* sequence (SKL in one-letter code) at the very C-terminus. PTS1 containing proteins are recognized by the cytoplasmic receptor Pex5 and are imported into peroxisomes in their fully folded form. The PTS2 signal is a sequence of nine amino acids and can be located near the N-terminus or internally and recognized by the soluble receptor Pex7. PTS2 exhibits the consensus sequence (R/K)–(L/V/I)–X_5–(H/Q)–(L/A). The importance of the 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.

3.14 Nucleus

The nucleus is the controlling center of eukaryotic cell. It contains most of the genetic materials of cell. Most eukaryotic cells have one nucleus (*uninucleate*) each, but some have many nuclei (*multinucleate*) and certain cells, such as mature red blood cells, do not have it. Paramecia (unicellular ciliate protozoa) have two nuclei - a *macronucleus* and a *micronucleus*. Genes in the macronucleus control the everyday functions of the cell, such as feeding, waste removal, and maintenance of water balance. Micronucleus controls the sexual reproduction.

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, nuclear matrix* and *chromosomes*.

Nuclear envelope and nuclear matrix

The nuclear envelope consists of two concentric membranes called the inner and outer nuclear membranes. The outer nuclear membrane is continuous with the endoplasmic reticulum, so the space between the inner and outer nuclear membranes, the *perinuclear space*, is directly connected with the lumen of the endoplasmic reticulum.

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complex then binds to myosin light chain kinase and activates it. Because this regulation involves myosin, it is known as *thick filament regulation.* Smooth muscle contraction also occurs in a calcium independent manner. In this situation, **Rho kinase** activates the regulatory light chain by phosphorylation.

3.15.8 Intermediate filaments

Intermediate filaments are ropelike cytoplasmic filaments of about 10-nm diameter. These filaments are found in many metazoans, including vertebrates, nematodes and molluscs but not in plants and fungi. Unlike the actin and tubulin proteins, the intermediate filament proteins are chemically heterogenous and show species-specific variations in molecular weight. The principal functions 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. All intermediate filaments share a common structural organization. The individual polypeptide of intermediate filament is an elongated molecule consisting of a non-α-helical N-terminal head domain, a central α-helical *rod domain* and a non-α-helical C-terminal tail domain. The central rod domain consists of long tandem repeats of a distinctive seven amino acid sequence called the *heptad repeat*. Polypeptide chain forms a parallel coiled coil dimeric structure with another. Two dimers then line up side by side to form an *antiparallel* tetramer of four polypeptide chains. Tetramer, the soluble subunit of intermediate filament, further organizes to form higher level organization. Tetramer is analogous to the $\alpha\beta$-tubulin heterodimer or G-actin. Unlike the actin or tubulin subunits, the intermediate filament subunits do not contain a binding site for a nucleoside triphosphate. The antiparallel arrangement of dimers implies that the tetramer, and hence the intermediate filament that it forms, is a non-polarized structure.

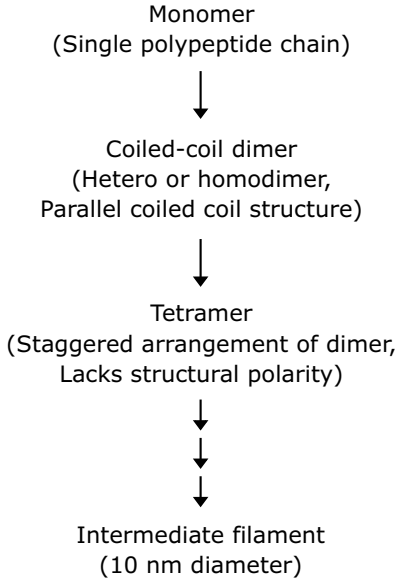

Figure 3.64 A model of intermediate filament construction.

Intermediate filament proteins are classified into four major types based on their sequences and tissue distribution: *nuclear, vimentin-like, epithelial* and *axonal.*

Types	Component polypeptides	Features
Nuclear	Lamins A, B and C	Most ubiquitous group of intermediate filaments and found exclusively in the nucleus. Lamins form a network structure that lines the inside surface of the inner nuclear membrane termed *nuclear lamina*.

Vimentin-like	Vimentin	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.
	Desmin	Desmin expressed in skeletal, cardiac and smooth muscles.
Epithelial	Type I keratins (acidic)	The largest group of intermediate filament proteins.
	Type II keratins (basic/neutral)	Keratins are obligatory heterodimers containing equimolar amounts of type I plus type II keratin polypeptide chains.
Axonal	Neurofilament (NF-L, NF-M and NF-H)	Forms 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.

3.16 Cell junctions

Many cells in tissues are linked to one another and to the extracellular matrix at specialized contact sites called cell junctions. The cell junctions are critical to the development and functions of multicellular organisms. Cell junctions can be classified into three functional groups: occluding junctions, anchoring junctions and communicating junctions.

1. Occluding junctions

Occluding junctions seal cells together in an epithelium in a way that prevents even small molecules from leaking from one side of the sheet to the other (i.e. forms permeability barrier across epithelial cell sheets). These junctions are of two types– tight junction and septate junction.

Tight junctions (or zonula occludens) are cell-cell occluding junctions mediated by two major transmembrane proteins-*claudins and occludin.* Claudins and occludins associate with intracellular peripheral membrane proteins called ZO proteins. Tight junctions make the closest contact between adjacent cells and 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. **Septate junctions** are the main occluding junctions in invertebrates.

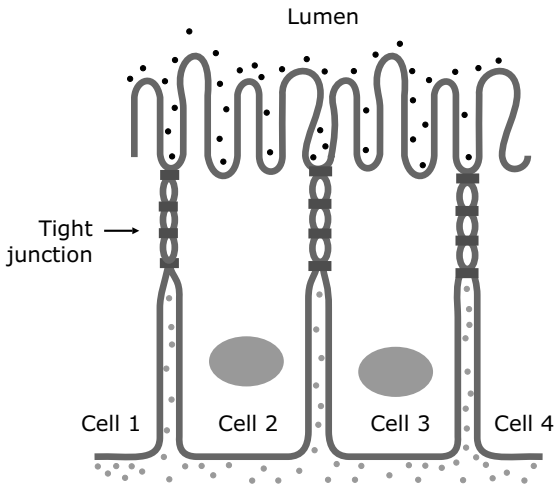

Figure 3.65 Tight junctions allow cell sheets to serve as barriers to solute diffusion. Schematic drawing showing how a small extracellular molecule present on one side of an epithelial cell sheet cannot traverse the tight junctions that seal adjacent cells together.

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3.21 Cell Cycle

The cell cycle is an ordered series of events. It is the sequence of events by which a cell duplicates its genome and eventually divides into two daughter cells. The cell cycle has two main phases - *interphase* and *M-phase*. The period of actual division, corresponding to the visible mitosis, is called **M phase** (mitosis phase). The interphase is the time during which the cell is preparing for division by undergoing both cell growth and DNA replication in an orderly manner. The interphase is further subdivided into;

G1 phase (Gap 1, the period between the end of mitosis and the start of DNA replication);

S phase (Synthesis, the period during which DNA synthesis occurs); and

G2 phase (Gap 2, the gap period following DNA replication and preceding the initiation of the mitotic prophase).

Cells may withdraw from the cycle into G0 (G zero) or re-enter from it. Cells in G0 state can remain for days, weeks, or even years before resuming proliferation. Most cells in our body are in G0 state. Neurons and skeletal muscle cells are present in *terminally differentiated* G0 state, in which cell division never occurs.

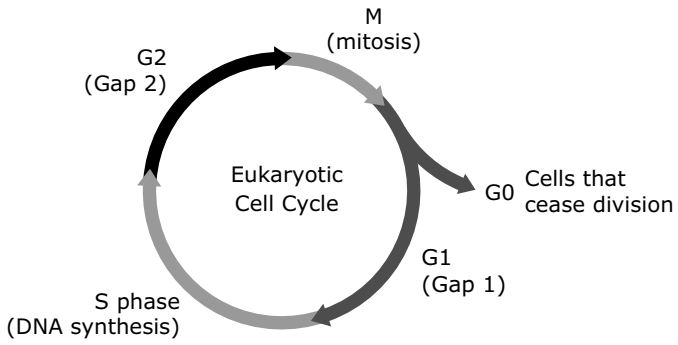

Figure 3.92 The four successive phases of a standard eukaryotic cell cycle. During interphase the cell grows continuously; during M phase it divides. DNA replication is confined to the part of interphase known as S phase. G1 phase is the gap between M phase and S phase; G2 is the gap between S phase 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.

Approximately 95% of the cell cycle is spent in interphase. The duration of the three stages (G1, S and G2) varies from species to species, and also from cell to cell within a species. Although the length of all phases of the cycle is variable to some extent, by far, the greatest variation occurs in the duration of G1. Its length is adjusted in response to growth conditions. In most cells, the whole of M phase takes only about an hour, which is only a small fraction of the total cycle time. For a typical rapidly proliferating human cell in culture with a total cycle time of approximately 24 hours, the G1 phase might last about 11 hours, S phase about 8 hours, G2 about 4 hours and M about 1 hour. Thus, the interphase, though called the resting phase, is very active phase during which the cell is preparing for division by undergoing both cell growth and DNA replication in an orderly manner.

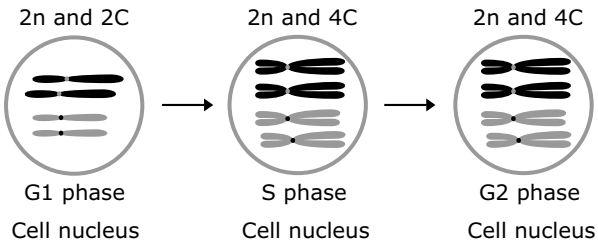

Figure 3.93 Change in number of chromosomes and amount of DNA during interphase.

During S phase, DNA replication takes place. Thus the amount of DNA per cell doubles. If the initial amount of DNA is denoted as 2C, then it increases to 4C. However, there is no increase in the chromosome number; if the cell had diploid (2n) number of chromosomes at G1, even after S phase the number of chromosomes remains the same, i.e. 2n. The M phase starts with the nuclear division, corresponding to the formation of daughter nuclei (*karyokinesis*) and usually ends with division of cytoplasm (*cytokinesis*).

Cell cycle control system

The engines that drive the progression from one step of the cell cycle to the next are a series of protein complexes composed of two subunits: **cyclin** and **Cyclin Dependent protein Kinase** (abbreviated CDK). Cyclin is a regulatory component, whereas CDK is catalytic and acts as protein kinase. Cyclins are so named because they undergo a cycle of synthesis and degradation in each cell cycle. In every eukaryote, there is a family of structurally and functionally related cyclin proteins. There are four classes of cyclins, each defined by the stage of the cell cycle at which they bind CDKs and function: **G1-cyclins**, **G1/S-cyclins**, **S-cyclins** and **M-cyclins**.

Cyclins bind to CDK molecules and control their ability to phosphorylate appropriate target proteins. Each cyclin is rapidly synthesized during a specific phase of the cell cycle and is again promptly broken down after it serves its purpose. They are broken down not only because they are no longer needed, but also because the breakdown is required for the cell cycle to transition to the next step.

Cyclin-dependent protein kinases (CDKs) also constitute a family of functionally related protein kinases. Kinases are enzymes that add phosphate groups to target substrates. CDKs are so named because their activities are regulated by cyclins. They catalyze the phosphorylation of specific serine and threonine residues of specific target proteins.

The target proteins for CDK phosphorylation are determined by the associated cyclin. Because different cyclins are present at different phases of the cell cycle, different phases of the 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 enzyme *phosphatases*.

In fission yeast, *Schizosaccharomyces pombe,* a single cyclin and single CDK drive all cell-cycle events whereas in budding yeast, *Saccharomyces cerevisiae,* a single CDK protein binds more than one classes of cyclins (G1-cyclins, G1/S-cyclins, S-cyclins and M-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, G1/S-cyclins, S-cyclins and M-cyclins. Accordingly, the different cyclin-CDK complexes form which are termed as G1-CDK, G1/S-CDK, S-CDK and M-CDK. Each of the different cyclin-CDK complexes serves as a molecular switch that triggers a specific cell cycle event.

