BASICS TO LEARN BIOCHEMISTRY

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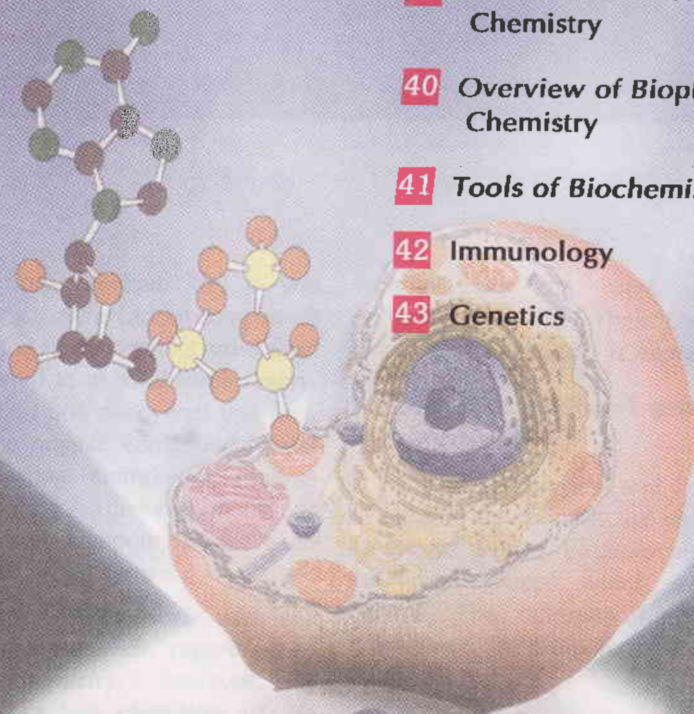


Section VII

39 Introduction to Bioorganic Chemistry

Carbon, the official spokesperson of organic chemistry, speaks :

"I am the unique and versatile element;
Capable of forming covalent C—C chains;
To produce unlimited number of compounds;
Thus, I am the mother of organic molecules."

As life comes from previous life, it was believed for a long that the carbon compounds of organisms (hence the name organic) arose from life only. This is referred to as ***vital force theory***. Friedrich Wohler (1825) first discovered that urea ($NH_2—CO—NH_2$), the organic compound, could be prepared by heating ammonium cyanate (NH_4NCO), in the laboratory. Thereafter, thousands and thousands of organic compounds have been synthesized outside the living system.

Organic chemistry broadly deals with the chemistry of carbon compounds, regardless of their origin. Biochemistry, however, is concerned with the ***carbon chemistry of life only***. The general principles of organic chemistry provide strong foundations for understanding biochemistry. However, biochemistry exclusively deals with the reactions that occur in the living system in aqueous medium.

Most common organic compounds found in living system

The organic compounds, namely carbohydrates, lipids, proteins, nucleic acids and vitamins are the most common organic compounds of life. Their chemistry has been discussed in Section I (***Chapters 1-7***).

Common functional groups in biochemistry

Most of the physical and chemical properties of organic compounds are determined by their functional groups. Biomolecules possess certain functional groups which are their reactive centres. The common functional groups of importance in biomolecules are presented in ***Table 39.1***.

Common ring structures in biochemistry

There are many homocyclic and heterocyclic rings, commonly encountered in biomolecules. A selected list of them is given in ***Fig.39.1***.

Homocyclic rings : Phenyl ring derived from benzene is found in several biomolecules (phenylalanine, tyrosine, catecholamines). Phenanthrene and cyclopentane form the backbone of steroids (cholesterol, aldosterone).

Table 39.1 Common functional groups of importance in biomolecules

Functional group Name	*Group*	*General structural formula*	*Type of compound*	*Examples of biomolecule(s)*
Hydroxyl	$-OH$	$R-OH$	Alcohol	Glycerol, ethanol
Aldehyde	$-\overset{\overset{O}{\Vert}}{C}-H$	$R-\overset{\overset{O}{\Vert}}{C}-H$	Aldehyde	Glyceraldehyde, glucose
Keto	$-\overset{\overset{O}{\Vert}}{C}-$	$R_1-\overset{\overset{O}{\Vert}}{C}-R_2$	Ketone	Fructose, sedoheptulose
Carboxyl	$-\overset{\overset{O}{\Vert}}{C}-OH$	$R-\overset{\overset{O}{\Vert}}{C}-OH$	Carboxylic acid	Acetic acid, palmitic acid
Amino	$-NH_2$	$R-NH_2$	Amino acid	Alanine, serine
Imino	$-\overset{\overset{H}{\vert}}{N}-$	$R-\overset{\overset{H}{\vert}}{N}-$	Imino acid	Proline, hydroxyproline
Sulfhydryl	$-SH$	$R-SH$	Thiol	Cysteine, coenzyme A
Ether	$-O-$	R_1-O-R_2	Ether	Thromboxane A_2
Ester	$-\overset{\overset{O}{\Vert}}{C}-O-R_1$	$R_2-\overset{\overset{O}{\Vert}}{C}-O-R_1$	Ester	Cholesterol ester
Amido	$-\overset{\overset{O}{\Vert}}{C}-N{<}^{R_1}_{R_2}$	$R_3-\overset{\overset{O}{\Vert}}{C}-N{<}^{R_1}_{R_2}$	Amide	N-Acetylglucosamine

Coenzyme Q is an example of benzoquinone while vitamin K is a naphthoquinone.

Heterocyclic rings : Furan is the ring structure found in pentoses. Pyrrole is the basic unit of porphyrins found in many biomolecules (heme) while pyrrolidine is the ring present in the amino acid, proline. Thiophene ring is a part of the vitamin biotin. The amino acid histidine contains imidazole.

Pyran structure is found in hexoses. Pyridine nucleus is a part of the vitamins-niacin and pyridoxine. Pyrimidines (cytosine, thymine) and purines (adenine, guanine) are the constituents of nucleotides and nucleic acids. Indole ring is found in the amino acid tryptophan. Purine and indole are examples of fused heterocyclic rings.

ISOMERISM

The compounds possessing identical molecular formulae but different structures are referred to as ***isomers.*** The phenomenon of existence of isomers is called isomerism (*Greek* : isos—equal; meros—parts). Isomers differ from each other in physical and chemical properties. ***Isomerism is partly responsible for the existence of a large number of organic molecules.***

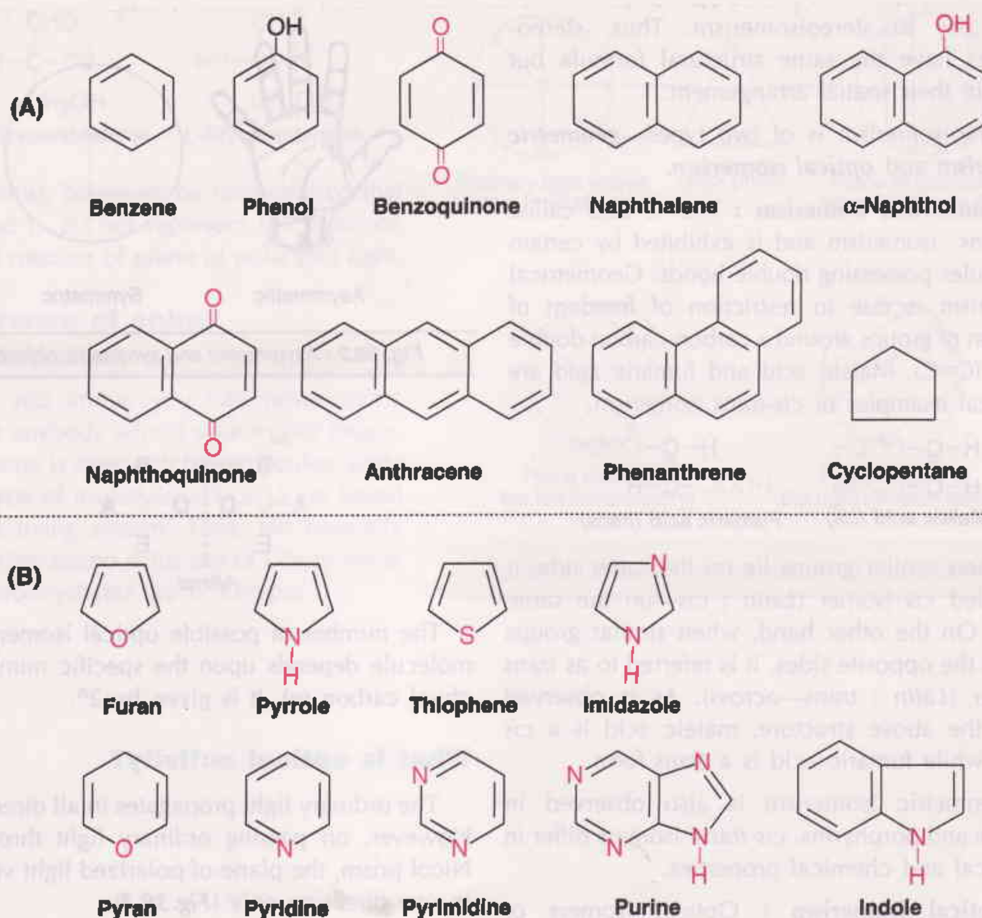
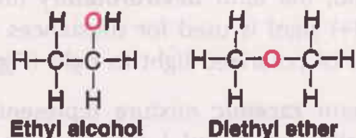


Fig. 39.1 : Common ring structures found in biomolecules (A) Homocyclic rings (B) Heterocyclic rings.

Consider the molecular formula—C_2H_6O. There are two important isomers of this—ethyl alcohol (C_2H_5OH) and diethyl ether (CH_3OCH_3) depicted next.



Isomerism is broadly divided into two categories-structural isomerism and stereoisomerism.

Structural isomerism

The difference in the arrangement of the atoms in the molecule (i.e. molecular framework) is responsible for structural isomerism. This may be due to variation in carbon chains *(chain isomerism)* or difference in the position of functional groups *(position isomerism)* or difference in both molecular chains and functional groups *(functional isomerism).*

Structural isomerism, as such, is more common in general organic molecules. ***Tautomerism,*** a type of structural isomerism, occurs due to the migration of an atom or group from one position to the other e.g. purines and pyrimidines (Chapter 5).

Stereoisomerism

Stereoisomerism (*Greek* : stereos—space occupying) is, perhaps, more relevant and important to biomolecules. The differential space arrangement of atoms or groups in molecules

gives rise to stereoisomerism. Thus, stereoisomers have the same structural formula but differ in their spatial arrangement.

Stereoisomerism is of two types—***geometric isomerism*** and ***optical isomerism.***

Geometrical isomerism : This is also called *cis-trans* isomerism and is exhibited by certain molecules possessing double bonds. Geometrical isomerism is due to restriction of freedom of rotation of groups around a carbon-carbon double bond (C=C). Maleic acid and fumaric acid are classical examples of *cis-trans* isomerism.

When similar groups lie on the same side, it is called *cis* isomer (*Latin* : cis—on the same side). On the other hand, when similar groups lie on the opposite sides, it is referred to as *trans* isomer (*Latin* : trans—across). As is observed from the above structure, maleic acid is a *cis* form while fumaric acid is a *trans* form.

Geometric isomerism is also observed in sterols and porphyrins. *cis-trans* isomers differ in physical and chemical properties.

Optical isomerism : Optical isomers or enantiomers occur due to the presence of an ***asymmetric carbon*** (a chiral carbon). Optical isomers differ from each other in their optical activity to rotate the plane of polarized light.

What is an asymmetric carbon?

An object is said to be symmetrical if it can be divided into equal halves e.g. a ball. Objects which cannot be divided into equal halves are asymmetric, e.g. hand (***Fig.39.2***). An asymmetric object cannot coincide with its mirror image. For instance, left hand is the mirror image of right hand and these two can never be superimposed. In contrast, a symmetrical object like a ball superimposes its image.

A carbon is said to be ***chiral*** (*Greek* : hand) or asymmetric when it is attached to four different groups. Their mirror images do not superimpose with each other.

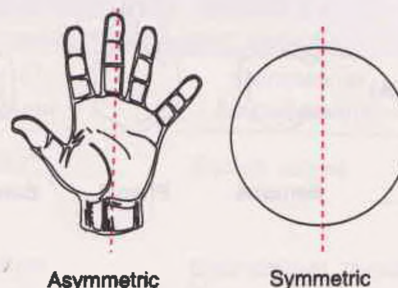
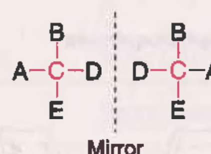


Fig. 39.2 : Asymmetric and symmetric objects.



The number of possible optical isomers of a molecule depends upon the specific number of chiral carbon (n). It is given by 2^n.

What is optical activity?

The ordinary light propagates in all directions. However, on passing ordinary light through a Nicol prism, the plane of polarized light vibrates in one direction only (***Fig.39.3***).

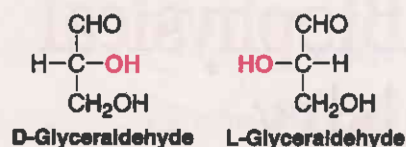
Certain organic compounds (optical isomers) which are said to exhibit optical activity rotate the plane of polarized light either to the left or to the right.

The term ***levorotatory*** (indicated by l or (–) sign) is used for the substances which rotate the plane of polarized light to the left. On the other hand, the term ***dextrorotatory*** (indicated by d or (+) sign) is used for substances rotating the plane of polarized light to right (***Fig.39.3***).

The term ***racemic*** mixture represents equal concentration of d and l forms which cannot rotate the plane of polarized light.

Configuration of chiral molecules

While representing the configuration of chiral molecules, the configuration of ***glyceraldehyde*** is taken as a ***reference standard***.



It must, however, be remembered that D- and L- do not represent the direction of the rotation of plane of polarized light.

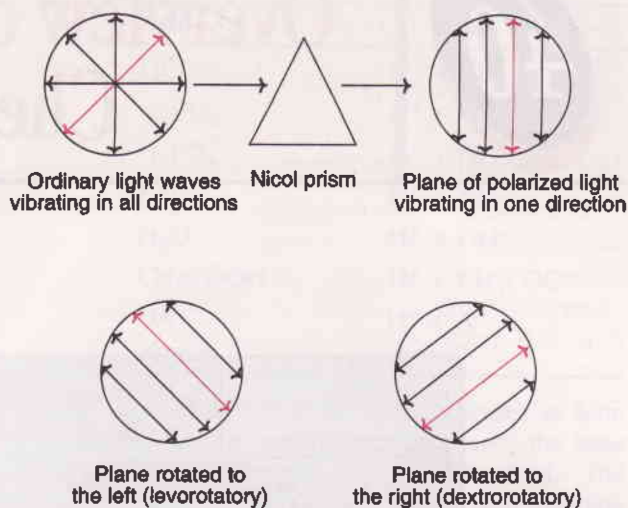


Fig. 39.3 : Diagrammatic illustration of optical activity.

