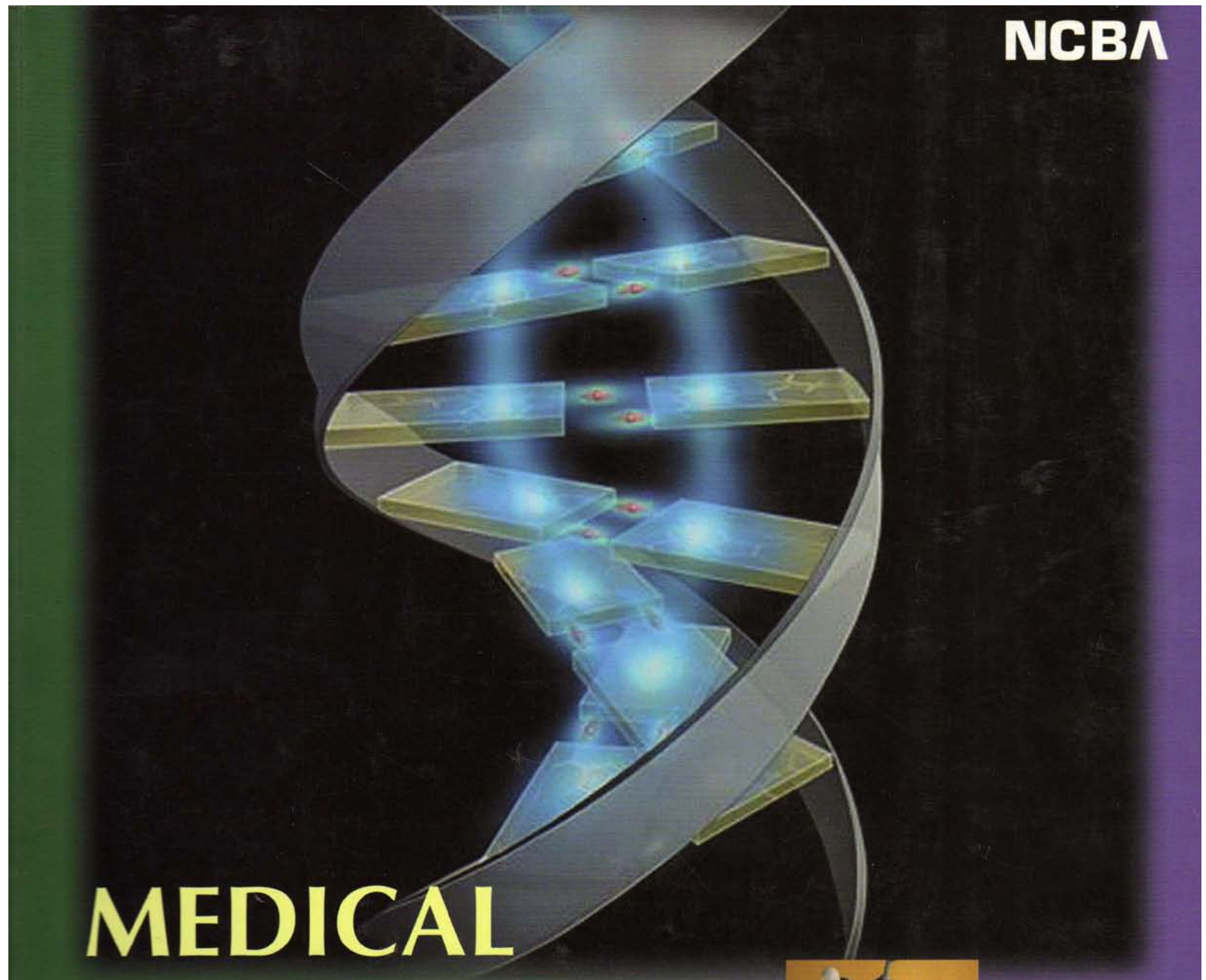
MEDICAL BIOCHEMISTRY AND BIOTECHNOLOGY

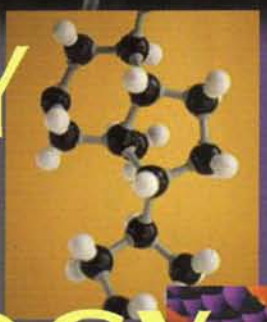
For Medicine and Allied courses

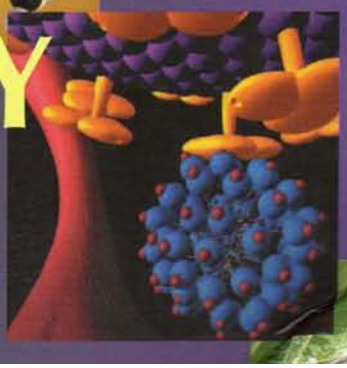
Mohammed Amanullah

NCBA



MEDICAL BIOCHEMISTRY *and* BIOTECHNOLOGY





Dr Mohammed Amanullah

REGD. OFFICE

8/1 Chintamoni Das Lane, Kolkata 700 009, India
email: ncbapvtltd@eth.net

OVERSEAS

27 Catherine Court, Chase Road, Southgate, London, N14 4RB

EXPORT

212 Shahpur Jat, New Delhi 110 049
email: ncbaexp@ncbapvtltd.com

BRANCHES

208B Peacock Lane, Shahpur Jat
New Delhi 110 049
email: ncbadel@ncbapvtltd.com

House No. 3-1-315, 1st Floor
Nimboliadda, Kachiguda, Hyderabad 500 027
email: ncbahydb@ncbapvtltd.com

Shop Nos. 3 & 4, Vinayak Towers
681/B Budhwar Peth, Appa Balwant Chowk
Pune 411 002
email: ncbapune@ncbapvtltd.com

GSS Shopping Complex, 1st Floor
Opposite College Post Office, Convent Road
Ernakulam 682 035
email: ncbaernk@ncbapvtltd.com

MEDICAL BIOCHEMISTRY AND BIOTECHNOLOGY

First Published: 2011

PUBLISHER AND TYPESETTER

New Central Book Agency (P) Ltd
8/1 Chintamoni Das Lane, Kolkata 700 009

PRINTER

New Central Book Agency (P) Ltd
Web-Offset Division, Dhulagarh, Sankrail, Howrah

COVER DESIGNER

Soumen Paul

COVER PRINTER

Liba Graphics, Kolkata

TECHNICAL EDITOR

Dr Mita Sen, MBBS

PROJECT TEAM

Prabhat Jas, Suman Dutta, Dipan Roy

ISBN: 978-81-7381-615-4

Price: Rs 585.00

ACKNOWLEDGEMENTS

A text book on any subject cannot be written without the help and assistance of a number of people. They include peers, contemporaries, colleagues, students, technical and non-technical assistants, etc. Therefore, it has to be acknowledged that I owe much to many people, who I will not able to thank individually. Yet, I feel that I need to put on record my sincere gratitude to the following people for their guidance, help and assistance.

The seed for the preparation of this textbook was sown in my mind by one of my students, Mr. Sridhar, who suggested that I should compile all my earlier lectures and notes into a book for the benefit of undergraduate students, who find it difficult to comprehend the complexities of other books on the subject. I am thankful to him for his suggestion.

I am deeply indebted to Dr. B. M. Rudresh, Professor & Head, Department of Biochemistry, AIMedical College, Mandya, India for his guidance and encouragement. I am grateful to Dr. Naseem Begum and Dr. Mohammed Muzaffar Shakeel for their valuable suggestions. I am thankful to all my colleagues, friends and relatives for their moral support.

I acknowledge that the material contained in this book is sourced from the standard literature available. I have tried to compile as much of the subject as necessary, and as little as possible, so as to fulfill the needs of undergraduate students of Medicine and Allied Sciences.

This book would not have been completed, but for the constant support and assistance of my wife Razia Rashida Parveen, who has been with me, through thick and thin, at every step and every stage of our married life. She has been deeply involved in the preparation of the manuscript and illustrations of this book. I am thankful to her for everything.

I am thankful to my parents for providing me with the best of education and upbringing. Their love and affection have been a constant source of strength throughout my life.

Finally, I supplicate myself to Almighty God for His Grace and Mercy. All Praise is due, to Him Alone.

Dr. Mohammed Amanullah 14th March 2012

CONTENTS

1 CHAPTER

BIOCHEMISTRY

SCOPE AND IMPORTANCE IN MEDICAL SCIENCES

Biochemistry is the answer to an innocent mind questioning itself as to—What is life? What is growth? What is maturity? What is a disease? And finally, what is death? In short, answers to two of these most important questions can be cited here.

Q **What is life?**

Ans Life is an organized state of a few elements.

Q **What is Death?**

Ans Death is a state of disorganization of these elements.

It is said that man is made out of mud and there are about 93 elements in the mud or the earth's crust. Among these many elements, only a few of them arrange in a specific order to give rise to life. Any disorder in these elements results in a disease which may lead to death. Therefore, Biochemistry is the study of elements which make life and the fashion in which they are arranged. It also explains the functioning of these elements which trigger and continue the process of life. The extent of de-arrangement of the life making elements, may either lead to disease or cause death.

Medical professionals undertake Biochemistry in order to deal specifically with different methodologies of prevention and rectification of various disorders thus bringing back the body to function normally thereby helping in leading a healthy life and delaying the process of death.

Human body is made up of myriad of molecules (union of elements). What are these molecules? What are their properties and characteristics? How are they aligned with each other to make possible the living state? In this book we will study the molecules forming the living matter and correlation of biological function with the molecular structure. From its study, Biochemistry has come to the conclusion that, matter in an appropriate physico-chemical state of organization acquires the traits of living substances.

Let us now list out exactly those elements that form life. The elements that give rise to life can be grouped into two categories—

1. **Major elements:** The elements which are found in large quantities in the human body are major elements. They include—carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), sulphur (S) and calcium (Ca).

2. **Minor elements:** The elements which are found in lesser quantities in the human body are minor-elements. They are further classified into two groups—
   (a) **Macro-elements:** They include the elements that are present in relatively appreciable amounts in the body, viz., iron (Fe), sodium (Na) and potassium (K).
   (b) **Micro-elements:** They include the elements that are present in minute quantities in the body, viz., cobalt (Co), copper (Cu), magnesium (Mg), manganese (Mn), arsenic (Ar), lead (Pb) and iodine (I).

These elements combine in different ratios and proportions to make a molecule. For instance C, H, and O combine in 1:2:1 ratio to make carbohydrates. Similarly, C, H, O and N combine to form proteins and lipids. Nucleic acids are the result of combination of C, H, O, N and P. Carbohydrates, proteins, lipids and nucleic acids, grouped under a category called macromolecules, form the basic components that make life possible. In addition to these molecules there are micromolecules like hormones, vitamins and minerals that also play a vital role in the process of life. Among the macromolecules, proteins and nucleic acids are known as informational macromolecules and the other two, viz., carbohydrates, lipids are non-informational macromolecules. The areas of study of Biochemistry are the study of structure of these molecules, their properties, their functions, their inter-relationship and their metabolism. In order to study these characters it becomes necessary to isolate them, separate them, purify them and bring them into a suitable form so that it can easily be analyzed and characterized. Hence, Biochemistry also deals with methods and techniques to isolate, purify and characterize various compounds.

Biochemistry deals not only at the molecular level but also at the level of the cell. A cell can suitably be defined as the theatre of life, where each and every artist (molecules) play their role (functions) exactly as the director (DNA) wants, and the director directs exactly as that appreciated and enjoyed by the audience (cell organelles). Biochemistry has proved that the above cooperation exists well for normal functioning of a cell and thereby of a living organism. A little misunderstanding between them leads to disturbances in the cell and finally may lead to death of the living organism.

The main purpose of all the efforts of Biochemistry is to benefit humans in all forms, particularly in diagnosis and treatment of various diseases. For example, measurement of serum levels of isoenzymes helps in the diagnosis and degree of various tissue disorders. Lactate dehydrogenase (LDH) has five isoenzyme forms, of which LDH_1 and LDH_2 levels in the serum increase following a myocardial infarction (heart attack). Due to the knowledge of enzyme inhibition, it has become possible to treat various diseases. Some bacteria do not require preformed folic acid (a vitamin for humans) as growth factor because they can make it if P-aminobenzoic acid (PABA), one of the components of folic acid, is available. Thus PABA is a vitamin for these bacteria. If sulphanilamide is administered, it competes with PABA in the enzymatic synthesis of folic acid due to its structural similarity and inhibits the growth of pathogenic bacteria requiring PABA. Likewise, allopurinol, a drug, is used to treat Gout, a disease of the joints, where there is an abnormal accumulation of uric acid crystals. Allopurinol competitively inhibits xanthine oxidase, the enzyme responsible for converting purines into uric acid.

Investigations of diabetes mellitus are completely based upon the laboratory tests in Biochemistry labs, where the presence of sugar in urine is tested by Benedict's test. Similarly, investigation of other disorders like albuminuria, lactosuria, etc. are carried out here.

Due to the recent knowledge of cell receptors for various substances it has become possible to treat hormonal disorders and to kill cancerous cells without affecting other cells. Toxic substances are encapsulated in membranous cells having complimentary surface receptors to that of cancerous cells. These capsulated cells when injected gets attached to the cancerous cells due to their complimentarity and release the toxic substances into the cancerous cells destroying them.

The technique of enzyme immobilization has helped in the treatment of various in born errors of metabolism. The most recent advancement in the technique of "gene manipulation" has helped in the large scale, artificial synthesis of peptide hormones like insulin and synthesis of enzymes making the use of micro-organism. Hence it can be concluded that Biochemistry is the tool to make diagnosis of certain diseases.

The scope of Biochemistry is as vast as the sky. It is not limited to a particular aspect. There is no end to Biochemistry research, it is growing and growing thousand times, as fast as a malignant tumour.

2 CHAPTER

GENERAL CHEMISTRY

If we look at our body we can see that it is made up of flesh and bones. But definitely, a question comes to our mind as to what is this flesh and bones made up of? The answer to this question is given by biochemistry. In anatomy you study, as to how the different organs of our body are arranged and in physiology you study the functions of these organs, but biochemistry shows what they are made up of and how do they function.

Human body can be divided into a number of divisions or parts, the least particle being the electron, which cannot be divided further. The electron is negatively charged particle. Similarly, there is a positively charged particle called proton and neutral particle known as neutron which also cannot be divided further. The electron, proton and neutron collectively form the atom (if named in particular is called as an element).

A second question arises in our mind as to how and wherefrom did these particles come into living beings. The answer to this question from the developing science is that before the formation of the earth, a bunch of fire got separated from the sun containing, solemnly these particles, viz., electrons, protons and neutrons. On gradual cooling of this fire the electrons, protons and neutrons combined in different numbers to give rise to a substance called atom or element. Up till now 93 different kinds of elements are found in earth's crust. The periodic table of elements is given below—

Atomic Number ← 6 C 12 → Atomic Weight
CARBON

Major element
Macro-element
Micro-element
Noble gases

^{1}H 1 HYDROGEN																	^{2}He 4 HELIUM
^{3}Li 7 LITHIUM	^{4}Be 9 BERYLLIUM											^{5}B 11 BORON	^{6}C 12 CARBON	^{7}N 14 NITROGEN	^{8}O 16 OXYGEN	^{9}F 19 FLUORINE	^{10}Ne 20 NEON
^{11}Na 23 SODIUM	^{12}Mg 24 MAGNESIUM											^{13}Al 27 ALMUNUM	^{14}Si 28 SILICON	^{15}P 31 PHOSPHORUS	^{16}S 32 SULPHUR	^{17}Cl 35 CHLORINE	^{18}Ar 40 ARGON
^{19}K 39 POTASSIUM	^{20}Ca 40 CALCIUM	^{21}Sc 45 SCANDIUM	^{22}Ti 48 TITANIUM	^{23}V 51 VANADIUM	^{24}Cr 52 CHROMIUM	^{25}Mn 55 MANGANESE	^{26}Fe 56 IRON	^{27}Co 59 COBOLT	^{28}Ni 59 NECKEL	^{29}Cu 64 COPPER	^{30}Zn 65 ZINC	^{31}Ga 70 GALL UM	^{32}Ge 73 GERMANIUM	^{33}As 75 ARSENIC	^{34}Se 79 SELLENIUM	^{35}Br 80 BROMINE	^{36}Kr 84 KRYPTON
^{37}Rb 85 RUBIDIUM	^{38}Sr 88 STRONTIUM	^{39}Y 89 YITRIUM	^{40}Zr 91 ZIRCONIUM	^{41}Nb 93 NEOBIUM	^{42}Mo 96 MOLYBDENUM	^{43}Tc 98 TECHNITIUM	^{44}Ru 101 RUTHENIUM	^{45}Rh 103 RHODIUM	^{46}Pd 106 PALLADIUM	^{47}Ag 108 SILVER	^{48}Cd 112 CADMIUM	^{49}In 115 INDIUM	^{50}Sn 119 TIN	^{51}Sb 122 ANTIMONY	^{52}Te 128 TELLURIUM	^{53}I 127 IODINE	^{54}Xe 131 XENON
^{55}Cs 133 CESIUM	^{56}Ba 137 BARIUM		^{72}Hf 178 HAFNIUM	^{73}Ta 181 TANTALIUM	^{74}W 184 TUNOSTEN	^{75}Re 186 RHENIUM	^{76}Os 190 OSMIUM	^{77}Ir 192 IRIDIUM	^{78}Pt 195 PLATINUM	^{79}Au 197 GOLD	^{80}Hg 201 MERCURY	^{81}Tl 204 THALLIUM	^{82}Pb 207 LEAD	^{83}Bi 209 BISMUTH	^{84}Po 209 POLONIUM	^{85}At 210 ASTHTINE	^{86}Rn 222 RADON
^{87}Fr 223 FRANC UM	^{88}Ra 226 RAD UM		Lanthanides														
			Actinides														

Structure of an atom: Where there are many numbers of electrons, protons and neutrons, there are a number of possibilities of their being combined with each other. Let us start with number one. Supposing there is one electron, you know it is negatively charged, so it will combine with a positively charged proton to form an atom. To recognize this atom it is given a particular name. The atom or the element with one electron and one proton is known as hydrogen. Likewise an atom with a combination of two electrons, two protons and two neutrons is known as helium atom. Lithium has three numbers of each, etc. etc.

Atomic number: The atomic number of an atom is the number of electrons in that atom (or net positive charge on that atom).

Atomic weight: It is the mass of an atom. The elements most commonly found in our body are – hydrogen, oxygen, carbon, nitrogen, phosphorus, sulphur, etc.

The final question is: *Why and how do these elements form the different organs of our body*? So the answer is: All the atoms except ^{2}He, ^{10}Ne, ^{18}Ar, ^{36}Kr, ^{54}Xe and ^{86}Rh (inert elements/noble gases) are unstable, hence in order to attain stability they combine with each other and thereby also attain the noble gas configuration. In order to attain stability they either gain, loose or share electrons from, to or with other atoms respectively.

1. **Sharing of electrons:** Hydrogen having one electron shares an electron with another hydrogen so that both of them can now have two electrons each which is a stable configuration as that of helium.

The aggregation of two or more atoms is known as a molecule. In the hydrogen molecule, a force of attraction develops due to sharing of electrons, which holds the two hydrogen atoms together. This force is known as a chemical bond. A bond formed by mutual sharing of electrons is known as a covalent bond.

   (a) **Single bond:** If one electron from each of the sharing atoms are contributed for the bond formation, then a single bond results (C—C).

   (b) **Double bond:** If two electrons from each of the sharing atoms are contributed for bond formation, then a double bond is formed (C = C).

   (c) **Triple bond:** If three electrons are shared (C≡C).

2. **Unequal sharing of electrons or coordinate bond:** Here both the electrons for sharing between the two atoms are contributed by one atom only. For example in the formation of ammonium ion (NH_4^+) from ammonia and proton (hydrogen ion) two electrons are contributed by NH_3.

There is a second type of coordinate bond wherein the sharing electrons are pulled more towards one of the atoms. For example, in water molecule H—O—H, the electrons are more towards oxygen atom than towards hydrogen atom. Hence the bond formed due to unequal sharing of electrons is known as co-ordinate or native or semi-polar bond.

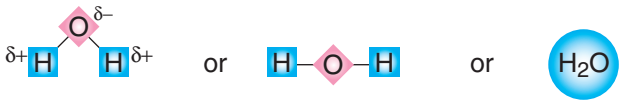
3. **Transfer of electron (gain or loss of electrons):** Sodium (Na) contains an electron more than its neighbour inert gas (neon), and chlorine (Cl) contains an electron less than its neighbour inert gas (Argon). Hence the Na atom donates an electron to Cl to form NaCl (sodium chloride). A bond formed by the complete transfer of one or more electrons of an atom to another atom is known as ionic bond.

The following are a few important molecules found in biological systems—

- The combination of carbon and hydrogen is known as **"hydrocarbon"** (—CH—).
- The group of carbon compounds containing an —OH are called as **"alcohols"** (—CHOH).
- The group of compounds containing 'C' double bond 'O' with one hydrogen and one carbon are called **"aldehydes"** (—CHO). (C = O with 2H also).
- The group of compounds containing 'C' double bond 'O' with two 'C' substitutes are called **"ketones"** (C—CO—C).
- Compounds containing carboxylic group are called **"carboxylic acids"** (-COOH).

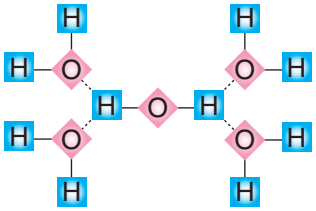
These molecules along with a few other elements combine to give rise to carbohydrates, proteins, lipids, nucleic acids etc. which in turn contributes to the structure and life of an organism.

WATER



Water is the major component of our body. It constitutes to about 70% of the total body mass. Most of the reactions in the cell are carried out in aqueous medium (water).

Water is made up of oxygen and two hydrogen atoms. Oxygen has a tendency to pull the electrons more towards itself, thereby becoming electronegative and leaving the hydrogens electropositive. This results in the creation of a dipole due to which each water molecule is surrounded by four other water molecules.



The bond between 'H' of one water molecule and 'O' of the other is known as hydrogen bond.

Properties:

(1) It has a high boiling point when compared to other liquids.
(2) It has a high specific heat of vaporization.
(3) High melting point.
(4) The pH of water is 7.

Specific heat of water is one calorie, it is, therefore, best suited to maintain constant temperature of the body with varying environmental temperature.

The heat of vaporization of 540 cal/gram at 100 ^{o}C is helpful in maintaining the body temperature, i.e., large amount of body heat is lost with only a small amount of water being vaporized from the surface of the skin.

ACIDS AND BASES

An acid is a proton- donor and a base is a proton- acceptor (Bronsted-Lowry theory).

Acids:

$$HCl \rightarrow H^{+} + Cl^{-}$$

$$CH_3COOH \rightarrow CH_3COO^{-} + H^{+}$$

Weak acids are those which have a slight tendency to give up protons. e.g., acetic acid. On the other hand, strong acids give up protons readily. e.g., HCl.

Bases:

$$NaOH \rightarrow Na^{+} + OH^{-}$$

$$H_2O \rightarrow H^{+} + OH^{-}$$

There are strong and weak bases similar to that of acids. e.g., NaOH is a strong base which releases hydroxyl ions very easily, and water is a weak base as it is a poor source of hydroxyl ions.

pH

pH is defined as the negative logarithm of the hydrogen ion concentration in a media.

$$pH = -Log_{(10)}[H^+]$$

In simple terms it is a value that gives the amount of hydrogen ions present in a solution. This value is expressed in a reverse or negative form, i.e., higher the pH value lower is the hydrogen ion concentration and lower the pH value higher is the hydrogen ion concentration. The pH of all the solutions ranges between 0 and 14 only. pH of value 7.0 is neutral and pH ranging from 0 to 6.9 is acidic and 7.1 to 14 is basic or alkaline. The water molecule dissociates as—

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

The hydrogen ion concentration in pure water was found to be 0.0000001 moles/litre.

This can also be written as $\frac{1}{10000000}$ or 1×10^{-7}.

Taking log of the above number we get = –7.

Negative logarithm of the above number = 7.

The negative logarithm of the hydrogen ion concentration is known as pH. Therefore, the pH of water is 7. To calculate the pH of any weak dissociable acid, the following equation is derived—

$$HA \Leftrightarrow H^+ + A^-$$

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

$$-\log[H^+] = -\log K_a - \text{Log}\frac{[HA]}{[A^-]}$$

$$pH = pK_a + \log\frac{[A^-]}{[HA]}.$$

This is known as the **"Henderson-Hasselbalch equation"**.

The normal pH of blood plasma ranges between 7.35 and 7.45, average being 7.4. The intracellular pH of the tissues is 7.25 to 7.35 averaging to 7.30 and pH of extracellular fluid is 7.30 to 7.40 with an average of 7.35. A decrease in the pH of blood is termed as acidosis and an increase in the pH of blood is termed as alkalosis. Alkalosis is more fatal than acidosis.

BUFFERS

A buffer solution is one which resists the changes in pH of a solution upon the addition of small amount of acid or alkali. Buffer solutions are a mixture of—

(a) Weak acid and its salt (or its conjugate base).

(b) Weak base and its salt (or its conjugate acid).

(c) Weak acid and weak base. e.g., weak acid CH_3COOH and its base CH_3COONa.

There are two important chemical buffers that act in the biological system, they are—

1. **Bicarbonate buffer:** Contains a mixture of carbonic acid (H_2CO_3) and bicarbonate (HCO_3^-). This maintains the pH of blood and extracellular fluid.
2. **Phosphate buffer:** Consists of a mixture of HPO_4^- and $H_2PO_4^-$. It maintains a pH of 6.86, hence it is more active intracellulary.

In addition to these two chemical buffers, the human body has proteins (albumin, haemoglobin, etc.) that maintain the pH of the biological system.

CALCULATING PH

Using Hydrogen Ions to Calculate pH: Calculate the concentration of hydrogen (H^+) ions by dividing the molecules of hydrogen ions by the volume, in liters, of the solution. Take the negative log of this number. The result should be between zero and 14, and this is the pH. For example, if the hydrogen concentration is 0.01, the negative log is 2 i.e. the pH. For a 0.1 M solution of an acid: 0.1 M = 10^{-1} M. The negative log of 10^{-1} = 1 i.e. the pH.

pH + pOH = 14. pOH is log of [OH^-], or the negative log of the hydroxide ion concentration. If you were told that the pOH was 9.3 and asked to calculate the pH you should 14 – 9.3 = 4.7. The pH is 4.7.

Question 1: Calculate the pH of 6.9×10^{-4} M HNO_3

Answer: There is only one mol of [H^+] and the hydrogen ion concentration given is 6.9×10^{-4}. To find the pH take the antilog of [H^+] = ·9984124777 i.e. rounded pH is 1.0

Using Hydroxide Concentration to Calculate pH : The pH of a solution can also be determined by finding the pOH. Determine the concentration of the hydroxide ions by dividing the molecules of hydroxide by the volume of the solution. Take the negative log of the concentration to get the pOH. Then subtract this number from 14 to get the pH. For example, if the OH^- concentration of a solution is 0.00001, take the negative log of 0.00001 which will be 5. This is the pOH. Subtract 5 from 14 and you get 9. This is the pH.

Question 2: Calculate the pH of 4.5×10^{-4} M $Ba(OH)_2$

Answer: There are two mols of OH^- and the concentration of each mol is 4.5×10^{-4}. So multiply the concentration by 2 and we get = 9×10^{-4}. The value of OH^- is now 9×10^{-4}. Take the -log of OH- to obtain the pOH. $-\log(9 \times 10^{-4})$ = 3.045757491

The value of pOH is 3.045757491

To Get the value of pH by setting pH and pOH equal to 14

14 = 3.045757491 + pH

14 – 3.045757491 = pH

10.95424251 = pH

pH is 10.95424251

Calculation of pH of a buffer solution: Write the chemical reaction for the dissociation of your buffer solution. Determine the dissociation constant for the relevant acid or base. The dissociation constant is the ratio of dissociated ions to initial compound present at equilibrium, and is represented as Ka for an acid or Kb for a base.

Take the negative log (base 10) of your dissociation constant. If the solution is at equilibrium, this is the pH of your buffer solution. For example, hypobromous acid has a dissociation constant of 2×10^{-9}. $\log(2 \times 10^{-9}) \sim = -8.699$, so the pH of a hypobromous acid buffer would be approximately 8.699.

Calculating pH using a calculator: If you are told to find the pH of an acid whose concentration is 0.0045 M, you should type in 0.0045 then hit the "log" button, then the +/- button. You will get 2.3 for the pH of the acid.

If you are told to find the Molarity (concentration) of an acid whose pH is 6.5, then type in 6.5 then the +/- button, and then do the anti-log. On most calculators it is just hitting the 2^{nd} button then the "log" button. Your answer will be: 3.16×10^{-7} M.

COLLOIDS

A colloidal solution is one that contains the solute particle of the size of 1 millimicron to 200 millimicron.

Tyndall phenomenon: When light passes through a colloidal solution it looks like a milky yellow solution due to scattering of light by the colloidal particles, this is known as **Tyndall effect**.

There are two types of colloidal solutions—(1) Suspensoids or lyophobic and (2) Emulsoids or lyophilic.

Suspensoids: Contain particles of insoluble substances like metals and some inorganic salts.

Emulsoids: Are the solution of proteins, carbohydrates, etc.

Measurement of particle size: The size of the colloidal particles can be measured by any of the following methods—

1. Measurement of sedimentation constants.
2. X-ray analysis.
3. If the particle is a single molecule of the substance then its size is given by its molecular weight. e.g., Proteins.

OSMOSIS

If two solutions of different concentration are separated by a semi-permeable membrane then the solute particles (permeable) move from the solution of higher concentration to that of the lower concentration or/and the solvent moves from the solution of lower concentration to the higher concentration, this phenomenon is known as **osmosis**.

The pressure that should be applied to prevent the osmosis is known as **osmotic pressure**. The osmotic pressure of the blood plasma is termed as **oncotic pressure**.

Transport across membranes: Most of the substances are transported across the membranes in the intestine or other parts of the body due to osmotic difference. However, the biological membranes are impermeable to most molecules that help in retaining ionized metabolites within the cell and prevent them from diffusing out. Cells require nutrients for their activity, so their intake and output are sometimes against the gradient (osmosis) and are mediated by specific active transport systems. The entry of some substance like ATP into organelles in the cell (like mitochondria) also requires active transport. It is called mediated or facilitated transport.

Protein molecules in the membrane play a crucial role in the process of transport. The specific protein serving the role of transport is called **transport system, carriers** or **translocases**. Mediated or facilitated transport may be active or passive. The transport increases with the concentration of substrate till a certain level after which the capacity of carrier molecule becomes saturated and no further increase in transport occurs.

1. In active transport the substance is usually transported against the concentration gradient.
2. The transportation requires expenditure of energy usually breakdown of ATP.
3. The transport is uni-directional. e.g., in the erythrocytes, sodium ion is transported out of the cell and potassium ion into the cell.

Mechanism of transport: Specific carrier protein (*P*) contains specific sites for specific substance (*S*). '*P*' is positioned on one side of the membrane that can take up '*S*' to form '*P-S*' complex. '*P*' diffuses across the membrane to the other surface or undergoes rotation or conformation change, so that the binding site faces the other side of the membrane. The '*S*' gets discharged. This is passive transport and occurs in either direction, depending on the concentration of the substance on either side of the membrane.

RADIOACTIVITY

Certain atomic nuclei are less stable, and in order to achieve stability they emit radiations and hence are said to be radioactive. The radiations are due to throwing off a stream of electrons. In order to attain stability the nucleus gives of protons and neutrons which ultimately results in the loss of electrons. This process of disintegration of nucleus is known as radioactive decay. Due to the loss of electrons the atomic number of the element is changing, i.e., it is being converted to entirely a new element. This process of conversion of an element to another is known as **transmutation**.

There are three types of radiations—(1) Alpha rays (2) Beta rays, and (3) Gamma rays.

1. **Alfa rays:** They are composed of 2 protons and 2 neutrons (helium nucleus). Uranium, a radioactive element, gives off alpha rays.

$$_{238}U^{92} \rightarrow {}_{232}Th^{90} + {}_{4}He^{2}$$

Thorium is again unstable and hence it also gives off radiations and gets converted to another element and finally a stable element lead ($_{207}Pb^{82}$) is formed.

2. **Beta rays:** They are composed of electrons.
3. **Gamma rays:** Almost similar to X-rays.
4. **Cosmic rays:** These are the fourth type of rays which originates from the sun and most of which is prevented from entering the earths atmosphere by the ozone layer (O_3). Some of the cosmic rays that escapes the ozone layer and enters the earth's atmosphere is disintegrated by the gases to electrons, protons, neutrons, positrons and the nucleus.

Unit of radioactivity: The unit of radioactivity is **curie**.

$$1 \text{ curie} = 3.7 \times 10^{10} \text{ disintegrations/sec}$$

Half-life: It is the time required for a radioactive element to reduce half of its radioactivity.

U = 1700 years and C = 5570 years.

Detection and measurement of radiations

1. **Scintillation counter:** It consists of a plate made up of fine sulfide phosphor, when radiations hit this plate it will give a tiny flash of light which is magnified and counted by a suitable device.
2. **Geiger counter:** A tube called Geiger-Muller tube is used to detect radiations. This tube is filled with a gas which on radiation gets ionized and the ions are conducted through a wire to a detector.

Artificial radioactivity: Radioactivity can be induced artificially by bombarding neutral elements with radiations.

$$_{14}N^{7} + {}_{4}He^{2} \rightarrow {}_{17}O^{8} + {}_{1}H^{1} \text{ (from uranium) (radioactive oxygen isotope)}$$

$$_{11}B^{5} + {}_{4}He^{2} \rightarrow {}_{13}C^{6} \text{ (carbon isotope)}$$

$$_{14}N^{7} \rightarrow {}_{14}C^{6} \text{ (radioactive isotope of carbon, } \beta\text{-particle elimination)}$$

Uses

(1) $_{131}I^{53}$ (normal is $_{127}I^{53}$) iodine 131 is radioactive and it is used to detect the physiology of thyroid gland. A known amount of NaI_{131} in urine is measured. If it is nearing the amount given, then it can be conferred that the thyroid gland is not functioning properly, and if the amount of ^{131}I in urine is far less than the amount provided, then the thyroid gland function is normal. Due to its normal functioning, the thyroid gland has absorbed maximum iodine and thereby a lesser amount of iodine is excreted in the urine. In some cases one of the lobes of the thyroid gland may be normal but the other may be abnormal, in such cases the urine analysis for iodine may not serve the purpose. The best way to measure thyroid ^{131}I uptake is by scintillation counter.

(2) Radioactive ^{32}P (normal is ^{31}P) is used to detect cancerous cells. Cancer cells take more phosphorous. Hence, radioactive phosphate is injected intravenously and after some time a photosensitive plate (or emulsion) is kept in close contact with the tissue. If the tissue is cancerous a dark spot is observed.

(3) In order to assess the normal function (absorption) of the gastro-intestinal tract, radioactive fatty acids, iron (^{59}Fe), and vitamin B_{12} ($Co^{57, 58 \& 60}$) are given orally and blood samples are analyzed at regular intervals.

(4) They are also used to trace the metabolic intermediates. ^{14}C is used to trace the metabolic intermediates in the metabolism of food stuffs. This helps in identifying the position of the carbon during the metabolism of that food stuff. Recent study on the metabolism of food stuffs using ^{14}C is for the detection of genetically defective enzymes.

The use of radioactive substances is not restricted only to make diagnosis but it is also used to treat certain diseases like hyperthyroidism, cancers, certain cardiac diseases and pulmonary diseases. It is also used for the pasteurization of milk and food.

Harmful effects: Radiations stops DNA synthesis, acts as uncoupler of oxidative phosphorylation, reduces ATP synthesis and cytochromes, causes pyknosis, vacuolization of cells, giant cell formation, mitotic delay, chromosomal breaks and altered permeability is also observed.

3 CHAPTER

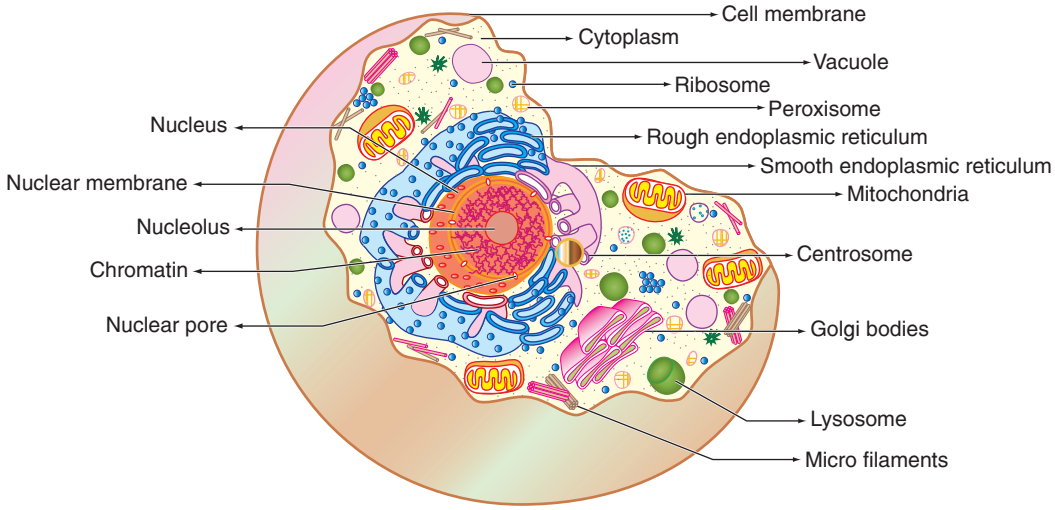
CELL

STRUCTURE AND FUNCTION

Cell is a compartment where all the activities of life takes place. There are two basic types of cells in nature, viz., prokaryotic cells and eukaryotic cells.

PROKARYOTIC CELLS	EUKARYOTIC CELLS
Prokaryotes are the simplest cells without a nucleus and cell organelles.	Eukaryotes are sophisticated cells with a well defined nucleus and cell organelles.
Prokaryotic cells are the smallest cells (1-10 µm).	The cells are comparatively larger in size (10-100 µm).
Unicellular and earliest to evolve (~4 billion years ago), still available.	Unicellular to multicellular in nature and evolved ~1 billion years ago.
The cell wall is rigid.	The cell membrane is semipermeable and flexible.
These cells reproduce asexually.	These cells reproduce both asexually and sexually.
They include bacteria and archaea.	Include the animals, plants and fungi.
Some species are highly evolved pathogens e.g., *Borrelia burgdorferi*.	Size ranges from tiny yeasts to giant sequoias, dinosaurs, etc.

A group of cells forms tissue, various tissues forms an organ and different organs make up the body. The structure and components of a human cell are given below—



Cell membrane: Thin layer of protein and fat that surrounds the cell is the cell membrane. It is semipermeable, allowing some substances to pass into the cell and blocking others.

Cytoplasm: Jelly–like material present outside the nucleus in which the cell organelles are located. It is the site of protein synthesis and many metabolic events. The cytoplasm contains many enzymes for general metabolism. It contains fibre of the cytoskeletal system, which organizes cytoplasmic structure.

Mitochondria: Spherical to rod-shaped organelles with a double membrane. The inner membrane is infolded many times, forming a series of projections (called cristae). The mitochondrion is knows as the **power house** of the cell as it generates ATP (adenosine triphosphate), the energy currency of the cell.

Ribosome: Small organelles composed of RNA-rich cytoplasmic granules that are sites of protein synthesis. Ribosome size is measured in Svedberg (*S*) units; derived from sedimentation in ultracentrifuge (used before electron microscopes were available). In prokaryotes the ribosomes are made of **30S** and **50S** subunits, assemble into **70S** ribosome whereas in eukaryotes the ribosomes are made of **40S** and **60S** subunits, assemble into **80S** ribosome. In bacteria they occupy 25% of cell volume and use 90% of cell energy. Less in many specialized eukaryotic cells but still are the dominant activity of almost all the cells.

Nucleus: It is a spherical body containing many organelles, including the nucleolus. It controls many of the functions of the cell (by controlling protein synthesis) and contains DNA (in chromosomes). The nucleus is surrounded by the nuclear membrane. It is the locus of DNA/RNA synthesis and protein assembly. It contains **chromatin** i.e., **DNA-protein complexes**. Chromatin can condense into chromosomes during cell division.

Nuclear membrane: The nuclear membrane is a double layered structure surrounding the nucleus containing many **nuclear pores**. These pores allow different materials to move in and out of nucleus. The pores have octagonal 'doors' made of protein which open and close on either side depending on specific signals. Pore diameter is about 10 nanometers (10×10^{-9} m), smaller than the diameter of a complete ribosome. They can open up to as much as 26 nm in response to certain signals. Some signals allow motion in but not out, other signals control reverse transport.

Nucleolus: The nucleolus is present within the nucleus. Some cells have more than one nucleolus. It is the **assembly plant for ribosomes**. Ribosomal proteins are made in cytoplasm and transported back into the nucleus. Ribosomal RNA is made in the nucleus. These two elements are integrated inside nucleolus to create ribosomal subunits. These are then exported out of nucleus through nuclear pores.

Centrosome: A small body located near the nucleus, also called the 'microtubule organizing centre'. It has a dense center and radiating tubules. The centrosomes are where microtubules are made. During cell division (mitosis), the centrosome divides and the two parts move to opposite sides of the dividing cell. The centriole is the dense centre of the centrosome.

Endoplasmic reticulum

Rough endoplasmic reticulum (rough ER): A vast system of interconnected, membranous, infolded and convoluted sacks that are located in the cell's cytoplasm (the ER is continuous with the outer nuclear membrane). Rough ER is covered with ribosomes that give it a rough appearance. Rough ER transports materials through the cell. It synthesizes proteins in sacks called cisternae for export or movement to different cell organelles like the golgi body, or inserted into the cell membrane but not to cytoplasm. The transport proteins designated for export carry a peptide signal at growing end, causing growing protein to move to ER (docking), insert peptide into membrane, translocate growing polypeptide chain across ER membrane.

Smooth endoplasmic reticulum (smooth ER): A vast system of interconnected, membranous, infolded and convoluted tubes that are located in the cell's cytoplasm (the ER is continuous with the outer nuclear membrane). The space within the ER is called the ER lumen. Smooth ER transports materials through

the cell. It contains enzymes which produces and digests lipids (fats) and membrane proteins; smooth ER buds off from rough ER, moving the newly-made proteins and lipids to the golgi body, lysosomes and membranes. It detoxifies drugs and poisons (in liver).

Golgi body: A flattened, layered, sac-like organelle that looks like a stack of pancakes. It is also called the golgi apparatus or golgi complex. It is located near the nucleus. It produces the membranes that surround the lysosomes. The golgi body packages proteins and carbohydrates into membrane-bound vesicles for **export** from the cell. Functions as intracellular 'post office' for sorting new proteins made on rER. Vesicles containing protein pinch off from ER, fuse with cis face of golgi. Inside golgi, oligosaccharide chains on proteins are modified. Vesicles pinch off from trans face of golgi, carry proteins to several possible destinations: export (out of cell), lysosomes, peroxisomes, cell membrane, etc.

Lysosomes: These are round organelles surrounded by a membrane where the digestion of cell nutrients takes place due to presence of the digestive enzymes. They contain ~40 hydrolytic enzymes such as lipases, proteases, nucleases, etc. which break down organic polymers of all types. Lysosomes continuously break down old proteins, foreign materials, and many wastes. They also bring about phagocytosis, a process in which foreign materials are brought into the cell and 'chewed up'. Sometimes lysosomes open up in cell itself causing death of the cell termed as **apoptosis**, hence are called **suicide bags** of the cell.

Vacuole: Fluid-filled, membrane-surrounded cavities inside a cell. The vacuole fills with food being digested and waste material that is on its way out of the cell. There are specialized vacuoles which function to store fat as fat droplets (TAG).

Peroxisomes: These are single membrane oval or spherical cellular organelles. They are also called as microbodies. They contain catalase enzyme. Peroxisomes are involved in the oxidation of long chain fatty acids and synthesis of plasmalogens and glycolipids.

Cytoskeletal system: It provides internal fibrous structure to cells because cell is not 'just a bag in a bubble', it contains lots of internal fibres or internal 'skeleton'. It is not rigid like bone, instead it is capable of being assembled, allows cell movement, cell division, internal motion of the organelles and is broken down in minutes. The cytoskeletal system is composed of microtubules and microfilament.

Microtubules: The microtubules has the largest diameter among the fibres found in the cytoplasm of all eukaryotes. It involves many structures: Cilia, flagella, spindle fibres that polymerize from centrioles during mitosis/meiosis. They are made of the protein called tubulin and polymerizes into hollow tubules of 25 nm diameter.

***Cilia and flagella*:** They are organelles of locomotion. Both of them contain 9 double rings of microtubules, 2 central microtubules, two motor proteins, i.e., motor protein 1-dynein and motor protein 2-kinesin, which allow motion along microtubules.

Microfilaments (actin): Another kind of fiber found in cytoplasm of most eukaryotes. Involved in muscle contraction, cell support, pinching off of daughter cells after mitosis.

Extracellular matrix (ECM): Animal cells do not have cell walls, but have ECM, i.e., a meshwork of macromolecules outside plasma membrane. It consists mainly of glycoproteins (proteins with oligosaccharide chains), especially collagen. Some cells are attached directly to ECM by bonding to collagen or fibronectin.

Intracellular junctions: In multicellular organisms, adjacent cells are held together by several types of specialized junctions.

1. **Tight junctions:** Specialized 'belts' that bind two cells tightly to each other, prevent fluid from leaking into intracellular space.
2. **Desmosomes:** Intercellular 'rivets' that create tight bonds between cells, but allow fluids to pass through intracellular spaces.

Gap junctions: Formed by two connecting protein rings embedded in cell membrane of adjacent cells. Allows passage of water, small solutes, but not proteins, nucleic acids.

4 CHAPTER

CARBOHYDRATES

Carbohydrates are the polyhydroxy aldehydes or ketones or their derivatives.

They contain C, H and O in the ratio of 1:2:1.

Their general formula is $C_n(H_2O)_n$ [n = 3 (minimum)]

They are commonly known as sugars.

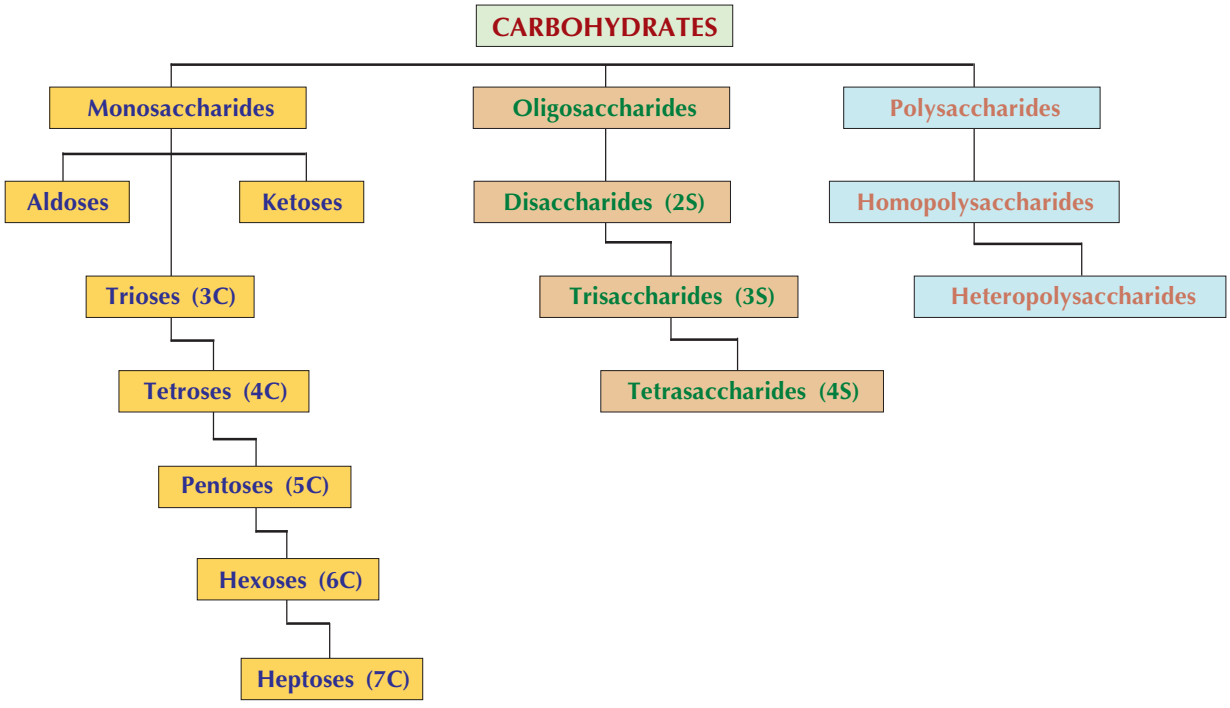
Importance

(1) They are the main source of energy to human beings–glucose, starch, etc.

(2) They help in cell-cell recognition–the blood groups A, B, O.

(3) They act as lubricants–mucus in the gastrointestinal tract.

Classification of carbohydrates

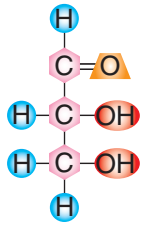
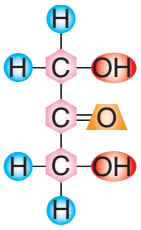
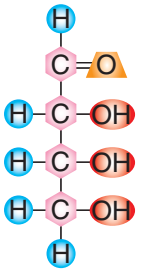
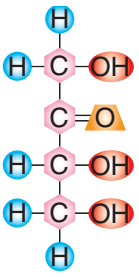
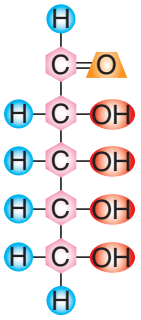
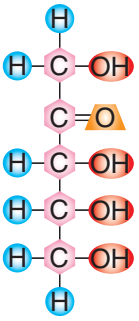


MONOSACCHARIDES

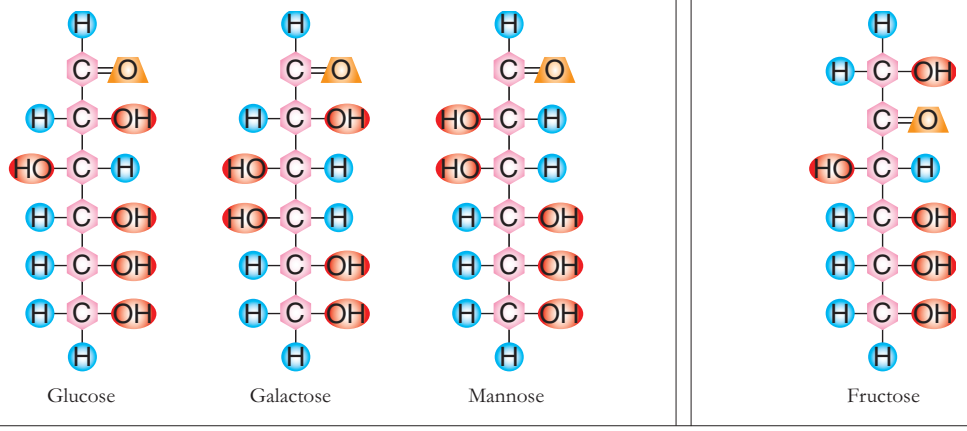
They are the sugar units that cannot be further hydrolyzed into simpler units. There are two major classes of monosaccharides.

1. **Aldoses:** Sugars containing an aldehydic group are known as aldoses. e.g., Glucose, galactose, mannose, ribose and glycerose.
2. **Ketoses:** Sugars containing a ketonic group are known as ketoses. e.g., Dihydroxyacetone, fructose and sedulopheptulose.

Depending upon the number of carbon atoms, aldoses and ketoses are further classified as—

ALDOSE SUGAR	KETOSE SUGAR
(a) Trioses: Sugars containing three carbon atoms	
 Glyceraldehyde	 Dihydroxyacetone
(b) Tetroses: Sugars containing four carbon atoms	
 Erythrose	 Threose
(c) Pentoses: Sugars containing five carbon atoms	
 Ribose Others 2-Deoxyribose Arabinose Xylose Lyxose	 Ribulose

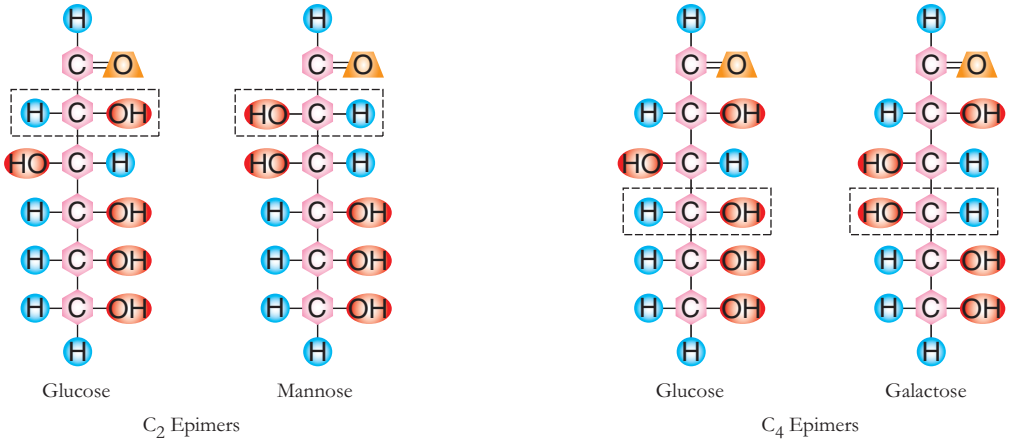
(d) Hexoses: Sugars containing six carbon atoms



(e) Heptoses: Sugars containing seven carbon atoms: An aldo-heptose is sedoheptose and a keto-heptose is sedoheptulose.

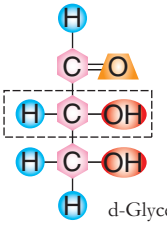
Physical characters of monosaccharides:

1. **A symmetric carbon atom / chiral centre:** A carbon atom substituted by four different groups or atoms is known as asymmetric carbon atom. All carbohydrates except dihydroxyacetone have one or more asymmetric carbon atoms.
2. **Isomers:** Two compounds having the same molecular formula but different structural formula are known as isomers. The number of isomers can be calculated from the number of chiral centres (n). The general formula is 2^n. Glucose has four asymmetric carbon atoms, i.e., $n = 4$, so $2^4 = 16$ isomers are possible for glucose.
   (a) *Epimers:* When sugars differ only in the configuration around one specific carbon atom they are called epimers. e.g., Glucose and mannose are epimers at C_2 whereas glucose and galactose are epimers at C_4.



(b) *Enantiomers*: Non super-imposable mirror images are known as enantiomers. e.g., D and L sugars.

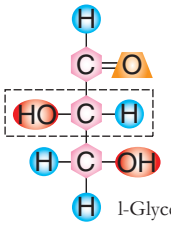
Explanation: When white light (which is a mixture of different wavelengths) is passed through a Nichol prism, then the emerging light will be of a single wavelength and this is known as plane polarized light. When this plane polarized light is passed through a solution containing carbohydrate, the light is deflected either towards the right or left, which depends upon the configuration of atoms around the chiral centre.



d-Glyceraldehyde

When the solution containing glyceraldehyde with the configuration in the figure given below around the chiral centre is taken, wherein the –OH group on the asymmetric carbon atom is towards right, when written on paper in the straight line projection form, then the light is deflected towards right. Hence this glyceraldehyde is known as dextrorotatory sugar or compound and is designated as *d*-sugar (+ sugar).

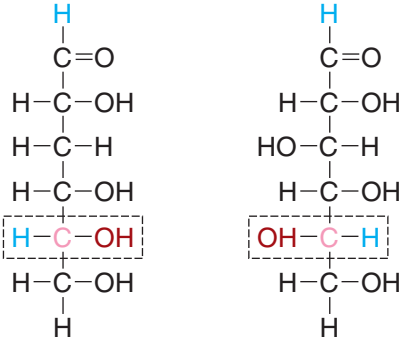
When the solution containing glyceraldehyde with the following configuration around the chiral centre is taken, wherein the –OH group on the asymmetric carbon atom is towards left, when written on paper in the straight line projection form, then the plane polarized light is rotated towards left. Hence this glyceraldehyde is known as levorotatory sugar or compound and is designated as l-sugar (– sugar).



l-Glyceraldehyde

Glyceraldehyde has only one chiral centre or asymmetric carbon atom, whereas tetroses, pentoses and hexoses have more than one chiral centre. In such cases the rotation of the plane polarized light is dependent upon many factors, viz., the configuration around each of the chiral centre, the solvent in which it is present, temperature, etc. Under such circumstances there is no relation between the configurations of the sugar to the rotation of the plane polarized light. Hence other compounds (sugars, amino acids, etc.) are grouped into two categories namely *D* series compounds and *L* series compounds.

D series compounds are those compounds that contain the reference group on the right side of the last chiral centre from the functional group. If glucose is taken, the functional group is the aldehydic group (–CHO) and the chiral centre furthermost from it is the 5th carbon atom and the reference group –OH is present on the right side of the straight chain. Hence it is known as *D*-glucose. This glucose may or may not be dextrorotatory. It may also be levorotatory. If this glucose is dextrorotatory then it is designated as *D*-(+)-glucose and if this glucose is levorotatory then it is designated as *D*-(–)-glucose.



D-Glucose *L*-Glucose

L series compounds are those compounds that contain the reference group on the left side of the last chiral centre from the functional group. If the –OH group is present at the left side on 5th carbon of the straight chain form of glucose then it is known as L-glucose. This glucose may or may not be levorotatory. It may also be dextrorotatory. If this glucose is dextrorotatory, then it is designated as *L*-(+)-glucose and if this glucose is levorotatory then it is designated as *L*-(–)-glucose.

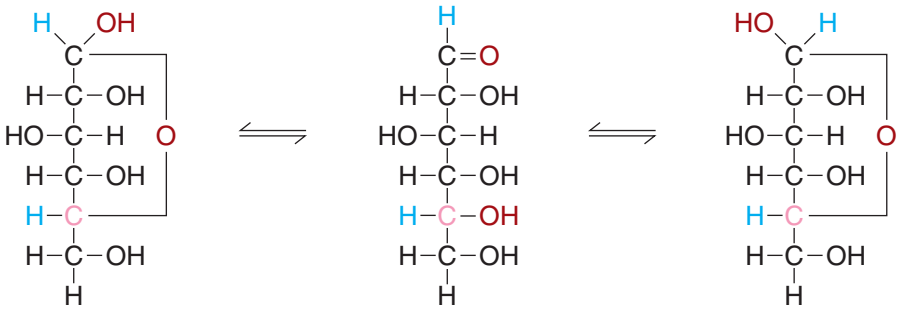
Racemic mixture: A solution containing equal number of *d* (+) & *l* (–) forms of a sugar is known as a racemic mixture.

(c) *Anomers*: Sugars differing at the anomeric carbon atom are known as anomers.

Explanation: When an aldehydic group (or carbonyl carbon) reacts with an alcoholic group, then it results in the formation of a hemiacetal. Carbohydrates contain both aldehydic (carbonyl) and alcoholic groups

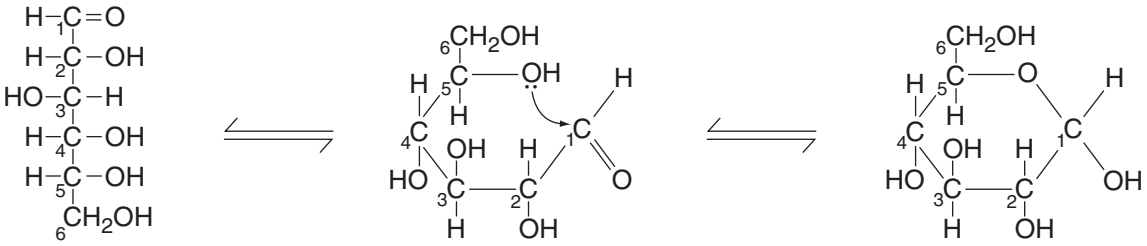
within the molecule. Hence it is possible that the aldehydic group present at the 1st carbon atom of the sugars can react with any of the alcoholic groups present on the other carbon atoms, thus resulting in the creation of an additional chiral centre at the 1st carbon atom and this chiral centre is now known as the anomeric carbon atom. Sugars differing at this anomeric carbon atom are known as anomers.

Two anomers for each of the sugars are possible. If the –OH group on the anomeric carbon atom is towards the right then it is known as alpha (α) anomer. If the –OH group on the anomeric carbon atom is towards the left, then it is known as beta (β) anomer or β-sugar.



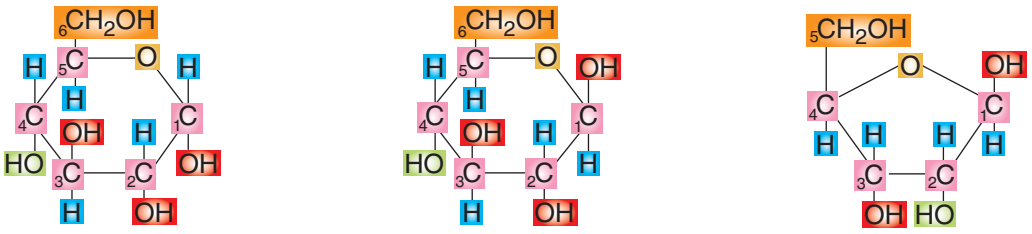
α *D* Glucose *D* Glucose β *D* Glucose

3. **Ring structures of carbohydrates:** Aldehydic group on 1st carbon atom of sugars can react with the alcoholic group on 4th carbon atom in pentoses and 4th or 5th carbon atoms in hexoses, forming an hemiacetal (as explained under anomers). This results in the formation of a cyclic ring structure. If the 1st and the 4th carbon atoms are involved in the hemiacetal formation, then the resultant ring structure is a five membered ring that resembles another compound known as furan. Hence the name of the resultant carbohydrate ring structure is furanose ring.

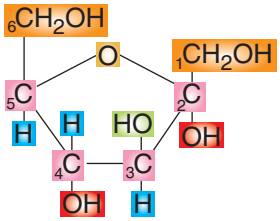


If the 1st and the 5th carbon atoms of the same sugar are involved in the hemiacetal formation then the resultant ring structure is a five membered ring that resembles another compound known as pyran. Hence the name of the resultant carbohydrate ring structure is pyranose ring.

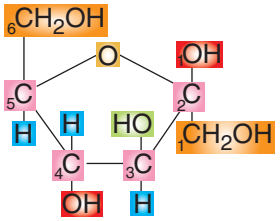
Among the carbohydrates, trioses and tetroses do not involve in the ring formation owing to their short length. Pentoses always forms the furanose ring structure, whereas hexoses can form both furanose and pyranose ring structures. The following are the ring structures of a few monosaccharides.



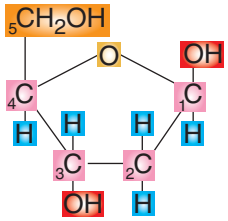
α-*D*-Glucopyranose β-*D*-Glucopyranose β-*D*-Ribofuranose



α-*D*-Fructofuranose



β-*D*-Fructofuranose



2-Deoxyribofuranose

4. **Mutarotation:** Change in the specific rotation of an optically active compound without any change in its other properties is knows as mutarotation.

Explanation: Glucose crystallized from cold water is *α-D*-glucose and it shows a specific rotation of $\{\alpha\}_D^{20} = +112.2°$. If it is dissolved in water, the specific rotation gradually changes with time and reaches a stable value of 52.7°. This change in specific rotation is because *α-D*-glucose isomerizes to *β-D*-glucose via a straight chain intermediate and finally an equilibrium mixture of about 1/3rd of *α-D*-glucose, 2/3rds of *β-D*-glucose and a little of straight chain form is formed. This change in specific rotation is known as mutarotation.

Similarly, *β-D*-glucose, which can be obtained on crystallization from pyridine shows a specific rotation of $\{\alpha\}_D^{20} = +19°$. When this is dissolved in water its rotation gradually changes and finals to 52.7°. This is again due to mutarotation and formation of *α*, *β* and straight chain forms of glucose in an equilibrium of 1/3 : 2/3 : 0/1(*n*).

Chemical reactions of carbohydrates:

1. **Reducing action of sugars:** In alkaline medium, the aldehydic or ketonic group of sugars can reduce a number of substances (metals) like copper, silver, mercury and bismuth. Copper salts are reduced to cuprous hydroxide or oxide in solution. The sugars are identified in the urine and blood based upon this principle. Benedict's reagent is commonly used for the detection of sugars in urine.
   (a) *Reducing sugars*: Sugars having a free aldehydic or ketonic group are known as reducing sugars. e.g., glucose, fructose, galactose and all other monosaccharides. Among disaccharides maltose and lactose are reducing sugars.

***Benedict's test*:** This is a semi quantitative test most commonly used for the detection of the percentage of sugar in urine. Benedict's test is carried out in a mild alkaline media. Hence weak reducing agents like uric acid and creatinine in urine cannot reduce Benedict's reagent. Therefore, this test is very specific for glucose or other reducing sugars in urine.

Principle: Cupric ions (hydroxide) in the Benedict's reagent are kept in solution as alkaline citrate complex. When Benedict's reagent is heated with the reducing sugar, the cupric ions are reduced to cuprous ions (oxide), which are less soluble in water, and hence they precipitate out of the alkaline solution as cuprous oxide.

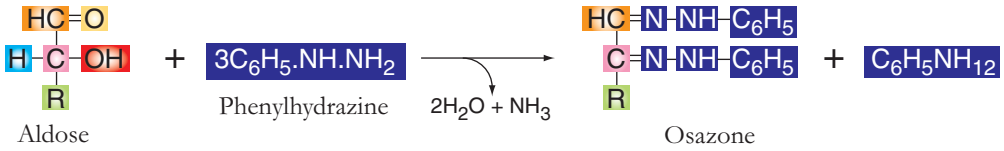
Procedure: 5 ml of Benedict's reagent is taken in a clean dry test tube and it is heated to boil. Upon confirmation that there is no formation of precipitate, 8 drops of urine is added to it and is heated for 2 more minutes. Formation of a coloured precipitate, after addition of urine is a positive indication. The colour of the precipitate depends upon the percentage of reducing sugar.

Percentage of reducing sugar (in gm)	Colour of the precipitate
0.5 - 0.9	**Green**
1.0 - 1.4	**Yellow**
1.5 - 1.9	**Orange**
2.0 and above	**Brick red**

(b) *Non-reducing sugars*: Sugars that do not have a free aldehydic or ketonic group are called as non-reducing sugars. e.g., sucrose and trehalose.

Note: Though polysaccharides have at least one free aldehydic or ketonic group, but still they are non-reducing sugars owing to their larger molecular size and complexity of the structure. Hence the aldehydic or ketonic group is not available for the reducing action.

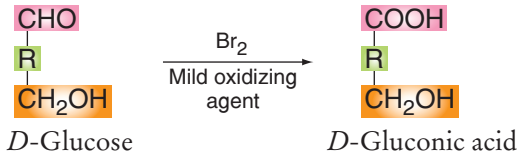
2. **Formation of osazones:** Phenylhydrazine reacts with reducing sugars to form osazones. It involves carbonyl carbon and the adjacent carbon. Osazone is a crystalline compound and is used as an identification test for sugars.



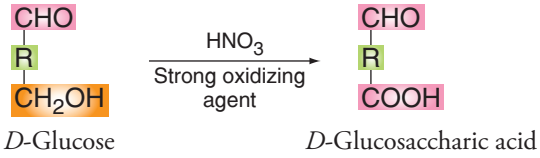
Fructose and glucose forms a broom stick shaped crystal in 3 and 5 minutes respectively. Maltose forms star shaped crystals in 20 minutes whereas lactose forms puff shaped crystals in 30 minutes time.

3. **Oxidation of sugars**

(a) *Mild oxidizing agent* like bromine oxidizes the aldehydic group of carbohydrates converting it to an acid group.

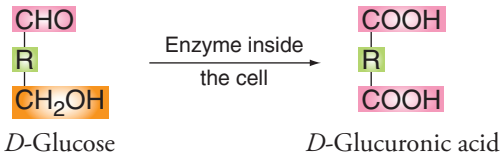


(b) *Strong oxidizing agent* like nitric acid oxidizes the primary alcohol of the carbohydrates forming saccharic acids.



Galactose forms mucic acid, which in insoluble in water. This forms an identification test for galactose known as mucic acid test.

(c) *Enzymes*: Inside the cell, the enzymes oxidize both the aldehydic and primary alcoholic groups of the carbohydrates forming uronic acids.



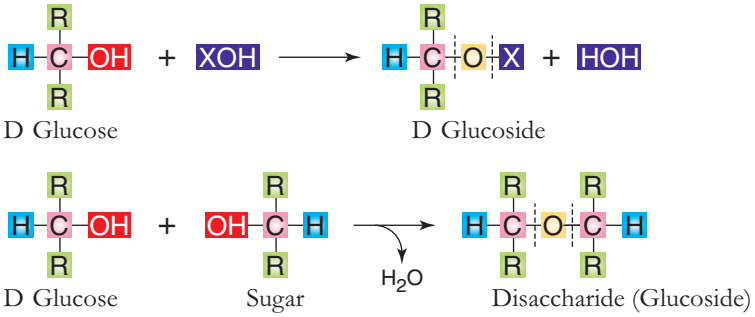
D-glucuronic acid is a component of structural materials like chondroitin sulphate, mucoitin sulphate and glycoproteins (proteoglycons). It plays an important role in detoxification of bile pigments. *L*-glucose forms ioduronic acid.

4. **Dehydration with strong acids:** Concentrated H_2SO_4 removes the adjacent –OH groups as water (H_2O) forming furfural form pentoses and hydroxymethyl furfural from hexoses.

Furfural Hydroxymethyl Furfural

Furfural condenses with α-naphthol in presence of alcohol forming a purple violet coloured complex. This is the principle of Molisch's test which is a common identification test for all carbohydrates.

5. **Derived sugars:** Substances formed from sugars on oxidation, reduction or addition/replacement of any group are called derived sugars.
   (a) *Amino sugars*: The hydroxyl group at the second carbon of a sugar is replaced by an amino group to form an amino sugar. e.g., glucosamine, galactosamine.
   (b) *Deoxy sugars*: These sugars are formed due to removal of one of the oxygen from the alcoholic group. e.g., 2-Deoxy-ribose, here the 'O' of the 2nd alcoholic group is removed. It is present in DNA. *L*-fucose is 6-Deoxy *L*-galactose, *L*-rhamnose is 6-Deoxy L-mannose.
   (c) *Oxidation products of carbohydrates*: Uronic acids and saccharic acids are also derived sugars.
6. **Formation of glycosides with alcohol:** When two alcoholic groups react with each other, a glycoside is formed. Carbohydrates contain many alcoholic groups. Hence two carbohydrates can react with alcoholic groups of one another sugars, forming glycosides. Union of two carbohydrates is known as a disaccharide, three is trisaccharide and many is a polysaccharide.



Medically important glycosides: There are some glycosides, other than carbohydrates discussed in this chapter, that are medically important, some of them are—

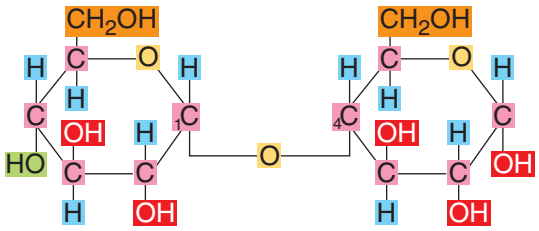
(i) *Digitonin*: This is a cardiac glycoside.
(ii) *Saponin*: A plant glycoside used as an immunostimulating agent.
(iii) *Phlorhizin*: Also a plant glycoside used in kidney functions.

DISACCHARIDES

Sugars containing two monosaccharide units linked by glycosidic bond are known as disaccharides. The three most common disaccharides are discussed below:

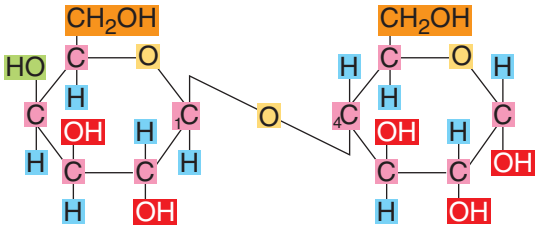
1. **Maltose:** It contains two *α-D*-glucose units linked by *α*-1→4 glycosidic linkage. Chemically it is named as *α-D*-glucopyranosyl-(α-1→4)-*α-D*-glucopyranose. It is also known as malt sugar. It is the

product of starch hydrolysis. It is a reducing sugar and forms star shaped osazone crystals.



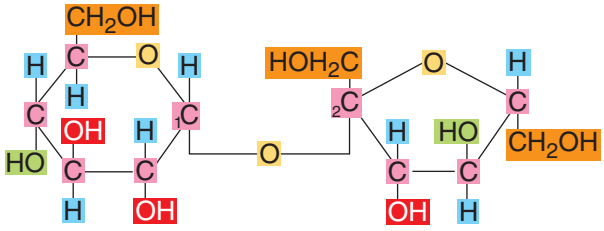
Maltose

2. **Lactose:** Made up of *β-D*-galactopyrasone and *α-D*-glucopyranose linked through *β*-1→4 glycosidic linkage. Chemically it is called as *β-D*-galactopyranosyl-(β-1→4)-*α-D*-glucopyranose. It is present in milk and hence called milk sugar. It is a reducing sugar and forms puff shaped osazone crystals.



Lactose

3. **Sucrose:** Contains *α-D*-glycopyranse and *β-D*-fructofuranose linked through *α*-1→2 glycosidic linkage. Its chemical name is α-D-glucopyranosyl-(α-1→2)-*β-D*-fructofuranose. It is the common table sugar obtained from sugar cane hence the name *cane sugar*. As it is a non-reducing sugar it does not form osazones. It is also known as invert sugar.



Sucrose

Invert sugar: Sucrose is known as invert sugar because sucrose is dextrorotatory with a specific rotation of +62.5°. On hydrolysis by an enzyme – sucrase or invertase, it gives a mixture of glucose and fructose. This mixture exhibits a net specific rotation of –19°, i.e., levorotatory. The phenomenon by which dextrorotatory sugar is converted to a levorotatory sugar is known as invert sugar.

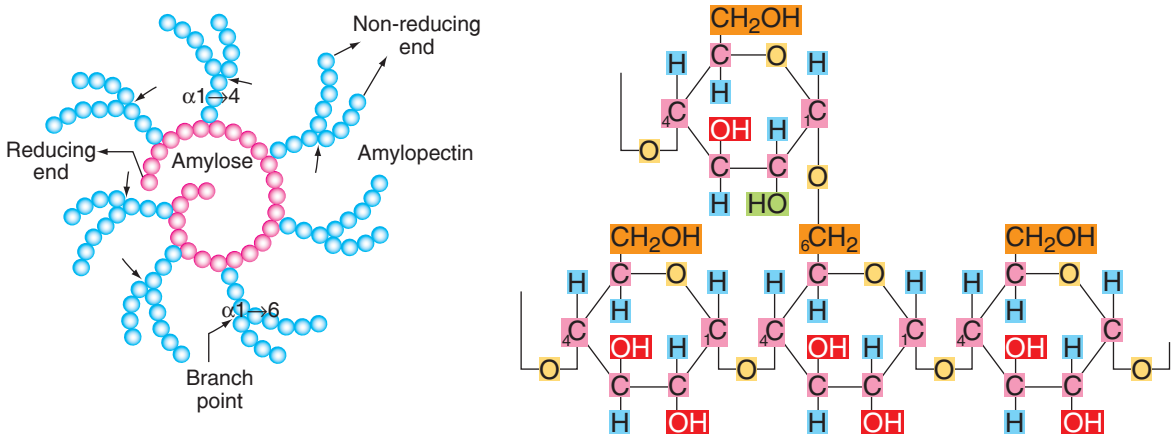
Chocolate companies use invertase enzyme in the preparation of toffees in order to increase the taste and commercial value. In the preparation of these toffees solid sucrose caramel is made and coated with the enzyme-sucrase or invertase over which cocoa is engorged. The enzyme hydrolyses solid sucrose into liquid glucose and fructose which is seventy times sweeter, thereby attracting the consumer.

POLYSACCHARIDES

Carbohydrates made up of 10 or more monosaccharide units are called as polysaccharides. They are also known as glycans. They are further classified as homopolysaccharides and heteropolysaccharides.

Homopolysaccharides: Those polysaccharides which contain only one kind of monosaccharide unit are called homopolysaccharides. e.g., starch, glycogen, cellulose, dextran, inulin, agar, chitin, etc.

1. **Glycogen:** It is known as animal starch. It is made up of α-*D*-glucose units linked by α-1→4 linkages in the linear and α-1→6 linkages at the branching points. It is highly branched. Glycogen is the storage form of energy (glucose) in each and every cell of the human body. Liver and muscle contain the highest amount of glycogen. At least 5% of glycogen is present in each cell even under severe fasting/starvation condition. It gives a red colour with iodine.
2. **Starch:** It is made up of α-*D*-glucose units, hence known as glucosan. It is composed of amylose and amylopectin. Amylose is coiled and unbranched. The α-*D*-glucose units are linked by glycosidic linkages. Amylopectin is uncoiled and is highly branched. It has α-1→4 linkages in the linear chain and α-1→6 at the branching points. It is the chief carbohydrates present in plants and forms the main source of dietary energy sources to humans. It gives a blue colour with iodine.



3. **Cellulose:** Made up of β-*D*-glucose units linked by β-1→4 glycosidic linkages. It is unbranched. It is the most abundant carbohydrate in nature. It forms the woods of the plant. Cellulase enzyme is absent in human being and hence it becomes non-utilizable. However, it adds to the bulk of the food and helps in the gastric motility.
4. **Dextran:** It is produced by yeasts and bacteria. It is made up of α-*D*-glucose linked by α-1→6 glycosidic linkages. The branching points are at 1-2, 1-3 and 1-4. It absorbs water to form gels. It is used as plasma substitute.
5. **Inulin:** It is a fructosan. It cannot be metabolized by the body, hence used to assay glomerular filtration rate (G.F.R.) in the study of kidney function.
6. **Agar:** It is a sulphated galactose. It dissolves in hot water. It gels on cooling, thereby forming a solidified medium in tissue culture studies.
7. **Chitin:** *N*-acetylglucosamine (chitosamine) linked by β-1→4 linkages. Present in the exoskeleton of invertebrates like cockroach and crab.

Heteropolysaccharides: Polysaccharides made up of two or more kinds of monosaccharide units. e.g., pectins and mucopolysaccharides.

Most of them are branched and exist in conjugation with proteins and hence called proteoglycans. The carbohydrate part is called glycosaminoglycan. They have a repeating disaccharide unit which is acetylated or sulphated. There are two major types of heteropolysaccharides.

(a) **Pectins:** They are composed of galacturonic acid, galactose and arabinose.

(b) **Mucopolysaccharides:** They are sticky polysaccharides (mucin like). The various types of mucopolysaccharides and their composition is given below—

Mucopolysaccharide	Composition	Importance
1. Hyaluronic acid	1. *D*-Glucuronic acid 2. *N*-Acetyl-D-glucosamine	Occurs in synovial fluid, skin and vitreous humour.
2. Chondroitin sulphate	1. *D*-Glucouronic acid 2. *N*-Acetyl-D-galactosamine sulphate	Component of cartilage, tendons and skin (connective tissue).
3. Dermatan sulphate	1. *L*-Ioduronic acid 2. *N*-Acetyl-D-galactosamine sulphate	Present in skin.
4. Keratan sulphate	1. *D*-galactose 2. *N*-Acetyl-D-glucosamine sulphate	Component of cartilage and cornea.
5. Heparin	1. *D*- Ioduronic acid (sulphated) 2. *N*-Acetyl-D-glucosamine (sulphated)	Present in most cells especially in liver, lungs and arterial walls.

5 CHAPTER

LIPIDS

Lipids are heterogenous group of compounds related either directly or indirectly (potentially) to fatty acids. They are insoluble in water but soluble in fat solvents like ether and benzene.

FATTY ACIDS

They are the monocarboxylic acids with a long hydrocarbon chain. The minimum number of carbon atoms required to be called as fatty acid is 4.

Fatty acid

There are two types of fatty acids (1) Saturated fatty acids, (2) Unsaturated fatty acids.

1. **Saturated fatty acids:** These fatty acids contain only single bonds along the length of the carbon chain, i.e., all the carbon atoms are fully saturated with hydrogen atoms.

The fatty acids found in the human body contain odd number (4, 6, 8, ···) of carbon atoms. All these are solids at room temperature. The various saturated fatty acids and the numbers of carbon atoms are:

Name of the fatty acid		Carbon Nos .	Name of the fatty acid		Carbon Nos.
1.	Butyric acid	4	4.	Palmitic acid	16
2.	Lauric acid	12	5.	Stearic acid	18
3.	Myristic acid	14	6.	Arachidic acid	20

2. **Unsaturated fatty acids:** These fatty acids contain one or more double bonds along the length of the hydrocarbon chain.

In human tissues, the double bond is of –cis type (i.e., the substituents are on the same side of the double bond). To represent the position of the double bond in a fatty acid, it is represented as Delta *n* (Δ^n), where *n* shows the position of double bond between the *n*th carbon atom and the carbon atom next to it towards the omega (last) carbon atom. Unsaturated fatty acids are all liquids at room temperature. They are further classified depending upon the number of double bonds present per fatty acid as—

(a) **Monounsaturated fatty acids:** They contain only one double bond per fatty acid.

Name of the fatty acid		Number of carbon atoms	Position of the double bond
1.	Palmitoleic acid	16	cis Δ^9
2.	Oleic acid	18	cis Δ^9

(b) **Polyunsaturated fatty acids:** They contain two or more double bonds along the length of the hydrocarbon chains.

Name of the fatty acid		No. of carbon atoms	No. of double bonds	Position of the double bond
1.	Linoleic acid	18	2	cis $\Delta^{9, 12}$
2.	Linolenic acid	18	3	cis $\Delta^{9, 12, 15}$
3.	Arachidonic acid	20	4	cis $\Delta^{5, 8, 11, 14}$

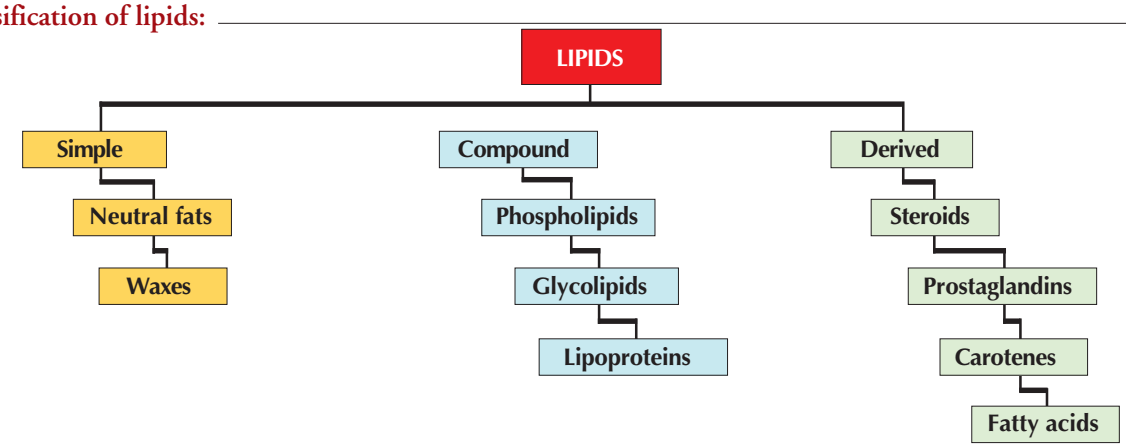
The polyunsaturated fatty acids are also known as essential fatty acids as they cannot be synthesized in the body and hence must be taken through the diet.

The other classes of fatty acids are—

(a) **Cyclic fatty acids:** Chaulmorgic acid

(b) **Hydroxy fatty acids:** (i) Saturated—Cerebronic acid and (ii) Unsaturated—Ricinoleic acid.

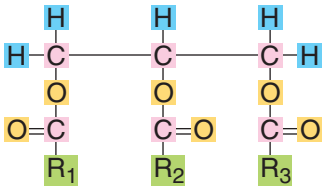
Classification of lipids:



SIMPLE LIPIDS

They are esters of fatty acids with alcohol. Depending upon the alcohol, they are further classified as—

1. **Neutral fats:** These are esters of fatty acids with glycerol (a trihydric alcohol). These are known as triacylglycerols (TAG) or triglycerides.



Triacylglycerol

R_1, R_2, and R_3 are the three fatty acids. All the three may be the same or different. If all the three *R*s are the same, then it may be, Tripalmitin-3 palmitic acids esterified with glycerol. Tristearin-3 stearic acids esterified with glycerol.

If the '*R*' groups are different then it is spelled out as Palmeto-stearo-olein indicating that glycerol is esterified with palmitic acid, stearic acid and oleic acid.

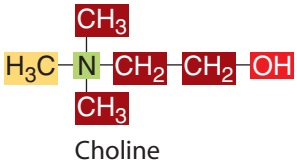
2. **Waxes:** These are esters of long chain fatty acids with long chain alcohol. e.g., Bee wax is ester of oleic acid (18 carbons) and oleyl alcohol (18 carbons).

COMPOUND LIPIDS

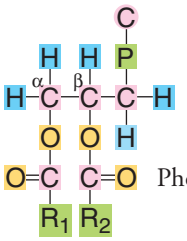
Simple lipids in combination with some other group are called compound lipids. Depending upon the group attached (prosthetic group) the compound lipids are further classified as—

1. **Phospholipids:** They contain a phosphoric acid as the prosthetic group. Depending upon the alcohol present they are further classified as—
   (a) **Glycerophospholipids:** They contain the alcohol-glycerol. The components of glycerophospholipids are glycerol, two fatty acids (the one at α-position is saturated fatty acid and the other at β-position is unsaturated), phosphoric acid and a base. Glycerol, fatty acids and phosphate together form a phosphatide to which a base is attached. Depending upon the base present there are various glycerophospholipids.

➠ **Phophatidyl choline or lecithin:** Here the base is choline



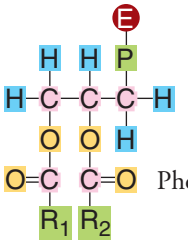
Choline



Phosphatidyl choline (Lecithin)

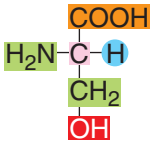
➠ **Phosphatidyl ethanolamine or cephalin:** Here the base is ethanol amine, attached through – OH group.

Ethanolamine

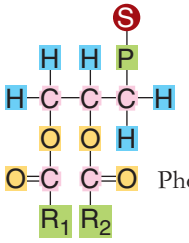


Phosphatidyl ethanolamine (Cephalin)

➠ **Phosphatidyl serine:** Here the base is the amino acid serine.

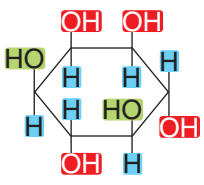


Serine

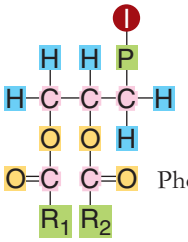


Phosphatidyl serine

➠ **Phosphatidyl inositol:** Here the base is inositol.

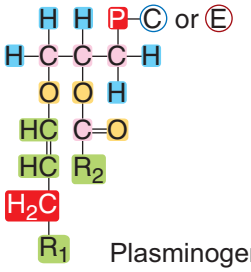


Inositol



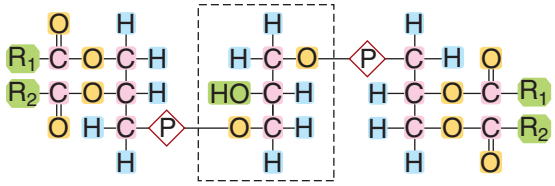
Phosphatidyl inositol

➠ **Plasminogen:** Here one of the fatty acids of the phosphatide is replaced by a long chain aldehyde which is in an enolic form. The base may be choline or ethanolamine.



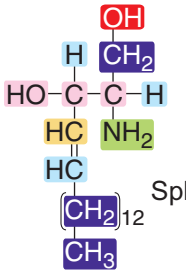
Plasminogen

➠ **Cardiolipin or diphosphatidyl glycerol:** Here two phosphatide groups are linked together through a glycerol.



Diphosphatidyl-glycerol (Cardiolipin)

(b) **Sphingophospholipids:** These phospholipids have sphingol as the alcohol. Sphingol is an amino alcohol with a chain length of 18 carbons having a double bond at trans delta 4 position. An example of sphingophospholipid or sphingolipid is sphingomyelin, which contains a fatty acid at the amino group (and this combination, i.e., sphingol and fatty acid is known as ceramide), a phosphoric acid at the primary alcohol and the base choline is attached to this phosphate group.



Sphingophospholipid

2. **Glycolipids:** These lipids contain a carbohydrate attached to the sphingol at the primary alcohol. They are also known as glycosphingosides or cerebrosides.
   (a) **Glucocerebrosides:** If the sugar is glucose, then they are called as glucocerebrosides.
   (b) **Galactocerebrosides:** If the sugar is galactose then they are called as galactocerebrosides.
   (c) **Gangliosides:** These are complex sphingolipids made up of several sugar units, viz., glucose, galactose, galactosamine and *N*-acetyl-neuramic acid or sialic acid.
3. **Lipoproteins:** These are lipids in conjugation with proteins. They mainly function for the transport of lipids (hydrophobic) through the blood (hydrophilic). The different types of lipoproteins and their composition is—

Name of the lipoprotein	Composition					Function in the body
	Protein		Triacyglycerol (TAG)	Phospholipid (PL)	Cholesterol	
	Type	Percent				
Chylomicron	A,B,C,E	2	83	7	8	Transport digested lipids
Very low density lipoprotein (VLDL)	B,C,E	9	50	19	22	Transport TAG from liver to adipose tissue

Contd. to next page

Contd. from previous page

Name of the lipoprotein	Composition					Function in the body
	Protein		Triacyglycerol (TAG)	Phospholipid (PL)	Cholesterol	
	Type	Percent				
Low density lipoprotein (LDL)	Apo B	21	10	22	47	Transport cholesterol from liver to kidney
High density lipoprotein (HDL)	A,C,D,E	33	8	29	30	Blood scavengers for cholesterol

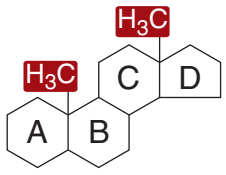
These lipoproteins are classified depending upon their densities in water. The density of a lipoprotein depends upon the fat content of that lipoprotein, more the fat content lower the density and hence it floats on the surface of water (vice versa). The protein part in the lipoprotein is known as *apoprotein*. The various types of apoproteins found in lipoproteins are apoprotein- A, B, C, D, E. Lipoproteins also constitute the combination of membrane proteins with membrane lipids.

DERIVED LIPIDS

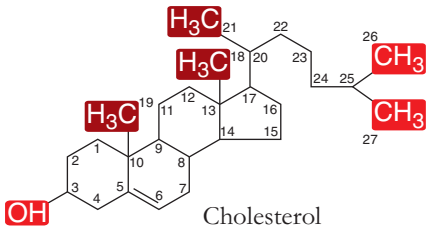
These are the compounds obtained on hydrolysis of simple and compound lipids. They also constitute all those compounds that are related to fatty acids. They include fatty acids, steroids, eicosanoids (prostanoids-prostaglandins, prostacyclins, thromboxanes-leukotrienes and lipoxins) carotenoids etc.

Steroids: All compounds containing the cyclo-pentano-perhydro-phenanthrene ring are called steroids. The most abundant steroids in the human body are the sterols, i.e., an alcohol (–OH) group is attached to the steroid nucleus. e.g., cholesterol, ergosterol, bile acids, sex hormones, adrenal cortical hormones and vitamin D_3.

Cholesterol is the major sterol in the body. It is a constituent of cell membrane and provides rigidity to it. Cholesterol acts as the precursor for all the other steroids in the body, viz., testosterone, estrogen, progesterone, vitamin-D, bile salts, corticosteroids, etc.

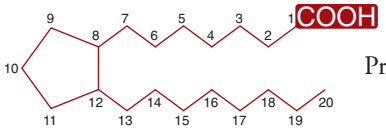


Cyclo pentano perhydro phenanthrene



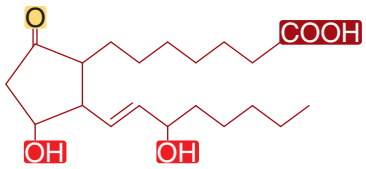
Cholesterol

Prostaglandins: They are the derivatives of polyunsaturated fatty acids, mainly the arachidonic acid (C_{20}) or even linoleic acid (C_{18}). They are 20 carbon fatty acids with a 5 membered ring.



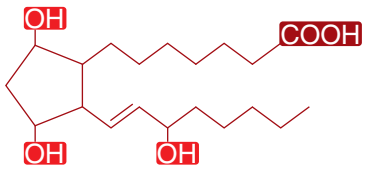
Prostaglandin

There are four types of prostaglandins PGE, PGF, PGA and PGB. But only PGE and PGF are important. The E group of prostaglandins contains a keto group at C-9, two –OH groups at C-11 and C-15 positions. The various types of PGE are E_1, E_2, E_3.



PGE

The PGF groups contain –OH group at all the three positions. The various types of PGF are F_1, F_2 and F_3. Among the subtypes there are two more sub-subtypes in each of the prostaglandins, i.e., α and β.



PGF

Functions of prostaglandins: Prostaglandins are synthesized in all tissues and cells except RBC. They act as local hormones and are destroyed immediately. Other functions include as under:

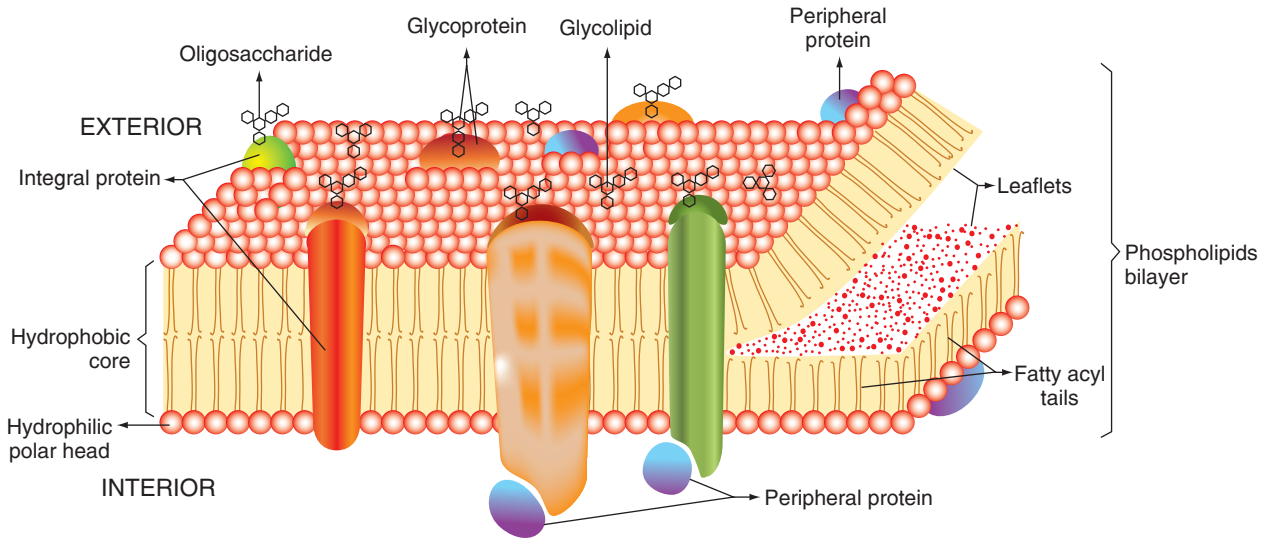
1. They act as vasopressors and hence lower the blood pressure.
2. They are used in induction of labour, termination of pregnancy and prevention of conception.
3. They facilitate fertilization of the ovum.
4. They are used in the treatment of gastric ulcers, as they diminish HCl secretion.
5. They are used to prevent inflammation.
6. They are used in asthma and congenital heart diseases.
7. They promote platelet aggregation (after conversion to thromboxanes).
8. PGE_2 acts as a messenger between hormone receptor and adenylate cyclase enzyme.

Chemical properties of lipids:

1. **Saponification:** Hydrolysis of TAG with KOH or NaOH is called saponification or soap formation. These soaps are the household soaps. Sodium soaps are hard and potassium soaps are soft. Detergents have acidic group like sulphuric acid attached to the fatty acids.
2. **Saponification number:** It is the number of milligrams of KOH required to saponify the free and combined fat in 1 gram of a given fat. A high saponification number indicates that the fat is made up of low molecular weight fatty acids and vice versa.
3. **Iodine number:** It is the grams of iodine required to saturate 100 grams of fat. It is an indication of unsaturation.
4. **Rancidity:** Fats contaminated with enzymes like lipase undergo partial hydrolysis and oxidation of unsaturated fatty acids at the double bonds. This is even brought about by the atmospheric moisture and temperature. Due to this, there is release of hydrogen peroxide giving a bad odour and taste to the fat. This fat is said to be rancid and the process is known as rancidity. Rancidity can be prevented by antioxidants like vitamin E, vitamin C, phenols, hydroquinones, etc.

Molecular structure of a cell membrane: Each cell and sub-cellular organelles are surrounded by a lipid bi-layer. 70 to 80 per cent are polar lipids and the remainder is mostly protein. The lipid part of

the membrane is polar or amphipathic lipid largely phosphoglycerides, some amounts of sphingolipids and a negligible amount of triacylglycerols. Cholesterol and cholesterol esters are also present in the membrane.



Membranes are very thin from 6 to 9 nm, flexible and fluid. They are freely permeable to water but impermeable to electrically charged ions like Na^+, Cl^- or H^+ and to polar but uncharged molecules like sugars. These impermeable substances are transported with the help of membrane transport proteins. On the other hand lipid soluble molecules readily pass through membranes.

6 CHAPTER

PROTEINS

Proteins are nitrogenous organic compounds of high molecular weight which play a vital or prime role in living organisms. They are made up of 20 standard α-amino acids. The main functions of proteins in human body are—

1. They serve as body building units. e.g., muscle proteins.
2. They provide support and protection to various tissues. e.g., collagen and keratin.
3. All chemical reactions in the body are catalyzed by proteinaceous enzymes. e.g., trypsin.
4. They transport various molecules and ions from one organ to the other. e.g., hemoglobin, serum albumin.
5. They store and provide nutrients. e.g., milk casein, ovalbumin.
6. They defend the body from harmful foreign organisms. e.g., immunoglobulins, fibrinogen.
7. They help to regulate cellular or physiological activity. e.g., hormones, viz., insulin, GH.

AMINO ACIDS

Amino acids are the building blocks of proteins. Among the thousands of amino acids available in nature, proteins contain only 20 different kinds of amino acids, all of them are *L*-alpha-amino acids. The same 20 standard amino acids make proteins in all the living cells, may it either be a virus, bacteria, yeast, plant or human cell. These 20 amino acids combine in different sequences and numbers to form various kinds of proteins. The number of proteins that can be had from these 20 amino acids can be calculated from 20 factorial, i.e., $20 \times 19 \times 18 \times 17 \times 16 \times \ldots\ldots\ldots \times 2 \times 1 = 2.4 \times 10^{18}$. In human beings alone there are more than 100 000 different types of proteins.

The general formulae for an amino acid can be written as 'R-CH-NH_2–COOH'. Depending upon the 'R' group present in the amino acid it is named accordingly. The 20 amino acids found in the proteins are known as primary or standard amino acids. In addition to these, some other amino acids are also found in proteins like 4-hydroxyproline, 5-hydroxylysine, 6-*N*-methyllysine, gamma carboxyglutamic acid and desmosine, all of these are derivatives of standard amino acids.

CLASSIFICATION OF AMINO ACIDS

I. **Depending upon the charge:** Amino acids can be broadly classified into three major groups (1) Neutral (2) Acidic and (3) Basic.

1. **Neutral amino acids:** Those amino acids that do not contain any charge on the 'R' group. They are further classified into the following categories—

(a) ***Aliphatic*:** Those amino acids whose 'R' group contains a chain of carbon atoms–Gly, Ala, Ser, Thr, Val, Leu, Ile, Asn, Gln.

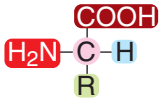
(b) ***Aromatic*:** Those amino acids whose 'R' group has a benzene ring–Phe, Tyr, Trp.

(c) ***Heterocyclic*:** The "R" group has a heterocylic ring, i.e., any of the ring structures which contain different atoms–Pro, His.