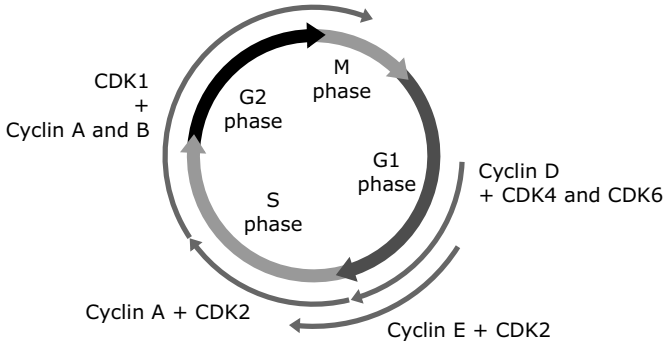

Figure 3.94 The cell cycle control system. Where and when do cyclins act on the cell cycle? Cyclin-CDKs complexes trigger the transition from G1 to S phase and from G2 to M phase by phosphorylating distinct sets of substrates. CDK1 and CDK2 bind to multiple cyclins (cyclin types A, B, D and E), whereas CDK4 and CDK6 only partner with D-type cyclins. Cyclins D and CDK4 or CDK6 regulate events in early G1 phase, cyclin E-CDK2 triggers S phase, cyclin A-CDK2 and cyclin A-CDK1 regulate the completion of S phase and cyclin B-CDK1 is responsible for M phase.

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Chapter 04

Prokaryotes and Viruses

4.1 General features of Prokaryotes

Prokaryotes (*pro* means before and *karyon* means kernel or nucleus) consist of *eubacteria* and the *archaea* (also termed as archaebacteria or archaeobacteria). The term eubacteria refer specifically to *bacteria*. *The informal name bacteria are occasionally used loosely in the literature to refer to all the prokaryotes, and care should be taken to interpret its meaning in any particular context.* Prokaryotes can be distinguished from eukaryotes in terms of their cell structure and molecular make-up. Prokaryotic cells have a simpler internal structure 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 distinguishing characteristics of prokaryotes and eukaryotes are as follows:

Features of prokaryotic organisms

True membrane bound nucleus	–	Absent
DNA complexed with histone	–	Absent
Number of chromosomes	–	One (mostly)
Mitosis and meiosis	–	Absent
Genetic recombination	–	Partial (unidirectional transfer of DNA)
Sterol in plasma membrane	–	Absent (Except *Mycoplasma*)
Ribosome	–	70S
Unit membrane bound organelles	–	Absent
Cell wall	–	Present in *most* of prokaryotic cells. In eubacteria, it is made up of peptidoglycan.

Features of eukaryotic organisms

True membrane bound nucleus	–	Present
DNA complexed with histone	–	Present
Number of chromosomes	–	More than one
Mitosis and meiosis	–	Present
Genetic recombination	–	By crossing over during meiosis
Sterol in the 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 circular, covalently closed DNA and 70S ribosomes in mitochondria and chloroplast support this theory.

Table 4.1 Similarities between 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

4.2 Phylogenetic overview

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 the 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 bp in length and possesses *signature sequences.* These sequences are conserved and found in the organisms of one taxonomic group but not in other groups.

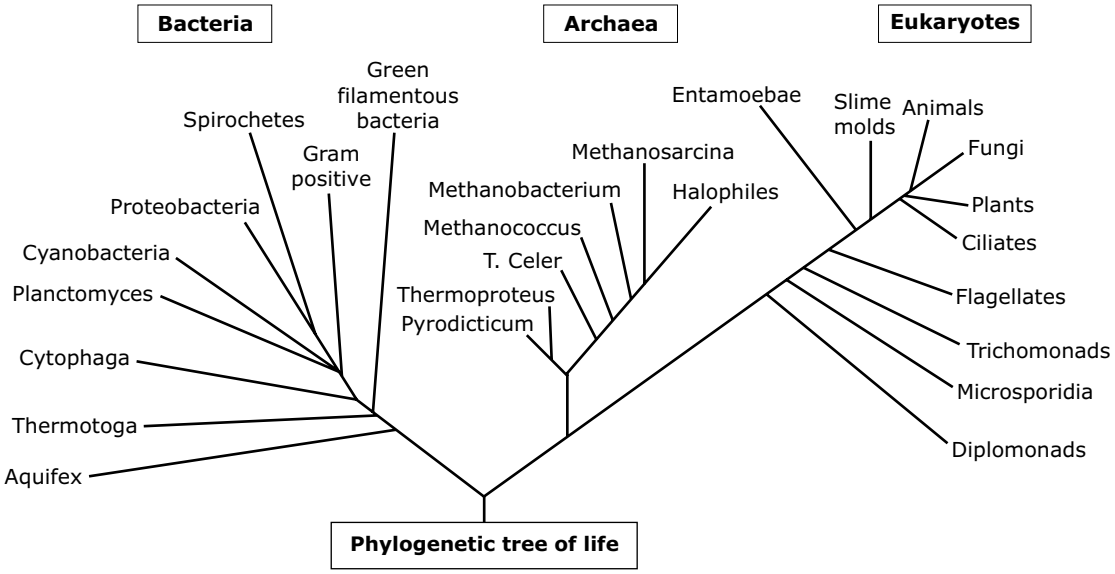

Figure 4.1 A phylogenetic tree of living things, based on RNA data (proposed by Carl Woese), showing the separation of bacteria, archaea, and eukaryotes from a common ancestor.

Based on ribosomal RNA signature sequences, 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* and the *Archaea* domains. Unlike Whittaker's five kingdom system, Woese's three domain system organizes biodiversity by evolutionary relationships.

4.3 Structure of bacterial cell

Bacteria (eubacteria) are microscopic, relatively simple, prokaryotic organisms whose cells lack a nucleus. Prokaryotes can be distinguished from eukaryotes in terms of their cell structure and molecular make-up. Prokaryotic cells are

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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. The *interval* for the formation of two cells from one is called a *generation* and the *time required* for this to occur is called the *generation time*.

$$\text{Generation time} = \frac{\text{t (time, in minutes or hours)}}{\text{n (number of generations)}}$$

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

$$\log N = \log N_0 + n\log 2$$

The number of generations (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 cell 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 a microscope. Whereas the 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 bacteria is 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 the growth of bacteria. On the basis of their response to major environmental factors microbes can be divided into various classes as given in the table 4.9.

Table 4.9 Effect of bacterial 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 growth between 20 and 30°C	*Pseudomonas fluorescens*
Thermophile	Can grow at 55°C or higher; optimum growth often between 55 and 65°C	*Thermus aquaticus*
Hyperthermophile	Has an optimum often between 80 and ~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 up to 10% for optimum growth	*Treponema pallidum*

4.6 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 as *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 a vertical gene transfer when they reproduce by binary fission. Prokaryotes can also do horizontal gene transfer when they pass the gene to other prokaryotic cells. Three mechanisms of horizontal gene transfer in prokaryotes have been discovered. These are *transformation, transduction* and *conjugation*.

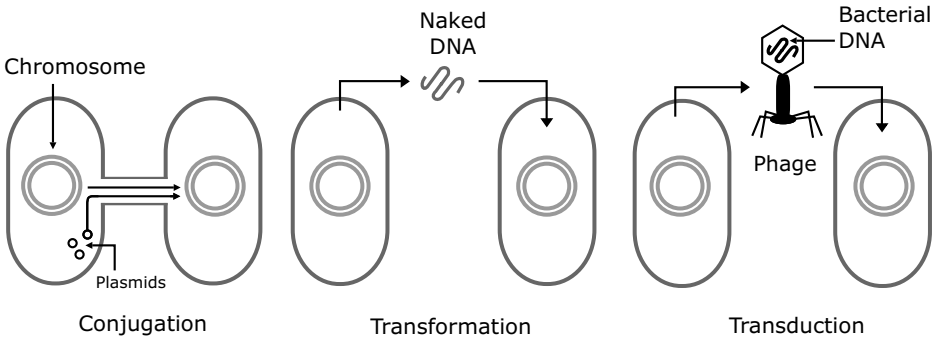

Figure 4.17 Conjugation is the transfer of genes between bacteria that depends upon direct cell-cell contact. Transformation is the uptake of a naked DNA molecule by a competent cell and its incorporation into the genome. Transduction is the transfer of bacterial genes by viruses.

The fragment of DNA that has been transferred during horizontal gene transfer 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

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4.6.2 Transduction

In transduction, bacteriophages function as vectors to transfer DNA from donor bacteria into recipient bacteria. The transfer of bacterial genes by phage was discovered by Lederberg and Zinder in 1951 in *Salmonella typhimurium*. 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.

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*).

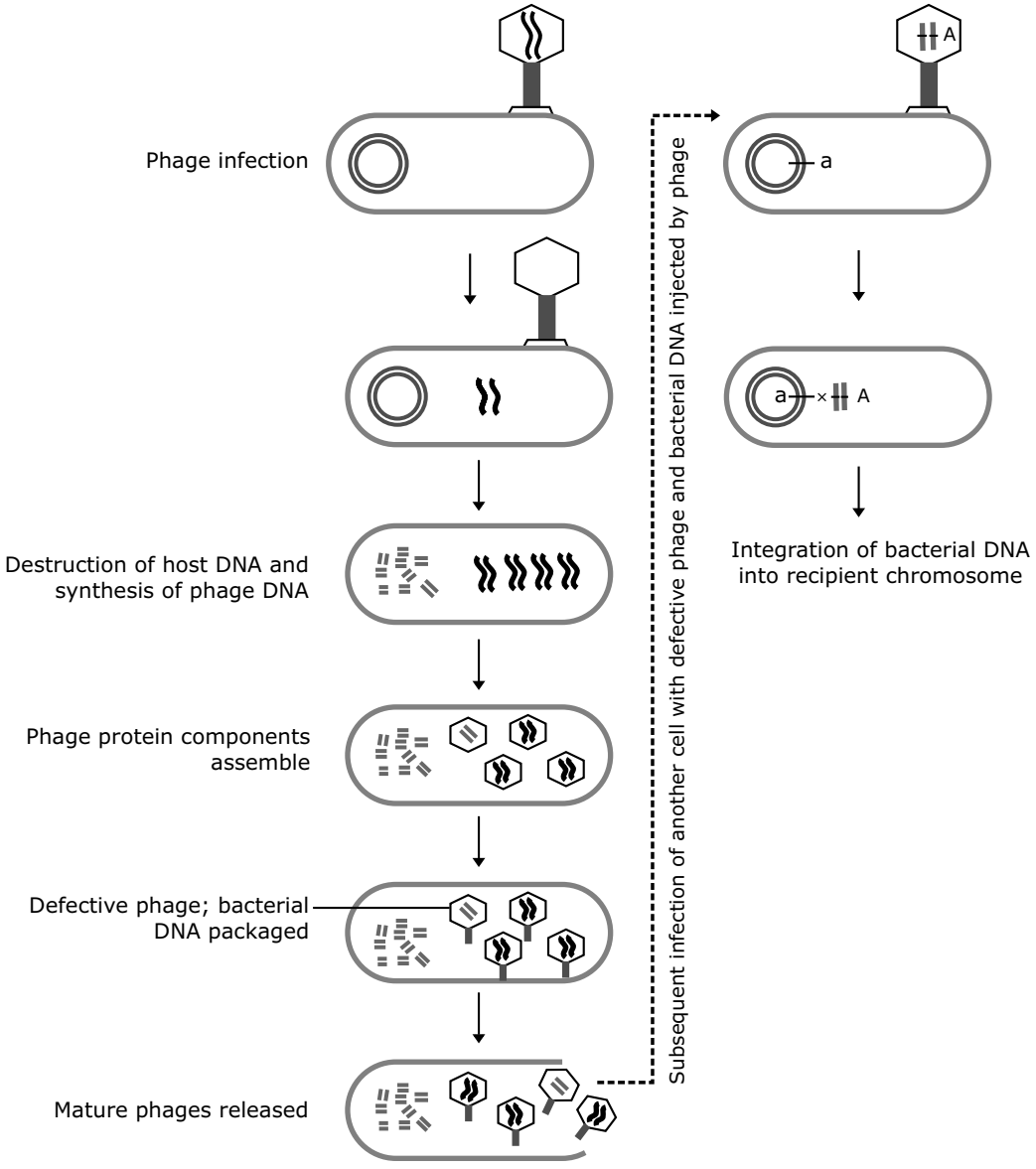

Figure 4.20 Generalized transduction.

The 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. The DNA remain double stranded during transfer and both strands are integrated into the endogenote. In abortive transduction, the donor DNA fragment does not integrate with endogenote and also 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.