Existence of chiral biomolecules

As you know, you can never come across anybody who is your mirror image. The same is true with biomolecules. Only one type of molecules (D or L) are found in the living system. Thus, the naturally occurring amino acids are of L-type while the carbohydrates are of D-type.

40 Overview of Biophysical Chemistry

The radioactive isotopes speak :

"We are elements of same atomic numbers;
Undergo decay to emit α or β or γ rays;
Exhibit identical chemical properties;
Exploited as tracers in biomedical research."

Isotope	Radiation	Half-life
^{3}H	β	12.2 years
^{14}C	β	5,700 years
^{22}Na	γ	2.5 years

The general laws and principles of chemistry and physics are applicable to biochemistry as well. It is, therefore, worthwhile to have a brief understanding of some of the basic chemical and physical principles that have direct relevance to life.

It must, however, be remembered that this chapter deals with quite unrelated topics to each other.

WATER

Water is the most abundant fluid on earth. It is justifiably regarded as the ***solvent of life***. As much as 70% of a typical cell is composed of water. The unique physical and chemical properties of water have profound biological importance. The structures of biomolecules (proteins, nucleic acids, lipids and carbohydrates) are maintained due to their interaction with water, which forms an aqueous environment. This is essential for sustaining life.

Structure of water

The H_2O molecule exists in a bent geometry. The bond angle of H—O—H is 104.5° and the O—H bond has a distance of 0.958A°. There exists electrical polarity in H_2O due to electronegativity (the power of an atom in a molecule to attract electrons) difference between H and O. This results in the polarization of a positive charge on H and a negative charge on O. Thus H_2O molecule, although carrying no net charge, possesses an electrical dipole. The polar character of water has tremendous biological significance.

Hydrogen bonds between H_2O molecules : The presence of electrical dipoles on H_2O molecules is responsible for their attraction. Hydrogen bonds are formed due to polarity between two atoms with different electronegativities. Thus, in H_2O, the transient negative charge on the O atom of one H_2O molecule and the transient positive charge on the H atom of another H_2O molecule attract each other to form a hydrogen bond. The water

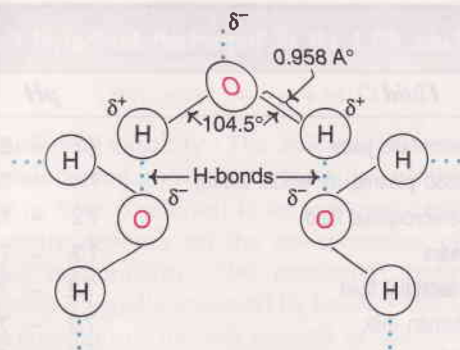


Fig. 40.1 : Diagrammatic representation of water structure along with hydrogen bonds.

molecules are interlinked with each other by profuse hydrogen bonding. The energy of each hydrogen bond is very small compared to that of a covalent bond. But the collective strength of H-bonds is due to their large numbers. Hydrogen bonds are important for the three-dimensional structures of biomolecules.

Water expands on freezing : Water is one of the very few substances that expands on freezing. Thus, ice has a density of 0.92 g/ml, while water at 0°C has density of 1.0 g/ml. For this reason, ice floats on water. And this property is essential to maintain water equilibrium in the environment, and to sustain life.

(Imagine that water contracted on cooling and becomes denser. In such a case, ice would sink to the bottom of seas and lakes and would never get exposed to sun rays. Thus, frozen water would permanently remain as ice. If this were to happen, earth would have a permanent ice age!)

ACIDS AND BASES

According to Lowry and Bronsted, an acid is defined as a substance that gives off protons while base is a substance that accepts protons. Thus, ***an acid is a proton (H^+) donor and a base is a proton acceptor.*** A few examples of acids and their corresponding bases are given in the next column.

Acid	Base
HCl	$H^+ + Cl^-$
H_2CO_3	$H^+ + HCO_3^-$
H_2PO_4	$H^+ + HPO_4^-$
NH_4^+	$H^+ + NH_3$
H_2O	$H^+ + OH^-$
CH_3COOH	$H^+ + CH_3COO^-$
HA (general)	$H^+ + A^-$ (general)

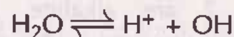
It is evident that an acid dissociates to form proton and base. On the other hand, the base combines with proton to form acid. The difference between an acid and its corresponding base (more commonly referred to as ***conjugate base***) is the presence or absence of a proton. In general, a strong acid has a weak base while a weak acid has a strong base. For instance, strong acid HCl has weak base Cl^-, weak acid HCN has a strong base CN^-.

Alkalies : The metallic hydroxides such as NaOH and KOH are commonly referred to as alkalies. These compounds do not directly satisfy the criteria of bases. However, they dissociate to form metallic ion and OH^- ion. The latter, being a base, accepts H^+ ions.

Ampholytes : The substances which can function both as acids and bases are referred to as ampholytes. Water is the best example for ampholytes.

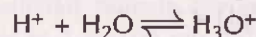
Dissociation of water

Water is a weak electrolyte and dissociates as follows.



$$H_2O \rightleftharpoons H^+ + OH$$

The proton reacts with another molecule of water to form hydronium ion (H_3O^+).



$$H^+ + H_2O \rightleftharpoons H_3O^+$$

For the sake of convenience, the presence of proton as H_3O^+ is ignored.

By applying the law of mass action for the dissociation of water.

$$K = \frac{[H^+][OH^-]}{[H_2O]}$$

Here K is a constant; the concentrations are expressed in molarity. Since the degree of dissociation is very small, the concentration of undissociated $[H_2O]$ may be taken as constant.

$$K_{\omega} = [H^+]\ [OH^-]$$

K_{ω} is the dissociation constant for water. Its value is 10^{-14} at 25°C.

$$[H^+]\ [OH^-] = 10^{-14}$$

In a neutral solution

$$[H^+] = [OH^-] = 10^{-7}$$

Hydrogen ion concentration (pH)

The acidic or basic nature of a solution is measured by H^+ ion concentration. The strength of H^+ ions in the biological fluids is exceedingly low. For this reason, the conventional units such as moles/l or g/l are not commonly used to express H^+ ion concentration.

Sorenson (1909) introduced the term pH to express H^+ ion concentration. ***pH is defined as the negative logarithm of H^+ ion concentration.***

$$pH = -\log\ [H^+]$$

The pH is a narrow scale, ranging from 0 to 14 which corresponds to 1 M solution to 10^{-14} M solution of $[H^+]$ concentration.

As explained under dissociation of water, pure water has an equal concentration of H^+ and OH^- ions i.e. 10^{-7} M each. Thus, pure water has a pH 7 which is neutral. Solutions with ***pH less than 7*** are said to be ***acidic*** while those with pH greater than 7 are alkaline. It must be remembered that the term acidic or alkaline are not absolute but only relative. Thus, a solution with pH 3.0 is more acidic when compared with a solution of pH 4.5.

A rise in H^+ concentration decreases pH while a fall in H^+ concentration increases pH. The reverse is true for OH^- concentration. The pH of a solution containing 1N $[H^+]$ is 0 while that containing 1N $[OH^-]$ is 14.

The pH of important biological fluids is presented in ***Table 40.1***.

Table 40.1 pH of important biological fluids

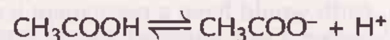
Fluid	*pH*
Pancreatic juice	7.5 - 8.0
Blood plasma (or whole blood)	7.35 - 7.45
Cerebrospinal fluid	7.2 - 7.4
Tears	7.2 - 7.4
Interstitial fluid	7.2 - 7.4
Human milk	7.2 - 7.4
Saliva	6.4 - 7.0
Intracellular fluid (cytosol)	6.5 - 6.9
Gastric juice	1.5 - 3.0
Urine	5.0 - 7.5

BUFFERS

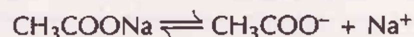
The pH of a given solution can be easily altered by the addition of acids or bases. ***Buffers are defined as the solutions which resist change in pH by the addition of small amounts of acids or bases.*** A buffer usually consists of a weak acid and its salt (e.g. acetic acid and sodium acetate) or a weak base and its salt (e.g. ammonium hydroxide and ammonium chloride). Several buffers can be prepared in the laboratory. Nature has provided many buffers in the living system.

Mechanism of buffer action

Let us consider the buffer pair of acetic acid and sodium acetate. Acetic acid, being a weak acid, feebly ionizes. On the other hand, sodium acetate ionizes to a large extent.

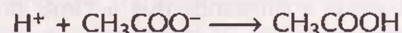


$$CH_3COOH \rightleftharpoons CH_3COO^- + H^+$$



$$CH_3COONa \rightleftharpoons CH_3COO^- + Na^+$$

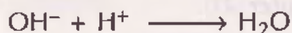
When an acid (say HCl) is added, the acetate ions of the buffer bind with H^+ ions (of HCl) to form acetic acid which is weakly ionizing. Therefore, the pH change due to acid is resisted by the buffer.



$$H^+ + CH_3COO^- \longrightarrow CH_3COOH$$

When a base (say NaOH) is added the H^+ ions of the buffer (acetic acid) combine with OH^- ions to form water, which is weakly

dissociated. Thus, the pH change due to base addition is also prevented by the buffer.



$$OH^- + H^+ \longrightarrow H_2O$$

Buffering capacity : *The **efficiency of** a buffer in **maintaining a constant pH** on the addition of acid or base is referred to as buffering capacity. It mostly depends on the concentration of the buffer components. The maximum buffering capacity is usually achieved by keeping the same concentration of the salt as well as the acid.*

For a comprehensive discussion on blood buffers, refer ***Chapter 21***.

SOLUTIONS

Solutions may be regarded as mixtures of substances. In general, a solution is composed of two parts—***solute*** and ***solvent.*** The substance that is dissolved is solute and the medium that dissolves the solute is referred to as solvent. The particle size of a solute in solution is < 1 nm.

The relative concentrations of substances in a solution can be measured by several ways.

Per cent concentration : This represents parts per 100. The most frequently used is weight per volume (w/v) e.g. 9% saline (9 g/100 ml solution). For expressing smaller concentration, mg (10^{-3}g), μg (10^{-6} g), ng (10^{-9} g) and pg (10^{-12} g) are used.

Parts per million (ppm) : This refers to the number of parts of a substance in one million parts of the solution. Thus 10 ppm chlorine means 10 μg of chlorine in 1 g of water.

Molarity (M) : It is defined as the ***number of moles of solute per liter solution.*** NaCl has a molecular weight of 58.5. To get one molar (1 M) or one mole solution of NaCl, one gram molecular weight (58.5 g) of it should be dissolved in the solvent (H_2O) to make to a final total volume of 1 liter. For smaller concentrations, millimole and micromole are used.

Molality : It represents the number ***of moles of solute per 1,000 g of solvent.*** One molal solution can be prepared by dissolving 1 mole of solute in 1,000 g of solvent.

Normality : Molarity is based on molecular weight while normality is ***based on equivalent weight.*** *One gram equivalent weight of an element or compound represents its capacity to combine or replace 1 mole of hydrogen. In general, the gram equivalent weight of an element or a compound is equal to its molecular weight divided by the total positive valence of the constituent ions.* Thus, for NaOH and KOH, the molecular and equivalent weights are the same, while, for H_2SO_4, equivalent weight is half of the molecular weight. The term milliequivalent per liter (mEq/l) is used for smaller concentrations.

COLLOIDAL STATE

Thomas Graham (1861), regarded as the 'father of colloidal chemistry', divided substances into two classes—***crystalloids*** and ***colloids.***

Crystalloids are the substances which in solution can freely pass (diffuse) through parchment membrane e.g. sugar, urea, NaCl. Colloids (*Greek* : glue-like), on other hand, are the substances that are retained by parchment membrane e.g. gum, gelatin, albumin. The above classification of Graham is no longer tenable, since any substance can be converted into a colloid by suitable means. For instance, sodium chloride in benzene forms a colloid.

Colloidal state : As such, there are no group of substances as colloids, rather, substances can exist in the form of ***colloidal state*** or ***colloidal system.*** Colloidal state is characterized by the particle size of 1 to 100 nm. When the particle size is <1 nm, it is in true solution. For the particle sizes >100 nm, the matter exists as a visible precipitate. Thus, the colloidal state is an intermediate between true solution and precipitate.

Phases of colloids : Colloidal state is heterogeneous with two phases.

1. **Dispersed phase** (internal phase) which constitutes the colloidal particles.

2. **Dispersion medium** (external phase) which refers to the medium in which the colloidal particles are suspended.

CLASSIFICATION OF COLLOIDS

Based on the affinity of dispersion medium with dispersed phase, colloids are classified as ***lyophobic*** and ***lyophilic*** colloids.

1. **Lyophobic** (*Greek* : solvent-hating) **:** These colloids do not have any attraction towards dispersion medium. When water is used as dispersion medium, the colloids are referred to as ***hydrophobic.***

2. **Lyophilic** (*Greek* : solvent-loving) **:** These colloids have distinct affinity towards dispersion medium. The term ***hydrophilic*** is used for the colloids when water is the dispersion medium.

The terms ***gel*** and ***sol*** are, respectively, used to jelly-like and solution-like colloids. ***Emulsions*** are the colloids formed by two immiscible liquids (e.g. oil + water). Frequently, emulsions can be stabilized by using agents known as ***emulsifiers.*** For instance, the protein casein acts as an emulsifier for milk.

Micelles are the aggregates of colloidal particles. Soap (sodium palmitate) in water is the classical example for the micelles formation.

Properties of colloids

1. **Brownian movement :** The continuous and haphazard motion of the colloidal particles is known as Brownian movement.

2. **Optical properties :** When light is passed through a colloidal solution, it gets scattered. This phenomenon is referred to as ***Tyndal effect.***

3. **Electrical properties :** The colloidal particles carry electrical charges, either positive or negative. The electrical charge may be due to ionization of the colloidal particles or adsorption of the ions from the medium, or both. The stability and precipitation of colloids is determined by the ionic charges they carry. The separation of charged colloids can be achieved by the analytical technique—electrophoresis (***Refer Chapter 41***).

4. **Osmotic pressure :** Since the colloidal particles are larger in size, their contribution to osmotic pressure is relatively less.

5. **Non-dialysable nature :** The colloidal particles, being larger in size, cannot pass through a membrane (cellophane or parchment). The membrane, however, allows dispersion medium and smaller particles to escape through the pores. This process is referred to as dialysis and is useful for the separation of colloids.