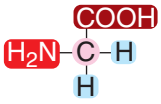
(d) ***Sulphur containing*:** Those amino acids which contain a sulphur atom–Cys, Met.

2. **Acidic amino acids:** Those amino acids that contain a negative charge or an acidic group–Asp, Glu.

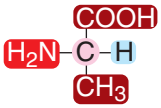
3. **Basic amino acids:** Those amino acids that contain a positive charge or a basic group–Arg, Lys and His.



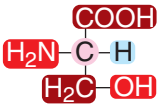
GENERAL FORMULA



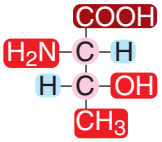
Glycine (Gly)
Mw = 75.08



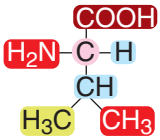
Alanine (Ala)



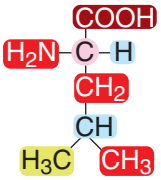
Serine (Ser)



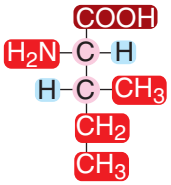
Threonine (Thr)



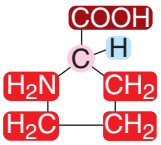
Valine (Val)



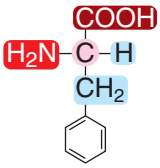
Leucine (Leu)



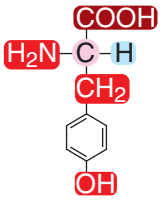
Isoleucine (Ile)



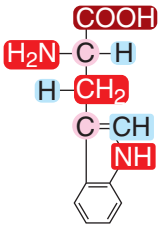
Proline (Pro)



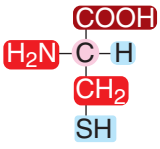
Phenylalanine (Phe)



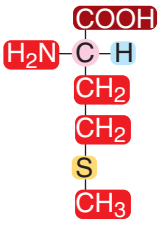
Tyrosine (Tyr)



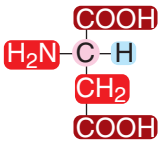
Tryptophan (Trp)
Mw = 206.27



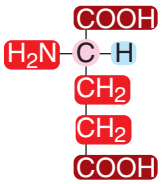
Cystine (Cys)



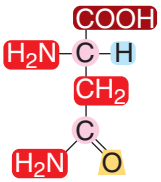
Methionine (Met)



Aspartic acid (Asp)

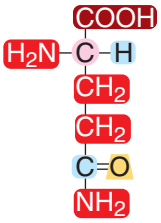


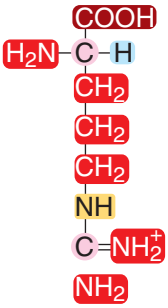
Glutamic acid (Glu)



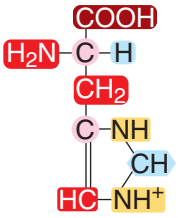
Asparagine (Asn)

Contd.


Glutamine (Gln)


Arginine (Arg)
Mw = 175.25


Lysine (Lys)


Histidine (His)

II. **Depending upon the solubility in water:** The amino acids can also be grouped into two different categories, depending upon their solubility in water. They are—

1. **Hydrophobic amino acids:** Amino acids insoluble in water are known as hydrophobic amino acids. They are—Ala, Val, Leu, Ile, Pro, Met, Phe, Trp.
2. **Hydrophilic amino acids:** Amino acids soluble in water are known as hydrophilic amino acids. They are—Gly, Ser, Thr, Cys, Tyr, Asp, Asn, Glu, Gln, Lys, Arg, His.

III. **Depending upon their nutritional requirements:** The amino acids are classified into two groups. They are—

1. **Essential amino acids:** Are those which cannot be synthesized by the human body and hence they should be taken through the diet. There are 10 essential amino acids. Among these amino acids, arginine and histidine are known as semi-essential amino acids.

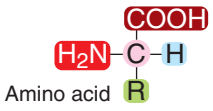
M – Methionine	**V** – Valine	**P** – Phenylalanine
A – Arginine	**I** – Isoleucine	**H** – Histidine
T – Threonine	**L** – Leucine	**Ly** – Lysine
T – Tryptophan		

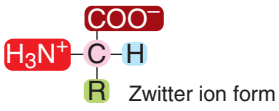
2. **Non-essential amino acids:** These acids are those that can be synthesized in the human body and are not required in the diet. These include gly, ala, ser, pro, tyr, cys, asp, asn, glu, gln.

REACTIONS OF AMINO ACIDS

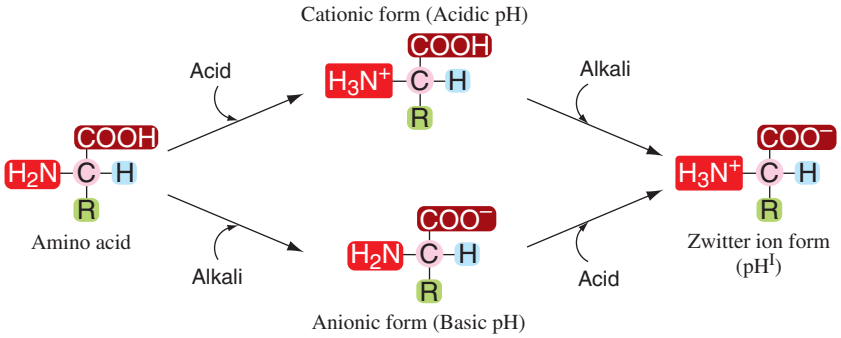
Physical characters of amino acids

1. **Zwitter ions:** Amino acids have an acidic group (–COOH group), i.e., a proton, donor. They also have a basic group (–NH_2 group), i.e., a proton, acceptor. A compound capable of both donating and accepting protons and thus able to act either as an acid or a base is known as amphoteric molecule. Amino acids have both anions and cations in solution and such compounds are called zwitter ions.


Amino acid


Zwitter ion form

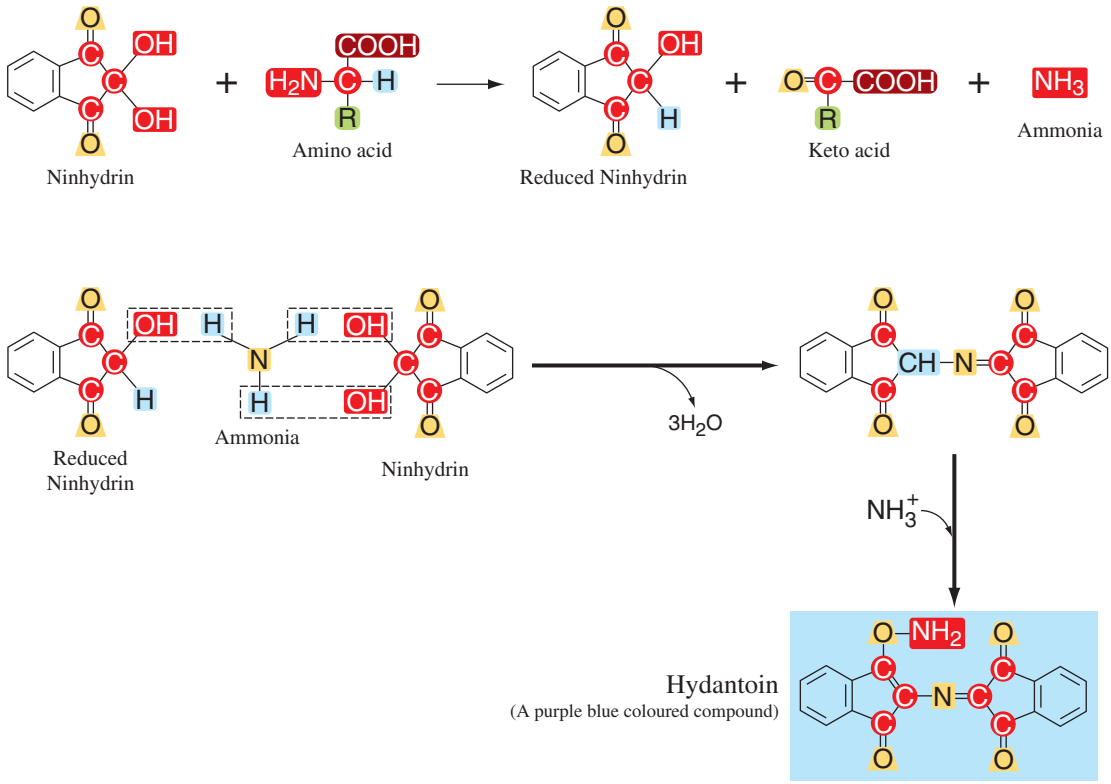
2. **Isoelectric pH (pH^I):** The pH at which the positive charge on the amino acid (or any other molecule) is equal to the negative charges, is known as isoelectric pH. At this pH the net charge will be zero and hence it does not move either to positive (anode) or to negative (cathode) electrode, when subjected to an electric field. At pH^I all the molecules exists in zwitter ion form.



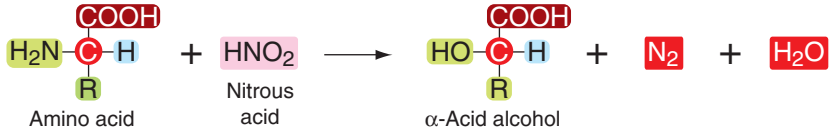
Chemical properties

1. Reactions due to amino group

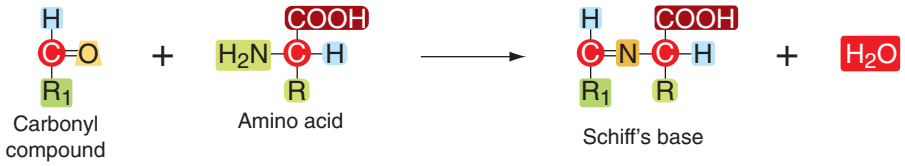
(a) ***Ninhydrin test*:** This test identifies or detects amino acids. If amino acids are heated with ninhydrin, they form a purple blue coloured compound, which is measured colorimetrically.



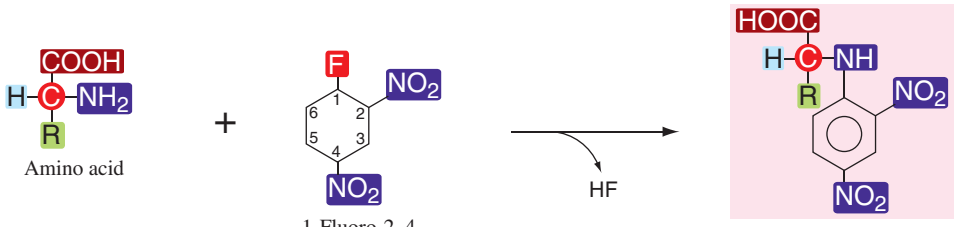
(b) ***Reaction with nitrous acid*:** It is a method by which amino acids are measured depending upon the amount of nitrogen released.



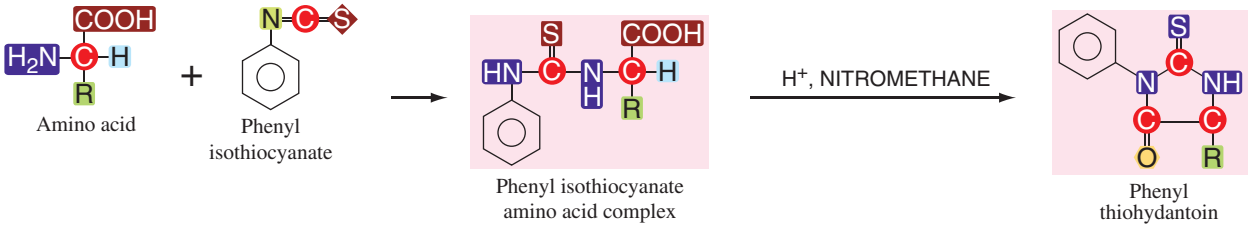
(c) ***Reaction with carbonyl compounds* (RCHO):** The amino group in the amino acids reacts with carbonyl compounds forming a Schiff's base.



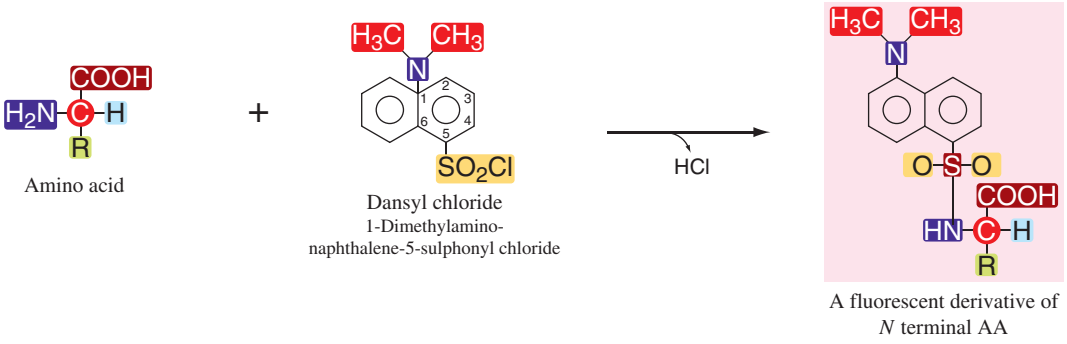
(d) ***Reaction with Sanger's reagent*:** Amino acids react with Sanger's reagent, i.e., 1-fluoro-2, 4-dinitrobenzene, forming a yellow coloured complex. This reagent is used to detect the *N*-terminal amino acid in the proteins.



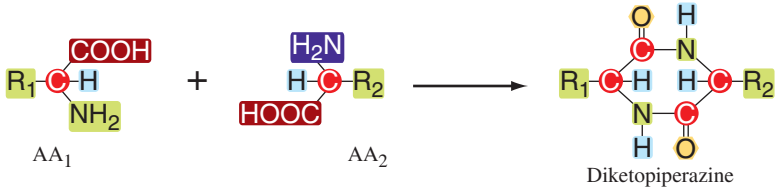
(e) ***Edmann's reaction*:** Edmann's reagent is phenyl isothiocyanate, which is also used to detect the *N*-terminal amino acid in a protein. It forms a purple coloured derivative.



(f) ***Reaction with dansyl chloride*:** Dansyl chloride, i.e., 1-dimethyl-amino-naphthalene-5-sulphonyl chloride forms a fluorescent derivate of the *N*-terminal amino acid of proteins. This is yet another reagent available for the detection of *N*-terminal amino acid.

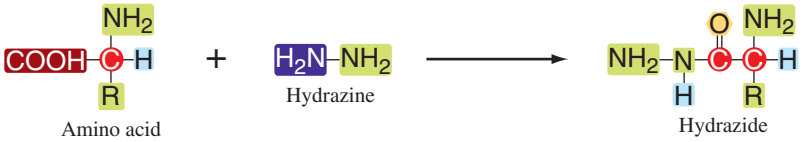


(g) *Condensation of two amino acids to form diketopiperazine*: Two amino acids react with amino groups of each amino acid and the carboxylic groups of the other amino acid forming a diketopiperazine.

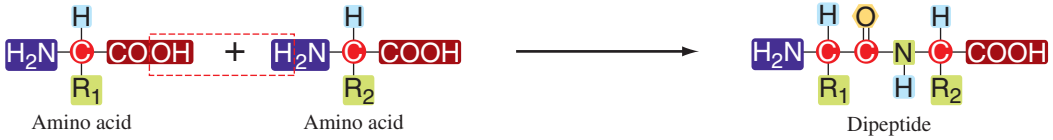


2. Reactions due to carboxylic group

(a) *Reaction with hydrazine*: Hydrazine is used to detect the C-terminal amino acid in proteins. It forms a complex with the amino acid by reacting with the carboxylic group.



3. **Reaction due to both amino and carboxylic group:** Due to the presence of both amino (basic) and carboxylic (acid) groups in amino acids, the amino group of one amino acid reacts with the carboxylic group of another amino acid to form a peptide bond.

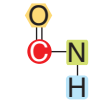


Polymerization of amino acids in a similar manner gives a polypeptide chain.

Peptide bond: The bond linking two amino acids is known as a peptide bond. It is formed due to reaction between an amino group of one amino acid and carboxylic group of another amino acid.

Peptide group: The group forming the peptide bond is known as peptide group. It has a double bond character and hence is very rigid in nature.

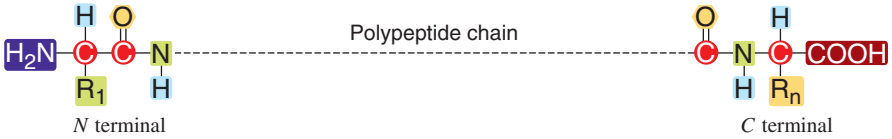
Polypeptide or peptide: A chain made up of two or more amino acids, linked by a peptide bond is known as a polypeptide or just a peptide.



Peptide group

Difference between a peptide and a protein: A peptide is that which has less than 50 amino acids or whose molecular weight is less than 5000 Daltons. A protein is that which has more than 50 amino acids or whose molecular weight is more than 5000 Daltons. This differentiation is based upon the immunological property of the two units Peptides are non-immunogenic, whereas proteins are immunogenic.

***N*-terminal and *C*-terminal of a protein:** The end of a protein or polypeptide where the amino group is free in known as *N*-terminal end and that amino acid whose amino group is free is known as *N*-terminal amino acid. Sanger's, Edmann's and Dansyl chloride are the reagents used to determine the *N*-terminal amino acids.



The end of the protein or polypeptide whose carboxylic group is free is known as *C*-terminal end and that amino acid whose carboxylic group is free in the protein is known as *C*-terminal amino acid. Hydrazine is used to detect the *C*-terminal amino acid. While representing a protein on paper, the *N*-terminal amino acid is written first (on the left) and the *C*-terminal amino acid is the last one (written at the right side of the paper).

Peptides of physiological importance

(a) **Glutathione:** It is a tripeptide made up of Glu, Cys and Gly. It is found in RBC and other tissues and functions to prevent oxidation of –SH groups of many enzymes.

(b) **Bradykinin and kallidin:** These are small polypeptides containing 9 and 10 amino acids respectively. They are formed by partial hydrolysis of plasma protein due to snake poisoning (venom). They are powerful vasodepressors and inhibitors of heart function. Others are tyrocidin, gramicidin, glucagon, insulin, oxytocin, etc.

Structure of protein: The structure of protein can be studied under four different levels of organization, viz., primary, secondary, tertiary and quaternary.

PRIMARY STRUCTURE OF PROTEIN

Primary structure of proteins refers to the total number of amino acids and their sequence in that particular protein.

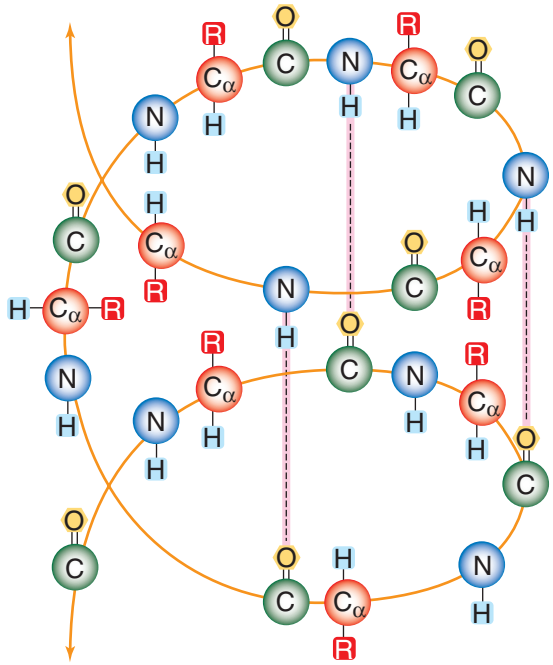
A fixed number of amino acids are arranged in a particular sequence. The sequence of amino acids in the protein determines its biological role. Different proteins have different sequences. Therefore, the study of total number and sequence of amino acids in a protein is the study of its primary structure. Primary structure differentiates normal protein from abnormal one. Normal adult haemoglobin (HbA) is made up of 2 α-chains and 2 β-chains. Each α-chain has 141 amino acids and each β-chain has 146 amino acids arranged in a specific sequence. Any change in the sequence results in an abnormal haemoglobin. Like in sickle cell haemoglobin (HbS), the amino acid valine is present at the 6th position of β-chain instead of glutamic acid in the normal haemoglobin.

SECONDARY STRUCTURE OF PROTEIN

It refers to the twisting of the polypeptide chain into a helical form. Three types of helical structures are found—(a) Alpha helix (b) Beta pleated and (c) Reverse turn.

1. **Alpha helix:** α means the first and the structure described below was the first among the helical structures to be discovered, hence known as alpha (α) helix. The salient features of this structure are as under:
   - Here the polypeptide is twisted or coiled to form a right handed helical structure.
   - The distance between each turn of the coil is 5.4 Å.
   - There are 3.6 amino acids per turn.

- The 'R' groups are seen protruding out of the helix.
- There are intra chain hydrogen bonding, wherein the hydrogen of –NH group combines with oxygen of –CO group of the 4th amino acid behind it. So every peptide group participates in hydrogen bonding.
- This type of structure is found in many proteins in combination with other structures. Pure α-helix structure is seen in hair protein, i.e., keratin.



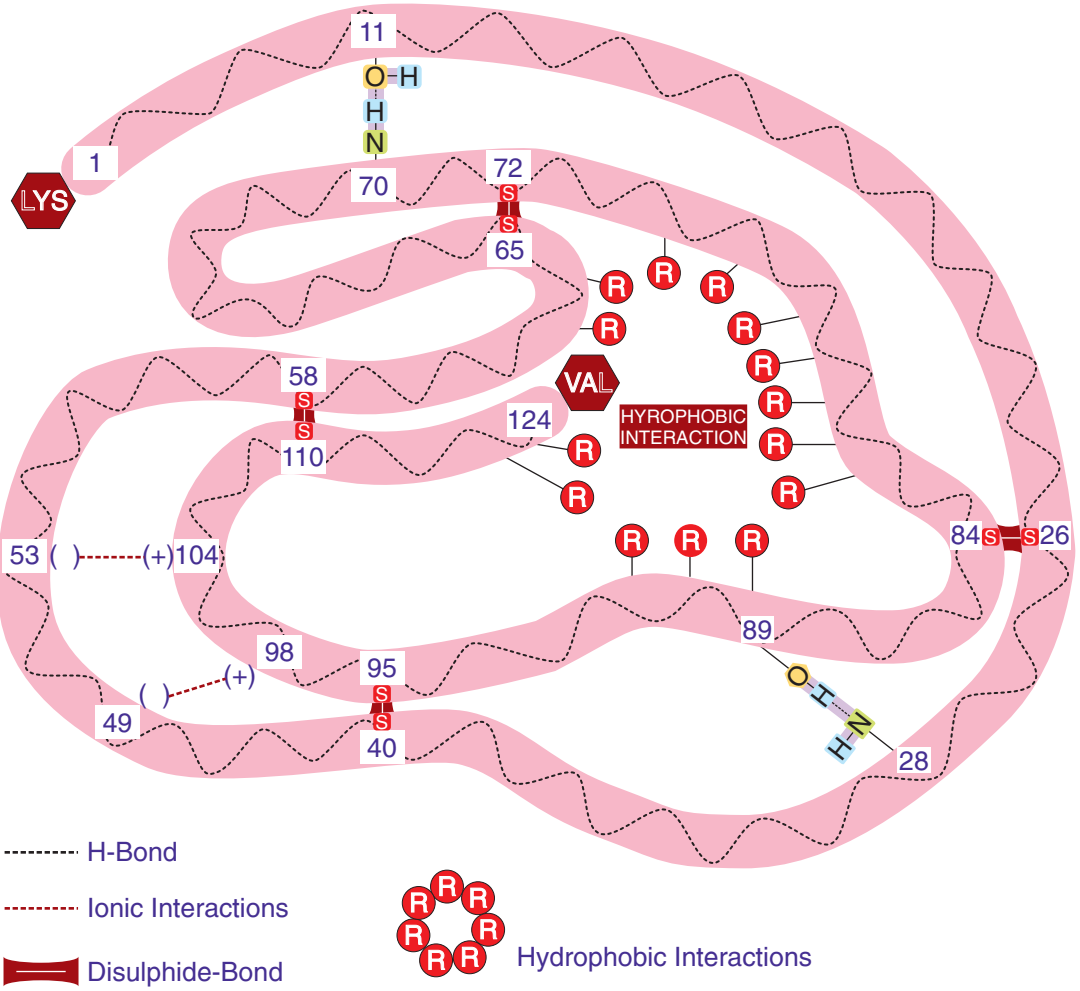
2. **Beta pleated:** β means the second and the structure described below was the second discovery after α helix. The salient features of this structure are—
   - Here the chain is not helical but zig-zag.
   - The distance between each turn is 7 Å.
   - Polypeptide chains are arranged side by side in the form of pleats.
   - There is inter-chain hydrogen bonding between the chains and each peptide group participates in hydrogen bonding.
   - The chains are anti-parallel to each other.
3. **Reverse turn:** Folds back on itself in reverse direction of the chain.

TERTIARY STRUCTURE OF PROTEIN

The helical form of polypeptide folds into spherical, globular, ellipsoidal or other conformation, which is called the tertiary structure of proteins. This folding is necessary for the biological activity of the proteins. e.g., enzymes, immunoglobulins. The tertiary conformation is maintained by four types of bonds—

1. **Hydrogen bonds:** Formed between hydrogen and an electronegative atom like oxygen or nitrogen in the 'R' group of amino acids.

2. **Ionic interactions:** Formed between acidic (glutamic and aspartic) and basic (arginine, lysine or histidine) amino acids.
3. **Disulphide bonds:** This is a strong bond formed between the sulphahydryl groups of two cysteine amino acids. The resultant dimer structure formed is known as cystine (an amino acid found in proteins only and not in free form).
4. **Hydrophobic interactions:** The 'R' groups of the hydrophobic amino acids aggregate together in the centre away from water, thereby developing a force of attraction between each "R" group and a force of repulsion from the water and these interactions are known as hydrophobic interactions.



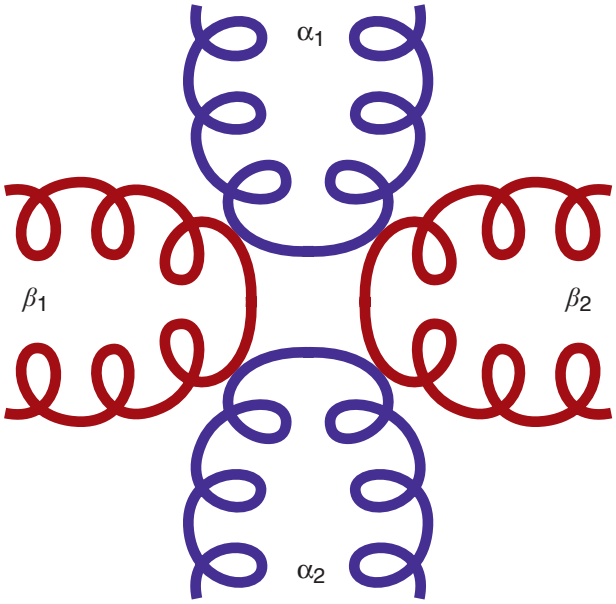
QUATERNARY STRUCTURE OF PROTEIN

Quaternary structure is exhibited by oligomeric proteins.

Oligomeric proteins: Are those which have two or more polypeptide chains.

Quaternary structure refers to the type of arrangement of the polypeptides in an oligomeric protein. These polypeptides are held together by either hydrogen bonds, ionic bonds or Vander Waals' forces. e.g., Haemoglobin has four polypeptide chains which are arranged in a particular fashion that is referred to the quaternary structure of haemoglobin. The Quaternary structure of haemoglobin describes that it is

made up of four polypeptide chains; two of which are α (α_1 & α_2) and the other two are β (β_1 & β_2). The two alpha chains are opposite to each other and adjacent to each β-chain. The α chains and the β chains are linked together by salt bridges.



Structure-function relationship in proteins: Haemoglobin plays a vital role in transport of oxygen from the lungs to the peripheral tissues and transport of carbon dioxide from the tissue to the lungs. There are three types of normal haemoglobin with the following polypeptides—

(1) Adult haemoglobin (Hb A) has 2α2β chains. (2) Foetal haemoglobin (Hb F) has 2α2γ chains. (3) Minor adult haemoglobin (Hb A_1) has 2α2δ chains.

The number of amino acids in α chains is 141 amino acids and the other chains, i.e., β, γ & δ chains have 146 amino acids. These chains are differentiated based upon the difference in the sequence of arrangement of the amino acids in the chains. The quaternary structure of haemoglobin creates a cavity in between the tetramer in which 2,3, diphosphoglycerate (DPG or BPG) is present forming a salt bridge with the amino terminal of β-chain that stabilizes the haemoglobin thereby lowering the affinity to oxygen.

In the lungs, the partial pressure of oxygen is high which results in binding of O_2 to one of the chains of Hb thereby rupturing the salt bridges between the four subunits. Subsequent oxygen binding (sigmoid curve of Hb-O_2 association) is facilitated by rupture of the salt bridges altering secondary, tertiary and quaternary structures thus allowing rotation of one α/β subunit with respect to another α/β chain thereby compressing the tetramer and release of DPG. This results in increasing its affinity towards oxygen (the R state of Hb).

In the peripheral tissues, CO_2 binds with the α-amino group of the amino terminal with its conversion from positive to negative charge which favours salt bridge formation between the polypeptide chains with return to the deoxy state (T-state), i.e., release of oxygen from Hb. Release of O_2 from the Hb is also facilitated by binding of DPG to the tetramer.

When a person takes off on a flight, the aeroplane slowly rises in altitude resulting in lowering of the O_2 tension due to which oxygenation of Hb is not possible. Thus the person feels hypoxic, but the physiological mechanism of the body starts decreasing the production of DPG, due to which Hb can bind the oxygen even at lower pressure of oxygen. Therefore, when the aeroplane reaches the maximum altitude and stays stable, the person feels comfortable. When Hb reaches the tissues DPG level increases enhancing release of oxygen. Similarly, the above process reverses, when a person dives deep into the sea. The high

O_2 pressure results in increased production of DPG facilitating oxygenation of Hb in the lungs and deoxygenation in the peripheral tissues.

PROPERTIES OF PROTEINS

1. **Denaturation:** Partial or complete unfolding of the native (natural) conformation of the polypeptide chain is known as denaturation. This is caused by heat, acids, alkalies, alcohol, acetone, urea, beta-mercaptoethanol.
2. **Coagulation:** When proteins are denatured by heat, they form insoluble aggregates known as coagulum. All the proteins are not heat coagulable, only a few like the albumins, globulins are heat coagulable.
3. **Isoelectric pH (pH^I):** The pH at which a protein has equal number of positive and negative charges is known as isoelectric pH. When subjected to an electric field the protein do not move either towards anode or cathode, hence this property is used to isolate proteins. The proteins become least soluble at pH^I and get precipitated. The pH^I of casein is 4.5 and at this pH the casein in milk curdles producing the curd.
4. **Molecular weights of proteins:** The average molecular weight of an amino acid is taken to be 110. The total number of amino acids in a protein multiplied by 110 gives the approximate molecular weight of that protein. Different proteins have different amino acid composition and hence their molecular weights differ. The molecular weights of proteins range from 5000 to 10^9 Daltons. Experimentally the molecular weight can be determined by methods like gel filtration, PAGE, ultra centrifugation or viscosity measurements.

CLASSIFICATION OF PROTEINS

Proteins are classified based upon (1) their solubility and (2) their structural complexity.

A. **Classification based upon solubility:** On the basis of their solubility in water, proteins are classified into—

1. **Fibrous proteins:** These are insoluble in water. They include the structural proteins. They have supportive function (e.g., collagen) and/or protective function (e.g., hair keratin and fibrin).
2. **Globular proteins:** They are soluble in water. They include the functional proteins. e.g., enzymes, haemoglobin, etc.

B. **Classification based upon structural complexity:** On the basis of their structural complexity they are further divided into (1) simple (2) conjugated and (3) derived proteins.

1. **Simple proteins:** Proteins which are made up of amino acids only are known as simple proteins. They are further sub-divided into—
   (a) ***Albumins*:** They are water soluble, heat coagulable and are precipitated on full saturation with ammonium sulphate. e.g., serum albumin, lactalbumin and ovalbumin.
   (b) ***Globulins*:** They are insoluble in water, but soluble in dilute salt solutions. They are heat coagulable and precipitate on half-saturation with ammonium sulphate. e.g., serum globulin and ovo-globulin.
   (c) ***Glutelins*:** They are insoluble in water and neutral solvents. Soluble in dilute acids and alkalies. They are coagulated by heat. e.g., glutelin of wheat.
   (d) ***Prolamines*:** Water insoluble but soluble in 70% alcohol. e.g., gliadin of wheat, proteins of corn, barley, etc.

(e) ***Histones*:** Water soluble, basic in nature due to the presence of arginine and lysine, found in nucleus. They help in DNA packaging in the cell. They form the protein moiety of nucleoprotein.

(f) ***Protamines*:** Water soluble, basic in nature, not-heat coagulable. Found in sperm cells, hence component of sperm nucleoprotein.

(g) ***Globins*:** They are water soluble, non heat coagulable. e.g., globin of haemoglobin.

(h) ***Albuminoids or scleroproteins*:** Insoluble in all neutral solvents, dilute acids or alkalies. e.g., keratin of hair and proteins of bone and cartilage.

2. **Conjugated proteins:** Proteins which are made up of amino acids and a non-amino acid/protein substance called the prosthetic group are known as conjugated proteins. The various types of conjugated proteins are—

(a) ***Chromoproteins*:** Here the non-protein part is a coloured compound in addition to the protein part. Ex. Haemoglobin has heme as the prosthetic group and cytochromes also have heme.

(b) ***Nucleoproteins*:** These proteins are bound to nucleic acids. e.g., chromatin (histones + nucleic acids).

(c) ***Glycoproteins*:** When a small amount of carbohydrate is attached to a protein it is known as glycoproteins. e.g., mucin of saliva. (Note: *Glycoproteins* have major amounts of protein and some amount of carbohydrates and *proteoglycans* contain major amounts of carbohydrates and little amount of proteins).

(d) ***Phosphoprotein*:** Phosphoric acid is present with the protein. Ex. Milk casein and egg yolk (vitellin).

(e) ***Lipoproteins*:** Proteins in combination with lipids. e.g., LDL, HDL.

(f) ***Metalloproteins*:** They contain metal ion in addition to the amino acids. e.g., haemoglobin (iron), ceruloplasmin (copper).

3. **Derived proteins:** They are the proteins of low molecular weight produced from large molecular weight proteins by the action of heat, enzymes or chemical agents.

Proteins → Proteans → Proteoses → Peptones → Peptides → Amino acids

7 CHAPTER

NUCLEIC ACIDS

The continuity of species since ages is being maintained without any amendments. It is due to the genetic material present in stable form in a cell. This genetic material is unique and specific for a given species. It exists in the form of a message or code which determines various activities, viz., number of cell divisions, time of organogenesis, size and shape of the various organs, gestation period, age at maturity, reproductive age, senescence and death. The genetic message is stored and expressed accurately, defining the individual species, distinguishing them from one another and assuring their continuity over successive generations. Any change in the message contained in the genetic material is called **mutation** and if expressed from generations to generations it results in evolution of species.

The genetic material is chemically termed as DNA, i.e., Deoxyribonucleic acid, one of the nucleic acids found in the cell. DNA is a linear polymer of nucleotide bases arranged in a unique sequence specific for individuals of different species. The message contained in the DNA, in the form of nucleotide bases encodes the instructions for forming all the other cellular components and provides a template for the production of identical DNA molecules to be distributed to the offspring when a cell divides. This chapter deals with the structural organization and functions of DNA and the other nucleic acid, viz., RNA, i.e., ribonucleic acid.

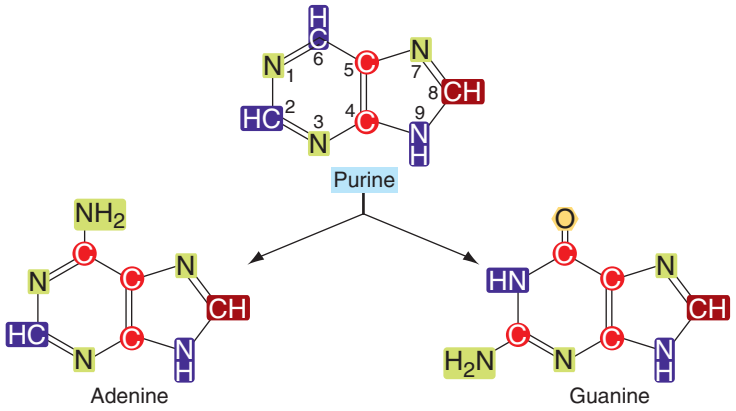
Knowledge of the structural organization of nucleic acids enables the understanding of genetic combinations, gene regulation, genetic improvement of species, genetic diseases, their diagnosis and treatment, modern biotechnological aspects like polymerase chain reaction, genetic engineering, DNA finger-printing technique and parental identification.

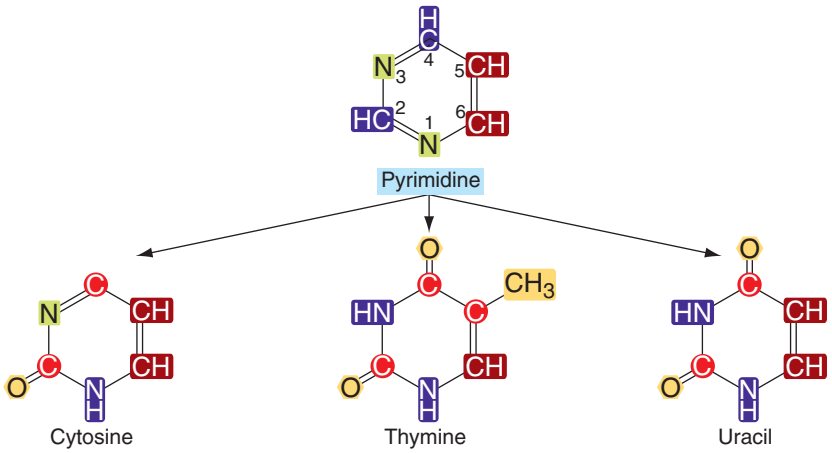
Chemistry: Nucleic acids are acidic compounds first discovered in the cell nucleus. Later they were found even in the cytoplasm. They are also present in the mitochondria and chloroplast. Nucleic acids are high molecular weight nitrogenous organic compounds which play an important role in storage, transmission and control of all the cellular activities. Nucleic acids are defined as polynucleotides, i.e., chain-like polymers of unto thousands of nucleotide units. Each nucleotide is a molecular complex of nucleoside and phosphoric acid.

NUCLEOSIDE

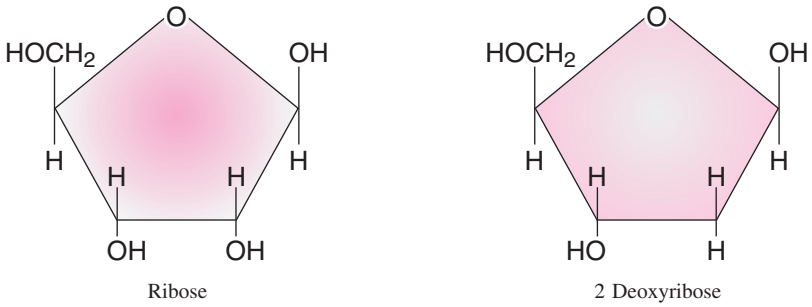
A nucleoside is composed of two components—(a) nitrogenous base and (b) five-carbon sugar (pentose).

1. **Nitrogenous bases:** The nitrogenous bases are derivatives of two parent heterocyclic compounds, i.e., purine and pyrimidine.





2. **Pentose Sugars:** The pentose sugar present in the nucleic acids is ribose in RNA and deoxyribose in DNA.



A nucleoside is formed by the linkage of the nitrogenous base to the –OH group at the first carbon of the pentose sugar through an *N*-glycosyl linkage. The binding of the two nitrogenous bases to the –OH group at the first position in ribose sugar differs. The purine bases are linked through the nitrogen

present at the ninth (9th) position and the pyrimidine bases are linked by the nitrogen present at the position one (1). The various nucleosides thus formed are—

Purines:

Adenine + Pentose = Adenosine

Guanine + Pentose = Guanosine

Pyrimidines:

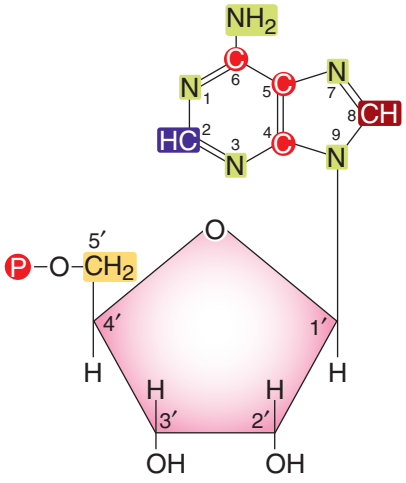
Cytosine + Pentose = Cytidine

Thymine + Pentose = Thymidine

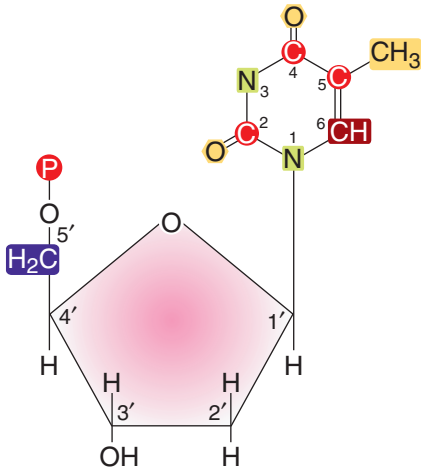
Uracil + Pentose = Uridine

NUCLEOTIDE

A nucleotide is formed by esterification of phosphoric acid to the –OH group present at the fifth (5th) position of the pentose sugar in a nucleoside.



Purine ribonucleotide



Pyrimidine deoxyribonucleotide

It can be observed in the nucleotide structures that, there are two cyclic rings–one pentose and the other nitrogenous base. While referring to any member in a particular ring, the number of the member will overlap in both the rings. Hence, in order to avoid confusion, and for convenience the members in the nitrogenous bases and the sugar are numbered differently. The members of the purine ring are numbered from 1 to 9 and that of pyrimidine ring from 1 to 6. On the other hand, the members of the pentose sugar are numbered as 1′, 2′, 3′, 4′ and 5′ (read as one prime, two prime, three prime, four prime and five prime).

Purines: Adenine + Ribose + Phosphoric acid → Adenylate (present in RNA)

Adenine + Deoxyribose + Phosphoric acid → Deoxyadenylate (present in DNA)

Guanine + Ribose + Phosphoric acid → Guanilate (present in RNA)

Guanine + Deoxyribose + Phosphoric acid → Deoxyguanilate (present in DNA)

Pyrimidines: Cytosine + Ribose + Phosphoric acid → Cytidylate (present in RNA)

Cytosine + Deoxyribose + Phosphoric acid → Deoxycytidylate (in DNA)

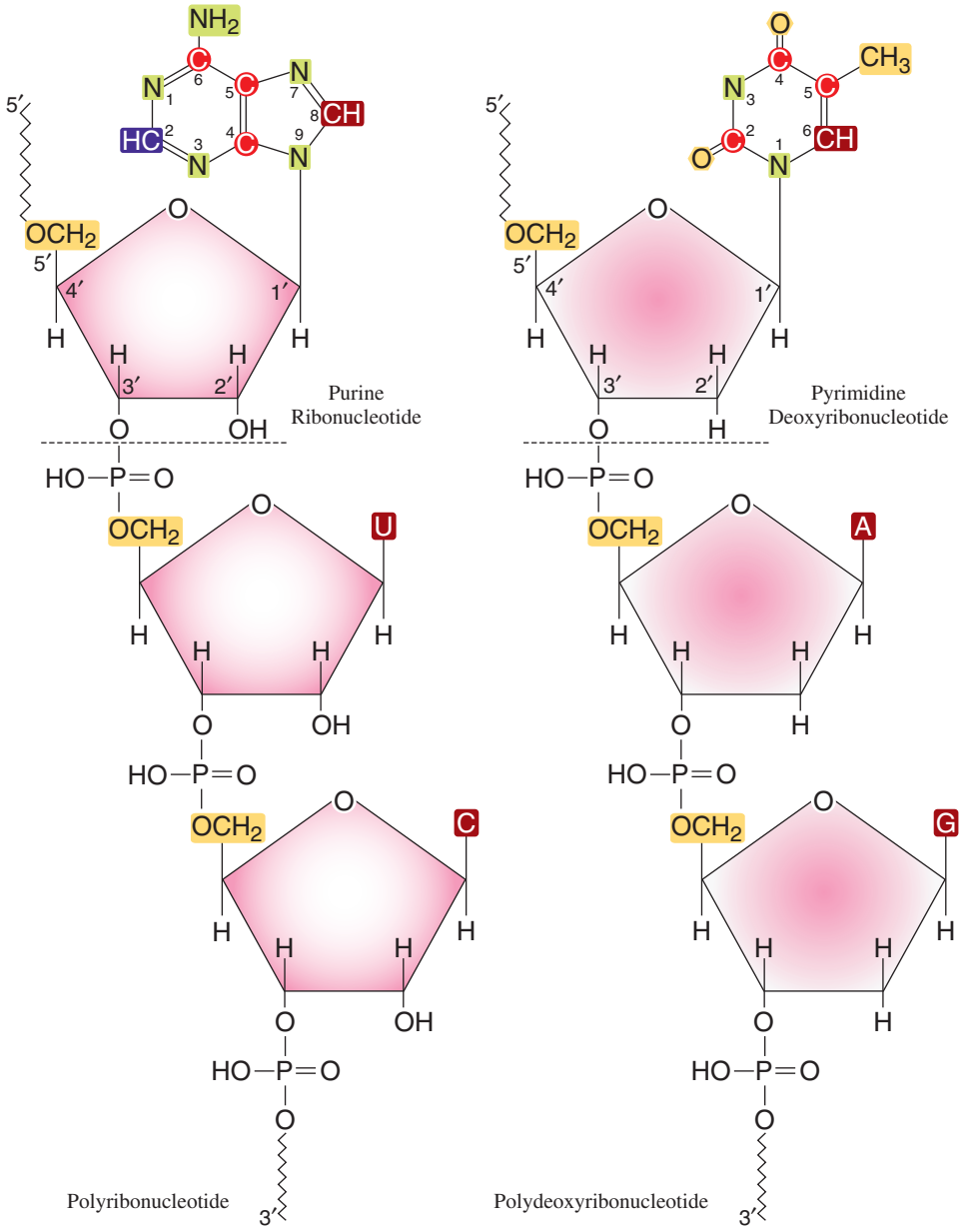
Thymidine + Deoxyribose + Phosphoric acid → Deoxythymidylate (in DNA)

Uracil + Ribose + Phosphoric acid → Uridylate (present in RNA)

Polynucleotide: A polynucleotide is formed by linkage of nucleotides through phosphodiester bonds formed between the hydroxyl group at 3′-carbon of the sugar of one nucleotide and the phosphate at the 5′-carbon of the other nucleotide. A polyribonucleotide forms an RNA and a polydeoxyribonucleotide forms a DNA. Thus every polynucleotide will have a free unbounded 3′ carbon (free –OH) at one end called the 3′-end, and at the other end the 5′ carbon containing the phosphoric acid is free and this end is called the 5′-end of that polynucleotide. While representing the nucleotide base sequence of DNA or RNA on paper it is represented in the 5′ → 3′ direction—

5′ –A–A–T–C–T–G–G–C–A–C–T– 3′

Nucleotides are linked together in a specific sequence, specific for a species DNA or RNA. The nucleic acids provide the script for everything that occurs in a cell, i.e., the amino acid sequence of every protein in a cell is specified by the cooperation of different RNA which in turn are specified by that cell's DNA. Thus the structure of every protein, and ultimately of cell constituents, is a product of information programmed into the nucleotide sequence of a cell's nucleic acid.

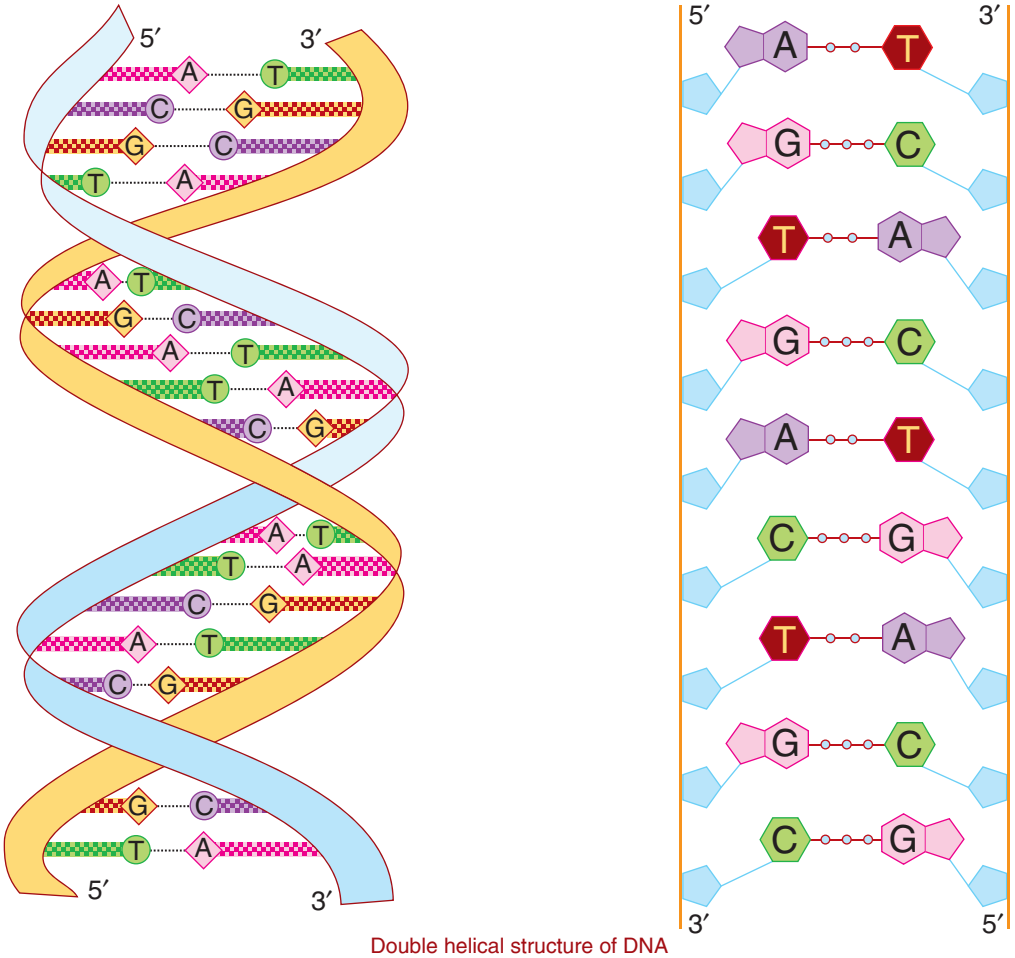


DEOXYRIBONUCLEIC ACID (DNA)

Deoxyribonucleic acid, also abbreviated as DNA, is the principal informational macromolecule of the cell, which stores, translates and transfers the genetic information. In the prokaryotes, the DNA is found mostly in the nuclear zone. In eukaryotes it is found in the nucleus, mitochondria and chloroplast. The present understanding of the storage and utilization of the cell's genetic information is based upon the discovery of the structure of DNA by Watson and Crick in 1953.

Double helical structure of DNA (Watson and Crick model): The three dimensional structure of DNA as proposed by Watson and Crick and the recent advances in it are summarized here—

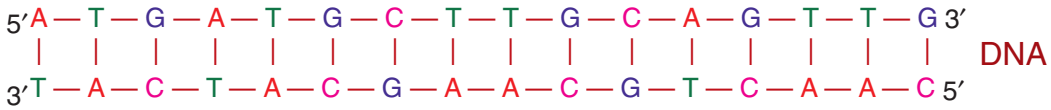
1. DNA is made of two helical chains coiled around the same axis, to form a **right-handed double** helix.
2. The two chains in the helix are **anti-parallel** to each other, i.e., the 5′-end of one polynucleotide chain and the 3′-end of the other polynucleotide chain is on the same side and close together.



Double helical structure of DNA

3. The distance between each turn is **3.6 nm** (formerly 3.4 nm).
4. There are **10.5 nucleotides** per turn (formerly 10 nucleotides).
5. The spatial relationship between the two strands creates **major** and **minor grooves** between the two strands. In these grooves some proteins interact.
6. The **hydrophilic** backbones of alternating deoxyribose and negatively charged phosphate groups are on the outside of the double helix.

7. The **hydrophobic** pyrimidine and purine bases are inside the double helix, which stabilizes the double helix of the DNA.
8. The double helix is also stabilized by **inter-chain hydrogen bond** formed between a purine and pyrimidine base.
9. A particular purine base, pairs by hydrogen bonds, only with a particular pyrimidine base, i.e., Adenine (A) pairs with Thymine (T) and Guanine (G) pairs with Cytosine (C) only.
10. Two hydrogen bonds pairs Adenine and Thymine (A = T), where as three hydrogen bonds pairs Guanine and Cytosine (G ≡ C).
11. The base pairs A = T and G ≡ C are known as **complementary** base pairs.
12. Due to the presence of complementary base pairing, the two chains of the DNA double helix are complementary to each other.



Hence the number of 'A' bases are equal to the number of 'T' bases (or 'G' is equal to 'C') in a given double stranded DNA.

13. One of the strands in the double helix is known as **sense strand**, i.e., which codes for RNA/proteins and the other strand is known as **antisense strand**.

Different structural forms of DNA: The DNA molecules exist in four different structural forms or organizations under different physiological conditions or in different cells or at different points in the same DNA.

Comparison between different structural forms of DNA

	A	B	Z	H
1.	Shorter and wider	Normal reference strand–Watson and Crick model	Longer and thinner	It is a long stretch (part) of DNA with alternating T and C or polypurine/polypyrimidine
2.	Right-handed double helix	Right-handed double helix	Left-handed double helix	Triple helix
3.	Distance between each turn is 2.3 nm	Distance between each turn is 3.6 nm	Distance between each turn is 3.8 nm	—
4.	11 base pairs per turn	10.5 base pairs per turn	12 base pairs per turn	—
5.	Stable in solutions devoid of water	Most stable under physiological conditions	Doubtful existence in physiological state	Helps in gene regulation

Functions of DNA: The base sequence of the DNA constitutes the informational signal called the *genetic material*. This nucleotide base sequence enables the DNA to function, store, express and transfer the genetic information. Hence it programs and controls all the activities of an organism directly or indirectly throughout its life cycle.

(a) DNA stores the complete genetic information required to specify (form) the structure of all the proteins and RNA's of each organism.

(b) DNA is the source of information for the synthesis of all cellular body proteins. Some of the proteins are structural proteins and some are enzymes. These enzymes arrange micromolecules to form macromolecules. These macromolecules are arranged to form supramolecular complexes or cell organelles which associate to form cells. These cells group to form tissues which in turn forms different organs of a body, specifically peculiar to that organism during foetal development, growth and repair. Hence DNA programs in time and space the orderly biosynthesis of cells and tissue components.

(c) It determines the activities of an organism throughout its life cycle, i.e., the period of gestation, birth, maturity, senescence and death.

(d) It determines the individuality and identity of a given organism.

(e) It duplicates (replicates to form two daughter DNA) itself and transfers one of the copy to the daughter cell during cell division, thus maintaining the genetic material from generation to generation.

Packaging of DNA within the cells: The length of DNA molecules existing in a particular cell is much longer than the long dimensions of the cell or the organelle where they exist. The contour length (i.e., its helix length) of a double stranded DNA can be calculated from the molecular weight, presuming the average molecular weight of each nucleotide pair to be about 650 Da and there is one nucleotide pair for every 0.36 nm of the duplex. Accordingly the length of the smallest DNA is 1938.96 nm, belonging to the ϕX174 virus (in duplex form), whose particle long dimension is 25 nm. On the other hand the total contour length of the entire DNA in a single human cell is about 2 metres and the cell nucleus is just 5-10 nm in diameter. These long DNA molecules are very tightly compacted, so as to fit into the cell. This packing is possible due to DNA getting further coiled into different fashions. The linear double helical DNA called the relaxed DNA, bends or twists upon its own axis, which is called as DNA coiling. This coiled DNA further coils upon itself to form the DNA supercoil, just like the telephone cord wire from the base of the phone to the receiver. The degree of DNA supercoiling depends upon the type of the cell/organelle to be packed and is coiled in such a manner that DNA can easily be accessible to the enzymes/proteins for all of its functions like replication and transcription. There are possibilities of two types of DNA supercoiling (a) *Solenoidal*, wherein the DNA coils in a spherical fashion upon itself and (b) *Plectonemic*, wherein the DNA coils back upon its reverse length in the form of pleats. Further the supercoiling and packing of DNA differ in the prokaryotes (i.e., those lacking a true nuclear envelope) and in the eukaryotes (i.e., those having a nuclear membrane).

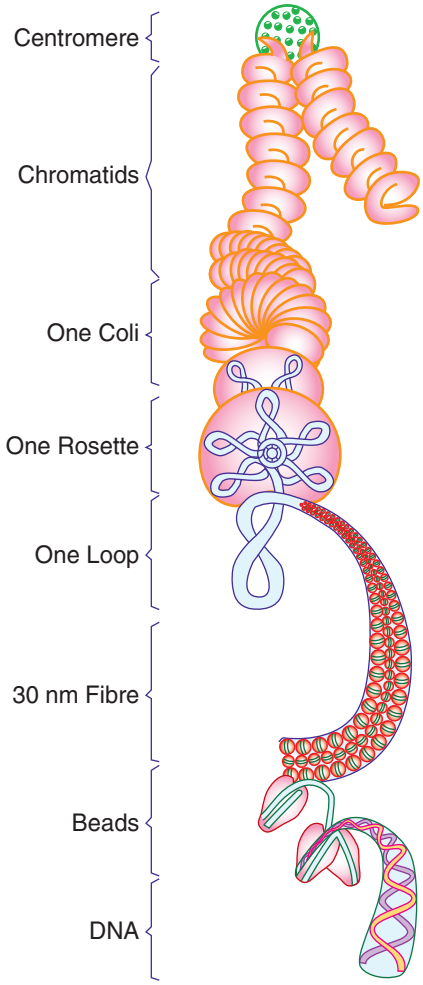
(a) **Packaging of viral DNA:** Though the viral DNA is much smaller than a bacterial or eukaryotic DNA, its contour length is much bigger than the long dimensions of the viral particle in which they are found. The DNA of bacteriophage T2 is 3500 times its particle diameter. The long dimensions of different viral particle/the contour length of their DNAs respectively in nanometres is T2-210/65520; Lamdaphage-190/17460; Ti-78/14376 and ϕX174 (in duplex form)-25/1938. In order to get themselves packed within the particle, most of these viral DNAs are linked covalently by the ends and, therefore, form an endless belt and thus become circular. Some of the single stranded DNA (ϕX174) becomes double stranded and circular.

(b) **Packaging of bacterial DNA:** The length of *E.coli* cell is 2 micrometres and its complete DNA molecule (the complete DNA molecule of an organism is called the chromosome) is 1.7 mm long, which exists as a single, covalently closed double stranded circular molecule, coiled and packed within nucleon of the cell. This circular chromosome is organized in a scaffold-like structure, which folds the chromosome into looped domains. These domains are further coiled around some basic proteins, called Hu proteins (Mw = 10 000). In addition to the nucleosomal DNA the bacteria contains some small circular supercoiled non-chromosomal DNA-called plasmids.

(c) **Organization and packing of eukaryotic DNA:** All the chromosomal DNA of an eukaryotic cell is embedded in a membranous cellular organelle called the nucleus. The eukaryotic DNA,

in the nucleus is linear and not circular. In the non-dividing resting cell all the DNA of the cell forms a fine filamentous network in the nucleus called the chromatin. During cell division the chromatin network is subdivided into defined number and shaped chromosomes, their diploid number (pairs) depends upon the species of organism. The normal chromosome number in humans is 46 (23 pairs). Each chromosome has a central axis called the centromere, from which two arms of DNA project out and each is referred to as chromatid. Each chromosome differs in size and shape within a given organism.

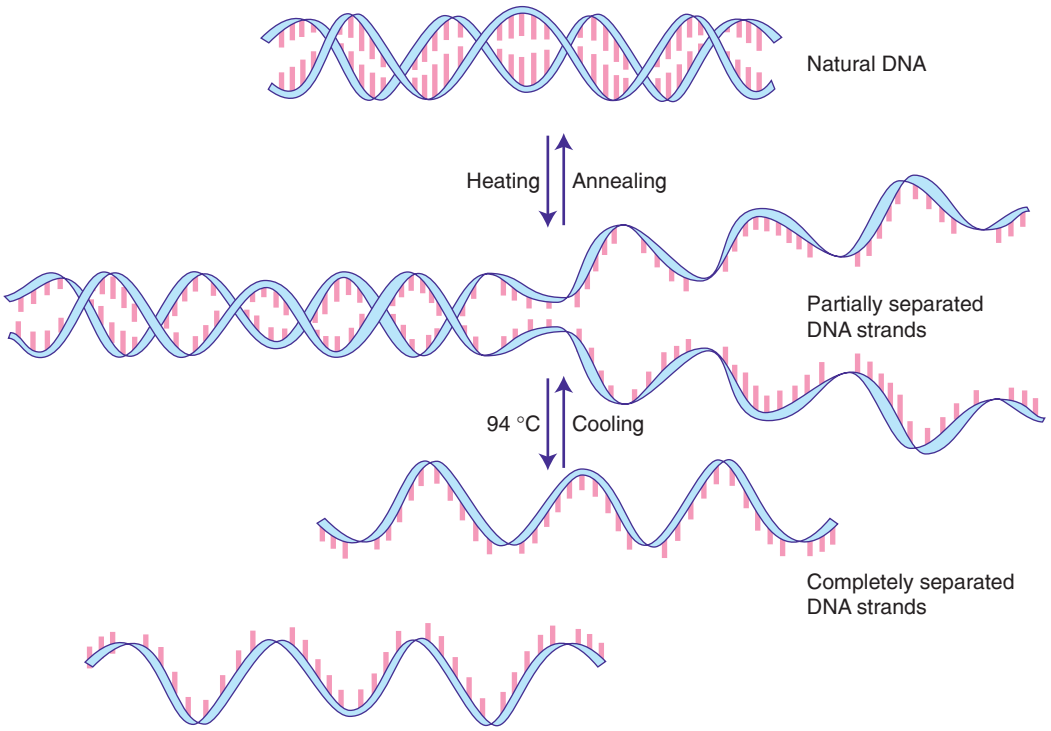
The 2 metre long eukaryotic human cell DNA is to be packed in the cell of about 5-10 micrometre in diameter. In order to facilitate its package, the helical DNA molecule is bound, tightly around beads of basic proteins called **histones**, which are spaced at regular intervals. The complexes of histones and DNA are called nucleosomes. Each nucleosome contains eight histone molecules, two copies each of H_2A, H_2B, H_3 and H_4 winding 146 base pairs of DNA. Between the two nucleosomes there is a spacer DNA of 54 base pairs with a single histone molecule (H_1). Wrapping DNA around a nucleosome compacts it to about seven-fold. These nucleosomes are organized very close together to form a structure, simply called 30 nm fibre. This provides 100-fold compaction of DNA. The 30 nm fibre then forms plectonemic pleats, called loops. Six such loops are bounded by scaffold attachment to proteins (histone-H_1/topoisomerase-II) to give rise to a cluster of loops called rosette. 30 such rosettes bunch together to form a single coil, 10 such coils (like phone cord) forms a chromatid and the two chromatids are linked together by a highly repetitive base sequence rich in AT base pairs called **satellite DNA**, which is the centromere. Two chromatids with a centromere form a chromosome.



Packaging of eukaryotic DNA

Physical properties of DNA

(a) **Denaturation:** When DNA is subjected to extremes of pH or temperatures above 80 to 90 degree centigrade, it gets denatured and the double helical structure is unfolded due to disruption of hydrogen bonds between the bases and the hydrophobic interactions of the bases. Finally, the two strands separate completely from each other. This is melting of DNA. The temperature at which a given DNA is denatured to about 50% is known as T_M. Different DNA melts at different temperatures, which depends upon the G ≡ C content of that DNA. Higher the G ≡ C content, higher is the melting temperature (T_M) and vice-versa. When the temperature or pH is slowly brought back to normal biological range, the two strands will automatically rewind or anneal and will again form the same double helical structure. If the temperature is suddenly cooled down, then the two strands remain separated and exist as single strands.



Denaturation and renaturation of DNA

(b) **Buoyant density:** When DNA is centrifuged at high speeds in a concentrated solution of caesium chloride–(CsCI), the CsCl will form a density gradient (ascending) and the DNA will remain stationary or buoyant at a point in the tube where its density is equal to the density of CsCI at that point. Different DNA will have different densities, which again depend upon the G ≡ C content of that DNA. Higher the G ≡ C content, higher is the buoyant density of that DNA and vice versa.

Measurement of these two characters, viz., melting temperature and buoyant density will enable us to calculate the proportions of G ≡ C and A = T pairs in that DNA, which indirectly helps in deducting the gene sequence.

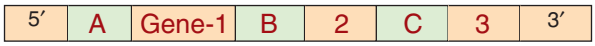
RIBONUCLEIC ACID (RNA)

A Ribonucleic acid is made up of a number of ribonucleotides (polyribonucleotide). A ribonucleotide is made up of (1) ribose sugar (2) nitrogenous bases—A, U, G, C [Note that uracil (U) is present instead of thymine (T) found in DNA] and (3) phosphoric acid. There are three types of RNA functioning to

express the genetic information contained in the DNA. They are (a) messenger RNA (mRNA) (b) transfer RNA (tRNA) and (c) ribosomal RNA (rRNA). In addition to these major types of RNA there are many specialized RNA with regulatory and catalytic function that are known as small nuclear RNA (snRNA) and heteronuclear RNA (hnRNA) respectively. Some of the viral particles contain RNA as their genetic material (i.e., genome). Thus RNA molecules not only carry and express genetic information, but they can also act as catalysts (enzymes). All the RNA molecules are derived from information permanently stored in DNA by a process called transcription, wherein the enzyme system converts the genetic information of a segment of DNA into an RNA strand with a base sequence complementary to one of the DNA strands. Hence RNA is single stranded.

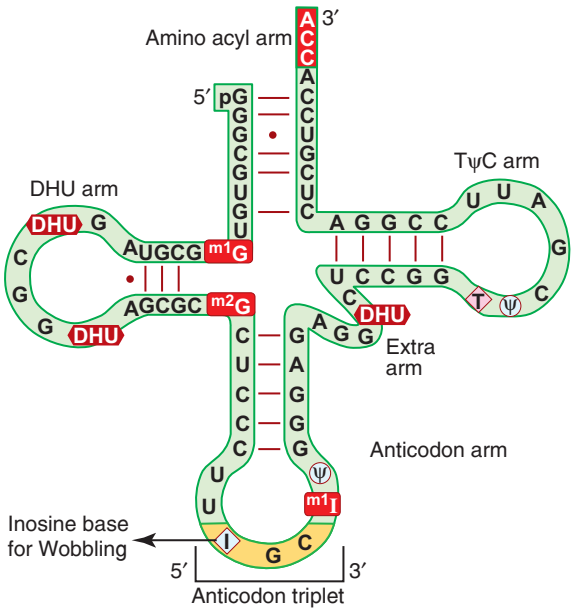
1. **Messenger RNA (mRNA):** It carries the genetic message from the chromosomes (DNA) to the ribosome (site of protein synthesis). This RNA is formed in the nucleus (or nuclear zone in prokaryotes) containing complementary base sequence to a part of one strand of DNA (gene). The base sequence in the mRNA specifies the amino acid sequence in the polypeptide chains. In prokaryotes a single mRNA molecule codes either for one polypeptide chain, hence called **monocistronic**, or it may code for more than one polypeptide, thus called **polycistronic**. In eukaryotes, most of the mRNAs are monocistronic.

Monocistronic-mRNA



Polycistronic-mRNA
(A, B, C are intergenic spaces of genes 1, 2 and 3)

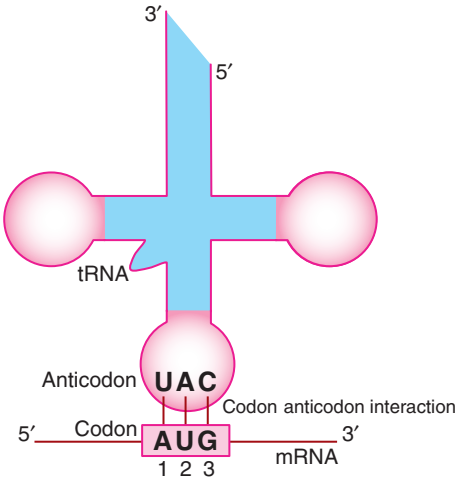
2. **Transfer RNA (tRNA):** It is an adapter molecule that reads the information encoded in the mRNA and accordingly transfers the appropriate amino acid to the growing polypeptide chain during protein synthesis. There is at least one kind of tRNA for each amino acid. Some amino acids have two or more specific tRNAs. At least 32 tRNA's are required to recognize all the amino acid codons (64 – 3 = 61). Most cells have to about 40-50 distinct tRNAs. Some tRNA's can recognize more than one codon (due to wobbling). tRNAs have between 73 and 93 nucleotides in their structures. Eight or more nucleotides are unusual modified bases which are methylated derivatives of the principal bases. Most tRNA's have guanylate (pG) at 5′ end and have the base sequence CCA at 3′ end. These are single stranded and there is maximum intrachain base pairing which gives it a clover leaf shape structure, with four arms. The four arms of tRNA are (i) Amino acyl arm (AA arm) (ii) Anticodon arm (iii) Dihydrouridine arm (DHU arm) and (iv) Ribothymidine - pseudouridine arm (TΨC Arm).



Structure of alanine tRNA

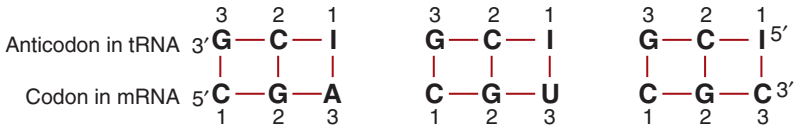
The amino acyl arm carries a specific amino acid esterified to its carboxyl group at the 2′ or 3′ hydroxyl group of the adenosine present at the 3′ end of the tRNA. The anticodon arm contains the anticodon, i.e., a sequence of three nucleotide bases complementary to the genetic code in mRNA for that particular

amino acid. The two RNAs are paired anti-parallel, i.e., the first base of the codon (5′ → 3′) pairs with the third base of the anticodon (3′ → 5′) creating codon-anticodon interaction as shown.



Codon-anticodon interaction

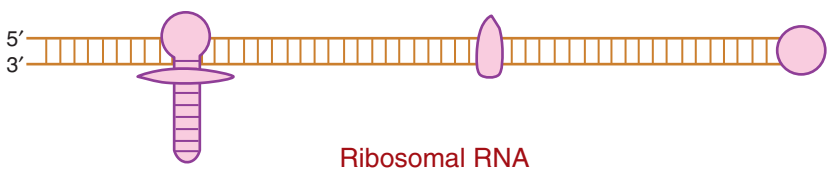
There are totally 64 codons formed by multiple combinations of any three nucleotide bases out of the four nucleotides forming the nucleic acids. At least one codon specifies one amino acid and some amino acid have more than one codon, but the number of different tRNA's for each amino acid is not the same as the number of its codons. This is because some tRNAs contain the first anticodon nucleotide inosinate (I), which can base pair by hydrogen bonds with three nucleotides, viz., U, C and A. Therefore, all those amino acid codons which differ at the third nucleotide will have the same tRNA. This is known as **wobbling**. e.g., there are six codons for arginine, but only four different types of $tRNA^{Arg}$ are available in the cell. One of these tRNA contains the anticodon (3′) GCI (5′) and will pair with three codons for arginine.



The other three codons for arginine, viz., CGG, AGA and AGG have different tRNAs.

The DHU arm contains the unusual nucleotide dihydrouracil and the TΨC arm contains ribothymidine (T) and pseudouridine (Ψ) which has an unusual carbon-carbon bond between the base and pentose sugar. The functions of DHU arm is recognition of its proper aminoacyl-tRNA synthetase and TΨC arm is involved in binding of the aminoacyl-tRNA to the ribosomal surface.

3. **Ribosomal RNA (rRNA):** Ribosomal RNA molecules in association with proteins forms the seat of protein synthesis or the protein synthesis machinery called the ribosome. The RNAs are designated depending upon their sedimentation coefficients. The rRNAs found in prokaryotes are 5S, 23S and 16S. Those found in eukaryotes are 5S, 18S 28S, and 5.8S. rRNA is single stranded with intra-chain hydrogen bonding.



Ribosomal RNA

Nucleotides of biological importance: Nucleotides in addition to the formation of nucleic acids have many other functions in the cell. They act as energy barriers, they are components of coenzymes, act as chemical messenger, etc. Structure and function of a few biologically important nucleotides are mentioned below:

1. **Adenosine-Triphosphate (ATP):** Adenosine triphosphate or ATP is generally known as energy currency of the cell, as it takes up energy from energy yielding processes and donates it to energy requiring cell processes. It is composed of the purine base adenine, attached to the first carbon of the ribose sugar and the 5th carbon of the sugar contains three phosphate groups. The first phosphate is bonded through an ester linkage, whereas the other two phosphates are linked through anhydride linkage. Hydrolysis of each of the anhydride linkage yields energy of 7.4 kcal/mol, hence they are known as high energy phosphate bonds. The hydrolysis of the third phosphate bond (ester linked, alpha bond) yields only 4 kcal/mol of energy. Hence it is not a high energy bond.
2. **Adenosine-Diphosphate (ADP) and Adenosine-Monophosphate (AMP):** ADP and AMP are the hydrolysis products of ATP. They take part in a variety of cellular reactions. ADP participates in energy yielding reactions, thereby capturing the energy in the form of high energy bond and getting converted to ATP.

   Example: Phosphoenol pyruvate + ADP — PYRUVATE KINASE → Pyruvate + ATP

   ADP is converted to ATP by the energy released during electron transport chain in the mitochondrial respiration. ADP and AMP act as positive allosteric enzyme modulator for many enzymes of energy yielding reactions.
3. **Guanidine-Triphosphate (GTP), Cytidine-Triphosphate (CTP), Uridine-Triphosphate (UTP):** GTP, CTP and UTP act as energy currency of the cells, but in a limited number of reactions. To quote an example for each—

   Succinyl-CoA + GDP — SUCCINATE THIOKINASE → Succinic acid + GTP

   Glucose-1-phosphate + UTP — PYROPHOSPHORYLASE →UDP-Glucose + pp

   Diacylglycerol + CTP — KINASE → CDP-diacylglycerol + pp
4. **Adenosine 3′, 5′-Cyclic Monophosphate (cAMP) and Guanosine 3′, 5′-Cyclic Monophosphate (cGMP):** cAMP and cGMP act a second messengers formed from ATP and GTP by the action of the enzymes adenylate cyclase and guanilynate cyclase respectively. They are formed in response to hormones like epinephrine and serves regulatory function within the cell (e.g., Activation of glycogen phosphorylase). Structurally both are ribonucleotides of adenine and guanine respectively, wherein the phosphate present at the 5′ position is bonded to the 3′ –OH, thereby forming an additional cyclic ring.
5. **S-Adenosyl-Methionine (adoMET or SAM):** adoMET is synthesized from ATP and methionine by the action of the enzyme methionine adenosyl transferase, wherein the amino acid methionine is attached to the 5′ end of the adenosine nucleoside. adoMET is used for methyl group transfers in the synthesis of amino acids and nucleotide metabolism.
6. **Nicotinamide Adenine Dinucleotide (NAD), Nicotinamide Adenine Dinucleotide Phosphate (NADP) and Flavine Adenine Dinucleotide (FAD):** NAD, NADP and FAD act as coenzymes in many oxidation-reduction reactions. They take up electrons and H^+, and get converted to

$NADH + H^+$, NADPH and $FADH_2$ during oxidative reaction and in turn give up electrons and H^+ during reductive reactions.

Differences between DNA and RNA

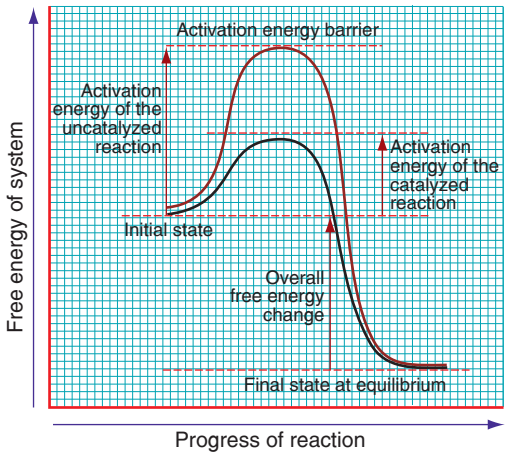
DNA	RNA
The sugar present is 2-deoxyribose sugar.	The sugar present is ribose sugar.
Thymine is the base present only in DNA.	Uracil is the base present instead of thymine.
It is double stranded.	It is single stranded.
The different structural forms of DNA are A, B, Z and H.	There are three types of RNA, viz., mRNA, tRNA, and rRNA.
It stores and transfers the genetic material.	It helps in the expression of the genetic information.
It has got only genetic character.	It has got both genetic and catalytic activities.

8

ENZYMES

Enzymes are proteinaceous (and even nucleic acids) biocatalyst which alter (generally enhance) the rate of a reaction.

Free energy of activation and effect of catalysis: A chemical reaction like substrate to product, will take place when a certain number of substrate molecules at any instant, possess enough energy to attain an activated condition called the **"transition state"** in which the probability of making or breaking a chemical bond to form the product is very high. **"Free energy of activation"** is the amount of energy required to bring all the molecules in one gram mole of a substrate at a given temperature to the transition state.



In presence of a catalyst, the substrate combines with it to produce a transient state having a lower energy of activation than that of the substrate alone. This accelerates the reaction. Once the product is formed, the enzyme (catalyst) is free to combine with another molecule of the substrate and repeat the process. Though there is a change in the free energy of activation in presence of an enzyme, the overall free energy change of the reaction remains the same whether the reaction is catalyzed by an enzyme or not.

CLASSIFICATION OF ENZYMES

Classification of enzymes are based upon (1) the reaction catalyzed, (2) the presence or absence at a given time, (3) the regulation of action, (4) the place of action and (5) their clinical importance.

1. **Classification based upon the reaction catalyzed:** Enzymes are broadly divided into six groups based on the type of reaction catalyzed. They are (1) Oxido-reductases (2) Transferases (3) Hydrolases (4) Lyases (5) Isomerases and (6) Ligases.
   - (a) **Oxido-reductases:** Enzymes which bring about oxidation and reduction reactions.

   Ex. Pyruvate + NADH—LACTATE DEHYDROGENASE → Lactate + NAD^+

   Glutamic acid + NAD—GLUTAMATE DEHYDROGENASE → α-ketoglutarate + NH_3 + NADH
   - (b) **Transferases:** Enzymes which catalyze transfer of groups from one substrate to another, other than hydrogen. Ex. Transaminase catalyzes transfer of amino group from amino acid to a keto acid to form a new keto acid and a new amino acid.

   Ex. α-Ketoglutarate + Alanine—ALANINE AMINOTRANSFERASE → Glutamate + Pyruvate

   Aspartate + α-Ketoglutarate —ASPARTATE AMINOTRANSFERASE → Oxaloacetate + Glutamate
   - (c) **Hydrolases:** Those enzymes which catalyze the breakage of bonds with addition of water (hydrolysis). All the digestive enzymes are hydrolases. Ex. Pepsin, trypsin, amylase, maltase.
   - (d) **Lyases:** Those enzymes which catalyze the breakage of a compound into two substances by mechanism other than addition of water. The resulting product always has a double bond.

   Ex. Fructose-1-6-diphosphate—ALDOLASE → Glyceraldehyde-3-phosphate + DHAP
   - (e) **Isomerases:** Those enzymes which catalyze the interconversion of optical and geometric isomers.

   Ex. Glyceraldehyde-3-phosphate—ISOMERASE → Dihydroxyacetone phosphate
   - (f) **Ligases:** These enzymes catalyze union of two compounds. This is always an energy requiring process (active process).

   Ex. Pyruvate + CO_2 + ATP—PYRUVATE CARBOXYLASE → Oxaloacetate + ADP + Pi
2. **Classification based upon the presence or absence at a given time:** Two types are identified—
   - (a) **Inducible enzymes:** Those enzymes that are synthesized by the cell whenever they are required. Synthesis of these enzymes usually requires an inducer.

   Ex. Invertase, HMG-CoA reductase, β-galactosidase and enzymes involved in urea cycle.
   - (b) **Constitutive enzymes:** Enzymes which are constantly present in normal amounts in the body, irrespective of inducers.

   Ex. Enzymes of glycolysis.
3. **Classification based upon the regulation of enzyme action:** They are of two types—
   - (a) **Regulatory enzymes:** The action of these enzymes is regulated depending upon the status of the cell. The action of regulatory enzymes is either increased or decreased by a modulator at a site other than the active site called the **"allosteric site"**.

   Ex. Phosphofructokinase (PFK) and glutamate dehydrogenase.
   - (b) **Non-regulatory enzymes:** The action of these enzymes is not regulated.

   Ex. Succinate dehydrogenase.
4. **Classification based upon the place of action:** Depending upon the two sites of action, they are divided into—
   - (a) **Intracellular enzymes:** Enzymes that are produced by the cell and act inside the same cell are known as intracellular enzymes.

   Ex. All the enzymes of glycolysis and TCA cycle.

(b) **Extracellular enzymes:** Enzymes produced by a cell but act outside that cell independent of it.

Ex. All the digestive enzymes viz. trypsin, pancreatic lipase etc.

5. **Classification based upon their clinical importance**

(a) **Functional plasma enzymes:** Enzymes present in the plasma in considerably high concentration and are functional in the plasma due to the presence of their substrate it plasma.

Ex. Serum lipase, blood clotting enzymes.

(b) **Non-functional plasma enzymes:** Enzymes present in the plasma in negligible concentration and have no function in the plasma due to the absence of their substrate in it. Non-functional plasma enzymes are of diagnostic importance.

Enzymes are named in 4 digits by the enzyme nomenclature commission, wherein the

1st digit refers to main classification

2nd digit refers to sub-classification

3rd digit refers to sub-sub classification

4th digit refers to that particular enzyme

Ex. 2.7.3.2 is adenosine triphosphate-creatine phosphotransferase (creatine kinase).

MECHANISM OF ENZYME ACTION

An enzyme (or protein) should be in its native conformation to be biologically active. The three dimensional conformation of enzymes have a particular site where the substrate binds and is acted upon, this site is called the active site. The active site is earmarked into two specific areas (1) binding site—where the substrate binds and (2) catalytic site—where the enzyme catalysis takes place. The amino acids present at the active site are tyrosine, histidine, cysteine, glutamic acid, aspartic acid, lysine and serine. In aldolase, lysine is present at the active site. In carboxypeptidase, two tyrosine residues are present at the active site. Ribonuclease has two histidines at the active site.

Michaelis and Menten established the theory of combination of enzyme with substrate to form the enzyme-substrate complex. According to this, the enzyme combines with the substrate on which it acts to form an enzyme-substrate complex. Then, this enzyme is liberated and the substrate is broken down into the product of the reaction.

E [Enzyme] + S [Substrate] → ES [Enzyme-Substrate complex] → E + Product

The ES complex is also called as 'Michaelis Menten complex'.

Enzymes accelerate the rate of chemical reaction by four major mechanisms viz.

1. **Proximity and orientation:** The enzyme binds to the substrate in such a way that the susceptible bond is in close proximity to the catalytic group and also precisely oriented to it resulting in the catalysis.
2. **Strain and distortion or induced fit model:** Binding of the substrate induces a conformational change in the enzyme molecule which strains the shape of the active site and also distorts the bounded substrate, thus bringing about the catalysis. The binding of the substrate to the enzyme will bring about a change in the tertiary or quaternary structure of enzyme molecule, which destabilizes the enzyme. In order to attain stability, the enzyme distorts the substrate thereby forming the reaction product.
3. **General acid-base catalysis:** The active site of the enzyme has amino acids that are good proton donors or proton acceptors, this results in acid-base catalysis of the substrate.
4. **Covalent catalysis:** Some enzymes react with their substrates to form very unstable, covalently joined enzyme-substrate complexes, which undergo further reaction to form the products.

FACTORS AFFECTING ENZYME ACTION

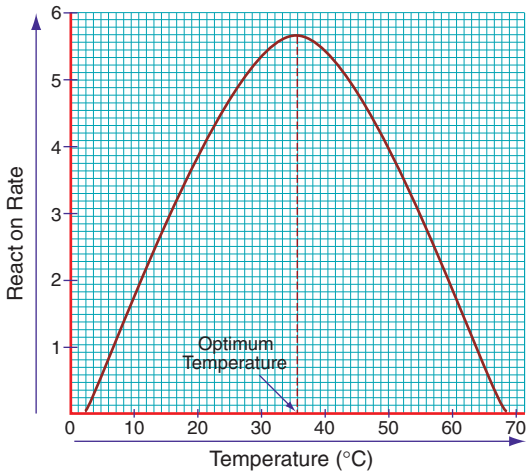
The factors influencing the rate of the enzyme catalyzed reaction are—

1. Temperature
2. pH
3. Substrate concentration
4. Enzyme concentration
5. Concentration of products
6. Light
7. Ions

1. **Effect of temperature:** When all the other parameters are kept constant (i.e. at their optimum level), then the rate of enzyme reaction increases slowly with increase of temperature till it reaches a maximum. Further increase in temperature denatures the protein resulting in decrease in the enzyme action and a further increase in temperature may totally destroy the protein.

Optimum temperature: The temperature, at which the enzyme activity is maximum, is termed as the optimum temperature.

Most of the enzymes are totally inactive at 0° C to 4° C, their activity starts at 10° C and slowly increases reaching its maximum capacity at its optimum temperature. Majority of the enzymes in the human body have their optimum temperatures between 37° C and 40° C. Beyond this temperature the enzymes become less active and may loose their activity completely at higher temperatures. In fever, rise in temperature increases the metabolic activity due to increase in enzymatic action. Decrease in the temperature leads to hypothermia which is seen in organ transplantation and open heart surgery.

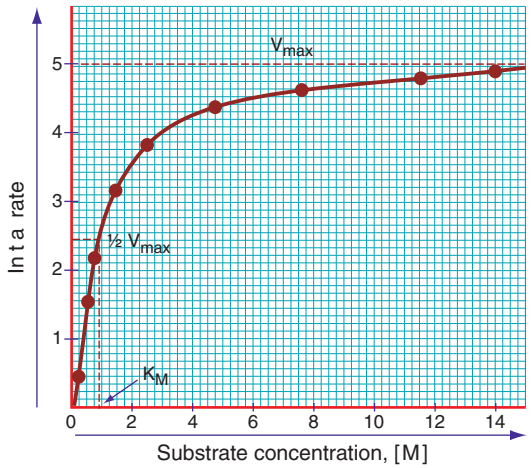


However, life exists in very cold regions and also in hot springs, indicating that the same enzyme that exists in human cell, for instance the enzymes of glycolysis and TCA cycle have their optimum temperatures at extremes of temperatures. Thus refrigeration bacteria exists with the optimum temperature of its enzymes being near 4°C. Likewise bacteria surviving in hot springs have the enzymes with their optimum temperatures nearing hundred(s) degree Celsius ex. the optimum temperature of Taq polymerase is 72°C.

***Vant Hoff's coefficient*:** It is the coefficient which explains that for every 10°C rise in temperature the enzyme activity increases 2 fold till the optimum temperature is reached.

2. **Effect of pH:** When all the other parameters are kept constant, the velocity of an enzyme catalyzed reaction increases till it reaches the optimum pH and then decreases with further increase/ decrease in pH. The activity is maximum for most of the enzymes at the biological pH of 7.4. Optimum pH for pepsin is 1.5, acid phosphatase is 4.5 and for alkaline phosphatase it is 9.8.

3. **Effect of substrate concentration:** When all the other parameters are kept constant including the enzyme concentration, then, as the substrate concentration increases the rate of reaction increases steadily, till the enzyme is saturated with the substrate. At this stage the reaction rate does not increase and remains constant. When a graph is plotted with velocity versus substrate concentration it gives a hyperbolic curve.



This is because, as the concentration of substrate is increased, the substrate molecules combine with all available enzyme molecules at their active sites till no more active sites are available. Thus at this stage, substrate only replenishes the sites when the products are liberated and cannot increase the rate of reaction.

Michaelis Menten constant:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

V → Velocity at a given concentration of the substrate
V_{max} → Maximum velocity with excess of substrate
[S] → Concentration of the substrate at velocity V
K_m → Michaelis-Menten constant for the enzyme

When the velocity is half the maximum velocity then,

$$V = ½V_{max}$$

$$\frac{V_{max}}{2} = \frac{V_{max}[S]}{K_m + [S]}$$

Dividing by V_{max} we get

$$\frac{1}{2} = \frac{[S]}{K_m + [S]}$$

$$K_m + [S] = 2[S]$$

$$K_m = [S]$$

Definition: K_m is defined as the substrate concentration at which the velocity of the enzyme catalyzed reaction is half the maximum velocity.

- A high K_m value indicates weak binding between the enzyme and the substrate.
- Low K_m indicates strong binding.

Limitations of Michaelis-Menten equation

- ✓ This equation enables the calculation of approximate value of the maximum velocity and not the accurate value.
- ✓ It holds good for enzymes which have active site only and not the allosteric site.
- ✓ It calculates the K_m for monosubstrate reactions and not for multisubstrate reactions.
- ✓ It is used to know the velocity of non-regulatory enzymes but not of regulatory enzymes.

Inorder to overcome the above limitations a Lineweaver-Burke plot is drawn so as to establish a relation between the reciprocals of substrate concentration and velocity.

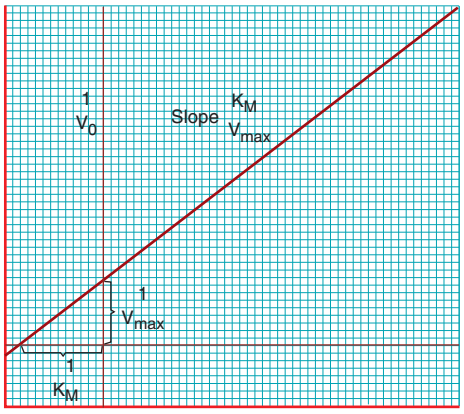
Lineweaver-Burke plot: Inverting the Michaelis-Menten equation, we get

$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]}$$

or,
$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{[S]}{V_{max}[S]}$$

or,
$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

This equation is similar to the mathematical equation i.e., y = ax + b, which gives a straight line plot. Here $y = 1/V$; $a = K_m/V_{max}$; $x = 1/S$; $b = 1/V_{max}$.



By this equation we can calculate accurately—

- ❖ The velocity of any enzyme catalyzed reaction
- ❖ The rate of reaction where more than one substrate is present.
- ❖ The velocity of all the enzymes.

Regulatory enzymes give a sigmoid curve and non-regulatory enzymes give a hyperbolic curve.

4. **Effect of enzyme concentration:** As the enzyme concentration increases, the rate of reaction increases steadily in presence of an excess amount of substrate, the other factors being kept constant. A linear curve is produced.
5. **Effect of products:** When the product is more in the reaction mixture, then the rate of reaction decreases due to feedback inhibition.
6. **Effect of light:** The speed of activity of various enzymes changes in different wavelength of light ex. blue light enhances the activity of salivary amylase whereas, U.V. light decreases the velocity.
7. **Effect of ions:** Presence or absence of particular ions enhances or reduces the activity of enzymes ex. Pepsinogen is converted to pepsin in presence of H^+ ions. Kinases act in presence of Mg^{+2} ions.

ENZYME SPECIFICITY

Enzymes are very specific in their reaction. They either act on one particular substrate or catalyze one particular reaction. Accordingly enzyme specificity is of two types.

1. **Reaction specificity:** These enzymes are specific for the type of reaction they catalyze, irrespective of the substrate on which they act. Thus different enzymes bring about different reactions on the same substrate i.e. enzymes are specific for one particular reaction no matter which substrate it may be ex. amino acids are acted upon both by amino acid oxidase which oxidizes the amino acids to keto acids and decarboxylase that removes carbon dioxide from them.
2. **Substrate specificity:** These enzymes are specific for the substrate upon which they act. This is further classified as follows.
   (a) **Absolute specificity:** These enzymes are highly specific and act on one particular substrate only and no other substrate. Ex. Urease, catalase, aspartase.
   (b) **Relative specificity:** These enzymes act on one particular bond. Ex. D-amino acid oxidase.
   (c) **Group specificity:** These enzymes act on only one particular group.
      - ***Pepsin*:** Is a proteolytic enzyme that acts on peptide bonds contributed by aromatic amino acids like tyrosine, tryptophan and phenylalanine.
      - ***Trypsin*:** Is specific for basic amino acids. Hence it cleaves peptide bonds contributed by lysine and arginine.
      - ***Aminopeptidase*:** Acts on peptide bond near the free amino end.
      - ***Carboxypeptidase*:** Specific for free carboxylic group.
      - ***Amylase*:** Specific for α-1→4 glycosidic linkages.
   (d) **Stereo specificity:** These enzymes act on one particular stereo isomer.
      - ✓ ***Succinate dehydrogenase*:** Is specific for the stereo isomer fumarate i.e. cis form of double bond.
      - ✓ ***Cellulase*:** Is specific for β glycosidic linkage.
      - ✓ ***L-amino acid oxidases*:** Act on L-amino acids only and not on D-amino acids.

Coenzymes: They are non protein, heat stable, low molecular weight dialyzable organic compounds that are required for the action of enzymes. Generally vitamins act as coenzymes ex. biotin, pyridoxine etc. Enzyme along with a co-enzyme is known as **'holoenzyme'** and that without a co-enzyme is an **'apoenzyme'**.

Apoenzyme (protein) + Co-enzyme (non-protein) → Holoenzyme (active enzyme protein)

Holoenzyme may contain an organic or inorganic compound (metal ions) or both. If organic substances are present with enzymes then they are known as **'co-enzymes'** and if inorganic substances are acting with the enzymes then they are called as **'co-factors'** (Mg, Mn, Zn, Co, Se, etc.). The role of co-enzymes is—

(i) They act as co-substrate or second substrate ex. Pyruvate + NADH → Lactate + NAD^+. NADH acts as a coenzyme or second substrate.

(ii) They help in transferring of groups either hydrogen or groups other than hydrogen.

(iii) Specific activity of a co-enzyme is the number of units of co-enzyme present in one milligram of enzyme protein.

Enzyme unit or activity: One unit of enzyme activity is the amount of enzyme that converts 1.0 μM of the substrate per minute into the products at 25°C.

Specific activity of an enzyme: It is defined as the number of enzyme units per milligram of the protein.

Enzyme turnover number: The number of substrate molecules transformed per minute (unit time) by a single enzyme is known as enzyme turnover number. Carbonic anhydrase has the highest turnover number of 36,000,000.

First and second order reaction: A reaction in which there is only one substrate is termed as 1st order reaction. A reaction in which two substrates are involved to form a product is termed as 2nd order reaction, also known as bisubstrate reaction. This involves either single displacement (i.e. both substrates binding to two active sites in the enzyme at the same time) or double displacement (ping-pong displacement, wherein only one substrate binds to the enzyme active site at a given time, once this is released the other substrate binds).

Zymogen: The inactive form of an enzyme is known as zymogen or proenzyme. Pepsinogen and trypsinogen are the zymogens of pepsin and trypsin respectively.

Ribozyme: Ribonucleic acids that catalyze a reaction similar to that of enzymes are known as ribozymes. These ribozymes help in the processing of the newly transcribed RNA ex. small nuclear RNA (SnRNA) and heteronuclear RNA (hnRNA).

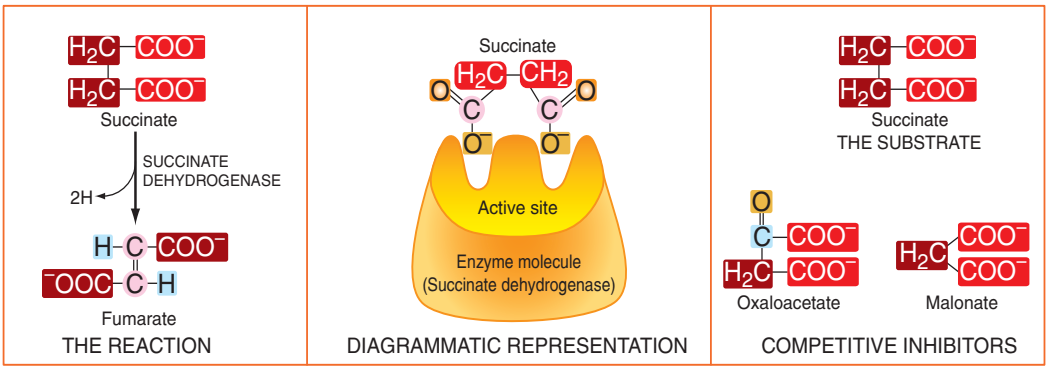
ENZYME INHIBITION

Alteration in the enzyme activity by specific substances other than non-specific substances like pH, temperature etc. is called enzyme inhibition. There are two types of enzyme inhibitions - (a) irreversible and (b) reversible.

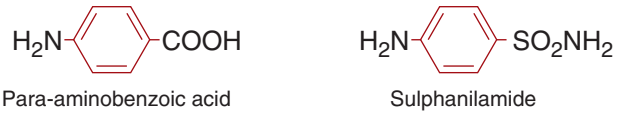
1. **Irreversible enzyme inhibition:** The activity of the enzyme is inhibited by covalent binding of the inhibitor at the active site. The enzyme inhibitor bond cannot be dissociated, so it is permanent and irreversible i.e. it cannot be reversed.
   - ❖ Aldolase is inhibited permanently by iodoacetate.
   - ❖ Di-isopropylflurophosphate (DFP), a component of nerve gas, inhibits most of the digestive enzymes permanently in human beings. Hence it is very poisonous.
   - ❖ Para chloro-mercuric benzoate (PCMB) inhibits the enzymes hexokinase and urease irreversibly.
   - ❖ Organic reagents like alkaloid reagents inhibit enzymes irreversibly.
2. **Reversible enzyme inhibition:** The inhibitors bind reversibly to the enzyme and so it is not permanent. The inhibition can be reversed by various mechanisms.
   (a) **Competitive enzyme inhibition:** It is a type of reversible inhibition in which there is competition between substrate and inhibitor for the active site of an enzyme because of the

structural similarity. All non-regulatory enzymes show competitive inhibition. Clinically competitive enzyme inhibition is of great importance since most of the drugs act by competitive inhibition.

(i) The enzyme succinate dehydrogenase's (SDH) substrate is succinic acid and the competitive inhibitors are oxalic acid, mallonic acid and glutaric acid. Among these, mallonic acid is the most potent competitive inhibitor of SDH.



(ii) Folic acid, a vitamin for human beings has para-amino benzoic acid (PABA) as one of its components. Whereas it is not a vitamin for microorganisms i.e., they cannot utilize preformed folic acid from external source, instead they synthesize their own folic acid from PABA. Sulpha drugs contain para-amino sulphonate which is structurally similar to PABA and hence competes for the enzyme active site of folic acid synthesis in microorganisms. If excess dose of sulpha drug is given, it results in inhibition of folic acid synthesis thus acting as an antibiotic. Human beings are not affected, because they do not synthesize folic acid.



(iii) Methanol is acted upon by the enzyme alcohol dehydrogenase forming formaldehyde which is highly poisonous. If ethanol if given to methanol intoxicated patients then ethanol competitively binds to alcohol dehydrogenase thereby preventing formation of formaldehyde.

(iv) Allopurinol is the competitive inhibitor of the enzyme xanthine oxidase whose substrate is hypoxanthine. Allopurinol prevents the formation of uric acid by competitive inhibition because it has structural similarity to hypoxanthine. This principle is used in the treatment of gout i.e. abnormal accumulation of uric acid crystals in the joint causing inflammation.

(v) Glaucoma is a condition in which there is accumulation of fluid in the lens resulting in enlargement of eye. This can be treated with **'acetazolamide'** which inhibits the enzyme carbonic anhydrase competitively. This prevents water formation and subsequent release of more water through the urine.

(b) Non-competitive enzyme inhibition: It is shown by regulatory enzymes, also called allosteric enzymes.