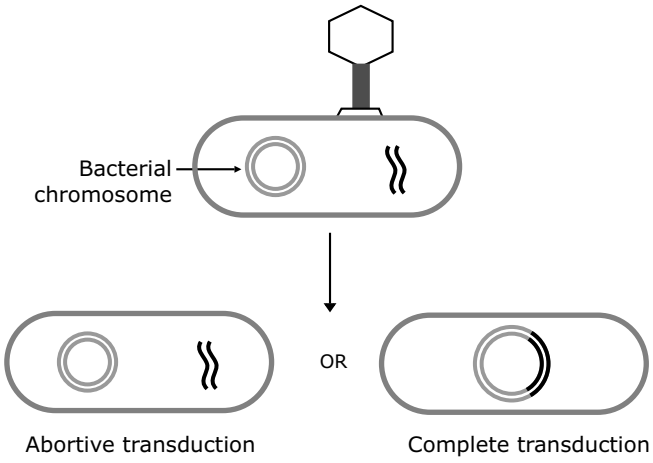

Figure 4.21 Complete and abortive transduction.

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 (*restricted*) 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 disintegrates 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* phages are usually plaque forming and are called λp*bio*, in which the 'p' stands for *p*laque forming.

If λd*gal* transducing particles lack phage genes required to grow lytically, then 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.

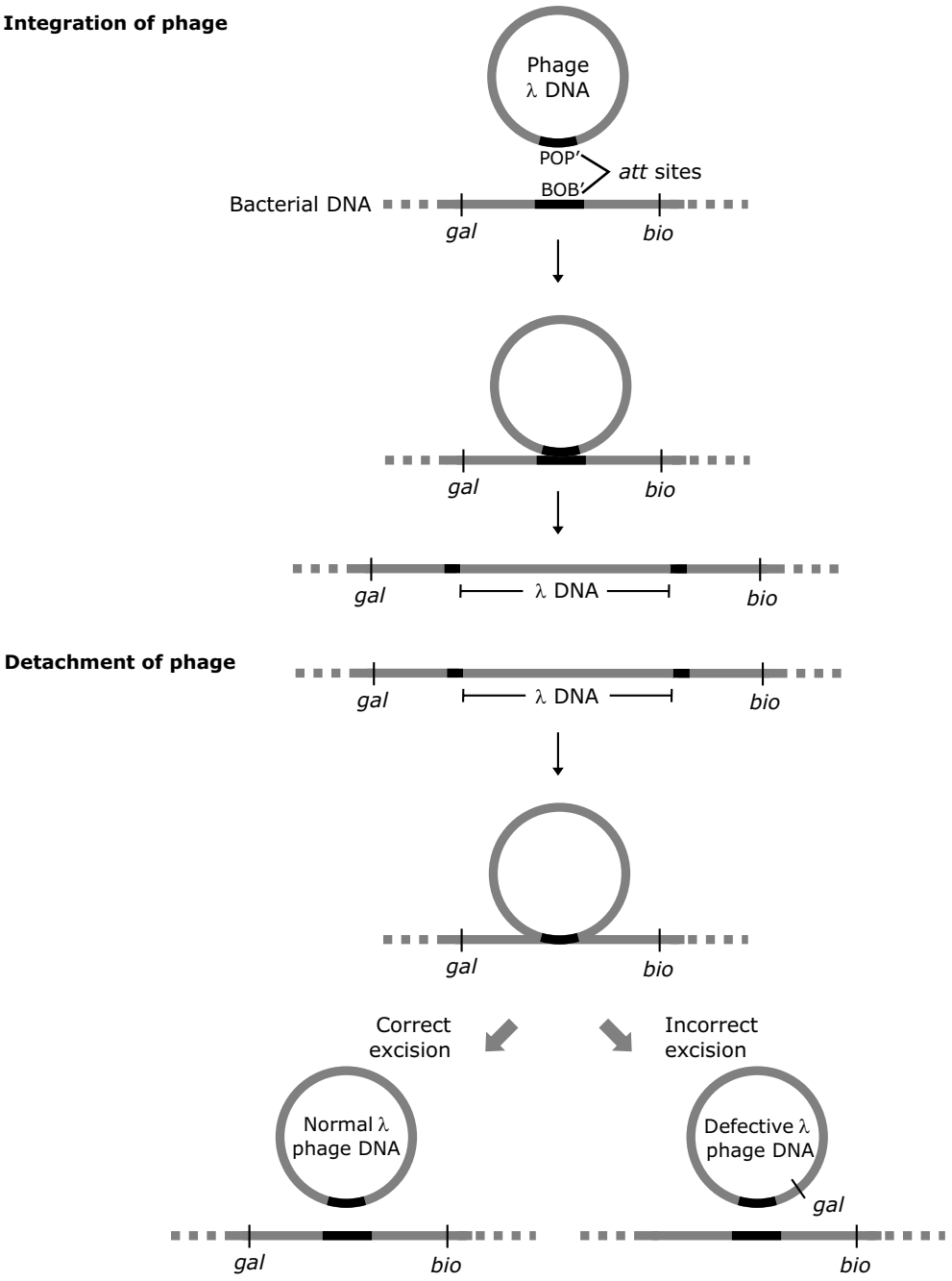

Figure 4.22 The production of defective lambda phage.

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 phage 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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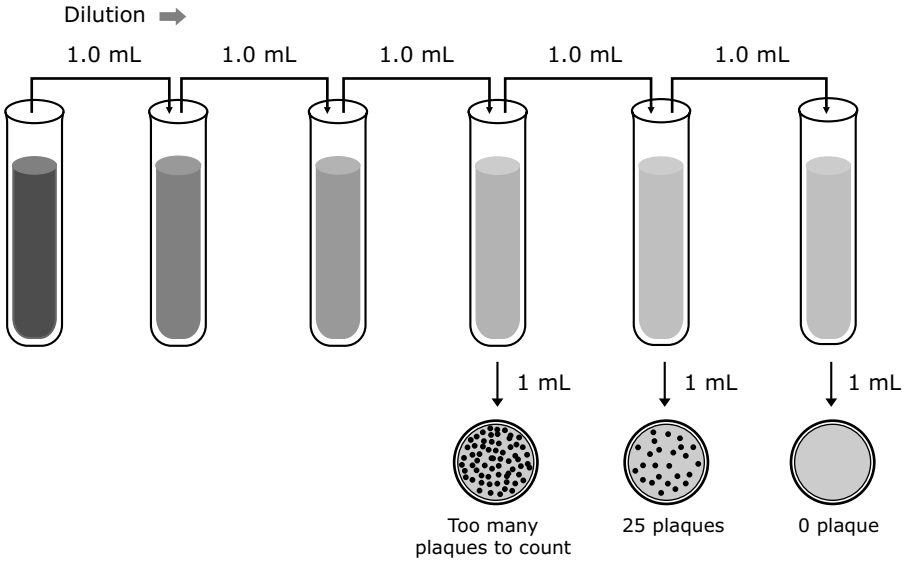
To obtain the PFU in a particular sample, the sample must be diluted. To make 10-fold (10^{-1}) dilution, one can mix 1.0 ml sample with 9.0 ml diluent. If 100 fold (10^{-2}) dilution is needed, 0.1 ml can mixed with 9.9 ml diluent. In the figure 4.40, dilutions (10^{-2}, 10^{-3}, 10^{-5}…) are analyzed using the plaque assay technique. 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 the following formula:

$$\text{(Plaque number/ml)} \times \text{(Dilution factor)}$$

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 25 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

$$(25/\text{ml}) \times (10^4) = 25 \times 10^4/\text{ml}$$



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 4.41 The plaque assay for bacteriophage analysis.

Multiplicity of infection

When phage and their bacterial host are mixed with each other then bacteria and phage collide randomly, leading to phage infection. The percentage of the bacterial cells that will be infected depends upon the concentration of the phage and bacteria. Higher concentration of the phage and bacteria leads to more infection. The efficiency of infection is affected not only by the concentration of the phage and bacteria but also by the ratio of phage to bacteria, the Multiplicity Of Infection (MOI). For example, if 1000 phages are added to 100 bacteria, there will be 1000/100 = 10 phage for every bacterial cell, and the MOI is 10. If only 500 phages had been added to the same number of bacteria, the MOI would have been 5. The MOI can be either high or low. If the number of phages greatly exceeds the number of bacterial cells to infect, the cells are infected at a high MOI.

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Total number of bacteriophage:

$$0.1\,\text{mL} \times \frac{4 \times 10^9\,\text{phage}}{\text{mL}} = 4 \times 10^8\ \text{bacteriophage}$$

Total number of cells:

$$0.5\,\text{mL} \times \frac{2 \times 10^8\,\text{cells}}{\text{mL}} = 1 \times 10^8\ \text{cells}$$

The MOI is then calculated as bacteriophage per cell:

$$\text{MOI} = \frac{4 \times 10^8\,\text{phage}}{1 \times 10^8\,\text{cells}} = 4\ \text{phage/cell}$$

Therefore, the MOI is 4 phage/cell.

4.12.4 Genetic analysis of phage

Phages are ideal for genetic analysis. They have a short generation time and are haploid. Phages multiply as clones in plaques and large number can be propagated on plates or in small volumes of liquid media. Mutant strains of phages can be stored for long periods also. Because of these advantages, phages were central to the development of molecular genetics, and important genetic principles such as recombination, complementation are most easily analyzed by phages.

*r*II locus of T4 phage

T4 is a virulent phage. The wild-type T4 phage gives small plaques (1.0–1.5 mm in diameter) and have a clear center surrounded by diffuse halo. This is called the wild type phage (or r^+ phage). One type of mutant T4 phage produced larger (2.0–2.5 mm in diameter) plaque with a sharp periphery due to rapid lysis of host: these are *r* (*rapid lysis*) *mutants*. Rapid lysis mutants fall into three classes: *r*I, *r*II and *r*III. Phages with a rapid lysis mutation can be distinguished by the appearance of their plaques on *E. coli* B indicator bacteria. The plaques formed by wild types r^+ phage have fuzzy edges because of the phenomenon called *lysis inhibition*, which delays lysis of the infected cells. However *r*-mutants do not show lysis inhibition, which causes them to form sharp edged, clear plaque. Among the *r* mutants, the *r*II class of mutants are very informative.

The host range of *r*II mutants and wild types phages are different. One type of bacterial strain, *E. coli* B allows both to grow, but plaques of different size result: wild type phages produce small plaques, and *r*II mutants produce large plaques. Another *E. coli* strain that carries the λ-prophage, designated *E. coli* K-12λ or K(λ), does not permit the growth of *r*II mutants, but it does allow wild type phage to grow. The *r*II mutants are then *conditional mutants*—namely, mutants that can grow under one set of conditions but not under another. *E. coli* B is said to be *permissive* for *r*II mutants, because it allows phage growth, whereas *E. coli* K(λ) is said to be *nonpermissive* for *r*II mutants, because it does not allow phage growth.

Complementation

Benzer studied the concept of *complementation*. He tried to find out whether the entire *r*II region of phage T4 acts as a single functional unit or whether it is made up of subunits that function independently. Therefore, he tested the mutations that he had mapped in the *r*II region to see whether various combinations of pairs of the mutations would restore the wild type phenotype. To do so, he carried out a mixed infection with different *r*II mutants (*mixed infection experiments*). Wild type *r*II was capable of lysing *E. coli* K(λ) and *r*II mutants cannot do so.

In the example described in the figure 4.42, two different mutant strains of a phage infect the same host cell. This host cell is normally a permissive host for the wild-type phage but cannot propagate either of the mutant phages by itself because they cannot make a gene product required for multiplication on that host. The outcome of the infection depends upon whether the mutations are in the same or different genes.

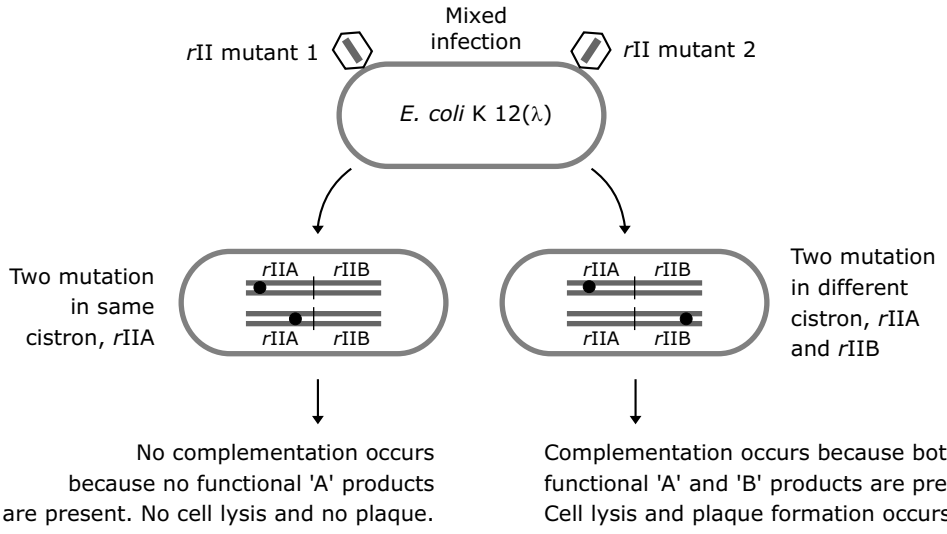

Figure 4.42 Complementation test: a schematic view of rII complementation. Two different mutants of rII are used to simultaneously infect *E. coli* K(λ) (mixed infection). Normally, an rII mutant cannot lyse *E. coli* K(λ). However, if the two different mutants can complement each other, then lysis and phage growth will result. If the two rII mutants cannot complement each other, then no lysis or phage growth will result.