6. **Donnan membrane equilibrium :** The presence of non-diffusible colloidal particles (e.g. protein) in the biological systems influences the concentration of diffusible ions across the membrane. This is an important phenomenon, the details of which are given on page 714.

Biological importance of colloids

1. **Biological fluids as colloids :** These include blood, milk and cerebrospinal fluid.

2. **Biological compounds as colloidal particles :** The complex molecules of life, the high molecular weight proteins, complex lipids and polysaccharides exist in colloidal state.

3. **Blood coagulation :** When blood clotting occurs, the ***sol*** is converted finally into the ***gel.***

4. **Fat digestion and absorption :** The formation of emulsions, facilitated by the emulsifying agents bile salts, promotes fat digestion and absorption in the intestinal tract.

5. **Formation of urine :** The filtration of urine is based on the principle of dialysis.

DIFFUSION

The molecules in liquids or gases are in continuous motion. ***Diffusion may be regarded as the movement of solute molecules from a higher concentration to a lower concentration.*** Diffusion is more rapid in gases than in liquids.

The smaller particles diffuse faster than the larger ones. The greater the temperature, the higher is the rate of diffusion.

Diffusion occurs in true solutions as well as in colloidal solutions.

Applications of diffusion

1. Exchange of O_2 and CO_2 in lungs and in tissues occurs through diffusion.

2. Certain nutrients are absorbed by diffusion in the gastrointestinal tract e.g. pentoses, minerals, water soluble vitamins.

3. Passage of the waste products namely ammonia, in the renal tubules occurs due to diffusion.

OSMOSIS

Osmosis (*Greek* : push) refers to the ***movement of solvent*** (most frequently water) ***through a semipermeable membrane.***

The flow of solvent occurs from a solution of low concentration to a solution of high concentration, when both are separated by a semipermeable membrane. In a strict sense, the semipermeable membrane is expected to be permeable to the solvent and not to the solute.

Osmotic pressure

Osmotic pressure may be defined as the excess pressure that must be applied to a solution to prevent the passage of solvent into the solution, when both are separated by a semipermeable membrane.

Osmosis is a ***colligative property*** i.e. a character which depends on the number of solute particles and not their nature. Osmotic pressure is directly proportional to the concentration (number) of the solute molecules or ions. Low molecular weight substances (e.g. NaCl, glucose) will have more number of molecules compared to high molecular weight substances (albumin, globulin) for unit mass. Therefore, the substances with low molecular weight, in general, exhibit greater osmotic pressure. Further, for ionizable compounds, the total osmotic pressure is equivalent to the sum of the individual pressures exerted by each ion. For instance, one molar solution of NaCl will exert double the osmotic pressure of one molar solution of glucose. This is because NaCl ionizes to Na^+ and Cl^- while glucose is non-ionizable.

The solutions that exert the same osmotic pressure are said to be ***isoosmotic.*** The term ***isotonic*** is used when a cell is in direct contact with an isoosmotic solution (0.9% NaCl) which does not change the cell volume and, thus, the cell tone is maintained. A solution with relatively greater osmotic pressure is referred to as ***hypertonic.*** On the other hand, a solution with relatively lower pressure is ***hypotonic.***

The term ***oncotic pressure*** is commonly used to represent the osmotic pressure of colloidal substances (e.g. albumin, globulin).

Units of osmotic pressure : *Osmole* is the unit of osmotic pressure. One osmole is the number of molecules in gram molecular weight of undissociated solute. One gram molecular weight of glucose (180 g) is one osmole. However, one gram molecular weight of NaCl (58.5 g) is equivalent to 2 osmoles, since NaCl ionizes to give two particles (Na^+, Cl^-).

Osmotic pressure of biological fluids is frequently expressed as ***milliosmoles.*** The osmotic pressure of plasma is 280–300 milliosmoles/l.

Applications of osmosis

1. **Fluid balance and blood volume :** The fluid balance of the different compartments of the body is maintained due to osmosis. Further, osmosis significantly contributes to the regulation of blood volume and urine excretion.

2. **Red blood cells and fragility :** When RBC are suspended in an isotonic (0.9% NaCl) solution, the cell volume remains unchanged and they are intact. In hypertonic solution (say 1.5% NaCl), ***water flows out of RBC*** and the cytoplasm shrinks, a phenomenon referred to as ***crenation.***

On the other hand, when the RBC are kept in ***hypotonic solution*** (say 0.4% NaCl), the cells bulge due to entry of water which often causes rupture of plasma membrane of RBC ***(hemolysis)***.

Osmotic fragility test for RBC is employed in laboratories for diagnostic purposes. For a normal human blood, RBC begin to hemolyse in 0.45% NaCl and the hemolysis is almost complete in 0.33% NaCl. ***Increased fragility*** of RBC is observed ***in hemolytic jaundice*** while it is decreased in certain anemias.

3. **Transfusion :** Isotonic solutions of NaCl (0.9%) or glucose (5%) or a suitable combination of these two are commonly used in transfusion in hospitals for the treatment of dehydration, burns etc.

4. **Action of purgatives :** The mechanism of action of purgatives is mainly due to ***osmotic phenomenon.*** For instance, epson ($MgSO_4$ $7H_2O$) or Glauber's (Na_2SO_4 $10H_2O$) salts withdraw water from the body, besides preventing the intestinal water absorption.

5. **Osmotic diuresis :** The high blood glucose concentration causes osmotic diuresis resulting in the loss of water, electrolytes and glucose in the urine. This is the basis of polyuria observed in diabetes mellitus. Diuresis can be produced by administering compounds (e.g. mannitol) which are filtered but not reabsorbed by renal tubules.

6. **Edema due to hypoalbuminemia :** Disorders such as kwashiorkor and glomerulonephritis are associated with lowered plasma albumin concentration and edema. Edema is caused by reduced oncotic pressure of plasma, leading to the accumulation of excess fluid in tissue spaces.

7. **Cerebral edema :** Hypertonic solutions of salts (NaCl, $MgSO_4$) are in use to reduce the volume of the brain or the pressure of cerebrospinal fluid.

8. **Irrigation of wounds :** Isotonic solutions are used for washing wounds. The pain experienced by the direct addition of salt or sugar to wounds is due to osmotic removal of water.

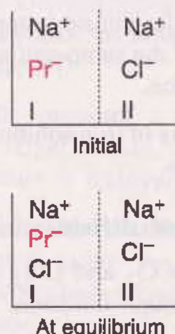


Fig. 40.2 : Diagrammatic representation of Donnan membrane equilibrium.

DONNAN MEMBRANE EQUILIBRIUM

When membrane is freely permeable to ions (say Na^+, Cl^-) and if the concentration of ions on both the sides is different, the ions freely diffuse to attain equal concentration. Gibbs-Donnan observed that the ***presence of a non-diffusible ion on one side of the membrane alters the diffusion of diffusible ions.***

In the molecule sodium proteinate (Na^+Pr^-), the protein (Pr^-) ion is non-diffusable through the membrane. Let us consider two sides of a compartment separated by a membrane. Initially, sodium proteinate is on side I while sodium chloride is on side II (***Fig.40.2***). Diffusible ions (Na^+, Cl^-) can freely pass through the membrane. On side I, Na^+ ions will balance the incoming Cl^- ions besides Pr^- ions, while on side II Na^+ ions have to balance only Cl^- ions. Therefore, the concentration of Na^+ on side I is greater than on side II. However, from the thermodynamical point of view, at equilibrium, the concentration of Na^+ Cl^- on both the sides should be the same.

Thus $Na^+ Cl^-$ (I) = $Na^+ Cl^-$ (II)

Since Na^+ (I) > Na^+ (II)

Cl^- (I) < Cl^- (II)

Consequently, the concentration of Cl^- ions should be greater on side II. Further, the total concentration of ions on side I is higher than on side II.

The salient features of Donnan membrane equilibrium are listed next.

1. The presence of a non-diffusible ion influences the concentration of diffusible ions across the membrane.

2. The concentration of oppositely charged ions (Na^+), is greater on the side of the membrane containing non-diffusible ions (Pr^-).

3. The concentration of similarly charged ions (Cl^-) is higher on the side of the membrane not containing non-diffusible ions (Pr^-).

4. The net concentration of total ions will be greater on the side of the membrane containing non-diffusible ions. This leads to a difference in the osmotic pressure on either side of the membrane.

Applications of Donnan membrane equilibrium

1. **Difference in the ionic concentrations of biological fluids :** The lymph and interstitial fluids have lower concentration of inorganic cations (Na^+, K^+) and higher concentration of anions (Cl^-) compared to plasma. This is attributed to the higher protein (Pr^-) content in the plasma.

2. **Membrane hydrolysis :** The relative strength of H^+ and OH^- ions and, therefore, the acidic or alkaline nature on either side of a membrane, is influenced by the presence of non-diffusible ions. This phenomenon is referred to as membrane hydrolysis. Donnan membrane equilibrium explains the ***greater*** concentration of ***H^+ ions in the gastric juice.***

3. **Lower pH in RBC :** The hemoglobin of RBC is negatively charged and, therefore, causes the accumulation of positively charged ions including H^+. Therefore, the pH of RBC is slightly lower (7.25) than that of plasma (7.4).

4. **Osmotic imbalance :** Donnan membrane equilibrium—which results in the differential distribution of ions in different compartments of the body—partly explains the osmotic pressure differences.

VISCOSITY

Liquid or fluid has a tendency to flow which is referred to as fluidity. The term viscosity may be defined as the ***internal resistance offered by a liquid or a gas to flow***. The property of viscosity is due to frictional forces between the layers while their movement occurs. Viscosity may be appropriately regarded as the internal friction of a liquid.

Liquids vary widely as regards their viscosity. For instance, ether has very low viscosity while honey and blood are highly viscous. Among the several factors that contribute to viscosity, density of the liquid, concentration of dissolved substances and their molecular weight and the molecular interactions are important. Increase in temperature decreases viscosity while increase in pressure increases viscosity to some extent.

Viscosity of colloidal solutions, particularly lyophilic colloids, is generally higher than true solutions.

Units of viscosity : The unit of viscosity is ***poise***, after the scientist Poiseuille, who first systematically studied the flow of liquids. A poise represents ***dynes/cm²***.

Applications of viscosity

1. **Viscosity of blood :** Blood is about ***4 times more*** viscous than water. The viscosity of blood is mainly attributed to ***suspended blood cells*** and colloidal ***plasma proteins***. As the blood flows through capillaries the viscosity decreases to facilitate free flow of blood. Blood viscosity is increased in polycythemia (elevation of RBC), while it is reduced in anemia and nephritis. A more viscous blood increases cardiac work load. When dehydration occurs, the viscosity of the blood increases.

2. **Viscosity change in muscle :** Excitation of the muscle is associated with increase in the viscosity of the muscle fibres. This delays the change in the tension of the contracting muscle.

3. ***Vitreous body*** : *This is an amorphous* viscous body located in the posterior chamber of the eye. It is rich in albumin and hyaluronic acid.

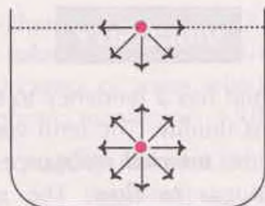


Fig. 40.3 : Surface tension of a liquid.

4. **Synovial fluid :** It contains hyaluronic acid which imparts viscosity and helps in the lubricating function of joints.

SURFACE TENSION

A molecule in the interior of a liquid is attracted by other molecules in all directions. In contrast, a molecule on the surface is attracted only downwards and sideways and not upwards (***Fig.40.3***). Due to this, the surface layer behaves like a stretched film. ***Surface tension is the force with which the molecules on the surface are held together.*** It is expressed as dynes/cm. Surface tension decreases with increase in temperature.

Due to the phenomenon of surface tension, any liquid occupies the minimum possible volume.

According to the principle of Gibbs-Thomson, the compounds which lower the surface tension get concentrated at the surface (or interface) layer while those compounds which increase surface tension get distributed in the interior portion of the liquid. In general, organic substances (proteins, lipids) decrease whereas inorganic substances (NaCl, KCl) increase surface tension.

Applications of surface tension

1. **Digestion and absorption of fat :** Bile salts reduce the surface tension. They act as detergents and cause emulsification of fat, thereby allowing the formation of minute particles for effective digestion and absorption.

2. **Hay's sulfur test :** This is a common laboratory test employed for the detection of bile salts in urine of jaundice patients. Sulfur powder, when sprinkled on the surface of urine possessing bile salts, sinks. This is in contrast to a normal urine where sulfur powder floats. Hay's test is based on the principle that bile salts in urine lower surface tension which is responsible for sulfur to sink.

3. **Surfactants and lung function :** The low surface tension of the alveoli keeps them apart and allows an efficient exchange of gases in lungs. In fact, certain surfactants, predominantly dipalmitoyl phosphatidyl choline (***dipalmitoyl lecithin***) are responsible for maintaining low surface tension in the alveoli. ***Surfactant deficiency causes respiratory distress syndrome in the infants.***

4. **Surface tension and adsorption :** Adsorption, being a surface phenomenon, is closely related to surface tension. Due to the coupled action of these two processes, the formation of complexes of proteins and lipids occurs in the biological systems.

5. **Lipoprotein complex membranes :** The structure of plasma membrane is composed of surface tension reducing substances, namely lipids and proteins. This facilitates absorption of these compounds.

ADSORPTION

Adsorption is a ***surface phenomenon.*** It is the process of accumulation of a substance (adsorbate) on the surface of another substance (adsorbent). Adsorption differs from absorption, as the latter involves the diffusion into the interior of the material.

The capacity of an adsorbent depends on the surface area. Therefore, porous substances serve as better adsorbents e.g. charcoal, alumina, silica gel. Adsorption is a dynamic and reversible process which decreases with rise in temperature.

Applications of adsorption

1. **Formation of enzyme-substrate complex :** For the catalysis to occur in biological system, formation of enzyme-substrate complex is a

prerequisite. This happens by adsorption of substrate on the enzyme.

2. **Action of drugs and poisons :** On adsorption at the cell surface, drugs and poisons exert their action.

3. **Adsorption in analytical biochemistry :** The principle of adsorption is widely employed in the chromatography technique for the separation and purification of compounds (enzymes, immunoglobulins).

ISOTOPES

Isotopes have revolutionized biochemistry when they became available to investigators soon after Second World War. ***Isotopes are defined as the elements with same atomic number but different atomic weights.*** They possess the same number of protons but differ in the neutrons in their nuclei. Therefore, isotopes (*Greek* : iso—equal; tope—place) occupy the same place in the periodic table. The chemical properties of different isotopes of a particular element are identical.