***Allosteric enzymes*:** These are the enzymes that contain a site other than the active site which is called **'allosteric site'**. The action of some enzymes is regulated by **'effectors'** which can bind reversibly to the enzyme molecule at specific sites other than the substrate binding site called the modulator site or the allosteric site.

There is no competition between substrate and inhibitor for the active site since the inhibitor or modulator binds at the modulator site or allosteric site. If the binding of the effector causes inhibition of the enzyme action then it is called a negative effector and the process is called **'allosteric inhibition'**.

If the enzyme reaction is activated by a modulator then it is called a positive modulator or effector and the process is called **'allosteric activation'**.

Ex. Phosphofructo kinase (PFK) is an allosteric enzyme of the glycolytic pathway.

✓ The positive modulators of this enzyme are AMP and ADP.

✓ The negative modulators of PFK are ATP and citrate.

Difference between regulatory and non-regulatory enzymes

Regulatory enzymes	Non-regulatory enzymes
They have two sites – active site and modulator site	They have only one active site
Gives a sigmoid curve	Gives a hyperbolic curve
Non competitive inhibition	Competitive inhibition
V_{max} varies	V_{max} remains the same
K_m remains the same	K_m varies

Antienzymes: These are substances (generally proteinacious in nature) that inhibit most of the digestive enzymes. ex. certain roundworms and hookworms survive in the intestine by secreting anti enzymes. Uncooked rice contains certain proteins that act as anti enzymes.

Reversible covalent modification: Enzyme activity can be regulated by reversible covalent modification. It is regulated by cyclic inter-conversion of enzyme into two forms—(i) modified form and (ii) unmodified form.

The inter-conversion is brought about by a **'converting enzyme'**. The process of activation and inactivation of the enzyme is generally brought about by covalent phosphorylation or de-phosphorylation of the target enzyme.

For example hormones like epinephrine, glucagon etc. bind to the hormone receptor site on the cell membrane and activates the enzyme adenyl cyclase, which in turn converts ATP to cyclic AMP (cAMP). This cAMP converts inactive protein kinase to active protein kinase ('a' form). This protein kinase phosphorylates many enzymes in the cell, some of which become active and yet some others become inactive. The inactive phosphorylase ('b' form) gets converted to active phosphorylase ('a' form) upon phosphorylation and affects the breakdown of glycogen to glucose. On the other hand glycogen synthase becomes inactive upon phosphorylation thereby inhibiting the formation of glycogen.

DIAGNOSTIC IMPORTANCE OF ENZYMES

Enzymes were classified into two groups based upon their clinical importance as **'functional plasma enzymes'** i.e., those enzymes present in the plasma in considerably high amounts and are functional in the plasma due to the presence of their substrate in it. Ex. serum lipase, blood clotting enzymes, and **'non-functional plasma enzymes'** i.e., those enzymes that are present in the plasma in negligible amounts and have no function in the plasma due to the absence of their substrate in it. Non-functional plasma enzymes are of diagnostic importance.

The non-functional plasma enzymes are present in higher concentration in tissues and very low concentration in the plasma i.e. in trace amounts, but their concentration in plasma increases immediately following tissue injury or destruction. If there is tissue damage leading to cell rupture then the enzymes

present in that tissue leaks into the blood leading to the increase in the concentration of these enzymes in the plasma. Increase in the level of non-functional plasma enzymes in the blood, indicates the disorder to the tissue where they exist. Different enzymes exist in different tissues in varying levels. Damage to a specific tissue releases a particular enzyme. Therefore estimation of enzymes in the plasma has a diagnostic importance.

The non functional plasma enzymes include lactate dehydrogenase (LDH), creatine phosphokinase (CPK), alanine amino transferase (ALT) or serum glutamate pyruvate transaminase (SGPT), aspartate transaminase (AST) or serum glutamate oxaloacetate transaminase (SGOT), sorbitol dehydrogenase, alkaline phosphatase, acid phosphatase, amylase, pancreatic lipase etc.

However functional plasma enzymes are already in higher concentration in the plasma, hence their decrease in the concentration in the plasma indicates malfunction of the organ where they are synthesized ex. blood clotting enzymes are synthesized in the liver; hence decrease in their concentration indicates liver dysfunction. Anyway an immediate assessment of the liver function cannot be made by this assessment because by the time the enzyme concentration in the plasma decreases (may take 4 to 5 days), the liver must have regained its normal vitality.

Some clinically important enzymes

Enzymes and their Concentration	Concentration increases in
Lactate dehydrogenase (LDH) – 60-12 IU/litre	Myocardial infarction, myopathy or muscle disorder. Also in leukemias, acute hepatitis, carcinomatitis.
Transaminases—	
(a) Aspartyl transaminase (AST) or Serum glutamyl oxaloacetate transaminase (SGOT) – 5-20 IU/litre	Myocardial infarction
(b) Alanine transaminase (ALT) or Serum glutamyl pyruvate transaminase (SGPT) – 5-15 IU/litre	Liver disorders
Creatine phosphokinase (CPK) – 10-60 IU/litre	Myocardial infarction, myopathy
Alkaline phosphatase – 4-17 King Amstrong (KA) units/100 ml	Bone disorders, obstructive jaundice, hyperparathyroidism
Acid phosphatase	Prostrate carcinoma
Isocitrate dehydrogenase	Brain tumor and meningitis, liver diseases
Amylase	Pancreatitis, parotitis (inflammation of parotid gland) intestinal obstruction, diabetes
Lipase	Pancreatitis or carcinoma of pancreas
Gamma glutamyl transpeptidase (g-GT)	Liver damage (indicator of alcoholism)

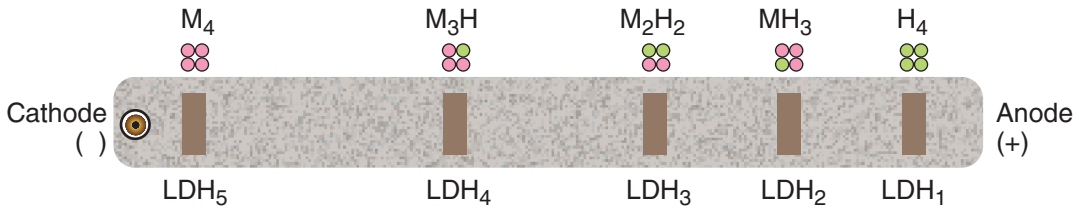
Diagnosis of myocardial infarction using enzyme assay: There are three main enzymes that are used in the diagnosis of myocardial infarction (1) Lactate dehydrogenase (LDH) (2) Creatine phosphokinase (CPK)—marker enzyme and (3) Transaminase (AST or SGOT).

(1) **Lactate dehydrogenase (LDH):** LDH catalyzes the inter conversion of pyruvate to lactate, a very important reaction of anaerobic glycolysis. Glycolysis occurs in each and every cell, in some cells it is always anaerobic (RBC) whereas in others it is aerobic sometimes and anaerobic at some other time (muscle tissue, liver, kidney etc). In other words LDH is present in each and every cell of the body. Therefore damage to any of the tissues of the body results in release of LDH into the plasma. Hence it becomes a difficult task to trace out the organ from which it has leaked.

However LDH exists in five isoenzyme forms i.e. multiple forms of the same enzyme (These enzymes bring about the same reaction but exhibit different physical characters like molecular weight, charge, electrophoretic mobility, K_m and isoelectric pH). The polypeptides in LDH are designated as 'H chain' and 'M chain'. All the isoenzyme forms of LDH are tetramer i.e. has four polypeptides in the following combinations.

(a) H_4 or LDH_1—Heart
(b) H_3M or LDH_2—RBC
(c) H_2M_2 or LDH_3—Brain and lungs
(d) HM_3 or LDH_4—Kidney
(e) M_4 or LDH_5—Liver and skeletal muscle

All these isomers have been successfully separated on Sodium Dodecyl Sulphate Polyacryl Amide Gel Electrophoresis (SDS-PAGE) and their banding pattern from the plasma is established as under—



LDH_1 or H_4 is predominantly present in the cardiac muscle, whereas the isoenzyme form LDH_5 or M_4 is more abundant in the skeletal muscle. These two enzymes have different Km values and Km is indirectly proportional to affinity (K_m α 1/affinity). The skeletal muscle enzyme M_4 has low K_m value for pyruvate and hence greater affinity for pyruvate resulting in high rate of conversion of pyruvate to lactate. The cardiac isoenzyme LDH_1 or H_4 has high K_m value for pyruvate hence lesser affinity for pyruvate, therefore low rate of conversion of pyruvate to lactate.

Thus the concentration of H_4 or LDH_1 isoenzyme form of lactate dehydrogenase increases in the plasma during myocardial infarction. The peak levels of LDH are maintained in the plasma for 6 days following the attack, after which it starts receding in its concentration.

(2) **Creatine phosphokinase (CPK):** This is known as the marker enzyme for the diagnosis of myocardial infarction or heart attack, because this is the first enzyme to increase within a short time in the blood plasma following a heart attack.

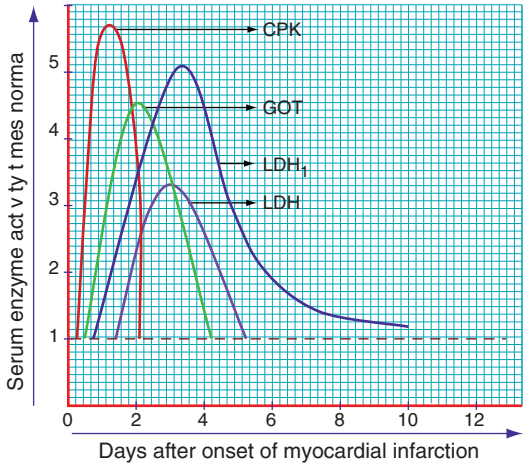
CPK is an enzyme that catalyzes the conversion of creatine to creatine phosphate, a high energy compound that works to supply energy during muscle contraction. Therefore this enzyme is present only in a few tissues like the cardiac muscle, skeletal muscle and the brain.

CPK also exists in various isoenzyme forms. It has two polypeptides 'B' & 'M' that form dimers in the following combinations to give rise to three isoenzymes of CPK.

MB – Predominant in cardiac muscle
BB – Predominant in brain
MM – Predominant in skeletal muscle

Thus estimation of the isoenzyme MB is indicative of heart attack. CPK maintains a higher concentration in the plasma for 1-2 days. The concentration of CPK after the first attack is 10 times more than the normal and if another attack occurs within a day or two the concentration further increases to 100 fold and a third attack within a short span of time raises the level of CPK to 300 fold which is lethal concentration.

(3) **Transaminases:** Among the two transaminases, aspartyl transaminase (AST or SGOT) increases in the plasma following an attack and the higher levels are seen in 4 to 5 days following an attack.



9

VITAMINS

Vitamins are organic substances required in minute quantities in the diet to maintain the normal physiological functions of the body.

Vitamins are classified as (1) water soluble vitamins and (2) fat soluble vitamins.

WATER SOLUBLE VITAMINS

The water soluble vitamins are B-complex vitamins and vitamin C. The B-complex group of vitamins includes—

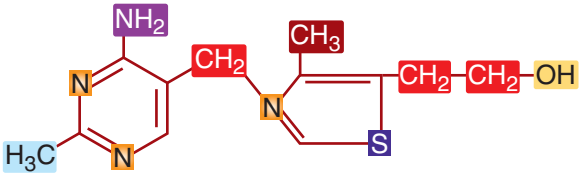
(1) Thiamine	(2) Riboflavin
(3) Niacin	(4) Pyridoxine
(5) Pantothenic acid	(6) Biotin
(7) Folic acid	(8) Lipoic acid
(9) Cyanocobalamine	(10) Choline
(11) Inositol	

THIAMINE

VITAMIN—B_1

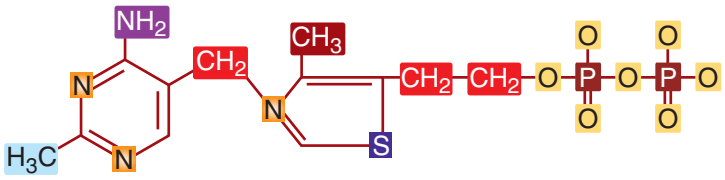
Vitamin B_1 is also called as Anti-Beri Beri vitamin and Anti-Neuritic vitamin.

Chemistry: Thiamine is present as a crystal of thiamine hydrochloride. Chemically thiamine contains a pyrimidine ring and a thiazole ring linked by a methylene bridge. Both the rings are substituted.



Thiamine

The alcohol group on the thiazole ring is esterified with two phosphoric acid molecules to form thiamine pyrophosphate (TPP). Thiamine pyrophosphate is the active co-enzyme form of thiamine.



Thiamine pyrophosphate

Source: The richest sources of thiamine are rice bran, wheat bran, whole grains, nuts, germinating seeds, pulses, beans, lentils, yeast, liver, eggs, fish, meat and milk.

Daily requirement: This vitamin cannot be stored in the body, as it is excreted in the urine, hence it should be provided daily in the diet. However the skeletal muscle can retain this vitamin for a short duration of time. Brain cannot retain any of the thiamine.

The daily requirement of thiamine depends upon the calorie requirement and the carbohydrate intake. For 3000 Kcal of energy 1.5 mg of thiamine is required per day. However—

- For normal adult males the daily requirement is from 1.5 to 2.0 mg/day.
- For adult females the daily requirement is from 1.0 to 1.2 mg/day.

Assay methods: Vitamin B_1 is oxidized to thiochrome which can be assayed by fluorescence. Other biological and microbiological procedures are also available for its assay.

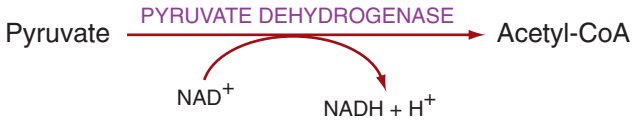
Metabolism: Vitamin-B_1 is freely absorbed by small intestine and drained into the liver. In the liver it is phosphorylated to thiamine pyrophosphate (TPP). This TPP coenzyme is present in all the tissues. There is no storage of this vitamin, hence regularly needed in the diet. About 10% of vitamin-B_1 taken in the diet is normally excreted in the urine.

Physiological functions and biochemical role: Thiamine is required for—

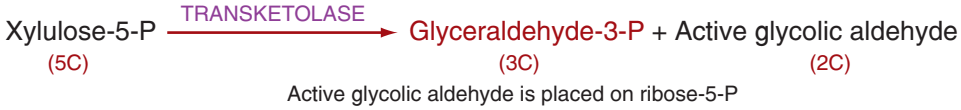
1. Utilization of carbohydrates in the body.
2. Maintenance of good appetite.
3. Normal brain metabolism.

Thiamine in its active coenzyme form i.e. as thiamine pyrophosphate and Mg^{2+} function as coenzyme for (1) Oxidative decarboxylation reactions and (2) Transketolation reactions. Ex.

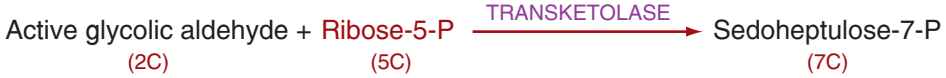
For oxidative decarboxylations:



For Transketolations:



Active glycolic aldehyde is placed on ribose-5-P



Deficiency diseases: Thiamine deficiency results in impaired utilization of carbohydrates due to which pyruvate and lactate accumulates in the cells. But this impairment is not uniform; the brain cells are more affected than the skeletal muscle. Blood brain barrier becomes permeable to pyruvate in rats. Erythrocyte transketolase levels are lower then normal.

The symptoms of thiamine deficiency are loss of appetite (anorexia). The clinical condition of thiamine deficiency is known as 'Beri-Beri', characterized by polyneuritis (various defects of nervous system), edema, cardio-vascular changes, weakness, muscular atrophy, headache, insomnia, gastro-intestinal disorders etc. Wernicke's encephalopathy or acute thiamine deficiency is seen in alcoholics.

Beri-Beri is of four types viz.—

(a) **Dry Beri-Beri:** In this nervous symptoms or polyneuritis predominates.

(b) **Wet Beri-Beri:** In this the symptoms are associated with edema and serous effusions.

(c) **Acute pernicious Beri-Beri:** In this the symptoms of heart are involved.

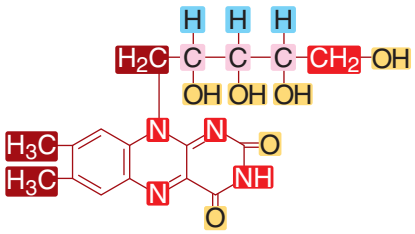
(d) **Mixed Beri-Beri:** All the above symptoms.

Another type of Beri-Beri is infantile Beri-Beri seen in breast fed children, whose mothers' milk is deficient in thiamine.

Antagonists: Pyrithiamine, oxythiamine and 2-n-butyl thiamine are the antagonists of thiamine. Foxes develop a type of paralysis called 'chastek paralysis' when they eat raw fish. It is caused by the presence of enzyme "thiaminase" in raw fish which destroys thiamine.

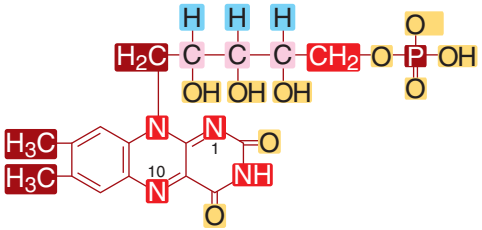
RIBOFLAVIN

Chemistry: Riboflavin is made up of substituted isoalloxazine ring. To this ring ribityl alcohol is attached.



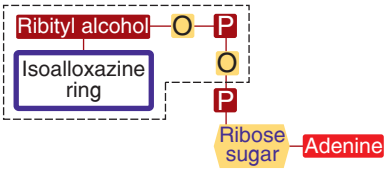
Riboflavin

The ribityl alcohol of the riboflavin is phosphorylated to form riboflavin phosphate or flavin mononucleotide (FMN).



Flavin mononucleotide (FMN)

Riboflavin is also linked to an adenosine nucleotide through a pyrophosphate linkage to form flavin adenine dinucleotide (FAD).



Flavin adenine dinucleotide (FAD)

FMN and FAD are the two active coenzyme forms of riboflavin.

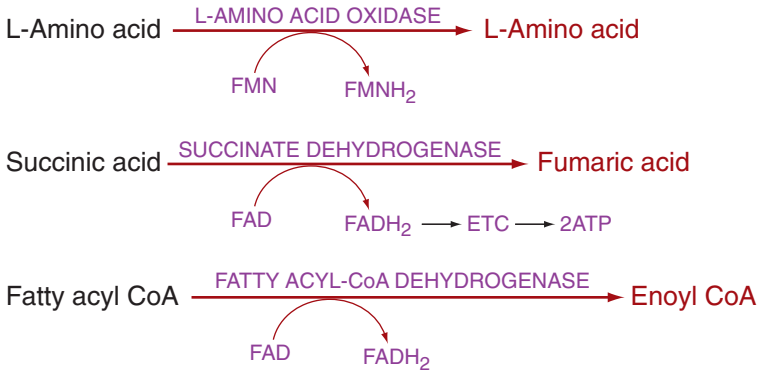
Source: Riboflavin is rich in animal sources like milk, liver, kidney, heart, egg yolk and in sprouts.

Daily requirement: The daily requirement of riboflavin depends upon the calorie requirement of an individual. For 1000 cal of energy 0.5 mg of riboflavin is recommended. On an average 1.5-2.0 mg/day of riboflavin is recommended.

Physiological functions of riboflavin

1. It is required for the regulatory functions of some hormones concerned with carbohydrate metabolism.
2. It stimulates the optic nerve in presence of light.

Coenzyme activities: FAD and FMN act as coenzyme for some enzymes called flavorproteins. They help in the oxidation-reduction reactions of cell metabolism. The hydrogens are transported by reversible reduction of the coenzyme by two hydrogen atoms added to the nitrogen (N) at positions 1 and 10. The enzyme reactions catalyzed are as follows—



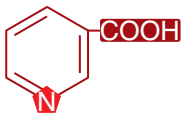
Deficiency diseases

(1) Characteristic lesions of the lips.
(2) Fissures at the angles of the mouth (cheilosis).
(3) Dermatitis of the face.
(4) Magenta tongue.
(5) Certain functional and organic disorders of the eye.

Antagonists: Dichlororiboflavin and isoriboflavin.

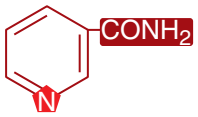
NIACIN

Chemistry: The chemical name of niacin is nicotinic acid. It contains a pyridine ring with a carboxylic acid.



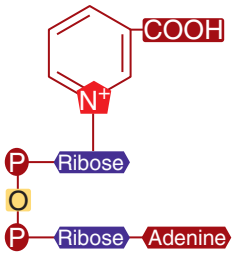
Nicotinic acid
(NIACIN)

Nicotinic acid is converted to its amide form called niacinamide or nicotinamide.



Nicotinamide
(NIACINAMIDE)

Nicotinamide combines with a ribose phosphate and later on gets esterified to an adenine nucleotide to form the '**Nicotinamide adenine dinucleotide (NAD^+)**'.



Nicotinamide adenine dinucleotide (NAD)

The ribose sugar of adenine may be phosphorylated to form '**Nicotinamide adenine dinucleotide phosphate ($NADP^+$)**'. NAD^+ and $NADP^+$ are the two coenzyme forms of niacin.

Source: Liver, fish, beans and peanuts. Niacin can also be formed in the human body form the amino acid - tryptophan. 60 mg of tryptophan can give rise to 1 mg of niacin.

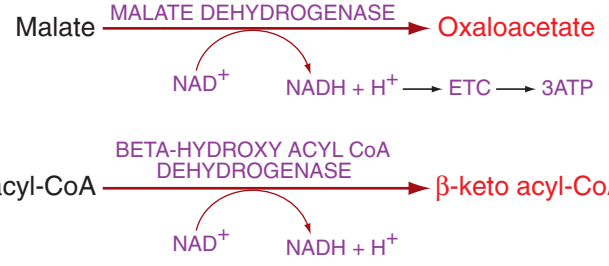
Daily requirement: 15-20 mg/day. Niacin requirements depend upon the quality and quantity of protein in the diet, as niacin can be formed from tryptophan.

Functions: Niacin in the form of NAD^+ and $NADP^+$ acts as coenzyme for a number of oxido-reductases. They act as electron acceptors during the enzymatic removal of hydrogen atoms.

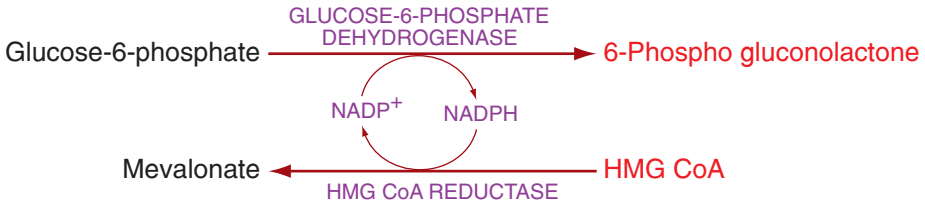
NAD^+ acts as coenzyme for dehydrogenases in oxidation of various food stuffs.

$NADPH^+$ acts as coenzyme for reductases in the synthesis of fatty acids and cholesterol.

Functions of NAD^+ as coenzyme



Functions of NADP as coenzyme (its formation and utilization)

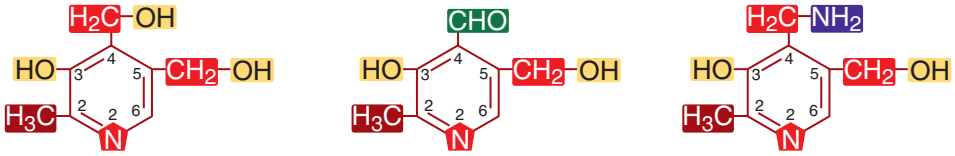


Deficiency diseases: The deficiency disease of niacin is pellagra. It is characterized by "3 Ds" i.e. Dermatitis, Diarrhoea and Dementia (headache, forgetfulness, depression, anxiety, etc.). If not treated it results in 4th 'D' i.e. Death.

PYRIDOXINE

VITAMIN – B_6

Chemistry: The chemical name of pyridoxine is 2-methyl-3-hydroxy-4, 5-dihydroxymethyl pyridine. The alcohol on 4th position may be oxidized to an aldehyde to form pyridoxal or it may be substituted by an amino group to form pyridoxamine. Hence vitamin B_6 exists in three forms i.e., **pyridoxine, pyridoxal** and **pyridoxamine**.



The – OH group at the 5th position may be phosphorylated to give **pyridoxal phosphate** and **pyridoxamine phosphate**. These are the two active coenzyme forms of this vitamin.

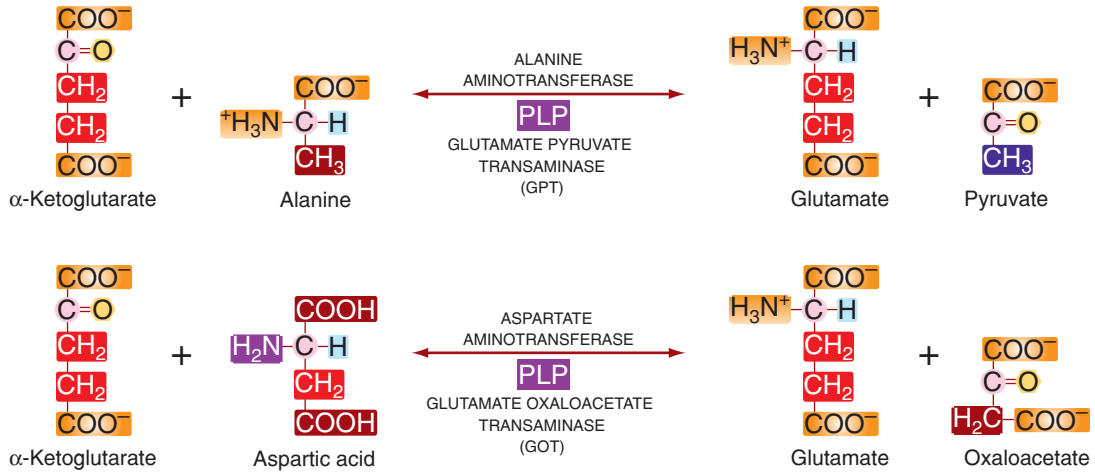
Source: Yeast, rice polishing, milk, meat, eggs, leafy vegetables and liver. Intestinal bacteria can also synthesize this vitamin.

Daily requirement: 1.6–2.0 mg/day.

FUNCTIONS

1. Pyridoxine is essential for the growth of infants.
2. Pyridoxal phosphate acts as coenzyme for—
   (a) Transamination reactions—takes up amino group to form pyridoxamine phosphate.
   (b) Decarboxylation—in the formation of dopamine (GABA).
   (c) Dehydration.
   (d) Desulphuration.
   (e) Deamination.
   (f) Trans-sulphuration.
   (g) Kynureninase enzyme in niacin synthesis.
   (h) Synthesis of serotonin, catecholamines and heme.

A Schiff's base is formed as an intermediate in the reactions catalyzed by pyridoxal phosphate.

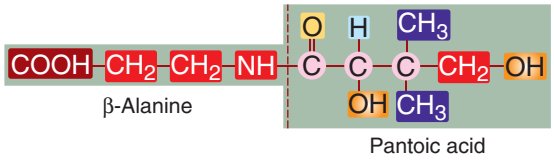


Deficiency diseases: Vitamin B_6 deficiency is rare. However irritability, depression, peripheral neuropathy, hypochromic microcytic anemia, cystathionuria, etc. may be seen.

Antagonists: Isonicotinic acid hydrazide (INH) and hydrazoline. Infants when treated with INH for tuberculosis suffer form convulsions because of structural resemblance of INH with vitamin B_6.

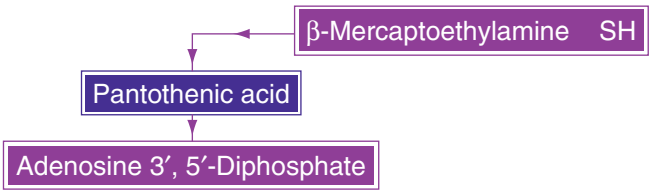
PANTOTHENIC ACID

Chemistry: Pantothenic acid contains β-alanine and pantoic acid joined by a peptide bond.



Pantothenic acid

The active coenzyme form of pantothenic acid is coenzyme-A. Coenzyme-A is formed by combination of pantothenic acid with adenine, ribose, phosphoric acid (Adenine nucleotide) and β-mercaptoethylamine.



Coenzyme-A

Source: Pantothenic in Greek means 'from everywhere'. It is so named because it is present in all the common food sources. Its richest source is honey. The intestinal flora also supplies considerable amount of this vitamin.

Food source: Cereals, nuts, oil seeds, egg, liver, wheat grain, legumes, rice polishing, milk, meat and fish are good sources of this vitamin. Vegetables like potato have relatively less amount of this vitamin.

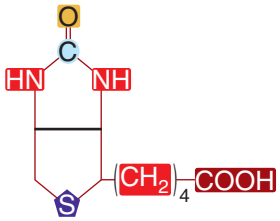
FUNCTIONS

1. Pantothenic acid is required for the growth of infants and children.
2. Coenzyme-A is required for the activation of acetate to acetyl-CoA.
3. Co-A is required to form succinyl-CoA for the citric acid cycle to operate normally.
4. Coenzyme-A is required for the activation of fatty acids for their oxidation.
5. Coenzyme-A is also required for fatty acid and cholesterol synthesis.

Deficiency diseases: As this vitamin is widely distributed, the deficiency rarely occurs. However nausea, vomiting, G.I tract disorders, irritability, inadequate growth, anemia, fatty liver, etc. may occur due to its deficiency. Impaired growth and reproduction, loss of hair, sensitivity to insulin, burning feet syndrome are other deficiency manifestations of this vitamin.

BIOTIN

Chemistry: It is a heterocyclic compound containing sulphur and a valeric acid side chain. It is attached to lysine residue of the enzyme through the valeric acid to form the active coenzyme i.e., biocytin.



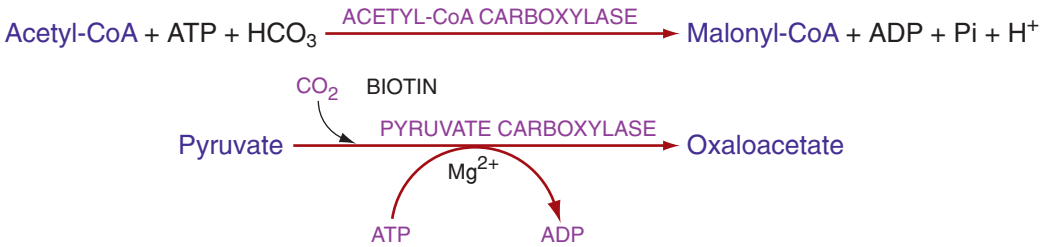
Biotin

Source: Egg, liver, fish, meat, beans, germinating seeds, intestinal flora, and peanut. Richest source is → honey. Poor source is → fruits and vegetables.

Daily requirement: Only if the intestinal flora is disturbed, the requirement of biotin ranges form 100-300 µg/day.

FUNCTIONS

Biotin is involved in CO_2 transfer and CO_2 fixation. Ex.

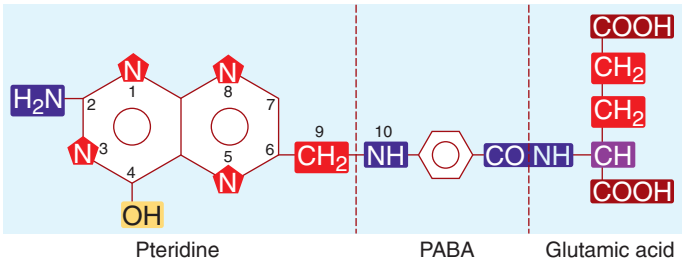


Deficiency diseases: Only if raw egg white which contains avidin is taken, it binds and inhibits the absorption of biotin that leads to deficiency. Signs include—alopecia, greying of hair and nervous symptoms.

FOLIC ACID

Chemistry: There are three components in folic acid viz. (1) Pteridine (2) Para aminobenzoic acid (PABA) and (3) Glutamic acid.

Pteridine nucleus comprises of pyrimidine and pyrazine rings. Further pteridine and PABA are collectively called pteroic acid. All the components are together known as pteroyl glutamic acid (PGA) i.e. folic acid.



Folic acid

Folic acid is readily absorbed by the intestine and not stored but undergoes reduction in the liver by the enzyme folic acid reductase that reduces folic acid to tetrahydrofolic acid (THF). This is the active coenzyme form of folic acid. This reaction requires $NADPH^+$ and ascorbic acid.

Folic acid —(FOLIC ACID REDUCTASE; NADPH + Ascorbic acid)→ Tetrahydrofolic acid

FUNCTIONS

The main function of folic acid is the transfer of one carbon units (moiety or fragments). The various one carbon moieties transferred by THF are—

1. $-CH_3$ = Methyl group
2. $-CH_2OH$ = Hydroxy methyl group
3. $-CH_2-$ = Methylene
4. $-CHO$ = Formyl
5. $-CH=$ = Methylidine or methenyl
6. $-COOH$ = Carboxyl
7. $-C=O$ = Carbonyl
8. $-CH=NH$ = Formimino

These one carbon moieties get attached either to the 'N' at position 5 or to the 'N' at position 10. They can get attached both to 5^{th} nitrogen and 10^{th} nitrogen. If the one carbon (1-C) moiety is attached to the 5^{th} nitrogen of folic acid then it is known as N^5-(1-C)-THF. If the one carbon moiety is attached to the 10^{th} nitrogen then it is N^{10}-(1-C)-THF. If the 1-carbon moiety is linked to both 5^{th} and 10^{th} nitrogen then it is called N^5-N^{10}-(1-C)-THF.

Some examples

1. N^5-Methyl tetrahydrofolate
2. N^5,N^{10}-Methylene tetrahydrofolate
3. N^5-Formyl tetrahydrofolate

The N^5 and N^{10} forms are inter-convertible. The one carbon moieties are derived from several sources and utilized to form several compounds.

One carbon moiety donors	One carbon moiety acceptors
Formimino group of formiminoglutamic acid (formed from histidine)	Positions 2 and 5 of purine ring
Methyl group of methionine	Glycine to form serine
Methyl group of betaine	Homocysteine to form methionine
Methyl group of methionine	Uracil to form thymine
Beta carbon of serine	Ethanolamine to form choline

Action of THF

Serine + THF ⇋ Glycine + N^5,N^{10}-Methylene-THF + H_2O

N^5, N^{10}-Methylene-THF + NADH + H^+ ⇋ N^5-Methyl-THF + NAD^+

N^5-Methyl-THF + Homocysteine ⇋ THF + Methionine

Source: The microorganisms of the intestinal tract can synthesize folic acid in considerable amounts. These microorganisms need PABA to synthesize this vitamin. Therefore if PABA is supplied in sufficient quantities the requirement of folic acid can be fulfilled. During antibiotic therapy the microflora die leading to deficiency of folic acid hence the exogenous food sources of folic acid like liver, eggs and leafy vegetables are required in the diet.

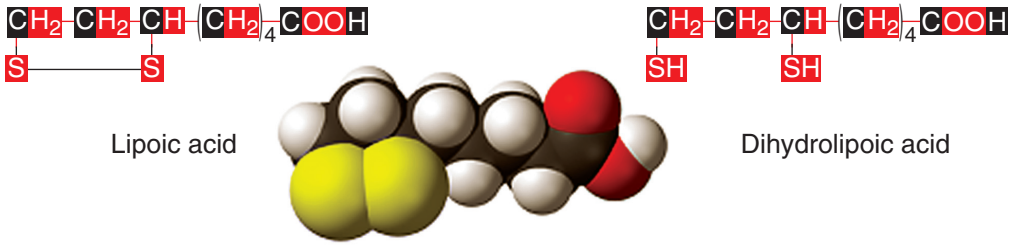
Daily requirement: As adult human liver can store to about 5-20 mg of folic acid the daily requirement of folic acid is about 300-400 µg, which can be easily supplied by the intestinal flora.

Deficiency disease: During treatment with sulphonamide drugs there will be deficiency of folic acid as the sulphonamide drugs competitively inhibit the synthesis of folic acid by the microorganisms. This competition is as a result of structural similarity between the drug and PABA. The deficiency results in poor synthesis of the pyrimidine thymine which in turn affects DNA synthesis, hence affects cell division. The signs of deficiency are magaloblastic anemia, glossitis and G.I tract disturbances and leucopenia.

Antagonists: Aminopterin, amethopterin, trimethoprim and methotrexate.

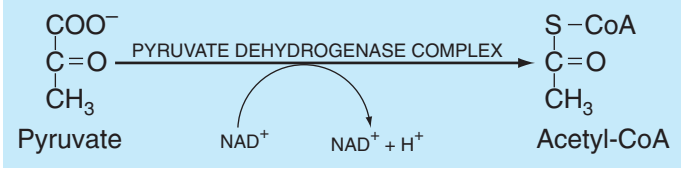
LIPOIC ACID

Chemistry: It is a sulfur containing fatty acid named 6, 8-dithiooctonoic acid. Dihydrolipoic acid is the reduced form of lipoic acid and is the active coenzyme form.



Biochemical functions

1. It is an essential cofactor for many enzyme complexes in aerobic metabolism, specifically the pyruvate dehydrogenase complex.



2. Other enzymes that use lipoic acid are 2-oxoglutarate dehydrogenase (OGDH) complex, branched chain oxoacid dehydrogenase (BCDH) complex, glycine cleavage complexes (GCV) and acetoin dehydrogenase (ADH) complex. Lipoate participates in transfer of acyl or methylamine groups in 2-oxoacid dehydrogenase (2-OADH) and glycine cleavage complexes (GCV), respectively.

3. Lipoic acid is an effective antioxidant and thus it prevents the symptoms of vitamin C and vitamin E deficiency. Dihydrolipoic acid is able to regenerate (reduce) antioxidants, such as glutathione, vitamin C and vitamin E, maintaining a healthy cellular redox state.
4. It increases the cellular uptake of glucose by recruiting the glucose transporter **'GLUT4'** to the cell membrane.
5. It acts as a good chelating agent for mercury.

Clinical uses:

- Intravenous administration of alpha lipoic acid (ALA) to people with acute and severe liver damage results in recovery of full liver function. Hence it is used successfully for the treatment of chronic liver disease (viral hepatitis, autoimmune hepatitis, etc).
- Use of ALA with various oral antioxidants results in the long term survival of patients with metastatic pancreatic cancer.
- Alpha lipoic acid has the ability to modify gene expression by stabilizing NF kappa B transcription factor, hence ALA is used for the treatment of various cancers for which no effective treatments exist.
- Intravenous ALA completely reverses the signs and symptoms of B-cell lymphoma.
- Its enhancement of glucose uptake by cells favours its use in diabetes.
- The use of carnitine and lipoic acid results in improved memory performance and delayed structural mitochondrial decay. As a result, it may be helpful for people with Alzheimer's disease or Parkinson's disease.
- It is used as a chelating agent in treatment of mercury intoxication. It is particularly suited to this purpose as it can penetrate both the blood-brain barrier and the cell membrane. Other chelators such as dimercaptosuccinic acid (DMSA) and 2,3-dimercapto-1-propanesulfonic acid (DMPS) are unable to cross the brain-blood barrier and to remove mercury from the brain.

Food sources: Lipoic acid is found in a variety of foods, notably kidney, heart and liver meats as well as spinach, broccoli and potatoes.

Dietary requirement: 60 mg/kg bw/day.

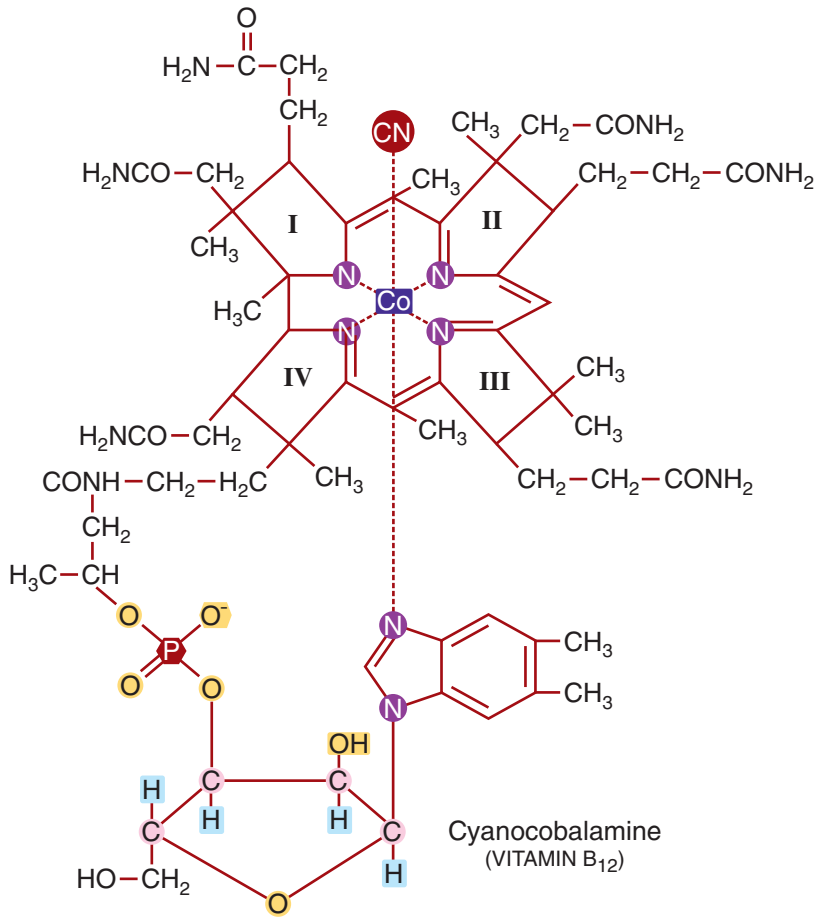
VITAMIN–B_{12}

COBALAMINE

Chemistry:

1. It has four pyrrole rings – I, II, III and IV.
2. Three rings are linked by methylene bridges.
3. I^{st} and IV^{th} rings are linked directly.
4. The tetrapyrrole ring structure of vitamin B_{12} is known as **'corrin'** ring system.
5. Cobalt atom is present in the centre linked by co-ordinate linkages to the nitrogens of the pyrrole rings.
6. The cobalt is also linked to cyanide. Hence it is called cyanocobalamine.
7. Cobalt is even linked to a ribonucleotide having the base 5,6-dimethylbenzimidazole.
8. The side chains of the corrin ring are much longer.

9. The side chain of the IVth ring is linked to the ribonucleotide.



Metabolism: Vitamin B_{12} is known as the 'extrinsic factor of castle'. Absorption of vitamin B_{12} requires an 'intrinsic factor of castle' secreted by the gastric glands. It is a glycoprotein and helps in the absorption of vitamin B_{12} from the intestine along with a releasing factor. B_{12} is transported in the plasma bound to specific carrier proteins called transcorrin-I and transcorrin-II.

The active coenzyme form of vitamin B_{12} is known as cobamide coenzyme.

FUNCTIONS

The coenzyme activity of cyanocobalamine is that of 1, 2-hydrogen shift and methyl group transfer.

Folic acid takes up the methyl group from the methyl donor and donates it to cobalamine and folic acid itself goes to folate pool to accept another methyl group. This is known as folate cycle.

1. Vitamin B_{12} is essential for the normal maturation and development of erythrocytes.
2. It is necessary for the synthesis of DNA.
3. It is required for the conversion of methyl malonyl-CoA to succinyl-CoA.

4. Methylation of homocysteine to methionine.
5. Inter conversion of glutamic acid and β-methyl aspartic acid.

Source: Neither plants nor animals can synthesize vitamin B_{12}. Only certain microorganisms can synthesize it. However as B_{12} is stored in the organs of various animals the sources of B_{12} are liver, egg, meat and fish.

Daily requirement: 5 µg/day.

Deficiency diseases: Megaloblastic anemia (pernicious anemia), demyelination, neurological lesions and infertility.

B–Complex group of vitamins and their coenzyme forms

Vitamin/ chemical name	Active coenzyme form	Biochemical role/ function	Reaction participating
B_1 Thiamine	Thiamine Pyrophosphate **(TPP)**	Oxidative decarboxylation reaction and Transketolation reaction	Oxidative decarboxylation Pyruvate —(PYRUVATE DEHYDROGENASE; NAD^+ / $NADH + H^+$)→ Acetyl-CoA Transketolase Xylulose-5-P + Ribose-5-P → Glyceraldehyde -3-P + Sedo-heptulose-5-P
B_2 Riboflavin	Flavin Mononucleotide **(FMN)** Flavin adenine Dinucleotide **(FAD)**	Oxidation reduction reactions	L-Amino acid —(L AMINO ACID OXIDASE; FMN / $FMNH_2$)→ L-Imino acid Succinic acid —(SUCCINATE DEHYDROGENASE; FAD / $FADH_2$)→ Fumaric acid Fatty acyl-CoA —(FATTY ACYL CoA DEHYDROGENASE; FAD / $FADH_2$)→ Enoyl CoA
B_3 Niacin (Nicotinic acid)	Nicotinamide Adenine Dinucleotide (NAD^+) Nicotinamide Adenine Dinucleotide Phosphate **($NADP^+$)**	Oxidation reduction reactions	Malate —(MALATE DEHYDROGENASE; NAD^+ / $NADH + H^+$)→ Oxaloacetate Glucose-6-phosphate —(GLUCOSE 6 PHOSPHATE DEHYDROGENASE; $NADP^+$ / $NADPH + H^+$)→ 6-Phospho gluconolactone Mevalonate ←(HMG CoA REDUCTASE)— HMG CoA
B_4 Biotin	Biocytin	CO_2 Transfer and CO_2 fixation	Pyruvate —(PYRUVATE CARBOXYLASE)→ Oxaloacetate
B_5 Pantothenic acid	Coenzyme-A	Acyl group transfer	Pyruvate —(PYRUVATE DEHYDROGENASE)→ Acetyl CoA Fatty acid —(FATTY ACYL CoA SYNTHETASE)→ Fatty acyl CoA

contd. to next page

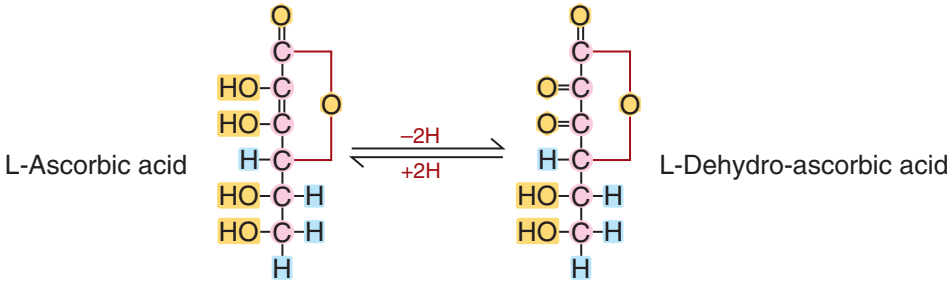
contd. from previous page

B_6 Pyridoxine	Pyridoxal Phosphate **(PLP)** Pyridoxamine Phosphate	**Transamination reactions** – (Takes up amino group to form Pyridoxamine) – Decarboxylation – Dehydration – Desulphuration – Deamination – Trans-sulphuration	α-Keto glutarate + Alanine $\xrightarrow[(GPT)]{\text{ALANINE TRANSAMINASE}}$ Glutamic acid + Pyruvate – In the formation of dopamine (GABA) – Kynureninase enzyme in niacin synthesis – Synthesis of serotonin, catecholamines and heme
B_7 Folic acid	Tetrahydrofolic Acid **(THF)**	**Transfer of one carbon units** (Moiety or fragments). One carbon units or moieties are: 1. $-CH_3$=Methyl group 2. $-CH_2OH$= Hydroxy methyl group 3. $-CH_2-$=Methylene 4. –CHO=Formyl 5. –CH= = Methylindine or methenyl 6. –COOH = Carboxyl 7. –C=O = Carbonyl 8. –CH=NH= Formimino	Deoxyuridylate $\xrightarrow{\text{THYMIDILATE SYNTHETASE}}$ Deoxythymidylate ***One example how THF acts*:** Serine + THF $\rightleftharpoons$ Glycine + N^5, N^{10}-Methylene THF + H_2O N^5, N^{10}-Methylene THF + NADH + H^+ $\rightleftharpoons$ N^5-Methyl THF + NAD^+ N^5-Methyl THF + Homocysteine $\rightleftharpoons$ THF + Methionine
B_8 Lipoic acid	Dihydro lipoic Acid **(DHL)**	Acyl group transfer and hydrogen carrier	Pyruvate $\xrightarrow{\text{PYRUVATE DEHYDROGENASE}}$ Acetyl CoA
B_{12} *Cobalamine* or Cyano-cobalamine	Cobamide Coenzyme (5′ Deoxy adenosyl cobalamine)	1, 2-Hydrogen shift – Vitamin B_{12} is essential for the normal maturation and development of erythrocytes – It is necessary for the synthesis of DNA – It is required for the conversion of methylmalony-CoA to succinyl-CoA – Methylation of homocysteine to methionine – Inter conversion of glutamic acid and β-methyl aspartic acid	Succinyl-CoA $\xrightarrow{\text{Mutase}}$ Methyl malonyl-CoA

VITAMIN–C

ASCORBIC ACID

Chemistry: It is a strong reducing substance. The structure resembles a hexose sugar. It is easily oxidized to dehydroascorbic acid.



Functions: It is concerned with the metabolism of connective tissue, particularly of collagen.

1. It maintains the redox potential of the cell.
2. Proline is converted to hydroxyproline in presence of vitamin C. Hydroxyproline is an important constituent of collagen.
3. It helps in the absorption of Iron from the intestine.
4. High doses of vitamin-C in the diet, reduces the duration and severity of common cold.

Source: The citrus fruits (lemons and oranges) are the richest sources. Other sources are fresh green vegetables, cabbage, lettuce, guavas, berries, melons and tomatoes.

Daily requirement: 30 mg for infants and 70 mg for adults.

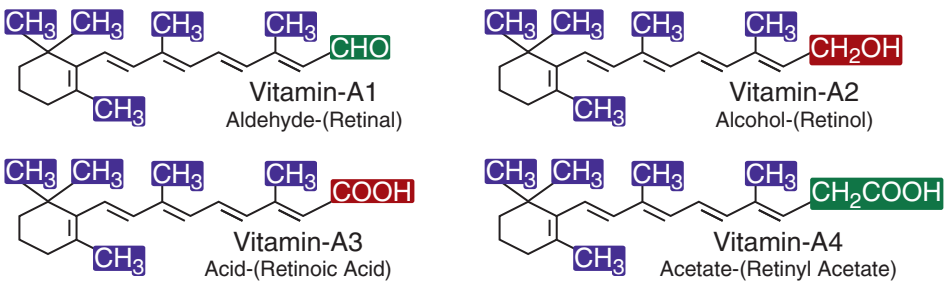
Deficiency diseases: Scurvy is the deficiency disease of vitamin C. The main defect is poor deposition of intercellular cement substance (i.e. collagen). The capillaries are fragile and so there is a tendency to hemorrhage. Wound healing is delayed due to deficiency in the formation of collagen. Gums are swollen and decay easily. There is poor dentine formation and tooth loss. Weak bones leading to fracture.

Fat soluble vitamin: Vitamins insoluble in water but soluble in fats or fat solvents are known as fat soluble vitamins. The fat soluble vitamins are vitamin A, D, E & K.

VITAMIN – A

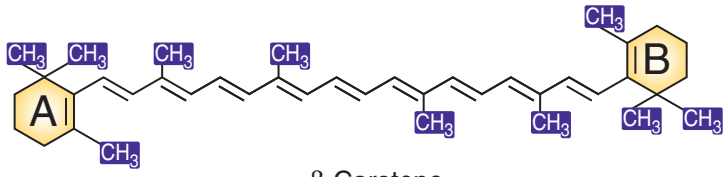
Chemistry: Vitamin A functions in the human body in four different forms—

1. Vitamin A1 known as Retinal—the aldehydic form
2. Vitamin A2 known as Retinol—the alcoholic form
3. Vitamin A3 known as Retinoic acid—the acid form
4. Vitamin A4 known as Retinyl acetate—the acetate form



Vitamin A, as such, is neither synthesized by the animals nor by the plants. Instead, plants synthesize the provitamins of vitamin A known as carotenoids that are converted to the active vitamin in the animal body. There are many carotenoids that includes α, β, γ carotenes and others. Among the various carotenoids; β-carotene is the most potent precursor of vitamin A.

β–Carotene is made up of two β-ionone rings connected through an eighteen member hydrocarbon chain, substituted by methyl groups at a few points.



β-Carotene

The provitamins (β-carotene) are converted into vitamin A in the intestinal wall in animals but in man this transformation takes place in liver. There is an enzyme called **β-carotene 15, 15'-oxygenase** in the liver of man and intestinal wall of other animals that cleaves β-carotene at the central position releasing two molecules of active vitamin A. This reaction takes place in presence of α-tocopherol (vitamin E) and it is a dioxygenase reaction in which molecular oxygen reacts with the 2 central carbon atoms of beta-carotene followed by cleavage of its central double bond to yield 2 molecules of vitamin A aldehyde (retinal). Vitamin A alcohol is then produced by reduction of the aldehyde in a NADH-dependent reaction catalyzed by retinene reductase.

Absorption and transport: The dietary sources of vitamin A to the humans and animals are via the conversion of beta-carotenes (plant sources) to vitamin A and hydrolysis of retinyl esters (animal sources) to retinol in the intestine. Retinol is absorbed in the cell membrane of the intestine, re-esterified inside the cell of the intestine and finally absorbed via the lymph. A significant amount of vitamin A is also absorbed directly into the blood circulation along with the other dietary fats that form chylomicrons. Vitamin A is stored in the liver as palmitate esters.

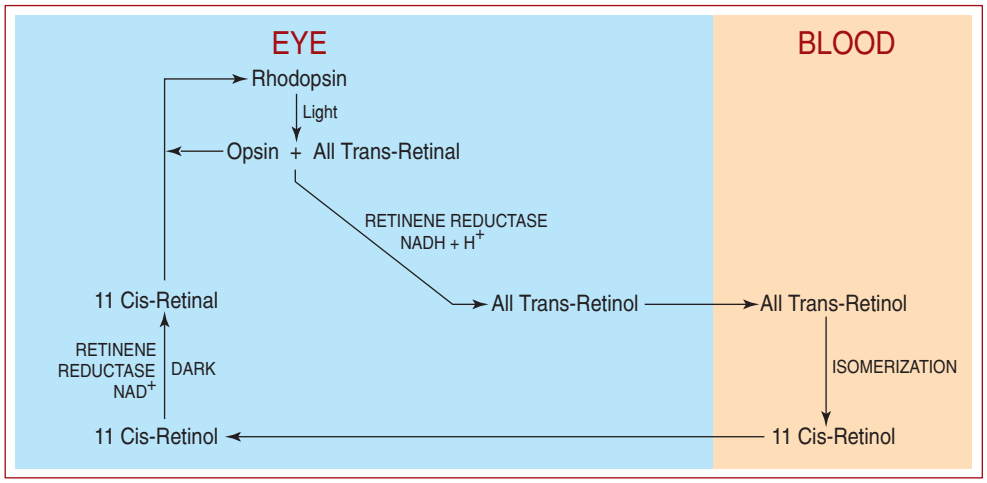
Vitamin A is transported from the storage organ (liver) to the organs of utilization (eye, skin etc) being bound to retinol-binding protein (RBP) which has a molecular weight of about 20,000 Da. It is also transported being bound to prealbumin in the blood.

BIOCHEMICAL FUNCTIONS

1. It is required for the normal vision and general growth of the body.
2. It accelerates the development of the nervous system and bones.
3. It maintains the structural integrity of the cell membrane and membranes of lysosomes and mitochondria. Thus it keeps the skin, kidney and other organs intact thereby preventing their degeneration.
4. It enhances the carbohydrate metabolism especially gluconeogenesis from lactate, acetate and glycerol.
5. It is also involved in muco-polysaccharide biosynthesis.
6. It enhances protein synthesis by activating aminoacyl-tRNA synthetases.
7. It accelerates the transcription and translation process in the cell.
8. It is also required for DNA metabolism.

Vitamin – A and vision (Rhodopsin cycle): There are two types of cells in the retina of the eye viz. rods (for dim light vision) and cones (for bright light vision). The rod cells contain rhodopsin (retinal pigment or visual purple). When light strikes the retina, rhodopsin is split into its protein component;

opsin and the non-protein; retinene (all trans-retinal). In the eye, the trans-retinal is reduced to trans retinol by the enzyme retinene reductase and NADH. The trans-retinol is inactive in the synthesis of rhodopsin and hence it passes into the blood, where it isomerizes into cis-retinol. In the dark or dim light the active cis-retinol enters the retina from the blood where it is oxidized to cis-retinal by the reverse action of retinene reductase and NAD. Now the cis-retinal combines with the protein opsin to give back rhodopsin and thus the cycle is repeated thereby helping in the normal vision of the eye.



Rhodopsin cycle

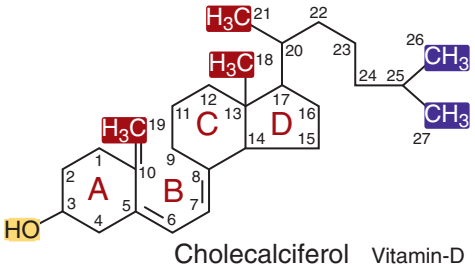
Deficiency diseases:

1. Nyctalopia—night blindness
2. Xeropthalmia—complete blindness
3. Colour blindness
4. Keratomalacia—Dryness of eye, skin and keratinization of respiratory, intestinal, urinary tracts, salivary glands and the genital system.

Hypervitaminosis: Nausea, vomiting and headache are the common symptoms of vitamin A excess.

VITAMIN – D

Chemistry: Vitamin-D is chemically known as calciferol. It is commonly known as antiricketic vitamin. It is a fat-soluble vitamin and exists in two forms—(1) vitamin D_2 (ergocalciferol) and (2) vitamin D_3 (cholecalciferol). Cholecaliferol is the most prominent form of vitamin D in humans. Vitamin D is similar to the classic steroid hormones. It is derived from cholesterol.



Cholecalciferol Vitamin-D

Daily requirement and source: The daily requirement of vitamin D is 100 units. Lactating women need 400 units/day. The rich dietary sources of vitamin D are cod liver oil, fish liver oil, egg yolk, milk and animal liver. Vitamin D_3 can be produced photochemically by the action of sunlight or ultraviolet light from the sterol precursor, 7-dehydrocholesterol which is present in the epidermis or skin. Vitamin D_3 can be endogenously produced and as long as an individual has access on a regular basis to sunlight there is no dietary requirement.

Absorption: The dietary vitamin D is absorbed from the upper part of small intestine and requires bile salts. Some of it is also absorbed along with other dietary fats through the chylomicrons. It is also absorbed by the skin if cod liver oil is massaged over the skin. Vitamin D formed by the ultraviolet radiation is also absorbed through the skin.

Metabolism: Cholesterol is dehydrated at the 7^{th} position to 7-dehydrocholesterol. In the skin, photoconversion of 7-dehydrocholesterol to cholecalciferol (vitamin D_3) takes place. Cholecalciferol is metabolised with the help of the enzyme D_3-25-hydroxylase in the liver to 25-hydroxycholecalciferol ($25(OH)D_3$), which is the major form of vitamin D circulating in the blood compartment. This is further metabolized in the kidney by the enzyme $25(OH)D_3$-1-hydroxylase (mediated by parathormone) to 1,25-Dihydroxycholecalciferol, this is the active form of vitamin D. Plasma vitamin D binding protein (DBP) carries vitamin D_3 and all of its metabolites to their various target organs through the blood. The production of $1,25(OH)_2D_3$ is modulated according to the calcium and other endocrine needs of the body. The chief regulatory factors are $1,25(OH)_2D_3$ itself, parathyroid hormone (PTH), and the serum concentrations of calcium and phosphate. Probably the most important determinant of the 1-hydroxylase is the vitamin D status of the individual. When circulating concentrations of $1,25(OH)_2D_3$ are low, production of $1,25(OH)_2D_3$ by the kidney is high, and when circulating concentrations of $1,25(OH)_2D_3$ are high, the output of $1,25(OH)_2D_3$ by the kidney is sharply reduced.

Biochemical functions: Vitamin D has three different sites of action i.e. intestine, bones and kidneys. The primary biochemical action of vitamin D is to regulate blood calcium. This is brought about by the following mechanisms—

1. Vitamin D increases the absorption of calcium and phosphorus from the intestine by decreasing the pH.
2. It increases the biosynthesis of calcium binding protein in the intestinal mucosal cells that helps in the transport of calcium through the intestine.
3. It reduces the excretion of calcium and phosphorus from the kidneys with the help of parathyroid hormone, thereby increasing the blood calcium levels.
4. When the serum calcium reduces, it promotes the mobilization of calcium from bones and releases it into the blood.
5. It aids in mineralization of bones (collagen).
6. It increases the citrate level of blood, bone, kidney and heart tissues and also excretion of citric acid.
7. It stimulates the activity of phytase which catalyzes the hydrolysis of phytic acid in the intestine.

Deficiency diseases: Human clinical disorders related to vitamin D can be considered as those arising because of—

- Altered availability of vitamin D
- Altered conversion of vitamin D_3 to $25(OH)D_3$ and then to $1,25(OH)_2D_3$.
- Variations in end organ responsiveness to $1,25(OH)_2D_3$ or possibly $25(OH)_2D_3$.

There are two major clinical disorders related to vitamin D deficiency, they are—

(1) **Rickets:** Bowed legs.

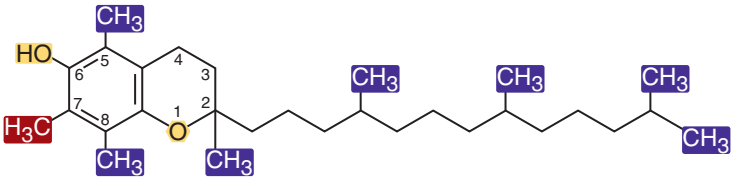
(2) **Osteomalacia:** Soft bones with tendency to break easily.

Hypervitaminosis: Nausea, vomition and headache are the common symptoms of vitamin D excess.

VITAMIN–E

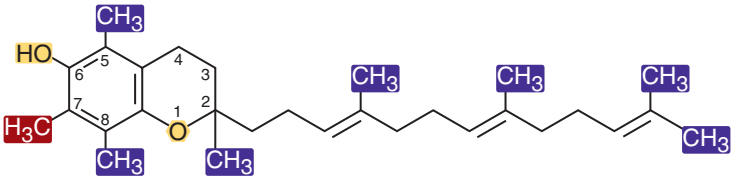
Introduction: Vitamin E is the anti-sterility factor. It is necessary for fertility of male and the birth process in the female, therefore it is called tocopherol (Greek word *Tokos* = child birth, *Pheros* = to bear and ol = alcohol).

Chemistry: Vitamin E is chemically known as α-tocopherol. It is an oily substance, heat stable, readily oxidized and acts as powerful antioxidant. Owing to its antioxidant property it protects other vitamins like vitamin A from oxidation. There are many derivatives of this vitamin viz. α, β, γ and δ due to the presence of different substituents on the aromatic ring at positions 5, 6, 7, and 8. α-tocopherol is 5,7,8-trimethyl derivative and has the highest vitamin activity. Vitamin E has a characteristic double ring structure called the chomane ring.



Vitamin E (α-Tocoferol)

The tocotrienols share the same ring structure, but have an unsaturated tail.



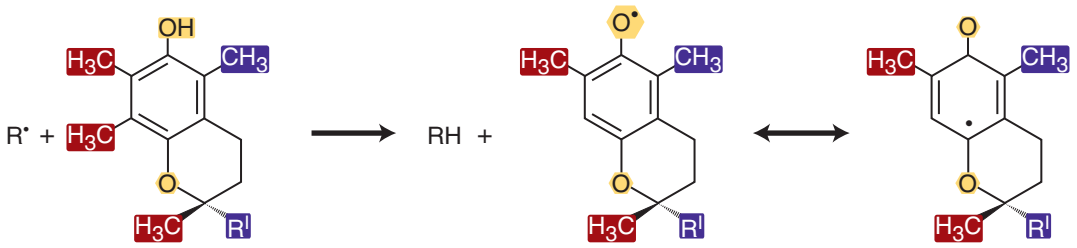
Tocotrienol structure

Position of methyl groups on aromatic ring	Tocopherol structure	Tocotrienol structure
5, 7, 8	alpha-Tocopherol	alpha-Tocotrienol
5, 8	beta-Tocopherol	beta-Tocotrienol
7, 8	tau-Tocopherol	tau-Tocotrienol
8	delta-Tocopherol	delta-Tocotrienol

Absorption and storage: Vitamin E is readily absorbed along with fat from the GI tract. It is metabolized to unidentified substances. It is absorbed to a great extent from salt solution. Bile is essential for vitamin E absorption, since bile contains bile salts which emulsify the fat by lowering the surface tension and theory favouring its absorption. It is stored in the liver, muscle and body fat stores.

Sources: Meat, liver, fish, chicken, vegetable oils, particularly wheat germ oil, corn oil, cotton seed oil and safflower oil are rich sources. Others are green leafy vegetables like spinach and lettuce and egg yolk.

Biochemical role: It has antioxidant activity. Tocopherols (vitamin E) can interrupt free radical chain reactions by capturing the free radical. This imparts to them their antioxidant properties. The free hydroxyl group on the aromatic ring is responsible for the antioxidant properties. The hydrogen from this group is donated to the free radical, resulting in a relatively stable free radical form of the vitamin.



Resonance-stabilized radical

Polyunsaturated fatty acids (constituent of cell membranes) are easily attacked by molecular oxygen resulting in the formation of peroxides. The tocopherols prevent this. Vitamin E and other antioxidants such as vitamin C, selenium, sulphur containing amino acids (cystine and methionine), ubiquinone, vitamin A and carotenes prevent lung tissue damage from atmospheric ozone and nitrogen dioxide.

Tocopherols prevent oxidation of vitamin A. It prevents enzymes in muscles, nerves and gonads from destruction. It also prevents the development of cerebral disorder. It is involved in heme synthesis. Thus the physiological role of vitamin E can be summarized as under—

1. It prevents peroxidation of polyunsaturated fatty acids in tissues and membranes.
2. It prevents haemolysis of erythrocytes by oxidizing agents like H_2O_2 and dialuric acid.
3. It prevents the degeneration of cellular and subcellular membranes rich in polyunsaturated fatty acids (PUFA).
4. It prevents poisoning of liver cells. The liver is exposed to carbon-tetrachloride, chloroform and other toxic chemicals.
5. It prevents demyelination of nerve fibres and prevents distortion of the axis of the nerves in the spinal cord.
6. Respiration in the mitochondria is depended upon the availability of vitamin E and the activators present in microsomal supernatant extraction of the cells also need this vitamin.
7. Requirement of vitamin E dependent on the amount of PUFA in diet and selenium status in the body. It spares the activity of selenium present in traces in the body.
8. It prevents hepatic necrosis produced by diet deficient in sulphur containing amino acids.
9. Its action as an antioxidant prevents rancidity.
10. It is important for reproductive physiology. In male rats that are deprived of vitamin E, the seminiferous epithelium undergoes irreversible degeneration and permanent sterility occurs. In females the deficiency of vitamin E does not affect the ovary. Ovulation, conception and implantation take place normally, but foetus dies in the uterus a few weeks after conception and undergoes resorption.

Deficiency diseases:

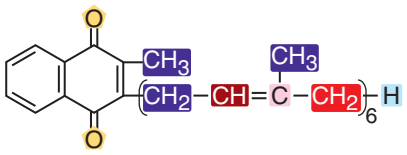
1. It causes discolouration of the enamel of the teeth due to oxidation of unsaturated fatty acids present in these structures to peroxide.
2. Anaemia is caused in monkeys due to lack of hemophysins in bone marrow, rather than by destruction of RBC.
3. Increased fragility of RBC.
4. Thrombocytosis and edema.
5. Permanent sterility in males and death of foetus in uterus after few weeks of implantation in females.
6. Necrosis of hepatic cells.

Daily requirement: Adults-10 mg/day. If PUFA in the diet is 1 gm/day then the requirement of vitamin E is as high as 35 gms/day. Pregnant or lactating women require greater amount of vitamin E.

Hypervitaminosis: Leads to nausea.

VITAMIN–K

It is chemically known as menaquinone (i.e. K_3) whereas K_1 and K_2 are napthaquinones (present in plants). Menaquinone is an anti-hemorrahagic vitamin i.e. it prevents hemorrahages by activating the process of blood clotting. Menadione is a synthetic analogue of vitamin K.



Vitamin K (Menaquinone)

Sources: Green leafy vegetables like cabbage, spinach etc. predominantly contain vitamin K_1 and are the best sources. Cauliflower, soyabean, wheatgerm etc. have proved to be good sources of this vitamin. Tops of carrots contain considerable amounts. Animal products contain very little although milk and eggs contain small amounts. Vitamin K_2 is produced by most bacteria present in the human intestine if it is not supplied in the diet.

Biochemical role: Vitamin K is necessary for proper formation of prothrombin (the blood plasma protein), the inactive precursor of thrombin which is an enzyme that converts the protein fibrinogen (of blood plasma) into fibrin, the insoluble fibrous protein that holds blood clots together.

1. Vitamin K increases the activity of many clotting factors.
2. It initiates the biosynthesis of the enzyme proconvertin of the liver cells which catalyzes the formation of prothrombin (precursor of thrombin protein).
3. It takes part in election transport chain.
4. It acts as a coenzyme for carboxylation of glutamate to γ-carboxyglutamate.

Deficiency conditions: Increased clotting time and decreased blood prothrombin levels are seen in vitamin K deficiency. There is continuous bleeding specially during delivery of the fetus.

Daily requirement: The average diet contains adequate amount of the vitamin being synthesized by the bacteria present in the intestine. Hence deficiency symptoms are not seen in healthy individuals except in new born infant fed on mother's milk when the mother's diet has low vitamin K content.

Hypervitaminosis: Very large doses of vitamin K are toxic.

10

BIOLOGICAL OXIDATION

This chapter gives the answer to the question as to **"How do the food we take in and the oxygen we respire, produce energy to continue the process of life"**. The simplest answer is that the food we take in is oxidized by the enzymes present in the body. During this process some reducing equivalents viz. NADH and $FADH_2$ are produced which are electron rich in nature. These reducing equivalents donate their electrons to the oxygen we respire in, during which energy is released to produce adenosine triphosphate (ATP). ATP is known as the energy currency of the cell and it brings about the biological process of life.

In order to understand the above process, we need to understand some basic terminologies. Oxidation and reduction can be defined in three different ways as under—

- **Oxidation** is 'Addition of oxygen' or 'removal of hydrogen' or 'removal of electrons'.
- **Reduction** is 'Removal of oxygen' or 'addition of hydrogen' or 'addition of electrons'.

Oxidizing agent or oxidant: An electron acceptor is an oxidizing agent or oxidant.

Reducing agent or reductant: An electron donor is a reducing agent or reductant.

Redox potential (E_0): The relative tendency of reductant to donate electrons as compared to hydrogen is termed as oxidation-reduction potential or **'redox potential'** (E_0).

The redox potential of hydrogen is taken as zero at pH 0 (-0.417), 25° C, in a solution of 1 molar concentration (1.0 atom of hydrogen).

- A compound having a negative value of E_0 is a better electron donor than hydrogen.
- A compound having a positive value of E_0 is a poor electron donor than hydrogen.

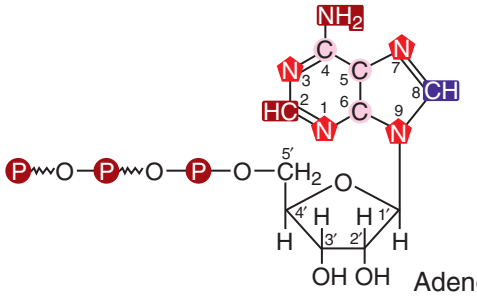
The electrons flow from compounds with negative value of redox potential to those with positive values of redox potential, because there will be loss of energy and thus, the compound becomes stable.

Free energy (F_0): Every chemical substance has a certain amount of energy built into it, which is the energy of the chemical bonds holding the atoms together. This is the free energy.

High energy compounds and energy rich bond (~): Any bond, which on hydrolysis gives a minimum free energy of 7.4 Kcal/mol, is known as energy rich bond and the compound which has an energy rich bond is known as high energy compound. Ex. ATP, pyrophosphate, 1,3-diphosphoglyceric acid, phosphoenol pyruvate, creatine phosphate and acetyl-CoA.

Adenosine triphosphate (ATP): ATP is also known as the **'energy currency'** of the living cell, because it transfers energy from energy yielding sources to the energy requiring cell processes. ATP has

two pyrophosphate bonds. On hydrolysis of each of the terminal two phosphate groups there is release of more than 7.4 Kcal/mol of energy but the third bond yields only 3 Kcal/mol of energy, hence it is not a high energy bond. On hydrolysis ATP is converted to ADP and to AMP.



Adenosine Triphosphate

Biological oxidation: Biological oxidation is catalyzed by enzymes which function in combination with coenzymes and / or electron carrier proteins. Different enzymes associated with biological oxidation are—

1. **Oxido-reductases:** These enzymes catalyze the removal of hydrogen from the substrate and add it to another substance, thus bringing about oxidation reduction reaction. Ex. Glyceraldehyde–3–phosphate dehydrogenase.
2. **Oxidases:** These enzymes catalyze the removal of hydrogen from the substrate and add directly to the molecular oxygen. Ex. Cytochrome oxidases, tyrosinase, uricase.
3. **Oxygenases:** These enzymes incorporate oxygen into the substrates.
   (a) ***Mono-oxygenases*:** Adds only one atom of oxygen to the substrate. These are also known as mixed function oxidases.
   (b) ***Di-oxygenases*:** Adds both the atoms of oxygen to the substrate. Ex. Homogentisic acid dioxygenase.
4. **Aerobic dehydrogenases:** These enzymes remove hydrogen from the substrate and add it either directly to oxygen or any other artificial acceptors like methylene blue. The product formed is hydrogen peroxide.
5. **Anaerobic dehydrogenases:** These enzymes use other substrates or substances to donate the hydrogen. They transfer hydrogens to some other hydrogen acceptor, but not directly to oxygen. Thus the hydrogen acceptors are NAD, FAD and FMN. Heme proteins like cytochromes also receive hydrogens. The cytochromes are **'b'**, **'c_1'**, **'c'**, **'a'** and **'a_3'**.
6. **Hydroperoxidases:** These enzymes have either hydrogen peroxide (H_2O_2) or organic peroxide as their substrate. There are two types of hydroperoxidases—(1) Peroxidase and (2) Catalase. Their prime function is destruction of H_2O_2.

ELECTRON TRANSPORT CHAIN

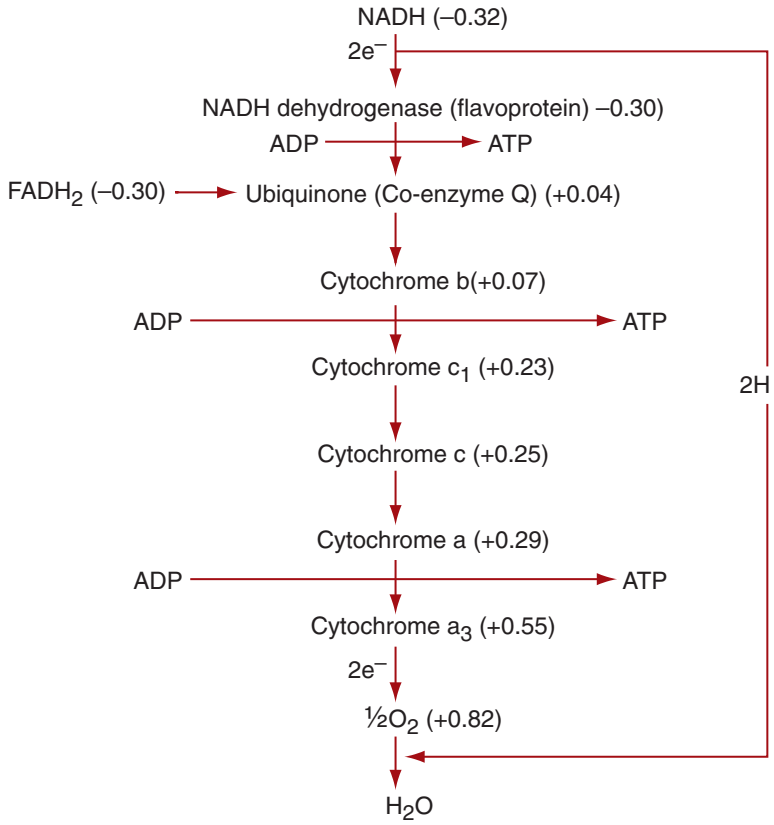
When electrons are transferred from the most electronegative system [(NADH or $FADH_2$) (–0.32V)] to the most electropositive system (+0.82V) (Oxygen), there will be liberation of all the energy at one time in an explosive manner. But, if they are transferred in a step wise manner through some intermediate systems then there will be slow release of energy and it can be captured by the cell to synthesize energy rich compounds. During biological oxidation, electrons are transferred through electron transport proteins which are arranged in a specific chain to form the electron transport chain (ETC), which is situated in the inner mitochondrial membrane.

Respiratory chain or ETC: Transfer of electrons from substrate to molecular oxygen through a chain of electron carriers is called electron transport chain or respiratory chain.

Mitochondria contains a series of catalysts forming the respiratory chain which are involved in the transfer of electrons and hydrogen and their final reaction is with oxygen to form water. The components of respiratory chain are arranged sequentially in the order of increasing redox potential. Electrons flow through the chain in a stepwise manner from lower redox potential to higher redox potential. Some amount of energy is liberated with transfer of electron from one component to another. Whenever there is a release of 7.4 Kcal of energy or a little more, then ATP formation takes place there. NADH forms 3 ATPs whereas FADH2 forms only 2 as it enters ETC at the site beyond the first site of ATP formation. The three sites of ATP formation in the ETC or respiratory chain are—

1. Between NADH dehydrogenase (flavoprotein) and ubiquinone (coenzyme Q).
2. Between cytochrome-b and cytochrome-c_1.
3. Between cytochrome-a and cytochrome-a_3 (cytochromes oxidase).

The components of ETC, their redox potential and their sequence is—



Phosphorylation: Esterification of a phosphate through a high energy bond (7.4 Kcal) is known as phosphorylation. Combination of inorganic phosphate (Pi) with any other compound through high energy bond is known as phosphorylation. Or formation of ATP from ADP and phosphate or NTP from NDP and P_i is known as phosphorylation. There are two types of phosphorylation—

1. **Substrate level phosphorylation:** Formation of high energy phosphate bond at the level of a substrate without the involvement of the respiratory chain is known as substrate level phosphorylation. Ex. Phosphoenolpyruvate is converted to pyruvate by pyruvate kinase where ATP is formed from ADP.
2. **Oxidative phosphorylation:** The enzymatic phosphorylation of ADP to ATP coupled with electron transport from a substrate to molecular oxygen is known as oxidative phosphorylation or respiratory chain phosphorylation.

Mechanism of oxidative phosphorylation: There are three theories or hypothesis, explaining the formation of ATP through electron transport chain. They are as follows—

1. **Chemical coupling hypothesis:** It states that a high energy compound is formed taking the energy liberated by electron transfer and this compound inturn phosphorylates ADP to ATP.
2. **Conformational coupling hypothesis:** There are many proteins in the wall of inner mitochondrial membrane; one of them is F_0F_1ATPase, which is responsible for the ATP production. According to this hypothesis the energy liberated from ETC brings a conformational change in the proteins of the membrane and is then transferred to F_oF_1ATPase which thereby also gets a conformation change and hence becomes unstable. In order to attain stability it provides energy for ATP synthesis.
3. **Chemi-osmotic hypothesis:** It states that electron transport pumps H^+ from the mitochondrial matrix across the inner mitochondrial membrane to the outer aqueous phase, thereby the matrix becomes basic and the outer phase becomes acidic. Due to this osmotic difference (i.e. more acidic outside and more basic inside the mitochondrial matrix) the H^+ influx (diffuse) into the matrix through a pore in the F_oF_1 ATPase which provides the energy for the ATP synthesis.

P/O ratio: The number of inorganic phosphates esterified per atom of oxygen consumed is known as P/O ratio. For NADH it is 3 and $FADH_2$ it is 2.

Formation and detoxification of H_2O_2: During ETC, O_2 accepts four electrons forming two H_2O. If by chance O_2 accepts only two electrons, the product formed is H_2O_2 and if it accepts only one electron then superoxide radical ($:O_2^-$) is formed. Both these damage the membrane structure by attacking the unsaturated fatty acids of the membranes.

Superoxide is detoxified as—

$$2O_2^- + 2H^+ \xrightarrow{\text{SUPEROXIDE DISMUTASE}} H_2O_2 + O_2$$

H_2O_2 is detoxified as—

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

Cytochrome-a_3: Cytochrome-a_3 is also known as cytochrome oxidase. It has two molecules of heme with long hydrocarbon side chains. To the other end of the heme, two copper atoms are attached which can directly react with oxygen to donate four electrons.

Inhibitors of ETC: Inhibitors of ETC are those which inhibit or stop the flow of electrons in the electron transport chain. Some of the inhibitors of ETC are—

(a) At the first site of ATP formation, **rotenone** and **barbital** inhibit the flow of electrons
(b) At the second site **antimycin-A** and **amytal** inhibits the flow of electrons.
(c) At the third site **cyanide** (Cn^-), **carbon monoxide** (CO) and **H_2S** gas inhibit.

Uncouplers of oxidative phosphorylation: Uncouplers are those substances which prevent oxidative phosphorylation (formation of ATP) though ETC is normally operating.

Due to the effect of uncouplers there is a continuous flow of electrons but there is no formation of ATP i.e. ETC is not coupled to the ATP formation, so the energy is dissipated as heat. Some of the uncouplers are—

1. **2, 4-Dinitrophenol (DNP):** It transfers protons across the mitochondrial membrane thereby diverting its flow from F_oF_1ATPase.
2. **Valinomycin:** It transfers K^+ ions, disturbing the osmotic pressure.
3. **Gramicidin:** It transfers Na^+ ions, across the membrane.

All the above three are known as **'ionophores'** i.e. those which disrupt the membrane permeability to ions, thereby uncoupling phosphorylation with ETC.

4. **Oligomycin:** It inhibits F_oF_1ATPase.
5. **Atractyloside:** It inhibits adenine nucleotide transport protein of the mitochondrial membrane which transport ATP in exchange of ADP.

Some of the mechanisms/applications of uncouplers are:

1. The mechanism by which body heat is increased during fever is by uncoupling.
2. Increase in the heat of the penis during erection is due to uncoupling.
3. Reduction in fat (weight) of obese persons is by the mechanism of uncoupling (banned).
4. Newly born infants have special type of mitochondria called brown fat mitochondria which are highly porous containing more cytochromes. They help in release of more heat by uncoupling, thus helping in maintaining the body temperature in the infants as they do not have sub-cutaneous fat resulting in loss of more heat.

Chemical warfare: It involves using the toxic properties of chemical substances to kill, injure or incapacitate an enemy.

The offensive use of living organisms (such as anthrax) is considered to be biological warfare rather than chemical warfare; the use of nonliving toxic products produced by living organisms, ex. toxins such as botulinum toxin, ricin, or saxitoxin is considered as chemical warfare. Chemical used in warfare is called a **'chemical warfare agent (CWA)'**. About 70 different chemicals have been used or stockpiled as chemical warfare agents during the 20th and 21st century. These agents may be in liquid, gas or solid form. Liquid agents are generally designed to evaporate quickly; such liquids are said to be volatile or have a high vapor pressure. Many chemical agents are made volatile so that they can be dispersed over a large region quickly. Chemical warfare agents are divided into lethal and incapacitating categories. A substance is classified as incapacitating if less than 1/100 of the lethal dose causes incapacitation, ex. through nausea or visual problems.

Classes: Chemical warfare agents are organized into several categories according to the manner in which they affect the human body. The names and number of categories vary slightly from source to source, but in general, different types of chemical warfare agents are—

Agent class	Agent name	Mode of action	Signs and symptoms
Nerve	Cyclosarin (GF), Sarin (GB), Soman (GD), Tabun (GA), Some insecticides, Novichok agents.	Inactivates enzyme acetylcholinesterase, preventing the breakdown of the neurotransmitter acetylcholine in the victim's synapses and causing both muscarinic and nicotinic effects.	Miosis (pinpoint pupils), Blurred/dim vision, Headache, Nausea, vomiting, diarrhea, copious secretions/sweating, Muscle twitching/fasciculations, Dyspnea, Seizures, unconscious.
Asphyxiant/ blood	Most Arsines, Cyanogen chloride, Hydrogen cyanide.	Arsine: Causes intravascular hemolysis that may lead to renal failure. Cyanogen chloride/hydrogen cyanide: cyanide directly prevents cells from utilizing oxygen. Cells use anaerobic respiration, creating excess lactic acid and metabolic acidosis.	Possible cherry-red skin, possible cyanosis, Confusion,Nausea, patients may gasp for air, Seizures prior to death and Metabolic acidosis.

Contd. to next page

Contd. from previous page

Vesicant / blister	Sulfur mustard (HD, H), Nitrogen mustard (HN-1, 2, 3) Lewisite (L) Phosgene, oxime.	Agents are acid-forming compounds that damage skin and respiratory system, resulting in burns and respiratory problems.	Severe skin, eye, mucosal pain and irritation, skin erythema with fluid blisters that heal slowly and may become infected. Tearing conjunctivitis, corneal damage. Respiratory distress and damage.
Choking / pulmonary	Chlorine, Hydrogen chloride, Nitrogen oxides, Phosgene.	Similar mechanism to blister agents in that the compounds are acids or acid-forming, but action is more pronounced in respiratory system, flooding it and resulting in suffocation; survivors often suffer chronic breathing problems.	Airway irritation, eye and skin irritation, Dyspnea, cough, sore throat, Chest tightness, Wheezing and Bronchospasm.
Lachrimatory agent	Tear gas, Pepper spray.	Causes severe stinging of the eyes and temporary blindness.	Powerful eye irritation.
Cytotoxic proteins	Non-living biological proteins such as: Ricin Abrin	Inhibit protein synthesis	Latent period of 4-8 hours, followed by flu-like signs and symptoms. Progress within 18-24 hours of inhalation: nausea, cough, dyspnea, pulmonary edema. Ingestion: gastrointestinal hemorrhage with emesis and bloody diarrhea; eventual liver and kidney failure.

11

CARBOHYDRATE METABOLISM

DIGESTION AND ABSORPTION

The dietary carbohydrates comprise of the polysaccharides viz. starch and glycogen, the disaccharides–lactose, maltose, sucrose and the monosaccharides like glucose, fructose etc. The complex poly and disaccharides are converted into simple monosaccharides which are absorbed by the body.

Digestion in the mouth: Saliva of the mouth contains salivary amylase (ptylin), whose optimum pH is 6.9 and requires Ca^{++} and Cl^{-} as activators. Salivary amylase acts on cooked starch and releases maltose. The digestion of starch in the mouth is not complete as the food stays here for a short duration of time. However salivary amylase continues its action in the stomach but the production of HCl and activation of pepsinogen to pepsin hydrolyses and deactivates amylase. There is no digestion of carbohydrates in the stomach as the enzymes for carbohydrate digestion are absent in the gastric secretions.

Digestion in the intestine: The pancreatic juice secreted in the intestine contains pancreatic amylase (diastase or amylopsin). It has an optimum pH of 7.1 and is activated by Cl^{-} ions. The pancreatic amylase acts both on cooked and uncooked starch and also on glycogen and converts it into erythro-dextrin, achro-dextrin and maltose. The pancreatic amylase acts only on α-1→ 4, glycosidic linkages, it cannot act on the α-1→ 6, glycosidic linkages, which are present at the branching points of starch and glycogen. These undigested branching points are known as isomaltose which are digested by an enzyme called isomaltase or α-dextrinase.

The disaccharides are hydrolyzed by respective disaccharidases, which are secreted by the intestinal mucosa. The disaccharide maltose is digested by the enzyme maltase to yield two glucose units. Lactase splits the disaccharide lactose into glucose and galactose. Sucrose is hydrolyzed by sucrase or invertase into glucose and fructose.

Cellulose cannot be digested by human beings as the enzyme cellulase (β-glycosidase) is absent, yet cellulose is specifically included in the diet so as to increase the bulk (fiber or roughage) of the food and thus help in the mobility of the food through the gastrointestinal tract.

The end products of carbohydrate digestion are glucose, fructose, galactose, mannose, ribose etc. some of the sugars are converted into glucose before absorption.

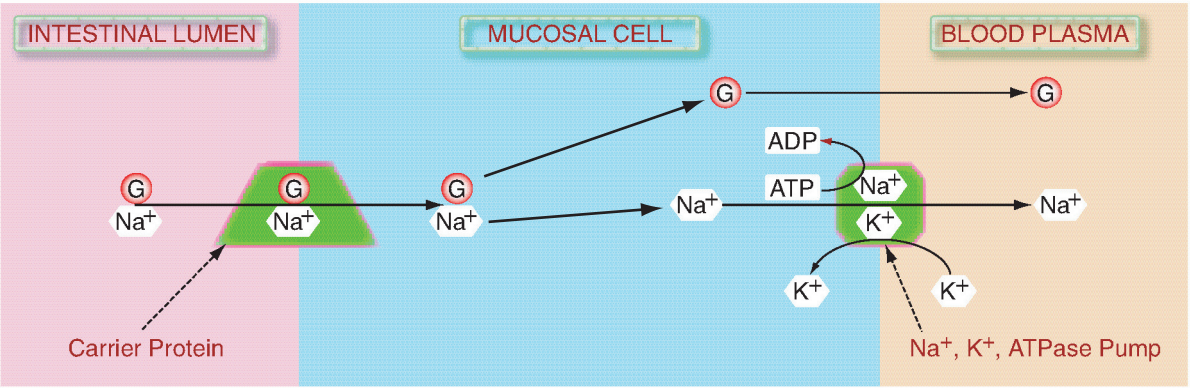
Absorption of carbohydrates: The monosaccharides are absorbed in the small intestine by three mechanisms—(1) Simple diffusion (2) Active transport and (3) Facilitated transport.

1. **Simple diffusion:** As the digestion proceeds, the concentration of glucose in the intestinal lumen increases more than the blood glucose level. This results in an **osmotic difference** between the

two, due to which glucose simply diffuses down hill from a region of higher concentration of glucose (lumen) to the region of lower concentration of glucose (blood).

2. **Active transport:** Simple diffusion continues till the concentration of glucose in the lumen equals to that of the blood, then glucose is transported by an active transport. Here glucose binds to a carrier protein, situated in the outer membrane of the intestinal wall. This carrier protein also binds two Na^+ ions. When both glucose and Na^+ are bound to the carrier protein it moves into the cell and releases glucose and Na^+ into the cytoplasm of the cell, from where glucose simply diffuses into the blood. To continue the active process Na^+ ions must be expelled out of the cell, so as to maintain a low concentration of Na^+ inside the cell, when compared to the lumen. Hence Na^+ is expelled out of the cell into the blood plasma, in exchange of K^+ through a Na^+, K^+, ATPase pump, which hydrolyses ATP for exchanging Na^+ with K^+. As this overall process requires energy it is known as active transport. So, during active transport glucose, moves against concentration gradient.

Galactose is also absorbed from the intestine in a similar manner as that of glucose active transport.



Mechanism of active transport

3. **Facilitated transport:** Fructose is transported by a carrier protein which does not require energy (ATP). Hence it is known as facilitated transport. This process is very slow.

Lactose intolerance: It is a genetic defect in which the enzyme lactase is deficient or absent. This leads to non-digestion of lactose and hence results in intolerance to lactose, thereby to milk. This disease is most common in the Asian population. Infants with inborn defect in the enzyme lactase are intolerant to milk and show symptoms like diarrhea and vomiting. Such infants are fed with artificial milk powder that is lactose free and contains some other added sweetener. Asian adults develop deficiency in the production of lactase in the later stages of life and thus feel aversion to intake of milk.

Diagnosis of lactase deficiency: Three tests are available for the diagnosis.

Hydrogen breath test: In a hydrogen breath test, after an overnight fast, 50 grams of lactose (in a solution with water) are swallowed. If the lactose is not digested, enteric bacteria metabolize it and produce hydrogen. This can be detected in the patient's breath by a clinical gas chromatograph or a compact solid state detector.

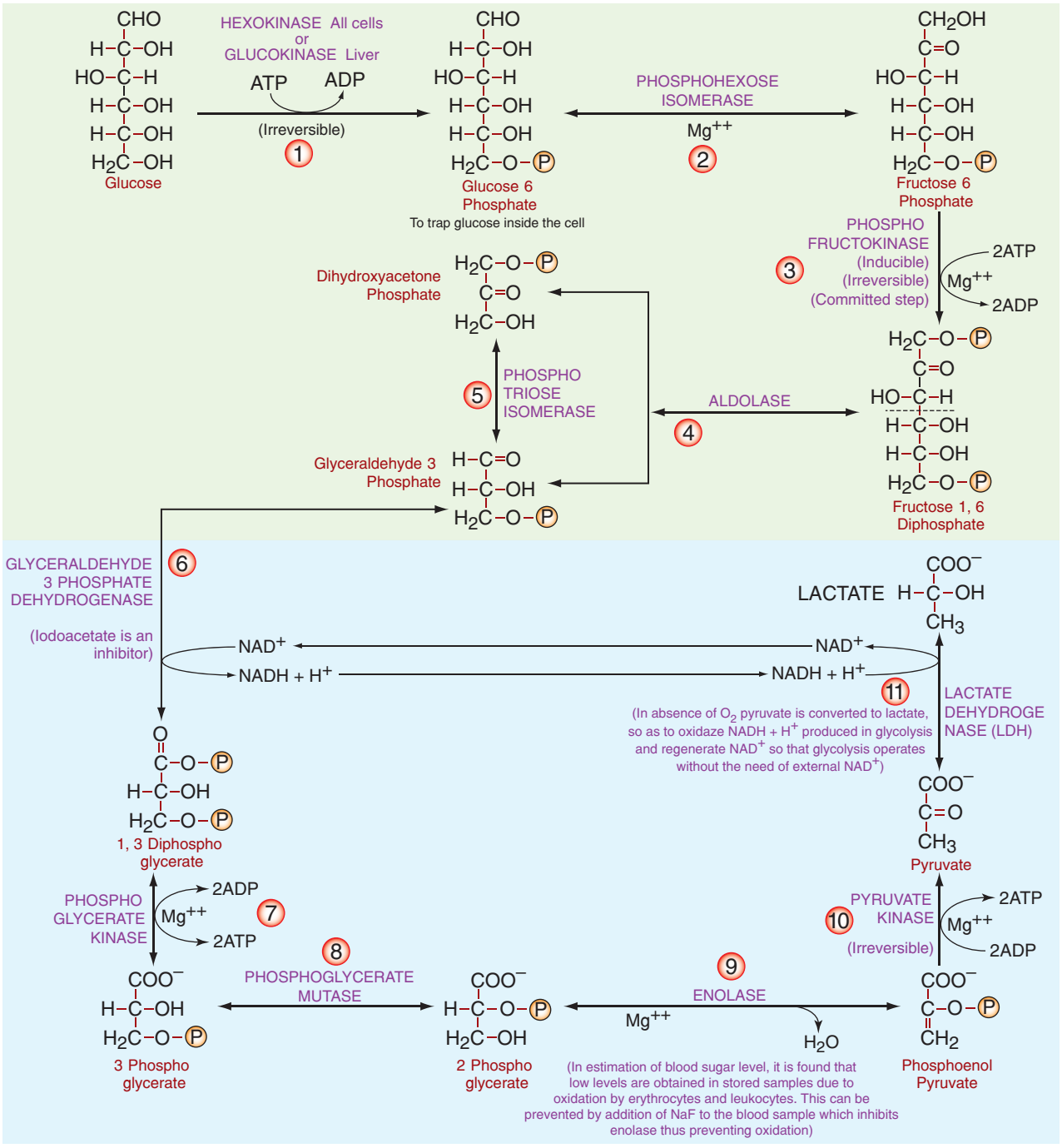
Intestinal biopsy: An intestinal biopsy can confirm lactose intolerance following discovery of elevated hydrogen in the hydrogen breath test. This test needs a highly specialized laboratory and expertise to measure lactase enzymes or mRNA in the biposy tissue, hence is not taken up routinely.

Stool acidity: Is used to diagnose lactose intolerance in small infants, for whom other forms of testing are risky or impractical.

Metabolism of carbohydrates in the cell: Metabolism is a complex process of breakdown and synthesis of the biomolecules inside the cell. Breakdown of molecules is known as catabolism and synthesis is termed as anabolism. The catabolic processes of carbohydrates include - (1) Glycolysis (2) Citric acid cycle (3) Glycogenolysis (4) Hexose monophosphate pathway and (5) Uronic acid pathway. The anabolic processes of carbohydrates include (1) Glycogenesis and (2) Gluconeogenesis.

GLYCOLYSIS

Glycolysis is the breakdown (lysis) of glucose to pyruvic acid under aerobic conditions and to lactic acid under anaerobic conditions.



Mechanism of glycolysis

Anaerobic glycolysis is also termed as Embden-Meyerhof pathway (EMP), after the scientists who proposed it. Glycolysis occurs in the cytosol of the cell and is initiated when the ATP level of the cell is low. It can be divided into two stages viz. (1) Preparatory phase and (2) Energy yielding phase.

In stage one, one molecule of glucose in converted into two molecules of D-glyceraldehyde-3-phosphate. Glucose is either cleaved from the glycogen molecule or enters the cell individually and is phosphorylated to glucose-6-phosphate by converting ATP to ADP with the help of the enzyme hexokinase/glucokinase. The phosphorylation of glucose serves two purposes. First, it makes the glucose molecule more reactive and ready for other reactions. Second, because phosphorylated compounds cannot pass through the cell membrane, phosphorylation keeps the glucose inside the cell. The six carbons in glucose-6-phosphate structure need to be rearranged to form fructose-6-phosphate so that it can split into two structures of 3 carbons each. The new compound, fructose-6-phosphate is phosphorylated again so that each of the 2, three carbon units have a phosphate group attached to them. The conversion of fructose-6-phosphate to fructose-1,6-disphosphate via phosphofructokinase is the primary regulation point of glycolysis. The final step of stage one is the splitting of fructose-1,6-disphosphate into 2 molecules of glyceraldehyde-3-phosphate.

Stage 2 of glycolysis is designed to liberate inorganic phosphate for the synthesis of ATP and to convert the glyceraldehydes into pyruvate. Glyceraldehyde is oxidized, in other words a hydrogen atom is removed from it, and phosphorylated to produce 1,3-diphosphoglycerate. The NADH carries the hydrogen to the electron transfer system for the production of 3 ATPs. In the next four reactions, four additional ATPs are synthesized (two each from both the three carbon compounds), before the final product of glycolysis i.e. pyruvate is formed. The three-carbon structure of pyruvate has several fates depending upon the energy state of the cell.

Calculation of ATPs produced per glucose molecule in glycolysis

Enzyme involved	Equivalent produced	No. of equivalents	Fate of the equivalent	ATPs/ equivalent	Total ATPs	Step in glycolysis
Glyceraldehyde-3-P-Dehydrogenase	NADH	2	Oxidized through ETC	3	6	6th step
Phosphoglycerate Kinase	ATP	2	—	2	2	7th step
Pyruvate Kinase	ATP	2	—	2	2	10th step
				Total ATP produced	**10 ATPs**	

ATPs utilized in glycolysis

Hexokinase	ATP	1	—	—	1	1st step
Phosphofructokinase	ATP	1	—	—	1	3rd step
				Total utilized	**2 ATPs**	

Net gain

ATPs produced	10	
ATPs utilized	2	
	Net gain	**8 ATPs**

Totally 8 ATPS are produced in aerobic glycolysis

In anaerobic glycolysis, NADH + H^+ is not oxidized through the electron transport chain, instead is oxidized by lactate dehydrogenase, hence no production of 6 ATPs i.e., ATPs are produced less in number.

ATPs produced in anaerobic glycolysis = 4 (7^{th} & 10^{th} step)
ATPs utilized in anaerobic glycolysis = 2 (1^{st} & 3^{rd} step)
Net gain of ATPs = 2 ATPs

Therefore, only two (2) ATPs are produced in anaerobic glycolysis of glucose.