If they are in different genes, each DNA provides one of the needed gene products and so the two mutations will complement each other and both mutant viruses can multiply. If, however, the two mutations are in the same gene, neither DNA will provide that gene product and so the mutations do not complement each other and neither mutant strain will multiply.

Tests of many different paired mutants allowed Benzer to separate the mutations into two groups, labeled A and B. All mutations in the A group complemented those in the B group, whereas no mutations in the A group complemented any other mutations in the A group and no mutations in the B group complemented any other mutations in the B group. Benzer found that all mutations in group A mapped in one half of the *r*II locus and that all mutations in group B mapped in the other half of the *r*II locus.

Lack of complementation is the diagnostic for mutations being in the same gene. Hence it must be concluded that the groupings *r*IIA and *r*IIB must represent different genes, located next to each other on the chromosome. Benzer called the *r*IIA and *r*IIB complementation groups *cistrons*, which he defined as the smallest functional genetic unit. We now know that Benzer's A and B cistrons represent two separate genes in what we originally referred to as the *r*II locus.

Fine structure analysis of *r*II locus

In the early 1950s, S. Benzer performed a detailed examination of a single *r*II locus in phage T4. By analyzing intragenic recombination he determined the detailed map of the *r*II locus. Recombination within a gene is called *intragenic recombination*. Because of the extremely detailed information provided from his analysis, Benzer's work was described as the *fine structure analysis of the gene*. Intragenic recombination between two *r*II mutations were measured by simultaneously infecting *E. coli* B with two different *r*II mutants and allowing the phage to multiply. The progeny phages were then plated to measure the frequency of recombinant types. If recombination can occur between the two *r*II mutations, two different recombinant type progeny would appear: double mutants and wild type. For example, if two *r*IIA mutants were first allowed to infect *E. coli* B, and if a recombination event occurred between the mutational sites in the A cistron, then wild type progeny viruses would be produced at low frequency. If samples of the progeny viruses from such an experiment were then plated on *E. coli* K(λ), only the wild type recombinants would lyse the bacteria and produce plaques. The percentage of recombinants can be determined by counting the plaques.

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Hepatitis virus

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	ssRNA; No envelope	2–6 week
Hepatitis B	dsDNA; enveloped	4–26 week
Hepatitis C	ssRNA; enveloped	2–22 week
Hepatitis D	ssRNA; enveloped	6–26 week
Hepatitis E	ssRNA; 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. Hepatitis D virus, classified as a *hepatitis delta virus*, is considered to be a subviral satellite because it can propagate only in the presence of the hepatitis B virus. Transmission of hepatitis D virus can occur either via simultaneous infection with hepatitis B virus (coinfection) or via infection of an individual previously infected with hepatitis B virus (*superinfection*). The hepatitis D virus genome consists of a single stranded, negative sense, circular RNA.

4.12.6 Plant viruses

Plant viruses exist in rod and polyhedral shape. Most plant viruses have genomes consisting of a single RNA strand of plus (+) sense 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 virus with ~2130 capsomeres arranged in a hollow right handed helix. It contains a single genetic RNA (ss, plus sense) of ~6400 nucleotides.

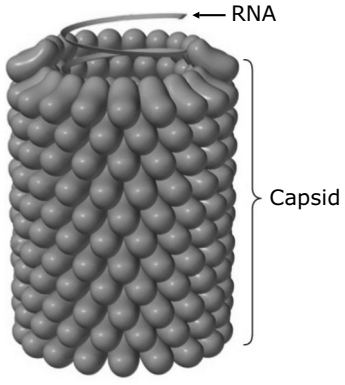

Figure 4.51 Tobacco mosaic virus has a rod-like appearance. Its capsid is made up of ~2130 capsomeres. One molecule of genomic ssRNA, 6400 nucleotides long, present in the centre of the capsid. The capsomere self-assembles into the rod like helical structure (16.3 capsomeres per helical turn) around the RNA.

4.13 Prions and Viroid

Prions are proteins. Prion proteins are designated as PrP. The word prion, coined in 1982 by Stanley B. Prusiner, is derived from the words protein and infection. The endogenous, normal cellular form is denoted PrP^{C} (for Cellular) while the disease-causing, infectious and misfolded form is denoted PrP^{Sc} (for Scrapie, after one of the diseases first linked to prions). Prions are glycosylated proteins and linked to the membrane by a GPI-linkage. The infectious form, PrP^{Sc}, is responsible for neurodegenerative diseases in animals including human. The normal cellular PrP^{C} form 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} results in the formation of amyloid fibrillous structure that is absent from the PrP^{C} form. The PrP^{Sc} form can perpetuate itself by causing the newly synthesized PrP protein to take up the PrP^{Sc} form instead of the PrP^{C} form.

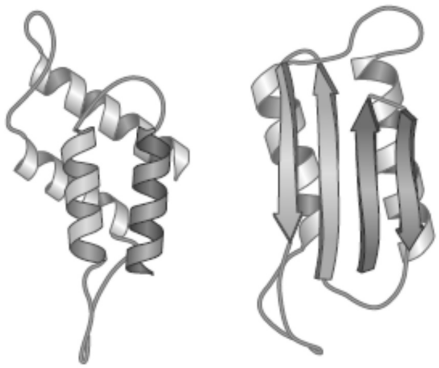

Figure 4.52 The normal prions (PrP^{c}) have a large percentage of α-helix, but the abnormal forms (PrP^{sc}) have more β-pleated sheets.

Prions are novel transmissible agents causing a group of neuro-degenerative diseases that can be perpetuated by inoculating animal with tissue extracts from infected one. Collectively, prion diseases are described as **spongiform encephalopathies**. No prion diseases of plants are known. In 1997, American scientist Stanley B. Prusiner won the Nobel Prize for this pioneering work with these diseases and with the prion proteins. **Kuru** was the first naturally occurring 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.

Table 4.23 Prion disease of human/animals

Disease	*Organism*
Creutzfeldt-Jakob	Human
Kuru	Human
Bovine spongiform encephalopathy (Also known as *Mad cow disease*)	Cow
Scrapie	Sheep
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 (discovered and named by Otto Diener) 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

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Chapter 05

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 resist infections 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 is typically divided into two categories—*innate* and *adaptive* immunity.

5.1 Innate immunity

Innate (native/natural) immunity is present since birth and consists of many factors that are relatively nonspecific—that is, it operates against almost any foreign molecules and pathogens. It provides the *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 enzymes like lysozyme inhibit the growth of most microorganisms. Similarly, respiratory and gastrointestinal tracts are lined by mucous membranes. Mucus membranes entrap foreign microorganisms. The respiratory tract is also covered by cilia, which are hair like projections of the epithelial-cell membranes. The synchronous movement of the cilia propels mucus-entrapped microorganisms out of these tracts. Similarly, the conjunctiva is a 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 proteins, cytokines, pattern recognition molecules, acute-phase proteins, cationic peptides, enzyme like lysozyme and many others.

Complement proteins

The complement proteins are soluble proteins/glycoproteins that are mainly synthesized by liver and circulate in the blood and extracellular fluid. They were originally identified by their ability to amplify and complement the

action of antibodies; 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 complement proteins 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 Receptors (TLRs) are a class of pattern recognition molecules that function exclusively as signaling receptors. It was originally identified as a protein involved in the establishment of dorso-ventral polarity in developing fly embryos. It is also involved, however, in the adult fly's resistance to fungal infections. There are at least 10 distinct TLRs in humans, which recognize lipopolysaccharide, peptidoglycan, zymosan and CpG DNA. 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. The TLR family proteins consist of extracellular leucine rich repeat (LRR) motifs and a cytoplasmic tail containing a Toll/IL-1 receptor homology (TIR) domain. The LRR motifs are responsible for ligand recognition and the TIR domain is essential for triggering intracellular signaling pathways.

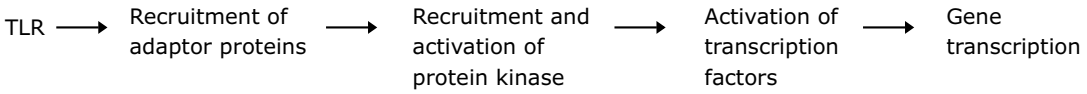

Figure 5.1 TLR and basic signaling mechanisms.

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 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

Many specialized cell types like *neutrophils, macrophages, monocytes, natural killer cells* participate in innate host defense mechanisms. Once a pathogen evades the physical and chemical barriers, these specialized cells play an important role in protection. Phagocytosis is a fundamental protective mechanism carried out by these cell types,

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5.6 Major–histocompatibility complex

Major Histocompatibility Complex (MHC) is a tightly linked cluster of genes present in every vertebrate species, the products of which play important roles 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 is highly **polymorphic** i.e. many alternative forms 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 set 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.

The MHC genes are traditionally divided into three classes:

Class I MHC genes,

Class II MHC genes and

Class III MHC genes.

MHC class I and class II genes encode the antigen-presenting MHC molecules. The peptide antigen-presenting MHC molecules are known as *classical* MHC molecules. Both classes also encode proteins that do not function in the presentation of peptide antigens to T-cells: these are known as *non-classical* MHC molecules. The concept of classical and nonclassical does not apply to class III.

Class I MHC genes

Class I MHC genes express glycoproteins called MHC molecules, sometimes referred to as MHC antigens 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 on 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.

Class II MHC genes

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 genes express MHC molecule *constitutively* (i.e. under all conditions) only on antigen presenting cells (APCs). APCs are classified as *professional* and *non-professional* antigen-presenting cells. Professional antigen-presenting cells are dendritic cells, macrophages, and B lymphocytes. Non-professional 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).

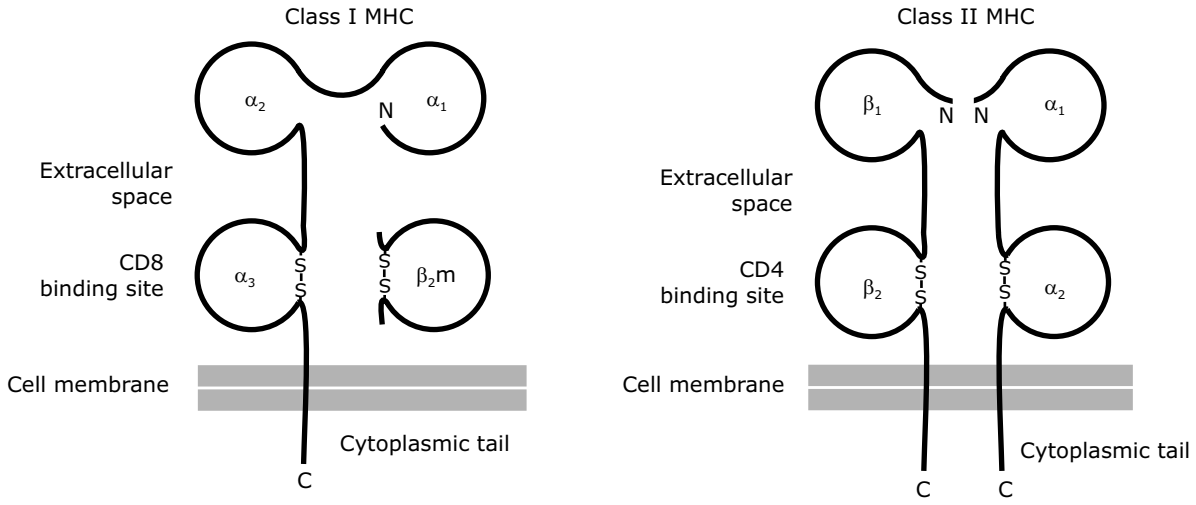

Figure 5.7 MHC class I molecules are composed of a polymorphic α chain noncovalently attached to the nonpolymorphic β_2 microglobulin (β_2m). The α chain is glycosylated. MHC class II molecules are composed of a polymorphic α chain non-covalently attached to a polymorphic β chain. Both chains are glycosylated.

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 A and B genes 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**.