Isotopes are of two types—***stable*** and ***unstable.*** The latter are more commonly referred to as radioactive isotopes and they are of particular interest to biochemists. Conventionally while representing isotopes, the atomic weight is written on upper left side of the element symbol.

Stable isotopes

They are naturally occurring and do not emit radiations (non-radioactive) e.g. deuterium (heavy hydrogen) ^{2}H; ^{13}C; ^{15}N; ^{18}O. Stable isotopes can be identified and quantitated by mass spectrometry or nuclear magnetic resonance (NMR). They are less frequently used in biochemical investigations.

Radioactive isotopes

The atomic nucleus of radioactive isotopes is unstable and, therefore, undergoes decay. The radioactive decay gives rise to one of the following 3 ionizing radiations.

1. **α-Rays**—an α particle possessing 2 protons i.e. helium nuclei.

2. **β-Rays**—due to the emission of electrons.

3. **γ-Rays**—due to emission of high energy photons.

The radiations emmitted by radioactive nuclei are characteristic of the isotope. For instance, ^{3}H, ^{14}C, and ^{32}P all emit β-particles in the respective energies of 0.018, 0.155 and 1.71 MeV.

The β and γ emitting radioisotopes are *employed* in biochemical research. These isotopes are produced in nuclear reactors. The simple chemicals so produced are then converted to *radiolabelled biochemicals* by chemical or *enzymatic synthesis.*

Units of radioactivity : *Curie* (Ci) is the basic unit of radioactive decay. It is defined as the amount of radioactivity equivalent to 1 g of radium i.e. 2.22×10^{12} disintegrations per minute (dpm). Millicurie (mCi) and microcurie (µCi), respectively, corresponding to 2.2×10^9 and 2.2×10^6 dpm, are more commonly used.

Half-lives of isotopes : The unstable radioisotopes undergo decay. The radioactivity gets reduced to half of the original within a fixed time. This represents the half-life which is characteristic for a given isotope.

Some of the commonly used radioactive isotopes in biochemical research with their characteristics are given in ***Table 40.2***.

Measurement of radioactivity of isotopes

Several techniques are in use for the detection of radioactivity of the isotopes. The most commonly employed in biochemical research are—***Geiger counters, liquid scintillation counter*** and ***autoradiography.*** Geiger counters are almost outdated. Liquid scintillation counters are now widely used.

In the liquid scintillation counter, the sample is *dissolved or suspended in a solution* containing one or two fluorescent organic compounds (fluors). The fluors emit a pulse of

TABLE 40.2 Commonly used radioisotopes in biochemistry

Isotope	*Radiation*	*Half-life*
^{3}H	β	12.2 years
^{14}C	β	5,700 years
^{22}Na	γ	2.5 years
^{32}P	β	14.5 days
^{35}S	β	87 days
^{45}Ca	β	164 days
^{59}Fe	β, γ	45 days
^{60}Co	γ	5.25 years
^{125}I	γ	60 days
^{131}I	β, γ	8.1 days

light when struck by radiation. The light, proportional to the radiation energy, can be detected. The advantage with liquid scintillation counter is that it can discriminate the particles of different energies. Thus, two or more isotopes can be simultaneously detected.

In autoradiography, the radiations are detected by its blackening of photographic film. This technique is commonly used for the detection of radioactive substances separated in polyacrylamide gel electrophoresis (PAGE).

Applications of radioisotopes in biochemistry

Radioactive isotopes have become indispensable tools of biochemistry. They can be conveniently used as tracers in biochemical research since the chemical properties of different isotopes of a particular element are identical. Therefore, the living cells cannot distinguish the radioactive isotope from a normal atom.

Radioisotopes are widely used in establishing the precursor-product relationships in metabolisms and understanding of the complex metabolic pathways.

A few important application of radioisotopes are

1. By the use of isotope tracers, the metabolic origin of complex molecules such as heme, cholesterol, purines and phospholipids can be determined. As early as 1945, it was established that nitrogen atom of heme was derived from glycine. This was done by feeding rats with (^{15}N) glycine and detecting (^{15}N) heme.

2. The precursor-product relationship in several metabolic pathways has been investigated by radioisotopes. e.g. Krebs cycle, β-oxidation of fatty acids, urea cycle, fatty acid synthesis.

3. Radioisotopes are conveniently used in the study of metabolic pools (e.g. amino acid pool) and metabolic turnovers (e.g. protein turnover).

4. Certain endocrine and immunological studies also depend on the use of radioisotopes e.g. radioimmunoassay.

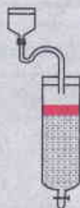
5. Radioisotopes are employed in elucidating drug metabolism.

Radioisotopes in diagnosis and treatment

Certain radioisotopes are used in the scanning of organs—thyroid gland (^{131}I), bone (^{90}Sr) and kidney (^{131}I hippuran).

Radioactivity has been employed in the treatment of cancers. This is based on the principle that radiations produce ionizations which damage nucleic acids. Thus, the uncontrolled proliferation of cells is restricted.

41 Tools of Biochemistry



The chromatography speaks :

"A technique I am, separating mixture of compounds;
To isolate, identify, characterize molecules, as desired;
Working on the principles of adsorption, partition, ion-exchange;
I am a key biochemical tool in laboratory experimentation."

Biochemistry is an experimental rather than a theoretical science. The understanding and development of concepts in biochemistry are a result of continuous experimentation and evidence obtained therein. It is no exaggeration to state that the foundations for the present (and the future, of course!) knowledge of biochemistry are based on the laboratory tools employed for biochemical experimentation. Thus, the development of sensitive and sophisticated analytical techniques has tremendously contributed to our understanding of biochemistry.

A detailed discussion on the tools of biochemistry is beyond the scope of this book. The basic principles of some of the commonly employed tools are described in this chapter. The reader must, however, ***refer Chapter 27***, for the following techniques related to molecular biology,and recombinant DNA technology

- Isolation and purification of nucleic acids
- Nucleic acid blotting techniques
- DNA sequencing
- Polymerase chain reaction
- Methods of DNA assay
- DNA fingerprinting or DNA profiling.

CHROMATOGRAPHY

Chromatography is one of the most useful and popular tools of biochemistry. It is an analytical technique dealing with the ***separation of closely related compounds from a mixture.*** These include proteins, peptides, amino acids, lipids, carbohydrates, vitamins and drugs.

Historical perspective

The credit for the discovery of chromatography goes to the Russian botanist Mikhail Tswett. It was in 1906, Tswett described the separation of plant leaf pigments in solution by passing through a column of solid adsorbents. He coined the term chromatography (*Greek* : chroma—colour; graphein—to write), since the technique dealt with the separation of colour *compounds (pigments). Coincidently,* the term Tswett means colour in Russian! Truly speaking,

chromatography is a misnomer, since it is no longer limited to the separation of coloured compounds.

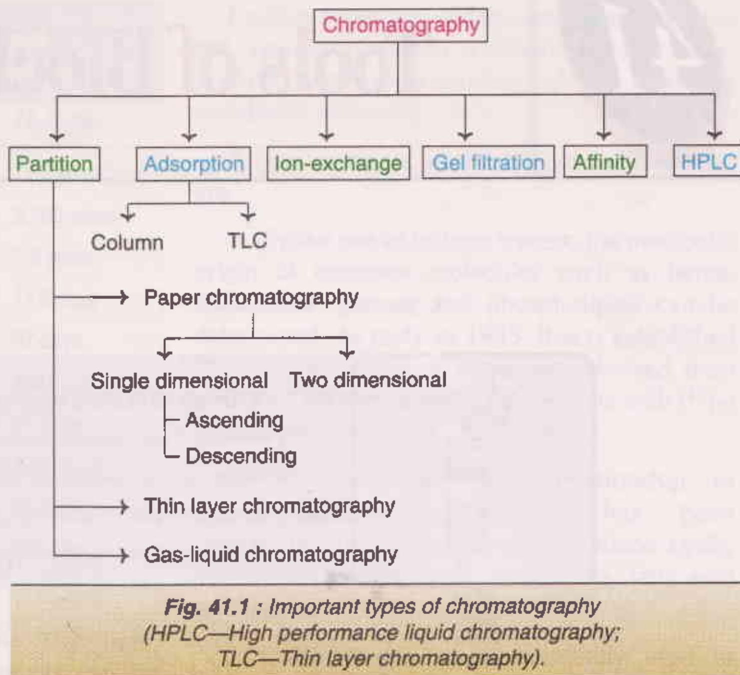


Fig. 41.1 : Important types of chromatography (HPLC—High performance liquid chromatography; TLC—Thin layer chromatography).

Principles and classification

Chromatography usually consists of a ***mobile phase*** and a ***stationary phase.*** The mobile phase refers to the mixture of substances (to be separated), dissoved in a liquid or a gas. The stationary phase is a porous solid matrix through which the sample contained in the mobile phase percolates. The interaction between the mobile and stationary phases results in the separation of the compounds from the mixture. These interactions include the physicochemical principles such as ***adsorption, partition, ion-exchange, molecular sieving*** and ***affinity.***

The interaction between stationary phase and mobile phase is often employed in the classification chromatography e.g. partition, adsorption, ion-exchange. Further, the classification of chromatography is also based either on the nature of the stationary phase (paper, thin layer, column), or on the nature of both mobile and stationary phases (gas-liquid chromatography). A summary of the different methods (classes) of chromatography is given in ***Fig.41.1***.

1. **Partition chromatography :** The molecules of a mixture get partitioned between the stationary phase and mobile phase depending on their relative affinity to each one of the phases.

(a) **Paper chromatography :** This technique is commonly used for the separation of amino acids, sugars, sugar derivatives and peptides. In paper chromatography, a few drops of solution containing a mixture of the compounds to be separated is applied (spotted) at one end, usually ~2 cm above, a strip of filter paper (Whatman No. 1 or 3). The paper is dried and dipped into a solvent mixture consisting of butanol, acetic acid and water in 4 : 1 : 5 ratio (for the separation of amino acids). The aqueous component of the solvent system binds to the paper and forms a stationary phase. The organic component that migrates on the paper is the mobile phase. When the migration of the solvent is upwards, it is referred to as ***ascending chromatography.*** *In* ***descending chromatography***, the solvent moves downwards (***Fig.41.2***). As the solvent flows, it takes along with it the unknown substances. The rate of migration of the molecules depends on the relative solubilities in the stationary phase (aqueous) and mobile phase (organic).

After a sufficient migration of the solvent front, the paper (chromatogram) is removed, dried and developed for the identification of the specific spots.

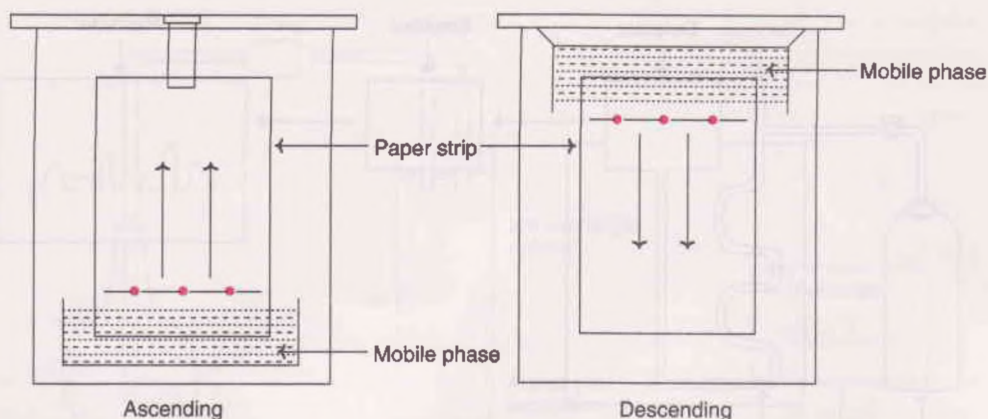


Fig. 41.2 : Paper chromatography—ascending and descending types.

Ninhydrin, which forms purple complex with α-amino acids, is frequently used as a colouring reagent. The chemical nature of the individual spots can be identified by running known standards with the unknown mixture.

The migration of a substance is frequently expressed as R_f value (ratio of fronts)

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

The R_f value of each substance, characteristic of a given solvent system and paper, often helps for the identification of unknown.

Sometimes, it is rather difficult to separate a complex mixture of substances by a single run with one solvent system. In such a case, a *second run is carried out by a different* solvent system, in a direction perpendicular to the first run. This is referred to as ***two dimensional chromatography*** which enhances the separation of a mixture into the individual components.

(b) **Thin layer chromatography (TLC) :** The principle of TLC is the same as described for paper chromatography (partition). In place of a paper, an inert substance, such as cellulose, is employed as supporting material. Cellulose is spread as a thin layer on glass or plastic plates. The chromatographic separation is comparatively rapid in TLC.

In case of ***adsorption thin layer chromatography,*** adsorbents such as activated silica gel, alumina, kieselguhr are used.

(c) **Gas-liquid chromatography (GLC) :** This is the method of choice for ***the separation of volatile substances*** or the volatile derivatives of certain non-volatile substances. In GLC, the stationary phase is an inert solid material (diatomaceous earth or powdered firebrick), impregnated with a non-volatile liquid (silicon or polyethylene glycol). This is packed in a narrow column and maintained at high temperature (around 200°C). A mixture of volatile material is injected into the column along with the mobile phase, which is an inert gas (argon, helium or nitrogen). The separation of the volatile mixture is based on the partition of the components between the mobile phase (gas) and stationary phase (liquid), hence the name gas-liquid chromatography. The separated compounds can be identified and

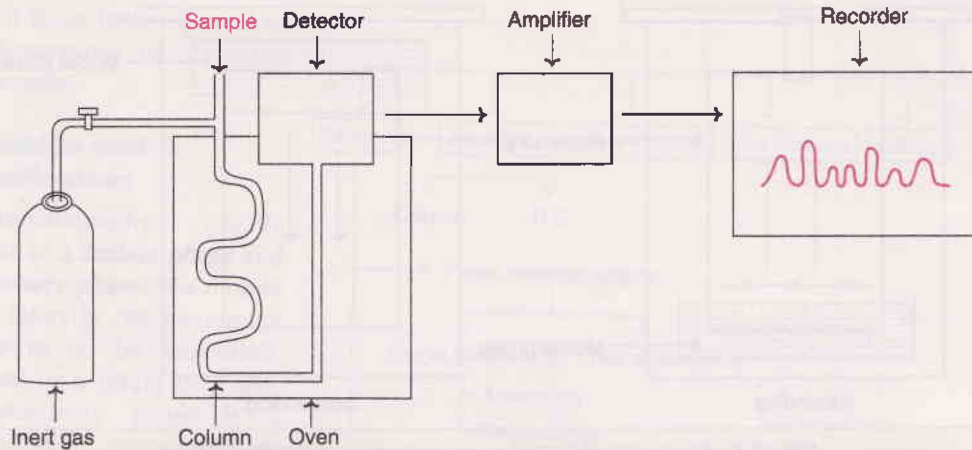


Fig. 41.3 : Diagrammatic representation of gas-liquid chromatography (GLC).

quantitated by a detector (*Fig.41.3*). The detector works on the principles of ionization or thermal conductivity.