Overall reaction stoichiometry/chemical summary of reaction

- **Overall reaction**

  Glucose + 2ATP + $2P_i$ + 2ADP + $2NAD^+ \rightarrow$ 2Pyruvate + 2NADH + $2H^+$ + 4ATP + $2H_2O$

- **Net reaction**

  Glucose + 2 P_i + 2ADP + $2NAD^+ \rightarrow$ 2Pyruvate + 2NADH + $2H^+$ + 2ATP + $2H_2O$

Under anaerobic conditions

- ✓ Glucose + 2 Pi + 2ADP $\rightarrow$ 2 Lactate + 2 ATP + $2H_2O$
  (Regenerates NAD^+ allowing reaction to continue in the absence of oxygen)

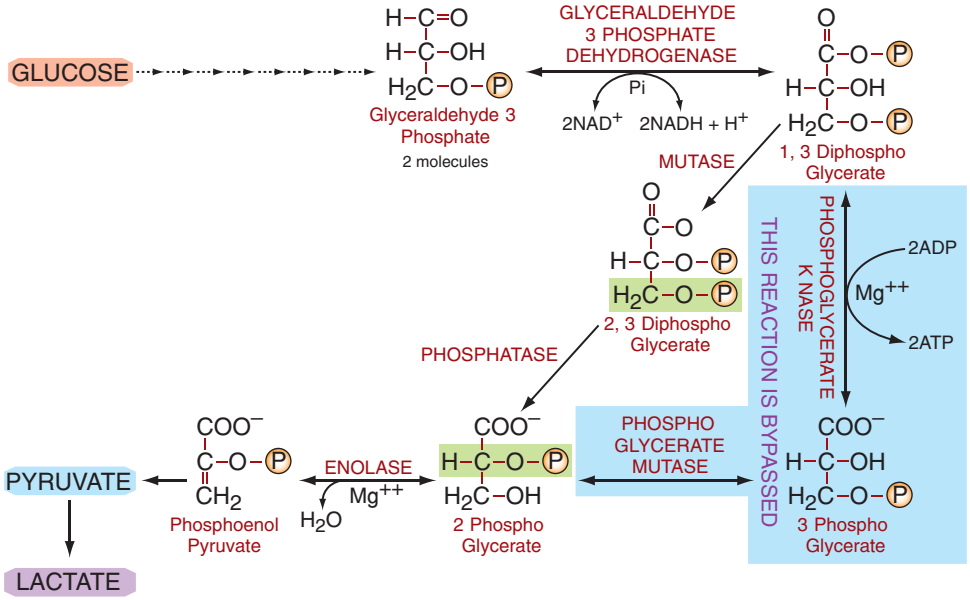
Anaerobic glycolysis generating lactate vs complete oxidation of glucose

Anaerobic glycolysis	Aerobic complete oxidation
Glucose $\rightarrow$ 2 Lactate + $2H^+$	Glucose $\rightarrow$ $6CO_2$ + $6H_2O$
$G^{o\prime}$ = –200 kJ/mol	$G^{o\prime}$ = –2870 kJ/mol
Incomplete oxidation = 93% of energy remains in lactate	Complete oxidation i.e., most of the free energy is released
Net yield of 2 ATP	Net yield of up to 38 ATP

Salient features of glycolysis: It is the main route for glucose metabolism. It occurs in all the cells of the body. Brain and RBC depend only on glucose for oxidation and production of energy. In brain aerobic glycolysis occurs whereas in RBC there is always anaerobic glycolysis (due to the absence of mitochondria), leading to the production of lactic acid. In skeletal muscle aerobic glycolysis occurs in normal conditions but during vigorous muscular contraction, anaerobic glycolysis is the major pathway for energy production. Although glycolysis can occur either aerobically or anaerobically, humans use aerobic glycolysis for about 90% of the time. Glycolysis can be initiated via glucose entering the cell from the blood or glucose arising from the breakdown of glycogen. In human muscle, glycolysis is almost always initiated from the breakdown of glycogen. Since the human brain does not store glycogen, glycolysis is initiated in this tissue from blood glucose. The initiation of glycolysis is regulated by the ATP concentration in the cytoplasm. When the concentration of ATP is high and ADP is low, glycolysis is inhibited. Specifically, the enzyme phosphofructokinase is inhibited by large ATP/ADP ratio. When the concentration of ATP is low and ADP is high, glycolysis is stimulated.

Glycolysis in RBC–The Rapaport-Luebering cycle: Erythrocytes metabolize excessive amounts of glucose by the glycolytic pathway. This generates much ATP which is not required and cannot be used by erythrocyte. Thus if ATP production by substrate phosphorylation is prevented by taking diversion pathway, it will—(1) reduce the production of ATP and (2) Supply 2,3-diphosphoglycerate required for the haemoglobin function which helps in deloading of oxygen in the tissues.

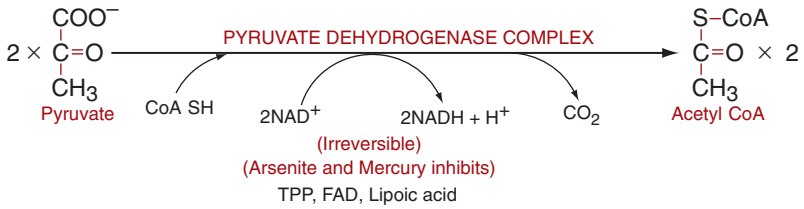
Hence 1,3-diphosphoglycerate formed in normal glycolysis is not converted to 3-phosphoglycerate, instead it takes a bypass route through 2,3-diphosphoglycerate, as under—



FATE OF PYRUVATE

Pyruvate is an important regulatory point for energy production. The ultimate fate of pyruvate depends on the energy state of the cell and the degree of oxidative phosphorylation taking place. When the energy state of the cell is low (high ADP; low ATP), pyruvate enters the TCA cycle as acetyl-CoA via the pyruvate dehydrogenase complex and oxidized completely to CO_2 & H_2O to yield energy. The pyruvate dehydrogenase complex is one of the most complex proteins in the body and consists of more than 60 subunits. When the energy state of the cell is high, the regulator of glycolysis is the enzyme phospho-fructokinase, and thus there is limited pyruvate in the cell. However, if pyruvate is present during the time of high-energy states, such as the liver metabolism of fructose, pyruvate is transformed into acetyl-CoA and is packaged as lipid. If oxygen to the cell is limiting, such as during intensive exercise, glycolysis proceeds anaerobically and pyruvate is converted to lactate by the lactate dehydrogenase enzyme. Finally, pyruvate can be converted into the amino acid alanine via transamination.

Pyruvate dehydrogenase complex is a multi-enzyme complex made up of 3 enzymes viz. (1) Pyruvate Dehydrogenase (2) Dihydrolipoyl Transacetylase and (3) Dihydrolipoyl Dehydrogenase. This reaction requires five coenzymes viz. (i) Thiamine pyrophosphate (ii) Lipoic acid (iii) Coenzyme-A (CoA) (iv) Flavin adenine dinucleotide (FAD) and (v) Nicotinamide adenine dinucleotide (NAD^+).



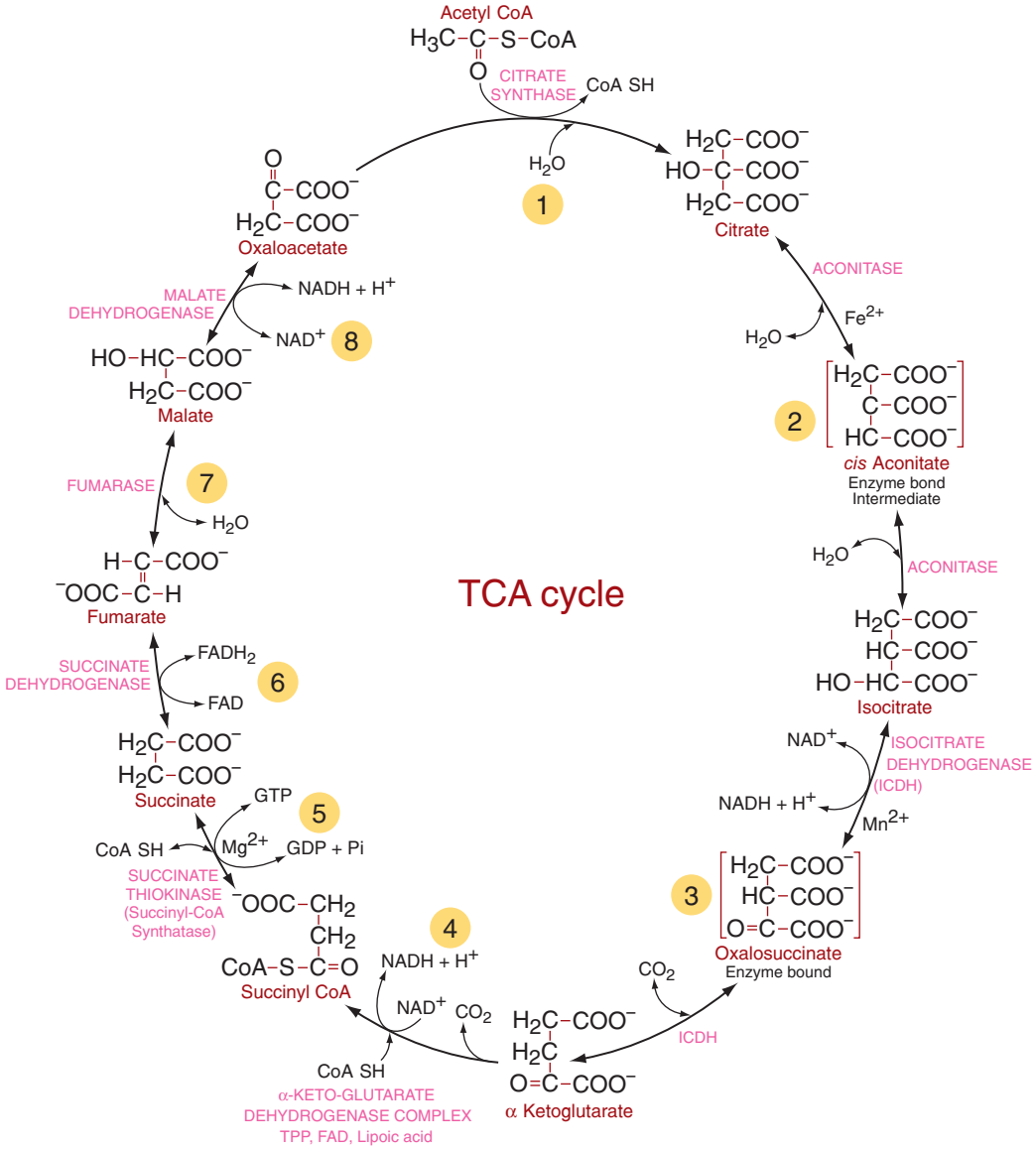
Acetyl-CoA formed in the above reaction may take part either in its oxidation to carbon dioxide and water, through TCA cycle, or formation of lipids, or synthesis of cholesterol etc. etc., which depends upon the nutritional state of the body and the type of the cell where it is formed.

KREB'S CYCLE / CITRIC ACID CYCLE / TCA CYCLE

Citric acid cycle also known as tricarboxylic acid (TCA) cycle is named after the scientist Sir Hans Krebs (1900-1981) who discovered it. He proposed the key elements of this pathway in 1937 and was awarded the Nobel Prize in Medicine for the discovery in 1953. Kreb's cycle is a set of continuous reactions (8 steps) occurring in a cyclic manner in the mictochondrial matrix in eukaryotes and within the cytoplasm in prokaryotes. Acetyl-CoA, the fuel of TCA cycle, enters the citric acid cycle inside the mitochondrial matrix, and gets oxidized to CO_2 and H_2O while at the same time reducing NAD to NADH and FAD to $FADH_2$. The NADH and $FADH_2$ can be used by the electron transport chain to create ATP.

Step 1—Condensation: In step 1, the two-carbon compound, **acetyl-S-CoA**, participates in a condensation reaction with the four-carbon compound, **oxaloacetate**, to produce **citrate**, a six carbon compound catalyzed by the enzyme **citrate synthase**. This is the first stable tricarboxylic acid in the cycle and hence the name TCA cycle.

Step 2—Isomerization of citrate: Step 2 involves moving the hydroxyl group in the citrate molecule so that it can later form an α-keto acid. This process involves a sequential dehydration and hydration



reaction, to form the **D-isocitrate** isomer (with the hydroxyl group now in the desired α-location), with **cis-aconitase** as the intermediate. A single enzyme, **aconitase** performs this two-step process.

Step 3—Generation of CO_2 by an NAD^+ linked enzyme: Oxidative decarboxylation takes place in the next reaction. The reaction is catalyzed by the enzyme **isocitrate dehydrogenase**. The reaction involves dehydrogenation to **oxalosuccinate**, an unstable intermediate which spontaneously decarboxylates to give **α-ketoglutarate**. In addition to decarboxylation, this step produces a reduced nicotinamide adenine dinucleotide (NADH) cofactor, or a reduced nicotinamide adenine dinucleotide phosphate (NADPH) cofactor.

Step 4—A second oxidative decarboxylation step: This step is performed by a multi-enzyme complex, the **α-ketoglutarate dehydrogenation complex**. The multi-step reaction performed by the α-ketoglutarate dehydrogenation complex is analogous to the **pyruvate dehydrogenase complex**, i.e. an α-keto acid undergoes oxidative decarboxylation with formation of an acyl-CoA i.e. succinyl-CoA.

Step 5—Substrate-level phosphorylation: Succinyl-CoA is a high potential energy molecule. The energy stored in this molecule is used to form a high energy phosphate bond in a guanine nucleotide diphosphate (GDP) molecule. Most of the GTP formed is used in the formation of ATP, by the action of nucleoside diphosphokinase.

Step 6—Flavin-dependent dehydrogenation: The succinate produced by **succinyl CoA-synthetase** in the prior reaction needs to be converted to oxaloacetate to complete the Kreb's cycle. The first step in the conversion is the dehydrogenation of succinate to yield fumarate facilitated by the enzyme **succinate dehydrogenase**. FAD is covalently bound to the enzyme (via a histidine residue) which is converted to $FADH_2$ that is oxidized through the ETC producing 2 ATPs.

Step 7—Hydration of a carbon-carbon double bond: Fumarate undergoes a stereo-specific hydration of the C=C double bond, catalyzed by **fumarate hydratase** (also known as **fumarase**), to produce L-malate.

Step 8—Dehydrogenation reaction that will regenerate oxaloacetate: L-malate (malate) is dehydrogenated to produce oxaloacetate by the enzyme **malate dehydrogenase** during which one molecule of NAD^+ is converted to NADH + H^+.

The formation of oxaloacetate completes the Kreb's cycle

The sum of all reactions in the citric acid cycle is

$$\text{Acetyl-CoA} + 2H_2O + 3NAD^+ + Pi + GDP + FAD \rightarrow 2CO_2 + 3NADH + GTP + CoASH + FADH_2 + 2H^+$$

Number of ATP's produced in one TCA cycle: The TCA cycle produces 3 NADH + H^+ and one $FADH_2$, these are known as the reducing equivalents. These reducing equivalents are oxidized through the electron transport chain. When NADH is oxidized through ETC it produces 3 ATPs and oxidation of $FADH_2$ through ETC produces 2 ATPs.

Calculation of ATPs produced in each round of citric acid cycle

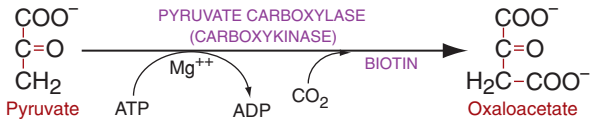
Enzyme involved	Equivalent produced	Fate of the equivalent	No. of ATPs produced	The step in TCA cycle
Isocitrate dehydrogenase	NADH	Oxidized through ETC	3	3rd step
α-Ketoglutarate dehydrogenase	NADH	- Do -	3	4th step
Succinate thiokinase	GTP	Converted to ATP	1	5th step
Succinate dehydrogenase	$FADH_2$	Oxidized through ETC	2	6th step
Malate dehydrogenase	NADH	- Do -	3	8th step
		Total ATP produced	**12 ATPs**	

Regulation of TCA cycle: The regulation of the TCA cycle is largely determined by substrate availability and product inhibition.

- NADH, a product of dehydrogenases in the TCA cycle, inhibits pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase and also citrate synthase.
- Succinyl-CoA inhibits succinyl-CoA synthase and citrate synthase.
- ATP inhibits citrate synthase and α-ketoglutarate dehydrogenase.
- Calcium is used as a regulator, it activates isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. This increases the reaction rate of many of the steps in the cycle, and therefore increases flux throughout the pathway.

Importance of citric acid cycle or amphibolic role of TCA cycle: TCA cycle is the common pathway for the oxidation of carbohydrates, fats and proteins (catabolic role). The anabolic role is synthesis of various carbohydrates, amino acids and fats. As it takes part both in anabolism and catabolism, it is said to be amphibolic pathway of metabolism.

Anaplerosis: It is the replenishment of the depleted intermediates of TCA cycle. As the TCA cycle takes part in the anabolic reactions, the intermediates of TCA cycle are utilized for the synthesis of various compounds. This results in the deficiency of one or more of the TCA cycle intermediates. In order to continue the TCA cycle, those intermediates, which are deficient, must be filled up by some other process and this process is known as anaplerosis. For example oxaloacetate is utilized for the synthesis of the amino acid aspartic acid and oxaloacetate is replaced via anaplerosis by carboxylation of pyruvate to oxaloacetate by the enzyme pyruvate carboxykinase.

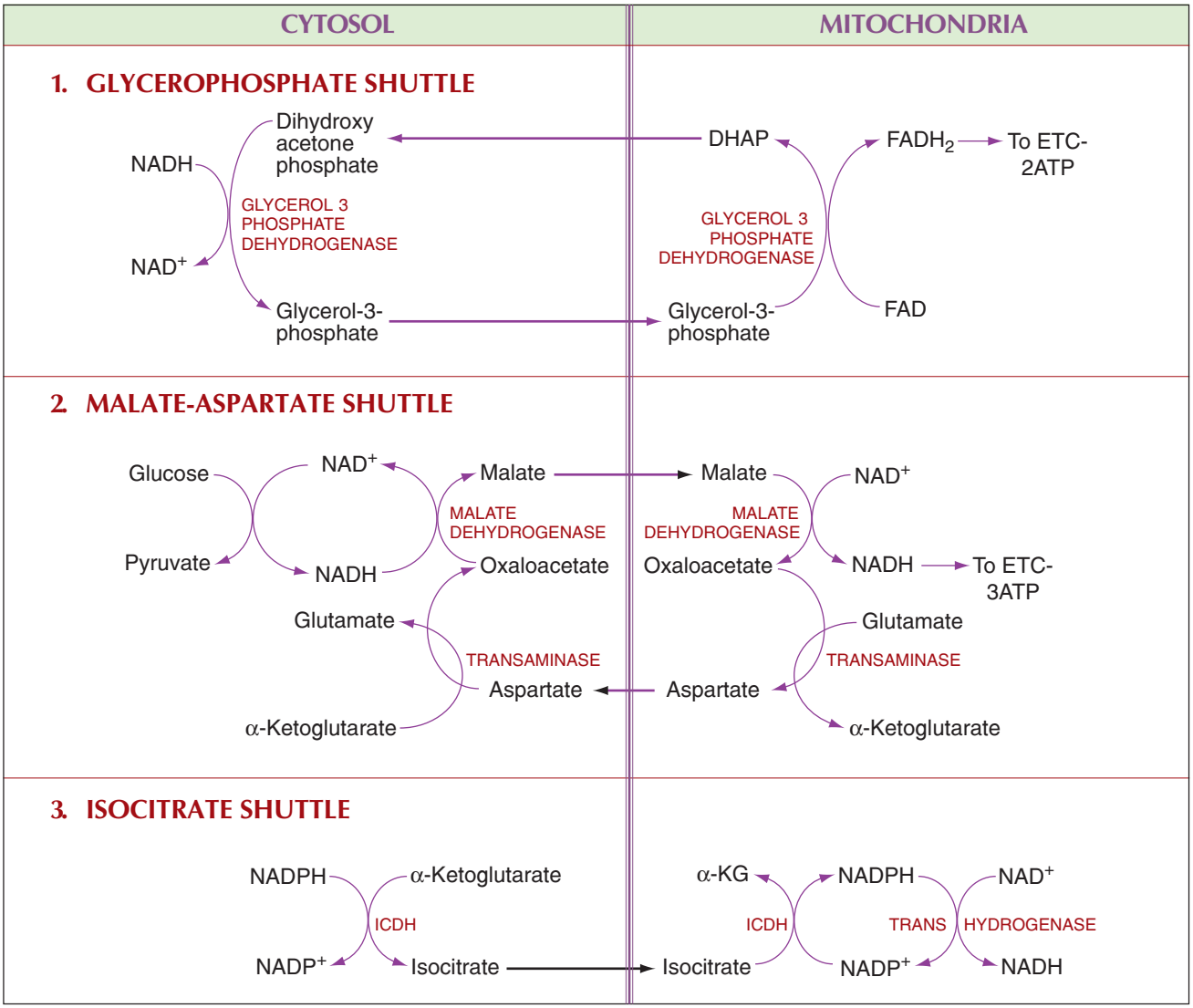


Total number of ATPs produced when glucose is completely oxidized to CO_2 and H_2O

(1)	Glycolysis	→	= 8 ATP
(2)	2 pyruvate → 2 acetyl-CoA → 2 NADH	→ 3×2	= 6 ATP
(3)	2 cycles of citric acid cycle for the 2 acetyl-CoA	→ 12×2	= 24 ATP
(4)	Total		= 38 ATP

- ✓ Total 38 ATPs are formed when one molecule of glucose is completely oxidized to CO_2 and H_2O.
- ✓ Net gain of 36 ATP is seen when NADH produced in glycolysis in the step catalysed by glyceraldehyde-3-phosphate dehydrogenase in the cytosol is transported to the mitochondria for oxidation in ETC, facilitated by glycerol phosphate shuttle instead of malate-aspartate shuttle.
- ✓ Net gain of 39 ATP does occur when glucose present in the glycogen is directly oxidized.

Shuttle systems: There are some reactions that take place in the cytosol which produce NADH. These NADH have to be oxidized through the electron transport chain situated in the inner mitochondrial membrane. NADH is not permeable to the mitochondrial membrane; therefore shuttle systems operate for its transport. There are three shuttle mechanisms—(1) Glycerophosphate shuttle (2) Malate-Aspartate shuttle and (3) Isocitrate shuttle.



Glycogen metabolism: Glycogen is a polysaccharide made up of glucose. It is the storage form of glucose in the body. Glucose requires more water for storage, but glycogen can be stored with much less amount of water hence glucose is stored as glycogen in the cell.

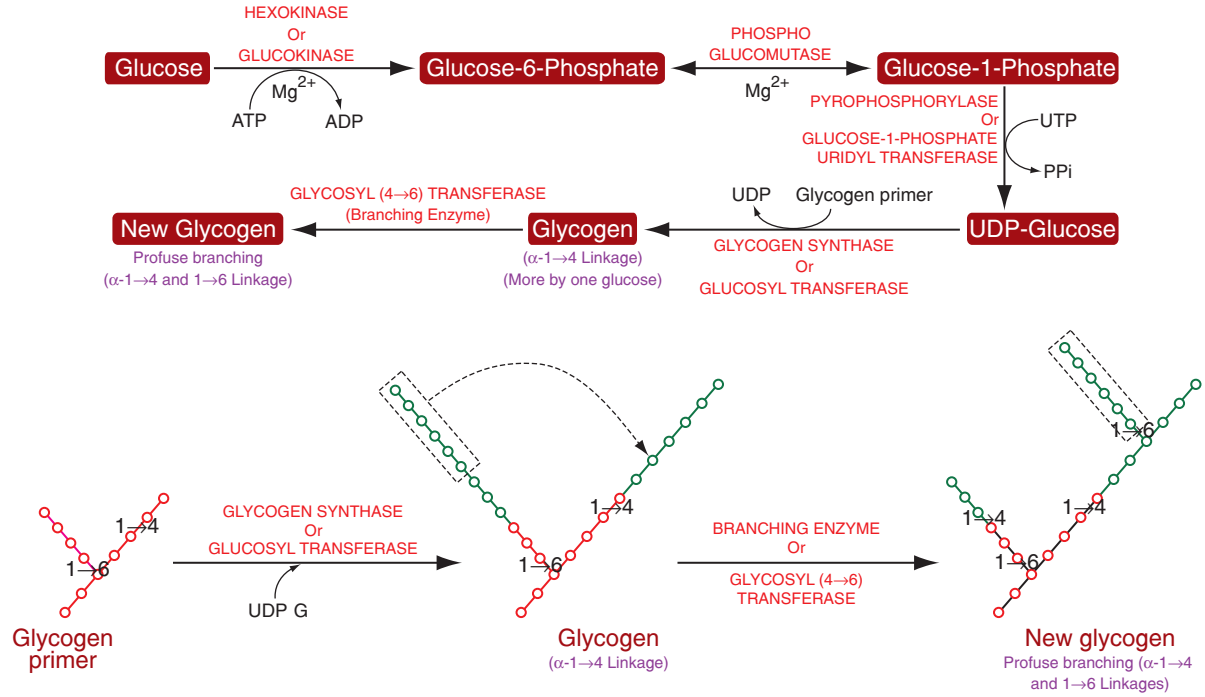
The largest amount of glycogen is stored in the liver and muscle. Liver glycogen provides glucose to other cells and maintains the blood glucose level in normal amounts. Muscle glycogen serves as readily available source of glucose during vigorous exercise, for glycolysis in the muscle itself. Glycogen metabolism includes glycogenesis and glycogenolysis.

GLYCOGENESIS

Synthesis of glycogen from glucose is known as glycogenesis. Glucose entraped in the cell as glucose-6-phosphate is mutated to glucose-1-phosphate by the enzyme phosphoglucomutase, which inturn is attacked to UTP by the enzyme glucose-1-phosphate uridyl transferase (pyrophosphorylase) forming UDP-glucose.

Glycogen synthase adds glucose to the glycogen primer (preformed glycogen with a few glucose units) by making α-1→ 4 glycosidic linkages and thus forms a linear chain of 10 to 12 glucose residues, all linked by α-1→ 4 glycosidic linkage. At this time another enzyme i.e. branching enzyme (glycosyl-(4 → 6)

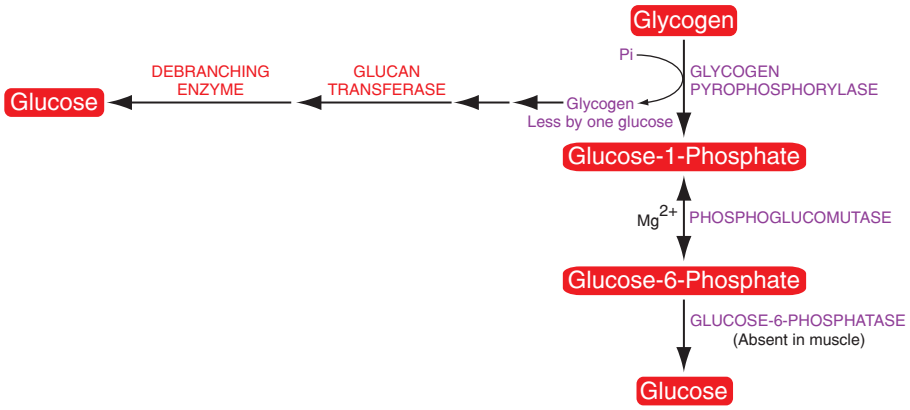
transferase) removes 6 to 7 glucose units from the linear chain and transfers them to the other chain and attaches by α-1→6 linkage, therefore creating a branching point. The process of addition of glucose by glycogen synthase to the linear chain and branching enzyme creating the branching points is repeated and thus glycogenesis is completed.



Mechanism of glycogenesis

GLYCOGENOLYSIS

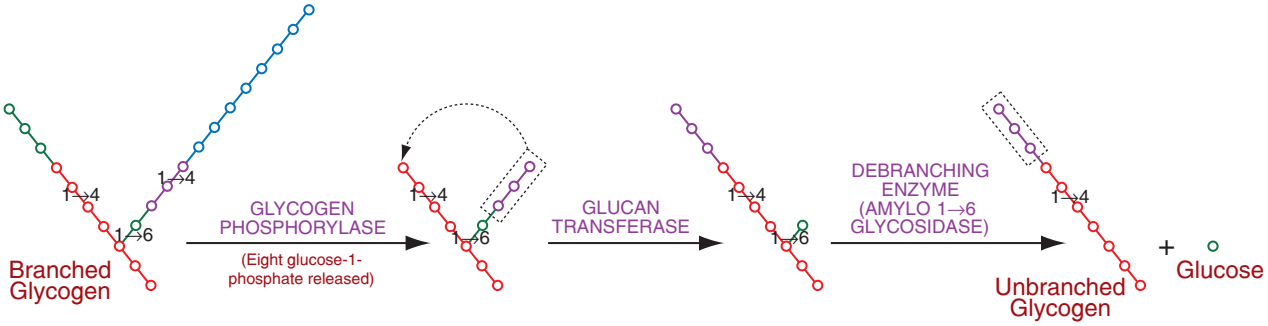
Breakdown of glycogen to glucose is known as glycogenolysis.

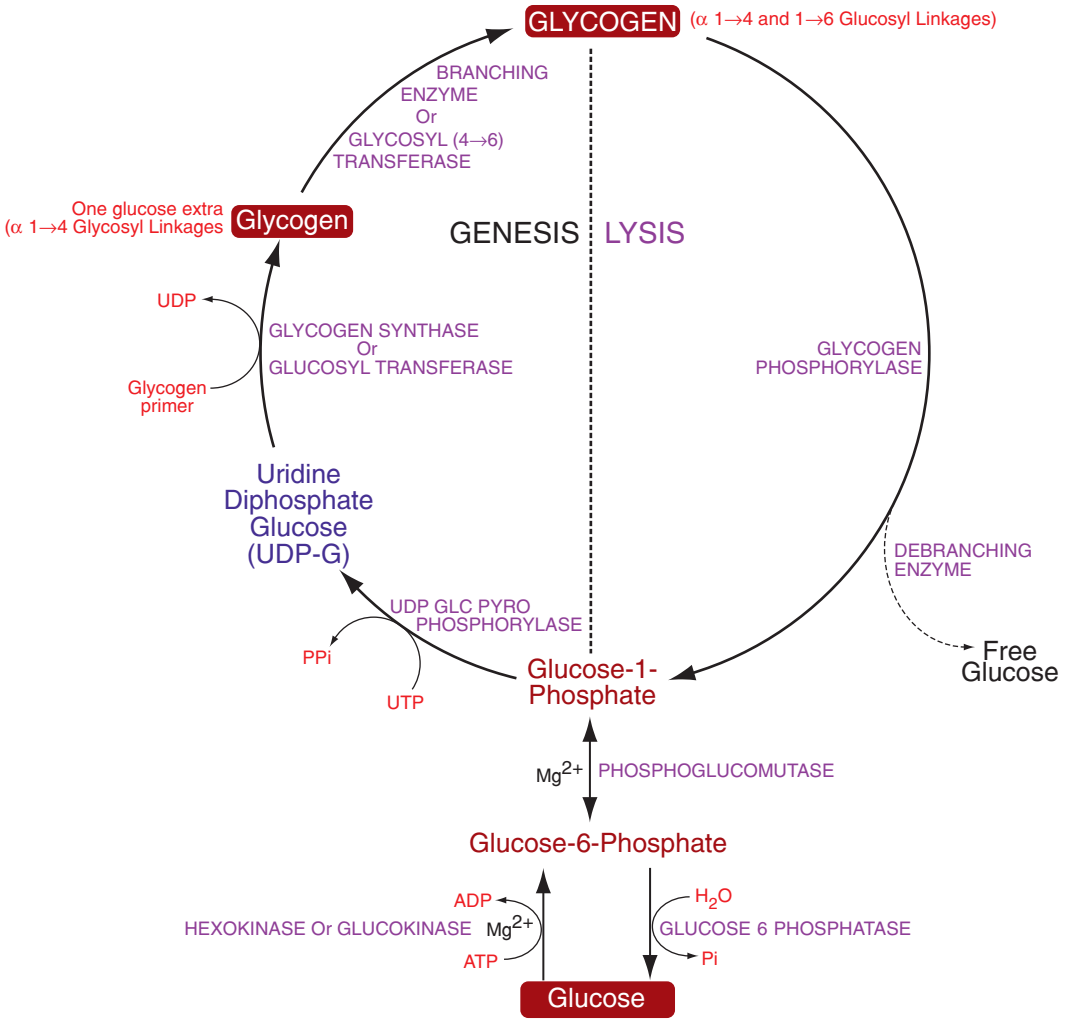


Mechanism of glycogenolysis

Glycogen phosphorylase is the key enzyme of glycogenolysis. It acts only on α-1→ 4 glycosidic linkages and thus releases glucose units one by one from the linear chain, till two or three or four glucose units near the branching point are left over. The remaining three glucose units linked by α-1→ 4 linkages are transferred to another linear chain by the enzyme glucan transferase, thus leaving one glucose residue linked with α-1→ 6 glycosidic linkage, which is acted upon by debranching enzyme (amylo-1,6-glycosidase) and thus releasing free glucose.

If glycogen is subjected to the action of phosphorylase alone, it will result in the formation of a glycogen molecule with each branch having only 4 glucose units which is called the 'limit dextrin'.

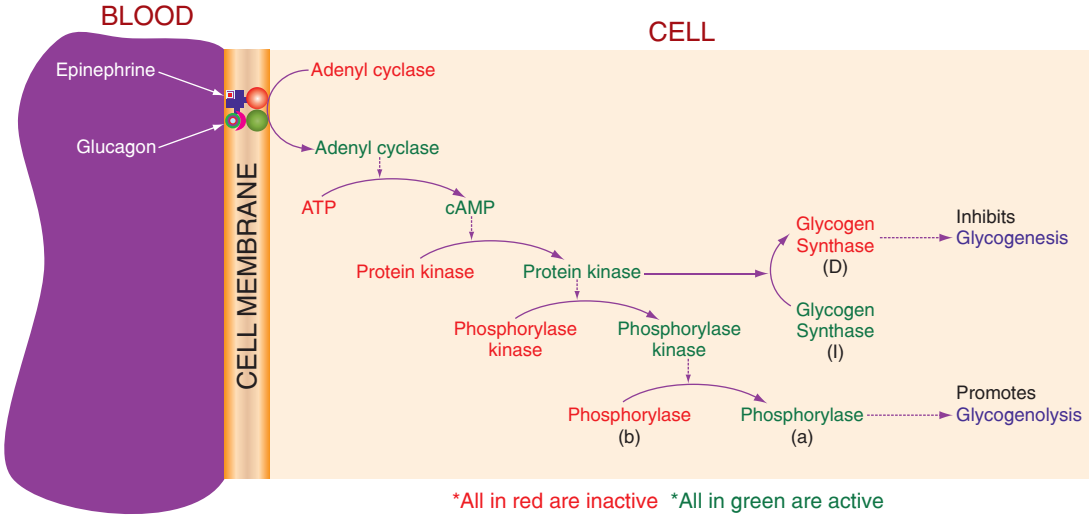




Overall picture of glycogen metabolism

Regulation of glycogen metabolism: Glycogen metabolism is reciprocally regulated, mainly by the action of hormones. At the time of shock and excitement, epinephrine stimulates glycogenolysis, both

in muscle and liver, whereas glucagon stimulates glycogenolysis only in the liver under hypoglycemic conditions. Insulin inhibits glycogenolysis and promotes glycogenesis.



Hormonal regulation of glycogen metabolism

Glycogen storage diseases: Glycogen storage diseases are a group of inherited disorders characterized by deficient mobilization of glycogen and deposition of abnormal forms of glycogen.

Various types of glycogen storage disorders

Type and common name	Enzyme deficient	Tissue in which glycogen accumulates
Type I – Von-Gierke's disease	Glucose-6-phosphatase	Liver and kidney – blood sugar levels are low and not increased by epineph rine or glucagon
Type II – Pompe's disease	α-1→4 and 1→6 Glucosidases	Lysosomes
Type III – Limit dextrinosis	Debranching enzyme	Forms limit dextrins
Type IV – Amylopectinosis	Branching enzyme	Unbranched glycogen – results in death in first ½ year
Type V – McArdle's syndrome	Muscle phosphorylase	Muscle – poor tolerance to exercise– blood lactate does not rise after exer cise or epinephrine injection
Type VI – Her's disease	Liver phosphorylase	Liver – hypoglycemia is seen

GLUCONEOGENESIS

Gluconeogenesis is the formation of glucose from non-carbohydrate sources.

Gluconeogenesis helps to maintain the glucose level in the blood, so that the brain, RBC and muscle can extract glucose from it to meet their metabolic demands when dietary glucose is low. This process is very much necessary in the body because brain and RBC utilizes only glucose as energy fuel.

The major non-carbohydrate precursors of glucose are lactate, glucogenic amino acids (all except leucine) and glycerol.

Lactate is formed by RBC in glycolysis because mitochondria are absent. Lactate is also formed by active skeletal muscle when the rate of glycolysis exceeds the rate of TCA cycle, the pyruvate formed is converted to lactate.

Amino acids are derived form proteins in the diet and during starvation, from the breakdown of proteins in skeletal muscle.

Glycerol is derived form the hydrolysis of triacylglycerols (TAG).

Gluconeogenesis occurs mainly in liver and kidney. It also occurs in brain and muscle to some extent.

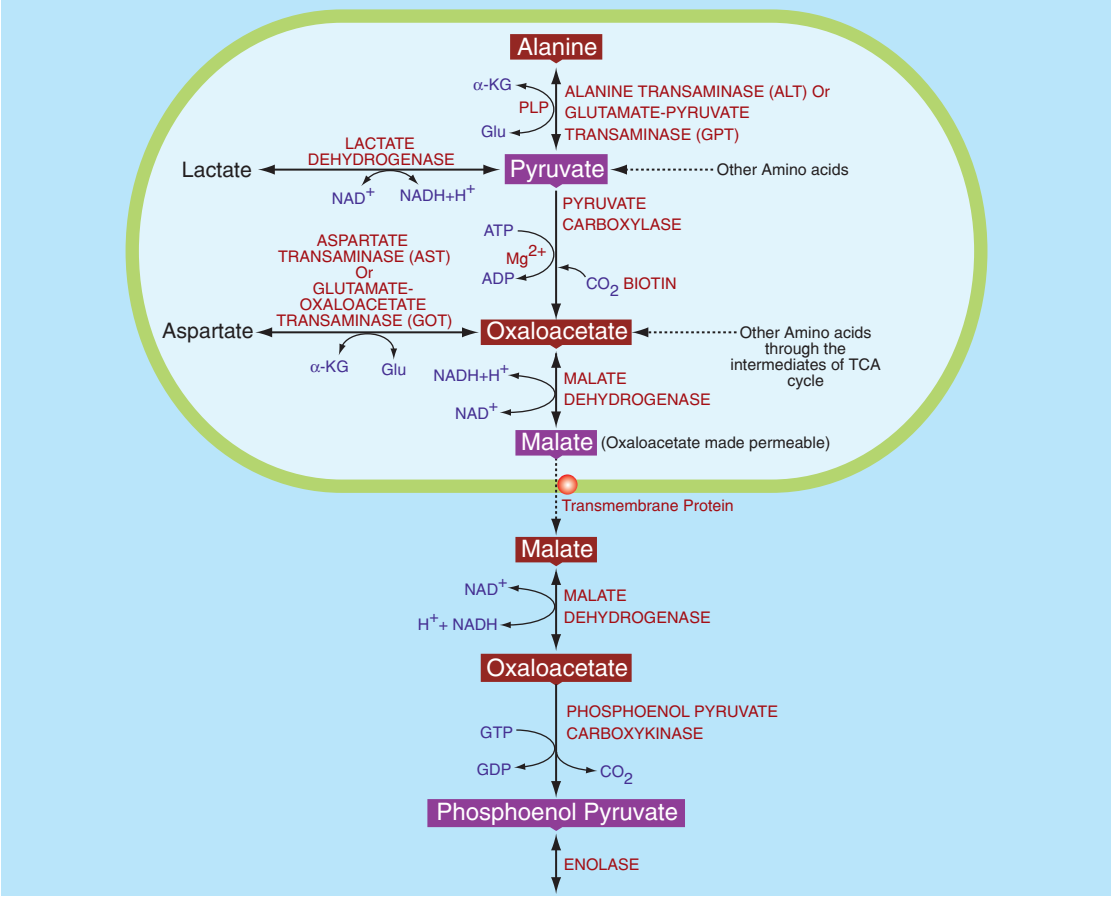
Gluconeogenesis occurs during

(1) Starvation, (2) To clear lactate formed in RBC and muscle, (3) When carbohydrates in the diet are low, (4) Pregnancy, (5) Lactation, (6) Febrile diseases.

Gluconeogenesis is almost the reversal of glycolysis excepting at three steps which are irreversible in glycolysis. These steps are reversed by enzymes known as the key enzymes of gluconeogenesis i.e. those enzymes specific for gluconeogenesis only but not for any other pathway. The key enzymes of gluconeogenesis are—

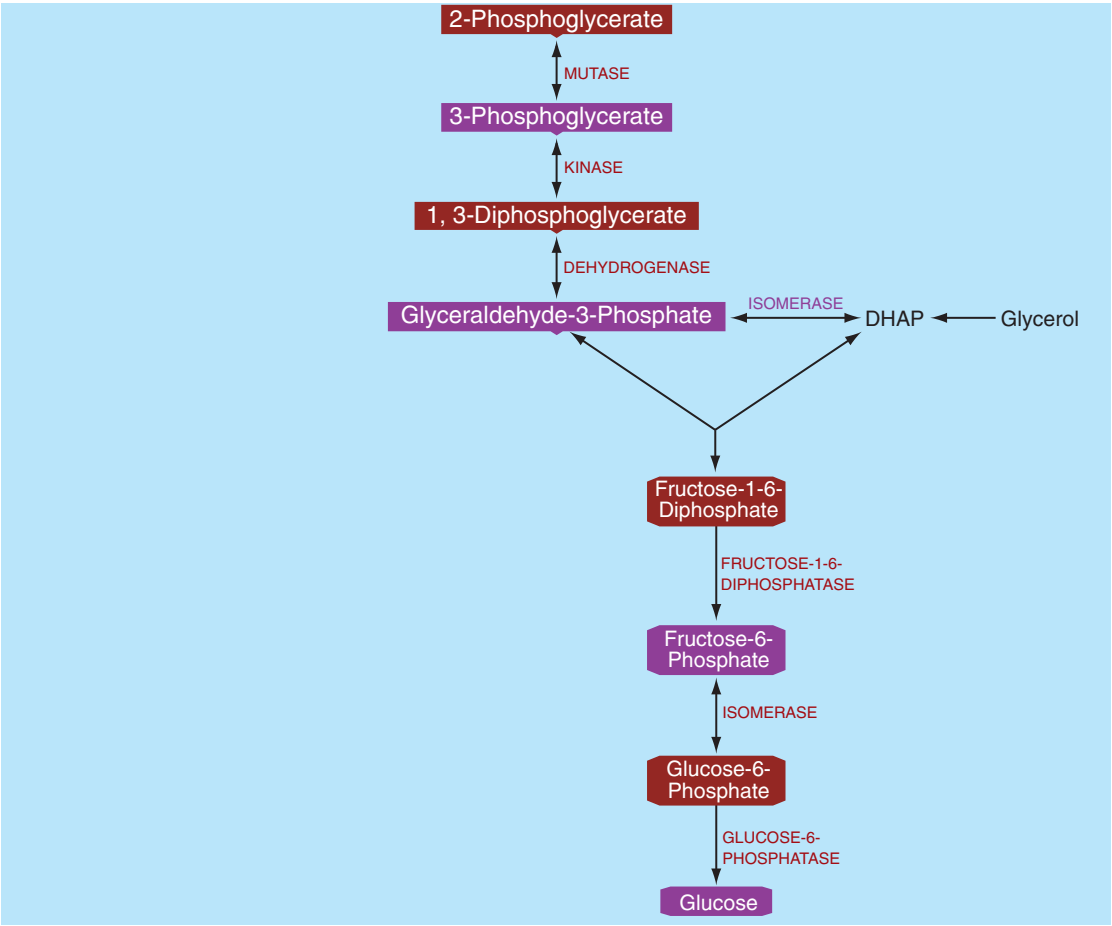
1. Pyruvate carboxylase (or carboxykinase)
2. Phosphoenol pyruvate carboxykinase
3. Fructose-1-6-diphosphatase
4. Glucose-6-phosphatase

The process of gluconeogenesis is as follows—



Contd. to next page

Contd. from previous page



Process of gluconeogenesis

Role of 2, 6-Biophosphate in gluconeogenesis Fructose 2, 6-bisphosphate (or fructose 2, 6-diphosphate), is a metabolite which allosterically affects the activity of the enzymes phosphofructokinase 1 (PFK-1) and fructose 1, 6-bisphosphatase (FBPase-1) to regulate glycolysis and gluconeogenesis. Fructose 2, 6-bisphosphate is synthesized and broken down by the bifunctional enzyme, phosphofructokinase 2/ fructose 2, 6-bisphosphatase (PFK-2/FBPase-2). The synthesis of Fructose 2, 6-bisphosphate is performed through the phosphorylation of fructose 6-phosphate using ATP by the PFK-2 portion of the enzyme. The breakdown of Fructose 2, 6-bisphosphate is caused by dephosphorylation, catalyzed by FBPase-2 to produce Fructose 6-phosphate and P_i. Fructose 2, 6-bisphosphate stimulates glucose breakdown further through reduction of gluconeogensis through allosteric inhibition of fructose 1, 6-bisphosphatase.

Hormones that regulate gluconeogenesis:

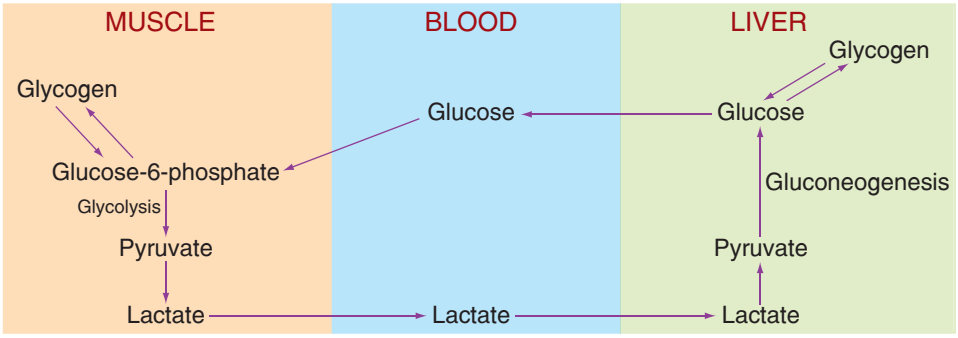
Gluconeogenesis is stimulated by—

1. Glucocorticoides
2. Thyroxine
3. Growth hormone
4. Epinephrine also stimulates but to a lesser extent

Gluconeogenesis is inhibited by—

1. Insulin

Cori's cycle: Conversion of muscle glycogen to liver glycogen through blood lactate and back to mucle glycogen through blood glucose is known as Cori's cycle.



Importance of Cori's cycle:

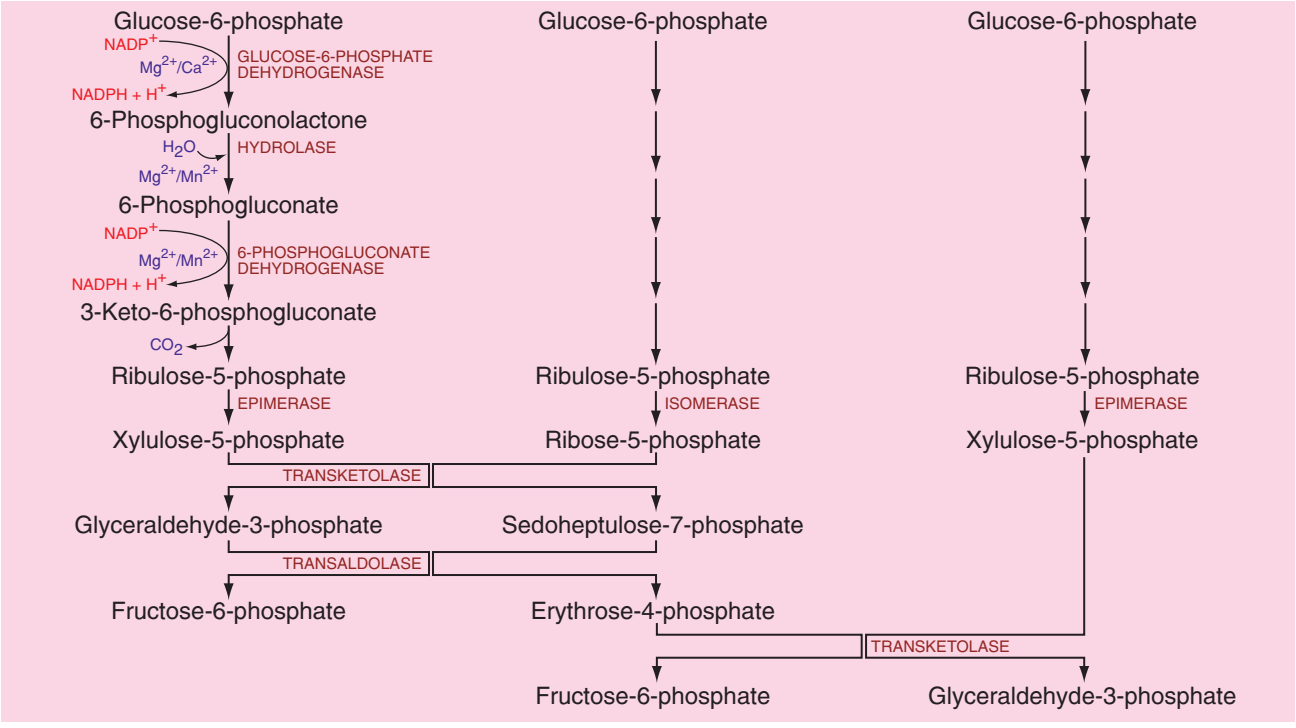
1. Regulation of blood sugar
2. Elimination of lactic acid from muscle

HMP PATHWAY OR PENTOSE PHOSPHATE PATHWAY

Hexose monophosphate shunt pathway or the HMP pathway is an alternative pathway for glucose oxidation. It neither utilizes nor produces ATP. The main purpose or significance of this pathway is—

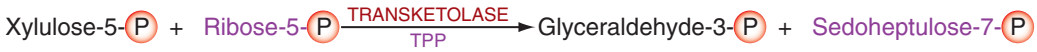
- It produces the reducing equivalents NADPH + H^+, for the synthesis of lipids (fatty acids and steroids) and keeps glutathione in reduced state in RBC.
- It generates ribose sugar (pentose phosphate) for the formation of nucleic acids.

The organs in which HMP pathway occurs are those which are actively concerned with lipid synthesis, like the adipose tissue, kidney, lactating mammary gland, liver, RBC, thyroid and gonads. It takes place in the cytosol. The steps involved in this pathway are—



Mechanism of HMP pathway

Transketolation reaction: Transfer of 2-carbon moiety i.e., active glycelaldehyde is known as transketolation. It is catalysed by the enzyme transketolase and the coenzyme is Thaimine pyrophosphate (TPP). In thiamine deficiency (also in pernicious anemia) transketolase activity is decreased in blood.

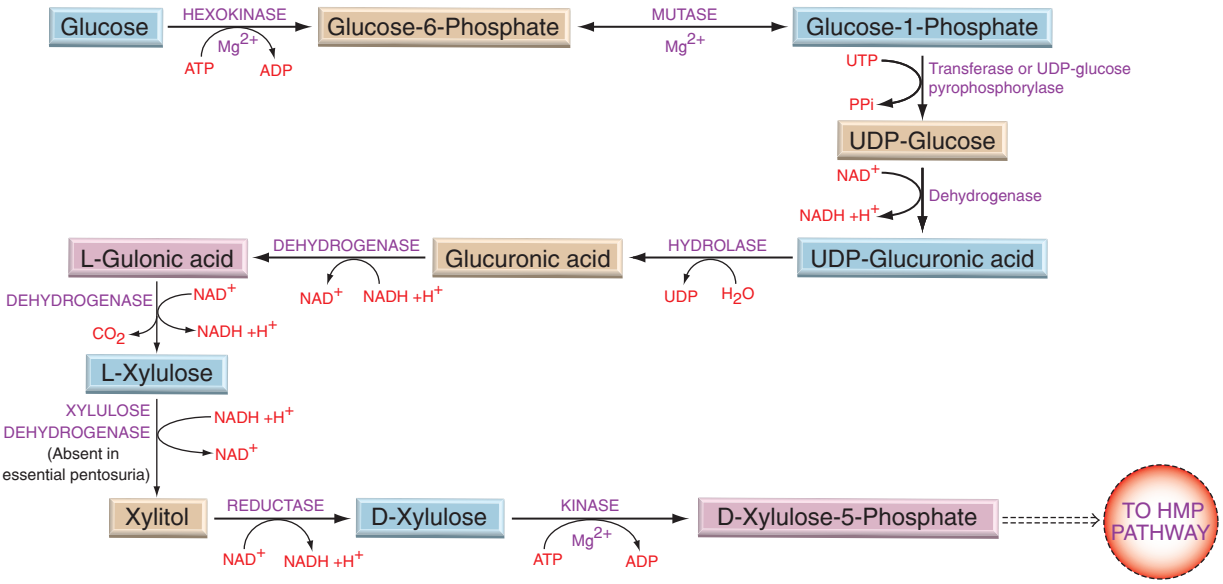


Transaldolation reaction: Transfer of 3-carbon moiety i.e., active dihydroacetone is known as transaldolation. It is catalysed by the enzyme transaldolase.

URONIC ACID PATHWAY

This is a synthetic pathway for the various uronic acids. Its importance is—

1. It produces glucuronic acid which takes part in detoxification of bile pigments, phenols, aromatic acids and steroid hormones.
2. It provides glucuronic acid and galacturonic acid for the formation of glycoproteins.
3. In lower animals this pathway leads to synthesis of ascorbic acids (vitamin C).

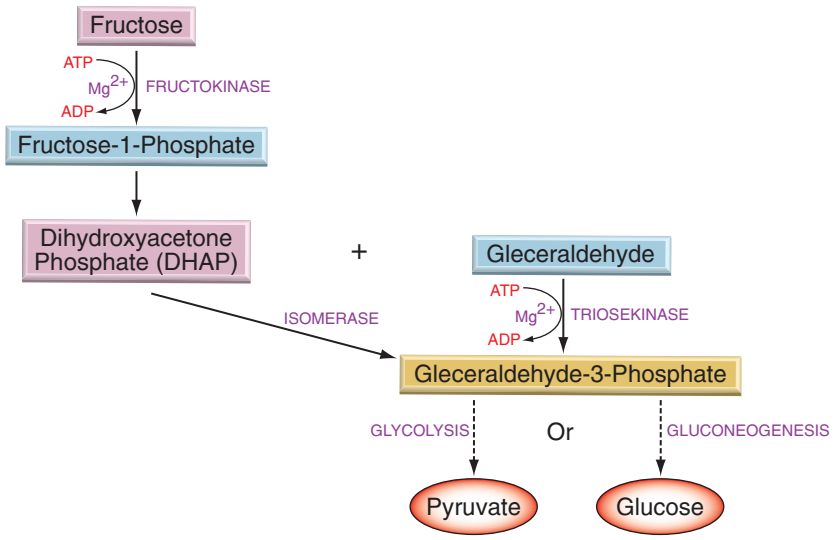


Mechanism of uronic acid pathway

Metabolism of fructose: In the diet fructose is obtained from fruits, honey and table sugar (sucrose). In human body it is the sugar of the semen and amniotic fluid.

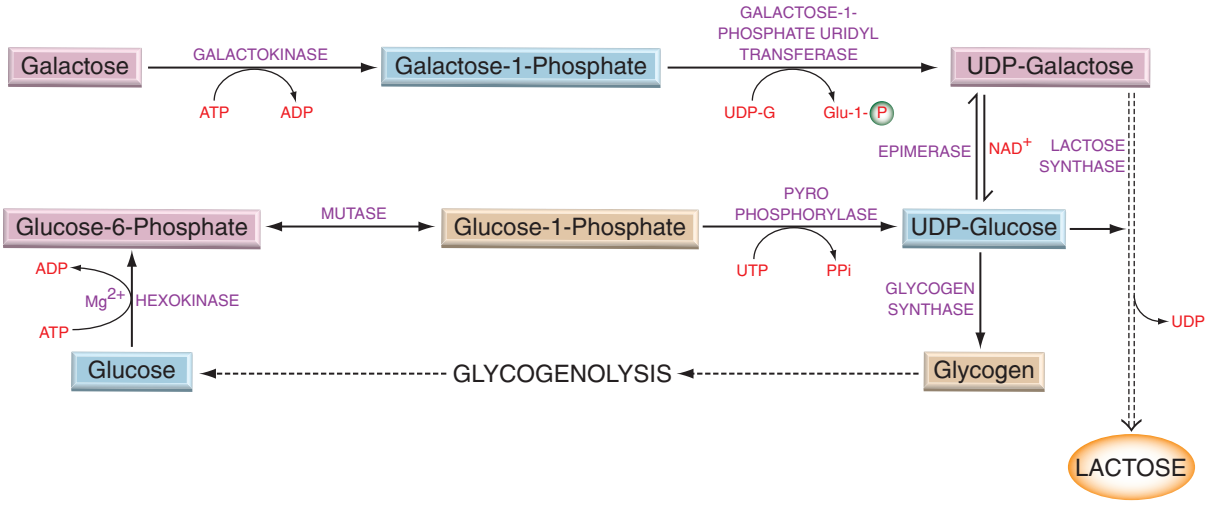
Essential fructosuria: It is a genetic defect in which there is excretion of fructose in the urine due to the lack of the enzyme fructokinase.

Fructose intolerance: A person shows disliking towards fruits and fructose rich diets due to the deficiency of the enzyme aldolase-B.



Metabolism of fructose

Metabolism of galactose and synthesis of lactose: In the diet, galactose is mainly derived from the milk sugar lactose. In the body it is converted to glycogen or may take part in the synthesis of the milk sugar lactose in lactating mammary gland.



Utilization of galactose and synthesis of lactose

Lactose intolerance type-II: This is due to the deficiency of the enzyme galactose-1-phosphate uridyl transferase, which results in the accumulation of galactose in the blood i.e. galactosemia and excretion in the urine i.e. galactosuria. Such infants are intolerant to lactose and hence to milk. They show symptoms like diarrhoea and vomiting on giving milk. Lactose free milk is the only remedy.

REGULATION OF BLOOD SUGAR LEVEL

Blood glucose hemostasis: After a meal, specially, rich in carbohydrates, the intestine adds glucose to the blood by absorption and this leads to an increase in the blood glucose level from 60-90 mg/100 ml to 100-140 mg/100 ml. A further increase in blood glucose is prevented mainly by the liver, which takes up most of the dietary glucose and stores it as glycogen by glycogenesis. Simultaneously, a hormone i.e. insulin, is released by the β-cells of islets of langerhans of the pancreas. This hormone helps in the uptake of glucose by other tissues, and thereby all the tissues start utilizing glucose as the primary metabolite for the production of energy by glycolysis. In each and every tissue, a small amount of glucose is stored as glycogen. Muscle stores much of glucose as muscle glycogen. When the storage of glucose saturates the liver, muscle and other tissues, then the adipose tissue convert the excess of glucose to fat by lipogenesis and store as triacylglycerols (TAG). Fats are also synthesized by the liver from excess of glucose and amino acids. Insulin aids in the entry of glucose in almost all the cells of the tissues of the body except five tissues viz., liver, brain, intestine, RBC and retina of the eye (hence causes cataract in diabetes mellitus patients).

If the glucose level in the blood increases further, more than 180 mg/ 100 ml (hyperglycemia) as occurs in intravenous injection of glucose or due to incapability of the cells to utilize glucose absorbed in normal amounts (insulin deficiency, defective insulin receptors or more of insulin antagonists i.e. diabetes mellitus, see details later in this chapter), then the kidney starts excreting glucose, a condition known as glucosuria. Glucosuria occurs when the capacity of the kidney to tolerate blood glucose is exceeded in the blood. This is known as renal threshold for glucose, the value for which is 180 mg/100 ml of blood. If the glucose concentration in the blood exceeds this value, it is excreted in urine. At 180 mg/100 ml of blood glucose the kidney reabsorbs 350 mg of glucose per minute, this is known as tubular maximum for glucose (T-G). Any amount of glucose being filtered by the glomerular filtrate more than this into the kidney per minute is excreted.

When the blood glucose level starts falling (hypoglycemia), as seen in fasting, starvation, shock, severe exercise, febrile disease, lactation, multiple pregnancies etc, the hormone glucagon is released by the α-cells of islets of langerhans of the pancreas. Glucagon stimulates the breakdown of glycogen in the liver (glycogenolysis) by activating liver phosphorylase. In conditions of shock and emotional excitement, epinephrine is released from the adrenal medulla which stimulates glycogenolysis in muscle by activating muscle phosphorylase. The maximum reserves of liver glycogen can maintain the blood glucose level within the normal range for 8-10 hours of fasting. After this, liver produces glucose by gluconeogenesis from lactate derived from muscle or RBC glycolysis and amino acids derived form muscle proteins.

The extent to which gluconeogenesis can provide glucose to the blood depends upon the muscle mass and the rate of glucose utilization, which in turn depends upon the fat content of the body. In normal healthy adult, gluconeogenesis can maintain blood glucose for abut 30-40 days of starvation. After which severe hypoglycemia and death occurs. In addition to liver, kidney can also take part in gluconeogenesis. The hormones which facilitate gluconeogenesis are glucocorticoids, GH, ACTH, and thyroid hormones.

Factors adding blood glucose	Factors removing blood glucose
Absorption of dietary glucose	Glycolysis by cells
Liver glycogenolysis	Glycogenesis
Gluconeogenesis	Lipogenesis
	Amino acid synthesis
	Renal excretion

ROLE OF BLOOD SUGAR AS AN AID TO DIAGNOSIS

The major sugar present in the blood is **Glucose**. There are three methods by which glucose in the blood can be measured viz. (1) Enzymatic method (2) Complex formation and (3) Reduction method.

1. **Enzymatic method:** The enzyme, glucose oxidase is used in the estimation of blood glucose. Glucose oxidase oxidizes glucose to gluconic acid, releasing hydrogen peroxide. Hydrogen peroxide then reacts with an oxygen acceptor, such as ortho-dianisidine, phenylamine-phenazone or any other chromogenic oxygen acceptor with the help of the enzyme peroxidase. The intensity of the color developed is directly proportional to the blood glucose level. This enzyme is very specific for glucose, hence the value obtained is true glucose value in the blood.
2. **Complex formation:** The carbonyl carbon is reacted with reagents like ortho-toluidine to form a coloured complex. The intensity of the colour developed is proportional to the carbonyl compounds present in the blood. Hence the value obtained is not a true glucose value; it is the value for all the carbonyl compounds present in the blood like glucose, fructose, galactose, and ribose. Since ortho-toluidine is carcinogenic, this method is discouraged.
3. **Reduction method:** This is based upon the reducing property of glucose. Due to the presence of free aldehyde group glucose easily reduces metal ions like copper, iron, bismuth. The reduced ions are then reacted with a colouring agent; the intensity of the colour developed is proportional to the amount of reducing substance present in the blood. There are many other reducing substances other than glucose present in the blood like – fructose, lactose, mannose, ribose, ascorbic acid (vitamin C) etc. Hence the values obtained are far from the actual amount of glucose in the blood. But this method is relatively economical; hence most of the diagnostic laboratories adopt this method (Folin-Wu–method) for the estimation of blood glucose.

Modern electronic gadgets for estimation of blood glucose: Most of them are based on a glucose oxidase-colorimetric reaction that occurs when a drop of blood is placed on a reagent–impregnated pad. The test pad contains the enzyme glucose, oxidase peroxidase, and color indicators. When whole blood is placed on the test pad, glucose is oxidized to gluconic acid and hydrogen peroxide with glucose oxidase acting as a catalyst. The hydrogen peroxide then oxidizes an oxygen acceptor in the presence of peroxidase to form a color change, the intensity of which is directly proportional to the amount of glucose in the blood sample. A reflectance photometer or an amperometric system is used to measure the reaction that takes place on the reagent strip. Hence, the reagent strip is inserted into the test chamber. When light shines on the reagent pad, light is reflected. This reflected light is measured electronically and a blood glucose concentration value is displayed.

Other systems use electrochemical methodologies. These monitors quantify glucose amperometrically by measuring the current that is produced when glucose oxidase catalyzes the oxidation of glucose to gluconic acid or when glucose dehydrogenase catalyzes the oxidation of glucose to gluconolactone. The electrons generated during this reaction are transferred from the blood to the electrodes. The magnitude of the resultant current is proportional to the concentration of glucose in the specimen and is converted to a readout displayed on the monitor.

Some blood glucose monitoring systems are based on a reflectometric hexokinase method. When blood is applied to the reagent strip, glucose is phosphorylated to glucose-6-phosphate. This is later oxidized with concurrent reduction of NAD to NADH. The NADH formed is directly proportional to the amount of glucose (any hexose) present in the sample. Then the NADH, in the presence of another enzyme, reduces the dye and a colored product is generated. The strip which is inserted in the photometer after application of sample, measures the reaction reflectance, uses an algorithm to calculate glucose and displays the result.

Normal blood glucose level under various nutritional states

1. **Fasting or post-absorptive state:** (i.e. 12-14 hours after a meal) ranges between 60-90 mg/100 ml of blood (true blood glucose, but by reduction methods it is 80-120 mg/100 ml).
2. **Post-prandial:** That is after meals (1-2 hours after the first meal) blood glucose level ranges between 100-140 mg/100 ml (or 5.5-8.0 mmols/L and 3.3-5.0 mmols/L, fasting state).
3. **Random blood glucose level:** It is measured any time after 1st, 2nd or 3rd meal that ranges between 100-180 mg/100 ml (or 5.5-10 mmols/L).

The level of glucose in the blood is maintained by various mechanisms of the body. The purpose of regulation of the blood glucose levels within the normal physiological limits is that, if the blood glucose level falls below 60-20 mg/100 ml, a condition known as hypoglycemia occurs. Hypoglycemia results in convulsions leading to profuse sweating, weakness, tremors, fainting, coma and finally death. All these conditions observed in hypoglycemia are due to insufficient supply of glucose to the brain, as brain uses glucose only as the source of energy and not any other substance.

When the blood glucose level rises above 180-360 mg/100 ml (a condition known as hyperglycemia) then it results in—

1. The excretion of glucose by the kidney, due to which the work load on the kidney increases, leading to kidney disorders and finally to kidney failure.
2. Due to the high concentration of glucose, the oncotic pressure (osmotic pressure of plasma) differs leading to unequal distribution of water and electrolytes.
3. Increased glucose concentration in the blood for a longer duration of time leads to various infections, as the microorganisms can grow easily. Delay in wound healing and post surgical recovery, are the other problems encountered due to excessive microorganism growth in hyperglycemia.
4. High blood glucose level leads to glycosylation of proteins of the blood vessels and capillaries, resulting in narrowing of the passage of the flow of blood (increases the blood pressure). Continuous glycosylation of the proteins disrupts the membranes and results in accumulation of blood clots and cholesterol, a condition known as atherosclerosis of arteriosclerosis. Glycosylation of the membranes also leads to reduced life of RBC, cell necrosis, kidney failure etc.

Abnormalities in the regulation of blood sugar level

Diabetes mellitus: Frequent urination tasting sweet.

It is a condition wherein the cells are incapable of utilizing glucose, due to which the blood glucose level increases (hyperglycemia) and subsequently, there is an increased excretion of glucose in the urine (glycosuria) and hence the urine tastes sweet.

Causes

1. Deficiency of insulin (Insulin Dependent Diabetes Mellitus—IDDM—i.e. type-I diabetes).
2. Excess of insulin antagonists like glucagon, epinephrine, GH etc.
3. Defective or absence of insulin receptors on the cells (Insulin Independent Diabetes Mellitus–IIDM—i.e. type-II diabetes).

Biochemical changes in diabetes mellitus: When the cells are incapable of utilizing glucose for energy production, they start utilizing amino acids and fats for the production of energy. There will be production of ketone bodies by the liver and if they are produced in excess it causes accumulation of ketone bodies in the blood (ketonemia) and subsequent excretion in the urine (ketonuria). Both the conditions together lead to diabetic ketosis. Due to this the pH of plasma is lowered. The excretion of glucose and

ketone bodies requires large amounts of ions and water leading to polyuria and weight loss. Hence the three main conditions seen in diabetes mellitus are—

1 **Polyuria:** Frequent urination leading to dehydration.
2 **Polydypsia:** Frequent intake of water due to frequent urination.
3 **Polyphagia:** Frequent intake of food due the starving condition of the cells.

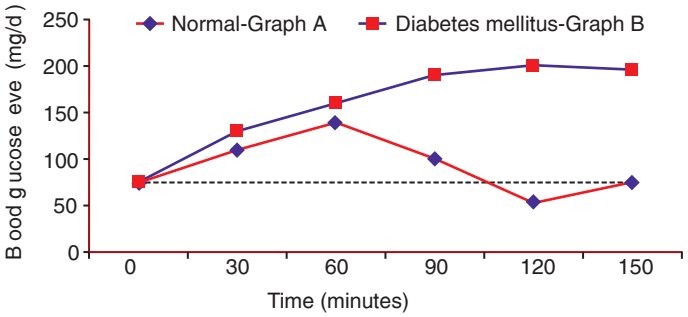
Classification of diabetes mellitus

1. **Juvenile or Hereditary:** Onset of diabetes mellitus in childhood is known as juvenile type. Here there is a defect in the gene synthesizing insulin or its receptors which is an inborn and hereditary character.
2. **Maturity onset type:** After maturity due to obesity or decrease in the function of the gene or due to other reasons, the insulin is not produced in normal amounts or if produced in normal amounts, cannot act on the target cells.
3. **Secondary diabetes:** Due to excess of insulin antagonists.

Detection and screening of diabetes mellitus

1. **Benedict's reducing sugar test:** This is the preliminary method for the detection of diabetes mellitus. Reducing sugars i.e. glucose in urine reduces copper to form a precipitate which depends upon the concentration of glucose. If 0.5% of glucose is present in urine, it forms a green coloured precipitate, 1.0% → yellow, 1.5% → orange, > 2.0% → brick red precipitate. Benedict's test is an easy and simple test to perform. Hence, at the first instance, detection of diabetes mellitus is done with urine sugar. If Benedict's test is positive then estimation of blood sugar is taken up. Further if blood glucose level is higher than the normal then diabetes mellitus is confirmed by the following tests.
2. **Glucose tolerance test (GTT):** Glucose tolerance test is done in patients, suspected with diabetes mellitus, but does not show any glucosuria because some persons have a very high renal threshold for glucose i.e. 250-300 mg/100ml. Secondly GTT is carried in those suspected diabetes mellitus cases whose blood glucose level shows around the normal values i.e. fasting blood glucose is less than 90 mg/100 ml but more than 60-70 mg/100 ml.

Glucose tolerance test is done with the patient being kept on a carbohydrate rich diet i.e. more than 150 gm/day, for three days prior to the experiment. The blood glucose is measured in fasting condition (12-14 hours). Then he is given an oral dose of 50-60 grams of glucose in 300 ml of water (i.e. 0.75 grams per kg body weight for Indian adult male or 1.0 gm/kg body weight for individuals of developed countries). Blood glucose is estimated every half an hour after ingestion of glucose and the ability of the individual to tolerate glucose is assessed, which gives an idea of diabetes mellitus. The result of glucose tolerance is plotted in a graph as follows—

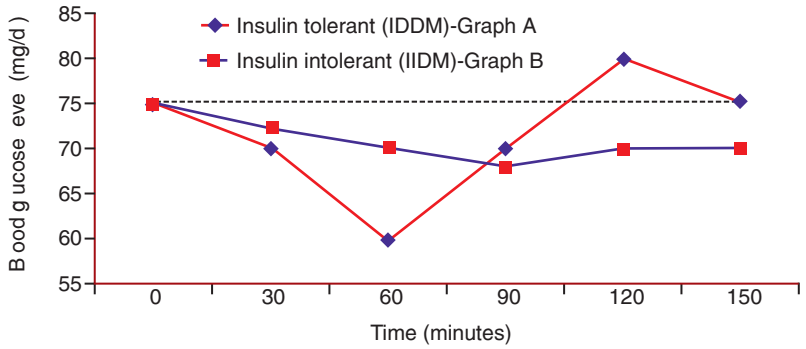


Graph—A: In normal persons the fasting blood glucose level will be at the lower limit i.e., 75 mg/100 ml. Upon providing oral glucose the blood glucose rises in one hour's time and falls below the fasting level in another half to one hour due to the secretion of insulin. Then, in about a time of 2½ hours it reaches back to normal. Such a person is normal and can tolerate glucose and is not a diabetic patient.

Graph—B: In diabetes the fasting blood glucose may be within the normal range but when glucose is given orally, there will a steep rise in blood glucose in ½ an hour and it increases further till one hour and it does not come down to normal level even by 2½ hours. Such patients are said to be intolerant to glucose and hence are diabetes mellitus patients.

If GTT is positive i.e. if the patient is a confirmed diabetes mellitus patient then further investigations are required to trace out the type of diabetes i.e. insulin dependent or independent. This can be done by performing (1) Insulin Tolerance Test (ITT) and (2) Glucagon Tolerance Test (GuTT).

Insulin tolerance test: Insulin is a hormone which is secreted by the β-cells present in islets of langerhans, of pancreas. It helps in the uptake of glucose by the cells. Insulin tolerance test is done with the patient being kept on a carbohydrate rich diet i.e. more than 150 gm/day for three days prior to the experiment. His blood glucose is measured in fasting condition (12-14 hours). Then he is given intravenous dose of 0.1 unit of insulin/kg body weight. Blood glucose is estimated every half an hour after injection of insulin and the ability of the individual to tolerate insulin is assessed, which shows whether the diabetes mellitus can be treated with insulin or not i.e. is it insulin responsive (IDDM) or not. The result of insulin tolerance is plotted in a graph as follows—

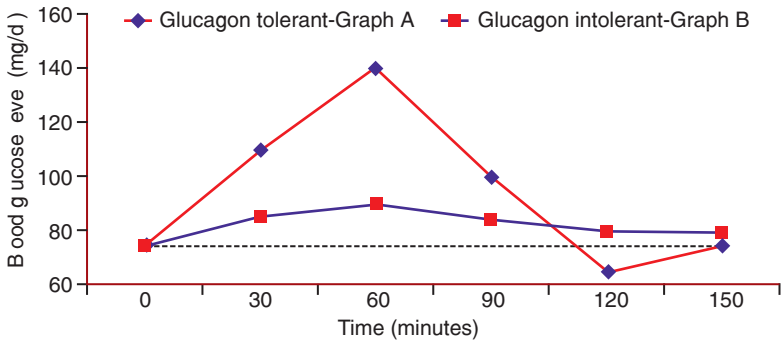


Graph—A: Injection of insulin in normal persons or insulin responsive diabetes mellitus (IDDM) persons will lead to a fall in the blood glucose level in one hour's time due to utilization of glucose by the cells aided by insulin and slowly increases and rises above the fasting level (base line) in another half to one hour due to the secretion of glucagon. Then in about the time of 2½ hours it reaches back to normal. Such a person is normal and can tolerate insulin. If the person is a confirmed diabetes mellitus case then it is insulin responsive diabetes mellitus or insulin dependent diabetes mellitus (IDDM).

Graph—B: In insulin non responsive diabetes mellitus patients there will not be any decrease in the blood glucose level or the decrease will be very marginal upon injection of insulin. This may be either due to excessive insulin antagonists or defective insulin receptors on the cell membrane (may also be due to antibodies to insulin-natural or injected one).

Glucagon tolerance test: Glucagon is a hormone that is secreted by the α-cells of islets of langerhans of pancreas which helps in the release of glucose from the cells. Glucagon tolerance test is done with the patient being kept on a carbohydrate rich diet i.e. more than 150 gm/day for three days prior to the experiment. The blood glucose is measured in fasting condition (12-14 hours) and then the patient is given intravenous dose of 30 μg of glucagon/kg body weight. Blood glucose is estimated every half an hour after injection of glucagon and the ability of the individual to tolerate glucagon is assessed, which shows

whether the diabetes mellitus is due to excessive insulin antagonists or not. The result of glucagon tolerance is plotted in a graph as follows—



Graph—A: Injection of glucagon in normal persons or insulin non-responsive diabetes mellitus persons will lead to increase in the blood glucose level in one hour's time due to release of glucose from the cells by the action of injected glucagon and slowly decreases and falls below the base line in another half to one hour due to cessation of the action of injected glucagon (upon degradation) or in normal non-diabetic persons there will be secretion and action of insulin. Then in about the time of 2½ hours it reaches back to normal. Such a person is normal and can tolerate glucagon. If the person is a confirmed insulin independent diabetes mellitus case, then it can hereby be confirmed that the person is not responding to insulin not because of excessive insulin antagonists but may be due to defective insulin receptors on the cell membrane or antibodies to insulin.

Graph—B: In diabetes mellitus patients with excessive insulin antagonists this type of graph is obtained wherein, there will not be any increase in the blood glucose level or the increase will be very marginal upon injection of glucagon. Thus it can be confirmed that the diabetes mellitus is due to excessive insulin antagonists like glucagons, growth hormone, epinephrine, glucocorticoids, testosterone (all the hormones except insulin are insulin antagonists).

Hemoglobin A1c: It is the main fraction of glycosylated hemoglobin (glycohemoglobin) that is the hemoglobin to which glucose is bound. Hemoglobin A1c is tested to monitor the long-term control of diabetes mellitus.

The level of hemoglobin A1c is increased in the red blood cells of persons with poorly controlled diabetes mellitus. Since the glucose stays attached to hemoglobin for the life of the red blood cell (normally about 120 days), the level of hemoglobin A1c reflects the average blood glucose level over the past 4 months. The normal level for hemoglobin A1c is less than 7%. Diabetics rarely achieve such levels, but tight control aims to come close to it. Levels above 9% show poor control, and levels above 12% show very poor control. It is commonly recommended that hemoglobin A1c be measured every 3 to 6 months in diabetes. Diabetics who keep their hemoglobin A1c levels close to 7% have a much better chance of delaying or preventing diabetes complications that affect the eyes, kidneys, and nerves than people with levels 8% or higher. A change in treatment is almost always needed if the level is over 8%. Lowering the level of hemoglobin A1c by any amount improves a person's chances of staying healthy. A 1% change in an A1c result reflects a change of about 30 mg/dl (1.67 mmol/L) in average blood glucose. For instance, an A1c of 6% corresponds to an average glucose of 135 mg/dl (7.5 mmol/L), while an A1c of 9% corresponds to an average glucose of 240 mg/dL (13.5 mmol/L).

Estimation: About 90% of hemoglobin is hemoglobin A, approximately 8% of hemoglobin A is made up of minor components that are chemically slightly different. These minor components include hemoglobin A1c, A1b, A1a1, and A1a2. Hemoglobin A1c (HbA1c) is a minor component of hemoglobin to

which glucose is bound. HbA1c also referred to as glycosylated or glucosylated hemoglobin has a chemical (electrical) charge on the molecule and the amount of the charge differs from the charges on the other components of hemoglobin. The molecule of HbA1c also differs in size from the other components. HbA1c may be separated by charge and size from the other hemoglobin A components in blood by a procedure called high pressure (or performance) liquid chromatography (HPLC). HPLC separates mixtures (e.g. blood) into its various components by adding the mixtures to special liquids and passing them under pressure through columns filled with a material that separates the mixture into its different component molecules. Because HbA1c is not affected by short-term fluctuations in blood glucose concentrations, for example, due to meals, blood can be drawn for HbA1c testing without regard to when food was eaten. Since HbA1c is not influenced by daily fluctuations in blood glucose concentration, it cannot be used to monitor day-to-day blood glucose concentrations and to adjust insulin doses nor can it detect the day-to-day presence or absence of hyperglycemia or hypoglycemia.

HbA1c may be increased falsely in certain medical conditions. These conditions include uremia (kidney failure), chronic excessive alcohol intake and hypertriglyceridemia. Medical conditions that may falsely decrease HbA1c include acute or chronic blood loss, sickle cell disease or thalassemia. Diabetes during pregnancy, commonly referred to as gestational diabetes, may falsely increase or decrease HbA1c.

Conditions in which blood glucose estimation is carried out for diagnosis

1. Blood glucose is estimated in order to diagnose diabetes mellitus, wherein hyperglycemia is observed.
2. While conducting glucose tolerance test for confirmation of diabetes mellitus—wherein hyperglycemia is observed.
3. While conducting insulin tolerance test in order to confirm the type of diabetes—hypoglycemia is seen relative to 0 hour in Insulin Dependent Diabetes Mellitus (IDDM) cases.
4. While conducting glucagon tolerance test to detect the type of diabetes—if hyperglycemia is seen it indicates that diabetes is due to defective insulin receptors and if there is no change in the blood glucose level, it indicates that the diabetes is due to excessive insulin antagonists.
5. Diagnosis of hypoinsulinism due to defective pancreas—wherein hyperglycemia is seen.
6. Detection of hyperinsulinism due to pancreatic tumours—wherein hypoglycemia is seen.
7. To assess glycogen storage diseases due to defective enzymes of glycogen metabolism in the liver—hypoglycemia is seen.
8. While performing epinephrine tolerance test—hyperglycemia is seen. If hypoglycemia is exhibited it indicates glycogen storage diseases.
9. During oral leucine administration test—hypoglycemia is seen, since leucine taken orally enhances insulin production.
10. During insulin therapy—hypoglycemia occurs. If hypoglycemia is very severe and persists for longer period, then it indicates that the insulin dose for treatment of diabetes mellitus is more than required and it has to be reduced.
11. Detection of excessive insulin antagonists—hyperglycemia.
12. During renal dysfunction hypoglycemia is seen due to non-reabsorption of filtered glucose by the kidney tubules. In total renal failure there is relatively hyperglycemia because glucose is neither filtered nor excreted.

13. In the assessment of hypocalcemia blood glucose estimation can be done, wherein there will be hyperglycemia because insulin cannot be released in absence of Ca and Zn from the islets of langerhans of pancreas.
14. To assess the intestinal status of carbohydrates, rich carbohydrate diet creates hyperglycemia and poor carbohydrate diet creates relative hypoglycemia.
15. Post exercise blood glucose level will be more i.e., hyperglycemia. In regular athletes there will be relative hypoglycemia.

12

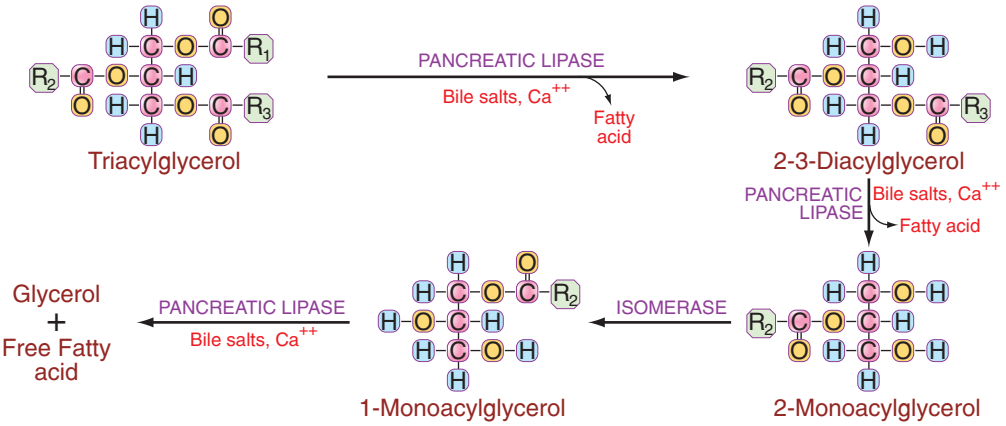
LIPID METABOLISM

DIGESTION AND ABSORPTION

Salivary juice produces a lipid hydrolyzing enzyme, especially in infants and children, known as lingual lipase. Lipids are not digested in the mouth, though lingual lipase is present because the high pH of the mouth does not favour the action of lingual lipase and also because the food remains here for a short period of time. As soon as the lipids enter the stomach, they get liquefied due to the heat of the stomach. Further, the lipids get emulsified due to the peristaltic movements of the stomach. The gastric juice contains gastric lipase, which is inactive at the low pH of the stomach, whereas lingual lipase is active at this pH and it hydrolyses triacylglycerols of short chain fatty acids (which are generally found in milk, so digestion in the stomach is seen only in children). The released fatty acids are absorbed via the stomach wall and enter the portal vein. The TAG with longer chain fatty acids dissolve and form fat droplets and finally enter the intestine.

In the intestine, the bile salts (sodium glycocholate and sodium taurocholate) and bile acids (cholic acid, chenodeoxycholic acid and cholesterol) help in emulsifying fats thereby making them susceptible to the digestive enzymes. The pancreatic secretions in the intestine contain pancreatic lipase which along with the help of a protein called co-lipase and lecithin acts on TAG at the water-oil interface.

Phospholipids are hydrolyzed by phosphorlipase A, B, C and cholesterol is hydrolyzed by cholesterol esterase. The end products of fat digestion are (1) Monoacylglycerols (MAG) (2) Diacylglycerols (DAG) (3) Triacylglycerols (TAG) (4) Free fatty acids and (5) Glycerol.



Digestion of lipids takes place for a longer duration of time. Until and unless digestion of fats has not taken place, other food materials (carbohydrates and proteins) cannot be digested because the fats will cover the food and prevent enzymes reaching the food (hence take a fatty meal while going on a long journey).

The digested lipids enter the intestinal epithelium by diffusion or by a process called **'pinocytosis'**. In the intestinal wall the free fatty acids and glycerol re-aggregate to form TAG i.e. here re-synthesis of digested TAG, phospholipids and cholesterol takes place (in order to maintain the concentration gradient of lipids). Now these lipids surround a little amount of protein around them to form chylomicrons. These chylomicrons enter the lacteals by reverse pinocytosis which then enter the thoracic duct and finally to the systemic circulation (sub-clavical vein). The chylomicrons of the circulation move, towards the adipose tissue, heart, kidney, liver and skeletal muscle. The capillary walls of these tissues contain an enzyme called lipoprotein lipase or clearing factor or serum lipase which hydrolyzes the lipids of the chylomicrons and help in their entry into the respective tissue. The lipids are stored as triacylglycerols mainly in the adipose tissue (sub-cutaneous tissue).

Abnormalities relating to lipid digestion:

1. **Steatorrhoea:** It is a condition wherein large amounts of lipids are excreted in faeces. This may be either due to the obstruction to the flow of bile in the intestine or due to defect in absorption of the digested lipids.
2. **Chyluria:** It is a condition wherein large amounts of chylomicrons are excreted in the urine. This may be due to obstruction in the transportation of chylomicrons form the lacteals to the thoracic duct due to which there will be an abnormal connection between the urinary tract and the lymphatic drainage system of the intestine.

LIPOLYSIS

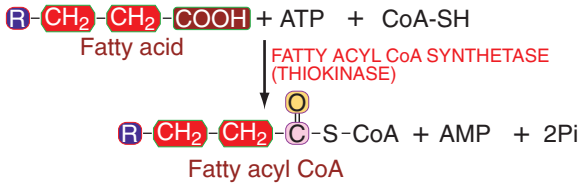
Lipolysis is a process of breakdown of lipids. Large amounts of fats are stored in adipose tissue as triacylglycerols (TAG). When the energy requirements of the body are not met by carbohydrates, then fats play an important role in the production of energy. The adipose tissue TAG break down into glycerol and free fatty acids (FFA) by the enzyme **'hormone-sensitive lipase'**. This lipase is sensitive to hormones (or is activated by those hormones) which tend to raise the blood glucose level. Ex. Glucagon, growth hormone, adrenaline, noradrenaline, ACTH, & TSH. This lipase is inhibited by the hormones that reduce the blood glucose level viz. insulin. The glycerol released from the TAG diffuses out into the blood and is utilized by the liver and kidney for synthesis of glucose by gluconeogenesis. The FFA released combine with albumin to form FFA-albumin complex which are transported through the blood to various tissues. However in the cytosol of each cell there is a little amount of fat droplets, these are degraded by the lipases to glycerol and FFA.

Knoop's β-oxidation of fatty acids: Oxidation of the fatty acids at the β-carbon atom to a carboxylic group is known as beta oxidation. This was proposed by the scientist Knoop, hence the name. The steps involved in the β-oxidation of fatty acids are—

1. Activation of fatty acids
2. Formation of α-β unsaturated acyl-CoA (Enoyl-CoA)
3. Formation of β-hydroxy acyl-CoA
4. Formation of β-keto acyl-CoA
5. Thiolytic cleavage of keto acyl-CoA

1. **Activation of fatty acids:** Oxidation of fatty acids can take place only if they are activated. The fatty acids are activated in the cytosol of the cell wherein the enzyme fatty acyl CoA synthetase

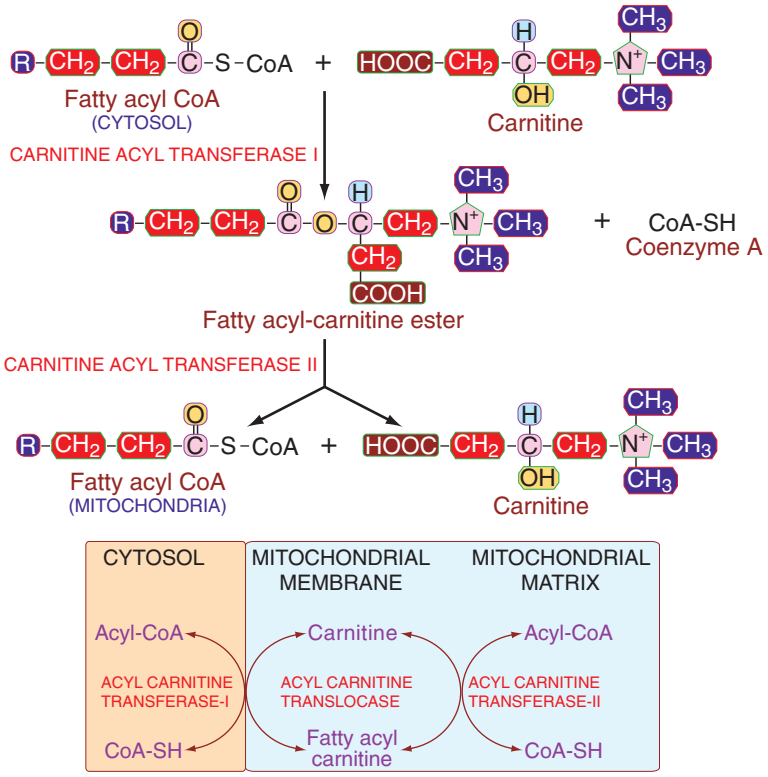
(thiokinase) condenses the fatty acids with coenzyme-A by esterification which requires two high energy bonds.



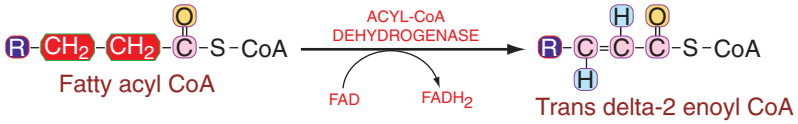
Further oxidation of fatty acids takes place in the mitochondrial matrix. The mitochondrial membrane is impermeable to the acylated fatty acids of the cytosol. Hence these are transported by carnitine mechanism after activation.

***Carnitine mechanism*:** Carnitine (beta-hydroxyl-gamma-trimethyl-ammonium butyrate) [$(CH_3)_3$–N^+–CH_2–CH(OH)–CH_2–COOH], is present in the inner mitochondrial membrane and it helps in the transport of fatty acids both form the cytosol to mitochondria and from mitochondria to the cytosol. The mechanism of carnitine transfer is as follows—

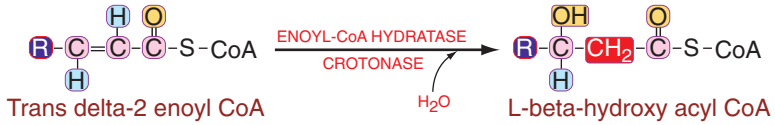
Carnitine reacts with activated fatty acids in presence of an enzyme carnitine acyl transferase-I, forming fatty acyl-carnitine complex and CoA-SH. Now this complex is easily transported through the inner mitochondrial membrane through a transport protein called carnitine acyl carnitine translocase. In the inner surface of the membrane another enzyme carnitine acyl transferase-II hydrolyzes the fatty acyl carnitine to give fatty acyl-CoA and carnitine. Thereby carnitine is free for reutilization and fatty acyl-CoA undergoes further oxidation in the mitochondrial matrix.



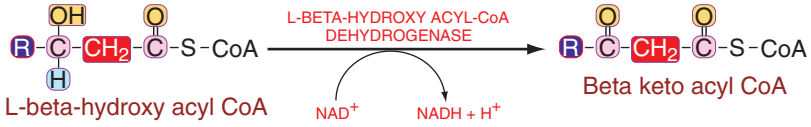
2. **Formation of α–β unsaturated acyl-CoA (Enoyl–CoA):** The fatty acyl-CoA undergoes dehydrogenation at the α and β-carbon atoms forming trans alpha-beta unsaturated acyl-CoA. These hydrogens are taken up by the coenzyme, Flavin Adenine Dinucleotide (FAD) which gets converted to $FADH_2$. Oxidation of $FADH_2$ through electron transport chain produces two ATPs. The enzyme is fatty acyl CoA dehydrogenase.



3. **Formation of β–hydroxy acyl-CoA:** Enoyl-CoA is then hydrated by the enzyme crotonase (Enoyl-CoA hydratase) which adds water across the double bond. The product formed is β-hydroxy acyl-CoA.

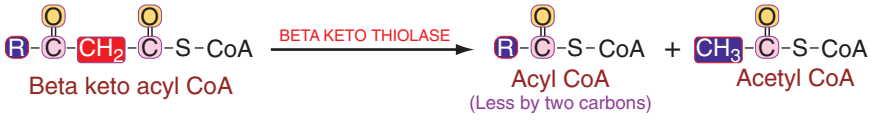


4. **Formation of β-keto acyl-CoA:** β-Hydroxy acyl-CoA undergoes another dehydrogenation of the β-oxidation process forming β-keto acyl-CoA. The enzyme is β-hydroxy acyl-CoA dehydrogenase and its redox potential allows it to use NAD^+ as the coenzyme releasing NADH + H^+ that produces 3 ATPs upon oxidation through ETC.

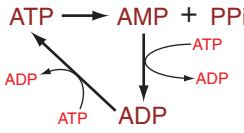


5. **Thiolytic cleavage of β–keto acyl-CoA:** β-Keto acyl-CoA is then cleaved between the α and β-carbon atom releasing an acetyl-CoA and a fatty acyl-CoA shortened by two carbon atoms. The enzyme is β-keto thiolase that uses the coenzyme A for adding to the newly formed acyl-CoA.

The fatty acids undergo oxidative removal of two carbon units from the carboxylic side in the form of acetyl-CoA, which repeats till the fatty acid is completely converted to acetyl-CoA.



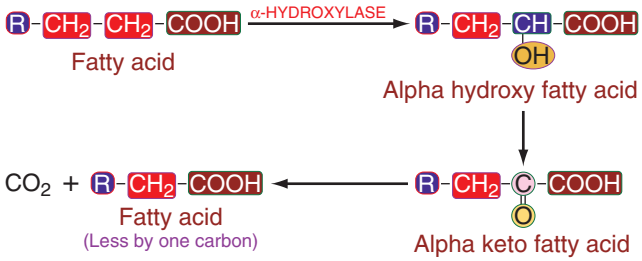
Calculation of total ATP produced when palmitic acid is completely oxidized to CO_2 and H_2O: 16 Carbon palmitic acid (i.e., the most abundant fatty acid in the human body) undergoes 7 such passes or cycles of β-oxidation, producing a total of 8 acetyl-CoA which are in turn oxidized through TCA cycle and the reducing equivalents produced are oxidized through ETC.

The number of ATPs produced by palmitic acid		
Name of the enzyme / process	**Number of reducing equivalents / ATP produced**	**Total number of ATP**
Fatty acyl CoA dehydrogenase	7 $FADH_2$ × 2	14
β-hydroxyacyl CoA dehydrogenase	7 NADH + H^+ × 3	21
	Total ATPs produced in β-oxidation	**35 ATP**
Acetyl CoA (8) oxidized through TCA cycle (each cycle=12 ATP)	8 × 12	96
	Total ATP produced both in β-oxidation and TCA cycle	**131**
ATPs utilized	Activation step ATP → AMP 	2
	NET GAIN OF ATPs	**129**
Totally 129 ATP's are produced when palmitic acid is oxidized completely		

α-Oxidation of fatty acids: Oxidation of the fatty acids at the alpha carbon atom is known as alpha oxidation. During α-oxidation there is removal of one carbon atom at a time from the carboxyl end of the molecule. Alpha oxidation takes place for the alpha hydroxy fatty acids which are found in the brain. During alpha oxidation, first a hydroxyl group is attached (if the fatty acid is devoid of alpha hydroxyl group) at the alpha carbon atom which is then oxidized to a keto group and then finally to a carboxylic group due to decarboxylation of the original carboxyl group giving rise to a fatty acid less by one carbon atom.

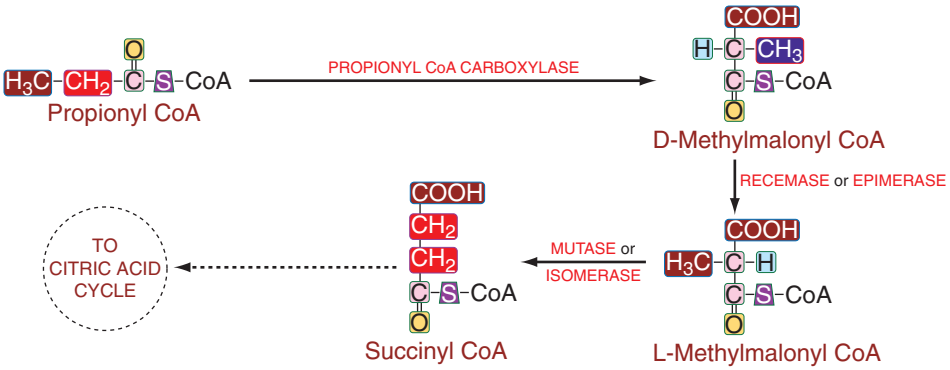
Characteristic features of α-oxidation:

1. It does not require CoA intermediates.
2. It does not generate high energy phosphate bonds.

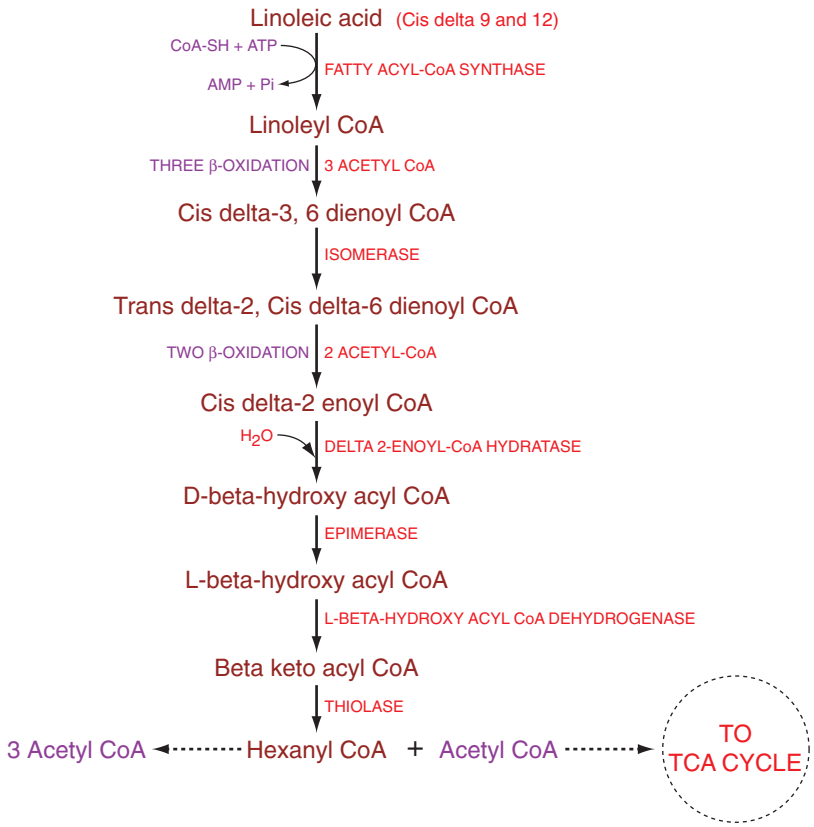


Omega oxidation: Oxidation of the omega carbon atom of the fatty acid is known as omega oxidation. During omega oxidation there is a successive removal of four carbon atoms at a time. It predominantly occurs in the liver, whenever there is a high demand of energy as in the case of lactation, severe diabetics and starvation. During omega oxidation first of all, with the help of hydroxylase enzyme a hydroxyl group is attached to the last carbon atom ($-CH_3$ i.e., methyl group of the omega carbon atom), which is then further oxidized to a carboxylic group to form an alpha omega dicarboxylic acid. Now beta oxidation occurs from both the ends producing two acetyl-CoA at a time.