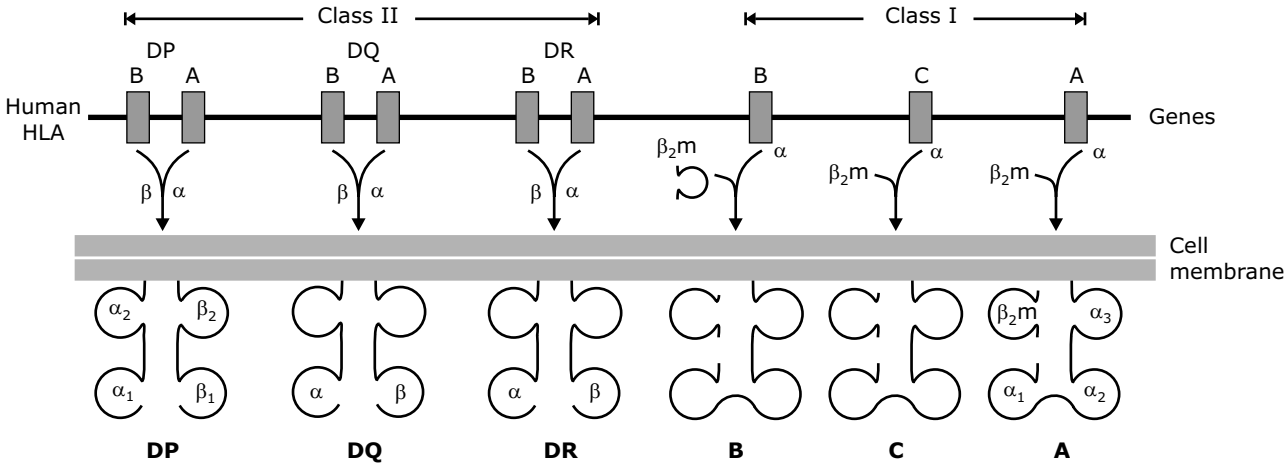

Figure 5.8 Simplified depiction of the human MHC showing regions and genes coding for polymorphic MHC class I and II molecules. β_2m encoded outside the MHC. Adapted and modified from Immunology A short Course, R. Coico et al., Wiley-Liss Publication.

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5.6.2 Antigen processing and presentation

T-cell receptor does not recognize free antigens. T-cell receptor recognize only antigen that is bound to *MHC molecules*. Both T_C and T_H cells can recognize antigen only when it is presented by a self-MHC molecule, an attribute called *self- 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 I MHC restricted.* 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 on the cell surface for T-cell recognition are known collectively as **antigen processing** and **presentation**.

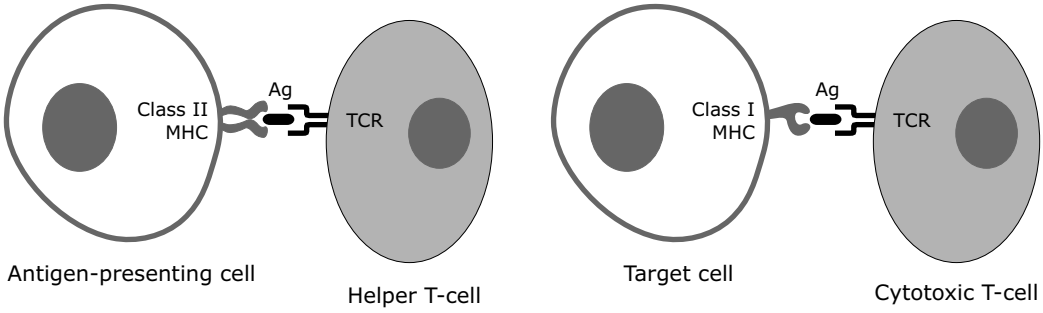
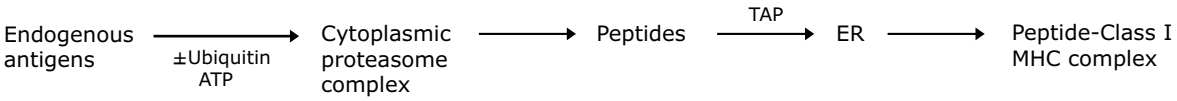


Figure 5.9 Recognition by T-cells of foreign peptide antigens bound to MHC proteins. Cytotoxic T-cells recognize foreign peptides in association with class I MHC proteins, whereas helper T-cells recognize foreign peptides in association with class II MHC proteins. In both cases, the peptide MHC complexes are recognized on the surface of an antigen-presenting cell or a target cell.

How do peptides derived from protein antigen get associated with MHC molecules? Protein antigens presented by APC belong 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.

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 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. The part of a processed antigen that binds to the MHC molecule is termed as *agretope*.



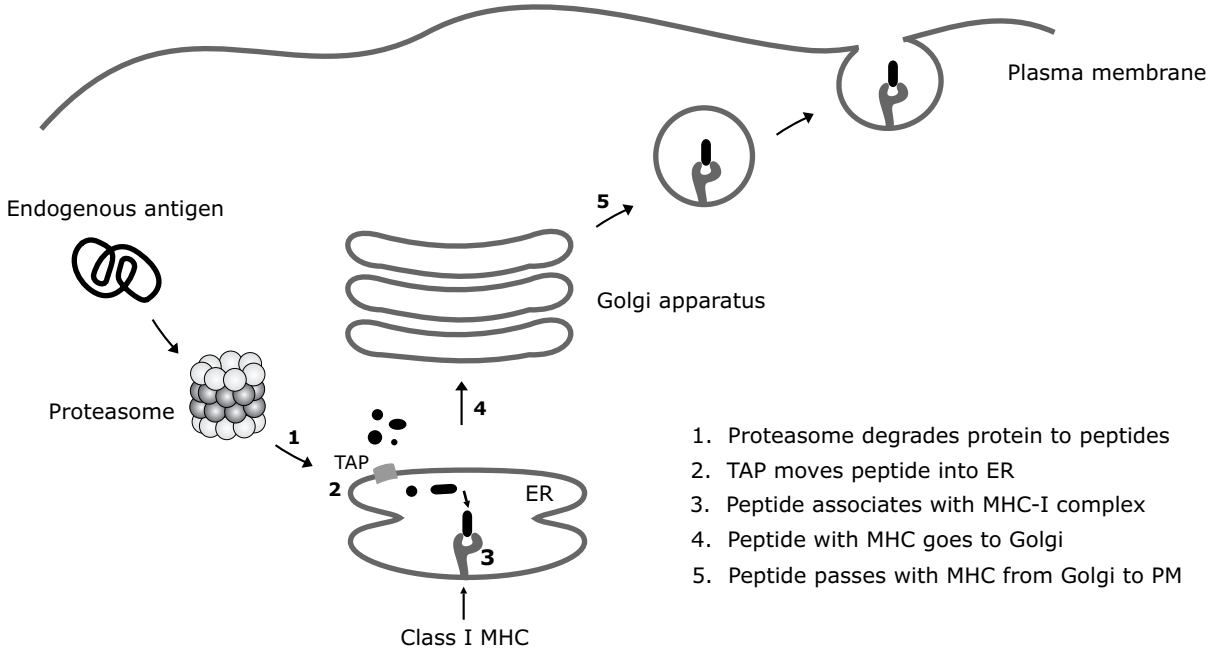


Figure 5.10 The processing of an endogenous protein antigen for presentation to a cytotoxic T-cell.

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 do 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. Another non-classical class II MHC HLA-DO may act as a negative regulator of class II antigen processing by binding to HLA–DM and inhibiting its role in the dissociation of CLIP from MHC.

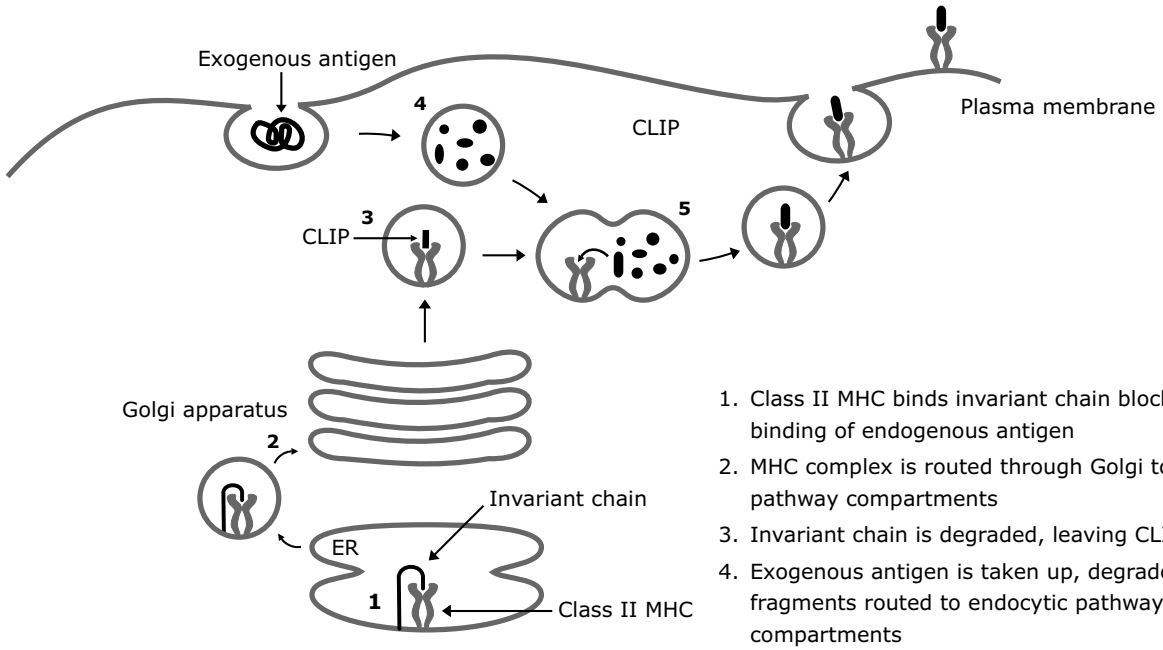


Figure 5.11 The processing of an exogenous protein antigen for presentation to a helper T-cell.

5.6.3 Laboratory mice

Mice are the most commonly used mammalian research model. They are common experimental animals in biology, primarily because they are mammals, are relatively easy to maintain and handle, reproduce quickly, and share a high degree of homology with humans. Laboratory mice include several inbred, outbred, knockout and transgenic mice strains. Many laboratory strains are *inbred*. An inbred strain is one that is produced using at least 20 consecutive generations of sister and brother or parent and offspring matings. The mating of two genetically related parents is called *inbreeding*. Inbreeding results in increased homozygosity. In contrast to inbred mice, outbred mice are usually heterozygous at many loci.

If mice are inbred (that is, have identical alleles at all loci), each H-2 locus will be homozygous because the maternal and paternal haplotypes are identical, and all offspring therefore express identical haplotypes. Inbred mouse strains are *syngeneic* or identical at all genetic loci. Two strains are considered *congenic* if they are genetically identical except at a single genetic locus.

Some inbred mouse strains have been designated as *prototype* strains and the MHC haplotype expressed by these strains is designated by an arbitrary italic superscript (e.g. H-2^a, H-2^b). If another inbred strain has the same set of alleles as the prototype strain, its MHC haplotype is the same as the prototype strain.

Table 5.5 H-2 haplotypes of some mouse strains

			H-2 alleles				
Prototype strain	*Other strains with the same haplotype*	*Haplotype*	*K*	*IA*	*IE*	*S*	*D*
CBA	AKR, C3H, C57BR	*k*	*k*	*k*	*k*	*k*	*k*
DBA/2	BALB/c, SEA, YBR	*d*	*d*	*d*	*d*	*d*	*d*
C57BL/10 (B10)	C57BL/6, C57L	*b*	*b*	*b*	*b*	*b*	*b*
A	A/He, A/Sn	*a*	*k*	*k*	*k*	*d*	*d*

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Resting mature B-cells express low levels of B7 and are poor APCs, whereas activated B-cells are very efficient APCs. One pair of adhesive molecules – CD54 (ICAM-1, intercellular adhesion molecule-1) and CD58 (LFA-3, leucocyte function-associated antigen-3) are also present on B-cell membrane, which stabilize the interaction of B-cell with T-cell.