Gas-liquid chromatography is sensitive, rapid and reliable. It is frequently used for the quantitative estimation of biological materials such as lipids, drugs and vitamins.

2. **Adsorption column chromatography :** The adsorbents such as silica gel, alumina, charcoal powder and calcium hydroxyapatite are packed into a column in a glass tube. This serves as the stationary phase. The sample mixture in a solvent is loaded on this column. The individual components get differentially adsorbed on to the adsorbent. The elution is carried out by a buffer system (mobile phase). The individual compounds come out of the column at different rates which may be separately collected and identified (*Fig.41.4*). For instance, amino acids can be identified by ninhydrin calorimetric

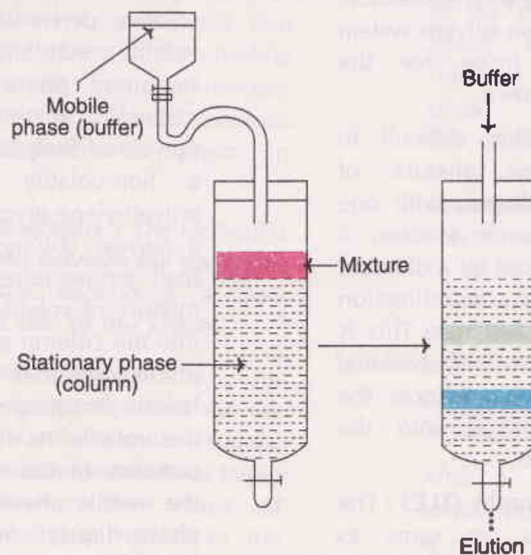


Fig. 41.4 : Diagrammatic representation of adsorption column chromatography.

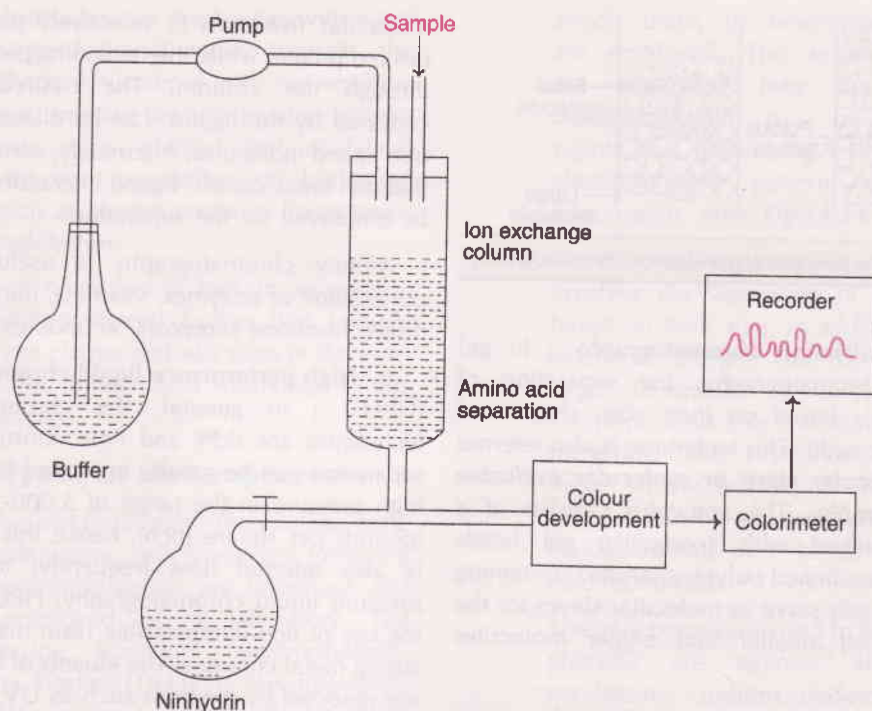


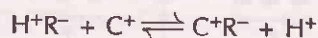
Fig. 41.5 : Diagrammatic representation of amino acid analyser.

method. An automated column chromatography apparatus—***fraction collector***—is frequently used nowadays.

3. **Ion-exchange chromatography** : Ion-exchange chromatography involves the separation of molecules on the basis of their electrical charges. Ion-exchange resins—***cation exchangers*** and ***anion exchangers***—are used for this purpose. An anion exchanger (R^+A^-) exchanges its anion (A^-) with another anion (B^-) in solution.

$$R^+A^- + B^- \rightleftharpoons R^+B^- + A^-$$

Similarly, a cation exchanger (H^+R^-) exchanges its cation (H^+) with another cation (C^+) in solution.



$$H^+R^- + C^+ \rightleftharpoons C^+R^- + H^+$$

Thus, in ion-exchange chromatography, ions in solution are reversibly replaced by ion-exchange resins. The binding abilities of ions bearing positive or negative charges are highly pH dependent, since the net charge varies with pH. This principle is exploited in the separation of molecules in ion-exchange chromatography.

A mixture of amino acids (protein hydrolysate) or proteins can be conveniently separated by ion-exchange chromatography. The amino acid mixture (at pH around 3.0) is passed through a cation exchange and the individual amino acids can be eluted by using buffers of different pH. The various fractions eluted, containing *individual amino acids*, are allowed to react with ninhydrin reagent to form coloured complex. *This is continuously* monitored for qualitative and quantitative identification of amino acids. The ***amino acid analyser,*** first developed by Moore and Stein, is based on this principle (***Fig.41.5***).

Several types of ion exchangers are commercially available. These include polystyrene resins (anion exchange resin, Dowex 1; cation exchange resin, Dowex 50), DEAE (diethyl aminoethyl) cellulose, CM (carboxy methyl) cellulose, DEAE-sephadex and CM-sephadex.

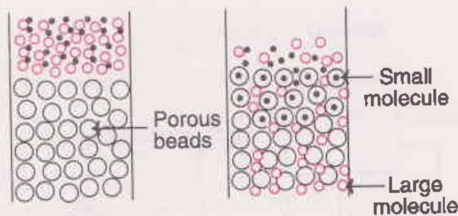


Fig. 41.6 : The principle of gel-filtration chromatography.

4. **Gel filtration chromatography :** In gel filtration chromatography, the separation of molecules is based on their size, shape and molecular weight. This technique is also referred to as ***molecular sieve or molecular exclusion chromatography.*** The apparatus consists of a column packed with spongelike gel beads (usually cross-linked polysaccharides) containing pores. The gels serve as molecular sieves for the separation of smaller and bigger molecules (***Fig.41.6***).

The solution mixture containing molecules of different sizes (say proteins) is applied to column and eluted with a buffer. The larger molecules cannot pass through the pores of gel and, therefore, move faster. On the other hand, the smaller molecules enter the gel beads and are left behind which come out slowly. By selecting the gel beads of different porosity, the molecules can be separated. The commercially available gels include Sephadex (G-10, G-25, G-100), Bio-gel (P-10, P-30, P-100) and sepharose (6B, 4B, 2B).

The gel-filtration chromatography can be used for an approximate determination of molecular weights. This is done by using a calibrated column with substances of known molecular weight.

5. **Affinity chromatography :** The principle of affinity chromatography is based on the property of specific and non-covalent binding of proteins to other molecules, referred to as ***ligands.*** For instance, enzymes bind specifically to ligands such as substrates or cofactors.

The technique involves the use of ligands covalently attached to an inert and porous matrix in a column. The immobilized ligands act as molecular ***fishhooks*** to selectively pick up the desired protein while the remaining proteins pass through the column. The desired protein, captured by the ligand, can be eluted by using free ligand molecules. Alternately, some reagents that can break protein-ligand interaction can also be employed for the separation.

Affinity chromatography is useful for the purification of enzymes, vitamins, nucleic acids, drugs, hormone receptors, antibodies etc.

6. **High performance liquid chromatography (HPLC) :** In general, the chromatographic techniques are slow and time consuming. The separation can be greatly improved by applying high pressure in the range of 5,000-10,000 psi (pounds per square inch), hence this technique is also referred (less frequently) to as high pressure liquid chromatography. HPLC requires the use of non-compressible resin materials and strong metal columns. The eluants of the column are detected by methods such as UV absorption and fluorescence.

ELECTROPHORESIS

The ***movement of charged particles*** (ions) ***in an electric field*** resulting in their migration towards the oppositely charged electrode is

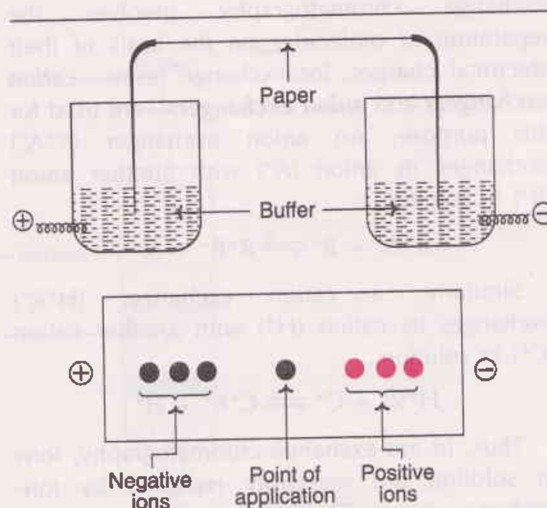


Fig. 41.7 : Diagrammatic representation of paper electrophoresis.

known as electrophoresis. Molecules with a net positive charge (cations) move towards the negative cathode while those with net negative charge (anions) migrate towards positive anode. Electrophoresis is a widely used analytical technique for the separation of biological molecules such as plasma proteins, lipoproteins and immunoglobulins.

The rate of migration of ions in an electric field depends on several factors that include shape, size, net charge and solvation of the ions, viscosity of the solution and magnitude of the current employed.

Different types of electrophoresis

Among the electrophoretic techniques, zone electrophoresis (paper, gel), isoelectric focussing and immunoelectrophoresis are important and commonly employed in the laboratory. The original moving boundary electrophoresis, developed by Tiselius (1933), is less frequently used these days. In this technique, the U-tube is filled with protein solution overlaid by a buffer solution. As the proteins move in solution during electrophoresis, they form boundaries which can be identified by refractive index.

1. **Zone electrophoresis :** A simple and modified method of moving boundary electrophoresis is the zone electrophoresis. An inert supporting material such as paper or gel are used.

(a) **Paper electrophoresis :** In this technique, the sample is applied on a strip of filter paper wetted with desired buffer solution. The ends of the strip are dipped into the buffer reservoirs in which the electrodes are placed. The electric current is applied allowing the molecules to migrate for sufficient time. The paper is removed, dried and stained with a dye that specifically colours the substances to be detected. The coloured spots can be identified by comparing with a set of standards run simultaneously.

For the separation of serum proteins, Whatman No. 1 filter paper, veronal or tris buffer at pH 8.6 and the stains amido black or bromophenol blue are employed. The ***serum proteins are separated into five distinct bands***—albumin, α_1-, α_2-, β- and γ-globulins (Refer ***Fig.9.1***). For the electrophoretic pattern of serum lipoproteins, refer ***Fig.14.34.***

(b) **Gel electrophoresis :** This technique involves the separation of molecules based on their size, in addition to the electrical charge. The movement of large molecules is slow in gel electrophoresis (this is in contrast to gel filtration). The resolution is much higher in this technique. Thus, serum proteins can be separated to about 15 bands, instead of 5 bands on paper electrophoresis.

The gels commonly used in gel electrophoresis are agarose and polyacrylamide, sodium dodecyl sulfate (SDS). Polyacrylamide is employed for the determination of molecular weights of proteins in a popularly known electrophoresis technique known as SDS-PAGE.

2. **Isoelectric focussing :** This technique is primarily based on the ***immobilization of the molecules at isoelectric*** pH during electrophoresis. Stable pH gradients are set up (usually in a gel) covering the pH range to include the isoelectric points of the components in a mixture. As the electrophoresis occurs, the molecules (say proteins) migrate to positions corresponding to their isoelectric points, get immobilized and form sharp stationary bonds. *The gel blocks can be stained and identified.* By isoelectric focussing, serum proteins can be separated to as many as 40 bands. Isoelectric focussing can be conveniently used for the purification of proteins.

3. **Immunoelectrophoresis :** This technique involves combination of the principles of electrophoresis and immunological reactions. Immunoelectrophoresis is useful for the analysis of complex mixtures of antigens and antibodies.

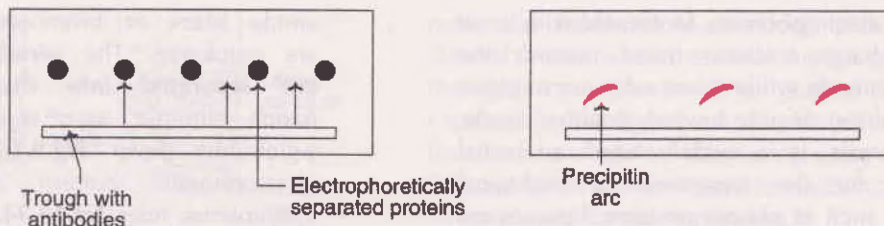


Fig. 41.8 : Diagrammatic representation of immunoelectrophoresis.

The complex proteins of biological samples (say human serum) are subjected to electrophoresis. The antibody (antihuman immune serum from rabbit or horse) is then applied in a trough parallel to the electrophoretic separation. The antibodies diffuse and, when they come in contact with antigens, precipitation occurs, resulting in the formation of ***precipitin bands*** which can be identified (***Fig.41.8***).

PHOTOMETRY—COLORIMETER AND SPECTROPHOTOMETER

Photometry broadly deals with the study of the phenomenon of light absorption by molecules in solution. The specificity of a compound to absorb light at a particular wavelength (***monochromatic light***) is exploited in the laboratory for quantitative measurements. From the biochemist's perspective, photometry forms an important laboratory tool for accurate estimation of a wide variety of compounds in biological samples. Colorimeter and spectrophotometer are the laboratory instruments used for this purpose. They work on the principles discussed below.

When a light at a particular wavelength is passed through a solution (incident light), some amount of it is absorbed and, therefore, the light that comes out (transmitted light) is diminished. The nature of light absorption in a solution is governed by ***Beer-Lambert law.***

Beer's law states that the amount of transmitted light decreases exponentially with an increase in the concentration of absorbing material (i.e. the amount of light absorbed depends on the concentration of the absorbing molecules). And according to Lambert's law, the transmitted light decreases exponentially with increase in the thickness of the absorbing molecules (i.e. the amount of light absorbed is dependent on the thickness of the medium).