Oxidation of fatty acids with an odd number of carbon atoms: Fatty acids with an odd number of carbon atoms are normally oxidized by beta oxidation but the last cycle of the beta oxidation produces acetyl-CoA and propionyl-CoA (a 3 carbon moiety). This propionyl-CoA is further oxidized in the following manner—



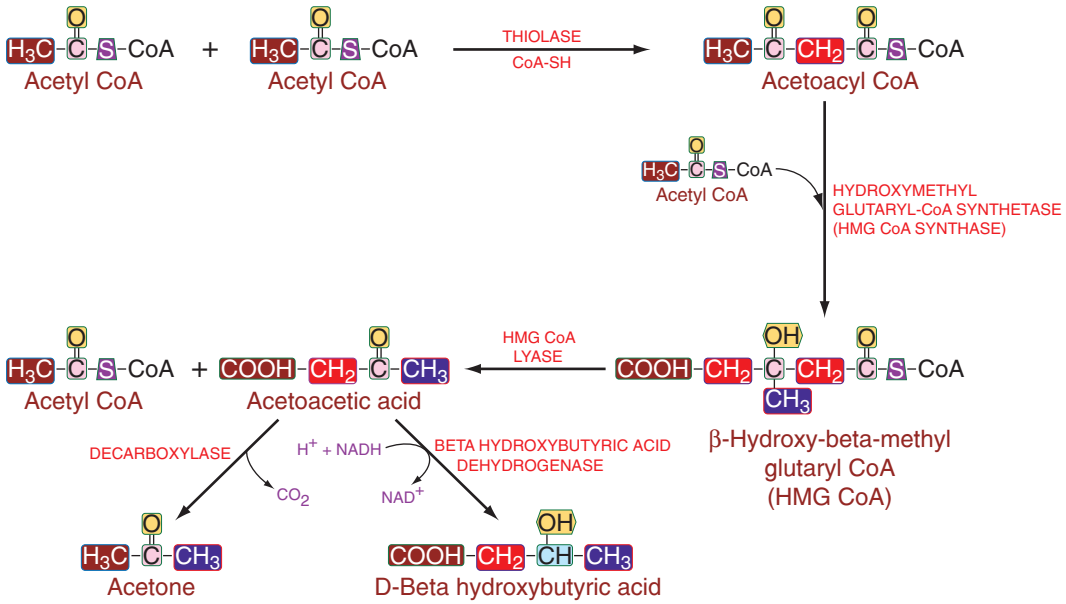
Oxidation of unsaturated fatty acids: The 18 carbon linoleic acid is an unsaturated fatty acid, containing two cis double bonds (1) between carbon atoms 9 & 10 (cis Δ^9) and (2) between carbon atoms 12 & 13 (cis Δ^{12}). In its oxidation, three cycles of beta oxidation take place giving rise to 3 acetyl-CoA and *cis*-delta-3,6-dienoyl-CoA, which is isomerised to trans-delta-2-*cis*-delta-6-dienoyl-CoA, which then undergoes two passes of beta oxidation forming two acetyl-CoA and *cis*-delta-2-enoyl-CoA. This is converted to D-β-hydroxy acyl-CoA by the enzyme delta-2-enoyl-CoA hydratase. D-β-hydroxy acyl-CoA cannot be acted upon by the stereo-specific enzyme; L-β-hydroxy acyl-CoA dehydrogenase. Hence it is epimerized by an epimerase to give L-β-hydroxy acyl-CoA and then normal beta oxidation continues till the fatty acid is completely converted to acetyl-CoA.



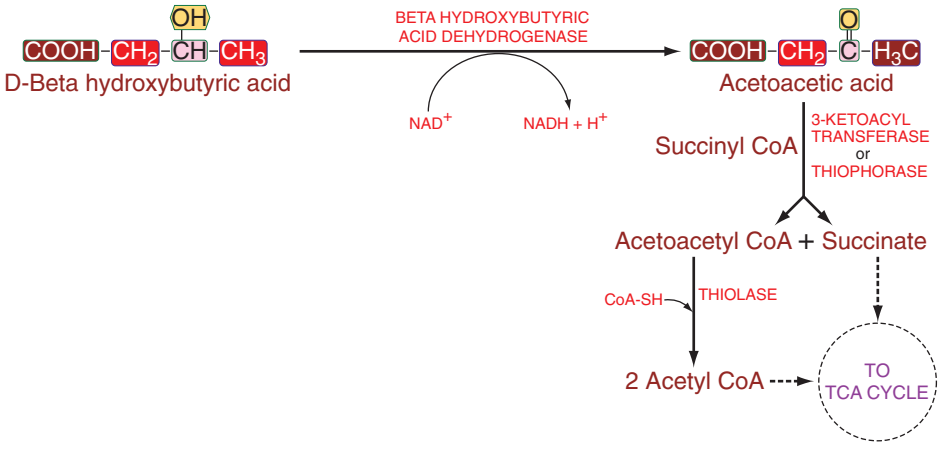
KETONE BODIES

There are three ketone bodies namely (1) Acetoacetic acid (2) Beta hydroxy butyric acid and (3) Acetone. Ketone bodies are also known as **'acetone bodies'**. Formation of ketone bodies is known as ketogenesis. The ketone bodies are synthesized in the liver even under normal conditions.

Synthesis and utilization of ketone bodies: The ketone bodies are synthesized in the liver by the following reaction mechanism—



These ketone bodies cannot be utilized by the liver because the enzymes needed to activate them are absent or low in activity and hence these ketone bodies are supplied to the peripheral tissues for oxidation. In the peripheral tissues the ketone bodies are utilized in the following manner—



Only acetoacetic acid and beta hydroxybutyric acid are easily oxidized by the extrahepatic tissue. Oxidation of acetone is difficult; hence it is excreted in urine in large amounts than other ketone bodies. Acetone is also eliminated through the lungs; hence starvation and diabetic patients show an alcoholic smell in their breath.

ROLE OF KETONE BODIES AS AN AID TO DIAGNOSIS

There are three ketone bodies viz., (1) Acetoacetic acid; (2) Beta hydroxy butyric acid; (3) Acetone. Ketone bodies are also known as Acetone bodies. Formation of ketone bodies is known as ketogenesis. The ketone bodies are synthesized in the liver even under normal conditions. The ketone bodies are produced from Acetyl CoA which comes from three sources. (1) Glucose (2) Fatty acids and (3) Amino acids.

Normal levels of ketone bodies in human beings: The normal level of ketone bodies in the blood of human beings is less than 1 mg/dl and is excreted in the urine to less than 1 gm/day.

Methods of estimation of Ketone Bodies: The presence or absence of ketone bodies in the urine is detected by Rothera's test. Rothera's test is also carried for the semi-quantitative estimation of ketone bodies in the blood, wherein the results obtained are—

(–ve) — Ketone bodies are less than 1 mg/100 ml of the blood

(+ve) — Ketone bodies are little more than 1 mg/100 ml of the blood

(++ve) — Ketone bodies are about 1.5 mg/100 ml of the blood

(+++ve) — Ketone bodies are more than 2 mg/100 ml of the blood

There are two methods for the quantitative estimation of ketone bodies in blood (1) Microdiffusion method (2) Enzymatic method.

The ketone bodies synthesized by the liver will continuously be utilized by the peripheral tissues. The peripheral tissues have a limited capacity to utilize the ketone bodies. If the production of ketone bodies by the liver exceeds the capacity of the peripheral tissues to utilize them, as in diabetics and starvation, then this results in accumulation of ketone bodies in blood; a condition known as ketonemia and consequently there will be an increased excretion of ketone bodies in urine; known as ketonuria. Both ketonemia and ketonuria together are known as ketosis.

Ketosis: Ketosis or keto acidosis is a condition in which there is an increased accumulation of ketone bodies in the blood (ketonemia) and consequently increased excretion in urine (ketonuria).

Biochemical changes in ketosis

1. Acetoacetic acid and Beta hydroxyl butyric acid are strong acids; their accumulation causes ketoacidosis, thereby lowering the pH of blood.
2. Buffering capacity is disrupted because bicarbonate of the blood decreases.

   The low pH caused by ketosis leads to disturbance in the normal buffering mechanism of the blood. This leads to emergence of yet another buffering system in the blood i.e., the muscle proteins are released and hydrolyzed to amino acids which are oxidized releasing ammonia (NH_3). NH_3 takes up H^+ ions to form NH_4^+ and thus compensates the acidity of blood. Ammonium ion is more destructive thereby causing more harm to the individual.
3. Along with ketone bodies large amounts of H_2O and Na^+ ions are lost leading to electrolyte imbalance and dehydration.

Symptoms: Depression, thirst, fatigue and coma.

Clinical conditions in which ketosis occurs

(1) **Starvation:** Includes both post fasting period i.e. 12-24 hours after meal or continuous starvation for days together. During this condition there may be lack of glucose leading to non entry of glucose into adipose tissue resulting in lowered glycolysis and low intermediates of glycolytic pathway. Low concentration of glyceraldehyde-3-phosphate, an intermediate of glycolysis cannot be converted to

glycerol phosphate and therefore there will be no re-esterification of fatty acids resulting in the release of fatty acids from adipose tissue into the blood. As the period of starvation increases, the glucose concentration in the blood decreases leading to increased release of fatty acids producing more amounts of ketone bodies much more than the peripheral tissues can use them, causing ketosis. The ketone bodies are utilized by all the extrahepatic tissues except brain in the initial stages. After three weeks of starvation, brain also shifts to the utilization of ketone bodies which leads to the destruction of brain cells due to ketosis.

(2) **Diabetes mellitus:** Though glucose is present in large quantities in the blood, it cannot be utilized by the cells, results in the release of fatty acids and overproduction of ketone bodies causing ketoacidosis.

(3) **Pregnancy:** During the third trimester of pregnancy, the demand for glucose is doubled and hence there will be overproduction of ketone bodies leading to ketoacidosis.

(4) **Lactation:** During lactation more energy is required because glucose is utilized for - (i) Production of lactose (ii) Formation of milk fat and (iii) Synthesis of milk protein casein.

This leads to depletion of glucose to adipose tissue resulting in more release of fatty acids producing more ketone bodies leading to ketosis. Ketosis is generally accompanied with low calcium levels which are referred to as milk fever. This is developed within hours in lactating mothers having twins or more babies which is characterized by sudden fall in blood pH and decrease in milk production.

(5) **Febrile diseases:** In fever causing disease there is a demand for glucose for the formation of antibodies thereby depleting glucose to adipose tissue leading to ketosis.

(6) **Heavy exercise:** Heavy physical exercise suddenly raises the level of ketone bodies and if the exercise is continued without intake of glucose then it may result in ketosis.

Control: Starvation ketosis can be controlled by injecting anti-ketogenic substances like glucose and glucose producers like glycerol and glucogenic amino acids like glycine, glutamic acid, alanine, serine etc.

BIOSYNTHESIS OF FATTY ACIDS

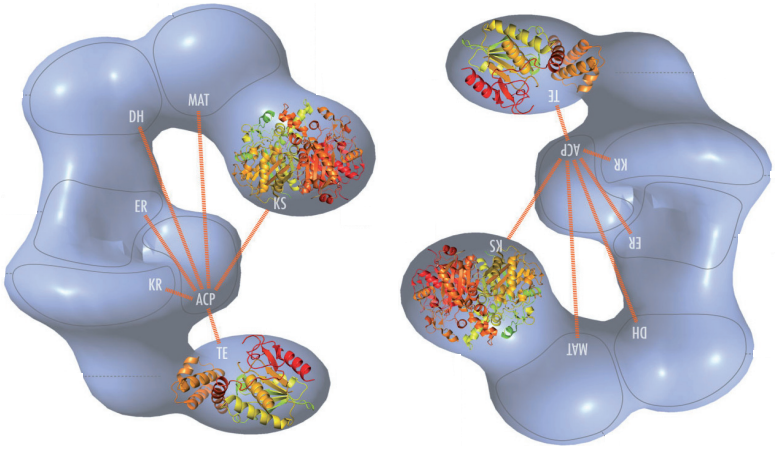
Fatty acids are synthesized in the liver and adipose tissue. They are also synthesized in other tissues but to a small extent. Palmitic acid (16 C) is the only fatty acid synthesized in the human body; all the other fatty acids are derived from this fatty acid. Acetyl-CoA serves as the source of carbon for the synthesis of fatty acids. The steps involved in fatty acid synthesis are—

Formation of malonyl-CoA from acetyl-CoA and bicarbonate: Malonyl-CoA is the compound that participates in each cycle of fatty acid biosynthesis and this is synthesized from acetyl-CoA. The enzyme acetyl-CoA carboxylase or carboxykinase is a biotin bound enzyme that takes up CO_2 and then transfers it to acetyl-CoA forming malonyl-CoA. This is the committed step in fatty acid synthesis, acetyl-CoA carboxylase is a regulated enzyme. A cell in a low energy state will not synthesize fatty acids.

In eukaryotes, synthesis of fatty acids takes place on a large, multifunctional enzyme complex called fatty acid synthase (FAS) formed from a single polypeptide chain. This complex is arranged as a dimer with the two units placed head to tail.

Mammalian FAS consists of two identical multifunctional polypeptides, in which three catalytic domains in the 'N-terminal' section—ketoacyl synthase (KS), malonyl / acetyl-transferase (MAT), and dehydrase (DH)), are separated by a core region of 600 residues from four 'C-terminal' domains—enoyl reductase (ER), ketoacyl reductase (KR), acyl carrier protein (ACP) and thioesterase (TE).

The fatty acid synthase has two types of active sulfhydryl groups. One is provided by phosphopantetheine group of an acyl carrier protein (ACP). The other sulfhydryl group is furnished by a specific cysteine residue of 3-ketoacyl-ACP synthase enzyme. The growing fatty acid chain is always attached to the phosphopantetheine group of ACP.

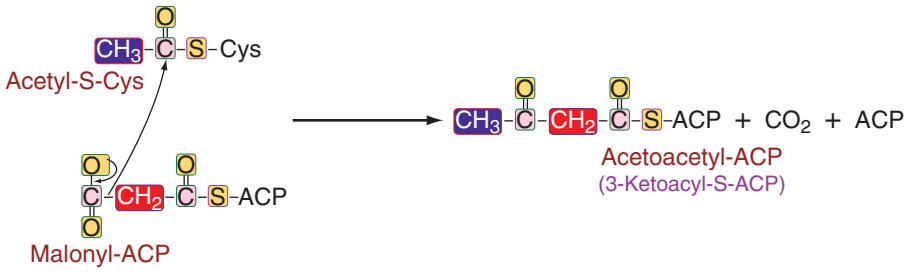


The first reaction of fatty acid synthesis is the transfer of the starting units, acetyl-CoA and malonyl-CoA, from coenzyme-A to the ACP. Acetyl-CoA is transferred to the –SH group of cysteine and malonyl-CoA is transferred to –SH group of ACP.

$$\text{Acetyl-CoA} + \text{KS-Cys-SH} \rightleftharpoons \text{Acetyl-S-Cys} + \text{CoA}$$

$$\text{Malonyl-CoA} + \text{ACP} \rightleftharpoons \text{Malonyl-S-ACP} + \text{CoA}$$

New fatty acid is formed by condensation between the acetyl-S-Cys, contained on the enzyme 3-ketoacyl synthase and the malonyl-S-ACP to form acetoacetyl-S-ACP with the help of the same enzyme 3-ketoacyl synthase.

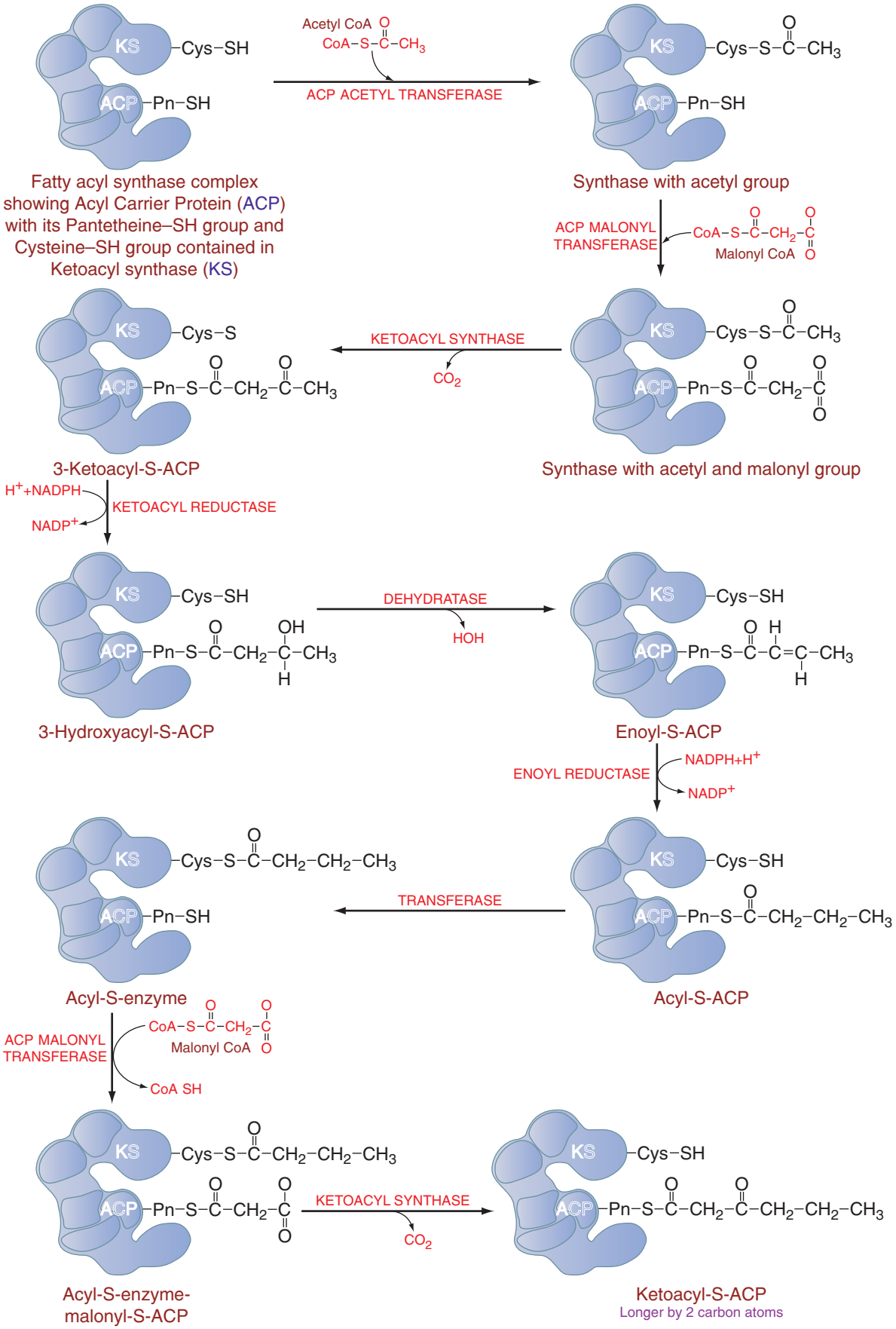


As in the case of degradation of fatty acids, their synthesis is performed through a cycle of four reactions. Each of these is essentially the opposite reaction of the degradation pathway, but it is not simply a reversal using the same enzyme systems.

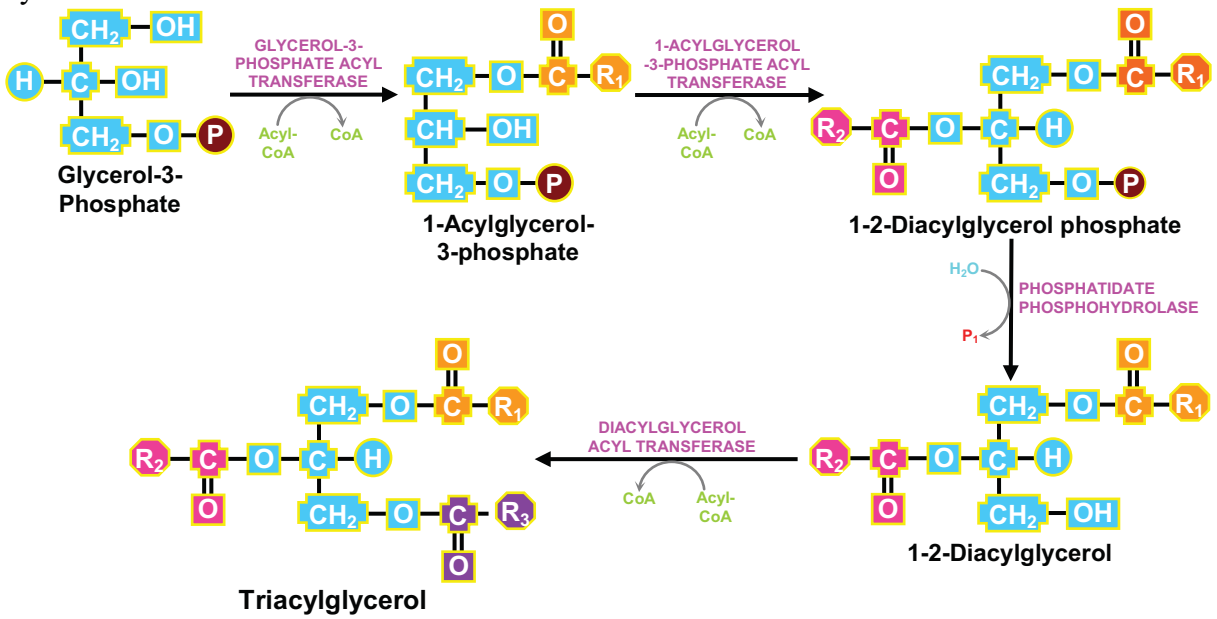
1. The acetoaceyl-S-ACP undergoes reduction at the carbonyl group to form 3-hydroxyacyl-S-ACP. This reaction is catalyzed by the enzyme 3-ketoacyl-ACP reductase which uses NADPH as electron donor.
2. 3-hydroxyacyl-S-ACP is dehydrated by 3-hydroxyacyl-ACP dehydratase to form trans-Δ^2-enoyl-S-ACP.
3. The last reaction of first cycle of fatty acid synthesis is conversion of enoyl-S-ACP to saturated acyl-S-ACP by the action of the enzyme enoyl-ACP reductase which uses another NADPH as electron donor.

4. The acyl group is now transferred from the phosphopantetheine-SH group to the cysteine-SH group i.e. yet another active group on the same enzyme complex. Thus phosphopanthetheine-SH group is now free to receive yet another malonyl-CoA.

Throughout these reactions, the fatty acid chain remains linked to the ACP on the fatty acid synthase. The cycle proceeds through 7 turns to yield palmitoyl-ACP, an acyl-ACP with 16 carbon atoms. Synthesis of fatty acids by the FAS complex stops after sixteen carbons have been added (palmitate) and further elongation and the addition of double bonds are carried out by other systems. The ACP bond is hydrolyzed with the help of the enzyme hydrolase, to release the free fatty acid which almost immediately reacts with coenzyme-A to form an acyl-CoA in the cell. During synthesis of fatty acids, NADPH is used as a reducing agent, provided by HMP pathway. The diagramatic representation of the steps involved in the biosynthesis of a fatty acid are given on next page.

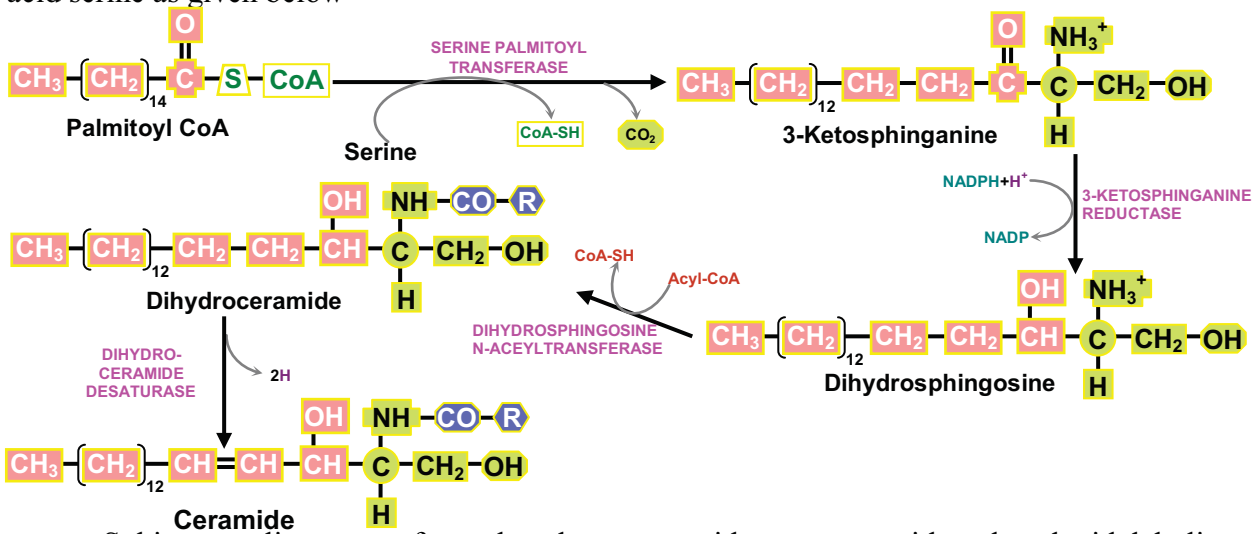

KS
ACP
Cys–SH
Pn–SH
Acetyl CoA
CoA–S–C–CH3
ACP ACETYL TRANSFERASE
Cys–S–C–CH3
Pn–SH
Fatty acyl synthase complex showing Acyl Carrier Protein (ACP) with its Pantetheine–SH group and Cysteine–SH group contained in Ketoacyl synthase (KS)
Synthase with acetyl group
ACP MALONYL TRANSFERASE
CoA–S–C–CH2–C
Malonyl CoA
Cys–S
Pn–S–C–CH2–C–CH3
KETOACYL SYNTHASE
CO2
Pn–S–C–CH2–C
3-Ketoacyl-S-ACP
Synthase with acetyl and malonyl group
H+ +NADPH
NADP+
KETOACYL REDUCTASE
OH
H
DEHYDRATASE
HOH
3-Hydroxyacyl-S-ACP
Pn–S–C–C=C–CH3
Enoyl-S-ACP
ENOYL REDUCTASE
NADPH+H+
NADP+
Cys–S–C–CH2–CH2–CH3
Pn–SH
TRANSFERASE
Pn–S–C–CH2–CH2–CH3
Acyl-S-enzyme
Acyl-S-ACP
ACP MALONYL TRANSFERASE
CoA–S–C–CH2–C
Malonyl CoA
CoA SH
Pn–S–C–CH2–C
KETOACYL SYNTHASE
CO2
Pn–S–C–CH2–C–CH2–CH2–CH3
Acyl-S-enzyme-malonyl-S-ACP
Ketoacyl-S-ACP
Longer by 2 carbon atoms

Biosynthesis of triacylglycerols: Two molecules of acyl-CoA, formed by the activation of fatty acids by acyl-CoA synthetase, combine with glycerol 3-phosphate to form phosphatidate (1,2-diacylglycerol phosphate). This takes place in two stages, catalyzed by glycerol 3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase. Phosphatidate is converted by phosphatidate phospho-hydrolase and diacylglycerol acyltransferase (DGAT) to 1,2-diacylglycerol and then to triacylglycerol. DGAT is the regulating enzyme in triacylglycerol synthesis.



Separately, choline and ethanolamine are activated due to phosphorylation by ATP followed by linkage to CTP resulting in the formation of CDP-choline or CDP-ethanolamine which then reacts with 1,2-diacylglycerol to form phosphotidylcholine or phosphotidylethanolamine respectively. Phosphotidylserine is formed from phosphatidylethanolamine directly by reaction with serine.

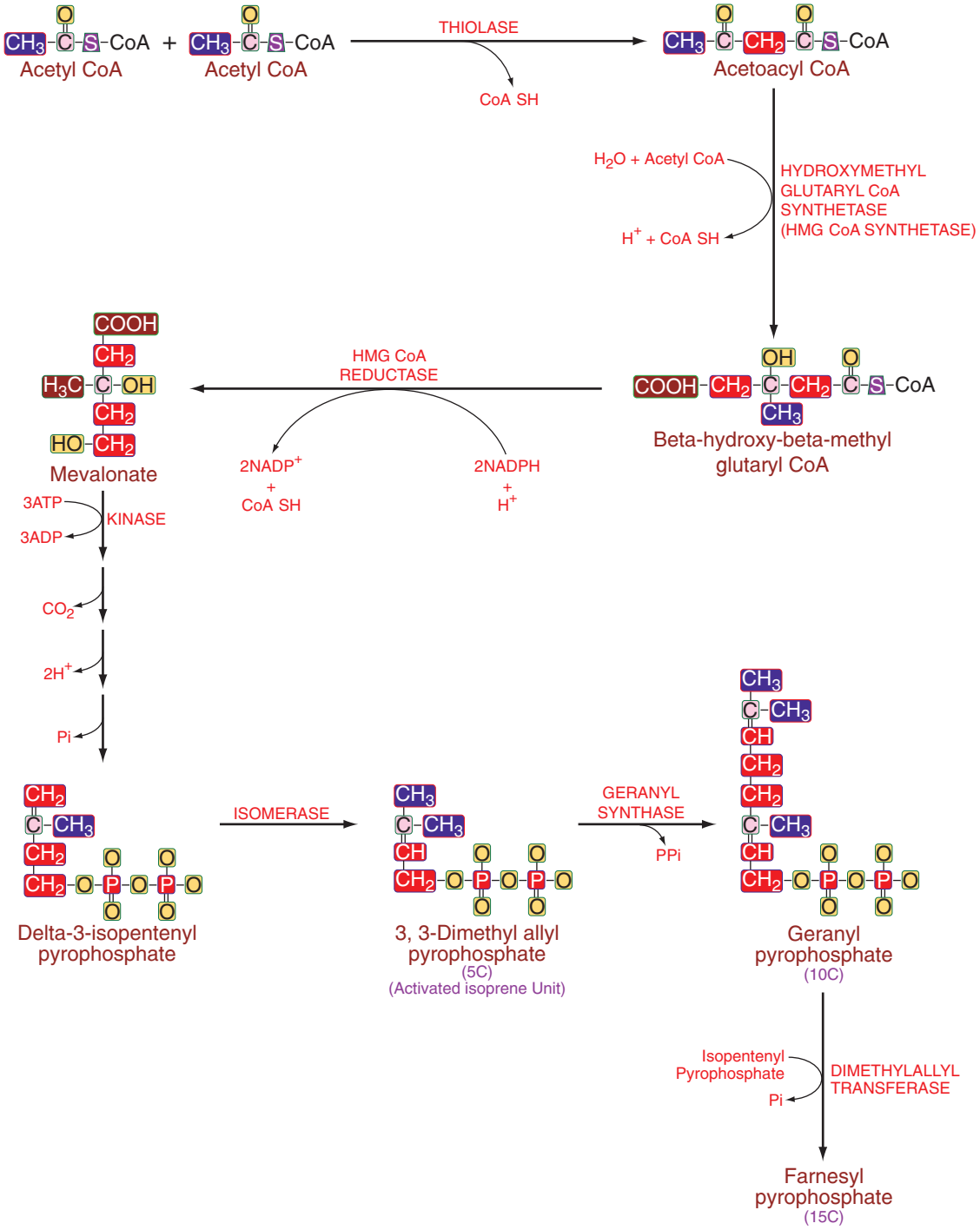
Biosynthesis of sphingolipids: All sphingolipids are formed from ceramide i.e. sphingol and fatty acid (Sphingol is an amino alcohol with a chain length of 18 carbons having a double bond at trans delta 4 position). Ceramide is synthesized in the endoplasmic reticulum from the amino acid serine as given below -

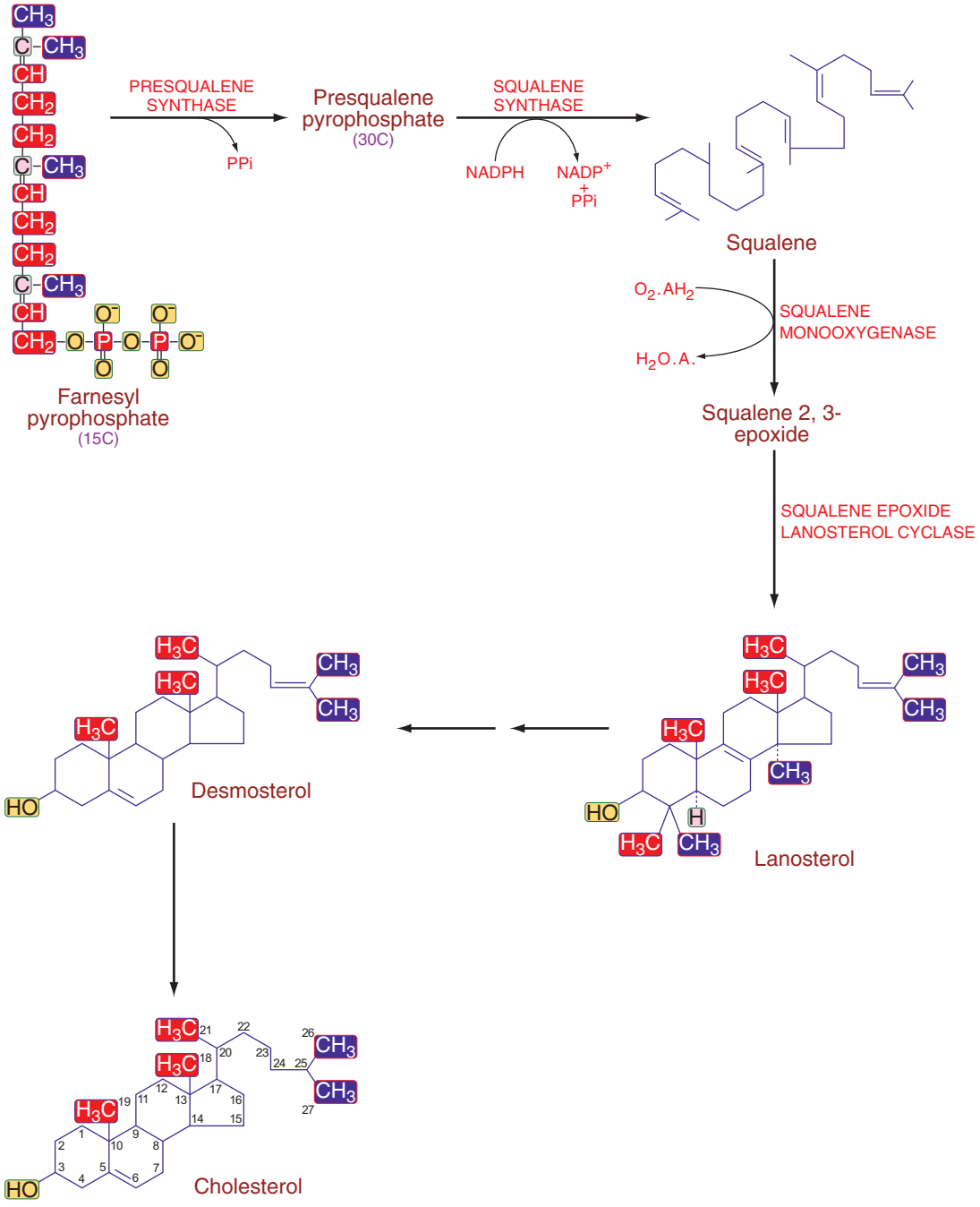


Sphingomyelins are formed when ceramide reacts with phosphatidylcholine. Glycophingolipids (cerebrosides) are glucosylceramide which is formed in a reaction between ceramide and UDP-glucose (UDPglc). Similarly galactoceramide is formed by reaction between ceramide and UDP-galactose (UDPgal). Gangliosides are formed from ceramide by stepwise addition of activated sugars i.e. UDPglc, UDPgal, sialic acid and N-acetyl-neuraminic acid.

BIOSYNTHESIS OF CHOLESTEROL

Average diet supplies about 0.3 grams of cholesterol/day, but over 1 gram of cholesterol is synthesized in the body. Liver is the main site of cholesterol synthesis. Other organs are adrenal glands, testis, ovaries, skin, adipose tissue, muscle and brain. The steps involved in the synthesis of cholesterol are—

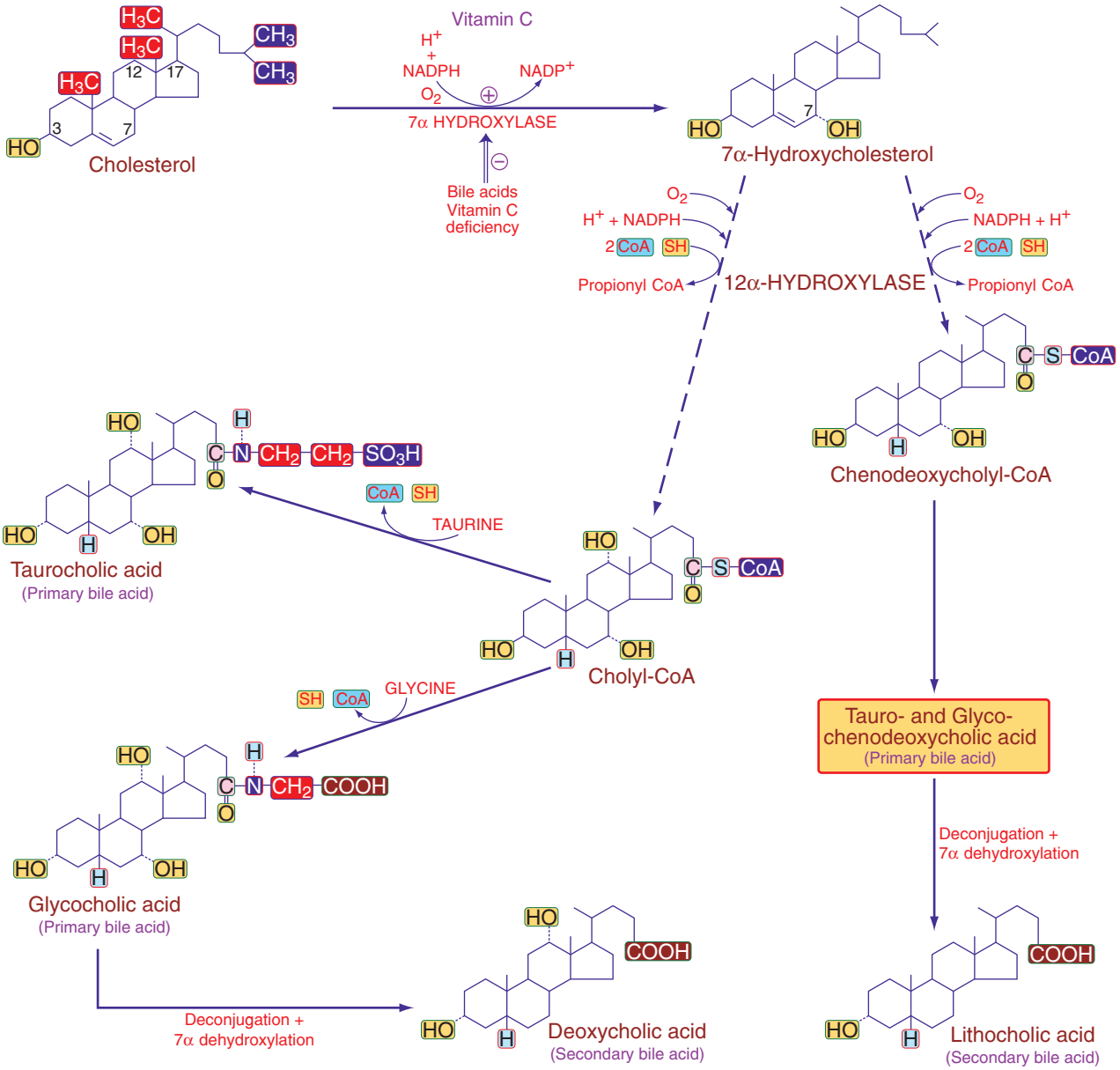




Two molecules of acetyl-CoA combine by thiolase to form acetoacetyl-CoA which takes up another acetyl-CoA forming HMG-CoA in presence of the enzyme HMG-CoA synthetase. HMG-CoA then undergoes the committed step (rate limiting step) catalyzed by HMG-CoA reductase to form mevalonate, a five carbon compound. Mevalonate is activated to an isoprene unit in a series of reaction steps. The isoprene unit then multiplies exponentially to form a 10 carbon geranyl pyrophosphate which in turn forms a 15 carbon farnesyl pyrophosphate and two farnesyl pyrophosphates combine to form a 30 carbon squalene. Squalene is further modified to lanosterol and then to desmosterol finally forming cholesterol.

Regulation of cholesterol biosynthesis: Cholesterol synthesis is regulated mainly at the HMG-CoA reductase step. Whenever there is excess of the end product cholesterol and its intermediate mevalonate there is feed back inhibition of HMG-CoA reductase. This enzyme activity is also regulated by phosphorylation (inactivated) with glucagon and epinephrine and dephosphorylation (activated). Other factors which control cholesterol biosynthesis are sterol carrier protein and cholesterol containing plasma lipoprotein.

Failure in the regulation of cholesterol biosynthesis is one of the factors involved in the pathological process of atherogenesis i.e. the formation of cholesterol and lipid rich deposits in arteries and arterioles. These deposits can limit blood flow and cause heart attack or strokes by depriving the tissue of an adequate supply of oxygen.



Biosynthesis and degradation of bile acids

Metabolism of bile acids:

Functions of bile acids: The bile acids are the glycocholic acid, taurocholic acid and glycochenodeoxycholic acid. They are good emulsifying agents, they convert fat into fat droplets and expose them to aqueous environment. Bile salts stabilize the smaller particles by preventing them from coalescing.

LIPOPROTEIN METABOLISM

Transport of lipids to various parts of the body through blood plasma: Lipids are hydrophobic and hence they cannot pass freely through the blood plasma which is hydrophilic. Thereby they combine with hydrophilic proteins, to form lipoproteins, so as to be transported through the blood to various organs. Various types of plasma lipoproteins are (1) Chylomicrons (2) Very low density lipoproteins (VLDL) (3) Low density lipoproteins (LDL) (4) High density lipoproteins and (5) Albumin-free fatty acid complex.

1. **Chylomicrons:** They are of intestinal origin. The protein component of chylomicrons is known as apoprotein-B. The formation of chylomicrons in the intestine takes place continuously. During fasting state the chylomicrons carry the lipids derived mainly from bile and intestinal secretions. The formation of chylomicrons increases after meals, especially after a fat rich meal, the plasma becomes opalescent (milky) due to high concentration of chylomicrons. The chylomicrons constitute of about two percent protein and 98% of lipids (mainly TAG from digested lipids). These go to adipose tissue, heart and skeletal muscle. Lipoprotein lipase or **'clearing factor'** an enzyme present in the capillary walls of these tissues hydrolyses the lipids of the chylomicrons and help in their deposition in the tissue. Their normal concentration in plasma is 100-250 mg/100 ml.
2. **Very low density lipoproteins:** These originate from liver. They mainly carry TAG formed or synthesized in the liver. The protein content is 9% and lipids are 91%. They go to exrahepatic tissue including adipose tissue where they are hydrolyzed by lipoprotein lipase. The concentration is 130-200 mg/100 ml of plasma.
3. **Low density lipoproteins (LDL):** These are the lipoproteins formed from chylomicrons and VLDL. The lipoprotein lipase hydrolyses only the TAG of chylomicrons and VLDL, leaving cholesterol and phospholipids as such. These remnants then surround some more amount of protein around them getting converted to LDL. LDL is also formed in the liver afresh. The cholesterol synthesized in the liver is transported through LDL. 50% of LDL are taken up by the extra-hepatic tissues like testes, ovaries, kidney, fibroblasts, lymphocytes and arterial smooth muscles which contain specific binding sites for LDL. The remaining 50% are taken up by liver. The protein content is 21% and lipid content is 79%. Their normal concentration in the plasma is 210-400 mg/100 ml.
4. **High density lipoproteins:** They are both of liver and intestinal origin. They are made up of 33% of proteins and 67% of lipids. The lipids are mainly phospholipids and cholesterol. The cholesterol in HDL is esterified by an enzyme lecithin-cholesterol acyltransferase, which transfers a fatty acid from lecithin to cholesterol to form the cholesterol ester. The cholesteryl ester of HDL is transferred to VLDL and LDL by a cholesteryl ester transfer protein. HDL takes up the cholesterol, from the tissue and transport them to liver, thus prevent Atherosclerosis. Hence they are also known as scavengers for cholesterol. Their normal concentration in the blood plasma is 50-130 mg/100 ml. The protein is apo-A & C.

Free fatty acids – albumin complex. They arise in the plasma from lipolysis of TAG in adipose tissue or as a result of the action of lipoprotein lipase during uptake of plasma TAG from chylomicrons and VLDL. The released free fatty acids combine with albumin to form free fatty acid–albumin complex. Their concentration during full fed state is about 0.02-0.03 micro equivalents/ml of blood plasma which rises to 0.5 during post absorptive state and in fasting condition it is 0.7 to 0.8. In uncontrolled diabetes

it is 2 micro eq./ml. About 50% of FFA-albumin is taken up by the extra-hepatic tissues like heart and skeletal muscle.

Disorders of plasma lipoproteins

Disorder	Abnormality	Problems
Abetalipoproteinemia	No chylomicrons, VLDL or LDL are formed because of defect in triacylglycerol transfer protein which prevents the loading of apo B with lipid	Intestine and liver acyl-glycerols accumulate
Familial α-lipoprotein deficiency	Low or absence of HDL	Atherosclerosis in later stages of life
Familial hypercholesterolemia	Defective LDL receptors	Due to reduced rate of LDL clearance there is hyper-cholesterolemia leads to atherosclerosis and coronary disease
Familial hypertriacyl-glycerolemia	Overproduction of VLDL due to hyperinsulinemia	High cholesterol leads to coronary heart disease. Seen in non-insulin dependent diabetes mellitus, obese & alcoholics
Familial hyperalpha-lipoproteinemia	Increased concentration of HDL	Rare condition that is beneficial to health, longevity

Fatty liver: Abnormal accumulation of fats in the liver is known as fatty liver. The lipid content of the liver is 5% under normal conditions. A rise in the lipid content of the liver to about 25% to 30% leads to fatty livers. This leads to disruption in the normal activities of the liver which may lead to jaundice. There are two types of fatty livers—

1. **Physiological fatty livers:** Physiological fatty livers arise due to increased mobilization of fatty acids to the liver, more than the liver can metabolize. Hence it leads to accumulation of fats in the liver causing fatty livers. The causes of physiological fatty liver may be starvation, diabetes, hypersecretion of hormones like epinephrine, glucagon and GH causing lipolysis.
2. **Pathological fatty livers:** In this case the fatty acids may be coming to the liver in normal amounts but the liver is not able to produce lipoproteins to clear the lipids which again leads to fatty livers. Non production of lipoproteins may be due to—
   - Defective liver due to alcoholism
   - Metabolic block in apoprotein synthesis
   - Infections of the liver
   - Deficiency of lipotropic factors

Lipotropic factors: Substances which prevent or relieve abnormal accumulation of lipids in the liver are called lipotropic factors or lipid clearing factors. The various lipotropic factors and their action are—

1. **Choline:** It serves as a base for the synthesis of phosphatidyl choline (lecithin), which is necessary for the formation of lipoproteins.
2. **Betaine and methionine:** Supply methyl groups for the synthesis of choline in the body.
3. **Glycine and serine:** Serve as precursors for the synthesis of choline.
4. **Folic acid and vitamin B_{12}:** Act as coenzyme for transfer of methyl groups in the synthesis of choline.
5. **Threonine:** An important amino acid of hepatic enzymes for the synthesis of choline.

Other lipotropic factors are essential fatty acids, pyridoxine, inosine, vitamin E and selenium.

Metabolism of adipose tissue: Adipose tissue is the sub-cutaneous tissue which mainly functions for the storage of fats. Fats are stored in the adipose tissue as fat droplets of triacylglycerols in large vacuoles. Under normal conditions the triacylglycerols of the adipose tissue are broken down to glycerol and free fatty acids. The glycerol so obtained, passes through the circulation to the liver for further oxidation. The free fatty acids get esterified to 3-phosphoglycerate, obtained from the glycolysis in the adipose tissue to form triacylglycerols. This is a continuous process in the adipose tissue and it occurs even under normal conditions i.e. under well fed conditions.

The glycerol obtained from the break-down of the TAG cannot be reutilized for the re-synthesis of TAG, hence it diffuses into the circulation. Only the 3-phosphoglycerate from glycolysis can be utilized for the re-synthesis of TAG. This is a well developed mechanism to differentiate between a well fed condition and starvation condition. During the well fed condition, glucose will be easily available for the adipose tissue, thereby glycolysis will be continuously operating and hence there is a continuous supply of 3-phosphoglycerate for the re-esterification of the free fatty acids. But, during the starvation period there is deficiency of glucose, therefore there will be deficiency in the availability of 3-phosphoglycerate ultimately resulting in non esterification of FFA which thus diffuses into the blood and combines with albumin to form the **'FFA-albumin'** complex. This inturn is transported to various organs for utilization. When again the subject is well fed there will be an easy availability of glucose which causes re-esterification of the FFA and thereby its supply is stopped to the other organs. The relationship of the availability and non availability of glucose to the supply and re-esterification of FFA is termed as the **'glucose-fatty acid cycle'**.

Brown adipose tissue: It is also a sub-cutaneous tissue and stores an excess amount of fat than the adipose tissue. This tissue appears reddish brown in colour, because it contains large amounts of mitochondria and each mitochondrion with an extra number of cytochromes than the normal mitochondria. As the cytochromes are reddish brown in colour (due to the porphyrin ring) the adipose tissue appears reddish brown in colour. Hence it is known as brown adipose tissue and the mitochondrion present in it is called brown fat mitochondria. The main purpose of this tissue is the production of heat. In the brown adipose tissue the catabolism of the TAG gives rise to only one ATP and large amounts of heat. The production of ATP is less but the production of heat is more because the brown fat mitochondria contain a protein called **'thermogenin'** which uncouples oxidative phosphorylation. In this way the brown adipose tissue regulates the body temperature.

Brown adipose tissue is generally found in new born infants and children on their neck and back side of their trunk. Infants and children generally have a very thin layer of sub-cutaneous fat pad, therefore there is no restriction for the loss of heat or there is no insulation for the prevention of loss of heat. Hence over production of heat by the brown adipose tissue helps in the regulation of the body temperature in children.

Brown adipose tissue is rarely found in adults. Such adults or persons can never become obese/fat because the extra carbohydrates and fats taken by such individuals are utilized by the brown adipose tissue and therefore lost as heat.

Normal levels of different lipids in the blood plasma (mg / 100 ml)

Lipid	Range	Average
Total lipids	360-820	570
Triacylglycerols (TAG)	80-180	142
Phospholipids	123-390	215
Cholesterol	107-320	200
Free fatty acids	6-16	12

13

PROTEIN METABOLISM

DIGESTION AND ABSORPTION

The major constituents of the food are carbohydrates, proteins and lipids. They are digested and absorbed in the stomach and intestine. Some of the digested/degraded components of the food stuffs may either be reutilized or may be excreted out. Chewing of food, movements of the stomach and intestine facilitate the grinding of the food materials and bring them in contact with gastric secretions.

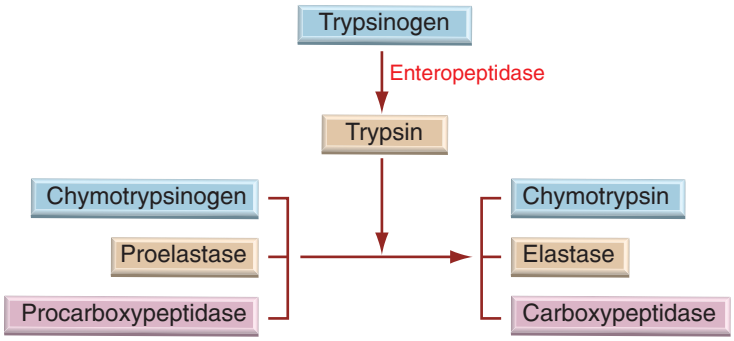
Proteolytic enzymes are absent in the salivary secretions, hence there is no digestion of proteins in the mouth. Proteolysis takes place in the gastro-intestinal tract (i.e. stomach and intestine). When the proteins enter the stomach they stimulate the secretion of the hormone called gastrin which in turn stimulates the secretion of HCl by parietal cells of the stomach and pepsinogen from the chief cells. Gastric juice is acidic i.e. the pH is 1.5–2.5. Acidic pH of the stomach has an antiseptic action that kills the bacteria and other microorganisms. At this pH the dietary proteins also get denatured. In presence of HCl, pepsinogen is converted to pepsin by autocatalysis resulting in removal of some of the amino acids from the amino terminal end. Pepsin is an endopeptidase for tyr, phe, trp. In the stomach the proteins are converted as follows—

Protein → Metaprotein → Proteone → Peptone → Peptide

As the food passes from the stomach to small intestine the low pH of the food triggers the secretion of the hormone 'secretin' into the blood. It stimulates the pancreas to secrete HCO_3 into the small intestine in order to neutralize HCl. The secretion of HCO_3 into the intestine abruptly raises the pH from 2.5 to 7.0. The entry of amino acids into the duodenum releases the hormone 'cholecystokinin' which in turn triggers the release of pancreatic juice (that contains many pancreatic enzymes like trypsinogen, chymotrypsinogen, procarboxypeptidases) by the exocrine cells of the pancreas (ecbolic and hydrolatic). Most of these enzymes are produced as zymogens (inactive enzymes) by the pancreas in order to protect the exocrine cells from being digested.

Subsequent to the entry of trypsinogen into the small intestine it is activated to trypsin by enterokinase secreted by the intestinal cells. Trypsin is formed from trypsinogen by the removal of hexapeptide from the N-terminal end. The newly formed trypsin activates the remaining trypsinogen. Trypsin is an endopeptidase, specific for (acts on) the peptide bonds contributed by the basic amino acids like arginine, histidine and lysine.

Chymotrypsin is secreted in an inactive from called chymotrypsinogen which is activated by trypsin. Chymotrypsin is an endopeptidase specific to aromatic amino acids i.e. phenylalanine, tyrosine, tryptophan.



Carboxypeptidase secreted as procarboxypeptidase is activated again by trypsin. It is an exopeptidase that cleaves the amino acids from the carboxy terminal end.

Aminopeptidase secreted as proaminopeptidase is once again activated by trypsin. It is an exopeptidase that cleaves the amino acids from the free amino terminal end.

Dipeptidase acts only on dipeptides and hydrolyses it into 2 amino acids.

Proteolytic enzymes and their action

Secreted in	Enzymes secreted	Action
Stomach	Pepsin	Converts complex proteins to small peptides
Pancreas	Trypsin	➢ Specifically acts on peptide bonds contributed by basic amino acids like arg, lys & his ➢ Activates trypsinogen to trypsin ➢ Procarboxypeptidase to carboxypeptidase, proelastase to elastase and proaminopeptidase to aminopeptidase
	Chymotrypsin	Specifically acts on peptide bonds contributed by aromatic amino acids like phe, tyr, trp
	Carboxypeptidase	Carboxy terminal amino acids
	Elastase	
Small intestine	Amino peptidase	Amino terminal amino acids
	Dipeptidase	Acts on dipeptides and releases free amino acids

Even after the action of all these enzymes most of the proteins remain undigested. Protein like collagen, fibrin etc, escape digestion and are excreted out.

Celiac disease: This is a rare disease caused due to genetic defect/absence of the enzyme required to hydrolyze the proteins containing N-glutamyl amino acids. Due to this the intestinal enzymes are unable to digest the water insoluble proteins present in wheat called gliadin which is injurious to the cells lining the small intestine.

In rare instances the inactive zymogen forms of the enzymes stored in the pancreas are prematured to active forms in the pancreas itself, which may be fatal to the pancreas. Antagonists called trypsin inhibitor, a protein secreted by the pancreas can be used to avoid such disaster.

Absorption of the digested proteins: There are four distinct carrier systems in the intestinal epithelium for the absorption of the digested proteins. These are—

(1) Carrier system for neutral amino acids
(2) Carrier system for basic amino acids
(3) Carrier system for acidic amino acids
(4) Carrier system for glycine and imino acid (proline)

The digested amino acids are carried across the mucosal cell membrane from the intestinal lumen to the cytoplasm of the cell by one of the above carrier systems specific to that particular amino acid. Absorption of amino acids is an up-hill process (i.e. against gradient as compared to the Na^+ absorption which is down hill i.e. along the gradient). There are four systems that operate to absorb amino acids from the mucosal cells into the blood. They are—

(1) A – system (alanine system)
(2) L – system (leucine system)
(3) Ly – system (lysine system)
(4) Ala-ser-cyst – system

Amino acids are taken up by the blood capillaries of the mucosa and are transported in the plasma to the liver. Some amounts of amino acids are also absorbed through the lymph. Glucagon stimulates the absorption of amino acids through 'A' system mediated by cAMP. Insulin stimulates the transcellular transport of amino acids to minimize the loss in the urine. The proximal tubule cells reabsorb and return them to the blood stream. It is done by glutathione, a tripeptide. Three ATPs are utilized for the absorption of each amino acid.

Absorbed amino acids do not stimulate antibody production whereas an intact protein absorbed becomes antigenic. Intestinal membranes allow the transport of proteins across them ex.—In a neonate the intestinal mucosa is permeable to γ-globulin (immunoglobulin) of colostrum. The immune system in a neonate is not well developed thus absorption of intact γ-globulin into the blood does not elicit any immune response instead it results in the defence of the neonate against infections. In adults, some proteins may be absorbed intact through the intestinal mucosa leading to the formation of antibodies and anaphylactic reactions or other such immunological phenomena after the absorption of those proteins. Thus in such cases allergies to food proteins occur.

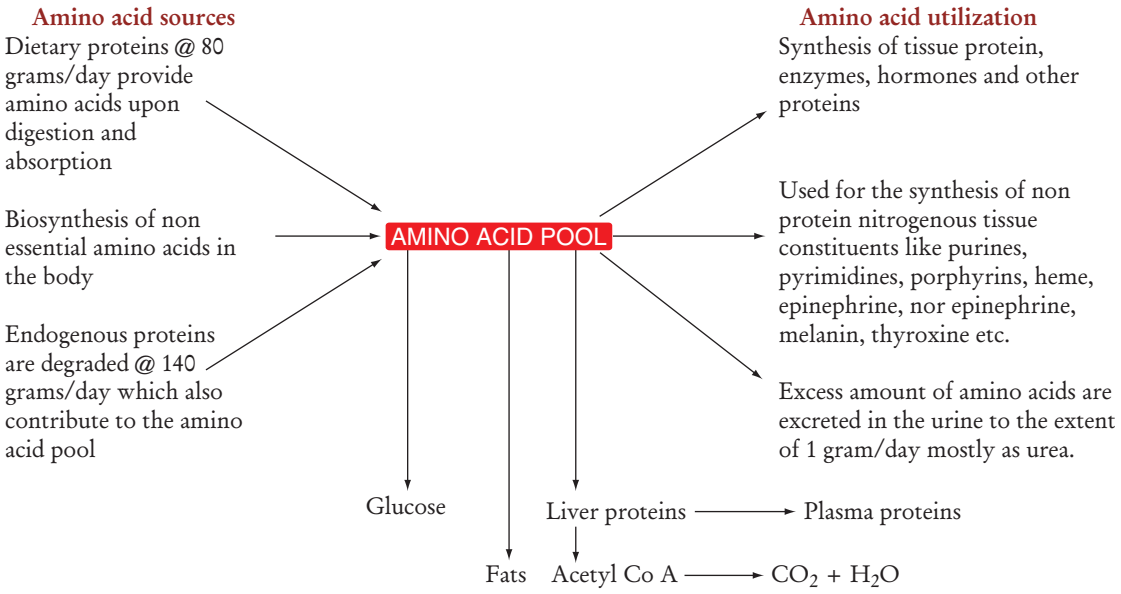
Protein turnover: Protein turnover is a continuous process of degradation and resynthesis of all cellular proteins.

Each day, human beings turn over 1 to 2% of their total body proteins i.e., about 2% of the body proteins are degraded and resynthesized every day. 75-80% of the amino acids released from the degraded proteins are reutilized for new protein synthesis and the nitrogen of the remaining 20-25% forms urea leaving the carbon backbone to be oxidized to intermediates of TCA or other metabolites. The rate at which proteins degrade depends upon the physiological state of the individual. The time required to reduce the concentration of a given protein to 50% of its original concentration is termed as **'half-life ($t_{½}$)'**. The half life of liver proteins ranges from 30 minutes to 150 hours. The half life of HMG CoA reductase is 0.5-2 hours whereas aldolase, lactate dehydrogenase and cytochromes have a half life of 100 hours. Hence it can be said that, almost all the proteins of the body are degraded within a span of 6-9 months and are replaced by new proteins.

Role of lysosomes in protein turnover: Lysosomes play an important role in the degradation of intracellular and extracellular proteins. The proteins from the circulation and those within the cell lose the oligosaccharides and are then internalized by the lysosomes and are degraded by proteases called cathepsins. The non-glycosylated proteins are degraded in the cytosol by ubiquitin, a small protein of 8.5 kDa in all eukaryotic cells. Ubiquitin forms a non-peptide bond with the N-terminal amino acid in the

protein with conversion of ATP to AMP. Thus, the life of the protein depends upon the type of amino acid present at the N-terminal end. If serine and methionine are present as the N-terminal amino acid then the life of the proteins is long and if aspartate and arginine are present then the life is short because ubiquitin acts fast on these amino acids.

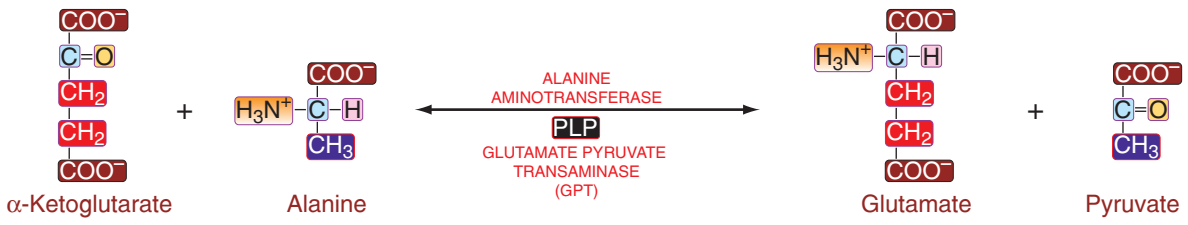
Various sources and products of amino acids in the body: The protein intake from exogenous sources amounts to about 80 grams/day. The dietary proteins are digested and absorbed contributing to the amino acid pool of the body. The endogenous proteins are hydrolysed to the extent of 140 grams/day which also contribute to the amino acid pool. The amino acids are used for the synthesis of tissue protein, enzymes, hormones and other proteins. They are also used for the synthesis of essential non-protein nitrogenous constituents like purines, pyrimidines, porphyrins, epinephrine, nor-epinephrine, melanin, etc. Further, amino acids also undergo oxidative degradation to carbon dioxide and water releasing energy or donate their carbon back bone for the formation of glucose and ketone bodies during starvation and other diseases. The ammonia released from the oxidized amino acids is converted to urea in the liver and excreted through the urine. Excess amino acids to the extent of about 1 gram/day are excreted in the urine.

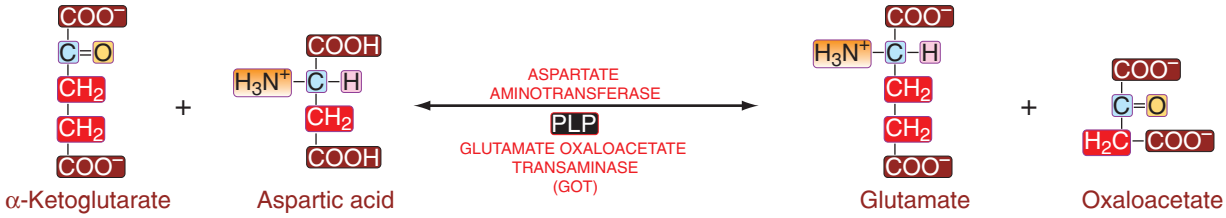


General reactions of amino acids:

1. **Transamination:** Transamination is a process of transfer of amino group reversibly from an amino acid to a keto acid.

   Transamination is a reaction in which the amino group from one amino acid is transferred to a keto acid to form a new keto acid and a new amino acid. The reaction is catalyzed by the enzyme transaminase. Coenzyme required is pyridoxal phosphate (PLP). It is a bi-substrate reaction. A Schiff's base is formed as an intermediate. The mechanism is called ping-pong reaction.



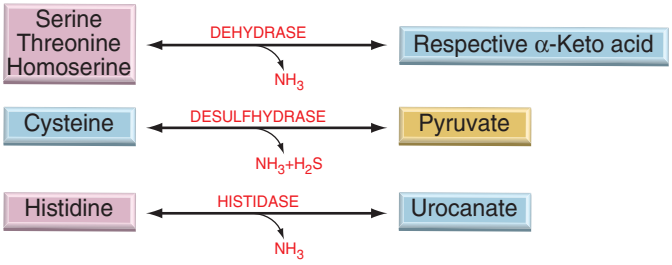


Generally three keto acids participate in this reaction. They are—1) Pyruvate (2) α-keto glutarate and (3) Oxalo acetic acid. Transamination reaction mainly serves two roles in the amino acid metabolism—(a) Inter conversion of amino acids and (b) To channel the amino group of amino acids ultimately to glutamate and aspartate.

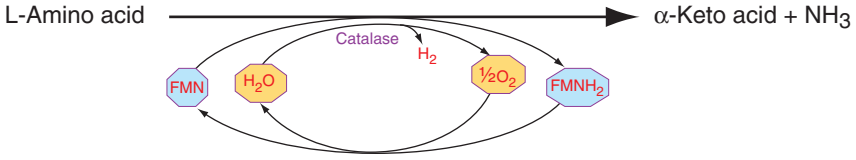
If these two amino acids are in excess than the requirement then the amino group is removed by deamination forming ammonium ion (NH_4^+) or urea which are excreted. All the amino nitrogen is ultimately concentrated on glutamate. Glutamate is the only amino acid in mammalian tissue that undergoes oxidative deamination at an appreciable rate.

2. **Deamination:** Removal of amino group from the amino acids is known as deamination. There are two types of deamination—(a) Non-oxidative and (b) Oxidative.

(a) *Non-oxidative deamination*: Removal of amino group without oxidation is known as non oxidative deamination. The enzyme is dehydrase, PLP acts as a cofactor. This occurs mainly on hydroxy amino acids like serine and threonine.



(b) *Oxidative deamination*: Removal of amino group following its oxidation is known as oxidative deamination. It takes place both in the liver and kidney. The coenzymes involved are FMN and FAD which are reduced to $FMNH_2$ and $FADH_2$. The enzyme is amino acid oxidase which is auto oxidizable i.e. the reduced $FMNH_2$ & $FADH_2$ are reoxidised directly by oxygen without the involvement of electron transport chain. Hydrogen peroxide is formed here, which is converted to water by catalase.



Glutamate dehydrogenase, one among oxidative deaminases is an allosteric enzyme. ATP, GTP and NADH act as negative modulators whereas ADP acts as a positive modulator. It converts glutamate to α-keto glutarate and ammonia.

3. **Transmethylation:** It is a process in which there is the transfer of methyl group from a donor to the acceptor.

$$\text{Serine} + \text{THF} \rightleftharpoons \text{Glycine} + N^5,N^{10}\text{-Methylene-THF} + H_2O$$

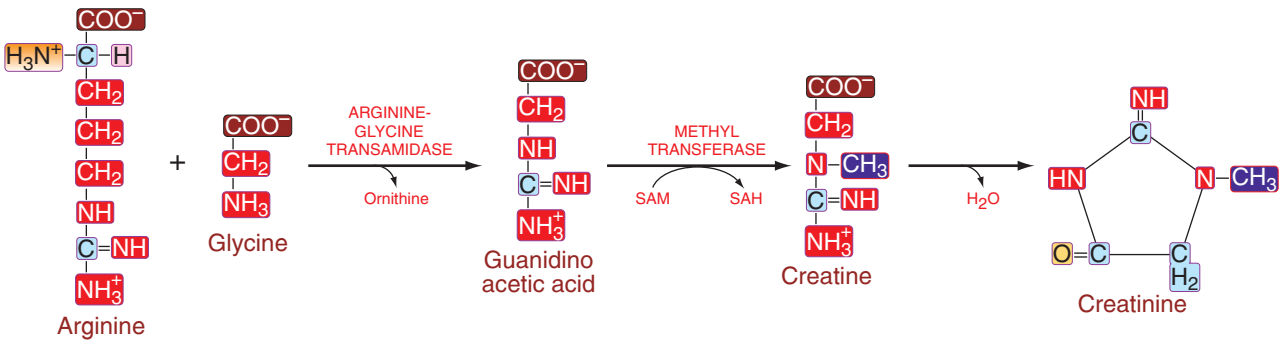
$$N^5,N^{10}\text{-Methylene-THF} + \text{NADH} + H^+ \rightleftharpoons N^5\text{-Methyl-THF} + NAD^+$$

$$N^5\text{-Methyl-THF} + \text{Homocysteine} \rightleftharpoons \text{THF} + \text{Methionine}$$

4. **Decarboxylation:** Reactions in which removal of CO_2 takes place from the carboxylic group of amino acids. Enzyme catalyzing the reaction is decarboxylase. Cofactor is PLP. An amine is formed due to decarboxylation. The amines so formed are known as '**biogenic amines**'. This is mainly confined to G-I tract where due to the putrefaction in intestine, toxic amines like tyramine, tryptamine, putrescine and cadaverine are formed. However some amines are useful.

Amino acids	Amine	Occurrence and significance
Histidine	Histamine	Affects blood pressure
Tyrosine	Tyramine	Contracts uterus
Tryptophan	Tryptamine	5-hydroxy tryptamine is serotonin, contracts smooth muscles
Glutamic acid	Gamma amino butyric acid (GABA)	Inhibits brain ganglion
Serine	Ethanolamine	Synthesis of phospholipids
3,4-di-hydroxy-phenylalanine	Dopamine	Precursor of adrenaline and nor adrenaline
Methionine	Spermidine	Ribosome and sperms
Lysine	Cadaverine	Product of putrefaction
Ornithine	Putrescine	Product of putrefaction
Arginine	Agmatine	Product of putrefaction

5. **Transamidation:** Transfer of amide group. The catalyzing enzyme is transamidase.



Metabolism of ammonia:

Formation of ammonia: Ammonia is formed in the tissues and also by the intestinal bacteria from the dietary proteins. All the ammonia is absorbed into the portal venous blood which generally contains

higher level of ammonia compared to systemic blood. Generally liver is virtually free from ammonia. Normal blood level is 10-12 μg/dl. Prompt removal of ammonia is essential, because even minute quantities of ammonia in blood are toxic to CNS. The symptoms called ammonia intoxication includes flapping tremor, slurring of speech and blurring of vision and in severe cases coma and death. It is similar to hepatic coma (with impaired hepatic function or any abnormality in portal and systemic veins), wherein increased ammonia level is seen. This can be corrected by surgery (shunting method).

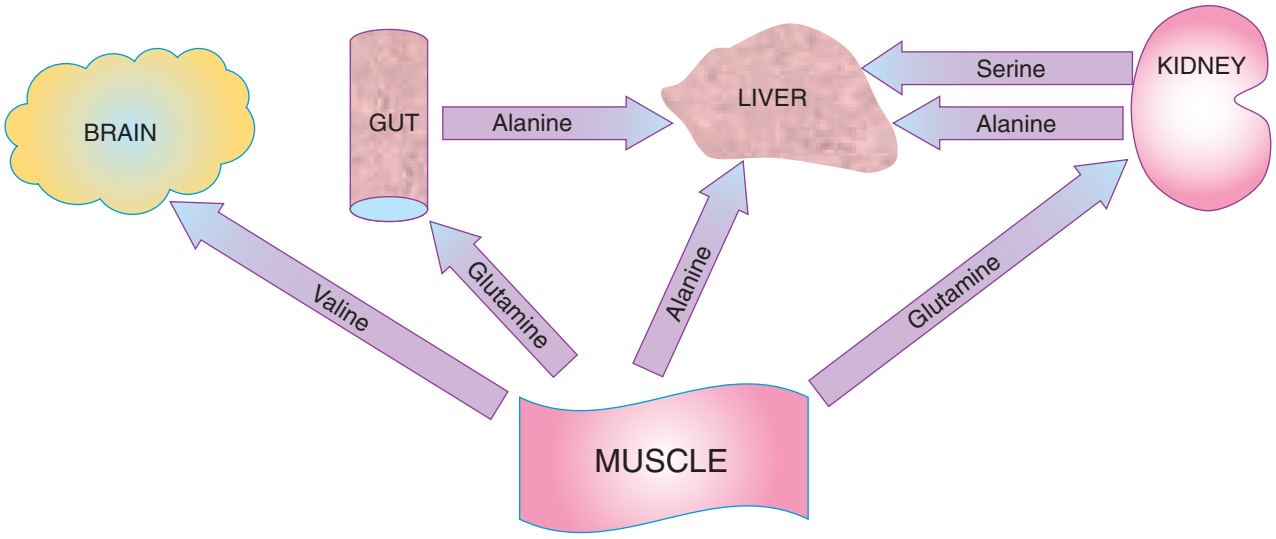
The ammonia content of blood in renal vein is more than renal artery indicating that kidney produces ammonia. The production of ammonia is very important in regulating the acid-base balance and conservation of cations like Na^+ and K^+ ions. This ammonia is formed by glutaminase enzyme in the kidney tubules.

Transport of ammonia: Removal of ammonia takes place by the following mechanisms—

1. Amination of α-keto acid to form amino acids.
2. Amidation of glutamic acid to glutamine.
3. Formation of urea in the liver.

Ammonia from the brain is released as glutamine and as a result more and more glutamine enters the liver. But the supply of glutamine to the brain is limited and when blood ammonia level is high, the intermediate of the TCA cycle i.e. α-ketoglutarate is used for the formation of glutamine. This results in depletion of the TCA intermediate which is replenished by anaplerosis from other amino acids by carbon dioxide fixation in the brain.

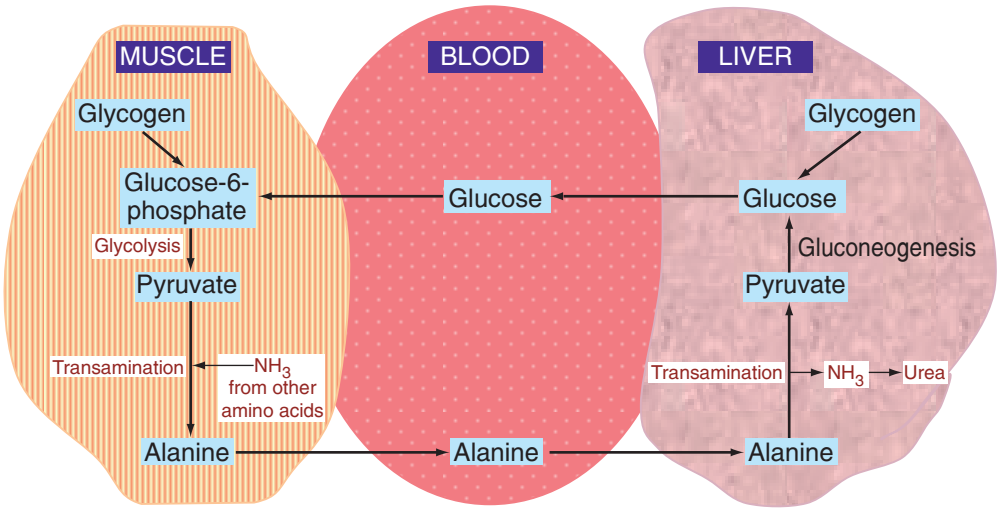
Inter organ amino acid exchange: Muscle tissue releases alanine and glutamine, whereas kidney releases serine, which are taken up by the splanchnic tissues (in liver). Hence, there is fairly close correspondence between the output of most amino acids from peripheral muscle and their uptake by the splanchnic tissues.



Liver takes up more of alanine and glutamine and less of valine, because the oxidation of branched chain amino acids is relatively more in the brain compared to other tissues. The uptake of valine by brain is more than other amino acids

Glucose-alanine cycle: The main purpose of this cycle is to transport ammonia from the muscle to the liver and to supply the glucose from the liver to the muscle.

Alanine is synthesized from pyruvate in muscle by transamination of glutamate, released into the blood stream and taken up by the liver where it is converted to glucose by gluconeogenesis and enters the blood stream that forms a source of glucose to muscle.



Conclusion: Ammonia formed in different tissues is transferred to the liver in its amide form, as glutamine from brain and peripheral tissue and from muscle as alanine. Ammonia is mainly transferred in the form of alanine, glutamine, aspargine and serine.

Excretion of ammonia: Ammonia is excreted in three different forms in various species, they are—

1. In fishes it is excreted as ammonia hence they are known as ammonotelic animals.
2. In uricotelic animals like birds and lizards, ammonia is excreted as uric acid.

In ureotelic animals including humans, ammonia is excreted as urea.

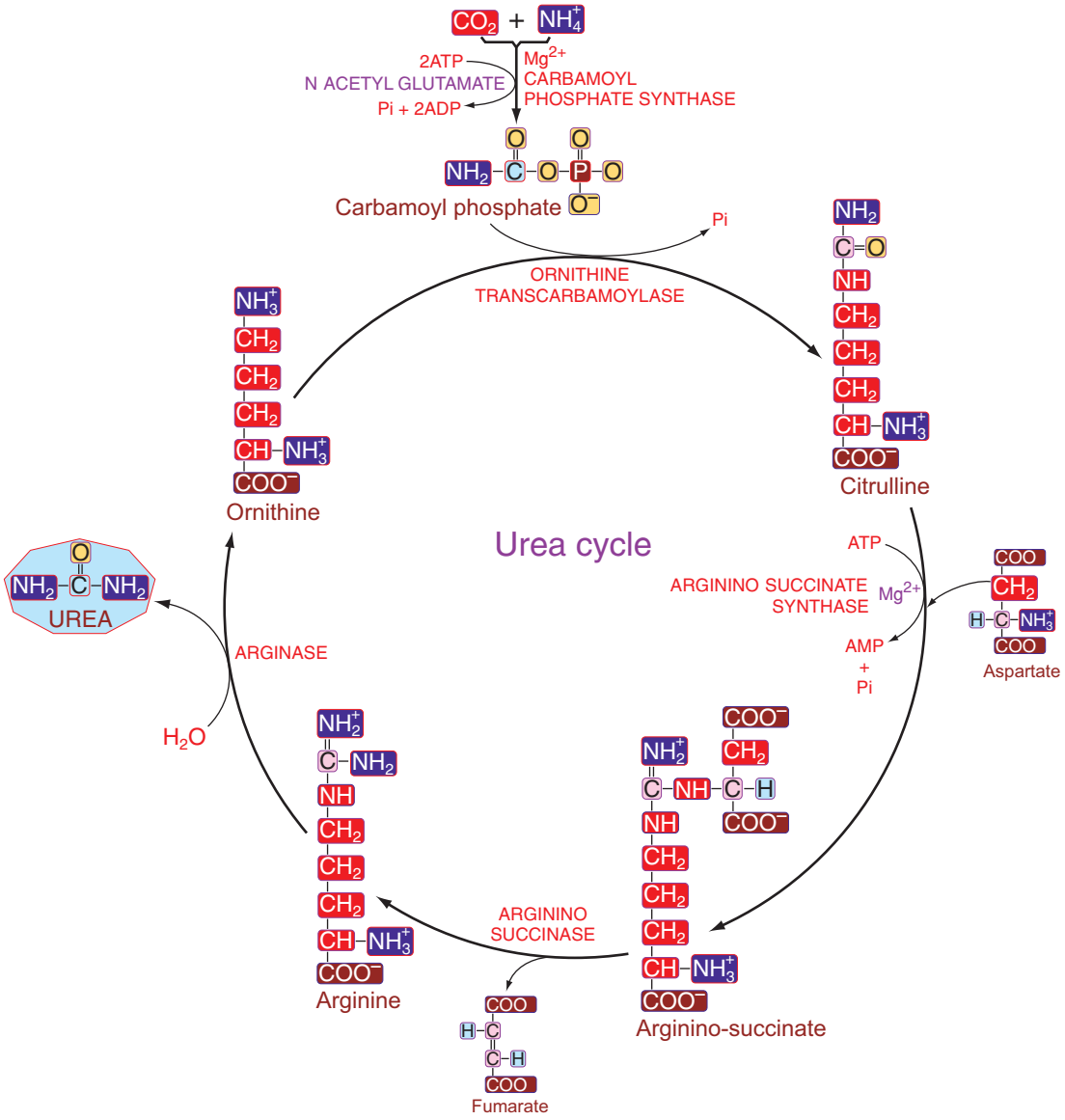
UREA CYCLE

Urea cycle was discovered by Krebs and a medical student Henseilet of Germany in 1932. Hence it is also known as Krebs Henseliet cycle. The normal blood urea level is 15–40 mg/dl. Every day 16.5 gms of nitrogen is excreted i.e. 20 to 40 grams of urea is excreted per day. Among this 95% is eliminated by the kidneys and the remaining 5% through the faeces. The major pathway of nitrogen excretion in human is as urea which is synthesized in the liver released into the blood and cleared by the kidney.

Steps of urea cycle:

1. **Formation of carbamoyl phosphate:** Condensation of ammonium ion with bicarbonate ion resulting in the formation of carbamoyl phosphate by the help of the enzyme carbamoyl phosphate synthase-I present in the liver mitochondria. It requires Mg^{2+} and a dicarboxylic acid i.e. N-acetyl glutamate. This step requires 2 ATPs.
2. **Synthesis of citrulline:** Carbamoyl phosphate formed in the first step combines with ornithine resulting in the synthesis of citrulline aided by the enzyme citrulline synthase or ornithine transcarbamoylase. Citrulline is easily permeable to the mitochondrial membrane and hence it diffuses into the cytosol.

3. **Synthesis of arginosuccinate:** In the cytosol, citrulline combines with the amino acid aspartate forming arginosuccinate catalyzed by the enzyme arginosuccinate synthase. It requires ATP which is hydrolysed to AMP resulting in utilization of two high energy bonds. Mg^{2+} acts as cofactor.



4. **Cleavage of arginosuccinate:** The enzyme arginosuccinase acts reversibly to cleave arginosuccinate into Arginine and fumarate. Fumarate enters the TCA cycle (the linkage between TCA and urea cycle is known as Krebs bi-cycle).
5. **Cleavage of arginine:** Arginine is lysed into ornithine and urea under the influence of the enzyme arginase. Hence arginine is known as semi-essential amino acid i.e. though it is synthesized in the body it is not available for protein synthesis. Ornithine is regenerated in this step and the urea cycle completes by the formation of urea. Ornithine and lysine are potent inhibitors of the enzyme arginase. Arginase is also present in testis, renal tubules, mammary gland and skin in minute quantities. The intermediate amino acids formed in the urea cycle i.e. ornithine, citrulline and arginosuccinate are known as non-protein amino acids.

The overall equation of urea formation is—

$$NH_4 + CO_2 + \text{Aspartate} + 3ATP \rightarrow \text{Urea} + \text{Fumarate} + 2ADP + 2P_i + AMP + PP_i$$

The urea cycle brings two amino groups and HCO_3 together to form urea. Thus toxic, insoluble ammonia is converted into non-toxic, water soluble, excretable urea. Hence, urea cycle disposes two waste products i.e. NH_4^+ and HCO_3^-. This fact suggests that urea cycle participates in the regulation of blood pH, which depends on the HCO_3^-/H_2CO_3. Though 3 ATPs are utilized, the ultimate cost of making a molecule of urea is 4 ATPs (one ATP is converted into AMP). The rate limiting steps of urea cycle are 1, 2, & 5.

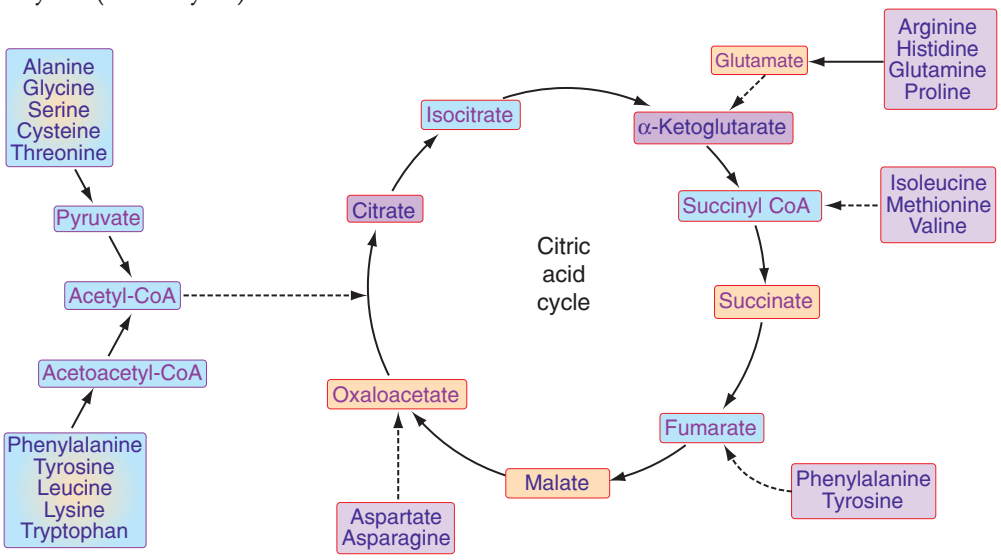
Metabolic disorders of urea cycle: Since urea cycle converts toxic ammonia to urea, disorders of this cycle lead to ammonia intoxication. This ammonia intoxication is more when there is block at step 1 or 2.

Common symptoms of the disorders of urea cycle are vomiting in infancy, avoidance of high protein diet, intermittent ataxia, irritability, lethargy and mental retardation.

1. **Hyper-ammonemia type-I:** Due to the deficiency of carbamoyl phosphate synthase-I. It is a familial disorder.
2. **Hyper-ammonemia type-II:** Due to the deficiency of ornithine transcarbamoylase. It is X-linked. Clinical finding is, the elevation of glutamine in the blood, CSF and urine.
3. **Citrullinemia:** More citrulline is excreted in the urine i.e. upto 1 to 2 gm/day, due to the defect in the enzyme argininosuccinate synthase.
4. **Arginino-succinic aciduria:** It is a rare recessive disease. Higher level of arginino-succinic acid in plasma and CSF. Usually present in the early age. Feeding arginine and benzoate promotes nitrogen excretion in these patients. This is due to lack of the enzyme argininosuccinase.
5. **Hyper-argininemia:** High level of arginine due to lack of arginase enzyme.

Glucogenic and ketogenic amino acids: Those amino acids which on oxidation give intermediate compounds, resembling those of carbohydrate metabolism and which may be converted to glucose are termed as glucogenic amino acids. Ex. Ala, Arg, Asp, Asn, Cys, Gly, Glu, Gln, His, Pro, Met, Ser, Tyr, Val, Lys. Those amino acids which form acetate or acetoacetate intermediates found during fatty acid metabolism are termed as ketogenic amino acids i.e. they can give rise to ketone bodies. Ex. Leu. The amino acids Ile, Lys, Phe, Tyr and Trp can give rise to both glucose and ketone bodies hence they are both glucogenic and ketogenic amino acids.

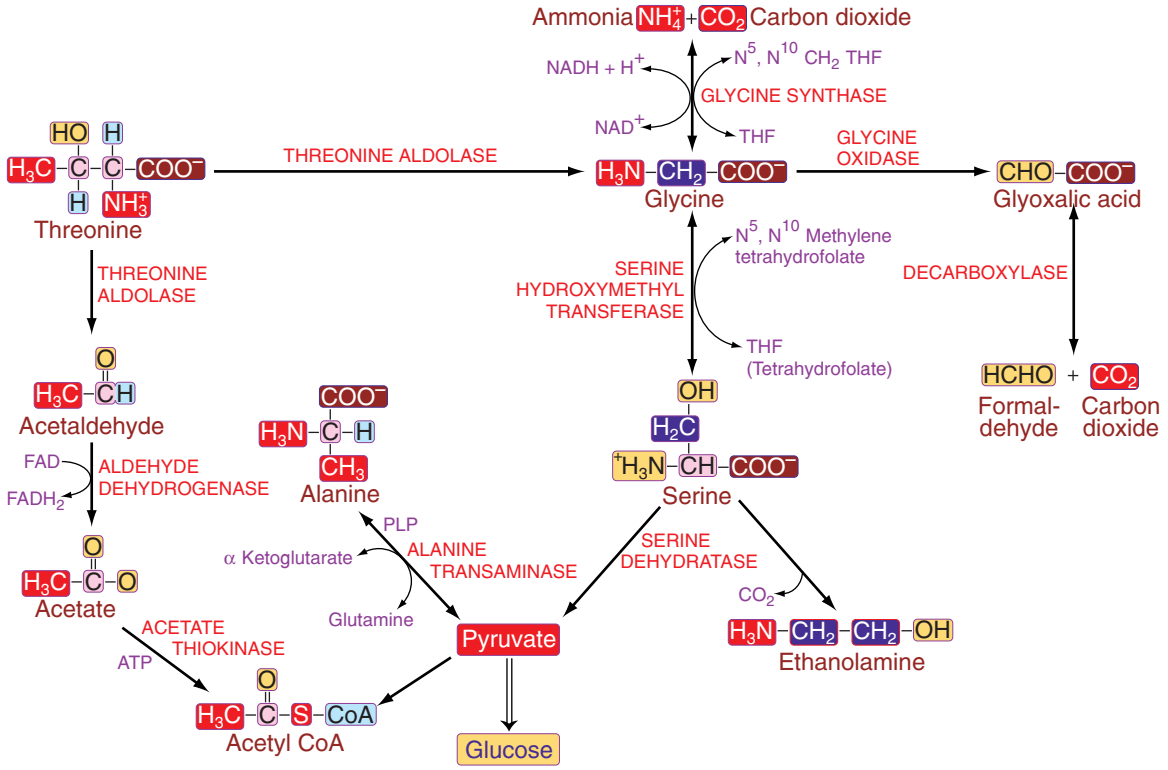
Oxidation of carbon skeleton of amino acids: Once ammonia is released from the amino acids the remnant carbon back bone undergoes various oxidative reactions to yield one or the other intermediates of citric acid cycle (TCA cycle) as shown below—



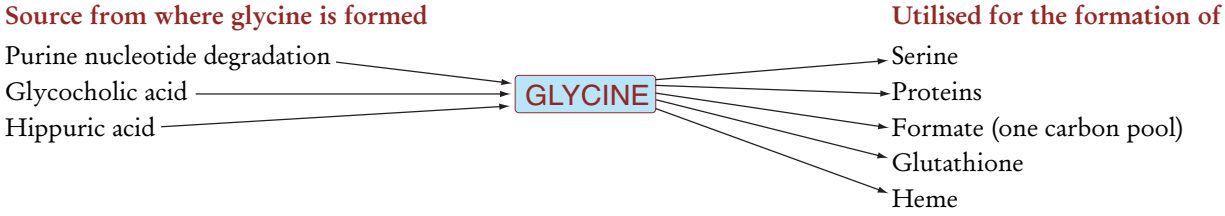
Amino acids entering TCA cycle

METABOLISM OF GLYCINE, ALANINE, SERINE, THREONINE

All these amino acids are glucogenic and share a common pathway. The reaction steps involved in their metabolism are—



Sources and utilization of glycine:



Metabolic disorders of glycine:

1. **Glycineuria:** It is a rare disease due to defect in renal reabsorption of glycine. 600 to 1000 mgs of glycine is excreted per day. Excess of this leads to oxalate renal stones.
2. **Primary hyperoxaluria:** Excess of oxalic acid is excreted because of the deficiency of decarboxylase or transaminase. So glyoxalic acid is converted to oxalic acid. The history of the disease is Nephrocalcinosis and recurrent infection of the urinary tract. Death occurs in childhood or early adulthood because of renal failure or hypertension.

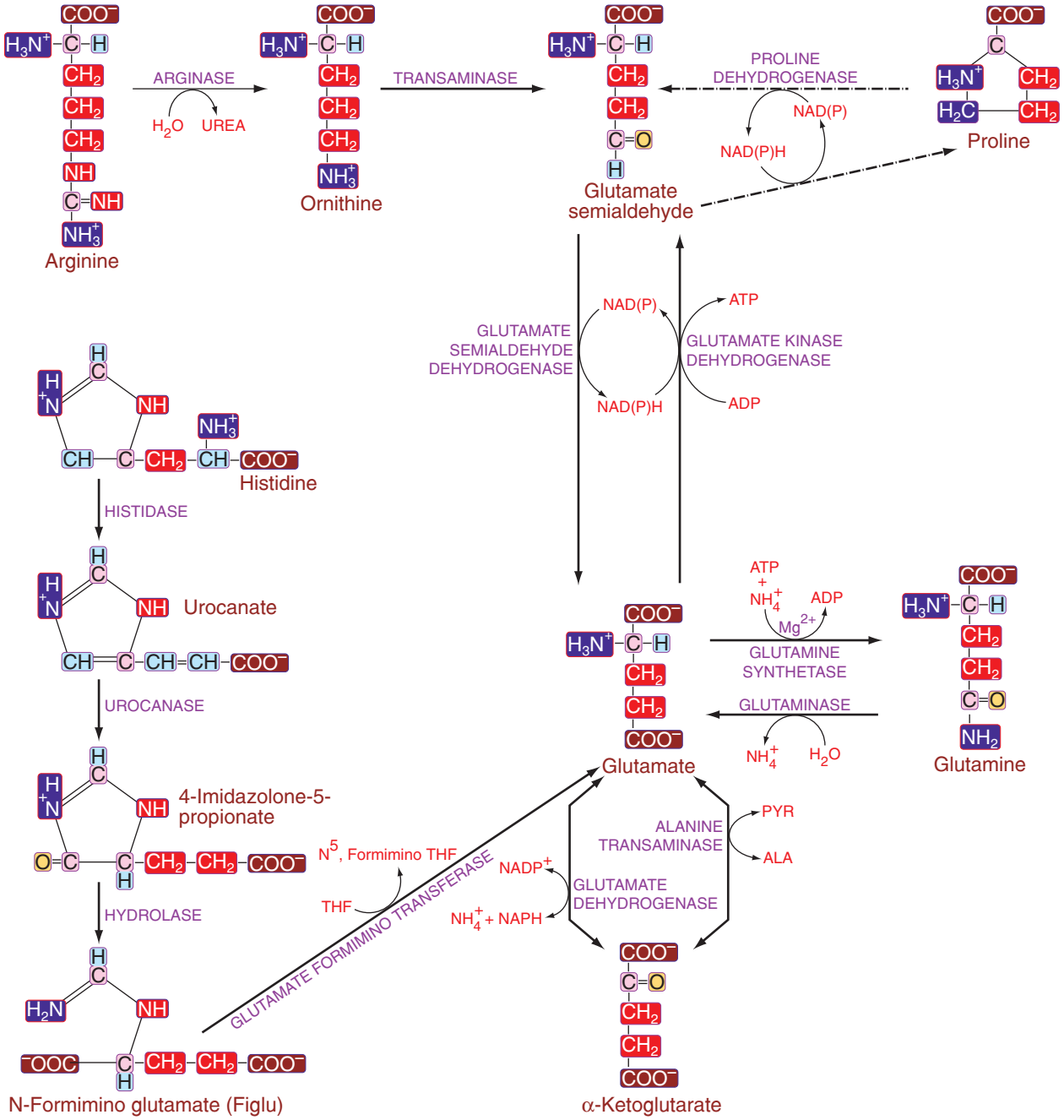
METABOLISM OF ARGININE, PROLINE, HISTIDINE, GLUTAMINE, GLUTAMATE

These are glucogenic amino acids and share a common pathway.

Importance of histidine metabolism: It is an essential and glucogenic amino acid. It serves as a source for one carbon moiety (formate). In pregnancy there is excess excretion of histidine in urine (pregnancy

histidinuria). It is not found in the urine of normal persons hence is of diagnostic importance for pregnancy. Decarboxylation of histidine produces histamine which increases the blood pressure. Antihypertensive drugs cause inhibition of production of histamine. RBC and liver contain a histidine derivative called ergathionine which is a reducing agent and gives false result for glucose level. Carnosine and anserine are formed by histidine and beta alanine present in the muscle. The excretion of these derivatives is of diagnostic importance in myopathy.

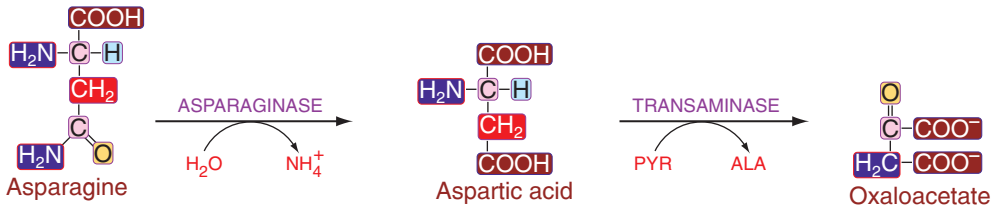
The reaction steps involved in their metabolism are—



Histidenemia: It is a metabolic disorder due to the deficiency of histidase enzyme. It is an inherited autosomal recessive disease. Clinical symptoms are mental retardation and a characteristic speech defect. Imidazole pyruvate is formed and excreted in urine which gives a false $FeCl_3$ test for phenylketonuria.

METABOLISM OF ASPARAGINE AND ASPARTATE

Asparagine and Aspartate produce oxaloacetate as the end product of their metabolism.



Importance of the amino acids glutamate and aspartate: Both are glucogenic. Both transport ammonia from tissues to liver in their amide form. Glutamate helps in the detoxification. Glutamate plays an important role in the transport of potassium (K^+) in the central nervous system (CNS). It forms gamma amino butyric acid (GABA) upon decarboxylation which regulates the neural activity (depresses its activity). It is a constituent of glutathione and folic acid. Aspartate is a constituent of purines and pyrimidines.