B-cell receptor signaling

The B-cell antigen receptor (BCR) is composed of membrane immunoglobulin (mIg) molecules and associated Igα/Igβ heterodimers. The mIg bind antigen, resulting in receptor aggregation, while the Igα/Igβ heterodimer transduce signals to the cell interior. Igα/Igβ has ITAMs (Immunoreceptor Tyrosine-based Activation Motifs) in the cytoplasmic tails. The BCR lacks intrinsic kinase activity but couples to Src family kinases such as Lyn, Blk, and Fyn. When antigen binds with BCR, the tyrosines in the ITAMs become phosphorylated by receptor-associated Src-family tyrosine kinases Blk, Fyn, or Lyn. The phosphorylated ITAMs are then able to bind with Syk (spleen tyrosine kinase) by virtue of their SH2 domains. This initiates the formation of a 'signalosome' composed of the BCR, the aforementioned tyrosine kinases, adaptor proteins (such as BLNK) and signaling enzymes (such as PLCγ2). Signals emanating from the signalosome activate multiple signaling cascades that involve kinases, GTPases, and transcription factors.

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* (T-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.

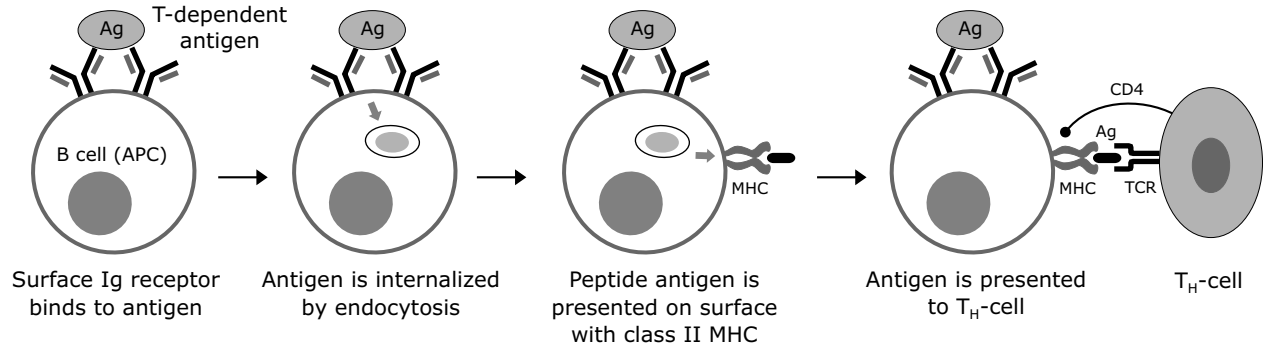

Figure 5.21 B-cell activation proceeds by two different routes, one dependent upon T_H-cells, the other not. The B-cell response to T-dependent antigens requires direct contact with T_H-cells, not simply exposure to T_H-derived cytokines.

In *T_H cells independent pathway*, antigens called *thymus-independent antigens* (T-independent antigens), 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 outer 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 5.7 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

Role of T_H cell in B-cell activation

Activation of B-cells by soluble protein antigens requires the involvement of T_H-cells. Activation of B-cells requires binding of antigen to B-cell mIg. Antigen crosslinks mIg, generating *signal* 1, which leads to increased expression of class II MHC and co-stimulatory B7. Antigen–antibody complexes are internalized by receptor-mediated endocytosis and degraded to peptides, some of which are bound by class II MHC and presented on the membrane as peptide–MHC complexes. T_H-cell recognizes antigen–class II MHC on B-cell membrane. This interaction activates T_H-cell. Interaction of CD40 (present on the B-cell membrane) and CD40L (present on the T_H-cell membrane) provides *signal* 2 for B-cell activation. Interactions between B7 of B-cell membrane and CD28 present on the T_H-cell membrane provide co-stimulation to the T_H-cell. In the presence of these signals, B-cell begins to express receptors for various cytokines (such as IL-2, IL-4, IL-5 and others) released from the T_H-cell. Binding of cytokines sends signals that support the differentiation.

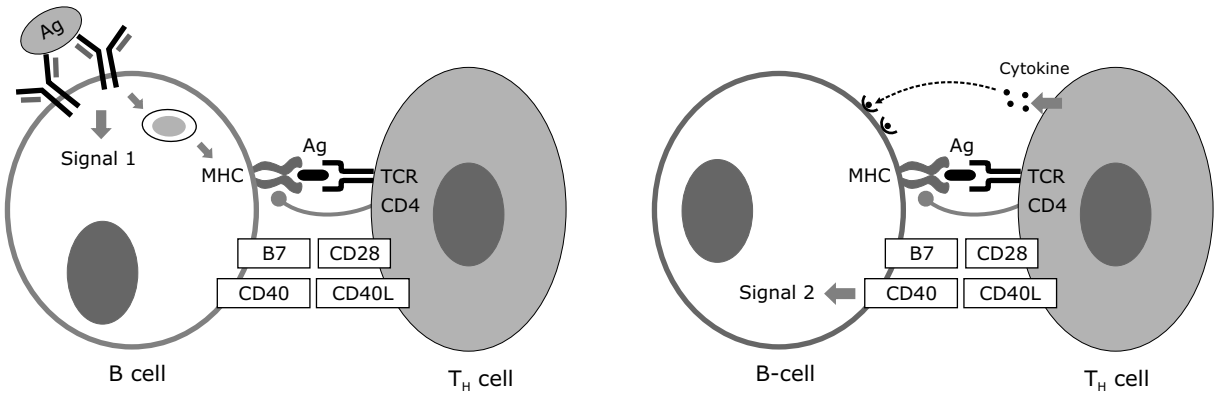

Figure 5.22 Two signals that activate a B-cell. The first is generated when a multivalent antigen binds and cross-links mIg. The second signal is provided by an activated T-cell, which binds to the B-cell both through its antigen receptor and via a separate interaction between CD40 on the B-cell and CD40L on the activated T_H cell. The bound T-cell then delivers cytokines and other signals to its partner B-cell to complete the activation process.

The B-cells after receiving the activating signals move into specialized regions of the lymph node or spleen to begin the process of differentiation into an antibody secreting **plasma cells** and **memory B-cells**. Some of these plasma cells die after the initial primary response is completed, whereas others take up long-term residence in the bone marrow, gut or other locations as long-lived plasma cells. Some antigen-stimulated B-cells migrate into the follicles of the lymph nodes and spleen where they divide and differentiate. As the follicle fills with proliferating B-cells, it develops into a *germinal center*. Within the germinal center, the Ig genes undergo class switching, in which μ constant regions are replaced by constant regions of other isotypes. The variable regions of genes undergo *somatic hypermutation* that result in the secretion of antibodies with altered sequences in their antigen-binding sites. Both class switching combination and somatic hypermutation are dependent on the activity of a germinal center enzyme, *activation-induced cytidine deaminase* (AID).

At the close of the primary immune response, memory B-cells remain that are the daughters of those cells that were stimulated during the primary response. Many of these progeny B-cells now carry mutated and selected B-cell receptors.

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5.9 Kinetics of the antibody response

Humoral immunity is mediated by serum antibodies which are the proteins secreted by the B-cells. 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 bound, 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 a 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 in the memory response are termed **memory cells**.

The primary response is slow and short lived with a long lag phase and low titer of antibodies that do 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.

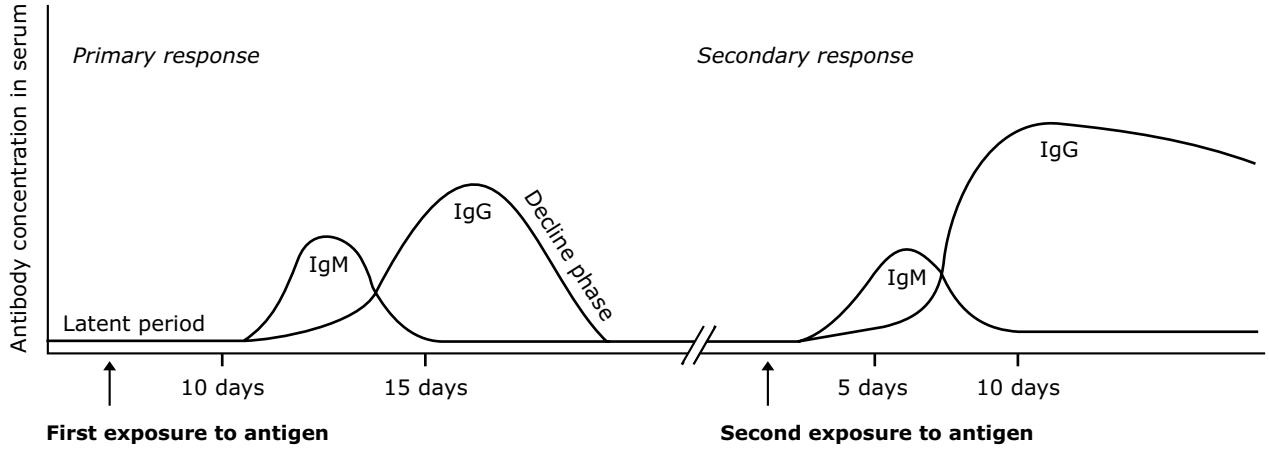

Figure 5.24 Antibody production and kinetics.

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. The IgG, IgE, and IgA molecules are collectively referred to as secondary classes of antibodies because they are thought to be produced only after antigen stimulation and because they dominate secondary antibody responses.

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 5.9 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

5.10 Monoclonal antibodies and Hybridoma technology

Antibodies produced ordinarily by infection or immunization are *polyclonal* because natural antigens have multiple epitopes, each of which generates clones of lymphocytes. This results in antisera containing antibodies from different clones of lymphocytes with specificities against different epitopes of the antigens. On the other hand, *monoclonal* antibodies (mAb) are monospecific antibodies. These antibodies are produced from clone of single lymphocyte directed against a single antigenic determinant or epitope. Such antibodies produced by a single clone and directed against a single epitope.

Hybridoma technology is a method of forming hybrid cell lines (called hybridomas) by fusing a specific antibody-producing B-cell with a myeloma cell (cancerous B-cell). The antibodies produced by the hybridoma are all of a single specificity and are therefore monoclonal antibodies. The production of monoclonal antibodies was invented by Cesar Milstein and Georges J. F. Köhler in 1975. They shared the Nobel Prize in 1984 for Medicine and Physiology.

Hybridomas are somatic cell hybrids produced by fusing antibodies forming spleen cells with myeloma cells. Antibody-producing B-cells normally die after several weeks in cell culture *in vitro*. Therefore, antibody-producing B-cells 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-cells, is then removed from the mouse, and the B-cells are fused with myeloma cells.

Hybridomas are selected by the use of a selective medium in which the myeloma cells die, but hybridomas survive. The most widely used selective systems involve the inclusion of the antibiotic *aminopterin* in the growth medium. This inhibits the *de novo* nucleotide synthesis pathway. Normal animal cells synthesize purine nucleotides and thymidylate for DNA synthesis by a *de novo* pathway requiring tetrahydrofolate. Aminopterin, acting as antifolate drug, blocks activation of tetrahydrofolate. However, normal cells survive in this medium as they are able to use the *salvage pathway* for nucleic acid synthesis. But if the cells are unable to produce the enzyme Hypoxanthine-Guanine Phospho Ribosyl Transferase (HGPRT), they are unable to utilize the salvage pathway and, therefore, die in aminopterin-containing medium.

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Chapter 06

Diversity of Life

6.1 Taxonomy

Taxonomy (arrangement by the rules) is the branch of biology that deals with *identification* (placement of a new organism into a previously described group), *nomenclature* (the naming of organisms) and *classification* (ordering of organisms into groups- can be phenetic or phylogenetic) of organisms. **Systematics** is the process of organizing taxonomic information about organisms into a logical classification that provides the framework for all comparative studies. It is the scientific study of biological diversity and its evolutionary history. Systematics and taxonomy are collectively referred to as the *systematic biology*.

Levels of taxonomy

There are three levels of taxonomy:

Alpha taxonomy	:	It is concerned with finding, describing and naming of organisms. This is the first and most basic step in taxonomy.
Beta taxonomy	:	It includes identification of natural groups and biological classes.
Gamma taxonomy	:	It includes study of evolutionary processes and patterns.

Organisms were first classified more than 2,000 years ago by Greek philosopher *Aristotle*. He classified organisms as either plant or animal. Modern biological classification began with the eighteenth century Swedish naturalist *C. Linnaeus.* He established a simple system for classifying and naming organisms. He developed a hierarchy (a ranking system) for classifying organisms that is the basis for modern taxonomy.