By combining the two laws (Beer-Lambert law), the following mathematical derivation can be obtained

$$I = I_0^{\varepsilon ct}$$

where I = Intensity of the transmitted light

I_0 = Intensity of the incident light

ε = Molar extinction coefficient (characteristic of the substance being investigated)

c = Concentration of the absorbing substance (moles/l or g/dl)

t = Thickness of medium through which light passes.

When the thickness of the absorbing medium is kept constant (i.e. Lambert's law), the intensity of the transmitted light depends only on concentration of the absorbing material. In other words, the Beer's law is operative.

The ratio of transmitted light (I) to that of incident light (I_0) is referred to as transmittance (T).

$$T = \frac{I}{I_0}$$

Absorbance (A) or ***optical density (OD)*** is very commonly used in laboratories. The relation between absorbance and transmittance is expressed by the following equation.

$$A = 2 - \log_{10} T = 2 - \log\% \ T$$

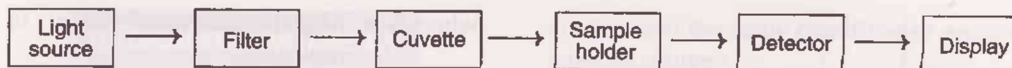


Fig. 41.9 : Diagrammatic representation of the components in a colorimeter.

Colorimeter

Colorimeter (or photoelectric colorimeter) is the instrument used for the measurement of coloured substances. This instrument is operative in the visible range (400-800 nm) of the electromagnetic spectrum of light. The ***working of colorimeter*** is ***based on the principle of Beer-Lambert law*** (discussed above).

The colorimeter, in general consists of light source, filter sample holder and detector with display (meter or digital). A filament lamp usually serves as a light source.The filters allow the passage of a small range of wave length as incident light. The sample holder is a special glass cuvette with a fixed thickness. The photoelectric selenium cells are the most common detectors used in colorimeter. The diagrammatic representation of a colorimeter is depicted in ***Fig.41.9***.

Spectrophotometer

The spectrophotometer primarily differs from colorimeter by covering the ultraviolet region (200-400 nm) of the electromagnetic spectrum. Further, the spectrophotometer is more sophisticated with several additional devices that ultimately increase the sensitivity of its operation severalfold when compared to a colorimeter. A precisely selected wavelength (say 234 nm or 610 nm) in both ultra-violet and visible range can be used for measurements. In place of glass cuvettes (in colorimeter), quartz cells are used in a spectrophotometer.

The spectrophotometer has similar basic components as described for a colorimeter (***Fig.41.9***), and its operation is also based on the Beer-Lambert law (already discussed).

FLUORIMETRY

When certain compounds are subjected to light of a particular wavelength, some of the molecules get excited. These molecules, while they return to ground state, emit light in the form of fluorescence which is proportional to the concentration of the compound. This is the principle in the operation of the instrument fluorometer.

FLAME PHOTOMETRY

Flame photometry primarily deals with the quantitative ***measurement of electrolytes*** such as sodium, potassium and lithium. The instrument, namely flame photometer, works on the following principle. As a solution in air is finally sprayed over a burner, it dissociates to give neutral atoms. Some of these atoms get excited and move to a higher energy state. When the excited atoms fall back to the ground state, they emit light of a characteristic wavelength which can be measured. The intensity of emission light is proportional to the concentration of the electrolyte being estimated.

ULTRACENTRIFUGATION

The ultracentrifuge was developed by a Swedish biochemist Svedberg (1923). The principle is based on the generation of centrifugal force to as high as 600,000 g (earth's gravity g = 9.81 m/s^2) that allows the sedimentation of particles or macromolecules. Ultracentrifugation is an indispensable tool for the ***isolation of subcellular organelles***, proteins and nucleic acids. In addition, this technique is also employed in the determination of molecular weights of macromolecules.

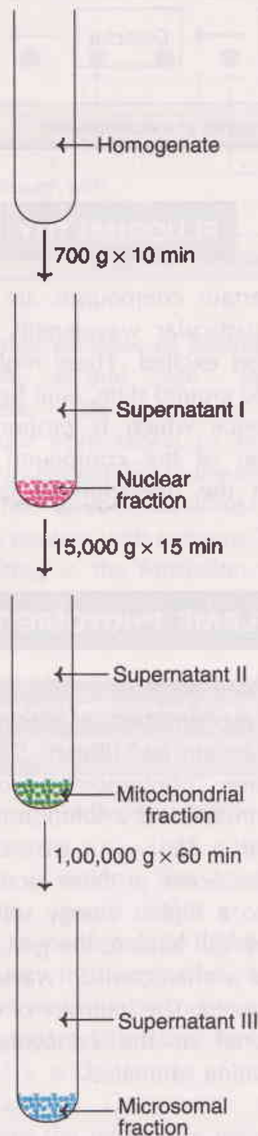


Fig. 41.10 : Separation of subcellular fractions by differential centrifugation.

The rate at which the sedimentation occurs in ultracentrifugation primarily depends on the size and shape of the particles or macromolecules (i.e. on the molecular weight). It is expressed in terms of ***sedimentation coefficient***(s) and is given by the formula.

$$s = \frac{v}{\omega^2 r}$$

where v = Migration (sedimentation) of the molecule

ω = Rotation of the centrifuge rotor in radians/sec

r = Distance in cm from the centre of rotor

The sedimentation coefficient has the units of seconds. It was usually expressed in units of 10^{-13}s (since several biological macromolecules occur in this range), which is designated as one ***Svedberg unit***. For instance, the sedimentation coefficient of hemoglobin is 4×10^{-13} s or 4S; ribonuclease is 2×10^{-13} s or 2S. Conventionally, the subcellular organelles are often referred to by their S value e.g. 70S ribosome.

Isolation of subcellular organelles by centrifugation

The cells are subjected to disruption by sonication or osmotic shock or by use of homogenizer. This is usually carried out in an isotonic (0.25 M) sucrose. The advantage with sucrose medium is that it does not cause the organelles to swell. The subcellular particles can be separated by differential centrifugation. The most commonly employed laboratory method separates subcellular organelles into 3 major fractions—nuclear, mitochondrial and microsomal (***Fig.41.10***).

When the homogenate is centrifuged at 700 g for about 10 min, the nuclear fraction (includes plasma membrane) gets sedimented. On centrifuging the supernatant (I) at 15,000 g for about 5 min mitochondrial fraction (that includes lysosomes, peroxisomes) is pelleted. Further centrifugation of the supernatant (II) at 100,000 g for about 60 min separates microsomal fraction (that includes ribosomes and endoplasmic reticulum). The supernatant (III) then obtained corresponds to the cytosol.

The ***purity*** (or contamination) of the subcellular fractionation can be ***checked by*** the use of ***marker enzymes.*** DNA polymerase is the marker enzyme for nucleus, while glutamate dehydrogenase and glucose 6-phosphatase are the markers for mitochondria and ribosomes, respectively. Hexokinase is the marker enzyme for cytosol.

RADIOIMMUNOASSAY

Radioimmunoassay (RIA) was developed in 1959 by Solomon, Benson and Rosalyn Yalow for the estimation of insulin in human serum. This technique has revolutionized the estimation of several compounds in biological fluids that are found in exceedingly low concentrations (nanogram or picogram). RIA is a highly sensitive and specific analytical tool.

Principle

Radioimmunoassay combines the principles of ***radioactivity*** of isotopes and ***immunological*** reactions of antigen and antibody, hence the name.

The principle of RIA is primarily based on the competition between the labelled and unlabelled antigens to bind with antibody to form antigen-antibody complexes (either labelled or unlabelled). The unlabelled antigen is the substance (say insulin) to be determined. The antibody to it is produced by injecting the antigen to a goat or a rabbit. The specific antibody (Ab) is then subjected to react with unlabelled antigen in the presence of excess amounts of isotopically labelled (^{131}I) antigen (Ag^+) with known radioactivity. There occurs a competition between the antigens (Ag^+ and Ag) to bind the antibody. Certainly, the labelled Ag^+ will have an upper hand due to its excess presence.

$$Ag^+ + Ab \longrightarrow Ag^+Ab$$

Ag → Ag^+

Ag-Ab

As the concentration of unlabelled antigen (Ag) increases the amount of labelled antigen-antibody complex (Ag^+-Ab) decreases. Thus, the concentration of Ag^+-Ab is inversely related to the concentration of unlabelled Ag i.e. the substance to be determined. This relation is almost linear. A standard curve can be drawn by using different concentrations of unlabelled antigen and the same quantities of antibody and labelled antigen.

The labelled antigen-antibody (Ag^+-Ab) complex is separated by precipitation. The radioactivity of ^{131}I present is Ag^+-Ab is determined.

Applications

RIA is no more limited to estimating of hormones and proteins that exhibit antigenic properties. By the use of ***haptens*** *(small molecules such as dinitrophenol, which, by themselves, are not antigenic)*, several substances can be made antigenic to elicit specific antibody responses. In this way, a wide variety of compounds have been brought under the net of RIA estimation. These include peptides, steroid hormones, vitamins, drugs, antibiotics, nucleic acids, structural proteins and hormone receptor proteins.

Radioimmunoassay has tremendous application in the diagnosis of hormonal disorders, cancers and therapeutic monitoring of drugs, besides being useful in biomedical research.

ENZYME-LINKED IMMUNOSORBANT ASSAY

Enzyme-linked immunosorbant assay (***ELISA***) is a non-isotopic immunoassay. An enzyme is used as a label in ELISA in place of radioactive isotope employed in RIA. ELISA is as sensitive as or even more sensitive than RIA. In addition, there is no risk of radiation hazards (as is the case with RIA) in ELISA.

Principle

ELISA is based on the ***immunochemical principles of antigen-antibody reaction.*** The stages of ELISA, depicted in ***Fig.41.11***, are summarized.

1. The antibody against the protein to be determined is fixed on an inert solid such as polystyrene.

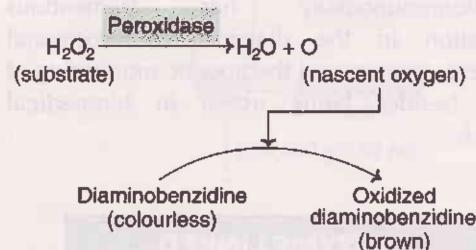
2. The biological sample containing the protein to be estimated is applied on the antibody coated surface.

3. The protein antibody complex is then reacted with a second protein specific antibody to which an enzyme is covalently linked. These enzymes must be easily assayable and produce preferably coloured products. Peroxidase, amylase and alkaline phosphatase are commonly used.

4. After washing the unbound antibody linked enzyme, the enzyme bound to the second antibody complex is assayed.

5. The enzyme activity is determined by its action on a substrate to form a product (usually coloured). This is related to the concentration of the protein being estimated.

The principle for the use of the enzyme peroxidase in ELISA is illustrated next.



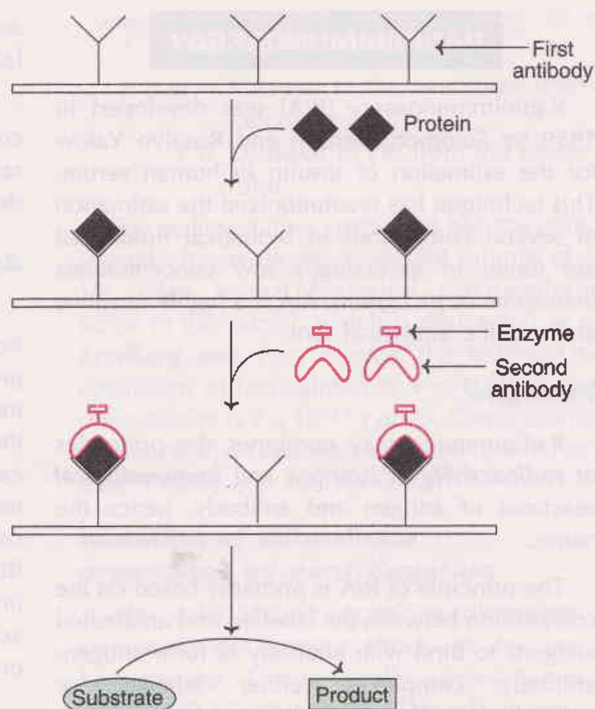


Fig. 41.11 : Diagrammatic representation of enzyme-linked immunosorbant assay (ELISA).

Applications

ELISA is widely used for the ***determination of*** small quantities of ***proteins*** (hormones, antigens, antibodies) and other biological substances. The most commonly used pregnancy test for the detection of human chorionic gonadotropin (hCG) in urine is based on ELISA. By this test, ***pregnancy*** can be detected within few days after conception. ELISA is also been used for the diagnosis of ***AIDS***.

HYBRIDOMA TECHNOLOGY

Conventional methods adopted in the laboratory for the production of antisera against antigens lead to the formation of heterogeneous antibodies. Among these antibodies a few may have the desired properties but are found with many other antibodies which undoubtedly are not required. A simple, convenient and desirable method for the large scale production of specific antibodies remained a dream for immunologists for a long period. In 1975, George Kohler and Cesar Milstein (Nobel Prize 1984) made this dream a reality. They created hybrid cells that will make unlimited quantities of antibodies with defined specificities, which are termed as ***monoclonal antibodies (McAb).*** This discovery, often referred to as hybridoma technology, has revolutionized methods for antibody production.