METABOLISM OF METHIONINE, CYSTEINE

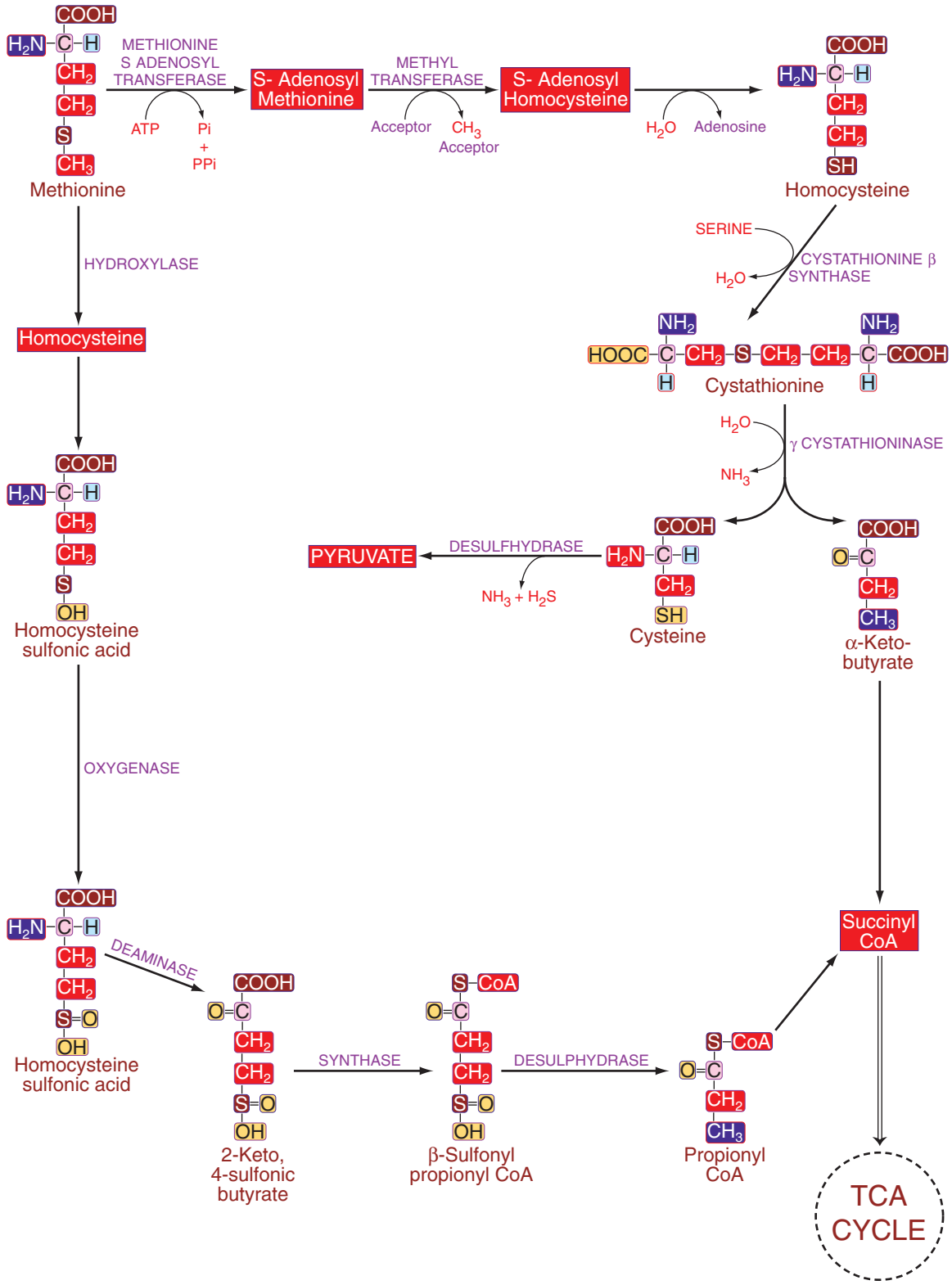
Methionine and cysteine are sulphur containing amino acids. Methionine is oxidized to propionyl-CoA which is converted to succinyl-CoA that enters the TCA cycle.

Metabolic importance of methionine:

1. Methionine along with cysteine maintains the secondary and tertiary structure of proteins.
2. It forms β-mercepto pyruvic acid which conjugates with cyanide to form thiocyanate and sulphite to form thiosulphate.
3. It is a constituent of scleroprotein.
4. It participates in detoxification of bromobenzene.
5. Methionine is the precursor for the synthesis of choline, creatine, glutathione, epinephrine and taurine which in turn forms the bile acid taurocholic acid.

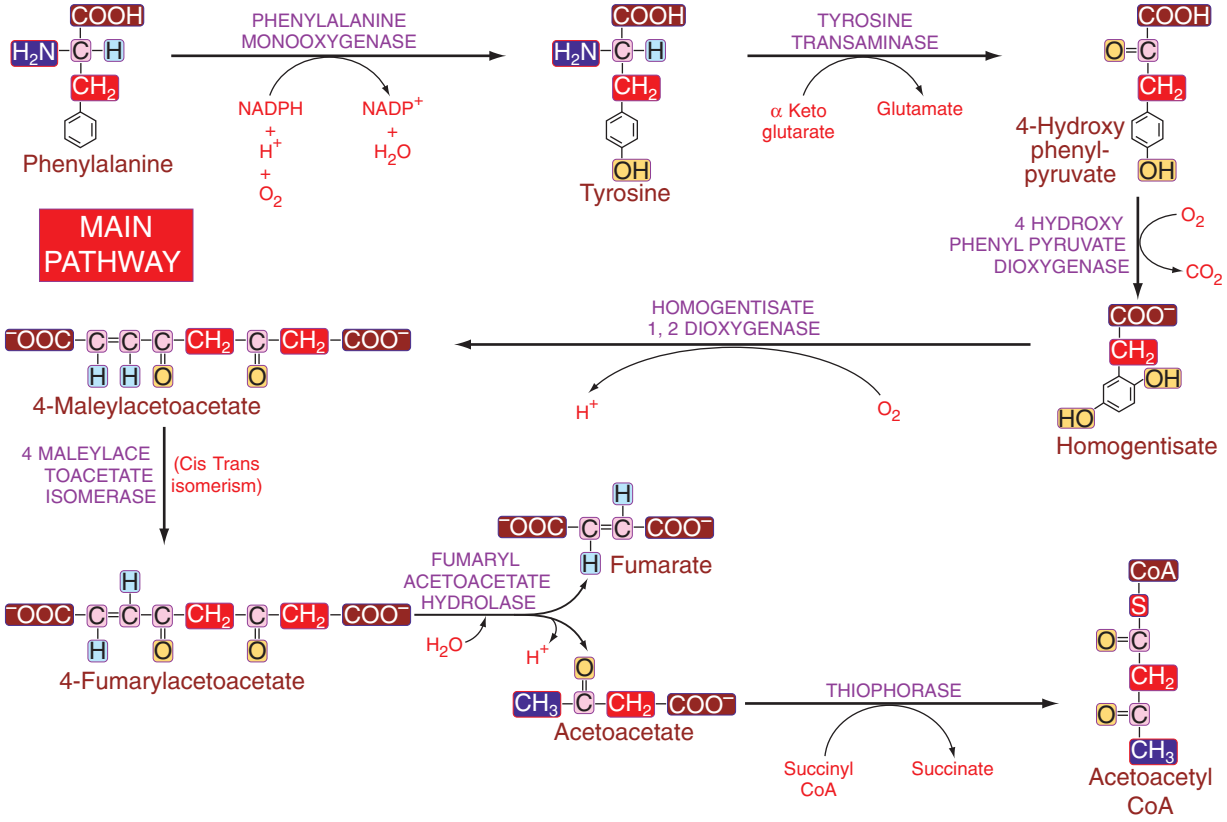
Metabolic disorders of sulphur containing amino acids:

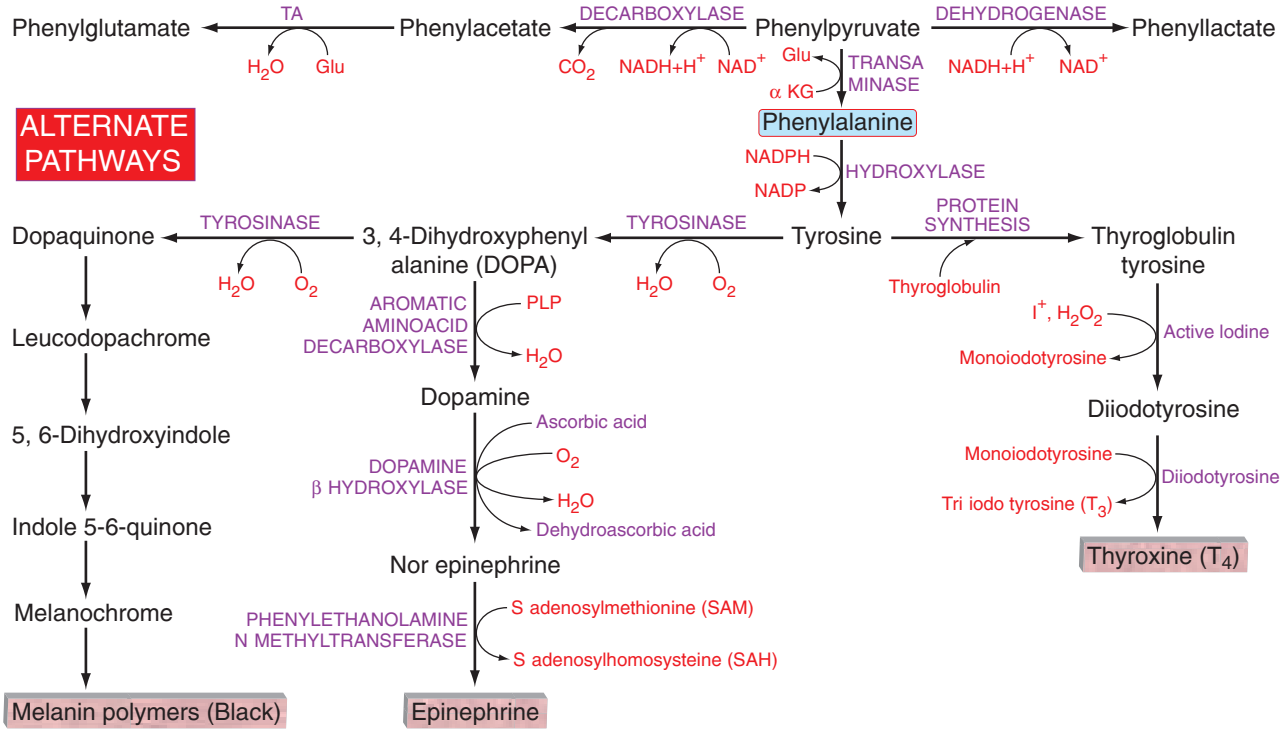
1. **Cystinuria (cystine-lysine in urine):** It is an inherited metabolic disease. Urinary excretion is 20-30 times more than the normal due to the failure in the re-absorption of cystine. Along with it lysine, homocysteine are also excreted. Hence cystinuria is a misnomer. Cystine-lysinenuria is the actual term to be used. But cystine deposits in the kidney forming cystine calculi. This may be a major complication of the disease, hence the name cystinuria. There may be intestinal transport defects also.
2. **Cystinosis (cystine storage disease):** Cystinosis is due to acute renal failure leading to generalized amino aciduria. Cystinosis is also an inherited disease. Cysteine crystals are deposited in many tissues and organs (particularly in the reticulo-endothelial system). The primary defect is due to the impaired lysosomal function.
3. **Homo-cystinuria:** It is also an inherited disease i.e. homocystinuria-1. The clinical symptoms are occurrence of thrombosis, osteoporosis, dislocated lenses in the eyes and frequently, mental retardation.


Methionine
METHIONINE S ADENOSYL TRANSFERASE
ATP
Pi + PPi
S- Adenosyl Methionine
METHYL TRANSFERASE
Acceptor
CH3 Acceptor
S- Adenosyl Homocysteine
H2O
Adenosine
Homocysteine
SERINE
H2O
CYSTATHIONINE β SYNTHASE
Cystathionine
HYDROXYLASE
Homocysteine
H2O
γ CYSTATHIONINASE
NH3
PYRUVATE
DESULFHYDRASE
NH3 + H2S
Cysteine
α-Keto-butyrate
Homocysteine sulfonic acid
OXYGENASE
DEAMINASE
Homocysteine sulfonic acid
2-Keto, 4-sulfonic butyrate
SYNTHASE
β-Sulfonyl propionyl CoA
DESULPHYDRASE
Propionyl CoA
Succinyl CoA
TCA CYCLE

METABOLISM OF PHENYLALANINE, TYROSINE

Phenylalanine is an essential amino acid. The steps involved in their oxidation are—





Metabolic disorders of phenylalanine and tyrosine metabolism

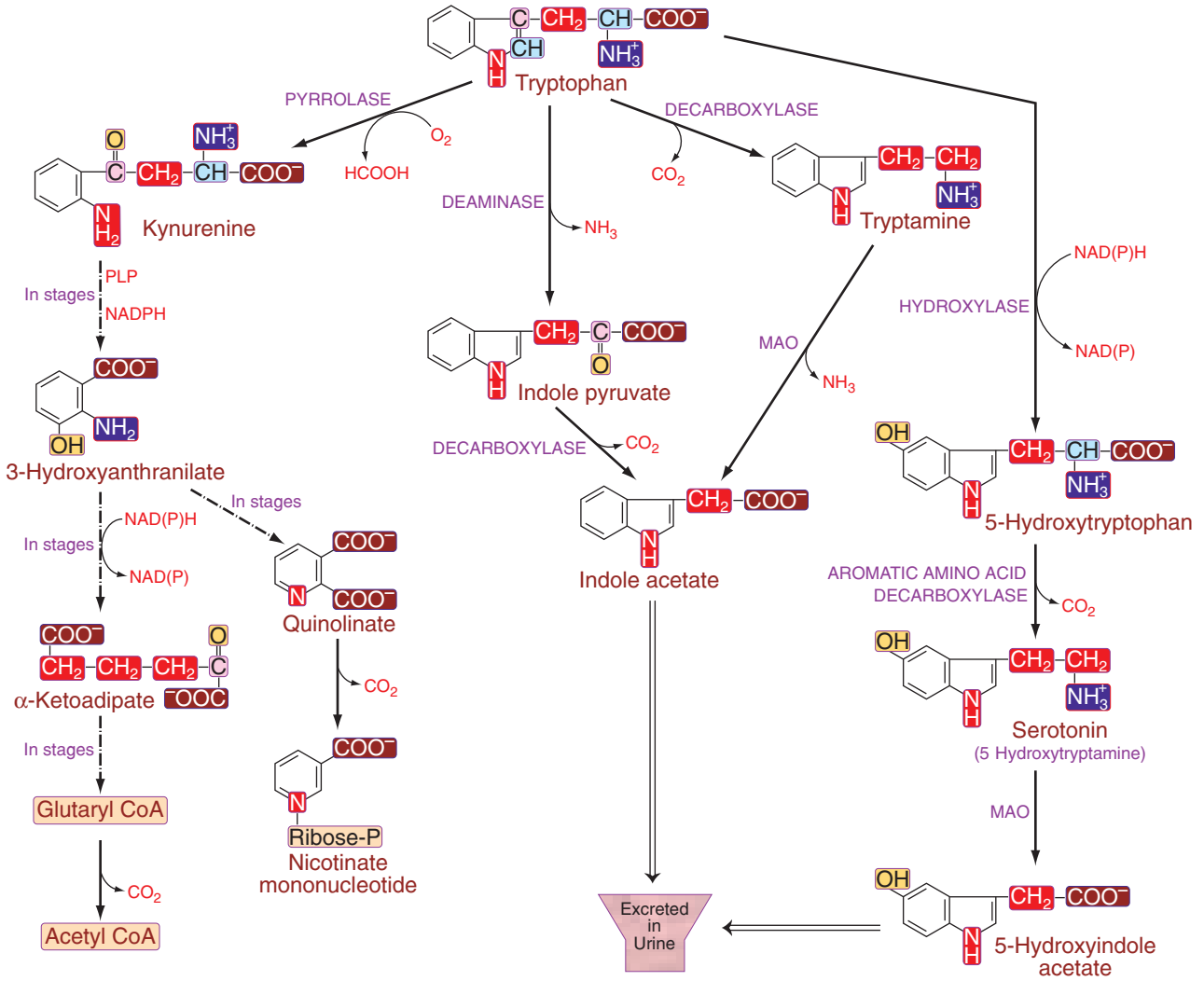
1. **Phenylketonuria:** It is a metabolic disorder with autosomal recessive inheritance. Due to defect in the enzyme phenylalanine hydroxylase (monooxygenase), phenylalanine accumulates in CSF, blood and in tissues. Alternative pathways start, leading to the formation of phenyl pyruvic and phenyl lactic acid. These inhibit the degradation of tyrosine and tryptophan. Therefore excretion of 5-hydroxy indole acetic acid and 5-hydroxy tryptamine (serotonin) in the urine is reduced. But tryptophan derivatives like indole acetic acid increases. Due to increase in phenylalanine in the cells they are not able to utilize other amino acids. Thus brain cells are deprived of essential nutrients for their maturation in the early stages. Even though it is not toxic to brain it shows neurological signs like irritability, convulsions, muscular hypertonia, tremors and retarded mental development. Phenylalanine competitively inhibits tyrosine resulting in blond hair, blur iris and fair skin. The skin becomes vulnerable to minor inflammatory lesions, rashes and eczema. Accumulation of phenyl acetic acid and lactic acid leads to musty body odour.

   Test for phenylketonuria: 10% $FeCl_3$ solution when added to patient's fresh urine results in emerald green colour which fades after 20 minutes.
2. **Tyrosinosis:** It is a very rare disease due to the deficiency of p-hydroxy phenyl pyruvic acid oxidase. So the latter is excreted in the urine, which turns dark on long standing. The black pigment is deposited in the sclera (between the cornea and the canthi), ear and nose cartilage which is termed as **'ochronosis'**. Ochronotic arthritis commonly involves shoulder and hips. Pigment is also deposited in the kidney leading to renal stones and nephrosis. There is no proper treatment for this; however ascorbic acid prevents the oxidation.
3. **Alkaptonuria:** Homogentesic acid oxidase is deficient.
4. **Albinism:** This is due to the deficiency of tyrosinase enzyme, which is an aerobic oxidase forming melanin in the melanosomes present in melanocytes of the skin. Albinism is of three types–in Type-I tyrosinase is absent, in type-II tyrosine cannot be transported to the melanosomes due to lack of permease and in type-III tyrosine normally converts to DOPA and quinone and thereafter the enzymes are absent. The condition is called oculocutaneous albinism. Clinically the skin is depigmented. It does not tan but burns on exposure to sunlight. The hair is white and silky. The iris is bluish or pinkish.

METABOLISM OF TRYPTOPHAN

It is an essential, glucogenic & ketogenic amino acid. The main pathway of tryptophan is formation of anthranillic acid and then glutaryl-CoA and acetyl-CoA. Small amount is also converted to niacin. 60 mg of tryptophan forms 1 mg of niacin. So tryptophan rich diets have a sparing effect on niacin requirement.

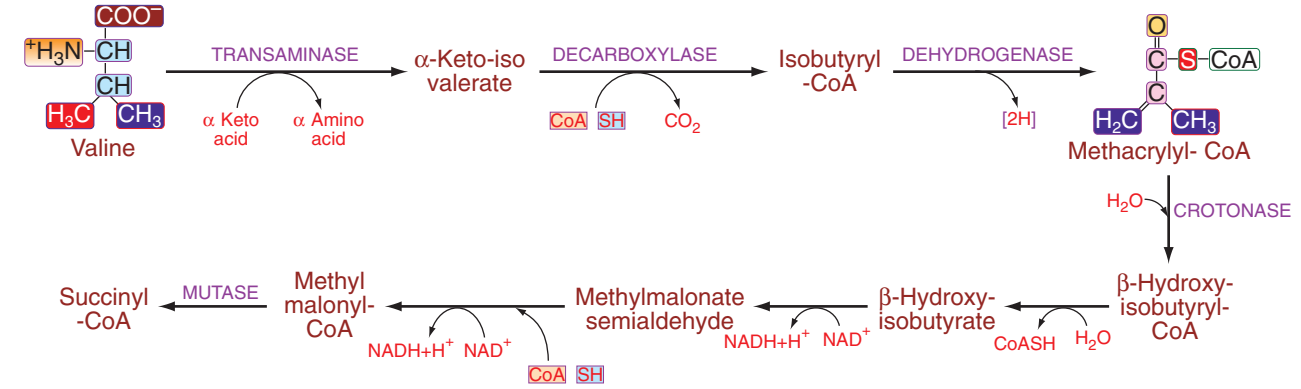
On decarboxylation, tryptophan is converted to 5-hydroxy tryptamine called serotonin. It is a potent vasoconstrictor, stimulates smooth muscle contraction and is a stimulator of cerebral activity. It is formed in intestinal epithelium, blood platelets and in the brain. In tumours like argentaffinomas, due to oxidation of serotonin there will be excess formation of 5-hydroxy indole acetic acid (5-HIAA) which is excreted in urine. Normal excretion of 5-HIIA is 7 mg/day. In malignant carcinoid it is 400 mg/day. Normally 1% of tryptophan is converted to serotonin. During carcinoid, 60% of this is converted to serotonin, so there is no formation of niacin. Symptoms like pellegra and negative nitrogen balance are seen. Seretonin in brain is in bound form. Reserpine as anti hypertensive drug releases serotonin and is thus available for monoamine oxidase which converts serotonin to 5-HIAA. Hence, reserpine brings depression of cerebral activity. Melatonin the hormone of the pineal body and the peripheral nerves of humans is synthesized from serotonin.

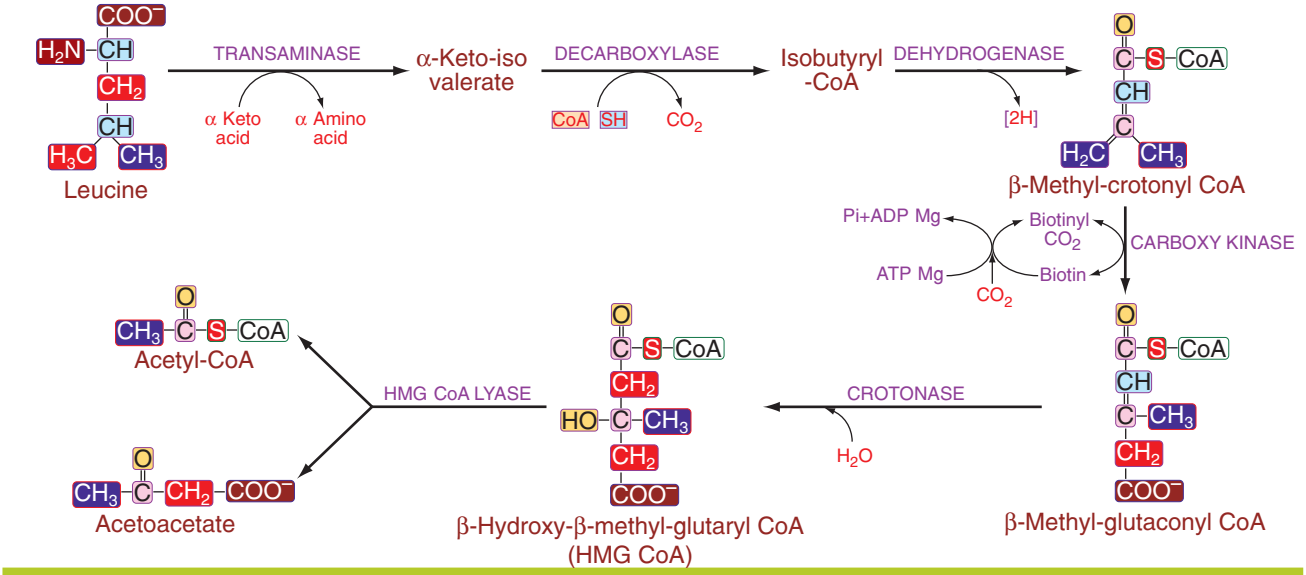


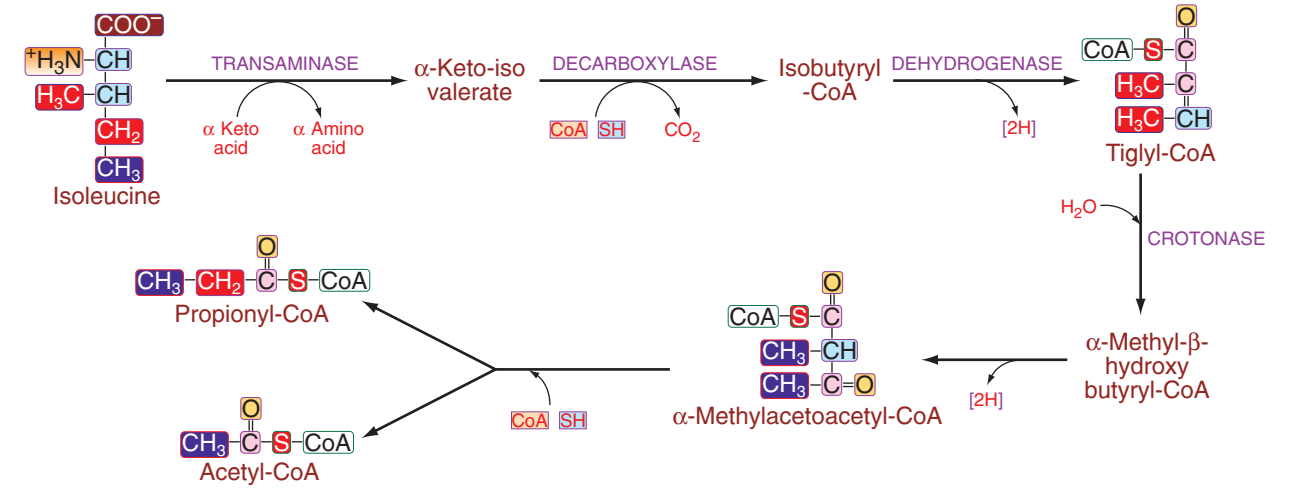
Hartnups' disease: It is a rare metabolic disease in which transport of mono amino mono carboxylic acids is defective. Accumulation of tryptophan results in formation of indole pyruvic and acetic acid, so amino aciduria is observed. Niacin formation is impaired. Symptoms exhibited are appearance of pellegra, skin rashes with intermittent cerebral ataxia and mental retardation.

METABOLISM OF VALINE, LEUCINE AND ISOLEUCINE

Maple syrup urine: It is a metabolic defect related to branched chain amino acids like leucine, isoleucine and valine due to deficient decarboxylase enzyme resulting in the accumulation of these amino acids in the CSF and blood. Synthesis of protein and lipoprotein is diminished. There is rapid degradation of brain. The smell of maple syrup in urine is attributed to keto acids formed from these branched chain amino acids upon transamination.

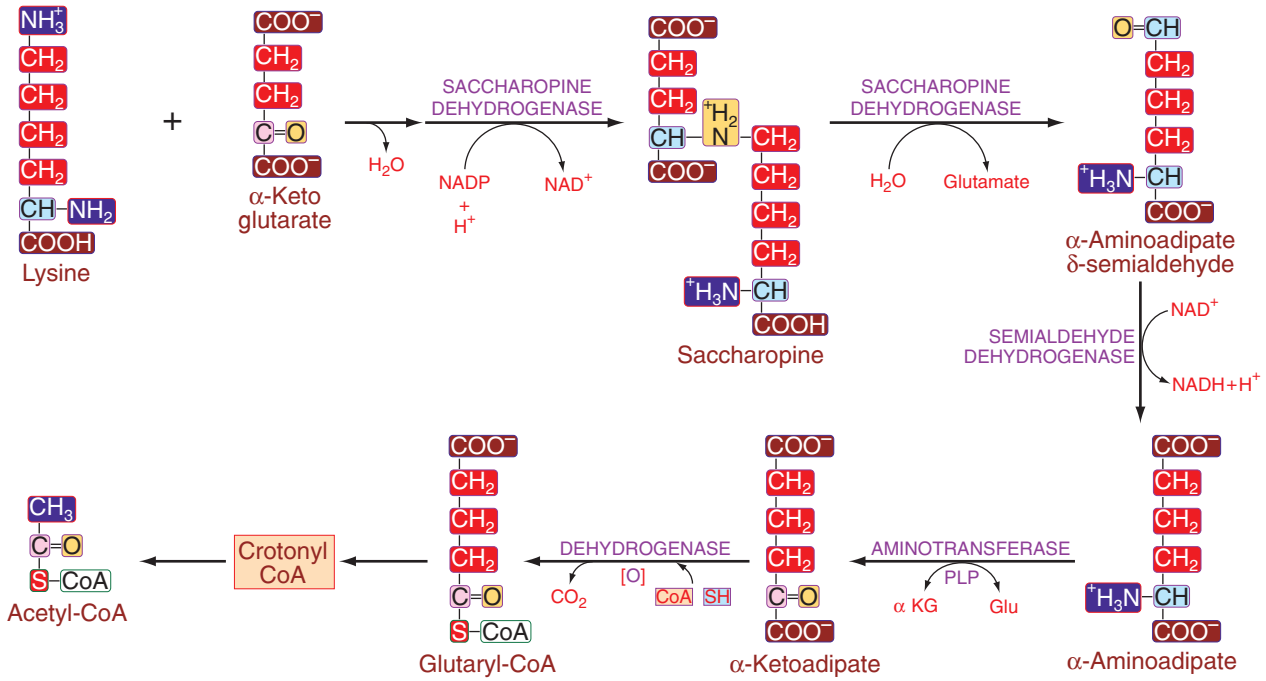

Valine
TRANSAMINASE
α Keto acid
α Amino acid
α-Keto-iso valerate
DECARBOXYLASE
CoA SH
CO2
Isobutyryl -CoA
DEHYDROGENASE
[2H]
Methacrylyl- CoA
H2O
CROTONASE
β-Hydroxy-isobutyryl-CoA
CoASH H2O
β-Hydroxy-isobutyrate
NADH+H+ NAD+
Methylmalonate semialdehyde
NADH+H+ NAD+
CoA SH
Methyl malonyl-CoA
MUTASE
Succinyl -CoA


Leucine
TRANSAMINASE
α Keto acid
α Amino acid
α-Keto-iso valerate
DECARBOXYLASE
CoA SH
CO2
Isobutyryl -CoA
DEHYDROGENASE
[2H]
β-Methyl-crotonyl CoA
Pi+ADP Mg
ATP Mg
Biotinyl
CO2
Biotin
CO2
CARBOXY KINASE
β-Methyl-glutaconyl CoA
CROTONASE
H2O
β-Hydroxy-β-methyl-glutaryl CoA (HMG CoA)
HMG CoA LYASE
Acetyl-CoA
Acetoacetate


Isoleucine
TRANSAMINASE
α Keto acid
α Amino acid
α-Keto-iso valerate
DECARBOXYLASE
CoA SH
CO2
Isobutyryl -CoA
DEHYDROGENASE
[2H]
Tiglyl-CoA
H2O
CROTONASE
α-Methyl-β-hydroxy butyryl-CoA
[2H]
α-Methylacetoacetyl-CoA
CoA SH
Propionyl-CoA
Acetyl-CoA

METABOLISM OF LYSINE

Lysine is an essential amino acid which is both glucogenic and ketogenic. It is not present in adequate amounts in cereal proteins. Hence its deficiency in vegetarians is quite common. It cannot be deaminated to its keto acid.



Lysine condenses with α-ketoglutarate forming a schiff's base and then by the action of dehydrogenase it is reduced to saccharopine which on further dehydrogenation finally forms adipic acid. Adipic acid sequentially produces acetyl-CoA. Metabolic disorder of lysine is rare. However hyperlysemia with associated hyperammonemea is seen.

Inborn errors of amino acid metabolism

Disorder	Metabolic defect (enzyme / other)
1. Urea cycle	
(a) Hyperammonemia type-I	Carbamoyl phosphate synthase-I
(b) Hyperammonemia type-II	Ornithine transcarbamoylase
(c) Citrullinemia	Argininosuccinate synthase
(d) Arginosuccinic aciduria	Argininosuccinase
(e) Hyperargininemia	Arginase
2. Glycine	
(a) Glycinuria	Defect in renal reabsorption
(b) Primary hyperoxaluria	Glycine transaminase

3. Cysteine, Cystine and Methionine (Sulphur containing amino acids)	
(a) Cystinuria	Defect in renal reabsorption
(b) Cystinosis	Impairment in cysteine utilization (defect in lysozomal function)
(c) Homocystinuria type-I	Cystathionine synthetase
(d) Homocystinuria type-II	N^5, N^{10}-Methylene THF reductase
(e) Homocystinuria type-III	N^5-Methyl THF-homocysteine methyltransferase
(f) Cystathionuria	Cystathioninase
4. Phenylalanine and Tyrosine	
(a) Phenylketonuria	Phenylalanine monooxygenase
(b) Tyrosinosis (tyrosinemia type-I)	Maleyl acetoacetate isomerase or fumaryl acetoacetate hydrolase
(c) Tyrosinemia type-II	Tryrosine transaminase
(d) Neonatal tyrosinemia	p-hydroxy phenylpyruvate dioxygenase
(e) Alkaptonuria	Homogentisate oxidase
(f) Albinism	Tyrosinase
5. Tryptophan	
(a) Hartnup's disease	Defective intestinal absorption
6. Valine, Leucine and Isoleucine (Branched chain amino acids)	
(a) Maple syrup urine	Branched chain α-keto acid dehydrogenase
(b) Intermittent branched chain ketonuria	Variant of the above enzyme (less severe)
(c) Hypervalinemia	Valine transaminase
(d) Isovaleric academia	Isovaleryl-CoA dehydrogenase
7. Histidine	
(a) Histidinemia	Histidase
8. Proline	
(a) Hyperprolinemia type-I	Proline oxidase

14

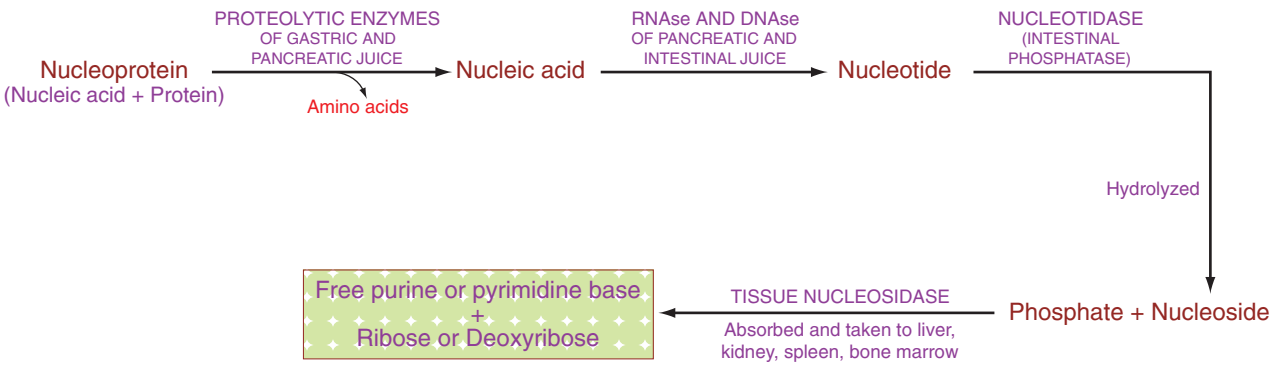
NUCLEIC ACID METABOLISM

Human beings are prototrophic for nucleic acids as these are synthesized in the body.

DIGESTION AND ABSORPTION

Nucleic acids are consumed in large quantities owing to their presence in all cells. These nucleic acids are not utilized by the body; instead they are digested, catabolized and excreted. They are taken in the form of nucleoproteins, which are conjugated proteins with amino acids constituting the protein part and nucleic acids constituting the prosthetic part.

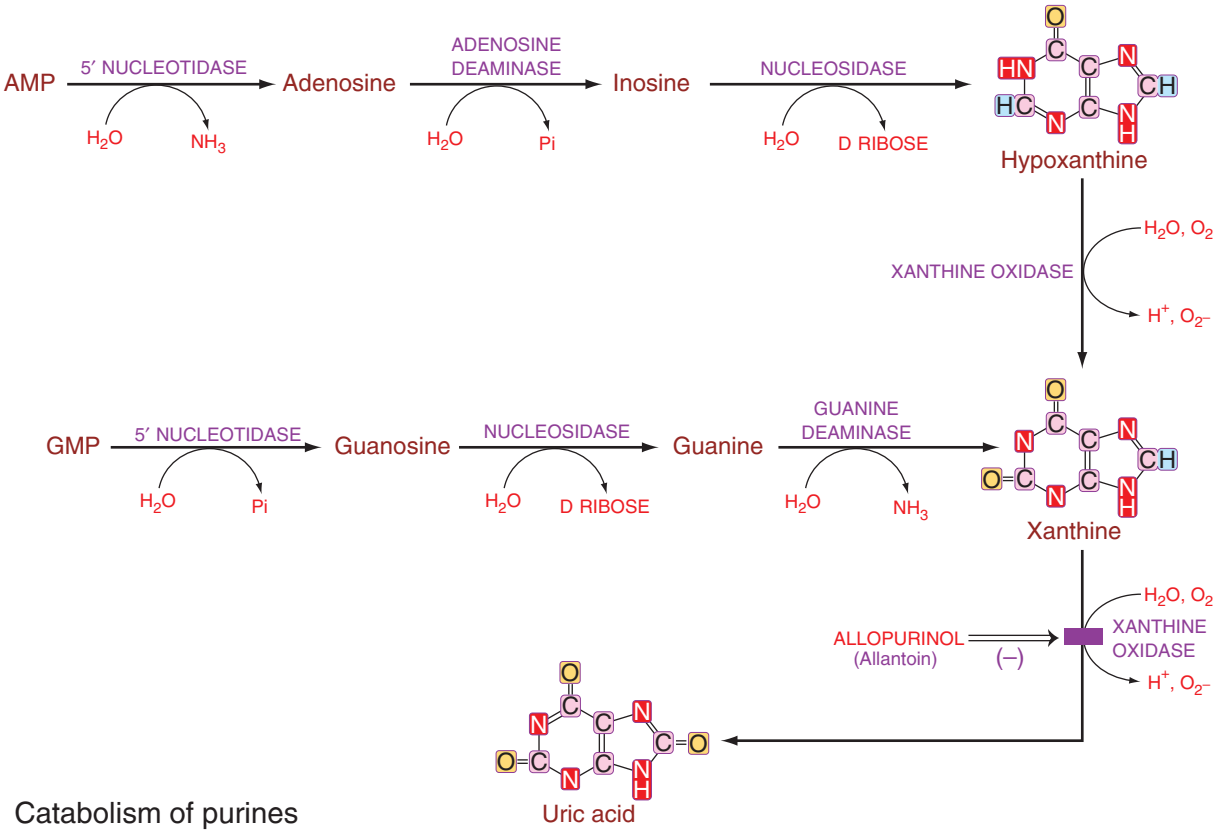
The protein part of nucleoprotein is acted upon by proteolytic enzymes of gastric and intestinal juices. Nucleic acids are acted upon by nucleases (ribonuclease and deoxyribonuclease) of pancreatic and intestinal juices to produce mononucleotides. Nucleotides are hydrolyzed to nucleosides by nucleotidases (intestinal phosphatase) Nucleosides are absorbed by intestinal mucosa to portal blood and transported to the liver and supplied through systemic circulation to other viscera. The enzyme nucleosidases liberate the free purine and pyrimidine base and ribose or deoxyribose from the nucleosides.



CATABOLISM OF NUCLEOSIDES

1. **Purine nucleoside – formation of uric acid:** End product of purine metabolism is uric acid (in primates including man and dog). In lower animals, birds and reptiles, uric acid is converted to

allantoin by the action of enzyme uricase. Liver, spleen, kidney, intestinal mucosa contain enzymes capable of acting on the purine ring in the free or combined state.



Catabolism of purines

Uric acid metabolism: Uric acid exists as sodium ureate in plasma. Maximum amount of sodium ureate that can dissolve in the blood plasma is about 7 mg/100 ml. At this point there will be saturation of blood with sodium ureate. Above this it gets precipitated. Uric acid concentration does not vary with greater intake of uric acid in the diet because on intake of higher concentration in the diet the synthesis of uric acid in the body will be inhibited. Hence there is a balanced amount of uric acid in the body.

Miscible pool of uric acid: Total amount of uric acid present in the body in the dissolved state in males is 3.4 to 7.0 mg/dl and in females it is 2.4 to 5.7 mg/dl.

Modes of excretion: It is excreted in urine by glomerular filtration. Amount excreted is 250 to 750 mgs/day. It is also excreted through bile, to about $1/5^{th}$ of the total amount into the intestine. In the intestine it will be converted to CO_2 and NH_3 either by action of intestinal flora or autoxidation of the uric acid. Estimation of blood uric acid in ureotelic animals has importance in the diagnosis of gout and Von-Gierke's disease.

Conditions varying the blood levels of uric acid

Gout: If the concentration of uric acid exceeds 7 mg/100 ml in the blood plasma, the uric acid gets precipitated as sodium ureate crystals that cannot be excreted hence gets deposited in the soft tissues. There is abnormal deposition in joints and tendons. This abnormal deposition of sodium ureate crystals

in soft tissue is known as TOPHI. Because of this the tissue gets degraded or degenerated at the bone joints leading to degeneration of neighbouring tissues as well. Degeneration causes inflammation of the joint.

***Gouty arthritis*:** Generally the metacarpal phalangeal joints and metatarsal phalangeal joints are affected by this gout. Knee joint is also affected. There are two types of gout—

1. ***Primary Gout*:** Enzyme 'PRPP synthetase' shows altered kinetics leading to overproduction of PRPP and hence overproduction of purines.
2. ***Secondary gout*:** This is due to—
   (i) Excess catabolism of purine as in polycythemia.
   (ii) Decreased excretion of uric acid as in renal failure.

Gout can be controlled by—

1. Uricosuric drugs like salicylates, cinchopher, adrenal cortical hormones, which cause increased excretion of uric acid in urine by decreasing its re-absorption.
2. Allopurinol (Allantoin) a structural analogue to hypoxanthine which competitively inhibits xanthine oxidase and decreases the production of uric acid.

Von-Gierke's disease: In this disease there is an overproduction of uric acid. The HMP shunt pathway is overactive due to the deficiency of glucose-6-phosphatase thereby producing excessive amounts of ribose-5-phosphate. This leads to the overproduction of PRPP, hence the uric acid.

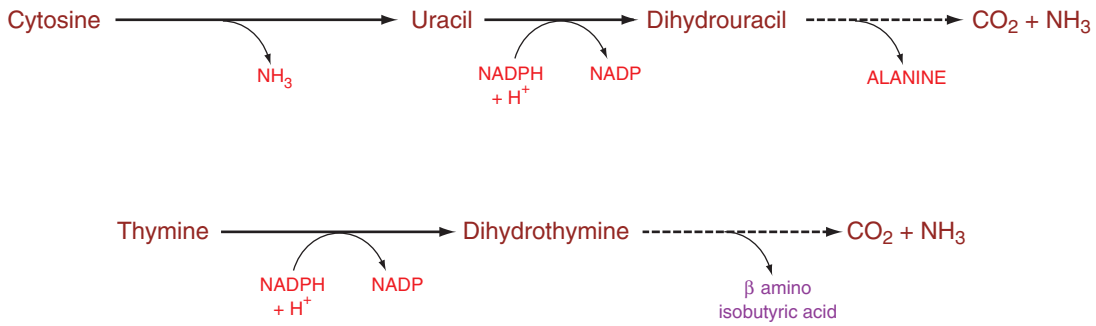
Methods of estimation: Phosphotungstic acid method, Fehling's method and Autoanalyzer.

Conditions varying the blood level of uric acids: Whenever there is cell death, the nucleic acids are released and converted to purines and finally uric acid is formed. The conditions where uric acid level increases in the blood are—(a) excessive tissue destruction and (b) gout.

***Excessive tissue destruction*:** Is seen in—

1. Old age
2. Febrile diseases
3. Hypoxia
4. Trauma
5. Kidney dysfunction or total renal failure
6. High non-vegetarian diets

2. **Pyrimidine nucleoside:** Pyrimidine nucleosides are catabolized in the liver. The products of breakdown of pyrimidine ring are ammonia and CO_2 which are converted into urea for excretion.



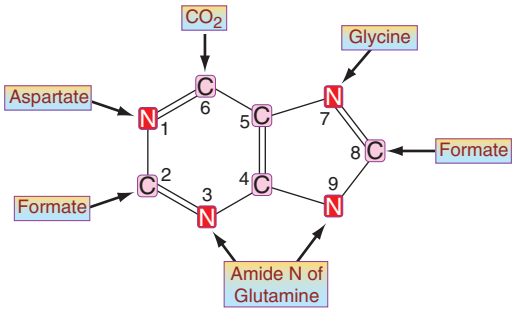
BIOSYNTHESIS OF PURINE NUCLEOTIDES

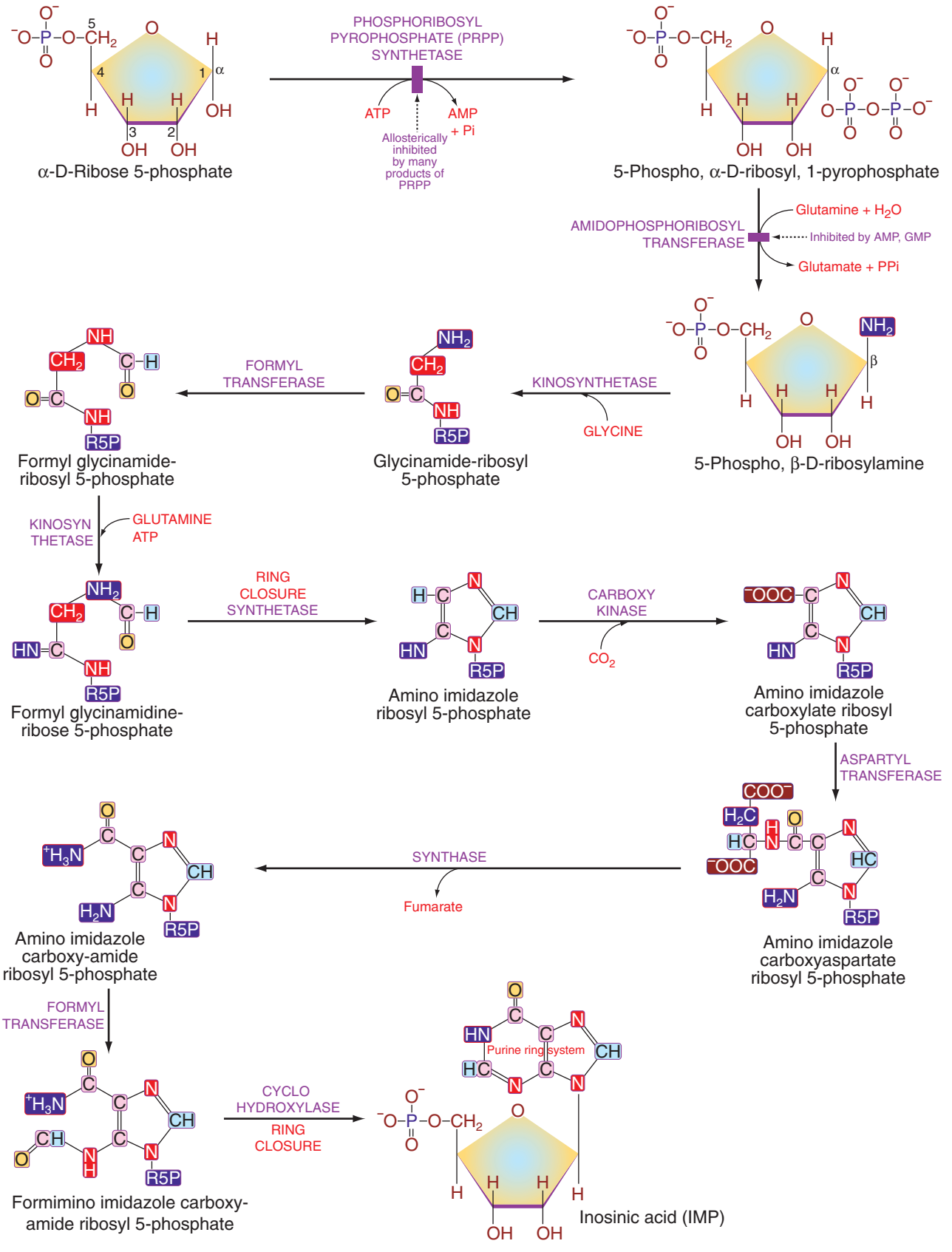
There are two pathways by which nucleotides are made available for the formation of nucleic acids (1) Denovo synthesis i.e. new synthesis and (2) Salvage process i.e. recycling of the bases.

DE NOVO SYNTHESIS

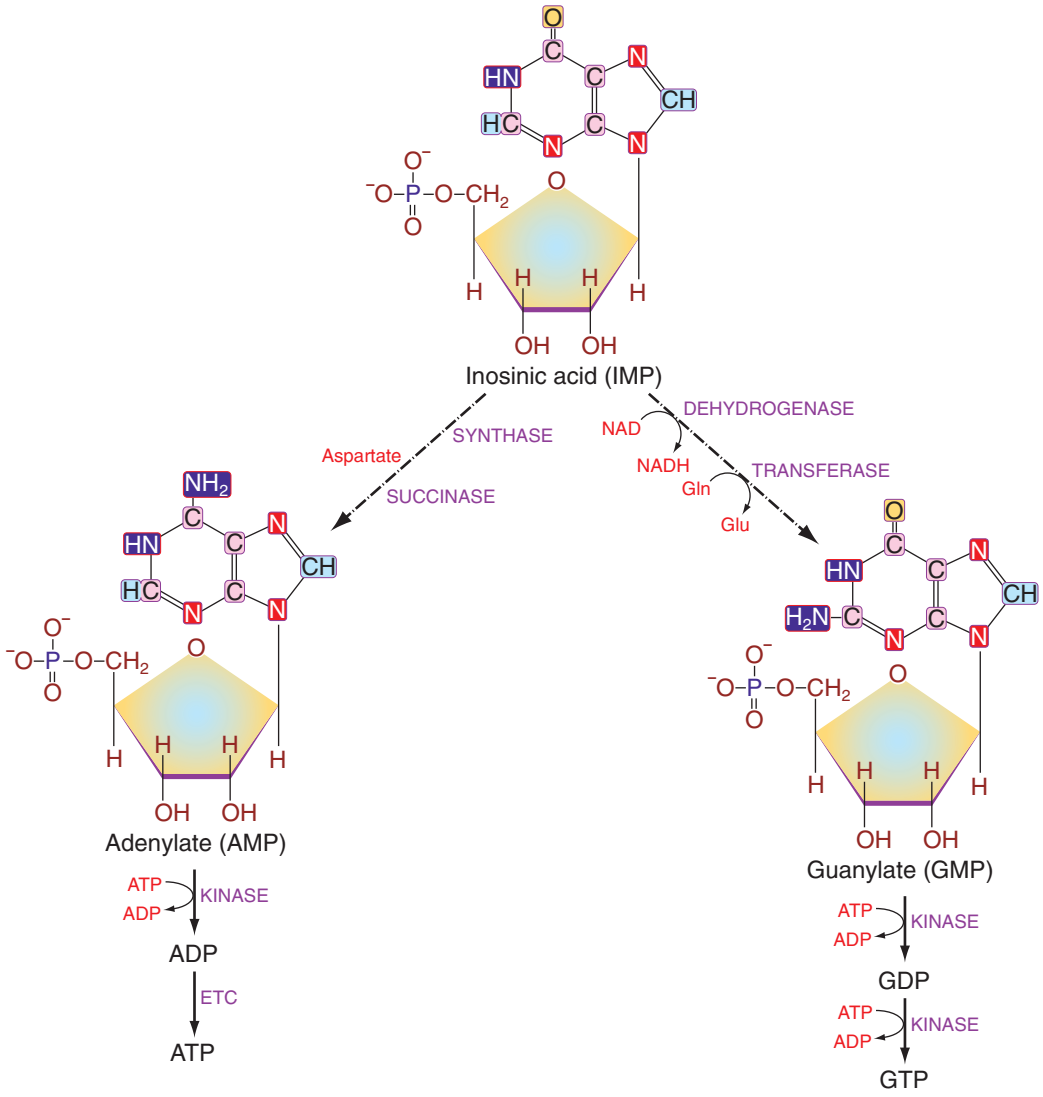
De novo (all over again) synthesis of purine nucleotides is synthesis of purines anew. The purine ring is synthesized along with the nucleotide i.e. attached to the ribose sugar provided from HMP pathway. This pathway supplies ribose sugar for the formation of the nucleotide. Activated form of D-ribose-5-phosphate serves as the starting material on which purine ring is build up step by step. Precursors of the members of purine ring are—

- ✓ N-1 is contributed by nitrogen of aspartate.
- ✓ N-3 and N-9 arise from amide nitrogen of glutamine.
- ✓ C-2 and C-8 originate from the formate.
- ✓ C-6 is embedded from respiratory carbon dioxide.
- ✓ C-4, C-5 and N-7 are taken up from glycine.

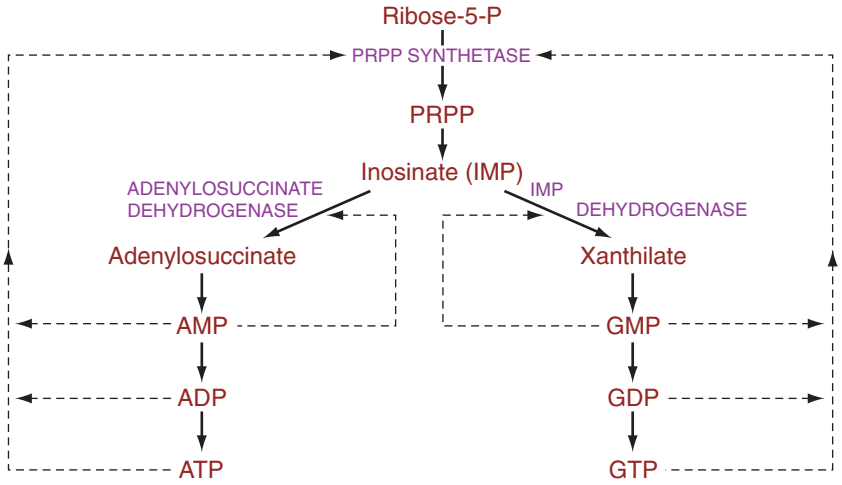




Purine biosynthesis



Regulation of purine nucleotide biosynthesis: Purine biosynthesis is regulated by feedback inhibition. This inhibition is in the 1st step. It is the committed step which is generally irreversible. Once the committed step is passed over, the product has to be formed. The different mechanisms by which it is regulated are—



SALVAGE PATHWAY

The de-novo synthesis does not occur in all the cells. Brain cells and leukocytes lack this mechanism. In these cells purine synthesis occurs by salvage pathway. Salvage pathway involves synthesis of purine nucleotides from free purine bases, which are salvaged from dietary sources and tissue breakdown. This pathway is promoted by the action of two enzymes which convert free purines into purine nucleotides for reuse. The enzymes are (1) Adenine phosphoribosyl transferase and (2) Hypoxanthine guanine phosphoribosyl transferase (HGPRT).

$$\text{Adenine} + \text{5-PRPP} \xrightarrow[\text{TRANSFERASE}]{\text{ADENINE PHOSPHORIBOSYL}} \text{AMP} + \text{PPi}$$

$$\text{Xanthine} + \text{5-PRPP} \xrightarrow{\text{HGPRT}} \text{XMP} + \text{PPi}$$

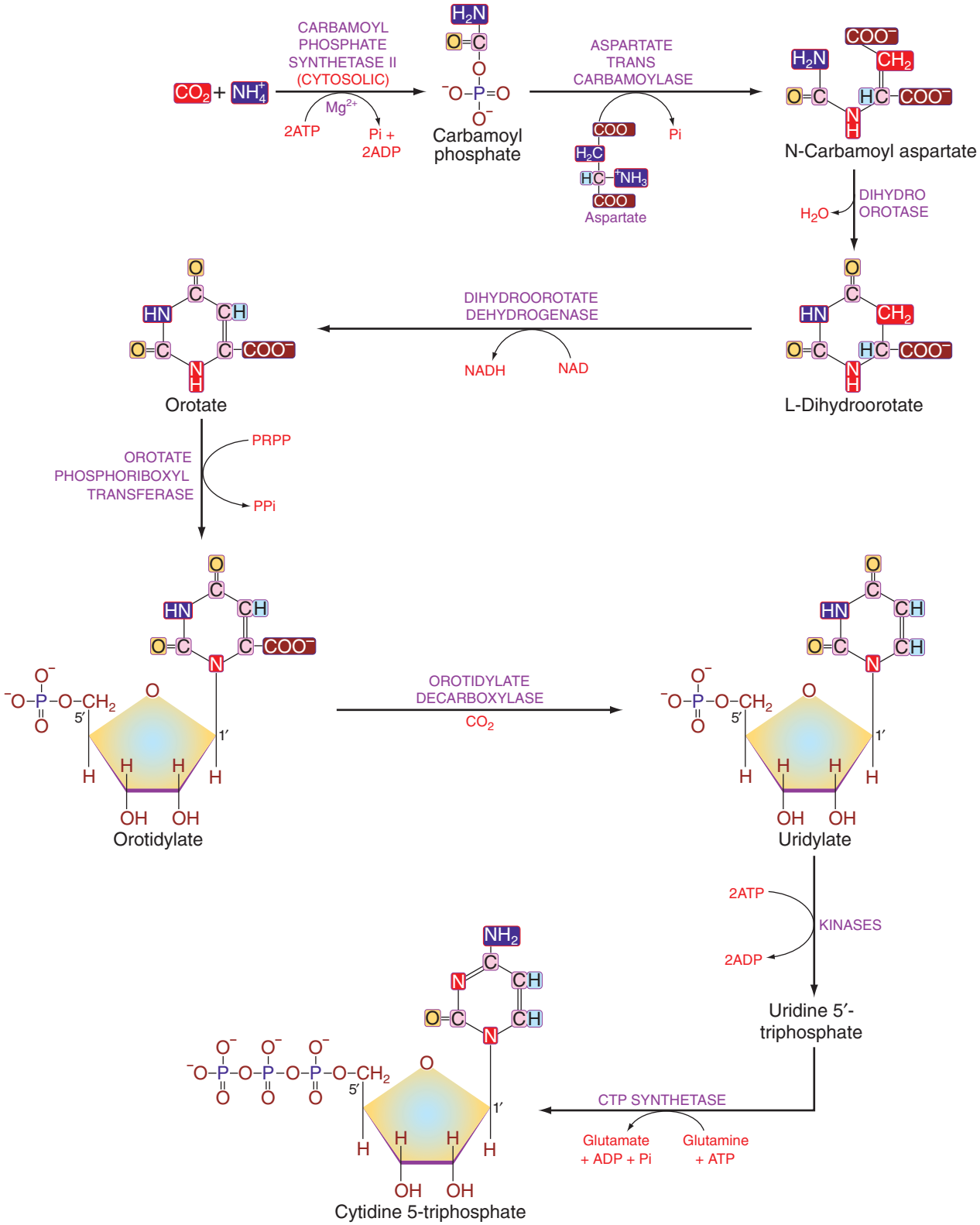
$$\text{Hypoxanthine} + \text{5-PRPP} \xrightarrow{\text{HGPRT}} \text{IMP} + \text{PPi}$$

$$\text{Guanine} + \text{5-PRPP} \xrightarrow{\text{HGPRT}} \text{GMP} + \text{PPi}$$

Lesch-Nyhan syndrome: This is a genetic disorder caused due to the deficiency of the enzyme **'Hypoxanthine Guanine Phospho Ribosyl Transferase (HGPRT)'**. When this enzyme is deficient, guanine, xanthine and hypoxanthine are not salvaged and hence degraded to uric acid. This is especially seen in male children. In female children the gene is recessive and is a carrier. It is a male dominant gene. Such males show (1) mental retardation and (2) tendency for self destruction.

BIOSYNTHESIS OF PYRIMIDINE NUCLEOTIDES

Pyrimidine nucleotide biosynthesis takes place in a different manner from that of purine nucleotides. The six membered pyrimidine ring is made first and then attached to ribose phosphate. The synthesis begins with carbon dioxide and ammonia combining to form carbamoyl phosphate catalysed by the cytosolic enzyme carbamoyl phosphate synthetase-II. Carbamoyl phosphate combines with aspartate to form carbamoyl aspartate aided by the enzyme aspartate transcarbamoylase. Dihydroorotate is formed from carbamoyl aspartate by removal of water and closure of the ring under the influence of the enzyme dihydroorotase. Dihydroorotase is oxidized to orotic acid by dehydrogenase which uses NAD^+ as the electron acceptor. Orotic acid is attached to ribose to yield orotidylic acid. Orotidylate is then decaroxylated to form uridylate. Uridylate is then converted to all the other pyrimidine nucleotides viz., CMP, UMP & TMP. The reaction steps involved in the biosynthesis of pyrimidine nucleotides are given under.



Pyrimidine biosynthesis

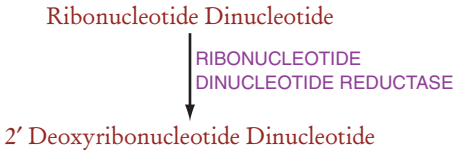
Regulation of pyrimidine biosynthesis: Regulation of pyrimidine biosynthesis is by feed back inhibition at the committed step i.e. the reaction catalyzed by the enzyme aspartate transcarbamoylase. This is negatively inhibited by the end product i.e. CTP. The second site is at carbamoyl phosphate synthase-II which is feed back inhibited by UMP.

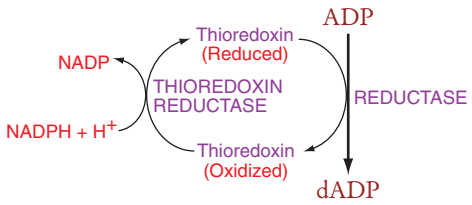
Orotic aciduria: It is a metabolic disorder of pyrimidine biosynthesis characterized by accumulation of orotic acid in blood and its increased excretion in urine. It is caused due to the deficiency of enzyme orotidylic acid phosphorylase and orotidylic acid decarboxylase or orotic phosphoribosyl transferase. This leads to non-conversion of orotic acid to UMP. This may even affect the synthesis of other nucleotides. It is generally found in children who show retarded mental development and growth as there is no proper synthesis of DNA. They show megaloblastic anemia. This can be overcome by injection of CTP and UTP.

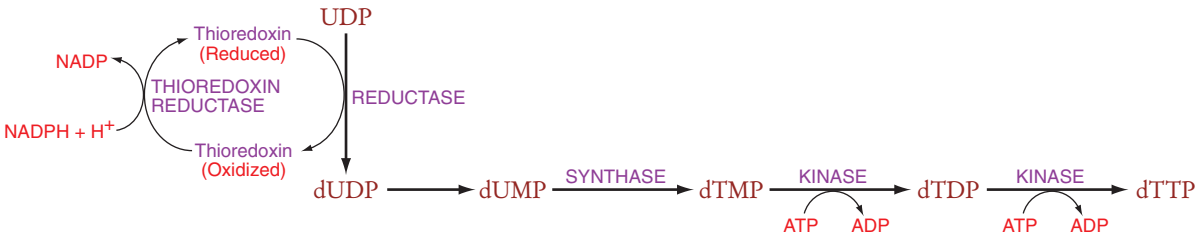
BIOSYNTHESIS OF DEOXYRIBONUCLEOTIDES

Deoxyribonucleotides are obtained from ribonucleotides. Thioredoxin is a protein which takes part in the conversion of ribonucleotides to deoxyribonucleotides.

Purine Deoxynucleotides:







Pyrimidine Deoxynucleotides:

15

HORMONES

Hormones are organic substances required in minute quantities produced by ductless glands directly into the blood and act on some other tissue (target tissue).

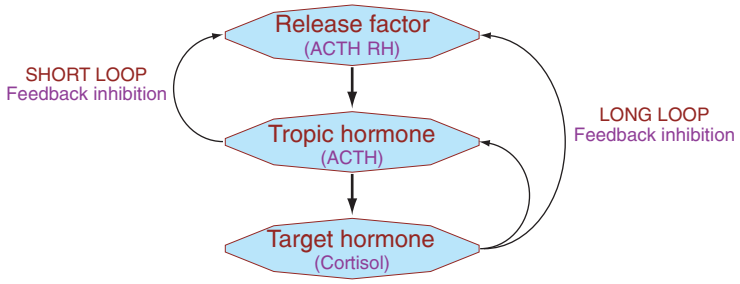
Endocrine glands and the hormones produced

Endocrine gland	Hormones produced
1. Pituitary gland	
a) Anterior pituitary (Adenohypophysis)	1. Growth Hormone (GH) 2. Thyrotropic or Thyroid Stimulating Hormone (TSH) 3. Adrenocorticotropic Hormone (ACTH) 4. Pancreotropic Hormone 5. Metabolic Hormone 6. Interstitial Cell Stimulating Hormone (ICSH) or Leutinising Hormone (LH) 7. Follicle Stimulating Hormone (FSH) 8. Prolactin or Luteotropic Hormone (LTH) 9. Melanocyte Stimulating Hormone (MSH)
b) Posterior pituitary (Neurohypophysis)	1. Oxytocin 2. Vasopressin or Antidiuretic Hormone (ADH)
2. Thyroid gland	1. Thyroxine (T_4) 2. Triiodothyronine (T_3) 3. Calcitonin
3. Parathyroid gland	1. Parathormone
4. Pancreas	
a) β-cells of islets of langerhans	1. Insulin
b) α-cells of islets of langerhans	2. Glucagon

5. Gastro-intestinal mucosa	
a) Stomach	1. Gastrin.
b) Intestine	1. Secretin. 2. Enterokinin. 3. Pancreozymin. 4. Cholecystokinin. 5. Enterogastrone. 6. Somatostatin.
6. Adrenal gland	
a) Adrenal cortex	1. Glucocorticoids—(i) Cortisol, (ii) Corticosterone 2. Mineralocorticoids—(i) Aldosterone 3. Androgens
b) Adrenal medulla	1. Epinephrine (Adrenaline) 2. Norepinephrine (Nor-Adrenaline)
7. Gonads	
a) Testis	1. Testosterone
b) Ovaries	1. Estrogen 2. Progesterone

In addition to these major glands, there are other few minor endocrine glands like the pineal gland, the thymus gland, placenta, kidney, corpus leutum etc.

The hypothalamic part of the central nervous system on receiving suitable information from the environment or other parts of the body produces the hormones known as the **'release factors'** which pass through the hypophyseal-hypothalamic portal vein to the master endocrine gland – the pituitary gland. This master gland produces the respective tropic hormone in response to the release factor. The tropic hormone will then stimulate another endocrine gland which will produce the specific hormone required for the action as per the information received by the CNS. This specific hormone or target hormone will finally act on the target tissue or the target cell.



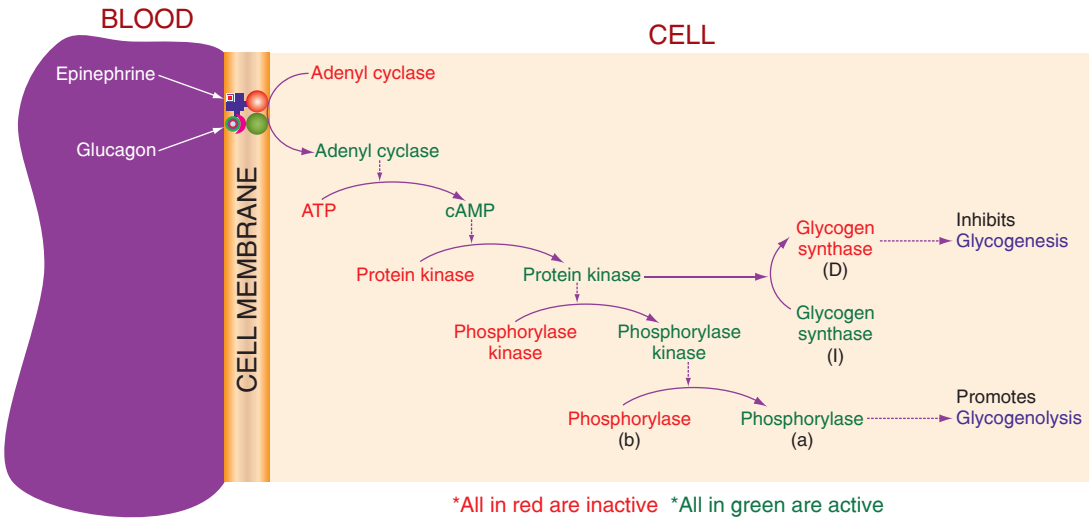
Example No. 1	General Scheme of hormone action	Example No. 2
Shock ↓	External stimuli ↓	Cold ↓
Hypothalamus ↓	Hypothalamus ↓	Hypothalamus ↓
ACTH-Release hormone ↓	Release factor ↓	Thyrotropic release factor ↓
Pituitary gland ↓	Pituitary gland ↓	Pituitary gland ↓
ACTH ↓	Tropic hormone ↓	Thyrotropic hormone (TSH) ↓
Adrenal gland ↓	Respective endocrine gland ↓	Thyroid gland ↓
Epinephrine ↓	Specific hormone ↓	Thyroxine ↓
Muscles ↓	Target tissues ↓	Various tissues ↓
Glycogenolysis	Metabolic action	Increased BMR

Classification of hormones: The hormones are divided into four classes—

1. **Peptide hormones:** All the hormones of the hypothalamus, pituitary, G.I. mucosa, and pancreas.
2. **Steroid hormones:** The hormones of adrenal cortex and gonads.
3. **Catecholamines:** Epinephrine and norepinephrine.
4. **Thyroid hormones:** Thyroxine and triiodothyronine.

Mechanism of hormone action:

1. **Enhancement of enzyme synthesis:** The steroid hormones and the thyroid hormones enter the cell and combine with the specific receptor protein to form **'receptor protein-hormone complex'**. This complex will then bind to a specific site on DNA and initiate or enhance the synthesis of mRNA which in turn synthesizes the protein i.e. enzymes. Therefore the cell reactions speed up.
2. **Change in cell permeability:** Hormones like insulin binds to a specific receptor on the cell membrane which results in alteration of the permeability of the cell to certain substances like glucose, amino acids and ions. The entry of these substances will bring a change in cell reactions.
3. **Action through a second messenger (cAMP):** Hormones like epinephrine, glucagon bind to a regulatory site on the cell membrane. On the inner side of this regulatory site, an enzyme known as **adenyl cyclase** is present that converts ATP to cAMP which then activates certain **protein kinases** that in turn will phosphorylate certain enzymes. Some enzymes on **phosphorylation** become active whereas some other enzymes become inactive. Certain reactions are therefore stimulated while others are inhibited.



Hormonal regulation of glycogen metabolism

Feedback regulation of hormone production: The production of hormones is regulated by feedback mechanism. There are two types of feedback regulation (1) Negative feedback regulation and (2) Positive feedback regulation.

1. **Negative feedback regulation:** When the target hormone is in little excess, then this excess hormone will inhibit the production of its tropic hormone. The tropic hormone in turn will stop the release of the release factors. The inhibitory action of the tropic hormone over the release factor is known as the short loop feedback. At other instances the target hormone can itself inhibit the release factor this is known as long loop feed back.
2) **Positive feedback regulation:** Estrogen stimulates the production of luteinizing hormone.

Secretion of hormones from endocrine glands: The peptide hormones and the catecholamines are secreted by a process called exocytosis. Steroid hormones are secreted by passive diffusion.

Circulation of hormones in blood: The water soluble peptide hormones and the catecholamines circulate in free form in the blood. Steroid and thyroid hormones circulate bound to specific proteins known as binding, carrier or transport proteins ex Thyroxine binding globulin (TBG).

Degradation and excretion of hormones: All the hormones are degraded and excreted. Peptide hormones are degraded in the liver and/or kidney. The catecholamines, steroids and the thyroid hormones are inactivated directly by enzymatic modification in the blood and/or in the liver.

Disorders of the endocrines: There are three major classes of endocrine disorders—

1. **Insufficient hormone production:** This may be due to abnormal function or destruction of the endocrine gland.
2. **Excess hormone production:** This is due to tumors of the gland.
3. **Altered tissue responsiveness to hormone effects:** (a) Immunity to hormones (b) Defective or absence of receptor (c) Inactive receptor hormone complex. These are the causes that lead to ineffectiveness of hormones though they are produced in normal amounts.

HYPOTHALAMUS

All the hormones of the hypothalamus are peptide hormones and they are known as release factors or releasing hormones. Their function is to stimulate the production of anterior pituitary hormones. Each of the anterior pituitary hormones has its own release factor ex. ACTH has ACTH-release hormone.

The neurons of the hypothalamus produce two hormones (1) Oxytocin (2) Vasopressin or Anti-diuretic Hormone (ADH).

The neurons extend to the posterior lobe of the pituitary where these two hormones are stored in the nerve endings. Neurophysin help in the transport of these hormones from hypothalamus to the posterior pituitary (Neurophysin I and II are proteins).

ANTERIOR PITUITARY

It is the master gland and controls the activities and development of other endocrine glands. The hormones produced by the anterior pituitary are known as tropic hormones. These are the hormones that stimulate the respective glands ex Thyrotropic hormone stimulates the thyroid gland for the production of thyroid hormones.

Tropic hormones and their site of action

Endocrine gland	Hormone produced	Site of action
1. Pituitary		
Anterior Pituitary (Adenohypophysis)	Growth Hormone (GH)	All tissues
	Thyrotropic or Thyroid Stimulating Hormone (TSH)	Thyroid gland
	Adrenocorticotropic Hormone (ACTH)	Adrenals
	Pancreotropic Hormone	Pancreas
	Metabolic Hormone	Liver
	Interstitial Cell Stimulating Hormone (ICSH) or Leutinising Hormone (LH)	Gonads
	Follicle Stimulating Hormone (FSH)	Gonads
	Prolactin or Leuteotropic Hormone (LTH)	Mammary gland
	Melanocyte Stimulating Hormone (MSH)	Skin

Growth hormone and prolactin do not act as tropic hormones; instead they act as the target hormones.

GROWTH HORMONE (GH)

It is a protein made up of 191 amino acids having a molecular weight of 22,000 Da. It has two disulphide bridges.

Stimuli: Sleep, stress, exercise, low blood glucose, high amino acid content in blood and starvation.

Actions:

1. It stimulates protein synthesis by enhancing amino acid uptake by the cells (this results in a positive nitrogen balance).
2. It helps in the retention of phosphorous, potassium, sodium and calcium.
3. It enhances lipolysis by accelerating the mobilization of fat from adipose tissue resulting in increased oxidation of fats in liver and muscle.
4. It inhibits glucose uptake by the extrahepatic tissue (hyperglycemic effect).
5. It increases liver glycogen by enhancing glycogenesis (via gluconeogenesis).
6. It stimulates the production of somatostatin (which aids in sulphur addition to cartilage, therefore GH is also known as somatotropin). This somatostatin inturn inhibits the release of GH.

(7) It increases synthesis of DNA and RNA in the tissues.
(8) It enhances erythropoiesis.
(9) It stimulates the growth of somatic tissue by enhancing the growth of cartilage; chondrogenesis and oesteogenesis.

Disorders: Excess production of GH in childhood results in gigantism, wherein the person shows a giant like appearance with abnormally long hands and legs.

Excessive production of GH after the usual age of full skeletal growth results in acromegaly and is due to a tumor of the adenohypophysis. The characters of acromegaly are broadened skull, hands and fingers. The soft tissue of the nose, lips, forehead and scalp are thickened.

Hyposecretion of GH results in dwarfism. It is characterized by short height, but fully developed mental and sexual ability.

PROLACTIN

LACTOGENIC HORMONE AND MAMMOTROPIN

It is a protein hormone with 199 amino acids having a MW of 23,000 Da. There are three intrachain disulphide bridges.

Stimuli: Pregnancy, nursing or breast stimulation, sleep and stress.

Actions:

1. **Effects on breast:** Development of the mammary tissue, production of milk (lactogenic) and ejaculation of milk.
2. **Amenorrhaeic effect:** It has an amenorrhaeic effect (absence of menses) on the reproductive system. Therefore breast feeding serves as a contraceptive method.
3. **Effect on corpus luteum:** It activates the corpus luteum and stimulates production of progesterone by the developed corpus luteum.

Excess: The excessive production of prolactin results in amenorrhea, galactorrhea (unnecessary discharge of milk) and enlargement of breast.

POSTERIOR PITUTARY

OXYTOCIN

(Greek: Rapid birth)

Chemistry: It is a nona-peptide (9 amino acids) with a disulphide (–S–S–) bridge between 1st and 6th cysteine amino acids. Union of these two cysteine molecules thus give rise to one cystine molecule hence it also considered as octa-peptide (8 amino acids).

Stimuli:

1. The neural impulses from the stimulation of nipples (termed as milk let down response).
2. Vaginal and uterine contractions (leading to rapid birth).
3. Estrogen stimulates oxytocin production whereas progesterone inhibits its production.

Actions:

1. Causes contraction of uterine smooth muscle at the time of child birth.
2. Stimulates the ejaculation of milk by producing constriction of specialized myo-epithelial cells.
3. Helps in the movement of spermatozoa in the female reproductive tract.

VASOPRESSIN OR ANTIDIURETIC HORMONE (ADH)

It is a nona-peptide (9 amino acids) with a disulphide (–S–S–) bridge between 1st and 6th cysteine amino acids. These two cysteine molecules unite thus giving rise to one cystine molecule hence it also considered as octa-peptide (8 amino acids).

Stimuli:

1. The osmotic pressure of the plasma, also known as oncotic pressure is the primary stimuli for the release of ADH. If the plasma is hypertonic then there is increased secretion of ADH. If the plasma is hypotonic the secretion of ADH is suppressed.
2. Low blood volume
3. Hypotension (low blood pressure)
4. Emotional stress
5. Drugs like nicotine

Actions:

1. It increases the rate of water re-absorption from the latter part of the distal convoluted renal tubules and collecting ducts (antidiuretic effect). This mechanism is adapted to control dehydration and to compensate the excess salt intake.
2. It causes constriction of smooth muscle, leading to the constriction of arteries and capillaries (vascular system). Due to the action of ADH as a vasoconstrictor it results in an increase in the blood pressure.
3. It is an inhibitor of gonadotropins especially LH.

Pathophysiology: Under production of ADH leads to diabetes insipidus. This is characterized by increased excretion of dilute urine. Normal excretion of urine is 1500 ml/day. In diabetes insipidus it rises upto 6 to 20 l/day. The condition is known as polyuria. This results in thirst leading to increased water intake.

The low secretion of ADH may be due to lesions of hypophysis or hypothalamus or due to non responsiveness of nephrons in the kidney, known as hereditary nephrogenic diabetes insipidus. Alcohol inhibits the release of ADH thereby causing diabetes insipidus. Overproduction results in water retention and hyponatremia.

THYROID GLAND

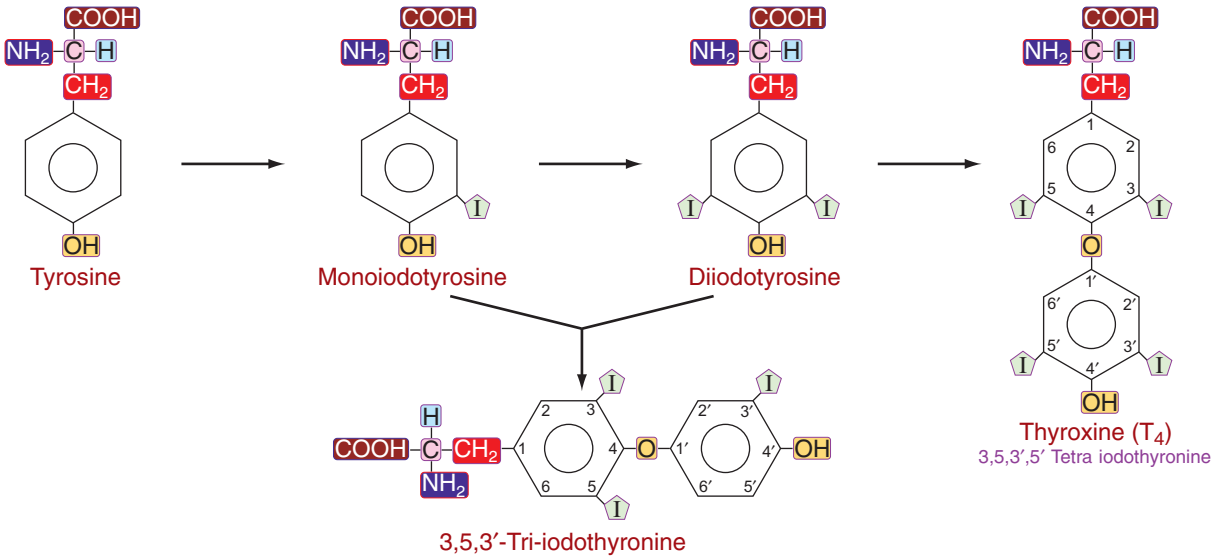
It is a pair of glands situated on either side of the trachea. The development and secretion of the thyroid gland is controlled by TSH.

THYROID HORMONES

The hormones secreted by thyroid are (1) Thyroxine (T_4) & (2) Triiodothyronine (T_3).

Synthesis of thyroid hormones: The thyroid hormones are synthesized from tyrosine in presence of iodine. Plasma contains iodine ion (I^-), which is taken up by the thyroid gland by an active transport mechanism, against the concentration gradient through Na^+, K^+–ATPase pump, which requires energy. In the gland, iodide is converted to active iodine (I or I^+) by the action of an enzyme thyroperoxidase which requires hydrogen peroxide.

In the thyroid gland there is a glycoprotein called thyroglobulin, Mw–660,000 Da, containing 5000 amino acids among which 140 are tyrosine residues. The tyrosine residues of this protein are iodinated to form T_3 and T_4. The steps of their synthesis on the protein are—



Out of the 140 tyrosine residues of the thyroglobulin some will be in the form of tetraiodothyronine, some will be in the form of triiodothyronine and some in the form of diiodotyrosine and monoiodotyrosine.

On stimulation with TSH the thyroglobulin is hydrolysed by proteolytic enzymes in the lysosomes and the free amino acids are released. The T_4, T_3, T_2 and T_1 released will enter the blood stream. T_1 and T_2 are taken back by the thyroid gland. T_4 and T_3 are transported through the blood to the target tissue by thyroxine binding globulin, prealbumin (transthyratin) and albumin. In the target tissue T_4 i.e. 3,5,3′,5′-tetraiodothyronine is converted to T_3 i.e. 3,5,3′-triiodothyronine and some to reverse T_3 i.e. 3,3′,5′-triiodothyronine. Only T_3 is the active species. The thyroid hormones are inactivated upon oxidation to triiodothyroacetic acid and finally deiodinated.

Functions of thyroid hormones: The target organ or tissues of thyroid hormones are almost all the tissues.

1. They stimulate Na^+K^+-ATPase thereby increasing the rate of oxygen consumption and heat production—BMR (except gonads, spleen and adult brain).
2. They increase the metabolism of carbohydrates, fats and proteins.
3. It causes increased absorption of glucose from intestine. It increases glycogenolysis in liver and muscle. It promotes gluconeogenesis. (Hyperglycemic effect).
4. It increases RNA synthesis, amino acid transport and protein synthesis.
5. In hypothermic conditions it uncouples oxidative phosphorylation by swelling mitochondria thereby producing heat.
6. They enhance the growth and development of many tissues; due to enhancement of protein synthesis by thyroid hormones.
7. It aids in the conversion of β-carotene to vitamin A.

Pathophysiology:

1. **Hypothyroidism:** It is a condition wherein there are insufficient amounts of T_3 & T_4. This may be due to (a) disease of thyroid gland (failure) or (b) disease of pituitary/hypothalamus or (c) deficiency of iodine.

***In children*:** Hypothyroidism results in cretinism, characterized by dwarfism, thick tongue and skin mental retardation and sexual undevelopment.

***In adults*:** Hypothyroidism results in myxoedema, which is characterized by thick, dry and waxy skin, dull mental ability and high sensitivity to cold.

In all the cases of hypothyroidism BMR is low (–20 to –40%) and hypercholesterolinemia is seen. T_3 and T_4 administration is the only remedy.

2. **Hyperthyroidism or thyrotoxicosis:** It is due to excessive production of thyroid hormones. Main cause is tumor of the gland.

Grave's disease: This is due to long acting thyroid stimulator (LATS) and LATS protector, which act similar to that of TSH resulting in over production of thyroid hormones due to which BMR increases (+20 to +80%), there will be loss of weight, increased heart rate, inability to sleep etc.

Treatment: Antithyroid drugs control Grave's disease.

Ex. (1) Thiocyanate, perchlorate and ouabain–inhibit iodine uptake by the gland.

(2) Thiourea and thiouracil–inhibit thyroperoxidase.

Goiter: Enlargement of thyroid gland is called goiter. It is due to (1) deficiency of iodine (2) tumors of thyroid (3) excess of TSH and (4) LATS.

Exophthalmic goiter: It is the bulging of the eyeballs out of the face, which is also due to hyperthyroidism.

PARATHYROID GLAND

A pair of glands present just behind the thyroids.

PARATHORMONE

They produce the hormone called parathormone or parathyroid hormone (PTH). It is a polypeptide of 84 amino acids with molecular weight of 9500 Da. It is synthesized as a preprohormone which is converted to prohormone by removal of 25 amino acids from the N-terminal end. Finally parathormone is formed by the removal of 6 more amino acids. It is secreted when blood calcium is low. Its secretion is inhibited when calcium is high in the blood. The target organs for parathormone are bone and kidney.

Functions:

1. Raises serum calcium by—
   (a) Formation of calcitriol in the kidney, which helps in the absorption of Ca^{2+} from the intestine.
   (b) Mobilization of Ca^{2+} from the bones.
   (c) Reducing the excretion of calcium by the kidney.
2. Decreases serum phosphate by increasing its excretion by the kidney.

Calcitonin: It is a hormone produced by the thyroid gland having 32 amino acids. It prevents the movement of Ca^{2+} from bone; it causes phosphate deposition in the bone and prevents phosphate excretion by the kidney. Therefore it is known as the parathormone antagonist.

Hypoparathyroidism: It occurs due to the accidental removal of parathyroid during neck or thyroid surgery. It can also occur due to auto-immune destruction of the gland. The resulting condition is known as tetany—due to which there is neuro-muscular irritability and hypocalcemia.

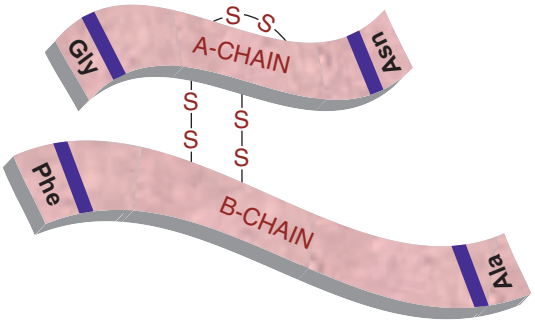
Hyperparathyroidism: Occurs mainly due to tumors of the gland. It results in high serum calcium, low serum phosphate, bone destruction and kidney stones.

PANCREAS

The islets of langerhans of the pancreas function as the endocrine cells. The alpha cells produce glucagon, beta cells produce insulin, D & F cells produce somatostatin and pancreatic polypeptides respectively.

INSULIN

It is a polypeptide with a molecular weight of 5734 Da. It is made up of two chains, chain 'A' having 21 amino acids and chain 'B' having 30 amino acids.



There are two interchain disulphide bonds (1) between A7 & B7 and (2) between A20 & B19. There is one intrachain disulphide bond in A chain between 6^{th} and 11^{th} amino acids.

Insulin is synthesized as a preproinsulin which is converted to proinsulin by the removal of 23 amino acids from the N-terminal end. Proinsulin is the inactive storage form of insulin which contains an additional 'C' chain containing 35 amino acids. Active insulin is formed due to cleavage of 'C' chain from proinsulin by proteolytic enzymes.

Stimulation for insulin secretion: High blood glucose, amino acids, fatty acids, ketone bodies and hormones like glucagon, GH, cortisol etc. Epinephrine inhibits insulin secretion by decreasing cAMP.

Target cells: The target cells for insulin action are mainly muscle, liver and adipose tissue. Other cells like lymphocytes, fibroblasts and mammary glands are also acted upon by insulin. It can be said that almost all the cells of the body are dependent upon insulin for uptake of glucose except brain, RBC, G.I tract, liver, retina of eye and pancreas itself.

Actions or effects of insulin:

1. Helps in the transport of glucose, amino acids, K^+, Ca^{2+}, P and nucleotides through the cell membrane.
2. It enhances the utilization of glucose by the cell by activating the enzymes of energy production (glycolysis) and of energy storage (glycogenesis and lipogenesis).
3. It inhibits the enzymes of glucose production in the cells (gluconeogenesis).
   All the above three mechanisms affect in lowering the blood glucose level (i.e. hypoglycemic effect).
4. It inhibits lipolysis.
5. It stimulates HMP shunt pathway resulting in production of NADPH for lipid synthesis.
6. It enhances protein synthesis.

Inactivation and degradation of insulin:

1. The hormone-receptor complex is taken up by the lysosomes of the target cells and proteolysis occurs.
2. In liver glutathione-insulin transhydrogenase reduces the disulphide linkages with reduced glutathione and thereby separating the A and B chains. The A and B chains are then cleaved by insulinase enzyme.

Diabetes mellitus: Details given under the chapter 'Carbohydrate Metabolism'.

Hyperinsulinism: Excessive production of insulin, mainly due to the tumors of the β-cells which results in hypoglycemia associated with sweating, tremors, fainting attacks. This can be relieved by injecting glucose or by oral supply of sugar.

GLUCAGON

It is a peptide of 29 amino acids having a molecular weight of 3485 Da. It is known as Hyperglycemic-Glycogenolytic Factor (HGF). It is secreted by the α-cells of the islets of langerhans in the pancreas. Hypoglycemia stimulates its production. It acts by activating hepatic adenyl cyclase thereby promoting glycogenolysis. It inhibits protein synthesis, fatty acids and cholesterol synthesis. It promotes gluconeogenesis, lipolysis and ketone body formation. Overall, it has hyperglycemic effect. Hyperglucagonism antagonizes the insulin action thereby causing hyperglycemia i.e. diabetes mellitus. Hypoglucagonism results in hypoglycemic convulsions and shock exhibiting sweating, shivering or fainting. It is inactivated in the liver by glucagonase.

ADRENALS

Adrenal glands are a pair of glands situated on the dorsal side of the kidney. Each gland is a composite of two structures (1) Adrenal medulla and (2) Adrenal cortex.

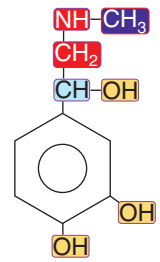
ADRENAL MEDULLA

They arise from the autonomic nervous system. The cells of the adrenals are called pheochromocytes or chromaffin cells (due to dichromate staining).

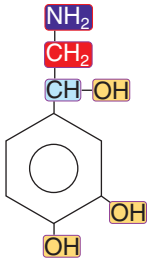
CATECHOLAMINES

The hormones produced by the adrenal medulla are known as catecholamines. They are—(1) Epinephrine (adrenaline) (2) Norepinephrine (nor-adrenaline).

These are synthesized from tyrosine and are stored in the chromaffin granules. Neural stimulation as a result of stress, fear, anger, exercise and hypoglycemia results in the secretion of these hormones. They circulate in plasma in free form or in loose association with albumin.



Epinephrine
(ADRENALINE)



Nor-Epinephrine
(NORADRENALINE)

Receptors: The receptors for catecholamines are known as adrenergic receptors. They are of two types viz. (1) Alpha (α-1 and α-2) and beta (β-1 and β-2). Epinerphrine acts through the alpha and beta receptors whereas norepinephrine acts only through the alpha receptor. They act mainly by varying the cAMP level.

Actions:

1. It enhances glycogenolysis in muscle and liver by activating adenyl cyclase.
2. It enhances gluconeogenesis.
3. It diminishes the glucose uptake by the cells, except brain and RBC.
4. It inhibits the secretion of insulin by lowering the cAMP.
5. It enhances lipolysis in adipose tissue.
6. It increases the blood pressure (by vasoconstriction and increasing the rate and force of contraction of heart).
7. It increases BMR.
8. It is a smooth muscle relaxant.

The catecholamines are produced under a threat and help the body in fighting against it by supplying fuel (glucose to brain and fatty acids to other tissues). This is known as fight or flight mechanism.

Metabolism and excretion: In the liver they are metabolized by Catechol-O-Methyl-Transferase (COMT) and Mono-Amine Oxidase (MAO) to form O-methylated and deaminated metabolites (Ex. Vanillylmandelic acid—VMA which is the major excretory product of catecholamines).

Pathophysiology: Tumors of the adrenal medulla cause excessive production of catacholamines resulting in hypertension. The condition is known as pheochromocytomas.

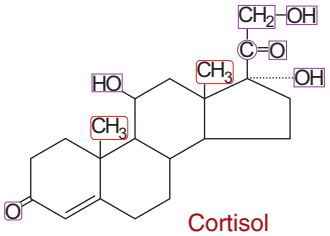
ADRENAL CORTEX

It surrounds the adrenal medulla.

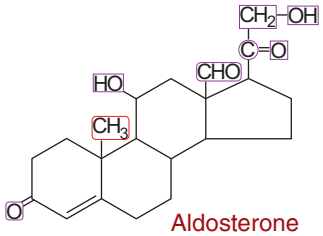
CORTICOSTEROIDS

The hormones produced by adrenal cortex are known as steroids or steroid hormones, as they contain the parent ring the cyclo-pentano-per-hydro-phenanthrene ring. It produces—

(1) Glucocorticoids → Cortisol, Corticosteroids
(2) Mineralocorticoids → Aldosterone
(3) Sex Hormones → Androgen



Cortisol



Aldosterone

These are synthesized from cholesterol, which is the first precursor of steroid synthesis. The development of adrenal gland is under the control of pituitary ACTH. It also regulates the synthesis and secretion of glucocorticoids. ACTH helps in the transport of cholesterol into the mitochondria where it is convert-

ed to pregnenolone. The synthesis of mineralocorticoids is regulated by renin-angiotensin system which changes the intracellular calcium level (increases) due to which the synthesis of aldosterone is stimulated.

Renin-angiotensin system: Renin, an enzyme, produced by the kidney, in response to low fluid (blood) volume and low sodium concentration, converts angiotensinogen, a protein produced by the liver to angiotensin-I which is converted to angiotensin-II by a converting enzyme. This angiotensin-II increases the intracellular calcium level thereby stimulating the aldosterone production. Angiotensin-II is a potent vasoconstrictor due to which the blood pressure increases. Angiotensin is inactivated by angiotensinases.

The cortical hormones are released immediately after the onset of sleep and their concentration reaches a maximum level in the morning. The cortical hormones circulate in the blood bound to Corticosteroid-Binding-Globulin (CBG) or transcortin.

Functions of cortical hormones (glucocorticoids):

1. It enhances gluconeogenesis.
2. It enhances glycogenesis, thus prepares the subject for increased survival and resistance to stress.
3. It releases amino acids from proteins of extra-hepatic tissues and thus provides a substrate for gluconeogenesis.
4. In some parts of the body it enhances lipogenesis whereas in others it suppresses lipogenesis.
5. It facilitates protein formation in the liver but protein degradation in muscle, adipose tissue and skin.
6. All the above actions are in part mediated by other hormones, whose release is facilitated by the steroid hormones.
7. Effects on immunity: Cortisol and certain synthetic steroids prevent or reduce inflammatory responses to physical, chemical or bacterial stimuli. This action serves as the basis for their use in treating a variety of inflammatory or immunologically mediated disorders. However, the response to infection is also reduced and this represents a significant drawback to their use.

Mineralocorticoids: They play a role in the retention of sodium, by facilitating the reabsorption of sodium from the distal convoluted tubules and collecting tubules of the kidney, in exchange of K^+, H^+ and NH_4^+. They also help in the retention of water.

Androgens (sex hormones):

1. If the androgens are present in excessive amounts, they lead to masculinization in females.
2. Adrenal cortex also produces estrogens and progesterones in small amounts.

Pathophysiology:

Hyperfunction of adrenal cortex: Tumors of adrenal cortex produce hyperadrenocorticism. This results in—

1. Hyperglycemia and glycosuria
2. Retention of sodium and water resulting in oedema and hypertension
3. Negative nitrogen balance
4. Hypokalemia and
5. Hirsuitism (Opposite sex characters).
6. ***Cushing's syndrome*:** Tumors of pituitary or adrenal result in obesity involving the face, neck and trunk known as buffalo type.

Hypofunction of adrenal cortex: It may be primary or secondary (pituitary). Clinical syndrome is Addison's disease. Excessive sodium and chloride loss, low blood pressure, hypoglycemia, and general

weakness are some of the symptoms. Inability to face minor stress resulting in "*crises and death*" is also a symptom exhibited.

Metabolism and excretion of cortical hormones: In the liver they are reduced to their tetrahydro derivatives and then conjugated with glucouronic acid and excreted through bile.

GONADS

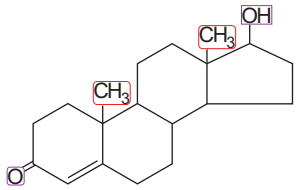
The gonads constitute the testis in males and the ovaries, corpus luteum and placenta in females.

TESTIS

Testosterone is the major hormone produced by the testis.

TESTOSTERONE

It is a 19 carbon compound. It is synthesized from cholesterol and pregnenolone is an intermediate in its synthesis. The synthesis and secretion of testosterone is controlled by LH and FSH (GTH). In the blood it is transported bound to Testosterone Estradiol Binding Globulin (TEBG) or Sex Hormone Binding Globulin (SHBG). In the target tissues, testosterone is converted into its active from i.e. dihydrotestosterone by the enzyme NADPH-5-α-reductase.



Testosterone

Functions:

1. It causes sex differentiation during foetal life.
2. It promotes the growth and function of the epididymis, vas deferens, prostate, seminal vesicle and penis.
3. It enhances spermatogenesis in adulthood.
4. It enhances and maintains the motility and fertilizing power of the sperms.
5. It favours development of secondary sexual features.
6. It is responsible for the male pattern behaviour.
7. It increases the secretion of sebaceous glands in the skin.
8. It has protein anabolic effect.
9. It depresses the estrogenic over activity in women with symptoms of dysmenorrhoea, painful breasts and stops lactation and menstruation.
10. It increases the activity of glycolytic enzymes.
11. It increases the rate of fatty acid synthesis.

Metabolism and excretion: In the liver it is converted to androsterone and then conjugated to form glucosiduronide and sulfate conjugates which are excreted in bile and urine.

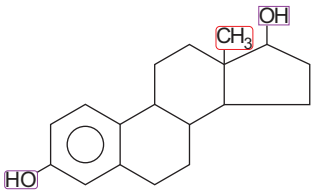
Lack of male hormones: Primary (testis defective) or it can be secondary (pituitary defect) results in failure to develop secondary sex characters, lack of musculinization, atrophy of sex organs in later life.

OVARIES

They produce estrogens and progesterone.

ESTROGENS

The most active estrogen is 17-beta-estradiol.



Estradiol

Synthesis and secretion of estradiol is controlled by GTH in addition to various other factors (FSH). In blood it is transported bound to SHBG.

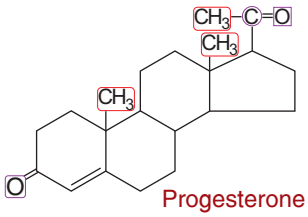
Functions:

1. Growth and maturation of female sex organs and maintenance of the reproductive capacity.
2. Stimulate the growth of follicles.
3. Increases the blood flow and transudation of water.
4. Increased cell division.
5. Regulates bone development (i.e. it stops bone development after a certain time (age) therefore females are shorter than males).
6. They regulate normal bone metabolism. Women after menopause develop osteoporosis.
7. It has a lipogenic effect (i.e. deposition of subcutaneous fat–a female's ankle is identified by its roundness due to disposition of subcutaneous fat).
8. It increases the level of HDL and LDL, thus prevents atherosclerosis. This is the reason why the incidence of heart attack is low in women compared to men.
9. It diminishes the sebaceous glands secretions.

Metabolism and excretion: Estradiol is converted to its inactive forms estrone and estriol which are conjugated to glucouronate and sulphates and finally excreted through bile and urine.

PROGESTERONE

It is produced by corpus leutum. During pregnancy it is also produced by the placenta. It is synthesized from cholesterol. It is also formed in the adrenal cortex as a precursor of corticosteroids. It circulates in the blood bound to cortisol binding globulin or transcortin.



Progesterone

Actions:

1. Acts on endometrium and helps in implantation of the fertilized ovum.
2. Helps in the growth of the breast.
3. It increases the BMR.
4. High concentrations of progesterone inhibit ovulation and hence it is used as a contraceptive.
5. It has a lipotropic (i.e. clearing lipids from liver) effect.

Female reproductive hormones and their action in reproductive physiology

Source	Hormone	Functions
Hypothalamus	Releasing hormones (Gn-RH, TRH)	Cause release of FSH, LH,TSH, from anterior pituitary.
Anterior pituitary	Somatostatin, prolactin inhibiting factor (PIF)	Inhibits release of GH. Inhibits prolactin release.
	Follicle stimulating hormone (FSH)	Stimulates follicular growth, estrogen secretion.
	Luteinizing hormone (LH)	Stimulates ovulation and corpus luteum (CL) function. Secretes progestrone, estrogen.
	Prolactin	Promotes lactation, stimulates corpus luteum function and progesterone secretion and may inhibit estrogen secretion.
Posterior pituitary	Oxytocin	Stimulates uterine contractions. Brings parturition.
Ovary	Estrogens	Promotes female sex behaviour, stimulates secondary sex characters and growth of reproductive tract and uterine contractions, mammary duct growth, controls gonadotrophin release. Stimulates calcium uptake in bone. Has anabolic effects
	Progesterone	Acts synergistically with estrogen in promoting estrus behaviour and preparation of reproductive tract for implantation, stimulates endometrial secretions, maintains pregnancy, stimulates mammary alveolar growth and controls gonadotrophin secretion.
	Relaxin	Loosening of symphysis pubis and sactroshiatic ligaments. Opens birth canal.
	Inhibin	Inhibits FSH release.
Corpus luteum	Progesterone	Prepares uterus for implantation of the embryo, maintains pregnancy and CL.
Placenta	Human chorionic gonadotrophin	Similar to LH activity.
	Placental lactogen	Growth hormone like activity. Maintains CL.
	Estrogen and progesterone	Same as those secreted by ovary.