6.1.1 Nomenclature

Nomenclature is the formal naming of a particular organism according to some standardized system. The fundamental principle of nomenclature is that each organism must have only one scientific name. In contrast to scientific names, many organisms also bear *common names* (also called vernacular names), which are generally used by people within a limited geographic region. Presently, the criteria for scientific naming of plants, algae and fungi are based on the rules and recommendations of the International Code of Nomenclature or ICN. It was formerly called the International Code of Botanical Nomenclature (ICBN); the name was changed at the International Botanical Congress in July 2011. Similarly, International Code of Zoological Nomenclature (ICZN) provides rules for naming animals. The scientific names of species are **binomials** (literally meaning two names). Swedish botanist Linnaeus (referred to as the *father of taxonomy*) first proposed the system of *binomial nomenclature* or *binary nomenclature*. According to binomial nomenclature, the first name of the binomial is the *genus name* (generic name). The second name of the binomial is the *specific epithet* or *species epithet* (*specific name* - a term used only in zoology). The first word denoting the genus starts with a capital letter while the specific epithet starts with a small letter. Binomial names are always either italicized or underlined to indicate their Latin origin. For example, *Mangifera indica* is the scientific name of mango. In this name *Mangifera* represents the genus while *indica,* is a specific epithet. A binomial name in which the name of the genus and that of the species are identical is called *tautonym*.

Nomenclature types

The scientific names must be associated with some physical entity, known as a *nomenclatural type* or simply *type*. A nomenclatural type is one particular specimen of an organism to which the scientific name of that organism is formally attached. Types are usually physical specimens that are kept in a museum or herbarium research collection, but it may also be an illustration. The type serves the purpose of acting as a reference for the name, upon which the name is based. There are different types of nomenclatural type:

Holotype : A holotype is the one specimen or illustration upon which a name is based.

Isotype : An isotype is a duplicate specimen of the holotype, collected at the same time by the same person from the same population.

Lectotype : A lectotype is a specimen that is selected from the original material to serve as the type when no holotype was designated at the time of publication, if the holotype is missing, or if the original type consisted of more than one specimen or taxon.

Neotype : A specimen later selected to serve as the single type specimen when an original holotype has been lost or destroyed, or where the original author never cited a specimen.

6.1.2 Classification

Classification is the arrangement of taxa into some type of order. The purpose of classification is to provide a system for cataloguing and expressing relationships between taxa. Classification is not a single step process but involves hierarchy of steps in which each step represents a category termed **rank**. Rarely, the term *taxonomic category* is used instead of *rank*. Every organism can be classified at seven taxonomic ranks- *kingdom*, *phylum, class, order, family, genus* and *species*. Each rank contains organisms with similar characteristics. The kingdom is the largest unit of classification. It splits into smaller units called *phyla* (singular, *phylum*). Phyla splits into *classes,* classes into *orders,* orders into *families*, families into *genera* (singular, *genus*) and genera into *species.*

Example:

Seven taxonomic ranks	*Taxa*
Kingdom	Plantae
Phylum (Division)	Magnoliophyta
Class	Liliopsida
Order	Arecales
Family	Arecaceae
Genus	*Cocos*
Species	*Cocos nucifera*

The various units of classification – kingdom, phylum, class and so on — are called *taxonomic categories* and together they make up the *taxonomic hierarchy.* A **taxon** is a defined group of organisms typically treated at a given rank. In the above example, Magnoliophyta is a taxon placed at the rank of phylum; Liliopsida is a taxon placed at the rank of class. As one progresses down the hierarchy, the number of organisms in each taxon decreases, and the similarities between them increases.

6.1.3 Biological species concept

Biologists almost universally agree that the species is a fundamental natural unit. However, biologists have not been able to agree on exactly how species should be defined. Taxonomists practically define species by means of morphological or phenetic characters. If one group of organisms consistently differs from other organisms, it will be defined as a separate species.

However, the most widely accepted species concept is the *biological species concept*. It defines species in terms of interbreeding. According to Ernst Mayr, *species are groups of interbreeding natural populations that are reproductively isolated from other such groups.* Reproductively isolated means that members of the species do not interbreed with

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Three domain system

The five-kingdom scheme divides living things into two fundamentally different groups – prokaryotes and eukaryotes. New data suggest that two kinds of prokaryotes diverged early in the history of life, and thus there are actually three major categories of organisms. A new classification system assigns more significance to the ancient split by creating a level of classification larger than a kingdom – a new category called a *domain*. The two domains of prokaryotes are Bacteria and Archaea. The domain Eukarya encompasses all of the kingdoms of eukaryotes. The *three-domain system* of biological classification was introduced by Carl Woese on the basis of differences in the signature sequences of 16S/18S rRNA genes of eubacteria, archaebacteria and eukaryotes.

History of taxonomic concepts

Linnaeus, 1735	2 Kingdoms	Animalia and Vegetabilia
Haeckel, 1866	3 Kingdoms	Protista, Plantae and Animalia
Chatton, 1937	2 Empires	Prokaryota and Eukaryota
Copeland, 1956	4 Kingdoms	Monera (prokaryotes), Protista, Plantae and Animalia
Whittaker, 1969	5 Kingdoms	Monera, Fungi, Protista, Plantae and Animalia
Woese *et al*, 1977	6 Kingdoms	Eubacteria, Archaea, Protista, Fungi, Plantae and Animalia
Woese and Fox, 1999	3 Domain systems	Eubacteria, Archaea and Eukaryotes

6.3 Protists

The German zoologist Ernst Haeckel first used the term *protista*. Protists are mostly unicellular (some are colonial and multicellular) eukaryotic organisms. Most eukaryotes are single-celled organisms. Protists are difficult to characterize because of the great diversity of the kingdom. These organisms vary in body form, nutrition and reproduction. Protists are capable of sexual, as well as asexual reproduction. They can be free-living, or may live symbiotically with another organism. The symbiosis can be mutualistic, where both partners benefit, or parasitic, where the protist uses its host as a source of food or shelter while providing no advantage to the other organism. Protists are more nutritionally diverse than other eukaryote groups. Some protists are photoautotrophs. Some are heterotrophs, absorbing organic molecules or ingesting larger food particles. Still others, called mixotrophs, combine photosynthesis and heterotrophic nutrition.

Despite the great diversity evident in this kingdom, scientists have been able to classify the protists into several groups. The protists can be classified into one of three main categories, animal-like, plant-like and fungus-like. Grouping into one of the three categories is based on an organism's mode of reproduction, method of nutrition and motility. The animal-like protists are known as the *protozoa*, the plant-like photosynthetic protists are the *algae*, and the fungus-like protists are the *slime molds* and *water molds*.

6.3.1 Protozoan protists

The protozoa are all unicellular heterotrophs, mostly free living. They obtain their nutrition by ingesting other organisms or dead organic materials. The word 'protozoa' comes from the *Latin* word for 'first animals'.

Features of protozoa:

- Unicellular (single celled) organisms.
- Aquatic, free-living or parasitic organisms.
- Nutrition is heterotrophic.
- Locomotion with the help of pseudopodia, cilia or flagella. The protozoans are grouped into various phyla based on their modes of locomotion. They may use cilia, flagella or pseudopodia. Some protozoans are non motile. The protozoans that use pseudopodia to move are known as *amoeboid protozoans*, those that use flagella are called *flagellated protozoans*, those that use cilia are known as the *ciliated protozoans* and those that do not move are called the *sporozoans*.

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6.5 Plantae

The kingdom *plantae* are multicellular eukaryotes with photosynthetic nutrition. Cells typically have cellulose wall, vacuole, plastids and several photosynthetic pigments which always include chlorophyll *a*. It can be broadly divided into:

Cryptogamae (plant without seeds): Algae (Thallophyta), Bryophytes and Pteridophytes.

*Phanerogamae (*plant with seeds): Gymnosperm and Angiosperm.

Bryophyta, pteridophyta, gymnosperm and angiosperm are termed as the *land plants* or embryophytes. One major innovation of land plants was the evolution of the *embryo.* The embryo is defined as an *immature sporophyte*. Land plants can be vascular (or tracheophyta) and non-vascular. During the early evolution of land plants, nonvascular land plants diverged *before* the vascular plants. Non-vascular plants are often informally called *bryophytes* (from the Greek *bryon,* moss, and *phyton,* plant). Bryophytes include the liverworts, hornworts and mosses. The terms *bryophyta* and *bryophyte* are not synonymous. Bryophyta is the formal taxonomic name for the phylum that consists solely of mosses.

6.5.1 Plant life cycle

The sexual life cycle in plants alternate between diploid (sporophyte) and haploid (gametophyte) phases. Sporophyte literally means *spore-plant*, and gametophyte means *gamete-plant*. The haploid gametophytic body produces gametes by mitosis whereas the diploid sporophytic phase produces meiospores (sexual spores) by the process of meiosis. The diploid phase produces the haploid phase by meiosis; the haploid gametes then fuse to make a zygote that starts another diploid phase. Three distinct versions of a generalized sexual life cycle occur among plants:

Haplontic life cycle

In *haplontic* life cycle, diploid sporophytic phase is represented only by the one-celled zygote. There are no free-living sporophytes. The dominant, photosynthetic phase is the free-living gametophyte. The haploid gametophyte produce gametes by mitosis. Fusion of gametes (termed *fertilization* or *syngamy*) forms diploid zygote. Meiosis occurs in the zygote (*zygotic meiosis*) which results in the formation of haploid meiospores. These spores in turn, germinate and divide by mitosis to form a haploid gametophyte body once again. Most green algae such as *Volvox, Spirogyra* and some species of *Chlamydomonas* represent this pattern of life cycle.

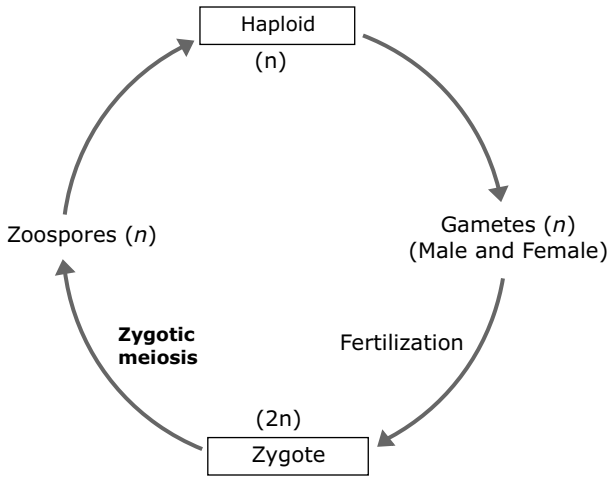

Figure 6.6 In *haplontic* life cycle, the zygote divides by meiosis (*zygotic meiosis*) to form haploid cells. Each of these cells divides by mitosis to produce a multicellular haploid individual that eventually gives rise to gametes by mitosis.

Diplontic life cycle

In *diplontic* life cycle, the zygote divides mitotically to produce a multicellular diploid individual. The diploid phase is the dominant, photosynthetic and independent phase. Certain cells of a multicellular diploid phase undergo meiosis to make gametes, not meiospores. Because meiosis produces gametes directly, it is called *gametic meiosis*. Fusion of gametes restores the diploid phase. This pattern of life cycle is not common in plants. Some brown algae such as *Sargassum, Fucus* follow this pattern of life cycle.

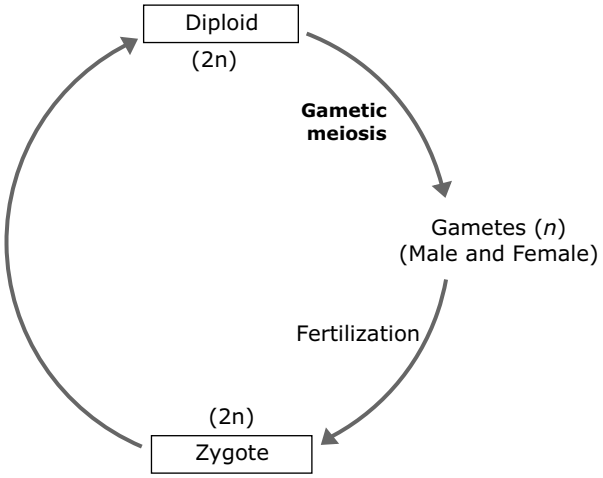

Figure 6.7 In *diplontic* life cycle, the haploid gametes are formed by meiosis (*gametic meiosis*) in a multicellular diploid individual and fuse to form a diploid zygote that divides to produce another diploid individual. This type of life cycle is characteristic of most animals and some protists, as well as some green and brown algae.

Haplo-diplontic life cycle

In the *haplo-diplontic* (also called diplobiontic) life cycle, diploid sporophyte produces haploid spores (meiospores) by the process of meiosis. Because meiosis produces spores directly, it is called *sporic meiosis*. The meiospores germinate and grow by mitosis into the multicellular haploid gametophytic phase of the cycle. The gametophytic bodies produce gametes by mitosis. Fusion of gametes gives diploid zygote.