Principle

This is based on the ***fusion between myeloma cells*** (malignant plasma cells) ***and spleen cells*** from a suitably immunized animal. Spleen cells die in a short period under ordinary tissue culture conditions while myeloma cells are adopted to grow permanently in culture. Mutants of

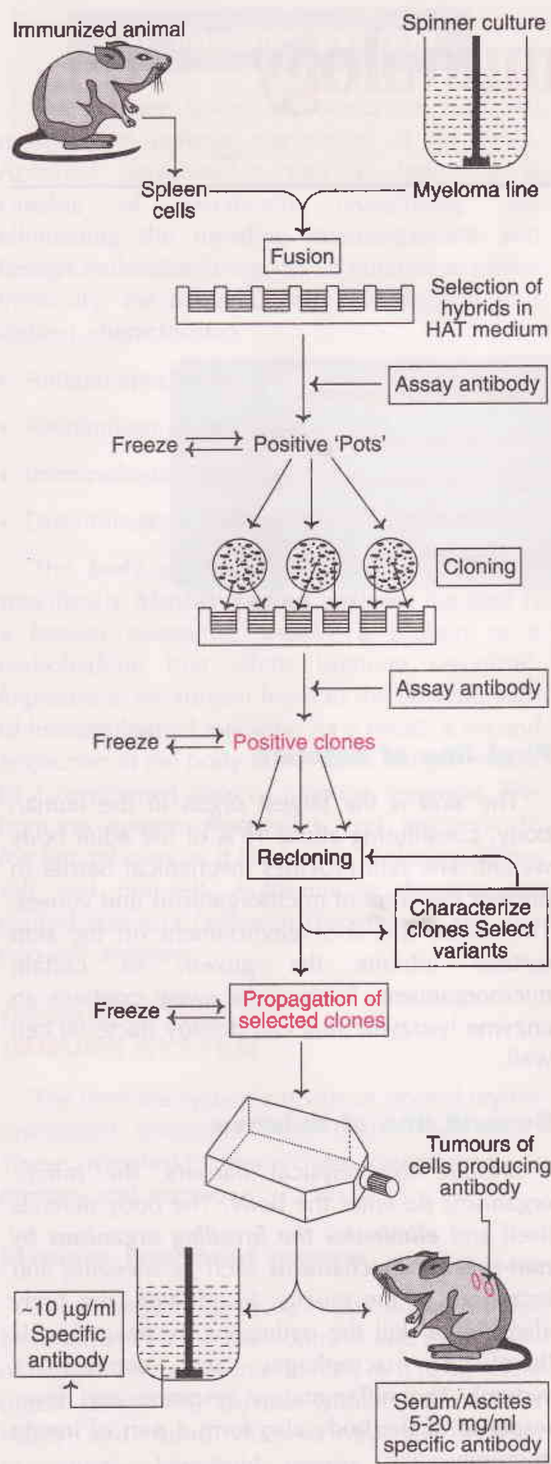


Fig. 41.12 : Basic protocol for the derivation of monoclonal antibodies from hybrid myelomas.

myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyltransferase (azaquinine resistant) or thymidine kinase (bromodeoxyuridine resistant). These mutants cannot grow in a medium containing aminopterin, supplemented with hypoxanthine and thymidine (HAT medium). Hybrids between the mutant myeloma cells and spleen cells can be selected and cultured in HAT medium.

From the growing hybrids, individual clones can be chosen that secrete the desired antibodies (monoclonal origin). The selected clones like ordinary myeloma cells can be maintained indefinitely. In short, the hybridoma technology for the production of monoclonal antibodies involves the following steps.

1. Immunization of appropriate animals with antigen (need not be pure) under study.

2. Fusion of suitable drug resistant myeloma cells with plasma cells, obtained from the spleen of the immunized animal.

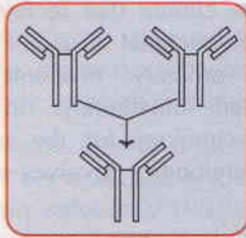
3. Selection and cloning of the hybrid cells that grow in culture and produce antibody molecules of desired class and specificity against the antigen of interest.

Hybridoma technology can make available highly specific antibodies in abundant amounts. The clones once developed are far cheaper than the traditionally employed animals (horses, rabbits) for producing antibodies. The clones developed from the hybrids will also ensure constancy of the quality of the product and will also avoid the batch to batch variation inherent in the conventional methods.

Applications of monoclonal antibodies

The antibodies produced by hybridoma technology have been widely used for a variety of purposes. These include the ***early detection of pregnancy***, detection and treatment ***of cancer***, diagnosis of leprosy and treatment of autoimmune diseases.

42 Immunology



The immunology speaks :

"I represent the defense system of the body;
Mainly composed of β-lymphocytes and T-lymphocytes;
Designed to eliminate invading microbes and moles;
My memory can distinguish self, and non-self."

Immunology deals with the study of immunity and immune systems of vertebrates. Immunity (immunis literally means exempt/free from burden) broadly involves the ***resistance shown***, and ***protection offered by the host organism against the infectious diseases***. The immune system consists of a complex network of cells and molecules, and their interactions. It is specifically designed to eliminate infectious organisms from the body. This is possible since the organism is capable of distinguishing the self from non-self, and eliminate non-self.

Immunity is broadly divided into two types — innate (non-specific) immunity and adaptive or acquired (specific) immunity.

INNATE IMMUNITY

Innate immunity is non-specific, and represents the inherent capability of the organism to offer resistance against diseases. It consists of defensive barriers.

First line of defense

The ***skin*** is the largest organ in the human body, constituting about 15% of the adult body weight. The skin provides mechanical barrier to prevent the entry of microorganisms and viruses. The acidic (pH 3-5) environment on the skin surface inhibits the growth of certain microorganisms. Further, the sweat contains an enzyme lysozyme that can destroy bacterial cell wall.

Second line of defense

Despite the physical barriers, the micro-organisms do enter the body. The body defends itself and ***eliminates the invading organisms by non-specific mechanisms*** such as sneezing and secretions of the mucus. In addition, the body also tries to kill the pathogens by phagocytosis (involving macrophages and complement system). The inflammatory response and fever response of the body also form a part of innate immunity.

THE IMMUNE SYSTEM

The immune system represents the third and most potent defense mechanism of the body. Acquired (adaptive or specific) immunity is capable of specifically recognizing and *eliminating the invading microorganisms and* foreign molecules (antigens). In contrast to innate immunity, the acquired immunity displays four distinct characteristics

- Antigen specificity
- Recognition diversity
- Immunological memory
- Discrimination between self and non-self.

The ***body*** possess tremendous capability to specifically ***identify various antigens*** (antigen is a foreign substance, usually a protein or a carbohydrate that elicits immune response). Exposure to an antigen leads to the development of immunological memory. As a result, a second encounter of the body to the same antigen results in a heightened state of immune response. The immune system recognizes and responds to foreign antigens as it is capable of distinguishing self and non-self. Autoimmune diseases are caused due to a failure to discriminate self and non-self antigens.

ORGANIZATION OF IMMUNE SYSTEM

The immune system consists of several organs distributed throughout the body (***Fig. 42.1***). These lymphoid organs are categorized as primary and secondary.

Primary lymphoid organs

These organs provide appropriate micro-environment for the development and maturation of antigen-sensitive lymphocytes (a type of white blood cells). The ***thymus*** (situated above the heart) and bone marrow are the central or primary lymphoid organs. T-lymphocyte maturation occurs in the thymus while B-lymphocyte maturation takes place in the bone marrow.

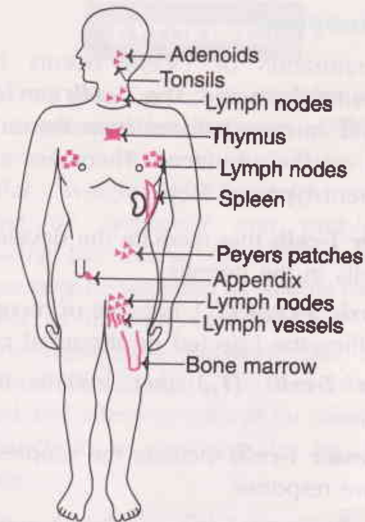


***Fig. 42.1 :** A diagrammatic representation of human lymphatic system.*

Secondary lymphoid organs

These are the sites for the initiation of immune response. e.g. spleen, tonsils, lymph nodes, appendix, Peyers patches in the gut. Secondary lymphoid organs provide the microenvironment for interaction between antigens and mature lymphocytes.

CELLS OF THE IMMUNE SYSTEM

Two types of lymphocytes namely B-cells and T-cells are critical for the immune system. In addition, several accessory cells and effector cells also participate.

B-lymphocytes

The site of development and maturation of B-cells occurs in ***b***ursa fabricius in birds, and ***b***one marrow in mammals. During the course of immune response, B-cells mature into plasma cells and secrete antibodies (immunoglobulins).

The B-cells possess the capability to specifically recognize each antigen and produce antibodies (i.e. immunoglobulins) against it. B-lymphocytes are intimately associated with ***humoral immunity. Immunoglobulins are described in Chapter 9.***

T-lymphocytes

The maturation of T-cells occurs in the thymus, hence the name. The ***T-cells*** can identify viruses and microorganisms from the antigens displayed on their surfaces. There are at least four different types of T-cells.

- ***Inducer T-cells*** that mediate the development of T-cells in the thymus.
- ***Cytotoxic T-cells*** (T_C), capable of recognizing and killing the infected or abnormal cells.
- ***Helper T-cells*** (T_H) that initiate immune responses.
- ***Suppressor T-cells*** mediate the suppression of immune response.

T-lymphocytes are responsible for the ***cell-mediated immunity***.

MAJOR HISTOCOMPATIBILITY COMPLEX

The major histocompatibility complex (***MHC***) represents a special group of ***proteins, present on the cell surfaces of T-lymphocytes***. MHC is involved in the recognition of antigens on T-cells. It may be noted here that the B-cell receptors (antibodies) can recognize antigens on their own, while T-cells can do so through the mediation of MHC.

In humans, the MHC proteins are encoded by a cluster of genes located on chromosome 6 (it is on chromosome 17 for mice). The major histocompatibility complex in humans is referred to as ***human leukocyte antigen (HLA)***. Three classes of MHC molecules (chemically glycoproteins) are known in human. Class I molecules are found on almost all the nucleated cells of the body. Class II molecules are associated only with leukocytes involved in cell-mediated immune response. Class III molecules are the secreted proteins possessing immune functions e.g. complement components (C_2, C_4), tumor necrosis factor.

THE COMPLEMENT SYSTEM

The complement system is composed of about 20 plasma proteins that 'complement' the function of antibodies in defending the body from the invading antigens. The complementary factors are heat labile and get inactivated if heated at 56°C for about 30 minutes. The complement system helps the body immunity in 4 ways

1. **Complement fixation :** The complement system binds to the foreign invading cells and causes lysis of the cell membranes.

2. **Opsonization :** The process of promoting the phagocytosis of foreign cells is referred to as opsonization.

3. **Inflammatory reaction :** The complement system stimulates local inflammatory reaction and attracts phagocytic cells.

4. **Clearance of antigen-antibody complexes :** The complement system promotes the clearance of antigen-antibody complexes from the body.

Nomenclature of complement system : The complement proteins are designated by the letter 'C', followed by a component number—C_1, C_2, C_3 etc.

Types of reaction : The complement system brings about two sets of reactions :

1. Antibody dependent classical pathway.
2. Antibody independent alternative pathway.

Each one of the pathways consists of a series of reactions converting inactive precursors to active products by serine proteases which resembles blood coagulation.

THE IMMUNE RESPONSE

The immune response refers to the series of reactions carried out by the immune system in the body against the foreign invader. When an infection takes place or when an antigen enters the body, it is trapped by the macrophages in lymphoid organs. The phagocytic cells which are guarding the body by constant patrolling engulf and digest the foreign substance. However, the partially digested antigen (i.e. processed antigen) with antigenic epitopes attaches to lymphocytes.

T-helper cells (T_H) play a key role the immune response (***Fig. 42.2***). This is brought out through

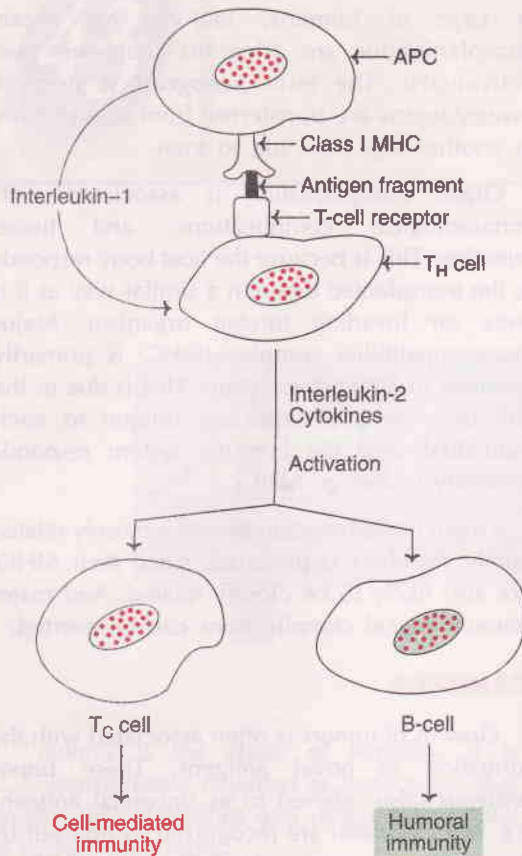


Fig. 42.2 : A diagrammatic representation of the central role of helper T cells (T_H cells) in immune response (APC—Antigen presenting cell; T_c cell—Cytotoxic T-cell).

the participation of antigen presenting cell (APC), usually a macrophage. Receptors of T_H cell bind to class II MHC-antigen complex displayed on the surface of APC. APC secretes interleukin-I, which activates the T_H cell. This activated T_H cell actively grows and divides to produce clones of T_H cells. All the T_H cells possess receptors that are specific for the MHC-antigen complex. This facilitates triggering of immune response in an exponential manner. The T_H cells secrete interleukin-2 which promotes the prolifiration of cytotoxic T cells (T_C cells) to attack the infected cells through cell-mediated immunity. Further, interleukin-2 also activates B-cells to produce immunoglobulins which perform humoral immunity.

CYTOKINES

Cytokines are a group of ***proteins*** that ***bring*** about ***communication between different cell types*** involved in immunity. They are low molecular weight glycoproteins and are produced by *lymphoid and non-lymphoid* cells during the course of immune response. Cytokines may be regarded as soluble messenger molecules of immune system. They can act as short messengers between the cells or long range messengers by circulating in the blood and affecting cells at far off sites. The latter function is comparable to that of hormones.

The term ***interleukin (IL)*** is frequently used to represent cytokines. There are more than a dozen interleukins (IL-I......IL_{12}), produced by different cells with wide range of functions. The main function (directly or indirectly) of cytokines is to ***amplify immune responses*** and inflammatory responses.

Therapeutic uses of cytokines

It is now possible to produce cytokines *in vitro*. Some of the cytokines have potential applications in the practice of medicine. For instance, IL-2 is used in cancer immunotherapy, and in the treatment of immunodeficiency diseases. IL-2 induces the proliferation and differentiation of T-and B-cells, besides increasing the cytotoxic capacity of natural killer cells.

A group of cytokines namely interferons can combat viral infection by inhibiting their replication.

IMMUNITY IN HEALTH AND DISEASE

The prime function of immune system is to protect the host against the invading pathogens. The body tries its best to overcome various strategies of infectious agents (bacteria, viruses), and provides immunity.

Some of the important immunological aspects in human health and disease are briefly described.