HORMONE RECEPTORS

A hormone receptor is a receptor protein on the surface of a cell or in its interior that binds to a specific hormone. The hormone causes many changes that take place in the cell.

Binding of hormones to hormone receptors often trigger the start of a biophysical signal that can lead to further signal transduction pathways, or trigger the activation or inhibition of genes.

Types of hormone receptors:

Peptide hormone receptors: Are often transmembrane proteins. They are also called G-protein-coupled receptors, sensory receptors or ionotropic receptors. These receptors generally function via intracellular second messengers, including cyclic AMP (cAMP), inositol 1,4,5-triphosphate (IP_3) and the calcium (Ca^{2+})–calmodulin system.

Steroid hormone receptors and related receptors: Are generally soluble proteins that function through gene activation. Their response elements are DNA sequences (promoters) that are bound by the complex of the steroid bound to its receptor. The receptors themselves are zinc-finger proteins. These receptors include those for glucocorticoids, estrogens, androgens, thyroid hormone (T3), calcitriol (the active form of vitamin D), and the retinoids (vitamin A).

Receptors for peptide hormones: With the exception of the thyroid hormone receptor, the receptors for amino acid derived and peptide hormones are located in the plasma membrane. Receptor structure is varied.

Some receptors consist of a single polypeptide chain with a domain on either side of the membrane, connected by a membrane-spanning domain. Some receptors are comprised of a single polypeptide chain that is passed back and forth in serpentine fashion across the membrane, giving multiple intracellular, transmembrane, and extracellular domains. Other receptors are composed of multiple polypeptides. Ex. The insulin receptor is a disulfide linked tetramer with the β-subunits spanning the membrane and the α-subunits located on the exterior surface.

Subsequent to hormone binding, a signal is transduced to the interior of the cell, where second messengers and phosphorylated proteins generate appropriate metabolic responses. The main second messengers are cAMP, Ca^{2+}, inositol triphosphate (IP_3), and diacylglycerol (DAG). Proteins are phosphorylated on serine and threonine by cAMP-dependent protein kinase (PKA) and DAG-activated protein kinase C (PKC). Additionally a series of membrane-associated and intracellular tyrosine kinases phosphorylate specific tyrosine residues on target enzymes and other regulatory proteins.

The hormone-binding signal of most, but not all, plasma membrane receptors is transduced to the interior of cells by the binding of receptor-ligand complexes to a series of membrane-localized GDP/GTP binding proteins known as G-proteins. The classic interactions between receptors, G-protein transducer, and membrane-localized adenylate cyclase are illustrated using the pancreatic hormone glucagon as an example. When G-proteins bind to receptors, GTP exchanges with GDP bound to the α-subunit of the G-protein. The G_a-GTP complex binds adenylate cyclase, activating the enzyme. The activation of adenylate cyclase leads to cAMP production in the cytosol and to the activation of PKA, followed by regulatory phosphorylation of numerous enzymes. Stimulatory G-proteins are designated Gs, inhibitory G-proteins are designated Gi.

A second class of peptide hormones induces the transduction of 2 second messengers, DAG and IP_3. Hormone binding is followed by interaction with a stimulatory G-protein which is followed in turn by G-protein activation of membrane-localized phospholipase C-γ, (PLC-γ). PLC-γ hydrolyzes phosphatidylinositol bisphosphate to produce 2 messengers viz. IP_3, which is soluble in the cytosol, and DAG, which remains in the membrane phase.

Cytosolic IP_3 binds to sites on the endoplasmic reticulum, opening Ca^{2+} channels and allowing stored Ca^{2+} to flood the cytosol. There it activates numerous enzymes, many by activating their calmodulin or calmodulin-like subunits.

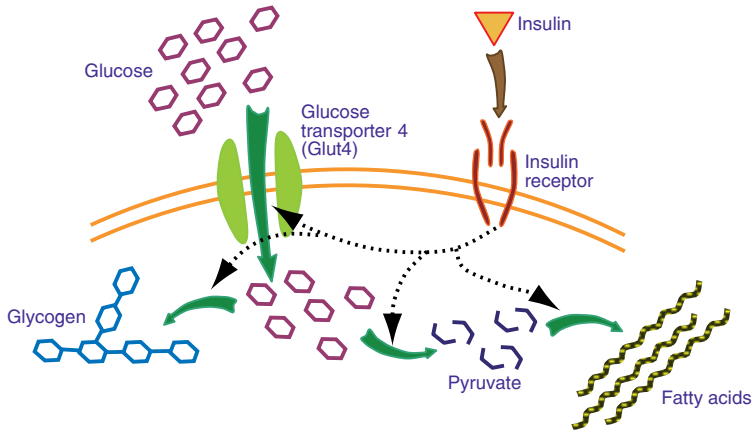
DAG has 2 roles–it binds and activates PKC, and it opens Ca^{2+} channels in the plasma membrane, reinforcing the effect of IP_3. Like PKA, PKC phosphorylates serine and threonine residues of many proteins, thus modulating their catalytic activity.

Insulin receptor: Is a transmembrane receptor that is activated by insulin. It belongs to the large class of tyrosine kinase receptors.

Two alpha subunits and two beta subunits make up the insulin receptor. The beta subunits pass through the cellular membrane and are linked by disulfide bonds. The alpha and beta subunits are encoded by a single gene (INSR). The insulin receptor has been designated as CD_{220} (cluster of differentiation 220).

***Function of insulin receptor–effect of insulin on glucose uptake and metabolism*:** Insulin binds to its receptor which in turn starts many protein activation cascades. These include—

- ✧ Translocation of Glut-4 transporter to the plasma membrane and influx of glucose
- ✧ Glycogen synthesis
- ✧ Glycolysis and fatty acid synthesis



Insulin receptors (a family of tyrosine kinase receptors), mediate their activity by causing the addition of a phosphate group to particular tyrosines on certain proteins within a cell. The 'substrate' proteins which are phosphorylated by the insulin receptor include a protein called 'IRS-1' for 'Insulin Receptor Substrate-1'. IRS-1 binding and phosphorylation eventually leads to an increase in the high affinity glucose transporter (Glut4) molecules on the outer membrane of insulin-responsive tissues, including muscle cells and adipose tissue, and therefore to an increase in the uptake of glucose from blood into these tissues. Briefly, the glucose transporter ($Glut_4$) is transported from cellular vesicles to the cell surface, where it then can mediate the transport of glucose into the cell. Glycogen synthesis is also stimulated by the insulin receptor via IRS-1.

***Pathology of insulin receptors*:** The main activity of activation of the insulin receptor is inducing glucose uptake. For this reason 'insulin insensitivity', or a decrease in insulin receptor signaling, leads to diabetes mellitus type 2 - the cells are unable to take up glucose, and the result is hyperglycemia (an increase in circulating glucose), and all the sequelae which result from diabetes. Patients with insulin

resistance may display acanthosis nigricans. A few patients with homozygous mutations in the INSR gene have been described, which causes Donohue syndrome or Leprechaunism. This autosomal recessive disorder results in a totally non-functional insulin receptor. These patients have low set, often protruberant ears, flared nostrils, thickened lips, and severe growth retardation. In most cases, the outlook for these patients is extremely poor with death occurring within the first year of life. Other mutations of the same gene cause the less severe Rabson-Mendenhall syndrome, in which patients have characteristically abnormal teeth, hypertrophic gingiva (gums) and enlargement of the pineal gland. Both diseases present with fluctuations of the glucose level—after a meal the glucose is initially very high, and then falls rapidly to abnormally low levels.

***Degradation of insulin and its receptors*:** Once an insulin molecule has docked onto the receptor and effected its action, it may be released back into the extracellular environment or it may be degraded by the cell. Degradation normally involves endocytosis of the insulin-receptor complex followed by the action of insulin degrading enzyme. Most insulin molecules are degraded by liver cells. It has been estimated that a typical insulin molecule is finally degraded about 71 minutes after its initial release into circulation

Glucagon receptor: It is a 62 kDa peptide that is activated by glucagon and is a member of the G-protein coupled family of receptors, coupled to Gs. Stimulation of the receptor results in activation of adenylate cyclase and increased levels of intracellular cAMP. Glucagon receptors are mainly expressed in liver and in kidney with lesser amounts found in heart, adipose tissue, spleen, thymus, adrenal glands, pancreas, cerebral cortex, and G.I. tract.

Steroid hormone receptors: Are proteins that have a binding site for a particular steroid molecule. Their response elements are DNA sequences that are bound by the complex of the steroid bound to its receptor. The response element is part of the promoter of a gene. Binding by the receptor activates or represses, as the case may be, the gene controlled by that promoter. It is through this mechanism that steroid hormones turn genes on (or off).

The DNA sequence of the glucocorticoid (a protein homodimer) response element is—

5′ –AGAACAnnnTGTTCT–3′

3′ –TCTTGTnnnACAAGA–5′

where n represents any nucleotide (a palindromic sequence)

The glucocorticoid receptor, like all steroid hormone receptors, is a zinc-finger transcription factor; there are four zinc atoms each attached to four cysteines.

For a steroid hormone to turn gene transcription on, its receptor must—

- ✓ Bind to the hormone
- ✓ Bind to a second copy of itself to form a homodimer
- ✓ Be in the nucleus, moving from the cytosol if necessary
- ✓ Bind to its response element
- ✓ Activate other transcription factors to start transcription

Each of these functions depends upon a particular region of the protein (Ex. The zinc fingers for binding DNA). Mutations in any one region may upset the function of that region without necessarily interfering with other functions of the receptor.

Nuclear receptor superfamily: The zinc-finger proteins that serve as receptors for glucocorticoids and progesterone are members of a large family of similar proteins that serve as receptors for a variety of small, hydrophobic molecules. These include other steroid hormones like the mineralocorticoid-aldoster-

one, estrogens, the thyroid hormone (T_3), calcitriol (the active form of vitamin D), retinoids–vitamin A (retinol) and its relatives–retinal/retinoic acid, bile acids and fatty acids. These bind members of the superfamily called Peroxisome Proliferator Activated Receptors (PPARs). They got their name from their initial discovery as the receptors for drugs that increase the number and size of peroxisomes in cells. In every case, the receptors consists of at least three functional modules or domains from N-terminal to C-terminal, these are—

- A domain needed for the receptor to activate the promoters of the genes being controlled
- The zinc-finger domain needed for DNA binding (to the response element)
- The domain responsible for binding the particular hormone as well as the second unit of the dimer

Receptors for thyroid hormones: Are members of a large family of nuclear receptors that include those of the steroid hormones. They function as hormone-activated transcription factors and thereby act by modulating gene expression.

***Thyroid hormone receptors bind DNA in absence of hormone*:** Usually leading to transcriptional repression. Hormone binding is associated with a conformational change in the receptor that causes it to function as a transcriptional activator.

Mammalian thyroid hormone receptors are encoded by two genes, designated alpha and beta. Further, the primary transcript for each gene can be alternatively spliced, generating different alpha and beta receptor isoforms. Currently, four different thyroid hormone receptors are recognized as–(i) α-1 (ii) α-2 (iii) β-1 and (iv) β-2.

Like other members of the nuclear receptor superfamily, thyroid hormone receptors encapsulate three functional domains—

- A transactivation domain at the amino terminus that interacts with other transcription factors to form complexes that repress or activate transcription. There is considerable divergence in sequence of the transactivation domains of alpha and beta isoforms and between the two beta isoforms of the receptor.
- A DNA-binding domain that binds to sequences of promoter DNA known as hormone response elements.
- A ligand-binding and dimerization domain at the carboxy-terminus.

***Disorders of thyroid hormone receptors*:** A number of humans with a syndrome of thyroid hormone resistance have been identified, and found to have mutations in the receptor beta gene which abolish ligand binding. Clinically, such individuals show a type of hypothyroidism characterized by goiter, elevated serum concentrations of T_3 and thyroxine and normal or elevated serum concentrations of TSH. More than half of affected children show attention-deficit disorder, which is intriguing considering the role of thyroid hormones in brain development. In most affected families, this disorder is transmitted as a dominant trait, which suggests that the mutant receptors act in a dominant negative manner.

Adrenergic receptors (or adrenoceptors)**:** Are a class of G-protein coupled receptors that are targets of the catecholamines. Adrenergic receptors specifically bind their endogenous ligands, the catecholamines adrenaline and noradrenalin (called epinephrine and norepinephrine), and are activated by these.

Many cells possess these receptors, and the binding of an agonist will generally cause a sympathetic response (i.e. the fight-or-flight response) viz. the heart rate will increase and the pupils will dilate, energy will be mobilized, and blood flow diverted from other, non-essential, organs to skeletal muscle. There are several types of adrenergic receptors, but there are two main groups viz. α-adrenergic and β-adrenergic.

α-Adrenergic receptors: These receptors bind noradrenalin (norepinephrine) and adrenaline (epinephrine). Phenylephrine is a selective agonist of the α-receptor. They exist as α_1-adrenergic receptors and α_2-adrenergic receptors.

β-Adrenergic receptors: These receptors are linked to Gs proteins, which in turn are linked to adenyl cyclase. Agonist binding thus causes a rise in the intracellular concentration of the second messenger cAMP. Downstream effectors of cAMP include cAMP-dependent protein kinase (PKA), which mediates some of the intracellular events following hormone binding.

Role in circulation: Epinephrine reacts with both α and β-adrenoreceptors, causing vasoconstriction and vasodilation, respectively. Although receptors are less sensitive to epinephrine, when activated, they override the vasodilation mediated by β-adrenoreceptors. The result is that high levels of circulating epinephrine cause vasoconstriction. Lower levels of epinephrine dominates β-adrenoreceptor stimulation, producing an overall vasodilation.

The mechanism of adrenergic receptors: Adrenaline or noradrenalin is receptor ligands to either α_1, α_2 or β-adrenergic receptors. α_1 couples to Gq, which results in increased intracellular Ca^{2+} which results in smooth muscle contraction. α_2 on the other hand, couples to Gi, which causes a decrease of cAMP activity, resulting in smooth muscle contraction. β receptors couple to Gs, and increase intracellular cAMP activity, resulting in heart muscle contraction, smooth muscle relaxation and glycogenolysis.

Functions of α-receptors: α-Receptors have several functions in common. They are—

- Vasoconstriction of arteries to heart (coronary artery)
- Vasoconstriction of veins
- Decrease motility of smooth muscle in gastrointestinal tract

Alpha-1 adrenergic receptor: Alpha1-adrenergic receptors are members of the G protein-coupled receptor superfamily. Upon activation, a heterotrimeric G-protein, Gq, activates phospholipase C (PLC), which causes an increase in IP_3 and calcium. This triggers all other effects. Specific actions of the β_1 receptor mainly involve smooth muscle contraction. It causes vasoconstriction in many blood vessels including those of the skin & gastrointestinal system and to kidney (renal artery) and brain. Other areas of smooth muscle contraction are for instance - ureter, vas deferens, hairs (arrector pili muscles), uterus (when pregnant), urethral sphincter, bronchioles (although minor to the relaxing effect of β_2 receptor on bronchioles). Further effects include glycogenolysis and gluconeogenesis from adipose tissue and liver, as well as secretion from sweat glands and Na^+ reabsorption from kidney.

Alpha-2 adrenergic receptor: There are 3 highly homologous subtypes of α_2 receptors viz. α_2A, α_2B, and α_2C. Specific actions of the α_2-receptor include—

- Inhibition of insulin release in pancreas
- Induction of glucagon release from pancreas
- Contraction of sphincters of the gastrointestinal tract

Beta-1 adrenergic receptor: Specific actions of the β_1 receptor include—

- Increase cardiac output, both by raising heart rate and increasing the volume expelled with each beat (increased ejection fraction)
- Renin release from juxtaglomerular cells
- Lipolysis in adipose tissue

Beta-2 adrenergic receptor: Specific actions of the β_2 receptor include—

- Smooth muscle relaxation, e.g. in bronchi
- Relaxes urinary sphincter and pregnant uterus
- Relaxes detrusor urinary muscle of bladder wall
- Dilates arteries to skeletal muscle
- Glycogenolysis and gluconeogenesis

- ✓ Contract sphincters of GI tract
- ✓ Thickened secretions from salivary glands
- ✓ Inhibit histamine-release from mast cells
- ✓ Increase renin secretion from kidney

Comparison of different adrenergic receptors

Receptor type	Agonist potency order	Selected action of agonist	Mechanism	Agonists	Antagonists
α_1:A, B, D	Adrenaline ≥ Noradrenaline >> Isoprenaline	Smooth muscle contraction	Gq: Phospholipase C (PLC) activated, IP3 and Calcium up	Noradrenaline Phenylephrine Methoxamine Cirazoline	(Alpha blockers) Phenoxy-benzamine Phentolamine Prazosin Tamsulosin Terazosin
α_2: A, B, C	Adrenaline ≥ Noradrenaline >> Isoprenaline	Smooth muscle contraction and neurotransmitter inhibition	Gi: Adenylate cyclase inactivated, cAMP down	Clonidine lofexidine Xylazine Tizanine Guanfacine	(Alpha blockers) Metoprolol atenolol
β_1	Isoprenaline > Adrenaline = Noradrenaline	Heart muscle contraction	Gs: Adenylate cyclase activated, cAMP up	Noradrenaline Isoprenaline Dobutamine	(Beta blockers) Metoprolol atenolol
β_2	Isoprenaline > Adrenaline >> Noradrenaline	Smooth muscle relaxation	Gs: Adenylate cyclase activated, cAMP up	Salbutamol Bitolterol Mesylate Formoterol Isoprenaline Levalbuterol Metaproterenol Salmeterol Terbutaline Ritodrine	(Beta blockers) Butoxamine propranolol
β_3	Isoprenaline = Noradrenaline > Adrenaline	Enhance lipolysis	Gs: Adenylate cyclase activated, cAMP up	L-796568	

16

WATER AND MINERAL METABOLISM

Water makes up to about 50-70% of the adult body weight and varies inversely with the fat content. This is distributed throughout the body as major component of the intracellular and extracellular fluids. Intracellular fluid amounts to about 50% of the body weight in lean persons and much less in obese persons. Extracellular fluids represent about 20% of the body weight, out of which 15% is in the interstitial fluid and 5% in blood plasma. CSF, ocular fluid, lymph and synovial fluids represent relatively small volumes.

Extracellular fluid: All cells of the body exist in fluid environment collectively called as extracellular fluid. This includes blood plasma, interstitial fluid and lymph. Plasma contains 7% protein, hepatic lymph slightly less and subcutaneous fluid 1%. They contain mainly NaCl, $NaHCO_3$, small amount of Ca, Mg, K, H, phosphate, sulphate, organic acids, non-electrolytes such as glucose, urea, lipids etc. pH value ranges from 7.35 to 7.45 under normal conditions. Several components of extracellular fluid are important in maintaining osmotic, anion-cation balance and hydrogen ion regulation. The cations (K^+, Ca^{++}, Mg^{++} and H^+) are present in comparatively very low concentration and exert profound influence on physiological processes. Interstitial fluid contains a higher total concentration of diffusible anions and lower concentration of cations than the plasma.

Intracellular fluid: Chief cations of intracellular fluid are potassium and magnesium. Less amount of Na is present. Calcium content although very little, but is biologically very important. Contains much more phosphate and sulphate ions as well as proteins than the extracellular fluid. Chloride ion is practically absent in intracellular fluid except in case of erythrocytes. Both Na and K ions are able to cross the membrane more freely under certain physiological and pathological conditions. Much of the magnesium is present as undissociated compound of protein and organic phosphate and therefore, is not in ionic form.

Exchanges between fluid compartments: Many of the substances which enter the body or produced by the cells are distributed to other tissues or excreted. Exchange systems are outlined as follows—

1. **Alveolar air-blood plasma:** This system provides for entrance of oxygen into and loss of CO_2 and water from the body.
2. **Plasma-Erythrocytes:** This system provides for ready exchange of oxygen, CO_2, water and certain anions (particularly Cl^- and HCO_3^-) in both directions.
3. **Plasma-Interstitial fluid:** These two media are separated by capillary wall, which are permeable to water, inorganic ions and small organic molecules (glucose, amino acids, urea etc) but not to large organic molecules such as proteins.

4. **Interstitial fluid-Intracellular fluid:** These two compartments are separated by the cell membranes, across which gasses in solution water and small uncharged molecules can diffuse. The small molecules ex. Glucose is not subjected to simple diffusion but is carried across cell membrane by active transport process. The permeability of electrolytes follows biological pump mechanisms. The membrane is also relatively impermeable to large molecules such as proteins.

WATER METABOLISM

Distribution of water in the body: Water is the major constituent of human body. The average body water is 50-70% of the body. Females have little less water than males.

1. Intracellular fluid is 50% of total body weight.
2. Extracellular fluid is 20% of the body weight.

Plasma	4-5%
Interstitial and lymph fluid	8%
Dense connective tissue, cartilage and bone	6%
Transcellular fluids(found in salivary glands, liver, pancreas, thyroid gland, gonads, skin, mucous membranes of the respiratory and gastrointestinal tracts, kidneys, fluid spaces in eye, CSF etc)	1.5%

Factors influencing the distribution of body water: The distribution of water is continuously changing. Osmotic forces are the principal factors for controlling the amount of fluid in various compartments of the body. These are maintained by the solutes of the body. Solutes are of three types.

1. *Organic molecules of small molecular size (glucose, urea, amino acids etc)*: Since these diffuse freely across the cell membrane, they are not important in the distribution of water. If they are present in large quantities, they can help retaining water.
2. *Organic substances of large molecular size (proteins)*: These substances can throw effect in the transport of fluids from one compartment to the other.
3. *The inorganic electrolytes*: These inorganic electrolytes are the most important both in the distribution and in the retention of body water.

***Intake and loss of body water*:**

A. **Water intake:** Water is supplied to the body by the following processes—
   1. Water taken orally.
   2. Along with food.
   3. Oxidation of food stuffs i.e. fats, proteins and carbohydrates yield water after combustion.

B. **Water loss:** Water is lost from the body by 4 routes
   1. Evaporation from lungs.
   2. Kidneys eliminate water as urine.
   3. The intestines excrete in the feces.
   4. Perspiration.

C. **Additional water loss in diseases:**
   1. Water loss is more in diarrhea and vomiting. These losses can be fatal in infants.
   2. In kidney disease, renal water loss is more.
   3. In fever, insensible losses may rise much higher than normal.
   4. Patients in high environmental temperatures sustain extremely high external water loss.

Water Balance: An equilibrium persists between the intake and output of water in the body. In addition to other factors, certain hormones such as ADH, vasopressin, oxytocin and aldosterone influence the regulatory mechanism.

Balance sheet of water

Water intake (ml/day)		Water loss (ml/day)	
Drinks	1350	Urine	1500
Solid food	900	Lungs	500
Oxidation of food	450	Skin	600
		Feces	100
TOTAL	2700	TOTAL	2700

There is a continuous excretion of water in the form of digestive juices from the body into the alimentary canal. This water (except 100 ml) is reabsorbed with the water of the food and drinks. The amount of this internal secretion is 7 to 10 liters/day.

Physiological functions of water:

1. **Specific heat:** Heat is required to raise the temperature of 1 gm of water through one degree Celsius is more than for almost any other solid or liquid. The high specific heat of water helps in minimizing the rise in body temperature due to the heat emitted out of chemical reactions.
2. **Latent heat of evaporation:** Water has the highest latent heat of evaporation than any other liquid. A certain amount of water can cause maximum cooling by evaporation, so that body temperature does not rise.
3. **Solvent power:** Water forms true solutions as well as colloidal solutions. Even water insoluble substances are made water soluble by the hydrotropic action. Therefore, it is the most suitable solvent for cellular components; water thus brings various substances in contact for chemical reactions to proceed.
4. **Dielectric constant:** Oppositely charge particles can coexist in water. Therefore, it is a good ionizing medium. This stimulates the chemical reactions.
5. **Catalytic action:** A large number of chemical reactions in the body are accelerated by water due to its ionizing power. All chemical reactions in the body proceed in presence of water only.
6. **Lubricating action:** Water acts as a lubricant in the body to prevent friction in joints, pleura, conjunctiva and peritoneum.

Regulation of passage of water:

1. If capillary pressure is increased, more water will flow into the tissues.
2. A fall in blood pressure helps in passage of water form the tissues to the blood.
3. If the plasma proteins are decreased, water will flow into the tissues.
4. Dilution of blood by excessive ingestion of water can lower the osmotic pressure of the plasma proteins and thus may increase capillary pressure.

Dehydration: When the loss of water exceeds the intake, the body's water content is reduced. This means that the body is in negative water balance and the condition is known as dehydration.

Causes:

1. **Primary dehydration:**
   (a) Deprivation of water during desert travel, extreme weakness and mental patients refraining to drinking water causes dehydration. Occurs more quickly in fever and in high environmental temperatures.

(b) Excessive water loss due to vomiting, prolonged diarrhoea, excretion of large quantities of urine and sweat. In water depletion, the concentration of extracellular fluid increases. Water is drawn from the cells and both extracellular and intracellular compartments shrink. Extreme thirst results; individual complains of hot and dry body. The tongue becomes dry.

2. **Secondary dehydration:** Concentration of electrolytes of the body fluids is maintained constant through the elimination or retention of water. The reduction or increase in the total electrolytes which affects, chiefly the basic radical Na (extracellular) or K (intracellular) and the acid radicals HCO_3 and Cl is accompanied by a corresponding increase or decrease in the volume of body water. This causes intracellular edema, slowing of circulation and impairment of urinal function. The individual becomes weak.
3. **Dehydration due to injection of hypertonic solution:** When a highly concentrated sugar or salt solution is injected into the body, the osmotic pressure of blood will increase. This results in the flow of fluid from the tissues into the blood until equilibrium sets in. Consequently, the blood volume increases. This increased blood volume soon returns to normal by the loss of excess material through urination. This causes a net loss of body water producing dehydration.

Effects of dehydration:

1. Loss of weight due to the reduction in tissue water.
2. Disturbances in acid-base balance.
3. Rise in the non-protein nitrogen of blood.
4. Rise in the plasma protein concentration and of chloride.
5. Rise in body temperature due to reduction in circulating fluid.
6. Increased pulse rate and reduced cardiac output.
7. Dryness, wrinkling and looseness of skin.
8. Exhaustion and collapse.

Correction of dehydration:

1. Ordinary NaCl solution may be given parenterally to repair the losses.
2. In the case of excretion of fluid high in Na and HCO_3 resulting fluid and electrolyte loss, a mixture of 2/3 isotonic saline and 1/3 Na lactate should be administered intravenously.
3. Dehydration in diabetes mellitus, Addison's disease, uremia, extensive burns and shock cannot be corrected by the above methods.

Water intoxication: Caused by excessive water retention due to renal failure, hypersecretion of ADH, excessive administration of fluids parenterally.

Symptoms: Headache, nausea and muscular weakness.

MINERAL METABOLISM

Living beings have organic and inorganic types of chemical constituents. The organic constituents i.e. proteins, carbohydrates, fats etc. are made up of C, H, O and N. The inorganic constituents described as **'minerals'** comprise of the elements present in the body other than C, H, O and N. Although they constitute a relatively small amount of the total body tissues, they are essential for many vital processes.

There are 31 elements present in the body. They are divided into two classes—(1) essential elements and (2) non-essential elements.

Essential elements: Those which are essential to maintain the normal living state of a tissue. They are again divided into two sub groups—

Macro elements: They are required to be present in the diet, more than 1 mg.

Ex. Ca, P, Mg, Na, K, Cl and S.

Micro elements: They are 8 in number and utilized in trace quantities (in microgram or nanogram). Hence they are called **trace elements**. These are Fe, Cu, Zn, Co, Mo, F, I and Mn.

Non-essential elements: They are 8 in number. They are present in tissues but their functions if any are not clearly defined. They include Al, B, Se, Cr, Br, As, Ti and Pb. Four additional elements, Ni, Tin, Vanadium and Silicon have been suggested as essential trace elements in nutrition but their implications for human nutrition are unknown.

The mineral elements present in the body are supplied in the diet. In poor diets consumed by a large majority of people, calcium and iron deficiency occur commonly. Iodine deficiency occurs in people living in certain hilly tracts, where the soil and water are deficient in iodine. In tropical countries, addition of sodium chloride in the diet is of great importance, because of the loss of NaCl in sweat. The deficiencies of other minerals do not occur normally in average diets.

- Sodium, potassium and chlorine are involved mainly in the maintenance of acid-base balance and osmotic control of water metabolism.
- Calcium, phosphorus and magnesium are constituents of bone and teeth.
- Phosphorus is the constituent of body cells of the tissues, such as muscles liver etc.
- Sulphur is present in cystein, methionine, thiamine, biotin, lipoic acid and coenzyme A.

CALCIUM

Source: Milk (0.2 gm/100 ml) and cheese are important dietary sources. Other sources are egg yolk, lentils, nuts, cabbage, cauliflower and asparagus, etc.

Requirement:

(1) Men and women after 18 years of age require 800 mg/day.
(2) During lactation and in pregnancy of 2^{nd} and 3^{rd} term 1.2 gm/day is required.
(3) Infants under 1 year require-360-540 mg/day.
(4) Children of 1-18 years need 800-1200 mg/day.

Absorption: Calcium is taken in the diet as calcium phosphate, carbonate, tartarate and oxalate. Calcium is absorbed actively in the upper small intestine. The active process is regulated by 1,25 dihydrocholecalciferol, a metabolite of vitamin D which is produced in the kidney in response to low plasma Ca++ concentrations. Absorption of calcium by the intestine is never complete. Ca is absorbed by an active transport process occurring mainly in the upper small intestine. Calcium absorption is influenced by the following factors—

1. Vitamin D promotes absorption of Ca.
2. Acidic pH favous calcium absorption because Ca salts (phosphate and carbonates) are quite soluble in acid solution and are relatively insoluble in alkaline solutions. Hence an increase in acidophilic flora, e.g. lactobacilli is recommended to lower pH which favours the absorption of Calcium.
3. Organic acids, lactose and basic amino acids in the diet favour calcium absorption.
4. Higher levels of proteins in the diet help to increase the absorption of calcium. On a high protein diet, about 15% of the dietary calcium is absorbed, compared with 5% absorption on a low protein diet. Certain calcium salts are much more soluble in aqueous solution of amino acids than in water and thus absorption of calcium is increased in presence of amino acids.
5. If calcium: phosphorus ratio is much high, $Ca_3(PO_4)_2$ will be formed and absorption of calcium is reduced. The optimal ratio for both elements is about 1:1 (1:2 to 2:1) and with ratios outside these limits, absorption is decreased. This is because of formation of insoluble calcium phosphate.

6. When fat absorption is impaired much free fatty acids are formed due to hydrolysis. These fatty acids react with free calcium to form insoluble calcium soap and then Ca is lost in faeces.
7. Absorption of calcium is inhibited by a number of dietary factors that cause formation of insoluble calcium salts, i.e. phytate (cereal grain), oxalate, phosphate and iron, etc.
8. High concentration of Mg in the diet decreases absorption of Ca.
9. Presence of excess fibre in the diet interferes with the absorption of Ca.
10. Percentage of calcium absorption decreases as its intake increases.
11. Parathyroid hormone increases the intestinal absorption of calcium.
12. Adrenal glucocorticoids diminish intestinal transport of Ca.
13. After the age of 55 to 60 there is gradual diminution of intestinal transport of calcium. During menopause many women develop negative calcium-phosphorus balance leading to a type of osteoporosis. This is usually accompanied by pain and fractures. The negative balance of calcium and phosphorous are markedly improved by administration of estrogen or by androgens such as testosterone. A combination of estrogen and androgen is more effective.
14. Kidney threshold regulates the blood calcium level. In a normal adult any extra calcium absorbed from the intestine is readily excreted in the urine. In hypercalcaemia kidney threshold also becomes abnormal.
15. Excess of iron also disfavours absorption of calcium and phosphorus, as ferric phosphate is highly insoluble. The net result is an upset in the Ca:P ratio.
16. Oxalate in certain foods precipitate calcium in the intestine as insoluble calcium oxalate. The phytic acids of food form insoluble salt with calcium and reduce calcium absorption.
17. Vitamin D increases calcium and phosphorus absorption from the intestine. Vitamin D promotes synthesis of specific calcium binding protein which participates in the active transport of calcium across the small intestinal mucosa. Lack of vitamin D, excess of phytates, low Ca/P ratio in diet, increased pH of upper intestine and malabsorption syndromes influence the amount of calcium absorption adversely.

Biological role:

Calcium is involved in the following biological processes—

1. **Constituent of bones and teeth:** Calcium along with phosphate constitutes the mineral part of the skeleton and teeth where it is present to the extent of 99% of the total calcium present in the body. It is primarily in the form of crystals of hydroxyapatite, while some is in combination with phosphate (calcium phosphate) in the form of amorphous crystals.
2. **Neuromuscular functions:** This involves excitability of nerve function, neural transmission, and contractility of cardiac and skeletal muscle. Normal concentration of calcium ions is required for the normal excitability of heart muscle.
3. **Blood coagulation:** It plays a vital role in blood clotting process since it activates the enzymic conversion of prothrombin into thrombin and production of thromboplastin. The removal of calcium from the blood can prevent blood coagulation and because of this reason EDTA, oxalates, citrates are used as anticoagulant because these ions can precipitate calcium into the respective insoluble salts.
4. **Membrane function:** It controls the permeability of all membranes and is often bound by lecithine in the membrane, i.e. it decreases the permeability and balances the opposite action of sodium and potassium capillary permeability. This involves transfer of inorganic ions across cell membranes and release of neurotransmitters at synaptic junction.
5. **Selected enzymatic reactions:** Calcium acts as activator for number of enzymes like ATPase, succinic dehydrogenase, lipase, etc. It also antagonizes the effect of magnesium on many enzymes. It releases cellular enzymes such as amylase from the parotid and increases the level of activity of

intracellular enzymes such as—Isocitric dehydrogenase, phosphorylase and Kinase and phosphofructokinase.

6. **Regulation of secretion of certain peptide hormones:** Pituitary hormones, parathyroid hormone, calcitonin and vasopressin are regulated through calcium ionic concentration. Calcium along with zinc plays a vital role in release of insulin from pancreas.

Calcium homeostasis: Normal blood values are 9.5-10.5 mg/100 ml. 35-45% of this is bound to proteins, mostly to the albumin fraction. In the extracellular fluid nearly all the calcium is in ionized form (55-65%). 0.5 (5-10%) mg is complexed to organic acids, phosphate, citrate, etc., while in renal failure, it may be complexed to other organic ions as well. The skeleton is in a dynamic state of equilibrium to maintain calcium homeostasis. 4-8 gm of calcium in bone is rapidly exchangeable with that in plasma and is present on the surface of the bone crystals–labile calcium storage pool. The remaining 99% of bone calcium is more firmly fixed in bone tissue and exchanges at a very slow rate.

Metabolism: The blood cells contain very little amount of calcium, most of the blood calcium is therefore, in the plasma, where it is present in 3 fractions—

(1) Ionized about 2 mg/100 ml.

(2) Non-diffusible (protein bound) above 3.5 mg/100 ml.

(3) A small amount as calcium complex of citrate and phosphate.

All these forms of calcium in the serum are in equilibrium with one another. A decrease in ionized calcium in the serum causes tetany. This may be due to an increase in the pH of blood or lack of calcium because of poor absorption from the intestine, decreased dietary intake, increased renal excretion as in nephritis or parathyroid deficiency.

Factors influencing blood calcium level:

1. *Parathyroid hormone*: In fasting condition or state there is no absorption from the intestine, the normal plasma Ca concentration is maintained by its rate of excretion and its mobilization from bones through the action of the parathyroid hormone.
2. *Vitamin D*: It enhances absorption of Ca from the intestine and thus maintains normal Ca concentration.
3. *Plasma proteins*: Half of the blood Ca (non-diffusible) is bound to plasma proteins and thus any decrease in these proteins will be accompanied by a decrease in the total calcium level.
4. *Plasma phosphate*: A reciprocal relationship exists between the concentration of Ca and phosphate ions in plasma. The marked increase in serum phosphate causes a fall in serum calcium concentration.
5. *Calcitonin*: An increase in the ionized Ca levels in the plasma is the stimulus for the production of calcitonin which then causes a deposition of Ca in bone.

Excretion: Calcium is excreted in the urine, bile and digestive secretion. About 75% of dietary calcium is absorbed and rest is excreted as fecal calcium. Nearly 10 g of Ca is filtered by the renal glomeruli in 24 hours. But only 200 mg appear in the urine, which is in the ionic state as well as in the complexes with citrate and other organic anions. A very small amount of Ca is excreted into the intestine after absorption. About 15 mg of Ca is excreted in the sweat. Vigorous physical exercise increases the loss of Ca by way of sweat.

Disease state: Calcium metabolism is highly influenced by parathyroid hormones. In hyperparathyroidism serum calcium rises (12-22 mg/100 ml) (hypercalcaemia), phosphatase activity is increased, urinary calcium is decreased and phosphorus rises in serum. The calcium, phosphorus ratio is important in

ossification. In the serum the product of calcium and phosphorus (in mg/100 ml) is normally 50 in children and may be below 30 during rickets. The following are the diseases related to calcium in the body.

(a) **Effects of parathyroid:**

1. ***In hyperparathyroidism***, the following changes occur
   - (i) Hypercalcemia (12-22 mg/dl).
   - (ii) Decrease in serum phosphate.
   - (iii) Diminished renal tubular reabsorption of phosphate.
   - (iv) Increased phosphatase activity.
   - (v) Renal urinary Ca and phosphorus found from bone decalcification and dehydration.
   - (vi) Extra Ca and P are lost from soft tissue and bones by increased bone destroying activity.
2. ***In hypoparathyroidism***, the following changes occur
   - (i) The concentration of serum Ca may drop below 7 mg/100 ml.
   - (ii) Increased serum phosphate and decreased urinary excretion of calcium and phosphorus.
   - (iii) Normal or occasionally raised serum phosphatase activity.
   - (iv) Normal acid-base equilibrium.
   - (v) Probably increased bone density.

(b) **Tetany:** Decreased ionized fraction of serum Ca causes tetany. This may be due to—

1. Increase in the pH of blood.
2. Poor absorption of Ca from the intestine.
3. Decreased dietary intake of Ca.
4. Increased excretion of Ca as in hepatitis.
5. Parathyroid deficiency.
6. Increased retention of phosphorus as in renal tubular disease.

Symptoms: Muscles lose tone and become flabby.
Affects the face, hands and feet.

(c) **Rickets:** This is characterized by faulty calcification of bones in children showing serum phosphate values of 1 to 2 mg/100 ml. This may be due to—

1. Vitamin D deficiency.
2. A deficiency of Ca and P in the diet or a combination of both.
3. Poor absorption of Ca from the intestine.
4. Parathyroid deficiency.
5. Increased alkaline phosphatase activity.

(d) **Osteoporosis:** This disease occurs in adults due to the following causes—

1. Decalcification of bones as a result of Ca deficiency in the diet.
2. Hypoparathyroidism.
3. Low vitamin D content of the body.

Symptoms: Fractures of the brittle bones occur even after minor accidents.
Pain due to fracture of vertebrae (may radiate round the trunk, to the buttocks or down the legs).

Renal rickets: It is a hereditary disease. It is called familial hypophosphatemia rickets. Affected persons show severe rickets with hypophosphatemia. The causes are (i) Defective transport of phosphate by the intestine and the renal tubules (ii) Lowered serum phosphorus and hyperphosphaturia (iii) Reduced

intestinal absorption of calcium and phosphorus. Vitamin D in ordinary doses does not relieve the disease. Hence, it is referred to as vitamin D resistant rickets.

PHOSPHORUS

Source: Phosphorus is present in nearly all foods therefore a dietary deficiency is not known to occur in man. Dairy products, cereals, egg yolk, meat, beans and nuts are usually rich sources. The daily average intake is 800-1000 mg and is about twice that of calcium.

Absorption: Like calcium, phosphorus is also absorbed by upper small intestine and factors influencing the absorption are also similar. The normal range for plasma inorganic phosphorus is 3.0-4.5 mg/dl. In children values are higher (5-6 mg/dl) and remain so uptill puberty.

Distribution: Phosphorus is distributed more widely than calcium. 15% is found in muscle and other soft tissues and 85% in the inorganic mineral phase of bone. It is an integral part of many macromolecules. Ex. Phospholipids, phosphoproteins and nucleic acids.

Functions: It has no physiological effects comparable to that of calcium but it has many other functions which are as follows—

1. Formation of bone and teeth.
2. Formation of phospholipids essential to every cell.
3. Formation of nucleic acids and derivatives.

Ex. Adenylic acid and is thus significant in (RNA and DNA) protein synthesis and from genetics point of view.

4. Formation of organic phosphates as intermediate in metabolic processes.

Ex. In glycolysis, Glucose + ATP → G-6-P + ADP.

5. Formation of energy rich phosphate compounds.

Ex. ATP (energy currency of the cell).

6. Both inorganic and organic phosphates can take part in buffering the cell.

Ex. Sodium-potassium-phosphates.

7. Formation of coenzymes.

Ex. TPP, NADP.

8. Formation of phosphoprotein.

Ex. Casein.

Excretion: Urinary excretion is equivalent to dietary phosphate intake. It varies diurnally, more being excreted at night. The usual daily loss is 600-800 mg, tubular resorption being 85-95%. Renal loss of phosphate can be of significant magnitude to lower serum phosphorus values and enhance osteoid demineralization.

Homeostasis: There is a greater fluctuation observed in blood phosphate values due to easy shift between extracellular fluid and intracellular compartments. Thus it is quite dependent on dietary phosphorus. Inorganic phosphate affects the net movement of calcium into and out of bone. Raised phosphate will lead to depression of the solubility of the calcium of bone crystals and thus shift equilibrium towards bone. In this manner it opposes the effect of the parathyroids. Ingestion of heavy dose of phosphate can lower serum calcium and increase excretion of calcium in urine. Lowered phosphorus on the other hand will make parathyroid activity more apparent.

Hormonal factors are not directly linked. However renal phosphate clearance is very vital in homeostasis and seems to be secondarily involved in certain endocrinopathies, e.g. involving parathormone, growth hormone and corticosteroids.

Disease state: The following are the disease states of phosphorus in the body.

1. In rickets, serum phosphate is as low as 1-2 mg/100 ml (There is a temporary decrease in serum P during absorption of carbohydrates and some fats).
2. Organic P content is low but inorganic content is high in the serum in diabetes.
3. P retention causes acidosis in severe renal diseases. This results in increase of serum P.
4. Serum P levels are increased in hypoparathyroidism and decreased in hyperparathyroidism and celiac disease.
5. In renal rickets, blood P is very low with an increased alkaline phosphatase activity.
6. The deficiency of vitamin D is the cause of low serum P and the defects in the calcification of bones (referred to as vitamin D resistant rickets).

MAGNESIUM

Source: Magnesium is present in milk, egg, cabbage, cauliflower etc.

Daily requirement: Infants—100-150 mg; Children—150-200 mg and Adults—200-300 mg.

Absorption: A greater part of the daily ingested Mg is not absorbed. A very high intake of fat, phosphate, calcium and alkalies diminish its absorption. Parathyroid hormone increases its absorption.

Distribution: Whole blood it is 2-4 mg/dl, CSF it is 3 mg/100 ml and muscle it is 2 mg/100 ml.

Functions:

1. 70% of the total magnesium content (21g) of the body is combined with calcium and phosphorus in the complex salts of bone. The remainder is in the soft tissues and body fluids. It is the principal cation of the soft tissue.
2. Magnesium ions act as activators for many of the phosphate group transfer enzymes.
3. It is found in certain enzymes, such as co-carboxylase.
4. It functions as a cofactor for oxidative phosphorylation.

Disease state: The following are the disease states of magnesium in the body.

1. Magnesium deficiency causes depression, muscular weakness and liability to convulsions. Its deficiency has also been observed in chronic alcoholics with low serum mg and muscular weakness.
2. Low in Kwashiorkor, causing weakness.

Low levels of Mg are reported in uremia, normal and abnormal pregnancy, rickets, growth hormone treatment, hypercalcemia and recovery phase of diabetic coma.

SODIUM, POTASSIUM, CHLORIDE

Substances whose solutions conduct an electric current are called 'electrolytes'. They are about 11 in general. Na, K, Ca and Mg are cations whereas Cl, HCO_3, HPO_4, SO_4, organic acids and proteins are anions. Among these sodium, potassium and chloride are important in the distribution and the retention of body water, thus have close relationship among them. Hence these three elements appear as a single question in the university exams.

Source: The most important source of Na and Cl in the diet is common table salt (NaCl). The good source of K are chicken, calf flesh, beef liver, dried apricot, dried peaches, bananas, the juice of orange and pineapple, potatoes etc.

Absorption: Normally Na, K and Cl are completely absorbed from the gastro-intestinal tract. About 95% of sodium which leaves the body is excreted in the urine.

Distribution: In the tissues both Na and K occur in a relatively large amount as compared to chloride and other inorganic salts as well as protein and organic salts. Sodium is present in extra cellular fluid and

in a very low concentration inside the cells whereas potassium is mainly found inside the cells and in a very low concentration in the extracellular fluid.

Functions of sodium and potassium: These electrolytes maintain normal osmotic pressure in the body and protect the body against excessive loss of fluid.

1. They maintain the acid base balance in the body. Sodium bicarbonate, sodium phosphate, potassium phosphate form the buffer system of extracellular and intracellular fluids.
2. They maintain normal water balance.
3. Na also functions in the preservation of normal excitability of muscle and the permeability of the cells. K inhibits **'muscular contraction'** in general.
4. High intracellular potassium concentrations are essential for several important metabolic functions, including protein biosynthesis by ribosomes.
5. Sodium and Potassium chlorides maintain the viscosity of blood. A number of enzymes including glycolytic enzymes, such as pyruvate kinase, require K^+ for maximal activity.
6. Na helps in the formation of the gastric juice. NaCl takes part in the series of reactions as a result of which HCl is manufactured by the stomach.
7. K of KHb in the red cells helps in carbon dioxide transport.
8. K ions inhibit cardiac contraction and prolong relaxation.
9. K ions exert important effect on the function of nervous system.

Functions of chloride:

1. It provides $2/3^{rd}$ of the anion of plasma and is the main factor for regulating body reactions.
2. NaCl and KCl are important agents in regulation of osmotic pressure in the body.
3. HCl of gastric juice is ultimately derived from the blood chlorides.
4. Chloride ions are essential for the action of ptyalin and pancreatic amylase.
5. It is essential in acid-base regulation. Chloride plays a role in the body by chloride shift mechanism.

Metabolism: The metabolism of these elements is influenced by the following factors—

Hormones: Mainly adrenocortical steroids and some of the sex hormones facilitate the retention of sodium and chloride in the body and excretion of potassium by kidneys in the urine. In adrenocortical deficiency, serum sodium decreases because excretion increases.

Temperature: When atmospheric temperature is high as in summer, large amounts of sodium and chloride are lost in perspiration (sweating) and this loss may be checked when temperature is low (in winter).

Renal function: In renal disease, with acidosis, Na and Cl ion excretion in urine is increased due to poor tubular reabsorption of sodium whereas that of K ion is decreased leading to hyponatraemia and hypochloraemia but hyperkalaemia.

Average requirement of Na and K in human body is 5-15 and 4 gm per day, respectively.

Disorders:

Hyponatraemia: On sodium deficient diet, young ones grow slowly, lack fat deposit, there is muscle and testicular atrophy, lung infection and deficiency of osteoid tissues. There will also be loss of water, which will be evident by rapid weight loss.

Hypokalaemia: Extreme potassium depletion in circulating blood causes hypokalemia in young one, they grow slowly and both sexes become sterile. The heart rate is slow, muscle weakness, irritability and

paralysis are seen. Bone growth is retarded and in becomes excessively fragile and kidney hypertrophy is exhibited.

Hyperkalaemia: Hyperkalaemic paralysis occurs due to excessive amount of potassium in blood. The disease is characterized by periodical attacks of weakness or paralysis. The symptoms of hyperkalaemia are chiefly cardiac and central nervous system depression. They are related to the elevated plasma potassium level and not to increase in intracellular potassium levels.

A dietary chlorine deficiency produces no symptom except a subnormal growth rate. Under normal dietary condition human beings are not subject to a deficiency of sodium, potassium or chlorine. However excessive diarrhoea, vomiting or extreme sweating over long period may bring about a NaCl deficiency.

Sometimes the metabolism of individual minerals is asked as a separate question in the university exams. Hence each one is described separately in detail, hereunder.

Sodium:

Physiological functions:

1. Major component of extracellular fluids and exists in the body in association with anions chloride, bicarbonate, phosphate and lactate.
2. In association with chloride and bicarbonate it plays a role in acid base equilibrium.
3. Maintains osmotic pressure of the body fluids and thus protects the body against excessive fluid loss.
4. Plays an important role in the absorption of glucose and galactose from small intestine.
5. Maintains normal water balance and distribution.
6. Maintains the normal neuromuscular function.
7. Functions in permeability of cells.

Distribution: About 1/3rd of the total sodium content of the body is present in the inorganic portion of the skeleton. Most of the sodium is present in the extracellular fluid.

Plasma	—	330 mg/100 ml	Muscles	—	60 to 160 mg/100 gm
Cells	—	85 mg/100 gm	Nerve	—	312 mg/100 gm

Daily requirement: Adults require 5-15 gms/day. In temperate region, NaCl intake is less. In tropical region, NaCl intake is more. Hypertension patients should not take more than 1 gm of Na per day.

Absorption: Normally, Na is completely absorbed from gastro-intestinal tract. Less than 2% is eliminated in feces. In persons suffering from diarrhoea, large amounts are lost in feces.

Excretion:

Urine	—	5-35 gm	Skin	—	25-50 mg
Stool	—	10-125 mg	Excessive loss of Na by sweating causes heat arrays.		

Disease state:

1. Adrenal cortical steroids regulate the metabolism of Na. Insufficiency of adrenal cortical steroids decreases serum Na level with an increase in sodium excretion.
2. In chronic renal disease when acidosis exists, Na depletion occurs due to poor tubular reabsorption of Na as well as to the loss of Na in the buffering acids.
3. In persons not adapted to high environmental temperature large amount of Na is lost in the sweat, developing muscular cramps of extremities, oedema, headache, nausea and diarrhoea.
4. Hyponatremia causes dehydration and reduced blood pressure, decreased blood volume and circulatory failure. This may be due to—
   (a) Prolonged vomiting and diarrhoea resulting in excessive loss of digestive fluid.
   (b) Chronic renal disease with acidosis due to poor tubular reabsorption of Na.

(c) Adrenocortical insufficiency.
(d) Loss of weight due to loss of water.

5. In hypernatremia, serum Na is high. This occurs in—
   (a) Hyperactivity of adrenal cortex as in Cushing's syndrome.
   (b) Prolonged treatment with cortisone and ACTH as well as sex hormones, this results in—
      (i) Increased retention of water in the body.
      (ii) Increase in blood volume.
      (iii) Increase in blood pressure.
6. Steroid hormones cause retention of Na and water in pregnancy.

Potassium:

Physiological functions:

1. Potassium is largely present in intracellular fluid and it is also present in small amounts in the extra cellular fluid because it influences the cardiac muscle activity.
2. It plays an important role in the regulation of acid-base balance in the cell.
3. It maintains osmotic pressure.
4. It functions in water retention.
5. It is essential for protein biosynthesis by ribosomes.
6. The glycolytic enzyme pyruvate kinase requires K^+ for maximal activity.

Sources: High content of potassium is found in chicken, beef, liver, bananas, orange juice, pineapple, yam, potatoes etc.

Distribution:

Plasma	—	20 mg/100 ml	Muscles	—	250-400 mg/100 g
Cells	—	440 mg/100 gm	Nerves	—	530 mg/100 g.

Daily requirement: Normal intake of K^+ in food is about 4 gm. It is so widely distributed that its deficiency is rare except in pathological condition.

Blood potassium: Normal level of serum K is 14-20 mg/100 ml. Erythrocytes contain large amounts of K which avoids hemolysis. Serum K decreases during increased carbohydrate utilization following glucose or insulin administration. Aldosterone decreases serum K.

Absorption: Normally, K is practically completely absorbed from gastrointestinal tract and less than 10% of K is eliminated in the feces. In subjects with diarrhea large amounts are lost in feces.

Excretion: K is normally eliminated almost entirely in urine and a small amount in the feces. Aldosterone exerts an influence on potassium excretion. In normal kidney function; K is very promptly and efficiently removed from the blood.

Disease state:

1. K is not only filtered by the kidney but is also secreted by the renal tubules. Excretion of K is greatly influenced by changes in acid-base balance and also by adrenal cortex. The capacity of kidney to excrete K is very great and therefore hyperkalaemia does not occur even after ingestion of K, if kidney function is impaired K should not be given intravenously unless, circulatory collapse and dehydration are corrected.
2. Hyperkalaemia occurs in patients in the following conditions.
   (a) Renal failure
   (b) Severe dehydration
   (c) Addison's disease due to decreased excretion of K by the kidney

K deficiency occurs in chronic wasting diseases like malnutrition, prolonged negative nitrogen balance, gastrointestinal losses and metabolic alkalosis.

Chlorine:

***Physiological functions*:**

1. As a component of sodium chloride, chloride ion is essential in acid-base balance.
2. As Cl^- it is also essential in water balance and osmotic pressure regulation.
3. It is also important in the production of HCl in the gastric juice.
4. Cl^- ion is an activator of amylase.

Sources: Mainly as NaCl salt (table salt).

Distribution:

Plasma	—	365 mg/100 ml	Muscle	—	40 mg/100 g
Cells	—	190 mg/ 100 mg	Nerve	—	171 mg/100 g
CSF	—	440 mg/100 ml			

Daily requirement: 5-20 gms. Excess consumption of NaCl increases blood pressure in hypertensive patients. Causes edema in protein deficiency.

Absorption: Normally Cl is practically completely absorbed from the GI tract.

Excretion: Cl is chiefly eliminated in the urine, also in sweat. Its concentration in sweat is increased in hot climates and decreased by aldosterone.

Diseases state:

1. Cl deficit also occurs when losses of Na are excessive in diarrhoea, sweating and certain endocrine disturbances.
2. Loss of Cl due to loss of gastric juice by vomiting or pyloric or duodenal obstruction.
3. Hypochloremia alkalosis may develop in Cushing's syndrome or after administration of ACTH or cortisone.

SULPHUR

Sources: Sulphur is taken mainly as cysteine and methionine present in proteins. Other compounds in the diet contribute small amounts of sulphur.

Absorption: Inorganic sulphate is absorbed as such from intestine into the portal circulation. Small amount of sulphide may be formed in the bowel by the action of bacteria, but if absorbed into the blood stream, it is rapidly oxidized to sulphate.

Sulphur in blood (serum):

Inorganic	—	0.5-1.1 mg/100 ml
Ethereal sulphate	—	0.1-1.0 mg/100 ml
Neutral sulphur	—	1.7-3.5 mg/100 ml

Physiological functions:

1. Sulphur is present primarily in the cell protein in the form of cysteine and methionine.
2. Cysteine plays important part in the protein structure and enzyme activity.
3. Methionine is the principal methyl group donor in the body. The 'activated' form of methionine, s-adenosyl methionine is the precursor in the synthesis of a large number of methylated compounds which are involved in intermediary metabolism and detoxification mechanism.

4. Sulphur is a constituent of coenzyme A and lipoic acid which are utilized in the synthesis of acetyl-CoA, malonyl CoA, Acyl-CoA and S-acetyl lipoate (involved in fatty acid oxidation and synthesis).
5. It is a component of a number of other organic compounds such as heparin, glutathione, thiamine, pantothenic acid, biotin, ergothionine, taurocholic acids, sulphocyamides, indoxyl sulphate, chondroitin sulphate, insulin, penicillin, anterior pituitary hormones and melanin.

Excretion: Excreted in urine in 3 forms. Total sulphate excretion may be diminished in renal function impairment and is increased in condition accompanied by excessive tissue breakdown as in high fever and increased metabolism.

Disease state:

1. Serum sulphate is increased in renal function impairment, pyloric and intestinal obstruction and leukemia.
2. Marked sulphate retention in advanced glomerulo-nephritis causes the development of acidosis.

Increase in blood indica (indoxyl potassium sulphate) may occur in uremia.

IRON METABOLISM

Iron is present in all organisms and in all the cells. It does not exist in the free state, instead is always present in organic combination, usually with proteins. It exists in two forms i.e. Fe^{2+} (ferrous) and Fe^{3+} (ferric). It serves as an oxygen and electron carrier and is incorporated into redox enzymes and substances which carry out the function of oxygen transport such as haemoglobin and cytochromes. Total iron content in normal adult is 4 to 5 grams. 60-70% is present in hemoglobin, 3% in myoglobin and 0.1% in plasma combined with β-globulin transport protein transferrin. Hemoprotein and flavoprotein make upto less than 1% of total iron. Rest is stored as ferritin.

Source:

Rich – Liver, heart, kidney, spleen.
Good – Egg yolk, fish, nuts, dates, beans, spinach, molasses, apples, bananas, etc.
Poor – Milk, wheat flour, polished rice, potatoes etc.

Daily requirement: Only about 10% of ingested iron is absorbed.

- Infants – 10-15 mg..
- Children – 1-3 years 15 mg.
- 4-10 years – 10 mg.
- Older children and adults of 11 to 18 years – 18 mg.
- 19 years and above – 10 mg.
- Females between 11 and 50 years of age and during pregnancy or lactation – 18 mg.
- After 51 years of age – 10 mg.
- In adult women the average loss of iron with blood during menstrual period is 16-32 mg per month or an additional loss of 0.5 to 1.0 mg per day. This amount is easily obtained from diet.
- In excessive menstrual blood loss and in chronic iron-deficiency anemia, a supplement of 100 mg of iron per day is sufficient to replenish.
- During growth, pregnancy and lactation iron demand is more.
- In healthy adult male or post menopause women dietary iron requirement is negligible unless any deficiency or loss of iron occurs.
- Iron deficiency occurs as a result of malabsorption from gastro-intestinal tract.
- A defect in hemoglobin synthesis in anemia is commonly found in copper deficiency.

Biologically active compounds that contain iron:

1. **Haemic compounds:** In these compounds the protoporphyrin is combined with iron to form haem (divalent iron) and haematin.

Ex. Haemoglobin, myoglobin, cytochromes, catalases and peroxidases.

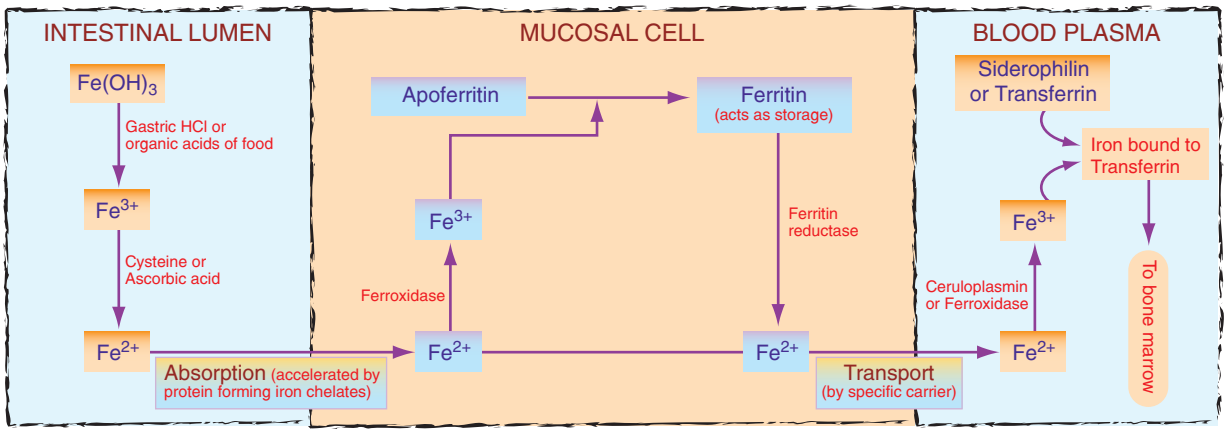
2. **Non-haemic compounds:** **These include** Transferrin (siderophilin) to transport iron, ferritin and haemosiderin which are the stored forms of iron and miscellaneous compounds like enzymes.

Absorption: Very little (less than 10%) of dietary iron is absorbed. Excretion in the urine is minimal. Infants and children absorb more iron as compared to adults. Iron deficiency in infants is due to dietary deficiency. Iron deficient children absorb approximately twice as much as normal children do.

Absorption mainly occurs in the duodenum and the proximal jejunum.

(a) Most of the iron in food occurs in the ferric form (Fe^{3+}), ex. either as ferric hydroxide or as ferric organic compounds. Acidic pH of the gastrointestinal tract favours the absorption whereas alkaline pH decreases it. In an acid medium, these compounds are broken down into free ferric ions or loosely bound organic iron, reducing substances such as –SH groups ex. cysteine and ascorbic acid which convert ferric iron into the reduced (ferrous) state, in this form iron is more soluble and should therefore be more readily absorbed.

(b) A diet high in phosphate, phytic acid and oxalic acid decreases iron absorption since these substances form the insoluble compounds with iron. Conversely, a diet very low in phosphate markedly increases iron absorption.



Mechanism of iron absorption

(c) The extent of absorption depends on the degree of saturation of the tissue, ex. anemic individuals absorb more than normal individuals.

(d) Iron absorption is enhanced by protein, possibly as a result of the formation of low molecular weight digestive products (peptides, amino acids) which can form soluble iron chelates.

(e) It is also increased in pernicious anaemia and in hypoplastic anaemia.

(f) Impaired absorption takes place in patients who have total removal of stomach or a removal of considerable amount of the intestine.

(g) Achlorhydria, administration of alkali, copper deficiency decrease iron absorption.

(h) Alcohol ingestion favours iron absorption.

MECHANISM OF IRON ABSORPTION

Ferrous ion on entering the mucosal cells is oxidized to ferric state and then combines with apoferritin forming ferritin which contains 23% of iron by weight. When apoferritin gets saturated with iron no

further iron can be taken up by the mucosal cells to store it in the form of ferritin. Heme enters the mucosal cells without being released from the porphyrin ring. Heme is broken down in the mucosa and iron appears in the plasma transferrin.

Transport: In the plasma, the iron is bound to transferrin which is only partially saturated. Plasma iron is also in exchange with interstitial and intra-cellular compartments. The iron in these compartments is generally referred to as 'labile iron pool' and is estimated to be in the order of 80 to 90 mg. Here the iron may stay briefly on the cell membrane before its incorporation into haem or storage compounds. Nearly all the iron released from the mucosal cell enter the portal blood mostly in the ferrous state (Fe^{2+}). In the plasma, Fe^{2+} is oxidized rapidly to the ferric state (Fe^{3+}) and then incorporated into a specific protein.

Storage: Stores of iron are maintained chiefly in the liver, spleen and bone marrow in the form of ferritin and haemosiderin. Women have lower stores than men and therefore, develop anaemia much more frequently than men. Iron stores are increased in haemochromatosis, severe haemolytic anaemias, aplastic anaemia and in persons receiving multiple blood transfusions, prolonged oral or parenteral iron therapy.

The normal content of protein bound iron (FBI) in plasma of males is 120-140 µg/100 ml; in females it is 90-120 µg/100ml. However, the total iron binding capacity (TIBC) is about the same in both sexes i.e. 300-360 µg/100 ml.

Excretion: Physiological excretion of iron is minimal. The normal routes of excretion are urine, bile, faeces, cellular desquamation, and sweat. Daily excretion in an adult male is estimated to be about 1 mg. In women of reproductive age, additional loss through menstruation averages to 1 mg per day.

Abnormal iron metabolism: Ferritin and hemosiderin, the storage forms of iron act as internal iron reserve to protect against sudden loss of iron by bleeding. Ferritin is present not only in the intestine but also in liver (about 700 mg) spleen and bone marrow. If excess iron is administered parenterally exceeding the capacity of the body to store it as ferritin, it accumulates in the liver as hemosiderin, a form of colloidal iron oxide in association with protein. Iron metabolism is disturbed mainly by the following causes—

(a) Decreased formation of hemoglobin.
(b) Decrease in circulating hemoglobin.
(c) Abnormalities in the serum iron concentration
(d) Abnormal deposition of iron-combining pigments in the tissues.

Physiological functions:

1. Iron functions mainly in the transport of oxygen to the tissues.
2. Involved in the process of cellular respiration.
3. Essential component of hemoglobin, myoglobin, cytochromes and the respiratory enzyme systems (cytochrome oxidase, catalase and peroxidase).
4. Non-heme iron is completely protein-bound (storage and transport).
5. Non-heme iron is utilized in the structure of xanthine dehydrogenase (xanthine oxidase) and succinate dehydrogenase and also in the iron sulphur proteins of the respiratory chain.

Iron deficiency: Iron deficiency is the commonest cause of nutritional anaemia and is prevalent all over the world.

Causes of iron deficiency:

(1) ***Dietary deficiency*:** The iron content in the diet is sufficient to meet the daily requirements, but excessive amount of phytates in cereals, is responsible for non-absorbability of this iron. Hence higher daily intake of iron is recommended for vegetarians.

(2) ***Lack of absorption*:** This may be seen in malabsorptive syndromes.

(3) *Increased demand*: This occurs during rapid growth in infancy and pregnancy.

(4) *Poor stores at birth*: These are found in premature birth and twin pregnancy.

(5) *Pathological blood loss*: With loss of 1g of haemoglobin 3.4 mg of iron is lost. Hook-worm infestation is the most important factor responsible for blood loss. Other sources of blood loss are bleeding piles, peptic ulcer, hiatus hernia, cancer of gastrointestinal tract, chronic aspirin ingestion, and oesophageal varices.

(6) *Iron deficiency anemia*: Iron deficiency anemia is widely prevalent among children, adolescent girls and nursing mothers. The hemoglobin content of the blood during iron deficiency anemia is 5 to 9 g/100 ml.

(a) *Women of child bearing age*: The clinical symptoms are breathlessness on exertion, giddiness and pallor of the skin. In severe cases, there may be edema of the ankles.

(b) *Weaned infants and young children*: The hemoglobin level is 5 to 9 g/100 ml of blood. The children are dull and inactive and show pallor of the skin. The appetite is poor and growth and development are retarded.

Treatment of iron deficiency anaemia: Anaemia responds to oral iron therapy. The commonly used preparations are ferrous sulphate, ferrous fumarate and ferrous gluconate. Iron dextran can be administered both intramuscularly and intravenously, iron sorbitex is given intramuscularly, and saccharated iron oxide is given intravenously.

Anemic women should take ferrous sulphate tablet. For a child below 12 months, a mixture of ferrous ammonium citrate sweetened with glycerine and for children of 1 to 5 years ferrous ammonium citrate mixture should be given for curing.

Iron overload: Hypersiderosis may occur as a primary disorder (Idiopathic haemochromatosis) or secondary with excessive entry of exogenous, iron into the body.

1. **Siderosis:** When excessive amounts of iron are released in or introduced into the body beyond the capacity for its utilization, the excess is deposited in various tissues, mainly in the liver. This may occur due to repeated blood transfusions, excessive breakdown of erythrocytes in hemolytic types of anemia and inadequate synthesis of hemoglobin as in pernicious anemia.
2. **Nutritional siderosis:** This disorder is found among Bantus in South Africa. Bantus cook their food in large iron pots and consume iron-rich food. The absorption of iron appears to be high, leading to the development of nutritional siderosis. Livers of the Bantus contain large amounts of iron.

Hemochromatosis: Hemochromatosis is a rare disease in which large amounts of iron are deposited in the tissues, especially the liver, pancreas, spleen and skin producing various disorders. Accumulation of iron in the liver, pancreas and skin produces hepatic cirrhosis, bronze diabetes and bronze-state pigment respectively.

COPPER

Source:

Richest sources: Liver, kidney, other meats, shell fish, nuts and dried legumes.

Poor sources: Milk and milk products. The concentration of copper in the fetal liver is 5-10 times higher than that in liver of an adult.

Daily requirements:

Infants and children	–	0.05 mg/kg body weight
Adults	–	2.5 mg

A nutritional deficiency of copper has never been demonstrated in man, although it has been suspected in case of nephrosis.

Absorption: About 30% of the normal daily diet of copper is absorbed in the duodenum.

Blood copper: The normal concentration of copper in serum is 90 µg/100 ml. Both RBC and serum contain copper. 80% of RBC copper is present as superoxide dismutase (erythrocuperin). Plasma copper occurs as firmly bound form and loosely bound forms. The firmly bound copper consists of ceruloplasmin. Loosely bound copper is called **'directly reacting copper'** and is bound to serum albumin. The plasma copper levels increase in pregnancy because of their estrogen content. Oral contraceptives have a similar effect.

Physiological functions:

1. It has important role in hemoglobin synthesis.
2. It is required for melanin formation, phospholipids synthesis and collagen synthesis.
3. It has a role in bone formation and in maintenance of the integrity of myelin sheath.
4. It is a constituent of several enzymes such as tyrosinase, cytochrome oxidase, ascorbic acid oxidase, uricase, ferroxidase I (ceruloplasmin), ferroxidase II, superoxide dismutase, amino oxidase and dopamine hydroxylase.
5. Three copper containing proteins namely cerebrocuperin, erythrocuperin and hepatocuperin are present in brain, RBC and liver respectively.

Excretion: Only 10 to 60 µg of copper is excreted in the urine. 0.5 to 1.3 mg is excreted through bile and 0.1 to 0.3 mg is excreted by intestinal mucosa into the bowel lumen.

Effects of copper deficiency:

1. Although iron absorption is not disturbed but the release of iron into the plasma is prevented due to the decreased synthesis of ceruloplasmin. As a result, hypoferremia occurs which leads to the depressed synthesis of heme developing anemia in severe deficiency of copper.
2. The experimental animals on a copper deficient diet lose weight and die.
3. In copper deficient lambs, low cytochrome oxidase activity results in neonatal ataxia.
4. Copper deficiency produces marked skeletal changes, osteoporosis and spontaneous fractures.
5. Elastin formation is impaired in the deficiency of copper. Because a copper containing enzyme plays an important role in the connective tissue metabolism, especially in the oxidation of lysine into aldehyde group which is necessary for cross linkage of the polypeptide chains of elastin and collagen.
6. Copper deficiency results in myocardial fibrosis in cows. It is suggested that reduction in cytochrome oxidase activity may lead to cardiac hypertrophy.

Disorders of copper metabolism: ***Wilson's disease*** **(*hepato-reticular degeneration*):** Wilson's disease is a rare hereditary disorder of copper metabolism. The following disorders have been observed in this disease.

(a) The absorption of copper from the intestine is very high (about 50 percent); whereas 2 to 5 percent copper is absorbed in normal subjects.

(b) Ceruloplasmin formation is very less. Hence a greater part of serum copper remains loosely bound to serum protein-notably albumin and therefore, copper can be transported to the tissues, such as brain and liver or to the urine.

(c) Excessive deposition of copper in the liver and the kidney causes hepatic cirrhosis and renal tubular damage respectively. The renal tubular damage results in the increased urinary excretion of amino acids, peptides and glucose.

IODINE

Source: Rich sources are sea water, marine vegetation and vegetables as well as fruits grown on the sea board. Plants grown at high altitudes are deficient in iodine because of its low concentration in the water. In such regions, iodide is commonly added to the drinking water or table salt in concentrations of 1:5000 to 1:200000.

Daily requirement:

Adults	—	100 to 150 µg
In adolescence and in pregnancy	—	200 µg

Distribution: Normal iodine content of body is 10 to 20 mg. 70 to 80% of this is present in thyroid gland. Muscles contain large amount of iodine. The concentration of iodine in the salivary glands, ovaries, pituitary gland, brain and bile is greater than that in muscle. Iodine in saliva is inorganic iodide, while most of the iodine present in tissue is in the organic form.

Blood Iodine: Practically all the iodine in the blood is in the plasma. The normal concentration in plasma or serum is 4 to 10 µg/100 ml. 0.06 to 0.08 µg/100 ml is in inorganic form, 4 to 8 µg/100 ml is in the organic form bound to protein, precipitated by protein precipitating agents. 90% of the organic form consists of thyroxine and the remainder tri and diiodothyronine. About 0.05% of thyroxine is in the free state. RBC contains no organic iodine.

Absorption: Iodine and iodide are absorbed most readily from the small intestine. Organic iodide compounds (diiodothyronine and thyroxine) are partly absorbed as such and a part is broken down in the stomach and intestines with the formation of iodides. Absorption also takes place from outer mucus membrane and skin.

Storage: 90% of the iodine of the thyroid gland is in organic combination and stored in the follicular colloid as 'thyroglobulin' a glycoprotein containing thyroxine, diiodthyronine and smaller amounts of triiodothyronine.

On demand these substances are mobilized and thyroxine as well as triiodothyronine is passed into the systemic circulation. They undergo metabolic degradation in the liver.

Excretion:

1. Inorganic iodine is mostly excreted by the kidney, liver, skin, lungs and intestine and in milk.
2. About 10% of circulating organic iodine is excreted in feces. This is entirely unabsorbed food iodine.
3. 40 to 80 % is usually excreted in the urine, 20 to 70 µg daily in adults, 20 to 35 µg in children. The urinary elimination is largest when the intake is lowest.
4. Urinary iodine is increased by exercise and other metabolic factors.

Physiological functions: Iodine is required for the formation of thyroxine and triiodothyronine hormones of the thyroid gland. These thyroid hormones are involved in cellular oxidation, growth, reproduction and the activity of the central and autonomic nervous systems. Triiodothyronine is more active than thyroxine in many respects.

Iodine deficiency:

1. In adults the thyroid gland is enlarged producing goiter. If treatment is started very early, the thyroid becomes normal. If treatment is delayed, the enlargement persists.
2. In children, severe iodine deficiency results in the extreme retardation of growth causing cretinism.

Prevention of goiter: Goiter can be prevented by the regular use of iodized salt or iodine added to the drinking water.

Goitrogenic substances in foods: Cabbage, cauliflower and radish contain substance like vinyl-2-thiooxazolidone which makes iodide present in the food unavailable by reacting with it. Such substances are called 'goitrogenic' substances.

SELENIUM

- Good dietary sources are kidney cortex, pancreas, pituitary and liver.
- It is rapidly absorbed mainly in duodenum.
- It is distributed in liver 0.44 µg/gm in skin 0.27 µg/gm and in muscle 0.37 µg/gm.
- In the cells it is present as selenocystine nad selenomethionine.
- Selenium along with Vitamin E plays an important role in tissue respiration.
- Selenium is involved in biosynthesis of coenzyme Q (ubiquinone), which is involved in respiratory chain.
- Selenium acts as an antioxidant providing protection against peroxidation in tissues and membrane.
- It is an essential component of glutathione peroxidase, an enzyme which catalyzes the conversion of reduced glutathione to its oxidized form.
- Selenium is excreted in faeces, urine and via exhalation.
- It causes toxic effect called selenosis.

17

ENERGY METABOLISM

Unit of energy: Calorie is the unit of energy which is equal to the amount of heat required to raise the temperature of one gram of water by one degree from 14.5° C to 15.5° C at one atmosphere pressure. A calorie is a measure of energy expenditure. The calories referred to in diet and exercise are kilocalories (Kcal) i.e. 1000 times the calorie.

MEASUREMENT OF CALORIFIC VALUE OF FOOD

The body requires energy for its internal and external work. This is provided by the oxidation of food e.g. carbohydrates, fats, and proteins. The foodstuffs contain varying amounts of carbohydrates, fats, and proteins and therefore, the energy obtained from different foods vary. This can be determined by two methods - direct and indirect.

Direct method: The caloric value of a foodstuff can be determined by measuring the heat produced when a given amount is completely burnt in oxygen. It is done in a **'bomb calorimeter'** where the oxygen is put in under considerable pressure. Since it requires a calorimeter of robust construction, it has been called a bomb calorimeter.

The one commonly used for the purpose is the 'Atwater' bomb calorimeter. It consists of a heavy steel bomb, with platinum or gold plated copper lining. It has a cover which is held tightly by a strong screw-collar. A weighed amount of the sample is placed and the bomb is charged with an oxygen valve. The valve is then closed and the bomb is immersed in a weighed amount of water. The burning of the sample is set off by an electric spark and the heat liberated is measured by the rise in temperature of the surrounding water by means of a differential thermometer which can read up to one-thousandth of a degree. Deduction of the heat arising out of accessory combustions is made in order to obtain the heat liberated in calories from the combustion of the actual sample.

Indirect method: The caloric value can also be determined indirectly by burning the food in oxygen in an oxycalorimeter. The volume of oxygen required to burn the food sample is measured and the caloric value is calculated. The energy production is accurately related to oxygen utilization. The calculation is based on the principle that when 1 litre of oxygen is utilized in the oxidation of organic nutrients, approximately 4.8 Kcal of heat is liberated. The measurement of oxygen consumption which is a relatively simple technique is now universally employed to estimate the metabolic rate. This is **'indirect calorimetry'**.

The energy obtained as a result of complete combustion is the potential energy but the energy liberated in the body is not the same, and this is called the physiological energy. Since carbohydrates and

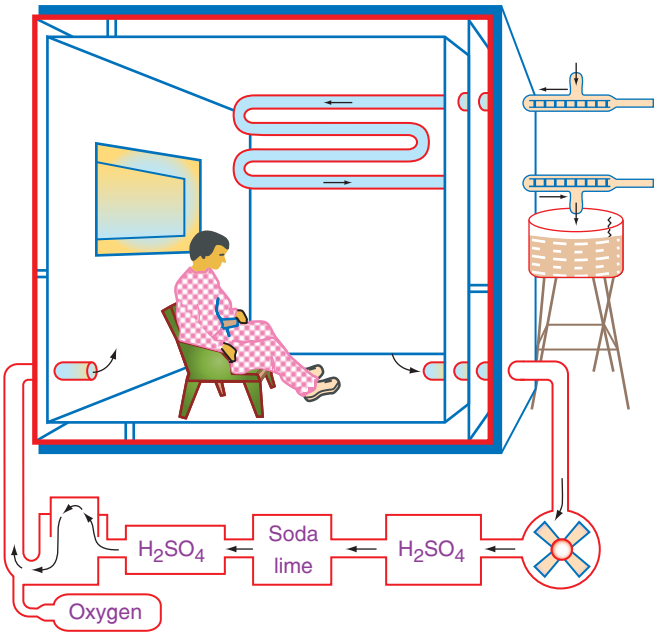
fats contain carbon, hydrogen and oxygen, they can be completely burnt to CO_2 and water and hence the potential energy is the same as the physiological energy. However, in the case of the proteins, the nitrogen is eliminated as urea etc. so the physiological energy is less than the potential energy.

Caloric values of various food stuffs

Food stuff	Kcal/gm in bomb calorimeter	Kcal/gm in the body
Carbohydrates	4.1	4.0
Fats	9.4	9.0
Proteins	5.6	4.0

MEASUREMENT OF ENERGY EXPENDITURE

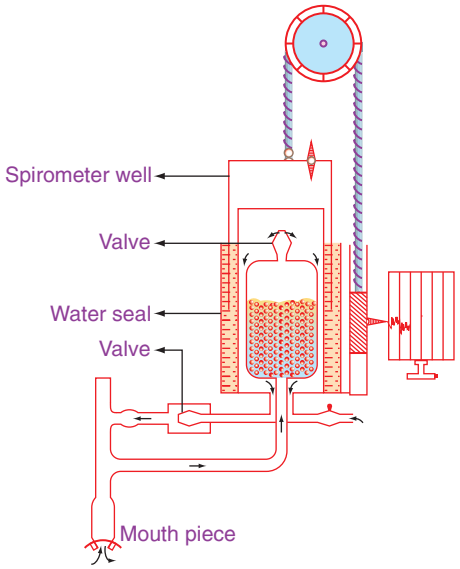
Total energy expenditure per unit time is called the metabolic rate. It can be measured directly or indirectly as in the case of calorific value of foods. In the direct method for the measurement of energy expenditure for individuals, the Atwater calorimeter is used which consists of a chamber in which a person could live and work for several days allowing at the same time the measurement of his total output of heat. The energy expenditure thus measured can be related to net energy intake which is the energy in food, minus the energy lost in urine and faeces. Atwater's experiments, measuring energy intake and energy output, lasted a number of days and he was able to demonstrate consistently a fair amount of agreement between the input and the output. Although nobody uses the human calorimeter these days on account of the difficulties of technique, Atwater's experiment was the first of its kind which demonstrated that the human body behaved like any engine running on combustion of fuels, thus taking the wind out of the sails of the theory of living matter possessing vital spirits.



Human calorimeter

The two indirect methods for measurement of energy expenditure in individuals commonly used are—

1. **The Benedict-Roth spirometer method:** This is a closed-circuit breathing apparatus which is filled with oxygen and has a capacity of about 6 litres. Oxygen is contained in a metal drum which floats on a water seal. The person whose O_2 consumption is to be measured breathes in oxygen through an inspiratory valve and breathes out into the drum through an expiratory valve and a soda-lime canister, so that the CO_2 produced is absorbed. As the O_2 is used up, the drum sinks and its movement is recorded on a moving paper mounted on a kymograph; from this, the rate of oxygen consumption can be read. The apparatus is accurate and simple to use. It has the disadvantage that it can be used only when the person is at rest or doing very light exercise.



Schematic diagram of Benedict-Roth spirometer used for measuring oxygen consumption

2. **Douglas bag method:** This is a canvas or plastic bag with a variable capacity, usually 100, 200 or 300 litres. The subject breathes through a mouth piece which contains inspiratory and expiratory valves. Room air is breathed in, but breathing out is into the Douglas bag so that all the expired air is collected in it. The bag is then emptied through a gas meter and a sample of the expired air is taken for analysis of O_2 and CO_2 content from which the rates of oxygen utilization and CO_2 production can be calculated. This method has the advantage that both O_2 consumption and CO_2 production can be measured at varying grades of activity or muscular exercise.

Person breathing out into the Douglas bag

RESPIRATORY QUOTIENT

The respiratory quotient (RQ) measures the ratio of the volume of carbon dioxide (V_c) produced by an individual to the volume of oxygen consumed (V_o). This is represented by the following equation—

$$RQ = \frac{Vc}{Vo}$$

This quotient is useful because the volumes of CO_2 and O_2 produced depend on which fuel source is being metabolized. Measuring RQ is a convenient way to gain information about the source of energy which a person is using. We can then compare the metabolism of a person under different environmental conditions by simply comparing RQ for various foods—

Carbohydrate	=	1.00
Fat	=	0.70
Protein	=	0.81
Mixed diet	=	0.85

Factors that increase RQ:

1. Hyperventilation
2. Metabolic acidosis leading to increase in carbon dioxide
3. Overfeeding leading to lipogenesis
4. Exercise

Factors that decrease RQ:

1. Hypoventilation
2. Mild starvation with ketosis
3. Diabetes with ketoacidosis or high rates of urinary glucose loss
4. Gluconeogenesis
5. Hypothermia via continued gluconeogenesis

BASAL METABOLIC RATE (B.M.R.)

Definition: Basal metabolic rate is the energy released when the subject is at complete mental and physical rest i.e. in a room with comfortable temperature and humidity, awake and sitting in a reclining position, 10-12 hours after the last meal. It is essentially the minimum energy required to maintain the heart rate, respiration, kidney function etc.

The B.M.R. of an average Indian man is 1750-1900 Kcal/day. In terms of oxygen consumption it would amount to about 15 litre/hr. Heavily built persons have higher BMRs, but the BMR per unit body weight is higher in the smaller built individuals ex. although the BMR of a man as given above is higher than that of a boy of 15 kg body weight that spends about 800 Kcal/day for its basal metabolism, the BMR per kg/day of man is about 30 Kcal, while that of the boy is about 53 Kcal/kg/day. The variable that correlates most with the BMR is the surface area of the body. Thus in case of both boy and man the BMR is around 1000 Kcal/m^2 body surface/day. In case of human beings body surface area can be calculated by the following formula—

$$S = 0.007184 \times W^{0.425} \times H^{0.725}$$

where

S = surface area in sq metres
W = body weight in kg and
H = height in cm

Factors influencing BMR: There are many factors that affect the BMR. These include body temperature, age, sex, race, emotional state, climate and circulating levels of hormones like catecholamines (epinephrine and norepinephrine) and those secreted by the thyroid gland.

1. **Genetics (Race):** Some people are born with faster metabolism and some with slower metabolism. Indians and Chinese seem to have a lower BMR than the Europeans. This may as well be due to dietary differences between these races. Higher BMR exists in individuals living in tropical climates. Ex. Singapore.
2. **Gender:** Men have a greater muscle mass and a lower body fat percentage. Thus men have a higher basal metabolic rate than women. The BMR of females declines more rapidly between the ages of 5 and 17 than that of males.
3. **Age:** BMR reduces with age i.e. it is inversely proportional to age. Children have higher BMR than adults. After 20 years, it drops about 2 per cent, per decade.
4. **Weight:** The heavier the weight, the higher the BMR, ex. the metabolic rate of obese women is 25 percent higher than that of thin women.
5. **Body surface area:** This is a reflection of the height and weight. The greater the body surface area factor, the higher the BMR. Tall, thin people have higher BMRs. When a tall person is compared with a short person of equal weight, then if they both follow a diet calorie-controlled to maintain the weight of the taller person, the shorter person may gain up to 15 pounds in a year.
6. **Body fat percentage:** The lower the body fat percentage, the higher the BMR. The lower body fat percentage in the male body is one reason why men generally have a 10-15% higher BMR than women.
7. **Diet:** Starvation or serious abrupt calorie-reduction can dramatically reduce BMR by up to 30%. Restrictive low-calorie weight loss diets may cause BMR to drop as much as 20%. BMR of strict vegetarians is 11% lower than that of meat eaters.
8. **Body temperature/health:** For every increase of 0.5° C in internal temperature of the body, the BMR increases by about 7 percent. The chemical reactions in the body actually occur more quickly at higher temperatures. So a patient with a fever of 42° C (about 4° C above normal) would have an increase of about 50 percent in BMR. An increase in body temperature as a result of fever increases the BMR by 14-15% per degree centigrade which evidently, is due to the increased rate of metabolic reactions of the body.
9. **External temperature:** Temperature outside the body also affects basal metabolic rate. Exposure to cold temperature causes an increase in the BMR, so as to create the extra heat needed to maintain the body's internal temperature. A short exposure to hot temperature has little effect on the body's metabolism as it is compensated mainly by increased heat loss. But prolonged exposure to heat can raise BMR.
10. **Glands:** Thyroxine is a key BMR-regulator which speeds up the metabolic activity of the body. The more thyroxine produced, the higher the BMR. If too much thyroxine is produced (thyrotoxicosis) BMR can actually double. If too little thyroxine is produced (myxoedema) BMR may shrink to 30-40 percent of normal rate. Like thyroxine, adrenaline also increases the BMR but to a lesser extent. Anxiety and tension may not show on the face but they do produce an increased tensing of the muscles and release of norepinephrine even though the subject is seemingly quiet. Both these factors tend to increase the metabolic rate.
11. **Exercise:** Physical exercise not only influences body weight by burning calories, it also helps raise the BMR by building extra lean tissue. (Lean tissue is more metabolically demanding than fat tissue.) So more calories are burnt even when sleeping.
12. **Pregnancy:** The BMR is not changed during pregnancy. The higher value of BMR in late pregnancy is due to the BMR of the foetus.

Significance of BMR:

1. The determination of BMR is the principal guide for diagnosis and treatment of thyroid disorders.
2. If BMR is less than 10% of the normal, it indicates moderate hypothyroidism. In severe hypothyroidism, the BMR may be decreased to 40 to 50 percent below normal.
3. BMR aids to know the total amount of food or calories required to maintain body weight.
4. The BMR is low in starvation, undernutrition, hypothalamic disorders, Addison's disease and lipoid nephrosis.
5. The BMR is above normal in fever, diabetes insipidus, leukemia and polycythemia.

SPECIFIC DYNAMIC ACTION (S.D.A.) OF FOOD

Metabolic rate is increased immediately after ingestion of food and remains so for 6 hours or more. This is measured by oxygen consumption of a resting man before and after eating, and is known as **'specific dynamic ation (S.D.A.)'** of foods. After most meals it usually amounts to an increase of 10-15%. Ingestion of proteins causes maximum increase, which is about 30%, carbohydrates increase it by 6%, and fats by 4%. This extra energy expenditure is not because of requirements of digestion and absorption of ingested food, which accounts for only a small proportion of energy, as intravenous injection of amino acids produces almost the same S.D.A. effect as oral ingestion. S.D.A. effect does not occur in an animal whose liver has been removed. Thus, the cause of S.D.A. of proteins is related to the process of deamination of the amino acids which occurs in the liver after their absorption. S.D.A. of fats is due to direct stimulation of metabolism by the liberated fatty acids, and that of carbohydrates is due to energy requirements for the synthesis of glycogen.

Prolonged fasting on the other hand produces a decrease in the metabolic rate. The reason for this is the reduction of body mass that occurs in starvation. It is an adaptive mechanism which helps the body in times of scarcity.

PRINCIPLES OF NUTRITION

Food: Food may be defined as any solid or liquid substance which when taken by the body provides it with necessary materials to enable it to grow, replace the wornout and damaged parts and provide energy to function normally. The daily food intake has a direct influence on the health and well being of an individual. Food is composed of different chemical elements.

Nutrients: Nutrients are molecules which the body uses to function appropriately and stay in a healthy condition.

Nutrition: It is the study of nutrients and their relationship with food and living beings.

Diet: The food that a person normally takes everyday.

Malnutrition: Malnutrition means an incorrect or imbalanced intake of nutrients.

Under-nutrition: Means insufficient total intake of nutrients.

Basic dietary components: There are five classes of basic components or nutrients—

(1) Carbohydrates (2) fats (3) proteins (4) vitamins (5) minerals and trace elements. The components under 1 to 3 are known as macronutrients, those under 4 and 5 are known as micronutrients.

Carbohydrates: Carbohydrates are not at all required in the diet. Yet, we consume large amounts of carbohydrates every day because they are the cheapest source of energy and easily available dietary sources. Carbohydrates are the main sources of energy to the body which provide about 70% of the daily calorie requirements. Carbohydrates, in addition to the supply of energy also serve as the components of cell membrane and receptors. Carbohydrates are very well synthesized in our body from non carbohydrate sources.

***Cellulose and stretch reflex*:** Cellulose (polysaccharide) materials present in the diet form the bulk (fiber) of the food and cannot be digested by human beings because of the absence of the enzyme cellulase. The cellulose helps in the movement of the food through the G.I tract. The cellulose material of the diet absorbs the waste from large intestine and while doing so it stretches the wall of the large intestine and as a result, defecation takes place. This is called stretch reflex.

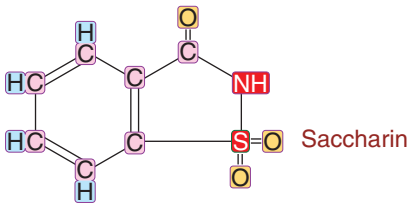
There is a metabolic disorder called diabetes mellitus wherein there is an increased level of glucose in the blood (hyperglycemia). Hyperglycemia affects the normal functioning of the kidney and brain leading to hypertension and other abnormalities. Hence, in diabetes mellitus patients, the blood glucose level should be kept under control, for which the carbohydrates should be restricted in the diet. The energy requirements can be met with proteins and to a little extent by fats. But as human beings first and the foremost food i.e. milk tastes sweet due to the presence of a carbohydrate lactose, he has a craving for sweet and hence he should be supplied with some non-carbohydrate sweeteners.

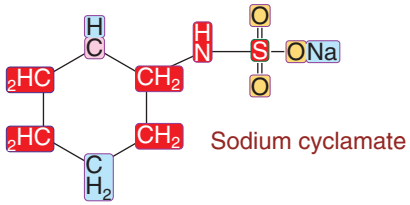
***Non-carbohydrate sweetners*:** Some of the non-carbohydrate sweeteners are saccharin, sodium cyclamate, monallin and aspartame. These sweeteners are also used in the milk powder supplied to children with lactose intolerance, wherein the milk is free from lactose but contains one of these sweeteners.

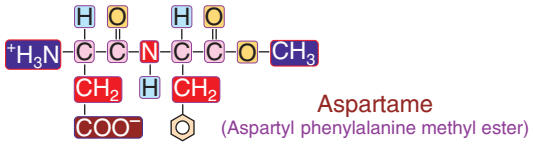
***Saccharin*:** It is 400 times sweeter than table sugar. Saccharin is commercially used in large scales though it is banned scientifically at present. It is used as sweetner in diabetes and obesity. But it is harmful to human beings. When saccharin is given in large quantities in drinks and food it causes cancer. However as the risk is too low it is still used as an artificial non-calorie sweetner in the diet drinks specially of those who are diabetis.

***Sodium cyclamate*:** It is 30 times sweeter than table sugar. It is a carcinogenic agent and has been banned from prepared foods.

***Aspartame*:** It is 180 times sweeter than table sugar. It is a methyl ester of a dipeptide of two amino acids that normally occur in protein (i.e., aspartate and phenylalanine). As it is non-toxic, it is used as a sugar substitute in many foods. The sugar free tablets available in the market are all made up of aspartame.







***Monellin*:** It is a protein with a molecular weight of 11,000. It is 2100 times sweeter than table sugar. Its sweetness is due to the three dimensional conformation of the polypeptide. But this cannot be used in prepared foods because it looses its sweetness on heating or denaturation.

***Fats*:** Fats are also not needed in our diet. But we consume it because of its high calorific values (1 g gives 9 Kcal) and also it can be easily stored with less amount of water and therefore occupy less space. These fats are also taken because of their essential fatty acid content. Essential fatty acids are those which cannot be synthesized in our body, hence they should be supplied through the diet. They are **'linoleic acid'** and **'linolenic acids'**. Arachidonic acid is also an essential fatty acid but it can be synthesized from linolenic acid. They are also a good source of fat soluble vitamins viz., A, D, E & K. Plant fats are

superior to animal fats because they contain more of polyunsaturated fatty acids i.e. essential fatty acids and less of cholesterol. The cholesterol in the diet should be restricted because excess intake of cholesterol leads to its deposition in the tissues thereby causing athlerosclerosis.

***Proteins*:** Proteins are needed for their content of certain amino acids that are essential to human body for the biosynthesis of proteins, body repair in adult and for body building and body repair in children. Human body proteins are made up of only 20 standard amino acids out of which nearly 10 amino acids can be synthesized in the body but the remaining 10 cannot be synthesized in the body hence they have to be supplied through the diet. Therefore they are known as essential amino acids. The essential amino acids are:

M – Methionine (semi essential)
A – Arginine (semi essential)
T – Tryptophan
T – Threonine
V – Valine
I – Isoleucine
L – Leucine
P – Phenylalanine
H – Histidine
Ly – Lysine

The nutritional value of a protein depends on two factors (1) Its content of the essential amino acids (2) its digestibility. The amino acid content of all the proteins is not the same. One will be deficient in one amino acid and the other in another type of amino acid. Some proteins are not completely digested to liberate all the amino acid contents, ex. the protein rich portions of wheat grains are not completely digestible.

***Nitrogen balance*:** It is a condition in which the intake of protein nitrogen (AA) exactly balances the loss of nitrogen in the urine and faeces. If the intake is more than the output due to nitrogen retention as tissue protein then the subject is said to be in positive nitrogen balance. If the intake is less than the output (as in old age and illness) then the subject is said to be in negative nitrogen balance.

***Biological value of protein*:** The biological value of a protein is a factor that is inversely proportional to the amount of a given protein source that must be consumed to keep an adult human in nitrogen balance.

If the protein taken in the diet has all the essential amino acids in good proportions, is completely digested and is completely absorbed then that protein is said to be a good protein or it is said to have 100% biological value. A protein of cent percent biological value should also completely replace the nitrogen lost in the urine. Generally animal proteins have higher biological value than plant proteins, because the animal proteins are much alike the human proteins and hence they have more digestibility and absorbability, for example egg and milk portions have their biological values near 100% (94% & 96% respectively).

Most of the plant proteins have low biological values and are said to be poor proteins. If two vegetable proteins are taken in combination, called succotash, then this mixture of proteins will have good biological value (though not 100%). Ex. the corn proteins are low in lysine but contain adequate amounts of tryptophan whereas bean proteins contain adequate amounts of lysine but are low in tryptophan. Neither is a good protein. But a mixture of the two is a good source of having balanced amino acids. If beans are taken in the breakfast and corn in the lunch (i.e. after 5-6 hours) then it will be of no biological value because amino acids cannot be stored. Therefore the biological value of vegetable proteins can be improved if taken along with animal proteins for the daily protein supply. It is recommended that 1/3rd to 1/2 of the proteins may be derived from animal proteins like egg, meat and milk.

Food stuff	Biological value (%)	Food stuff	Biological value (%)
Egg	94	Bengal gram	76
Rice	86	Fish	75
Mutton	60	Milk	96

***Protein sparing action of carbohydrates and fats*:** Carbohydrates and fats spare the proteins and make then available for anabolic or constructive purpose. Carbohydrates and fats supply the required energy and so proteins will not take part in energy metabolism, especially in patients needing tissue repair, this action is seen.

BALANCED DIET

A balanced diet can be defined as the nutrients required for sustaining and keeping the human body in metabolic health. It can also be defined as nourishment (food) required for maintaining normal life.

Balanced diet is one which contains all the food constitutes in proper proportion to meet the energy and nutritional requirements of the individual. The components of a well balanced diet will vary depending on age, sex, physiological needs such as pregnancy and lactation and nature of physical activity. While designing the quality and quantity of a balanced diet the total calories are distributed among 3 classes of food in the following proportion—

- Carbohydrates – 50-70%
- Fats – 20-30%
- Proteins – 10-15%

Proximate principles of diet: The proximate principles of diet are—

- Carbohydrates: the energy yielding substances.
- Fats: yield energy and act as insulating materials.
- Proteins: act as building materials and bear the wear and tear of the body.

Construction of a diet–the spectrum of food: The food is divided into four basic food groups—

1. ***Milk group*:** Two glasses of milk or servings of cheese, cottage cheese, ice creams, or other dairy products.
2. ***Meat group*:** Two servings of meat, fish, poultry or eggs, peas, beans or nuts.
3. ***Vegetable and fruit groups*:** Four servings of green or yellow vegetables, tomatoes, citrus fruits.
4. ***Bread and cereal group*:** Four servings of whole grains or fortified cereal products.

There is no single perfect food that provides all nutritional needs for everyone. The 40 different required nutrients occur in very different proportions in different foods. Therefore a variety, within each group is essential.

Functions of food:

1. ***Milk, egg and meat*:** They provide the essential amino acids and have high nutritional values ranging from 98-100%. Butter supplies the fat soluble vitamins A, D and E.
2. ***Cereals*:** Cereals supply vitamin B_1 and roughage (fibrous or cellulose material).
3. ***Essential fatty acids*:** Prevents atherogenic disorders. Vegetables contain phytosterol which helps in reducing serum total cholesterol.
4. ***Fruits*:** Citrus fruits like orange prevent diseases like scurvy, constipation etc. Fruits supply potassium required by the body to prevent diabetic coma.
5. ***Vegetables*:** Green leafy vegetables will help in the synthesis of Hb and provide some vitamins like vitamin A, folic acid, etc. Vegetables including green leafy vegetables prevent constipation by acting as roughage (cellulose materials).

Recommended dietary allowances (RDA): The food and nutritional board of National Academy of Sciences, National Research Council has developed a table of recommended daily dietary allowances (RDA) of various nutrients for optimum nutrition of infants, children, adults, pregnant & lactating women and various conditions of health and diseases to provide an ample safety margin of life.