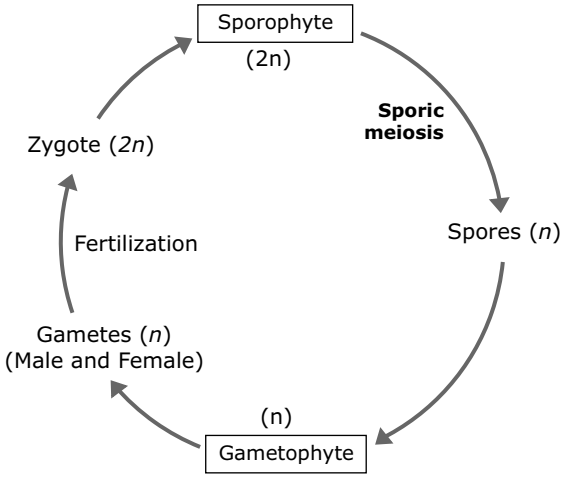

Figure 6.8 In *haplo-diplontic* life cycle, the diploid sporophyte individual, produces haploid spores as a result of meiosis (*sporic* meiosis). These meiospores do not function as gametes but germinate and grow by mitosis into the multicellular haploid gametophytic phase of the cycle. The gametophytic bodies eventually produce gametes that fuse to form diploid zygotes. These zygotes, in turn, differentiate into diploid individuals.

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The diploid zygote is the first stage in the sporophyte generation. The zygote divides by mitosis and develops into a multicellular *embryo*, the young sporophyte plant. Embryo development takes place within the archegonium. Eventually, the embryo grows into a mature sporophyte plant. The sporophyte bears spore-producing structure called the **sporangium**. Within the sporangium, *sporogenous cells* (or spores producing cells) divide by meiosis to form haploid meiospores. The sporophyte may produce one kind of spores or two morphologically different kinds of spores (termed *microspores* and *megaspores*). Plants that produce two different kinds of spores are called **heterosporous**; those that produce only one kind of spore are **homosporous**. If a plant forms both megaspores and microspores, each of these will be formed in different kinds of sporangium, *megasporangium* and *microsporangium,* respectively.

Among land plants, gametophyte and sporophyte differ in their dominance. In bryophytes, gametophyte is dominant, independent and photosynthetic. Whereas the sporophyte is short-lived multicelluler and totally or partially dependent on the gametophyte for nutrition. In pteridophytes, sporophyte is represented by a dominant, independent, photosynthetic, vascular plant body. It alternates with multicellular, independent but short-lived gametophyte.

6.5.4 Bryophytes

General features

1. Bryophytes are *amphibians* i.e. although majority of them are terrestrial, they require lots of moisture and humidity in their surrounding. Most bryophytes are small, compact, green plants. Like green algae, they produce chlorophylls *a* and *b*, starch, cellulose cell walls, and motile sperm. Bryophytes usually grow very slowly.
2. Plant organisation in bryophytes is of two types – Thalloid and Leafy. In leafy form the leaf and stem are simple, without vascular tissue and belong to the gametophytic generation. Thallus grows prostrate on the ground. Thallus as well as leafy form get attached to the substratum by delicate unbranched, unicellular hair like organs called rhizoids. Bryophytes lack well-developed vascular tissues and lignified tissues. As a result, they grow low to the ground and absorb water by capillarity. However, not all bryophytes are strictly nonvascular. For example, some mosses have a central strand of conducting cells that are functionally equivalent to xylem and phloem.
3. Organs such as leaves and roots of vascular plants are defined by the arrangement of their vascular tissues. Since bryophytes lack true vascular tissues, they also lack true leaves and roots. However, many bryophytes have structures that are similar and functionally equivalent to leaves, so they are often referred to as such.
4. Bryophytes get their nutrients from dust, rainwater, and substance dissolved in water at the soil's surface. Tiny rhizoids (hairlike extensions of epidermal cells) along their lower surface anchor the plants but do not specialized for absorption of water or minerals. Water and dissolved minerals move by capillarity over the surface of bryophytes.
5. The gametophytes dominate the life cycle.
6. The sporophyte is short-lived and unbranched, and produces a terminal sporangium. Although photosynthetic, the sporophyte is permanently attached to and partially dependent on the gametophyte. The sporophytes of bryophytes do not have a direct connection to the ground. Spores have a cutinized coat and are usually dispersed by wind.
7. The sex organs are jacketed and multicellular. Female sex organ in the form of archegonia appears for the first time in the bryophyta in the plant world. It is a flask shaped organ. The basal swollen part of the flask-shaped body is known as venter and the narrow canal like part as the neck. There is a sterile jacket surrounding the axial canal. Antheridium is the male sex organ. It is a multicellular structure which is ellipsoidal or club-shaped in outline. The sperms are biflagellate. Both the flagella are whiplash type.
8. Fertilization takes place in the presence of water. The fertilized egg is retained within the venter of the archegonium. The zygote undergoes repeated division to form an undifferentiated structure called the embryo.

A generalized life cycle of bryophytes

During sexual life cycle, sporophyte produces spores in sporangium. Haploid spores formed by meiosis begins the gametophytic generation. The spore germinates to form a gametophyte, which is more diverse in bryophytes than in any other group of plants. The gametophyte bears antheridia and archegonia. Antheridia produce sperms, and archegonia produce eggs. Sexual reproduction in bryophytes requires free water, because the sperm must swim to the egg. Sperms released from an antheridium do not swim randomly; rather, they are attracted by a gradient of a still-unidentified chemotactic substance(s) produced by an archegonium. A sperm fertilizes an egg and forms a diploid zygote, thus beginning the sporophytic generation of the life cycle. Bryophytes have *heteromorphic* alternation of generation; that is, the sporophyte and gametophyte are distinctly different.

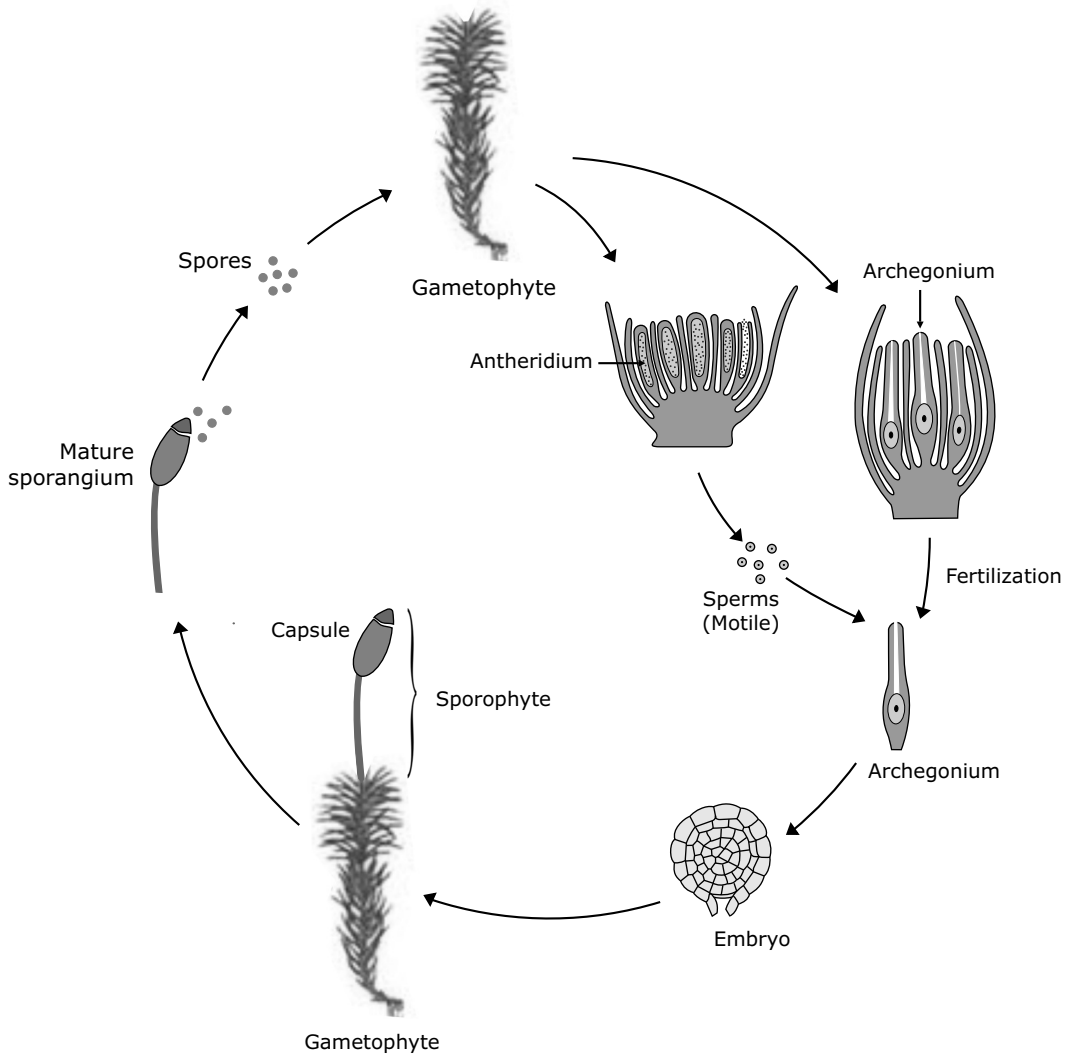

Figure 6.10 Sexual life cycle in *Funeria*. The spore germinates and produces a protonema (not shown in the figure) and the leafy stage of the gametophyte develops from the protonemal stage. The male and female sex organs, the antheridia and the archegonia respectively, are produced on the gametophytic plant. Haploid sperm are released from the antheridia and fertilize with a haploid egg present in an archegonium. The zygote develops into the diploid sporophyte. Typically sporophyte is differentiated into a foot, seta and spore forming capsule.

6.5.5 Pteridophytes

General features

1. The sporophyte of seedless vascular plants, which dominates the life cycle, is long-lived and often highly branched.
2. Sporophytes and gametophytes of seedless vascular plants are nutritionally independent of each other. Sporophytes are photosynthetic, and gametophytes are either photosynthetic or saprophytic (i.e., they obtain nutrition from dead and decaying organic matter).
3. Seedless vascular plants have a well-developed cuticle to minimize water loss. They also have stomata to allow gas exchange for photosynthesis, although bryophytes such as hornworts and mosses have stomata, but they are not well developed and do not function efficiently to prevent water loss. Most bryophytes lack a cuticle.
4. Seedless vascular plants produce chlorophylls *a* and *b*, carotenoids, starch, cellulose cell walls, and motile sperm. These features are also shared with bryophytes and many algae.
5. The life cycle of seedless vascular plants (pteridophytes) is similar to that of bryophytes and algae that exhibit sporic meiosis. The diploid sporophyte produces haploid spores by meiosis. Each spore germinates and grows into a gametophyte that produces gametes by mitosis. The gametes (egg and sperm) fuse to form diploid zygotes.
6. Eggs are produced in archegonia, and sperms are produced in antheridia.
7. Flagellated sperm swim through water to eggs. Like bryophytes, seedless vascular plants require free water for sexual reproduction.
8. The zygote germinates to produce a multicellular embryo that depends on the gametophyte for its nutrition. To complete the life cycle, the embryo grows into a mature sporophyte. A multicellular embryo also characterizes the bryophyte life cycle, but *it is absent in algae*.
9. Pteridophytes may be – *homosporous* i.e. all spores are the same, producing bisexual gametophytes or *heterosporous* - similar to flowering plants in that two types of spores are produced, which produce two types of gametophytes: *megaspores* (develop to form the egg-producing gametophyte or *megagametophyte*) and *microspores* (develop to form the sperm-producing gametophyte or *micro-gametophyte*). The nature of gametophyte development may be **endosporic** (within the spore wall as in flowering plants) or **exosporic** (spore germination with plant development outside the spore).
10. Each sporangium is protected by a multicellular jacket of non-reproductive cells. Spores are dispersed from sporangia by wind and are cutinized to resist desiccation.

A generalized life cycle of pteridophytes

A *fern* is a representative of the pteridophytes. Fern gametophytes are green, photosynthetic plants that live in relatively moist places and resemble the gametophytes of certain liverworts. They are flat, thin and heart shaped. Rhizoids project from their lower surface. Archegonia form on the lower surface of the gametophytes near the *apical notch*. The antheridia are formed on the lower surface. When the sperm are mature, they are released from the antheridia. They require the *presence of water* to make their way to the mouth of the archegonium. The sperm probably are attracted by chemicals released from the archegonium. Inside the archegonium, a sperm fuses with the single egg, producing a zygote.

The zygote begins to divide within the archegonium of the fern. Soon the growing sporophyte becomes much larger than the gametophyte and also nutritionally independent from it. Most ferns have more or less horizontal stems that creep along below the ground; subterranean stems of this kind are called **rhizomes**.

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