AUTOIMMUNE DISEASES

In general, the immune system is self-tolerant i.e. not responsive to cells or proteins of self. Sometimes, for various reasons, the ***immune system fails to discriminate between self and non-self***. As a consequence, the cells or tissues of the body are attacked. This phenomenon is referred to as ***autoimmunity*** and the diseases are regarded as autoimmune diseases. The antibodies produced to self molecules are regarded as ***autoantibodies***. Some examples of autoimmune diseases are listed.

- Insulin-dependent diabetes (pancreatic β-cell autoreactive T-cells and antibodies).
- Rheumatoid arthritis (antibodies against proteins present in joints).
- Myasthenia gravis (acetylcholine receptor autoantibodies).
- Autoimmune hemolytic anemia (erythrocyte autoantibodies).

Mechanism of autoimmunity : It is widely accepted that autoimmunity generally occurs as a consequence of ***body's response against bacterial, viral or any foreign antigen***. Some of the epitopes of foreign antigens are similar (homologous) to epitopes present on certain host proteins. This results in cross reaction of antigens and antibodies which may lead to autoimmune diseases.

ORGAN TRANSPLANTATION

The phenomenon of transfer of cells, tissues or organs from one site to another (in the same organism, ***autograft*** or from another organism ***allograft***) is regarded as organ transplantation. In case of humans, majority of organ transplantations are allografts (between two individuals). The term ***xenograft*** is used if tissues/organs are transferred from one species to another e.g. from pig to man.

Organ transplantation is associated with immunological complications, and tissue rejection. This is because the host body responds to the transplanted tissue in a similar way as if it were an invading foreign organism. Major histocompatibility complex (MHC) is primarily involved in allograft rejection. This is due to the fact that MHC proteins are unique to each individual, and the immune system responds promptly to foreign MHCs.

Organ transplantation between closely related family members is preferred, since their MHCs are also likely to be closely related. And major immunological complications can be averted.

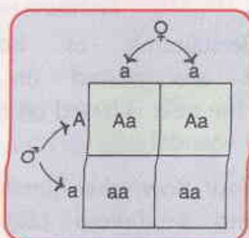
CANCERS

Growth of tumors is often associated with the formation of novel antigens. These ***tumor antigens*** (also referred to as oncofetal antigens e.g. α-fetoprotein) are recognized as non-self by the immune systems. However, tumors have developed several mechanisms to evade immune responses.

AIDS

Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus, is characterized by immunosuppression, secondary neoplasma and neurological manifestations. AIDS primarily affects the cell-mediated immune system which protects the body from intracellular parasites such as viruses, and bacteria. Most of the immunodeficiency symptoms of AIDS are associated with a reduction in CD_4 (cluster determinant antigen 4) cells.

43 Genetics



The genetics speaks :

"I am the science for the study of heredity,
With DNA as the chemical of inheritance;
Transmitting characters from parents to offsprings;
Mutations and chromosomal abnormalities result in diseases."

Genetics is the ***study of heredity***. It is appropriately regarded as the science that explains the similarities and differences among the related organisms.

The blood theory of inheritance in humans

For many centuries, it was customary to explain inheritance in humans through blood theory. People used to believe that the children received blood from their parents, and it was the union of blood that led to the blending of characteristics. That is how the terms 'blood relations', 'blood will tell', and 'blood is thicker than water' came into existence. They are still used, despite the fact that blood is no more involved in inheritance. With the advances in genetics, the more appropriate terms should be as follows

- ***Gene relations*** in place of blood relations.
- ***Genes will tell*** instead of blood will tell.

BRIEF HISTORY AND DEVELOPMENT OF GENETICS

Genetics is relatively young, not even 150 years. The blood theory of inheritance was questioned in 1850s, based on the fact that the semen contained no blood. Thus, blood was not being transferred to the offspring. Then the big question was what was the hereditary substance.

Mendel's experiments : It was in 1866, an Austrian monk named Gregor Johann Mendel, for the first time reported the fundamental laws of inheritance. He conducted several experiments on the breeding patterns of pea plants. Mendel put forth the ***theory of transmissible factors*** which states that inheritance is controlled by ***certain factors*** passed from parents to offsprings. His results were published in 1866 in an obscure journal Proceedings of the Society of Natural Sciences.

For about 35 years, the observations made by Mendel went unnoticed, and were almost

forgotten. Two European botanists (Correns and Hugo de Vries) in 1900, independently and simultaneously rediscovered the theories of Mendel. The year 1900 is important as it marks the beginning the modern era of genetics.

The origin of the word gene : In the early years of twentieth century, it was believed that the Mendel's inheritance factors are very closely related to chromosomes (literally coloured bodies) of the cells. It was in 1920s, the term ***gene*** (derived from a Greek word *gennan* meaning to produce) was introduced by Willard Johannsen. Thus, ***gene replaced the earlier terms inheritance factor or inheritance unit.***

Chemical basis of heredity : There was a controversy for quite sometime on the chemical basis of inheritance. There were two groups— the protein supporters and DNA supporters. It was in 1944, Avery and his associates presented convincing evidence that the chemical basis of heredity lies in DNA, and not in protein. Thus, ***DNA*** was finally identified as ***the genetic material.*** Its structure was elucidated in 1952 by Watson and Crick.

Importance of genes in inheritance—studies on twins

Monozygotic or ***identical twins*** contain the ***same*** genetic material — ***DNA or genes.*** Studies conducted on identical twins make startling revelations with regard to inheritance. One such study is described here.

Oskar Stohr and Jack Yufe were identical twins separated at birth. Oskar was taken to Germany where he was brought up by his grandmother as a Christian. Jack was raised by his father in Israel as a Jew. The two brothers were reunited at the age of 47. Despite the different environmental influences, their behavioural patterns and personalities were remarkably similar

- Both men had moustaches, wore two pocket shirts, and wire-rimmed glasses.
- Both loved spicy foods and tended to fall asleep in front of television.
- Both flushed the toilet before using.
- Both read maganizes from back to front.
- Both stored rubber bands on their wrists.
- Both liked to sneeze in a room of strangers.

Besides Oskar and Jack, many other studies conducted on identical twins point out the importance of genes on the inherited characters related to personality and mannerisms.

BASIC PRINCIPLES OF HEREDITY IN HUMANS

The understanding of how genetic characteristics are passed on from one generation to the next is based on the principles developed by Mendel.

As we know now, the human genome is organized into a diploid (2n) set of 46 chromosomes. They exist as ***22 pairs of autosomes*** and ***one pair of sex chromosomes (XX/XY).*** During the course of meiosis, the chromosome number becomes haploid (n). Thus, haploid male and female gametes — sperm and oocyte respectively, are formed. On fertilization of the oocyte by the sperm, the diploid status is restored. This becomes possible as the zygote receives one member of each chromosome pair from the father, and the other from the mother. As regards the sex chromosomes, the males have X and Y, while the females have XX. The sex of the child is determined by the father.

Monogenic and polygenic traits

The genetic traits or characters are controlled by single genes or multiple genes. The changes in genes are associated with genetic diseases.

Monogenic disorders : These are the ***single gene disease traits*** due to alterations in the corresponding gene e.g. sickle-cell anemia, phenylketonuria. Inheritance of monogenic disorders usually follows the Mendelian pattern of inheritance.

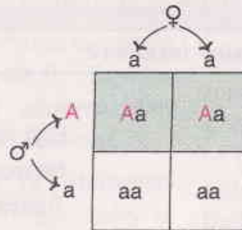
Polygenic disorders : The genetic traits conferred ***by more than on gene*** (i.e multiple genes), and the disorders associated with them are very important e.g. height, weight, skin colours, academic performance, blood pressure, aggressiveness, length of life.

(A) Autosomal dominant

PARENTS

Male (♂)
Genotype - Aa
Phenotype - Affected male

Female (♀)
Genotype - aa
Phenotype - Normal female



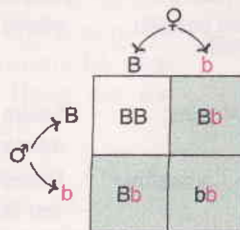
CHILDREN
Genotype ratio - 1 : 1 Aa to aa
Phenotype - 50% affected
-50% normal

(B) Autosomal recessive

PARENTS

Male (♂)
Genotype - Bb
Phenotype - Carrier male

Female (♀)
Genotype - Bb
Phenotype - Carrier female



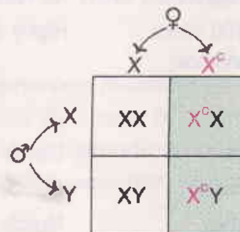
CHILDREN
Genotype ratio - 1 : 2 : 1 BB/Bb/Bb/bb
Phenotype - 25% affected
-25% normal
-50% carriers

(C) X-chromosome (sex chromosome)–linked inheritance

PARENTS

Male (♂)
Genotype - XY
Phenotype - Normal male

Female (♀)
Genotype - X^cX
Phenotype - Carrier female



CHILDREN
Genotype ratio - 1 : 1 : 1 : 1 XX/XY/X^cX/X^cY
Phenotype - 50% of males affected

Fig. 43.1 : *Patterns of inheritance-autosomal dominant, autosomal recessive and X-linked (**Note :** Genotype refers to the description of genetic composition, while phenotype represents the observable character displayed by an organism).*

PATTERNS OF INHERITANCE

The heredity is transmitted from parent to offspring as individual characters controlled by genes. The genes are linearly distributed on chromosomes at fixed positions called ***loci***.

A gene may have different forms referred to as ***alleles***. Usually one allele is transferred from the father, and the other from the mother. The allele is regarded as ***dominant*** if the trait is exhibited due to its presence. On the other hand, the allele is said to be ***recessive*** if its effect is masked by a dominant allele. The individuals are said to be ***homozygous*** if both the alleles are the same. When the alleles are different they are said to be ***heterozygous***.

*The pattern of inheritance of monogenic traits may occur in the following ways (**Fig. 43.1**).*

1. Autosomal dominant
2. Autosomal recessive
3. *Sex-linked.*

1. **Autosomal dominant inheritance :** A normal allele may be designated as ***a*** while an autosomal dominant disease allele as ***A*** (***Fig. 43.1A***). The male with Aa genotype is an affected one while the female with aa is normal. Half of the genes *from the affected male will* carry the disease allele. On mating, the male and female gametes are mixed in different combinations. The result is that ***half of the***

Table 43.1 Selected examples of genetic disorders (monogenic traits) in humans

Inherited pattern/disease	*Estimated incidence*	*Salient features*
Autosomal dominant		
Familial hypercholesterolemia	1 : 500	High risk for heart diseases
Huntington's disease	1 : 5000	Nervous disorders, dementia
Familial retinoblastoma	1 : 12000	Tumors of retina
Breast cancer genes *(BRAC 1 and 2)*	1 : 800	High risk for breast and ovarian cancers
β-Thalassemia	1 : 2500 (in people of Mediterranean descent)	A blood disorder; the blood appears to be blue instead of red
Autosomal recessive		
Sickle-cell anemia	1 : 100 (in Africans)	Severe life threatening anemia; confers resistance to malaria
Cystic fibrosis	1 : 2500 (in Caucasians)	Defective ion transport; severe lung infections and early death (before they reach 30 years)
Phenylketonuria	1 : 2000	Mental retardation due to brain damage
α_1-Antitrypsin deficiency	1 : 5000	Damage to lungs and liver
Tay-Sachs disease	1 : 3000 (in Ashkenazi Jews)	Nervous disorder; blindness and paralysis
Severe combined immunodeficiency disease (SCID)	Rare (only 100 cases reported worldwide)	Highly defective immune system; early death
Sex-linked		
Colour blindness	1 : 50 males	Unable to distinguish colours
Hemophilia (A/B)	1 : 10,000 males	Defective blood clotting
Duchenne muscular dystrophy	1 : 7000 males	Muscle wastage
Mitochondrial		
Leber hereditary optic neuropathy	Not known	Damage to optic nerves, may lead to blindness

children will be heterozygous (Aa) and ***have the disease***. Example of autosomal dominant inherited diseases are familial hypercholesterolemia, β-thalassemia, breast cancer genes.

2. **Autosomal recessive inheritance :** In this case, the normal allele is designated as ***B*** while the disease-causing one is ***a*** (***Fig. 43.1B***). The gametes of carrier male and carrier female (both with genotype Bb) get mixed. For these heterozygous carrier parents, there is one fourth chance of having an affected child. Cystic fibrosis, sickle-cell anemia and phenylketonuria are some good examples of autosomal recessive disorders.

3. **Sex (X)-linked inheritance :** In the ***Fig. 43.1C***, sex-linked pattern of inheritance is depicted. A normal male (XY) and a carrier female (X^cY) will produce children wherein, half of the male children are affected while no female child is affected. This is due to the fact that the male children possess only one X chromosome, and there is no dominant allele to mark its effects (as is the case with females). Colour blindness and hemophilia are good examples of X-linked diseases.

A selected list of genetic disorders (monogenic traits) due to autosomal and sex-linked inheritance in humans is given in ***Table 43.1***.

GENETIC DISEASES IN HUMANS

The pattern of inheritance and monogenic traits along with some of the associated disorders are described above (***Table 43.1***). Besides ***gene mutations***, ***chromosomal abnormalities*** (aberrations) also result in genetic diseases.

Aneuploidy : The presence of ***abnormal number of chromosomes*** within the cells is referred to as aneuploidy. The most common aneuploid condition is ***trisomy*** in which three *copies of a particular chromosome are present in a cell instead of the normal two e.g. trisomy-21* causing ***Down's syndrome***; trisomy-18 that results in ***Edward's syndrome***. These are the examples of autosomal aneuploidy.

In case of sex-linked aneuploidy, the sex chromosomes occur as three copies. e.g. phenotypically male causing Klinefelter's syndrome has XXY; trisomy-X is phenotypically a female with XXX.

EUGENICS

Eugenics is a ***science of improving human race based on genetics***. Improving the traits of plants and animals through breeding programmes has been in practice for centuries.

Eugenics is a highly controversial subject due to social, ethical, and political reasons. The proponents of eugenics argue that people with desirable and good traits (good blood) should reproduce while those with undersirable characters (bad blood) should not. The advocates *of eugenics, however, do not force any policy,* but they try to convince the people to perform their duty voluntarily. The object of eugenics is to ***limit the production of people who are unfit to live in the society***.

Eugenics in Nazi Germany

Germany developed its own eugenic programme during 1930s. A law on eugenic sterilization was passed in 1933. In a span of three years, compulsory sterilization was done on about 250,000 people, who allegedly suffered from hereditary disabilities, feeble mindedness, epilepsy, schizophrenia, blindness, physical deformaties, and drug or alcohol addiction.

The German Government committed many atrocities in the name of racial purity. Other countries, however do not support this kind of eugenics.