Formulation of a diet: A correct diet must provide for maintenance of the body as well as energy requirements, for growth and reproduction. The essential elements lost by the body by excretion must be replaced. The important factors are—

1. **Energy value:** The average caloric requirement of an adult male and female should be met by the food provided daily.
2. **Quality and quantity of the constituents of food:**
   (a) *Primary foods (proteins, fats and carbohydrates)*: Proteins, fats and carbohydrates are consumed in the ratio of 1:1:4. 3000 calories are provided by 100 grams of protein, 100 grams of fat and 400 grams of carbohydrates. It is advisable that 10-15% of the total calories should be obtained from protein, 20-30% from fat and 50-70% from carbohydrate.
   (b) *Secondary foods (vitamins and minerals)*: These are essential in the diet but in very minute quantities to enable utilization of primary foods.
   (c) *Water*: Although water is not a food, it is ordinarily consumed in the diet and serves a prime role in the health of the body. Hence it is one of the components of food.
3. **Variation in the diet:** There is a risk of missing some essential elements or vitamins in a varied diet. Eskimos live mainly on fish and meat and poor orientals chiefly live on rice with small amounts of fish and meat.
4. **Digestibility of the food:** The food is of no use if it is not digested in the alimentary canal. Digestibility is more concerned with absorbability. When fats and starch are largely used, vegetables and animal proteins are not absorbed. Absorption is enhanced with a mixed diet than when the substance is taken alone.
5. **Cooking:** Food consistency is considerably changed on cooking. Harmful organisms are destroyed. Cooking breaks down the connective tissue fibres of meat and makes meat easier to masticate and helps in digestion. Overcooking shrinks the coagulated protein and decreases the digestibility. Cooking increases the water content and digestibility of vegetables. Cellulose frame work is loosened and starch from starch grains is liberated. Fats are not changed much upon cooking. Cooking enhances the flavour of the food. However, vitamins B and C are destroyed when vegetables are cooked.
6. **Psychological factors:** Appetite is reduced by worry and anxiety. Digestion is also upset due to imperfect mastication and secretion of digestive juices. Consumption of food is increased while taken in pleasant surroundings and good company with different items.
7. **Cost:** Dietary food is much influenced by family income. When the income is good, consumption is high with all the protective foods. A poor income has poor protective foods. Lowest income group having low protective foods suffer form rickets and nutritional anemia. They are less resistant to infectious diseases.

Based upon the above it can be concluded that a balanced diet should contain proteins (70 gms), fats (50 gms), carbohydrates (440 gms), calcium (0.8 gms), phosphorus (1.4 gms), Iron (40 mg), vitamin A (1300 I.U.), Vitamin B1 (1.8 mg) and vitamin C (200 mg). In addition to this 2-3 liters of water and other elements must be included in the daily diet.

ENERGY REQUIREMENTS

Human beings are classified into 3 groups depending on the energy requirement.

1. **Sedentary people:** Require less calories of energy i.e. upto 2400 Kcal/day.
2. **People with moderate work:** Require about 2800 Kcal/day.
3. **Heavy workers:** Require about 3000 Kcal/day.

Pregnant and lactating mothers require additional 800 and 700 Kcal/day respectively.

Calculation of total calorie requirements and building a balanced diet:

A moderate medical student requires 2800 Kcal of energy/day–how to calculate it?

Weight of the person	→	55 kg
Height of the person	→	162 cms
Surface area of the person	→	1.55 sq.m
BMR	→	41 cal/sq m/hr
Basal energy required	→	BMR x surface area × 24 hrs 41 × 1.55 × 24 = 1525 cal
Specific dynamic action (SDA)	→	10% of basal energy requirement = 152.5 Cal
If a moderate medical student does 8 hours of moderate work (add 100 cal/hr)	→	= 800 cal
8 hours of sedentary work (add 40 cal/hr)	→	= 320 cal
Total basal energy	→	1525 + 800 + 320 = 2645
Total calorie requirement	→	2645 + 152.5 = 2797.5 ~ 2800 Kcal

Nutritive value of some common foodstuffs

Ingredients	Calories/100 gms	Ingredients	Calories/100 gms
Rice	345	Mutton	194
Wheat	341	Fish	87
Pulses	35	Egg	173
Leafy vegetables	34	Milk – Buffalo's	67
Orange	40	Butter milk	15
Banana	115	Sugar of sweets	400

Balanced diet for a vegetarian moderate medical student with a total calorie requirement of 2800 Kcal/ day as worked out by Indian Council of Medical Research (ICMR).

Dietary article	Quantity required (gms)	Calories / day
Cereals	475	1638
Pulses	80	287
Green leafy vegetables	125	42
Other vegetables	75	18
Roots and tubes	75	70
Milk	200	234
Oils and fat	38	342
Sugar or Jaggery	40	162
	Total	**2781 ~ 2800**

Recommended Daily caloric requirements

	Male	Female
Adults	**(70 kg)**	**56 kg)**
Sedentary (Tailors, shoemakers)	2400	2000
Moderate activity (farmers)	3000	2400
Heavy work (Navvies, lumberman, stone masons)	4500	3000
Light work (carpenters, painters)	2500	2000
Pregnancy (latter half)		2400
Lactation.		3000
Children		
16-20 years	3800	2400
13-15 years	3200	2600
10-12 years	2500	2500
7-9 years	2000	2000
4-6 years	1600	1600
1-3 years	1200	1200
Under 1 year	110/kg	110/kg

Increments in calorie requirements (above basal) per hour for different occupations:

Occupation or activity	Increased requirement (cal/hr)	Occupation or activity	Increased requirement (cal/hr)
Sitting quietly	35	Painter	145
Standing quietly	40	Carpenter	150
Reading loudly	40	Santing wood	380
Tailor	70	Walking (moderate)	235
Typing	75	Walking fast	550
House work	110	Walking upstairs	1000

Effect of pregnancy: A pregnant mother requires additional energy to maintain the metabolism of the foetus, build up a reserve of fat and additional maternal tissue. By the end of a pregnancy the mother gains approximately 1 kg protein, mostly in the uterus and products of conception and 4 kg of fat in her own fat depots. Energy will be required for the metabolism of the foetus and placenta, metabolism of additional uterine and breast tissues and for the extra work of heart and respiration. An extra amount of about 70,000 Kcal spread evenly at an almost constant rate over the last 2/3rds of pregnancy is generally required. An average woman at rest thus will need approximately 300-400 Kcal/day. During the last stages of pregnancy approximately 60 litres/day of extra oxygen, equaling approximately 300 Kcal, is consumed. If the mother continues her normal activities these are certain to cost her more than before pregnancy on account of the increase in her weight. Specific nutrient requirements are less easy to specify. The need for proteins is greatest in the last 10 weeks when it accumulates at a rate of 5-6 g/day.

Lactation: A lactating mother must also consume extra food to meet the energy cost of lactation. The efficiency of milk production is probably 80%, so that for the production of milk having an energy value of 80 Kcal, the mother must expend 100 Kcal, thus requiring the same amount of energy in her diet. A

healthy baby may require 850 ml of milk daily. At an energy value of 68 Kcal/100 ml of milk, this will mean making the provision of 578 Kcal/day. A lactating mother will thus require an extra diet of approximately 720 Kcal/day to meet the demands of lactation. However, the fat reserves which are carefully laid down during pregnancy are also handy for this energy requirement.

Metabolic changes in starvation: Fasting is a post absorptive state probably after 12-14 hours after the last meal. Starvation commences thereafter, during which the liver glycogen is broken down and falls to about 10% of its normal concentration and remains at that level almost during the entire remaining period of starvation. The blood glucose level remains at 80 mg/100 ml, increased mobilization of depot fats occurs. The ketone bodies level increases leading to ketoacidosis. The pH of the blood decreases due to decrease in bicarbonate. The R.Q comes from 0.82 to 0.70. Within a few days the excretion of urinary nitrogen increases, due to the increased breakdown of the protein even though adequate fat stores are available. This is to supply glucose to the brain by gluconeogenesis. The earliest proteins to be depleted are the enzymes of G.I tract, then the hepatic enzymes are involved and finally muscle starts loosing the proteins. In the starting about 100 grams of proteins are metabolized, by 4-6 weeks it falls to 12-15 grams per day because the brain develops the ability to utilize the ketone bodies i.e. beta hydroxybutyric acid. Once the fat stores are depleted the condition of the fasting person takes a rapid turn to worse.

Absence of food does not affect the normal functioning instead is helpful in the treatment of certain disorder of G.I. tract, diabetes mellitus and obesity.

Protein calorie malnutrition or protein energy malnutrition: When there is insufficient supply of dietary proteins, all the cells lack amino acids for their synthetic activities and growth slows down or stops.

The earliest feature of protein deficiency is indigestion and failure of absorption of food, because of lack of synthesis of digestive enzymes. As a result chronic diarrhea sets in. The liver cells fail to synthesize plasma albumin and this leads to water retention and oedema.

Skeletal muscles and RBC fail to maintain their structure and functions resulting in wasting of muscles and anemia. These clinical conditions comprise a spectrum of disease in early childhood and are collectively known as protein energy malnutrition. The diseases are marasmus and kwashiorkar.

Marasmus: It is a disease caused if the child is not fed a little extra over the mother's milk from the fourth month after birth. If the child is fed only on mother's milk for 18 months then it results in calorie malnutrition leading to marasmus. Marasmus also occurs if the child is fed with highly diluted bottle milk which lacks calories. It is also seen in the areas of famine and in children of poor families. The marasmic child looks very weak and thin. Hands and legs look like sticks. Head looks big and old. Muscles are weak with hypoproteinemia. The infant fed on a diet low in both protein and calories become undernourished and lose weight considerably with chronic diarrhea. If it is not corrected it leads to extreme wasting of subcutaneous fat and muscles. There is no oedema in marasmus.

Kwashiorkor: This condition is mostly seen at the age of 14 months due to protein deficiency. It is a disease caused due to **'weaning away the child from mothers milk'**, most probably to give way to the second child. Now the child is bottle fed or he will be fed with other foods which cannot supply the proteins required for the growth of the child. This results in the deficiency of proteins in the child (protein malnutrition). Due to the poor quality of the proteins in the diet the amino acids are not utilized for protein synthesis, instead are converted into fats, therefore there is a net increase in calories in the body which is stored as fats due to which the body of the infant becomes fat and rounded. The hands and the legs will be swollen due to the presence of large amounts of water (oedema). The muscles become thin and weak. The skin becomes pale and starts peeling off the body. Hairs become loose and come out easily on pulling. The biochemical changes occuring during kwashiorkor are hypoproteinemia, especially

hypoalbuminemia, due to which oedema occurs (unequal distribution of water between the tissue and body fluids). The other effects are anemia, fatty livers, hepatic cirrhosis, muscle wasting etc.

The chief characteristics of kwashiorkor are—

(a) Lack of appropriate cellular development.
(b) Oedema.
(c) Diarrhea.
(d) Poor growth.
(e) Low plasma protein level.
(f) Muscle wasting.
(g) Increased susceptibility to infection.

18

BLOOD AND BODY FLUIDS

Almost 3/4th of the globe is fluid in nature (water). Likewise about 70-80% of the human body is composed of various fluids mostly suspended in water, where all the intracellular, extracellular and intercellular reactions occur. The fluids in the body are—

1. Amniotic fluid surrounding a fetus
2. Aqueous humour or CSF
3. Bile
4. Blood and blood plasma
5. Breast milk
6. Cerumen also known as earwax
7. Cowper's fluid or pre-ejaculatory fluid
8. Chyle
9. Chyme
10. Female ejaculate
11. Interstitial fluid
12. Lymph
13. Menses
14. Mucus (including snot and phlegm)
15. Pleural fluid
16. Pus
17. Saliva
18. Sebum (skin oil)
19. Semen
20. Serum
21. Sweat
22. Tears
23. Urine
24. Vaginal lubrication
25. Vomit
26. Water

BLOOD

Blood is the major fluid present in the body that flows through the blood vessels (veins, arteries & capillaries) and helps to connect, communicate, supply and drain substances to and from various parts of the body. The total volume of blood in an adult male human being is about 5 litres. It has a specific gravity of 1.055 to 1.065. The pH of blood ranges from 7.35 to 7.45 average being 7.4, however the pH of blood varies from 7.3 to 7.5 without disturbing the normal functions of the body. The pH of intracellular fluid (cytoplasma) is 7.2.

Composition of blood: Blood is composed of—

(a) Blood cells which include—(i) Erythrocytes (ii) Leukocytes and (iii) Platelets.

(b) Blood plasma.

BLOOD PLASMA

It is the fluid remaining after the removal of blood cells. Plasma devoid of clotting factors is termed as serum i.e. plasma — fibrinogen = serum

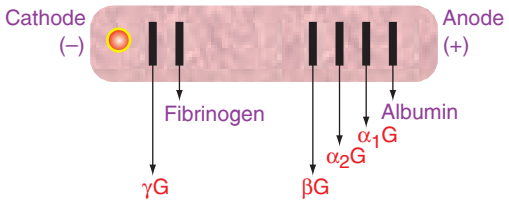
Plasma contains the plasma proteins, organic constituents (glucose, amino acids, fatty acids, urea, cholesterol etc.) and inorganic constitutes (phosphates, K, Na, Cl, and Fe). All these are suspended in a fluid form which constitutes about 90 % water.

Plasma proteins: Proteins in the plasma can be detected by Biuret test. The amount of protein is estimated by conducting microbiuret test. Upon estimation it is found that the amount of plasma proteins ranges from 6.5 to 7.5 gms/dl.

Methods to separate different types of plasma proteins:

1. Electrophoresis
2. Isoelectric focusing
3. Precipitation using salts
4. Precipitation using alcohol
5. Precipitation using heavy metals
6. Ultracentrifugation.

Electrophoresis: Plasma, which has a pH of 7.4 is rendered alkaline and brought to a pH of 8.6 and maintained by barbitone buffer. At pH 8.6, all plasma proteins will have a negative charge i.e. they exist in anionic form. A drop of this solution is applied on to a piece of filter paper (or a dish with polyacrylamide gel) and this filter paper is dipped in a buffer (barbitone). This strip is subjected to electrophoresis by applying electric current. When current is applied, all the proteins move towards anode (+ve). But different proteins move with different rates which depend on the intensity of charge present on them. Different proteins exhibit the various number of charges on them. Current is allowed to pass for a short time interval after which it is stopped. Paper is stained with a dye. On staining it shows a number of separate bands. The band close to anode corresponds to albumin, other bands in the order are α_1-globulin, α_2-globulin, β-globulin, fibrinogen and γ-globulin (closest to cathode). When the plasma proteins are separated on polyacrylamide gel both molecular weight and charge contribute to their separation in the same order.



Salting-out technique: Most of the proteins are insoluble in pure water. But when some amount of salt is added to this solution then these proteins become soluble, this is known as salting-in of proteins. When high amounts of the same salt is added to the same solution then the proteins precipitate out of the solution, this is known as salting-out of proteins. When protein water interaction increases the protein becomes soluble but when protein-protein interaction increases then protein precipitates out of the solution.

In the absence of the salt there is neither protein water interaction nor protein-protein interaction, thereby the protein is neither soluble nor is precipitated. Now, when a small amount of the salt is added, it associates with the protein molecule creating an atmosphere around the protein molecule thereby increasing protein-water interaction and thus making protein soluble in the water i.e. salting-in of proteins.

When the salt is added in high amounts, the ions of the salt start associating with the water molecules thereby reducing the protein-water interaction and increasing protein-protein interactions. As the protein-protein interaction increases, precipitation increases i.e. salting-out of proteins.

Concentrated salt solutions, like sodium sulphate, sodium chloride and ammonium sulphate etc. precipitate proteins by neutralization and dehydration, as well as absorption, thereby converting emulsoids into suspensoids, which results in the precipitation. Different proteins precipitate out with different salts at varying concentrations; this forms the basis of separation of proteins.

Name of protein	Precipitation with ammonium sulphate
Albumin	It does not precipitate at half saturation, instead it precipitates at full saturation
Casein	It is precipitated at half saturation
Peptone	It is precipitated neither at half saturation nor at full saturation

Precipitation using alcohols and heavy metals: Using different concentrations of alcohol different proteins are precipitated. Heavy metals like zinc (Zn), lead (Pb) and mercury (Hg) are used to precipitate the plasma proteins.

Fingerprinting technique: When polypeptide chains, obtained by enzymatic hydrolysis of a protein are subjected to two dimension paper chromatography or two dimensional paper electrophoresis or paper chromatography in one direction and paper electrophoresis in the other (i.e. 90 degrees to the first), give a particular pattern or map unique to that protein. As the pattern for each protein is unique this map is used as a fingerprint, which helps in the identification and separation of proteins and amino acids. Fingerprinting technique enables the determination of protein sequence and differentiation of abnormal protein from normal. Inorder to differentiate normal hemoglobin (HbA) from abnormal sickle cell hemoglobin (HbS), both the proteins are separately digested by trypsin. The hemoglobin digest is applied on a filter paper and subjected to electrophoresis in one direction and paper chromatography in the other direction. On treatment with ninhydrin, the map obtained for HbS differs in one position from the normal thereby indicating that one of the polypeptides has been changed. Separation and characterization of plasma proteins revealed the following proteins.

Albumin: It is present in highest concentration than other plasma proteins. Its concentration ranges from 3.5-5.5 gms/100 ml. It has the lowest isoelectric pH of 4.7. It migrates fastest in electrophoresis and precipitates last in salting out or alcohol precipitation methods. It is a simple protein synthesized in the liver. It is of nutritional value because it contains all essential amino acids. It coagulates on heating and precipitates on full saturation with ammonium sulphate.

Functions:

(a) Stores amino acids and supplies amino acids to extra hepatic tissues.
(b) Maintains the colloidal osmotic pressure of plasma (oncotic pressure).
(c) Deficiency of albumin leads to oedema because albumin is the major protein that regulates water content of the tissues.
(d) It is a carrier protein. It transports fatty acids, steroid hormones, calcium, thyroxine, toxic substances like bilirubin, urobilinogen and phenolic substances across the blood.
(e) It acts as a buffer.

Concentration of albumin decreases in hepatic disorders, malnutrition, increased excretion of albumin as seen in kidney disorders (nephrosis). Concentration of albumin increases in dehydration and insulin resistant diabetes.

Globulin: Its concentration is about 2-5 gms/dl. It is synthesized in the extra hepatic tissue (lymphoid). However a minor quantity is also synthesized in the hepatic tissue. The albumin/globulin ratio is 1.2 to 1.8, average being 1.5. Decreased AG ratio indicates decreased albumin. Increased AG ratio indicates decreased globulin.

Plasma proteins - properties, concentration, functions & variations in health and disease

Protein component	Conc. gms/dl	Functions	Increased in	Decreased in
1. **Prealbumin or transthyretin**	0.01–0.04	Binding and transport of thyroxine and acts as retinol biding protein	—	—
2. **Albumin:** synthesized in the liver, contains large amounts of essential amino acids, freely soluble in water–coagulate on heating and precipitates on full saturation with ammonium sulphate. pHI – 4 7. Mol. Wt. – 66,241. No. of a.a. = 575	3.5–5.5	Storage of amino acids, transport of Ca, fatty acids, thyroxine, steroids and toxic products like bilirubin, urobilinogen and some phenolic substance, regulation of osmotic pressure. Low albumin levels cause oedema and kwashiorkor. It acts as a buffer. Protein synalbumin is associated with it and it has an anti-insulin action	Severe dehydration. Its level is found to be high in insulin resistant diabetes	Haemodilution, malnutrition, cirrhosis of liver, nephrosis, protein loss in gastro-enteropathy, haemorrhage, burns. Moderate decrease in pregnancy, diabetes mellitus, tuberculosis, cancer and hyperthyroidism
3. **Globulins: (Total)**	3–6			
A. α_1-Globulins	0.3–0.6		Acute febrile condition, excess tissue destruction, inflammations	—
(i) Orosmucoid (α_1 acid glycoprotein: it contains a high amount of carbohydrate (N-acetyl glucosamine, fucose, mannose and galactose). It is non-heat coagulable and not precipitated by sulphosalysilic acid.	0.1	Transport of hexoseamines	Cancer, acute myocardial infarction and rheumatoid arthritis	Infective hepatitis, portal cirrhosis
(ii) α_1-glycoprotein	0.03	Inhibition of trypsin	—	—
(iii) α_1-lipoproteins (HDL)	0.25–0.4	Transport of phospholipids and cholesterol	—	—
(iv) Thyroxine binding globulin	0.01–0.02	Transport of thyroid hormones	—	—
(v) Transcortin	0.03–0.035	Transport of cortisol and corticosterol	—	—
(vi) Retinol binding globulin	0.03–0.6	Retinol transport—		
B. α_2-Globulins	0.4–0.9			
(i) α_2-glycoprotein	0.1	Inactivation of progesterone	—	—

Protein component	Conc. gms/dl	Functions	Increased in	Decreased in
(ii) α_2-macroglobulin	0.2	Anti-trypsin and anti-plasmin	—	—
(iii) Hepatoglobulin	0.1–0.26	Binds free hemoglobin and prevents its excretion	Pneumonia, tuberculosis, rheumatoid arthritis and nephritis; pregnancy	Pernicious anaemia, malaria, haemolytic conditions
(iv) Ceruloplasmin	0.01–0.06	Copper transport and ferroxide action	Pregnancy	Wilson's disease, nephrosis, severe hypo-proteinemia, vitamin K deficiency, obstructive jaundice, hepatocellular damage
(v) Prothrombin	0.02	Inactive form of thrombin	—	—
C. β-globulins	0.6–1.1			
(i) β-lipoprotein (LDL)	0.2–0.4	Transport of cholesterol and phospholipids	Nephrosis, myxoedema, xanthomatosis, atherosclerosis, hypertension, infective hepatitis, severe diabetes mellitus	Abetalipo-proteinemia (acanthocytosis), tuberculosis and hyperthyroidism
(ii) Transferrin (siderophilin)	0.2–0.4	Binds and transports iron	Chronic iron deficiency, pregnancy	Pernicious anemia, liver disease, chronic infections
(iii) Hemopexin	0.05–0.1	Binds and aids disposal of heme		
(iv) Plasminogen (Profibrinolysin)	0.05	Forms fibrin for fibrinolysis (splits fibrin to small peptides and amino acids)		
D. γ-globulins (Immunoglobulins-IgG, IgA, IgM, IgD, IgE)	0.8–17	Functions as antibodies	Cirrhosis of liver, multiple myeloma, bacterial, viral and protozoal infections	Agamma-globulinemia, nephrosis and agranulocytosis
E. Fibrinogen: It is a glycoprotein having a Mw, of 340,000. PH^I is 5.8. It is synthesized in the liver made up of six polypeptide chains linked by disulphide bonds. By the action of thrombin it is converted to fibrin.	0.2–0.4	Clotting of blood	Pregnancy, rheumatic fever, rheumatic arthritis, tuberculosis, pneumonia, pulmonary embolism, myocardial infarction and acute pancreatitis	Afibrinogenemia, abruption placenta and abortions

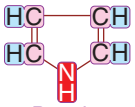
HEMOGLOBIN

It is an oxygen/CO_2 carrier protein present in the red blood corpuscles of blood. Hemoglobin is a conjugated chromoprotein having heme as its prosthetic group. Heme is the prosthetic group, not only of hemoglobin but also of myoglobin, cytochromes etc.

Hemoglobin is formed by the combination of heme with globin (protein). Globin is made up of four polypeptide chains (an oligomeric protein). Two of these polypeptides are known as alpha (α) and the other two are known as beta (β). Each alpha chain has 141 amino acids and each beta chain has 146 amino acids, which are arranged in a definite sequence. Its molecular weight is 65,000. Due to the characteristic folding of its tertiary structure each polypeptide forms a cup like structure with a pocket like area where the prosthetic group, heme is buried. Heme has iron, which is linked to the imidazole nitrogen of the histidine in positions 58 and 87 of the alpha chains. In the beta chain the heme iron is linked with histidine in positions 92 and 63. Altogether there are four heme groups in one hemoglobin molecule.

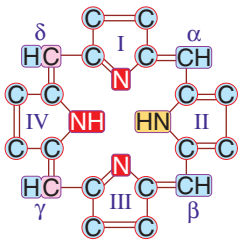
Heme: It is an iron-porphyrin compound. It is the prosthetic group embedded in the packet like structure formed by folding of the hemoglobin tertiary structure.

Porphyrin: Porphyrin is a complex compound with a tetrapyrrole ring structure. Pyrrole is a heterocyclic compound having the following structure.

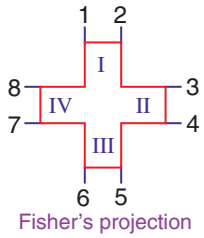


Pyrrole

4 pyrrole rings join together through methylidyne bridges (–CH=) to form a porphin.



Porphin



Fisher's projection formulae for a porphin

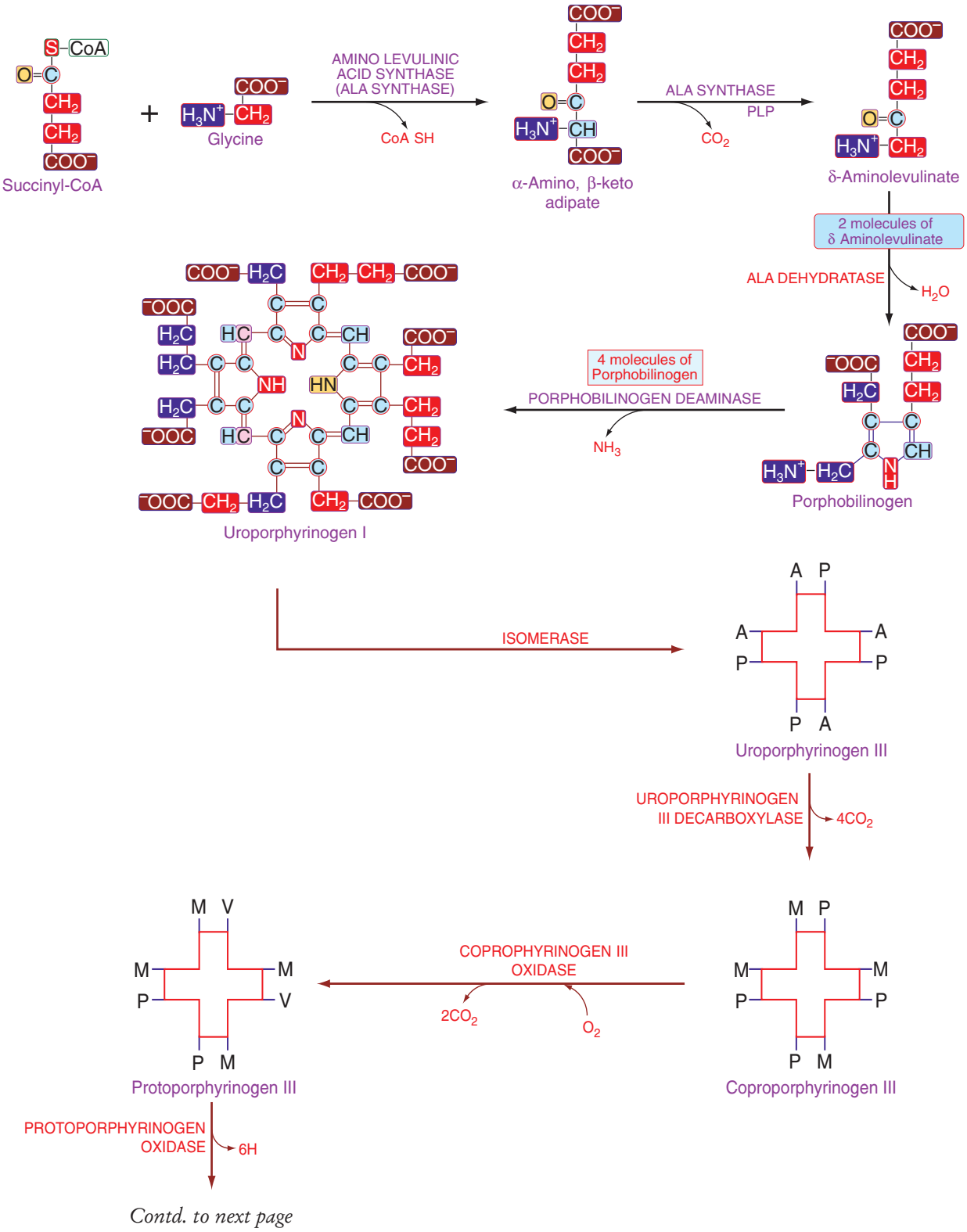
This porphin is substituted by different groups at positions numbered from 1-8 to form the porphyrin. Depending upon the groups (methyl, acetyl, propyl, butyl or venyl) present on these positions different types of porphyrins are identified, that will be seen during the synthesis of heme.

Properties of porphyrins:

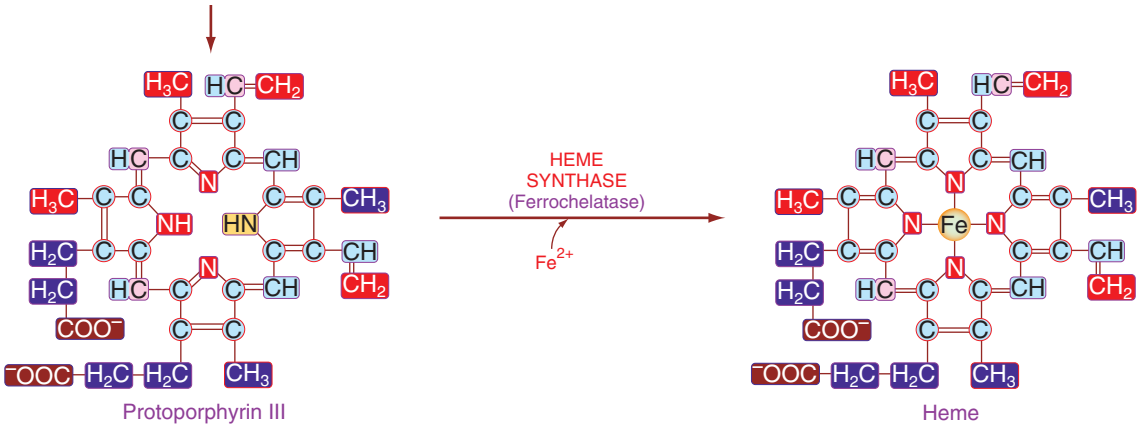
1. They act both as acids (–COOH) and bases ($–NH_2$).
2. Their isoelectric pH is between 3-4.5.
3. Porphyrins are fluorescent and coloured due to presence of alternating double bonds.
4. Porphyrinogens are colourless.

Biosynthesis of heme: Heme is an iron porphyrin structure, synthesized in the reticuloendothelial cells (bone marrow) of adult human being. Erythropoietin produced in kidney stimulates the formation, maturation and release of erythrocytes by bone marrow. Early stage of erythrocytic cells contain porphyrin, during the course of their development, porphyrin is converted to heme by addition of iron and then to hemoglobin by addition of protein, globin. The type of porphyrin present in heme is protoporphyrin-

III (also known as No. IX). It is synthesized starting from glycine and succinyl-CoA. Given below is the diagrammatic representation of biosynthesis of Heme where 'A' stands for acetyl group, 'P' stands for propyl group, 'M' for methyl group, and 'V' for venyl group.



Contd. to next page



Regulation of heme synthesis: The first enzyme in this sequence i.e. ALA synthase is the key regulatory enzyme for heme synthesis which is inhibited by heme the end product of the metabolism. ALA synthase is a regulatory enzyme. Heme acts as an aporepressor molecule and is a negative regulator for the synthesis of ALA synthase-I (heme inhibits the gene).

Inhibitors of heme synthesis: The following substances inhibit heme synthesis—

- Succinylacetone (SA) is an inhibitor of heme synthesis that acts on the enzyme aminolevulinic acid dehydratase.
- *N*-methyl mesoporphyrin IX blocks iron insertion into protoporphyrin IX and thus acts as an inhibitor of heme synthesis.
- Isonicotinic acid hydrazide (INH) is an inhibitor of 6-aminolevulinic acid synthase.

Disorders related to abnormalities in the synthesis of porphyrins:

Porphyrias: Porphyrias are a group of diseases in which there is an increased excretion of porphyrins or porphyrin precursors (intermediates of porphyrin synthesis). About 85% of heme synthesis occurs in erythrocyte precursors and 15% in liver. Therefore porphyrias are classified into two types (1) Erythropoietic and (2) Hepatic. Some of them are inherited while others are acquired.

Condition	Enzyme defective	Symptoms
1. Erythropoietic		
a. Congenital erythropoietic porphyria	Non-cooperativity between (a) Uroporphyrinogen-I synthase and (b) Uroporphyrinogen-II cosynthase	Darkening of urine upon standing, abdominal pain, vomiting, constipation, cardiovascular abnormalities
2. Hepatic		
a. Acute intermittent porphyria	Uroporphyrinogen-I synthase	Urine is red due to oxidation of porphobilinogen on exposure to light. Abdominal pain, vomiting, constipation and paralysis
b. Hereditary coproporphyria	Coproporphyrinogen oxidase	Cutaneous hypersensitivity to light
c. Variegate porphyria	Protoporphyrinogen oxidase	No symptoms
d. Porphyria cutanea tarda	Uroporphyrinogen decarboxylase	Chin is pink or brown coloured. Hepatic damage and accumulation of iron. Photosensitivity of skin
e. Toxic porphyria	Variable	—
3. Both erythropoietic and hepatic		
a. Protoporphyria	Ferro chelatase is absent, defective or less	

Hemoglobin derivatives: There are some derivatives of normal Hb that arise due to metabolic changes in the RBC. The various hemoglobin derivatives are—

1. **Oxyhemoglobin (HbO_2):** The main function of hemoglobin is to transport oxygen from the lung to the tissues. In lungs the partial pressure of oxygen is 100 mm of Hg, at this pressure hemoglobin is 95-96% saturated with oxygen. On binding with O_2 in the lungs hemoglobin is converted to oxyhemoglobin (HbO_2). O_2 is bound to heme iron.

$$Hb + O_2 \rightarrow HbO_2$$

2. **Reduced hemoglobin (HHb):** Oxyhemoglobin moves to the tissue where the partial pressure of O_2 is 26 mm of Hg due to which oxygen is released into the tissues and in turn H^+ binds to Hb and forms reduced hemoglobin.

$$HbO_2 + H^+ \rightarrow HHb + O_2$$

3. **Carbaminohemoglobin:** Hemoglobin also binds to CO_2 in the tissues. CO_2 is bound to the α-amino group at the N-terminal end of each of the four polypeptide chains of hemoglobin to form carbamino-hemoglobin. As one CO_2 binds O_2 is released.
4. **Methemoglobin:** In RBC the iron of hemoglobin is normally in ferrous (Fe^{2+}) form, but it is readily oxidized to the ferric (Fe^{3+}) form by hydrogen peroxide formed by RBC cell metabolism, to yield methemoglobin. Ferric iron is incapable of binding O_2 therefore the functions of hemoglobin are disturbed. Normally 1.7 to 2.4 % of total hemoglobin will be in the form of methemoglobin. Increase in the percent of methemoglobin is prevented by the peroxidase action of a naturally occurring peptide known as glutathione present in the RBC. Methemoglobin is dark brown in colour. The percent of methemoglobin can increase if the person consumes drugs like ferricyanide, nitrite, quinines, hydroxylamines, acetanilide and sulfonamide. Higher levels of methemoglobin is observed clinically in factory workers who inhale (or contact through skin) aromatic nitro and amino compounds and in patients taking large amounts of acetanilide and sulfonamides. The symptoms are cyanosis (blue skin) and dyspnoea (labored breathing).

***Importance of methemoglobin*:** Methemoglobin can be used to overcome cyanide poisoning. By injecting methemoglobin it combines with cyanide to form cyanomethemoglobin preventing cyanide poisoning.

5. **Carboxyhemoglobin:** Oxyhemoglobin can bind to carbon monoxide (CO). Even normal, non-oxygenated hemoglobin can bind with CO to form carboxyhemoglobin. **[Hb + CO → HbCO]**. CO has got an affinity of 200 times more than that of O_2 towards Hb. Hemoglobin can bind more readily to CO than to O_2. Even if there is a little amount of CO in air, it can displace oxyHb to form carboxyHb. Due to this there will be tissue hypoxia because the oxygen binding capacity is reduced and there is also reduced O_2 releasing capacity i.e. it cannot release O_2 though it may be bounded to O_2. City dwellers have at least 1% of carboxyhemoglobin which can increase to 8% depending upon the pollution. Over traffic can increase carboxyHb to 40% which leads to death. Clinically such patients show cherry red colour of skin. CO poisoning can be treated if high amount of O_2 is provided continuously at high pressure, then at such high concentrations and pressure HbCO is dissociated forming HbO_2 + CO. When treatment continues for 2 hours CO is expelled out.

Abnormal hemoglobins: There are three types of hemoglobins that are normally found in human beings, they are—

1. **HbA:** Found in normal adult human beings – contains 2α and 2β chains.
2. **HbA_2:** Found in some human beings and is considered normal – contains 2α and 2δ chains.
3. **HbF:** Foetal hemoglobin – found in growing foetus – contains 2α and 2γ chains.

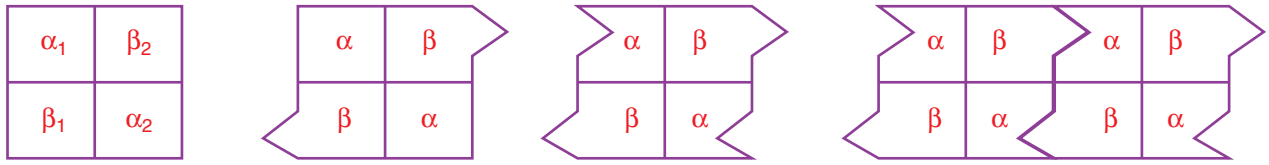
Each chain is synthesized by the information obtained from the gene for hemoglobin. α chain is synthesized from α genes of hemoglobin, β chain from β genes of hemoglobin likewise γ and δ from their respective genes. There are 2 pairs of α genes but only one pair each of β, γ and δ genes.

Abnormal hemoglobins arise due to mutation in the gene for the hemoglobin synthesis. There are about 300 abnormal hemoglobins. Some of them are those which have defect in α genes and some are with defective β chains.

Name of Hb	Abnormality present at position	Actual amino acid present in normal Hb	Replaced amino acids in abnormal Hb
Hemoglobin (Hb) with abnormal α chain			
HbI	16	Lysine	Glutamic acid
HbG	23	Glutamic acid	Valine
HbM_B	58	Histidine	Tyrosine
Hemoglobin (Hb) with abnormal β chain			
HbS	6	Glutamic acid	Valine
HbC	6	Glutamic acid	Lysine
HbM_S	63	Histidine	Tyrosine

Biochemistry of abnormality in the hemoglobins:

HbS or sickle cell hemoglobin: Sickle cell hemoglobin (HbS) arises due to the defect in β chain in which glutamic acid present at the 6th position is replaced by valine. Valine is also present naturally at position one. These two valine residues form hydrophobic interaction producing a sticky patch on HbS. Due to this replacement there is a sticky patch on HbS which appears on the oxy HbS. There is a complementary site to this sticky patch on deoxy HbS and also on deoxy HBA. The mechanism of biconcave RBC converting to sickle shape is given here under—



Four chains of normal Hb arranged in specific order | Change in structure of β chains in HbS with sticky patches | Complementary site on α chains in deoxy-HbS | Combination of two HbS molecules at the sticky patch and its complementary site

When hemoglobin molecules combine together in chains they form precipitates of HbS. The precipitate formed in the RBC sinks down and the biconcave shape of RBC is converted to sickle shape.

The life span of RBC is reduced to less than half (about 30 days). HbS is very unstable, due to which there is excessive hemolysis. This results in anemia called sickle cell anemia. The physiological changes observed in sickle cell anemia are - physical exertion, weakness, short of breath, leukemia and heart murmurs.

HbM or methemoglobin: The defect lies both in α and β chains. This is due to replacement of histidine residue in 58th position in α chain and 63rd position in β chain. Due to this replacement, the iron (Fe) present in the ferrous state is oxidized to ferric state. This ferric iron cannot bind oxygen. Therefore the oxygen carrying capacity is disrupted leading to anemia and hypoxia (low O_2 to tissues).

Thalassemias: The defect in thalassemias is the decreased rate of synthesis of one of the polypeptide chains of the globin molecule. One of the chains is synthesized in less amounts than the other due to the defect in DNA. There are two types of thalassemias—

1. **β-thalassemia:** β-thalassemia occurs due to the decreased synthesis rate of β-chain of globin. Due to the deficiency of β-chain the α-chains either combine among themselves forming α-4-globin or it can combine with γ or δ chains, thereby forming more of HbA2 and HbF. This results in the impairment of the transport of O_2 by Hb resulting in hypoxia. There are very low levels of Hb i.e. 2-3 g/100ml (hypochromic cells). The life span of such RBC is greatly reduced. The symptoms include anemia, growth retardation, wasting and fever.
2. **α-thalassemia:** α-thalassemia occurs due to the decreased rate of synthesis of α-chain of globin. But this is rarely seen due to the presence of two pairs of genes for α chain in the Hb gene. Due to lack of α chain, the β chain may combine either with δ, γ or among itself forming β_4, or $\beta_2\delta_2$ or $\beta_2\gamma_2$.

Glucose-6-phosphate dehydrogenase deficiency: It is an X-linked recessive hereditary disease characterised by abnormally low levels of the glucose-6-phosphate dehydrogenase enzyme (abbreviated G6PD or G6PDH). Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme in the pentose phosphate pathway, a metabolic pathway that supplies reducing energy to cells (most notably erythrocytes) by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turn maintains the level of glutathione in these cells that helps to protect the red blood cells against oxidative damage. G6PD converts glucose-6-phosphate into 6-phosphogluconolactone and is the rate-limiting enzyme of the pentose phosphate pathway.

Patients with G6PD deficiency are at risk of hemolytic anemia in states of oxidative stress. Individuals with the disease may exhibit nonimmune hemolytic anemia in response to a number of causes. This can be due to severe infection, medication and certain foods. Broad beans contain high levels of vicine, divicine, convicine and isouramil—all are oxidants.

In states of oxidative stress, all remaining glutathione is consumed. Enzymes and other proteins (including hemoglobin) are subsequently damaged by the oxidants, leading to electrolyte imbalance, membrane cross-bonding and phagocytosis and splenic sequestration of red blood cells. The hemoglobin is metabolized to bilirubin (causing jaundice at high concentrations) or excreted directly by the kidney (causing acute renal failure in severe cases).

Deficiency of G6PD in the alternative pathway causes the build up of glucose and thus there is an increase of advanced glycation end products (AGE). The deficiency also causes a reduction of NADPH which is necessary for the formation of Nitric Oxide (NO). The high prevalence of diabetes mellitus type 2 and hypertension in Afro-Caribbeans in the West could be directly related to G6PD deficiency.

Patients are almost exclusively male, due to the X-linked pattern of inheritance, but female carriers can be clinically affected due to lyonization where random inactivation of an X-chromosome in certain cells creates a population of G6PD deficient red cells coexisting with normal red cells. Although female carriers can have a mild form of G6PD deficiency (dependent on the degree of inactivation of the unaffected X-chromosome), homozygous females have been described; in these females there is coincidence of a rare immune disorder termed chronic granulomatous disease (CGD).

G6PD deficiency manifests itself in a number of ways—

- Prolonged neonatal jaundice

- Hemolytic crises in response to:
  - Certain drugs
  - Certain foods, most notably broad beans
  - Illness (severe infections)
  - Diabetic ketoacidosis
- Very severe crises can cause acute renal failure

Diagnosis: The diagnosis is generally suspected when patients from certain ethnic groups develop anemia, jaundice and symptoms of hemolysis after challenge to any of the above causes, especially when there is a positive family history.

Generally, tests will include:

- Complete blood count and reticulocyte count; in active G6PD, Heinz bodies can be seen in red blood cells on a blood film;
- Liver enzymes (to exclude other causes of jaundice);
- Lactate dehydrogenase (elevated in hemolysis and a marker of its severity)
- Haptoglobin (decreased in hemolysis);
- A 'direct antiglobulin test' (Coombs' test)–this should be negative, as hemolysis in G6PD is not immune-mediated;
- TSH measurement.

Pyruvate kinase deficiency: It is an inherited autosomal recessive genetic disorder which affects the survival of red blood cells. Pyruvate kinase deficiency is the second most common cause of enzyme-deficient hemolytic anemia, following G6PD deficiency.

A variety of mutations can lead to lowered production, activity, or stability of pyruvate kinase, an enzyme essential to glycolysis. A total lack of this enzyme's activity will be lethal.

Because the ability of erythrocytes to manufacture ATP depends on glycolysis, the cells become deficient in energy and hence are unable to maintain the activity of the basolateral Na^+/K^+-ATPase. This will result in an increase in intracellular [Na^+] which will cause water to diffuse passively into the red blood cells (RBC) and will lead to swelling. This swelling will lead to lysis of the RBCs and an increase in plasma bilirubin. The increase in plasma bilirubin will lead to jaundice and the lysis of the RBCs will lead to hemolytic anemia. The buildup of reaction intermediates can also increase the level of 2,3-bisphosphoglycerate (2,3BPG) in the cells and affect tissue oxygenation. This will cause a 'right shift' in the hemoglobin oxygen saturation curve, implying a decreased oxygen affinity for the hemoglobin and earlier oxygen unloading than under normal conditions.

Red blood cells use glycolysis as their sole energy source. In pyruvate kinase deficiency, the last step (phosphoenolpyruvate converted to pyruvate) of glycolysis is unable to occur. A discrepancy between red blood cell energy requirements and ATP generating capacity produces irreversible membrane injury resulting in cellular distortion, rigidity and lysis. This leads to premature erythrocyte destruction by the spleen and liver.

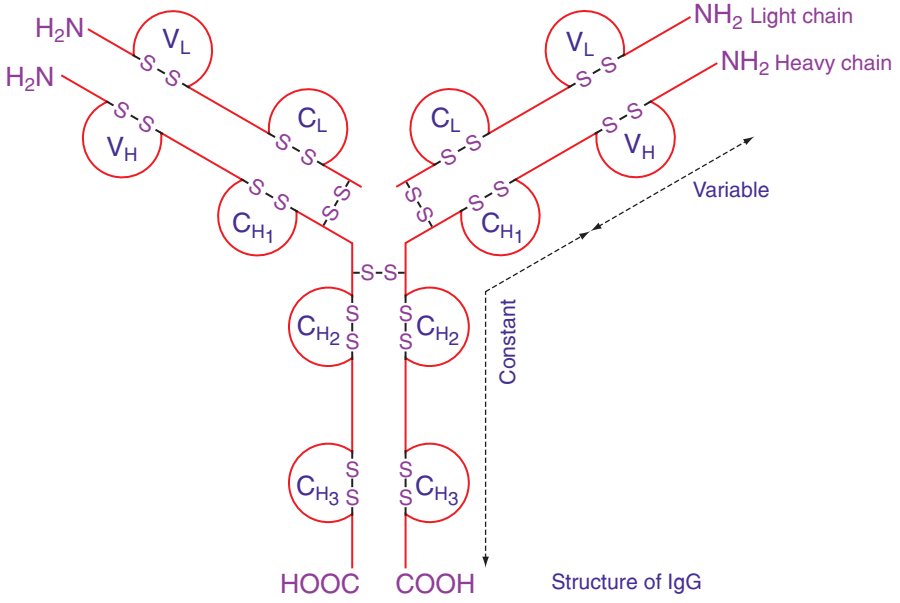
IMMUNOGLOBULINS

The ultimate aim of the immune organs of the body is to recognize and react specifically with the 'non-self' substance, i.e. antigen and destroy it. Nature has evolved a unique protein molecule distributed widely in the body more so in serum, to perform these two different functions. The immunoglobulins comprise of heterogeneous group of proteins, which account for approximately 20% of the total plasma proteins. Protein molecules that combine specifically with antigens are termed as antibodies; collectively, proteins with antibody activity are referred to as immunoglobulins.

They are produced in multicellular organisms in response to a foreign stimulus called antigen which may be a protein, a carbohydrate or any other substance. They are all produced by cells called B-lymphocytes.

Structural and functional characteristics of immunoglobulins (Igs): The immunoglobulin (Ig) belongs to a large group of closely related globular glycoproteins. It is composed of polypeptide (82-96 %) and carbohydrate (4-18 %). It is a three-dimensional protein molecule, which is bilaterally symmetrical. Antibody molecules are extremely heterogeneous, which is demonstrated by amino acid sequence, electrophoretic and serological methods.

Basic structure of an immunoglobulin: All immunoglobulin molecules consist of 2 identical light (L) chains and two identical heavy (H) chains, held together as a tetramer (L_2H_2) by interchain disulfide bonds. The arrangement of the four polypeptide chains in an immunoglobulin molecule gives it a "Y" shape. The half of the light chain towards N-terminal is called as variable region (V_L-variable light) and the other half towards C-terminal end is called as constant regions (C_L-constant light). Similarly one-quarter (1/4) of the heavy chain at the N-terminal end is called variable region (V_H-variable heavy) and one-thirds (1/3) at the C-terminal is called constant region (C_H-constant heavy). Each chain can be divided into domains or regions. In each of the two light chains there are two intrachain disulphide bonds giving rise to two domains, one each in variable and constant regions. Similarly there are four intrachain disulphide bonds in the heavy chain giving rise to four domains in each of the heavy chain. One domain appears in each variable region of the heavy chain and three in constant regions. When immunoglobins are subjected to papain digestion, 3 fragments are produced, two of them retain the ability to bind with the antigen and hence they are called antigen binding fragments (F_{ab}). The third one does not have such a capacity but it is easily crystallizable and hence called crystallization fragment (F_c). The point of papain cleavage is called the hinge region.



There are two types of light chains known by the Greek letters kappa (κ) and lambda (λ). An immunoglobulin has either κ or λ chain but not both. There are five (5) types of heavy chains designated as gamma (γ), alpha (α), meu (μ), epsilon (ε) and delta (δ). Depending upon the type of the heavy chain, five different types of immunoglobulins identified are (1) IgG (contains γ) (2) IgA (contains α) (3) IgM (contains μ) (4) IgE (contains ε) and (5) IgD (containing δ). Each type of heavy chain can combine with each type of light chain, but within the same antibody molecule, the two heavy and two light chains are of same type.

IgG: This is the immunoglobulin found in the highest concentration in the blood (172 i.u/ml) and is the main Ig responsible for fighting against diseases, because it is more specific for the antigen. It is a

monomer i.e. the "Y" structure described above, bearing a molecular weight of 150,000 daltons. The light chains have 214 amino acids and heavy chains have 446 AAs.

VL region is from 1 to 108 amino acids from N-terminal.

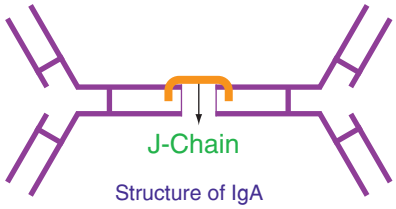
CL region is from 109 to 214 amino acids from the N-terminal.

VH region is from 1 to 118 amino acids from the N-terminal.

CH region is from 119 to 446 amino acids from the N-terminal.

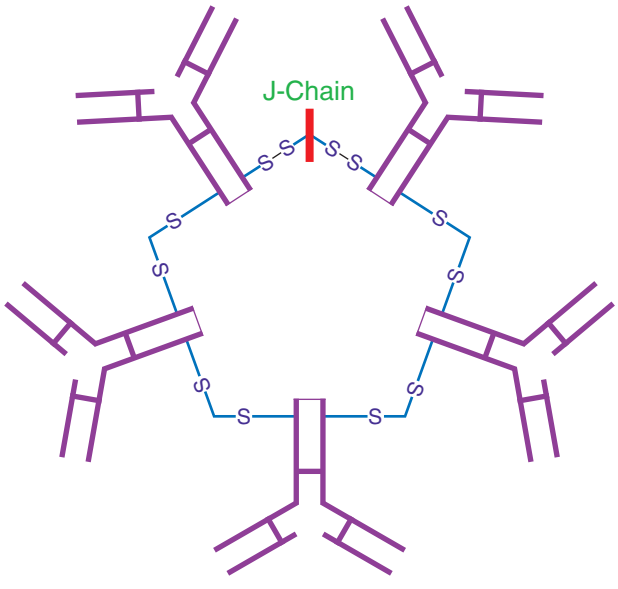
There are 4 subclasses of IgG viz. IgG_1, IgG_2, IgG_3, IgG_4, these differ in the number of inter-chain disulphide bonds between the heavy chains.

IgA: This is the second largest class of immunoglobulin in the serum with a concentration of 121 i.u./ml. It accounts for about 10-15% of the total immunoglobulins in contrast to IgG which is predominant (about 80%). IgA is present as a dimer i.e. two "Y" chains joined by a "J" chain linked through disulphide bonds. This dimer combines with another protein known as the secretory piece, which enables its secretion. IgA has a molecular weight of 160,000 daltons. IgA is an important immunoglobulin found in the seromucous secretions such as the saliva, secretions of respiratory and gastro-intestinal tract, tears, colostrum etc which prevents the entry of micro-organisms into the body.



Structure of IgA

IgM: IgM is also called as macroglobulin because it is the biggest molecule among all the immunoglobulins. It has a molecular weight of 900,000 daltons. It is a pentamer i.e. five "Y" molecules are linked together by intermolecular disulphide bonds with a single J-chain in between. Its concentration is 170 i.u/ml. This is the first antibody to be formed in response to any antigen, later on, the response shifts to IgG type which is more specific.



Structure of IgM

IgD: It has a molecular weight of 185,000 Daltons with a very negligible concentration in the serum. It is present on the surface of the lymphocytes and later shifts to IgM and IgG.

IgE: Its molecular weight is 200,000 Daltons and is present in very small amounts in blood to about 17-450 ng/ml (1 ng = 10^{-9} g). It has a cytophilic property and hence is bound to cells. The epsilon chain has receptors for binding to mast cells in the skin and elsewhere, hence IgE is found on these cells. The antigen-antibody reaction for IgE takes place on the cell surface which results in the degranulation of the mast cells and release of vasoactive amines. These compounds elicit symptoms characteristic of allergy e.g. vasodilation, oedema, rash, hay fever, asthma etc. IgE levels are raised in several tropical parasitic diseases. The histamine released by antigen-antibody reaction helps in ejection of the parasites.

Light chains: There exist two types of light chains; kappa (k) and lambda (λ). Light chain protein of the kappa type consists of 214 amino acids. All kappa chains have the same sequence in the carboxy terminal half of the molecule except that certain kappa chains have the amino acid leucine at position 191, while others have valine in this position. The amino acid sequence in the amino terminal half of the light chain is not same in different Ig. These two portions of the k light chain have been designated V_k and C_K respectively i.e. the segments of variable and constant amino acid sequence.

Light chain proteins of either k or λ type have the amino acid threonine at position 5, glutamine at position 6 and glycine at position 16. All kappa light chains have isoleucine at position 2 and leucine at position 11, all lambda light chains have proline at position 7 and glutamine at position 17. Certain residues do not change from one portion to another, but some portions of the variable region show great variability in sequence composition and these are known as hypervariable regions or complementarity determining regions (CDRs) each containing 6 to 10 amino acids. Three regions of the V_L segment show extreme sequence variability; these are located at positions 24 through 34; 50 through 56 and 89 through 97.

The proportion of k and λ chains in immunoglobulin molecules varies form species to species being about 2:1 in human. A given molecule of immunoglobulin has either both the light chains as kappa or both of them as lambda, but never both. In humans 65% of the light chains are kappa and 35% are lambda.

Heavy chains: Heavy chains consist of segments of variable (V) and constant (C) amino acid sequence composition; the V_H segment of IgG consists of approximately 115 amino acids and C_H segment of approximately 330. The C_H segment in turn consists of three regions of approximately 110 amino acids termed C_{H1}, C_{H2} and C_{H3}.

Four regions in V_H region show marked variability in amino acid sequence composition and constitute the hypervariable regions of heavy chains. These regions are located at positions 31 through 37, 51 through 68, 86 through 91 and 101 through 109. The hypervariabe regions (15–20%) are held in place by more conserved framework regions (80–85%). In an intact immunoglobulin, the hypervariable regions of a light chain and of heavy chain can be brought together in three dimensional space to form an antigen-binding surface. Because these sequences form a surface complementary to the three dimensional surface of a bound antigen, the hypervariable regions are also called complimentarity determining regions (CDRs).

The variable segment of the heavy chain also has certain residues, which appear in variant, for example, valine at position 2, leucine at 4, glutamine at 6 among others. In addition, certain residues permit the assignment of V_H segments to 4 subgroups. These four V_H seqments are not restricted to one class or subclass of heavy chains but may be found in association with the C_H regions of the γ, α, μ, δ or ε heavy chains.

IgG molecule treated with the enzyme papain, in the presence of cysteine separated the molecule into two major fragments, F_{ab} (fragment of antigen binding) which retained the antigen binding capacity of the intact molecule and F_c (fragment crystallizable) which did not combine with antigen but was readily cyrstallizable. Each fragment has a molecular weight of approximately 50,000 Daltons; F_{ab} fragment accounted for approximately 2/3rd and the F_c fragment for 1/3rd of the papain digested IgG.

J – Chains: Multimeric IgM and IgA also contain an additional 15 kD polypeptide joining (J) chain, which is disulphide bonded to the tailpieces, stabilizing the multimer. All membrane immunoglobulin molecules regardless of isotype, are believed to be monomeric, containing two heavy and two light chains.

Secretory component: The secretory IgA has fourth polypeptide chain i.e. the secretory component (SC). The J and SC chain differ from each other antigenically and in amino acid composition and also from light and heavy chains.

Hinge region: The basic four-chain structure of the immunoglobulin molecule is 'Y'-shaped. However, it may change from a 'Y' to a 'T' shape depending on the reaction conditions. This is facilitated by the existence of a hinge region located between C_H1 and C_H2 in certain isotypes. The hinge may contain from about ten (in α_1, α_2, γ_1, γ_2 and γ_4) to over 60 (in γ_3 and δ) amino acid residues. There are intrachain disulphide bonds in the V_L and C_L segments of the light chain and the V_H, C_H1, C_H2 and C_H3 segments of the heavy chain. Thus in each segment, the intrachain disulphide bond establishes a loop involving approximately 60 amino acids. The segments of polypeptide immunoglobulin chains with homologous amino acid sequences are the domains.

Light chains contain one variable domain (V_L) and one constant domain (C_L). Heavy chains contain one variable domain (V_H) and either three or four constant domains (C_H1, C_H2, C_H3 and C_H4) depending on the antibody class.

The constant region of heavy chain consists of three to four independent domains. Each chain is folded into recognizable loop-shaped zones created by intrachain disulphide bonds. These zones are termed as "domains', which are folded over. Those of light chains consist of only one domain. These domains are numbered from 1 to 3 or 4. Both chains of heavy and light chains contain several homologous units of about 110 amino acid residues within each unit i.e. domain, an intrachain disulphide bond from a loop of about 60 AAs.

BODY FLUIDS

Cerebrospinal fluid (CSF): It is a clear bodily fluid that occupies the subarachnoid space and the ventricular system around and inside the brain. Essentially, the brain floats in it. More specifically the CSF occupies the space between the arachnoid mater (the middle layer of the brain cover, meninges) and the piamater (the layer of the meninges closest to the brain). Moreover it constitutes the content of all intra-cerebral (inside the brain, cerebrum) ventricles, cisterns and sulci (singular sulcus), as well as of the central canal of the spinal cord. It is an approximately isotonic solution and acts as a 'cushion' or buffer for the cortex, providing also a basic mechanical and immunological protection to the brain inside the skull.

It is produced in the brain by modified ependymal cells in the choroid plexus. The cerebrospinal fluid is produced at a rate of 500 ml/day. Since the brain can only contain from 135-150 ml, large amounts are drained primarily into the blood through arachnoid granulations in the superior sagittal sinus. This continuous flow into the venous system dilutes the concentration of larger, lipoin-soluble molecules penetrating the brain and CSF.

Biochemical constitutens: The normal fluid is watery with low viscosity. Its specific gravity is 1.003 to 1.008. CSF pressure ranges from 60-100 mm H_2O or 4.4 –7.3 mm Hg, with most variations due to coughing or internal compression of jugular veins in the neck.

The CSF contains approximately 0.3% plasma proteins or 15 to 40 mg/dl. The proteins of CSF do not coagulate. The albumin globulin ratio is 3.1. In diseased condition there is an increase in protein, especially globulin. The protein content of CSF in inflammatory meningitis increases to about 125 mg to 1 g/100ml. In various brain disases like neurosyphilis, encephalitis, abscess, tumour the protein content is elevated to 20-300 mg/100 ml and fibrinogen is completely absent.

The glucose content of CSF is 50-85 mg/100 ml which is less than the plasma level. It increases in encephalitis, central nervous sytem syphilis, abscesses and tumors. It is decreased in purulent meningitis.

The lactic acid content of CSF ranges from 1.8 to 2.4 mg/dl. Measurement of lactic acid in CSF is done to differentiate between bacterial and viral meningitis. The concentration of lactic acid is elevated in conditions causing severe or global brain ischemia and anaerobic glycolysis.

Among the minerals, Ca is 4.1–5.9 mg/100 ml of CSF. Na and Cl are higher in CSF than in serum, whereas K and P are less than in serum. Chloride content is decreased in meningitis and unchanged in syphilis, encephalitis, poliomyeilitis and other diseases of the central nervous system. Chloride in CSF is decreased in tuberculous meningitis. Magnesium is about 5 mg/100 ml.

Functions: The cerebrospinal fluid has many putative roles including mechanical protection of the brain, distribution of neuroendocrine factors and prevention of brain ischemia. The prevention of brain ischemia is made by decreasing the amount of cerebrospinal fluid in the limited space inside the skull. This decreases total intracranial pressure and facilitates blood perfusion.

When CSF pressure is elevated, cerebral blood flow may be constricted. When disorders of CSF flow occur, they may affect not only CSF movement, but also the intracranial blood flow with subsequent neuronal and glial vulnerabilities. The venous system is also important in this equation. Infants and patients shunted as small children may have particularly unexpected relationships between pressure and ventricular size, possibly due to venous pressure dynamics. This may have significant treatment implications but the underlying pathophysiology needs to be further explored.

Cerebrospinal fluid can be tested for the diagnosis of a variety of neurological diseases. It is obtained by lumbar puncture, to count the cells in the fluid and estimate protein and glucose. These parameters alone may be extremely beneficial in the diagnosis of subarachnoid hemorrhage and central nervous system infections (such as meningitis). A cerebrospinal fluid culture examination shows microorganism that has caused the infection. By the detection of the oligoclonal bands, an ongoing inflammatory condition (for example, multiple sclerosis) can be recognized. A beta-2 transferrin assay is highly specific and sensitive for the detection of cerebrospinal fluid leakage.

Electrophoresis of CSF and its use in diagnosis: The proteins in the CSF are identified by isoelectric focusing (IEF) and crossed immunoelectrophoresis wherein forty distinct bands are seen. But only 22 CSF protein bands are formed after polyacrylamide gel electrophoresis (PAGE). These protein patterns are of great diagnostic importance. There is an abnormal alkaline gamma-globulin region in patients with multiple sclerosis. CSF protein abnormalities are found in patients with spinal muscular atrophy and with muscular dystrophy.

Two-dimensional gel electrophoresis is a technique with the capacity to resolve complex mixtures of thousands of proteins. Samples are subjected to IEF, then PAGE, to produce a gel pattern of proteins. The position of the proteins is determined by their isoelectric point (pI) and relative molecular mass (Mr). The stained density of each polypeptide on the gel is a function of its concentration. A highly sensitive stain like silver stain or Coomassie Brilliant Blue is required to identify the proteins in the gel.

CSF electrophoresis for the detection of oligoclonal bands is performed if there is suspicion of an inflammatory and/or demyelinating condition of the central nervous system. A concomitant serum sample for electrophoresis and protein estimation is mandatory for correct interpretation of the CSF results.

CSF is produced by the choroid plexus. The blood brain barrier acts as a molecular sieve excluding the passage of high molecular weight proteins, including immunoglobulin. Some inflammatory conditions of the central nervous system (CNS) result in increased production of immunoglobulins, and thus a raised CSF immunoglobulin level. These immunoglobulins have restricted specificity and thus restricted electrophoretic mobility, producing oligoclonal banding on CSF electrophoresis. Other causes of raised CSF immunoglobulin, with or without increase in other proteins include malignancy (such as lymphoma), hypergammaglobulinaemia (including serum paraproteins) and increased permeability of the blood-brain barrier.

Oligoclonal bands can be detected in up to 90% of those with multiple sclerosis (MS). They can also be found in other inflammatory and demyelinating conditions of the CNS, such as Guillain-Barre syndrome,

bacterial meningitis, viral encephalitis, subacute sclerosing panencephalitis (SSPE), neurosyphilis, chronic progressive myelopathies, optic neuritis and idiopathic polyneuritis. Repeated testing can aid in differential diagnosis. In the first three conditions, the oligoclonal bands are transient whilst in SSPE new bands may develop. In SSPE, antibodies specific for measles are detectable. In MS and its variants, including some progressive myelopathies and optic neuritis, the pattern tends to remain unchanged over time.

The detection of IgG oligoclonal bands in CSF in the absence of corresponding bands in serum, implies local production of IgG of restricted specificity, highly suggestive of an intracerebral inflammatory process. The commonest cause is multiple sclerosis (MS).

CSF is first concentrated because the protein levels are much lower than those of serum. An electrophoretogram (EPG), which separates proteins on the basis of their electrical charge is then performed, preferably in conjunction with a corresponding serum sample. Immunofixation with antisera to IgG confirms that the bands are Immunoglobulin G.

Oligoclonal bands are defined as two or more discrete, narrow immunoglobulin bands in the gamma region. They are usually faint unless the CSF immunoglobulin level is markedly elevated. In multiple sclerosis and other inflammatory conditions of the brain, the oligoclonal immunoglobulins are synthesised locally in the central nervous system, hence are present in CSF but not in serum. Very rarely, some patients with MS can also have oligoclonal banding in their serum but those in the serum are usually less prominent and less numerous than those in the CSF. If bands are prominent in both serum and CSF, changes are presumed to be secondary to other systemic conditions such as viral infections, malignancy or immune complex disease.

To distinguish raised CSF IgG due to local CNS production from leakage of serum into the CSF, our laboratory can compare CSF and serum IgG levels are with reference to albumin, a value known as the IgG index. A CSF IgG:albumin ratio higher than that of serum (raised IgG index) is indicative of local CNS production of IgG. A serum IgG:albumin ratio very much higher than that of CSF (low IgG index) is suggestive of hypergammaglobulinaemia or low serum albumin (normal is 0.26-0.70).

$$\text{IgG index} = \frac{\text{CFSF IgG} / \text{albu min}}{\text{Serum IgG} / \text{albu min}}$$

The presence of oligoclonal IgG bands in CSF together with a raised IgG index is highly specific for a demyelinating condition such as multiple sclerosis.

Normal CSF electrophoretogram

CSF-3 × oligoclonal bands

Amniotic fluid: It is the nourishing and protecting liquid contained by the amnion of a pregnant woman. The amnion grows and begins to fill, mainly with water, around two weeks after fertilization. After a further 10 weeks the liquid contains proteins, carbohydrates, lipids, phospholipids, urea and electrolytes, all of which aid in the growth of the fetus. In the late stages of gestation much of the amniotic fluid consists of fetal urine. The amniotic fluid increases in volume as the fetus grows. The amount of amniotic fluid is greatest at about 34 weeks after conception or 34 weeks ga (gestational age). At 34 weeks ga, the amount of amniotic fluid is about 800 ml. This amount reduces to about 600 ml at 40 weeks ga when the baby is born.

Amniotic fluid is continually being swallowed, 'inhaled' and replaced by 'exhaling' and through urination by the baby. It is essential that the amniotic fluid be breathed into the lungs by the fetus in order for the lungs to develop normally. Swallowed amniotic fluid contributes to the formation of meconium.

Analysis of amniotic fluid, drawn out of the mother's abdomen in an amniocentesis procedure, can reveal many aspects of the baby's genetic health. This is because the fluid also contains fetal cells which can be examined for genetic defects. It has been found that amniotic fluid is also a good source of non-embryonic stem cells. These cells have demonstrated the ability to differentiate into a number of different cell-types, including brain, liver and bone.

Amniotic fluid also protects the developing baby by cushioning against blows to the mother's abdomen, allows for easier fetal movement, promotes muscular/skeletal development and helps protect the fetus from heat loss.

The forewaters are released when the amnion ruptures, commonly known as when a woman's 'water breaks'. When this occurs during labour at term, it is known as 'spontaneous rupture of membranes' (SROM). If the rupture precedes labour at term, however, it is referred to as 'premature rupture of membranes' (PROM). The majority of the hindwaters remain inside the womb until the baby is born.

Too little amniotic fluid (oligohydramnios) or too much (polyhydramnios or hydramnios) can be a cause or an indicator of problems for the mother and baby. In both cases the majority of pregnancies proceed normally and the baby is born healthy but this isn't always the case. Babies with too little amniotic fluid can develop contractures of the limbs, clubbing of the feet and hands, and also develop a life threatening condition called hypoplastic lungs. If a baby is born with hypoplastic lungs, which are small underdeveloped lungs, this condition is potentially fatal and the baby can die shortly after birth.

Preterm premature rupture of membranes (PPROM) is a condition where the amniotic sac leaks fluid before 38 weeks of gestation. This can be caused by a bacterial infection or by a defect in the structure of the amniotic sac, uterus, or cervix. In some cases, the leak can spontaneously heal, but in most cases of PPROM, labor begins within 48 hours of membrane rupture. When this occurs, it is necessary that the mother receives treatment to avoid possible infection in the newborn.

Cytosol: The cytosol or **'cytoplasm'**, (often abbreviated as ICF [intracellular fluid]) which also includes the organelles) is the internal fluid of the cell, and where a portion of cell metabolism occurs. Proteins within the cytosol play an important role in signal transduction pathways and glycolysis. They also act as intracellular receptors and form part of the ribosomes, enabling protein synthesis.

In prokaryotes, all chemical reactions take place in the cytosol. In eukaryotes, the cytosol surrounds the cell organelles; this is collectively called the cytoplasm. The portion of cytosol in the nucleus is called nucleohyaloplasm. The cytosol also surrounds the cytoskeleton which is made of fibrous proteins (ex. microfilaments, microtubules and intermediate filaments). In many organisms the cytoskeleton maintains the shape of the cell, anchors organelles and controls internal movement of structures (e.g. transport vesicles).

The cytosol is composed of free-floating particles, but is highly organized on the molecular level. As the concentration of soluble molecules increases within the cytosol, an osmotic gradient builds up towards the outside of the cell. Water flows into the cell, making the cell bigger. To prevent the cell from bursting apart, molecular pumps in the plasma membrane, the cytoskeleton, the tonoplast or the cell wall (if present) are used to counteract the osmotic pressure. Cytosol consists mostly of water, dissolved ions, small molecules and large water-soluble molecules (such as protein). Cytosol has a high concentration of K^+ ions and a low concentration of Na^+ ions. Normal human cytosolic pH is (roughly) 7.0 (i.e. neutral), whereas the pH of the extracellular fluid is 7.4.

Interstitial fluid: Interstitial fluid (or tissue fluid, or intercellular fluid) is a solution which bathes and surrounds the cells of multicellular animals. It is the main component of the extracellular fluid, which also includes plasma and transcellular fluid. On average, a person has about 11 litres of interstitial fluid providing the cells of the body with nutrients and a means of waste removal. Plasma and interstitial fluid are very similar. Plasma, the major component in blood, communicates freely with interstitial fluid through pores and intercellular clefts in capillary endothelium.

Hydrostatic pressure is generated by the pumping force of the heart. It pushes water out of the capillaries. The water potential is created due to the inability of large solutes to pass through the capillary walls. This build-up of solutes induces osmosis. The water passes from a high concentration (of water) outside of the vessels to a low concentration inside of the vessels, in an attempt to reach equilibrium. The osmotic pressure drives water back into the vessels. Because the blood in the capillaries is constantly flowing, equilibrium is never reached. The balance between the two forces is different at different points in the capillaries. At the arterial end of the vessel, the hydrostatic pressure is greater than the osmotic pressure, so the net movement favors water and other solutes being passed into the tissue fluid. At the venous end, the osmotic pressure is greater, so the net movement favours substances being passed back into the capillary. This difference is created by the direction of the flow of blood and the imbalance in solutes created by the net movement of water favoring the tissue fluid. To prevent a build-up of tissue fluid surrounding the cells in the tissue, the lymphatic system plays a part in the transport of tissue fluid. Tissue fluid can pass into the surrounding lymph vessels and eventually end up rejoining the blood. Sometimes the removal of tissue fluid does not function correctly and there is a build-up. This causes swelling and can often be seen around the feet and ankles, ex. Elephantiasis. The position of swelling is due to the effects of gravity.

Composition: Interstitial fluid consists of a water solvent containing amino acids, sugars, fatty acids, coenzymes, hormones, neurotransmitters, salts, as well as waste products from the cells. The composition of tissue fluid depends upon the exchanges between the cells in the tissue and the blood. This means that tissue fluid has a different composition in different tissues and in different areas of the body. Not all of the contents of the blood pass into the tissue, which means that tissue fluid and blood are not the same. Red blood cells, platelets and plasma proteins cannot pass through the walls of the capillaries. The resulting mixture that does pass through is essentially blood plasma without the plasma proteins. Tissue fluid also contains some types of white blood cell, which help combat infection. Lymph is considered a part of the interstitial fluid. The lymphatic system returns protein and excess interstitial fluid to the circulation.

Interstitial fluid bathes the cells of the tissues. This provides a means of delivering materials to the cells, intercellular communication, as well as removal of metabolic waste.

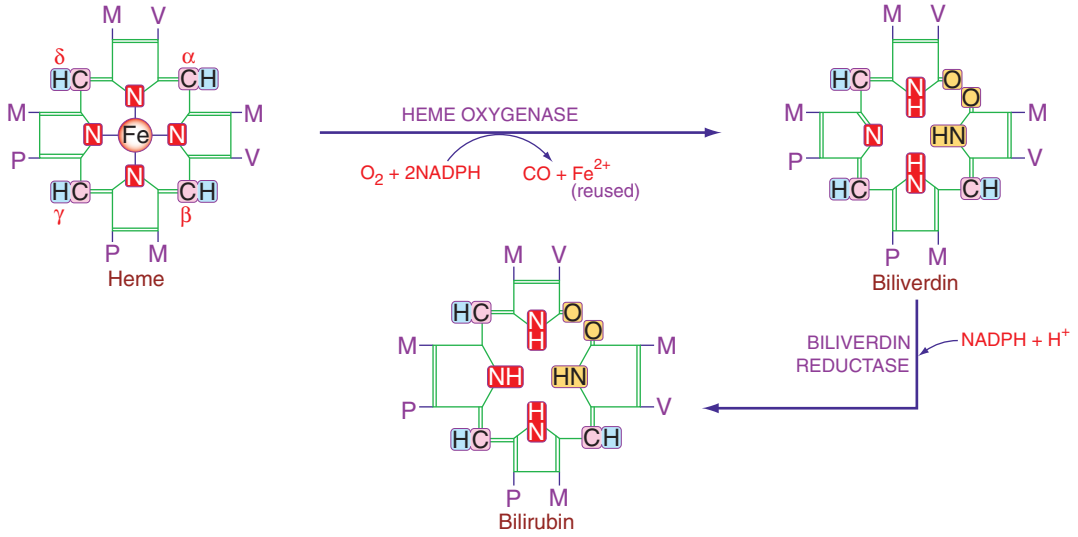
19

CLINICAL AND APPLIED BIOCHEMISTRY

Biochemistry is applied in each and every field of medical sciences. This is the basic subject that supports and enables the student to understand all the other disciplines of medicine viz. Physiology, Microbiology, Pathology, Pharmacology, Medicine and even Obstetrics & Gynaecology. Application of Biochemistry is not restricted to the learning aspect of medical sciences; instead it has a vast clinical application in diagnosis and prognosis of various diseases. Diagnosis is the basic aspect of any clinical treatment and to monitor its prognosis. Nevertheless to say, knowledge available in the medical field is due to basic research in Biochemistry.

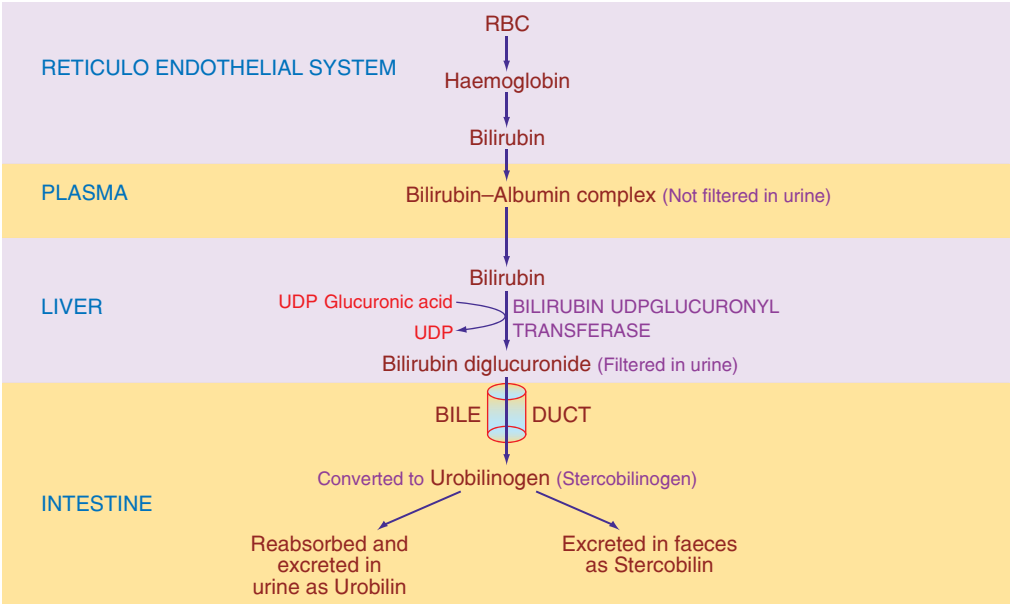
HEMOGLOBIN DEGRADATION AND JAUNDICE

Degradation of hemoglobin: The life span of hemoglobin is about the same as that of the erythrocytes i.e. 120 days. Red blood cells (RBC) are phagocytozed by macrophages in the reticuloendothelial system, mainly is spleen, liver and bone marrow. The diameter of the capillaries in these organs specially the spleen is less than the diameter of RBC, so every time the RBC passes through these capillaries, the cell membrane gets weaker and weaker due to friction between the cell membrane and capillaries and after 120 days the RBC membrane cannot withstand the pressure of the capillaries and thereby it ruptures releasing the hemoglobin. Haemoglobin undergoes cleavage of the methane bridge to form choleglobin, an iron pyrrole complex. This reaction is catalysed by methane oxygenase requiring NADPH and Fe^{2+}.



Then globin is removed forming verdohemochrome. Finally iron is removed by a mixed function oxidase called heme oxygenase producing biliverdin which is further reduced to bilirubin.

Bile pigments and their excretion: Bilirubin and biliverdin are known as the bile pigments. They are toxic substances hence should be excreted out. As they are water insoluble they cannot be excreted as such. Therefore they are converted into water soluble form in the liver. To pass from the reticuloendothelial system to liver they should pass through the hyrophillic blood. So they combine with albumin in the plasma forming albumin-bilirubin complex. This complex is transported to the liver. In the liver only bilirubin is taken up leaving the albumin free into the plasma. In the liver it is conjugated with glucuronic acid (UDP-G) by the enzyme UDP glucuronyl transferase to give rise to bilirubin monoglucuronide. To it, one more glucuronic acid is added to form bilirubin diglucuronide, by the action of the same enzyme UDP glucuronyl transferase. Glucuronic acid is taken up and UDP is left free. Now this bilirubin diglucuronide is water soluble and can be easily excreted out. For excretion it is transported into the intestine through the bile duct. In the intestine, 97% of bilirubin is converted into stercobilinogen which is colourless. This is then converted into stercobilin which gives brown colour to feces and is excreted out through the feces. Remaining 3% is reabsorbed and is excreted in urine as urobilin a yellow colour pigment.



Formation and excretion of bile pigments

Conjugated and unconjugated bile pigments: Bilirubin and biliverdin are also known as unconjugated bile pigments. Bilirubin mono and di-glucuronide are known as conjugated bile pigments.

Jaundice or icterus: Jaundice is caused due to accumulation of various forms of hemoglobin degradation products. Excess of these bile pigments leak out of the hemopoietic tissues and go into the blood circulation, from there they enter into the cells of peripheral tissue which becomes yellow. The normal concentration of bilirubin is less than 1 mg/100ml (17.1 µmol/L). If the concentration is increased then it is known as hyperbilirubinemia. When the concentration of bile pigments reaches 2 mg/100 ml of blood then the blood cannot hold anymore bilirubin. This results in the diffusion of bilirubin into the tissues. If this diffusion continues then it gives a yellow colouration to the tissue. This condition is known as jaundice or icterus.

Classification of jaundice: Jaundice is classified into three types—

1. **Haemolytic or preheptatic jaundice:** It is caused due to excessive haemolysis, thereby resulting in excessive production of bile pigments. When this exceeds the capacity of the liver to excrete, unconjugated bilirubin accumulates in the blood and causes jaundice.

2. **Infective or hepatic (parenchymatous) jaundice:** This is due to damaged liver due to alcoholism, other toxic substances or due to viral or bacterial infections wherein the parenchymatous cells are damaged. Liver fails to conjugate and excrete bilirubin produced in normal amounts. Here both conjugated and unconjugated bilirubin levels are raised in the blood. There will be more of urobilinogen in the urine.
3. **Obstructive or post-hepatic jaundice:** This is due to obstruction in the flow of bile in the bile ducts which results in increase in conjugated bilirubin in the blood.

Van Den Bergh test for jaundice:

1. **Direct Van Den Bergh test:** Conjugated bilirubin when reacted with Diazo reagent (i.e. Sodium nitrite and sulphanilic acid in presence of HCl catalyst) gives a purple red colour within one minute and in absence of alcohol. This is known as direct bilirubin and reaction is direct Van Den Bergh reaction. This type of reaction is seen only in obstructive jaundice.
2. **Indirect Van Den Bergh test:** Unconjugated bilirubin gives no colour with Diazo reagent. But when alcohol is added it gives purple red colour. This is known as indirect bilirubin and reaction is indirect Van Den Berg reaction which is seen in hemolytic and infective jaundice.

Conjugated hyperbilirubinemia:

(a) **Chronic idiopathic jaundice:** In this condition there is an excessive excretion of bile pigments in the urine. It is an autosomal recessive disorder. This disorder is not restricted to bile pigments but it also involves secretion of conjugated estrogens and other dyes like sulfobromophthalein. 80-90% of coproporphyrins excreted are of type-I instead of type-II.

(b) **Biliary tree obstruction:** In this there is a blockage of the hepatic or common bile ducts due to which conjugated bile pigments cannot be excreted and hence regurgitate into the hepatic veins and lymphatics which appears in blood and urine (choluric jaundice).

Unconjugated hyperbilirubinemia: This is seen in infants in whom the UDP glucuronyl transferase activity is not fully developed. Hence unconjugated bilirubin accumulates in blood. When it reaches over 25 mg/100 ml, it exceeds the capacity of albumin to bind it and gets released into the blood. This penetrates the blood-brain barrier of infants and causes **'kernicterus'** leading to mental retardation.

Crigler-Najjar syndrome: This is due to absence of UDP glucuronyl transferase activity in the liver cells. This is of two types, in type-I serum bilirubin level exceeds 20 mg/dl and in type-II liver can form bilirubin monoglucuronide but this cannot be further glucuronated due to absence of enzyme UDP glucuronyl transferase-II.

Gilbert's disease: There is defect in uptake of bilirubin by the liver due to lowered UDP-glucuronyl transferase activity.

Differential diagnosis of jaundice

Type of jaundice	Plasma		Urine			Faeces	
	Direct Van Den Bergh test	Total bilirubin	Bilinogen	Bilirubin	Bile salts	Bilinogen	Others
Pre hepatic (hemolytic)	–ve	Increased	Increased	Absent	Absent	Markedly increased	
Hepatic	+ve	Increased	Variable	+	+	Usually low	Variable
Post-hepatic (obstructive)	+ve	Markedly increased	Absent	++	++	Absent	Bulky, greasy, frothy

DISEASES AND DISORDERS OF THE LIVER

Many diseases of the liver are accompanied by jaundice caused by increased levels of bilirubin in the system. The bilirubin results from the breakup of the hemoglobin of dead red blood cells; normally the liver removes bilirubin from the blood and excretes it through bile.

1. **Hepatitis:** Inflammation of the liver, caused mainly by various viruses but also by some poisons, autoimmunity or hereditary conditions.
2. **Liver failure:** It is the inability of the liver to perform its normal synthetic and metabolic functions. There are two types of liver failure—
   (a) **Acute liver failure:** Is due to development of hepatic encephalopathy (confusion, stupor and coma) and decreased production of proteins (such as albumin and blood clotting proteins) within four weeks of the first symptoms (such as jaundice) of a liver problem. **'Hyperacute'** liver failure is said to be present if this interval is 7 days or less, while **'subacute'** liver failure is said to be present if the interval is 5-12 weeks.
   (b) **Chronic liver failure:** It occurs due to cirrhosis, which may be due to excessive alcohol intake, hepatitis B or C, autoimmune, hereditary and metabolic causes (such as iron or copper overload or non-alcoholic fatty liver disease).
3. **Cirrhosis:** Is the formation of fibrous tissue in the liver, replacing dead liver cells. The death of the liver cells can be caused by viral hepatitis, alcoholism or contact with other liver-toxic chemicals. This leads to progressive loss of liver function.

Ascites (fluid retention in the abdominal cavity) is the most common complication of cirrhosis and is associated with a poor quality of life, increased risk of infection. Cirrhosis is generally irreversible once it occurs, and treatment generally focuses on preventing progression and complications. In advanced stages of cirrhosis the only option is a liver transplant.

4. **Hepatic encephalopathy (or hepatoencephalopathy):** It is a reversible neuropsychiatric abnormality in the setting of liver failure, whether chronic (as in cirrhosis), or acutely.

With severe liver impairment, toxic substances normally removed by the liver accumulate in the blood and impair the function of brain cells. If there is also portal hypertension and subsequent bypassing of the liver filtration system of blood flowing in from the intestines, these toxic substances can travel directly to the brain without being modified or purified.

Due to the presence of scarring within the liver, cirrhosis leads to obstruction of the passage of blood through the liver causing portal hypertension. This means it is difficult for blood from the intestines to go through the liver to get back to the heart. Portal systemic anastomoses ('shunts') develop, and portal blood (from the intestinal veins) will bypass the liver and return to the heart via another route without undergoing first-pass detoxification by the liver.

Furthermore, in cirrhosis and other forms of liver disease the damaged liver will not be functioning as well as it should be, so even blood that does travel through the liver may not be as detoxified as it otherwise would be. In fact, if the degree of liver damage and malfunction is severe, then even in the absence of portal hypertension and the consequent bypassing of the liver by blood coming in from the intestines, hepatic encephalopathy will still occur. Such may well be the case, for example, following severe injury due to acetaminophen poisoning or acute viral infection (e.g. hepatitis A).

5. **Haemochromatosis:** Hereditary disease causing the accumulation of iron in the body, eventually leading to liver damage.
6. **Cancer of the liver:** Primary hepatocellular carcinoma or cholangiocarcinoma and metastatic cancers, usually from other parts of the gastrointestinal tract.

7. **Wilson's disease:** Hereditary disease which causes the body to retain copper.
8. **Primary sclerosing cholangitis:** Inflammatory disease of bile duct, mostly autoimmune.
9. **Primary biliary cirrhosis:** Autoimmune disease of small bile ducts.
10. **Budd-Chiari syndrome:** Obstruction of the hepatic vein.
11. **Gilbert's syndrome:** A genetic disorder of bilirubin metabolism, found in about 5% of the population.
12. **Glycogen storage disease type-II:** The build-up of glycogen causes progressive muscle weakness (myopathy) throughout the body and affects various body tissues, particularly in the heart, skeletal muscles, liver and nervous system.

There are also many pediatric liver diseases, including biliary atresia, alpha-1 antitrypsin deficiency, alagille syndrome and progressive familial intrahepatic cholestasis.

Liver function tests: A number of liver function tests are available to test the proper function of liver. These tests for the presence of enzymes in blood those are normally most abundant in liver tissue, metabolites or products. Some of the parameters used in liver function tests are—

Parameter	Significance	Normal value
Alanine transaminase (ALT)	ALT rises dramatically in acute liver damage, such as viral hepatitis or paracetamol (acetaminophen) overdose. Elevations are often measured in multiples of the Upper Limit of Normal (ULN).	9 to 60 IU/L
Aspartate transaminase (AST)	It is raised in acute liver damage, but is also present in red cells and cardiac and skeletal muscles and is therefore not specific to the liver. The ratio of AST to ALT is sometimes useful in differentiating between causes of liver damage.	10 to 40 IU/L
Alkaline phosphatase (ALP)	Alkaline phosphatase (ALP) is an enzyme in the cells lining the biliary ducts of the liver. ALP levels in plasma will rise with large bile duct obstruction, intrahepatic cholestasis or infiltrative diseases of the liver. ALP is also present in bone and placental tissue, so it is higher in growing children (as their bones are being remodelled) and elderly patients with Paget's disease.	30 to 120 IU/L
Total bilirubin (TBIL)	Increased in jaundice.	2 to 14 µmol/L
Direct bilirubin	Increase indicates normal liver.	0 to 4 µmol/L
Gamma- glutamyl transpeptidase (GGT)	Although reasonably specific to the liver and a more sensitive marker for cholestatic damage than ALP, gamma glutamyl transpeptidase (GGT) may be elevated with even minor, sub-clinical levels of liver dysfunction. It can also be helpful in identifying the cause of an isolated elevation in ALP. GGT is raised in alcohol toxicity (acute and chronic).	0 to 51 IU/L

Coagulation tests: The liver is responsible for the production of coagulation factors. The international normalized ratio (INR) measures the speed of a particular pathway of coagulation, comparing it to normal. If the INR is increased, it means it is taking longer than usual for blood to clot. The INR will

only be increased if the liver is so damaged that synthesis of vitamin K-dependent coagulation factors have been impaired. It is not a sensitive measure of liver function.

Serum glucose (BG, Glu): The liver's ability to produce glucose (gluconeogenesis) is usually the last function to be lost in the setting of fulminant liver failure.

Lactate dehydrogenase (LDH): Lactate dehydrogenase is an enzyme found in many body tissues, including the liver. Elevated levels of LDH may indicate liver damage.

DETOXIFICATION

The mechanism which converts the toxic substances into less harmful products and then into readily excretable form is known as detoxification mechanism. All the detoxification reactions occur in the liver and this constitutes one of the liver function tests.

Several unwanted and harmful substances get entry into the body either by absorption from the gastrointestinal tract (e.g. drugs) or by parenteral route. Some of the physiological substances normally produced in the body (e.g. hormones) also require to be eliminated regularly to prevent accumulation and prolonged and cumulative action. The foreign substances undergo some preliminary changes which are not different from the metabolic changes occurring to physiological substances. These are grouped into two phases. Phase-I includes—(1) Oxidation (2) Reduction and (3) Hydrolysis. Phase-II includes (4) Conjugation or synthesis or coupling with some substance in the body which will make it suitable for excretion. Some of the sample reactions for each of the class of reactions are—

1. Oxidation:

(i) **Alcohols:** Are oxidized to corresponding **acids**

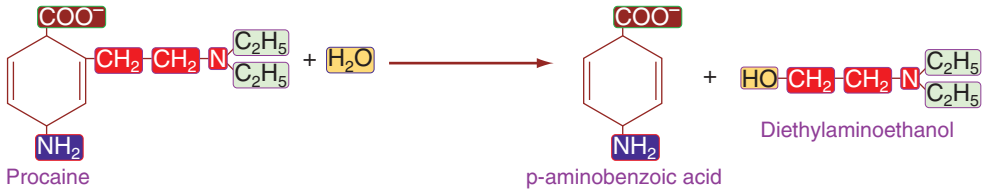
(ii) **Aromatic hydrocarbons:** Are oxidized to **phenols**

2. Reduction:

(i) **Aldehydes:** Are reduced to **alcohols**

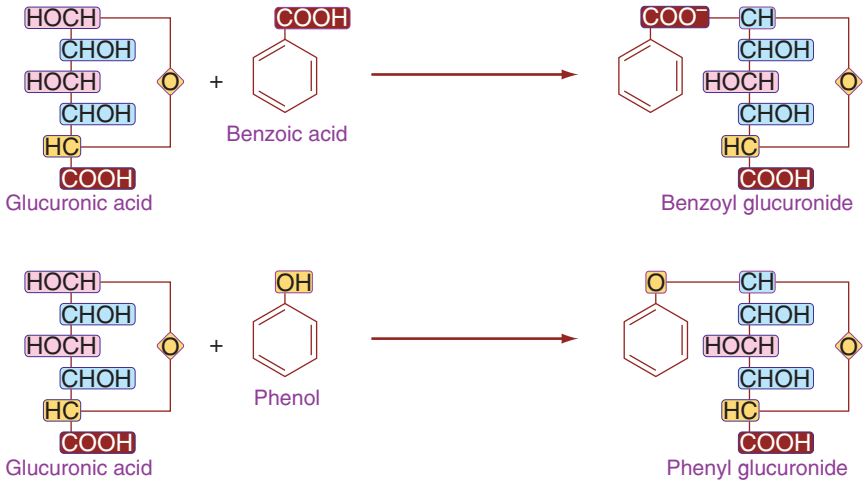
(ii) **Aromatic nitro compounds:** Are reduced to corresponding **amines**

3. **Hydrolysis:** Drugs like procaine and acetyl salicylic acid and cardiac glycosides like digitalin undergo hydrolysis.



4. **Conjugation:** The foreign substances as such or after suitable preparation by one or other of the process of oxidation, reduction and hydrolysis are usually conjugated with another substance before they are excreted. The following are some examples—

(i) **Glucuronic acids:** Aromatic acids (e.g. benzoic acid) and phenols are conjugated with glucuronic acid. The glucuronic acid is derived form uridine diphosphate glucuronic acid. The drug chloramphenicol and the bile pigments are among the important substances conjugated with glucuronic acid. Derivatives of steroid hormones also are conjugated with glucuronic acid before excretion.

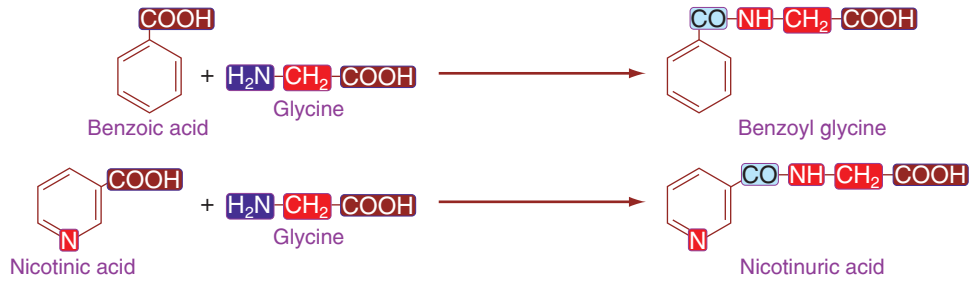


(ii) **Active sulfate:** Is used to conjugate phenolic compounds. The derivatives are called ethereal sulfates. An increase in their amount in urine signifies excessive intestinal putrefaction or stasis. Adrenal cortical hormones are also excreted after conjugation with sulfuric acid.

Yeast and mammalian liver contain enzymes that can activate inorganic sulfate by adding it to 3-phosphoadenosine-5-phosphate. Active sulfate is, adenine-3-phosphoribose-5-phosphosulfate. Incorpora-

tion of sulfate into sulfated mucopolysaccharides and conjugation of steroid hormones and others with sulfate are brought about after preliminary activation of sulfate.

(iii) **Glycine:** Is used to conjugate aromatic acids, cholic acid and also nicotinic acid. The formation of bile acid (glycocholic acid) from cholic acid is also brought about by conjugation with glycine.

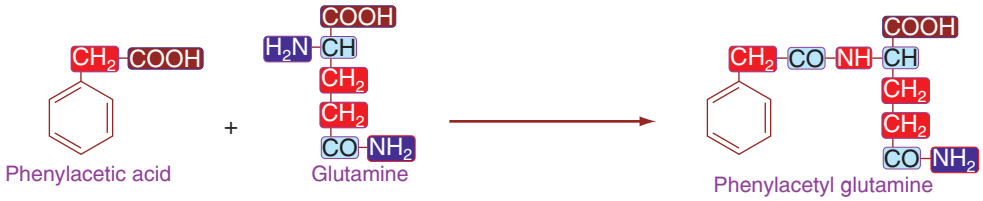


(iv) **Cysteine:** Is used in the conjugation of certain Aromatic compounds like benzene and halogenated ring compounds like bromobenzene.

The amino group is later acetylated to form what are known as mercapturic acids.

(v) **Acetic acid:** Is used to conjugate with aromatic amino compounds like sulfanilamide.

(vi) **Glutamine:** Is conjugated with phenylacetic acid to form phenylacetylglutamine.



(vii) **Methyl groups:** From active methionine (s-adenosyl methionine) are used for conjugation of certain pyridine and other heterocyclic nitrogen containing compounds like nicotinamide.

(viii) **Interaction of highly toxic cyanides:** With thiosulfate to form the relatively non toxic thiocyanates is also included under conjugation reaction. The enzyme which converts cyanide to thiocyanate is called 'rhodanase'.

DISEASES AND DISORDERS OF KIDNEY

Disturbance in the normal function of the kidneys can result from both congenital and acquired factors.

Congenital: Congenital hydronephrosis, congenital obstruction of urinary tract, duplicated ureter, horse-shoe kidney, polycystic kidney disease, renal dysplasia, unilateral small kidney, multicystic dysplastic kidney.

Acquired: Diabetic nephropathy, glomerulonephritis, hydronephrosis (the enlargement of one or both of the kidneys caused by obstruction of the flow of urine). Interstitial nephritis, kidney stones are a relatively common and particularly painful disorder. Kidney tumors (Wilms tumor, renal cell carcinoma), lupus nephritis, minimal change disease.

1. **Nephrotic syndrome:** In this syndrome the glomerulus gets damaged so that a large amount of protein in the blood enters the urine. Other frequent features of the nephrotic syndrome include swelling, low serum albumin and high cholesterol.
2. **Pyelonephritis:** It is infection of the kidneys and is frequently caused by complication of a urinary tract infection.
3. **Renal failure:** Renal failure or kidney failure is a situation in which the kidneys fail to function adequately.

Biochemically, it is typically detected by an elevated serum creatinine. In the science of physiology, renal failure is described as a decrease in the glomerular filtration rate. When the kidneys malfunction, problems frequently encountered are abnormal fluid levels in the body, deranged acid levels, abnormal levels of potassium, calcium, phosphate, hematuria (blood in the urine) and (in the longer term) anemia. Long-term kidney problems have significant repurcussions on other diseases, such as cardiovascular disease.

Classification: Renal failure can broadly be divided into two categories viz. acute renal failure and chronic kidney disease. The type of renal failure (acute vs. chronic) is determined by the trend in the serum creatinine. Other factors which may help differentiate acute and chronic kidney disease include the presence of anemia and the kidney size on ultrasound. Long-standing, i.e. chronic, kidney disease generally leads to anemia and small kidney size.

Acute renal failure: Acute renal failure (ARF) is a rapidly progressive loss of renal function, generally characterized by oliguria (decreased urine production, quantified as less than 400 ml per day in adults, less than 0.5 ml/kg/h in children or less than 1 ml/kg/h in infants); body water and body fluids disturbances and electrolyte derangement. ARF can result from a large number of causes.

Causes: Depending upon the cause, acute renal failure is grouped into pre-renal, renal and post-renal causes.

1. **Pre-renal (causes in the blood supply):**
   - Hypovolemia (decreased blood volume), usually from shock or dehydration and fluid loss or excessive diuretics use.
   - Hepatorenal syndrome in which renal perfusion is compromised in liver failure.
   - Vascular problems, such as atheroembolic disease and renal vein thrombosis (which can occur as a complication of the nephrotic syndrome).
   - Infection usually sepsis, systemic inflammation due to infection.
2. **Renal (damage to the kidney itself):**
   - Toxins or medication (e.g. some NSAIDs, aminoglycoside antibiotics, iodinated contrast, lithium).
   - Rhabdomyolysis (breakdown of muscle tissue)—the resultant release of myoglobin in the blood affects the kidney; it can be caused by injury (especially crush injury and extensive blunt trauma), statins, stimulants and some other drugs.
   - Hemolysis (breakdown of red blood cells)—the hemoglobin damages the tubules; it may be caused by various conditions such as sickle-cell disease, and lupus erythematosus.

- Multiple myeloma, either due to hypercalcemia or "cast nephropathy" (multiple myeloma can also cause chronic renal failure by a different mechanism).
- Acute glomerulonephritis which may be due to a variety of causes, such as anti-glomerular basement membrane disease/Goodpasture's syndrome, Wegener's granulomatosis or acute lupus nephritis with systemic lupus erythematosus.

3. **Post-renal (obstructive causes in the urinary tract) due to:**
   - Medication interfering with normal bladder emptying.
   - Benign prostatic hypertrophy or prostate cancer.
   - Kidney stones.
   - Due to abdominal malignancy (ex. ovarian cancer, colorectal cancer).
   - Obstructed urinary catheter.

Diagnosis: Renal failure is diagnosed when either creatinine or blood urea nitrogen levels in the blood which are elevated in an ill patient, especially when oliguria is present. Previous measurements of renal function may offer comparison, which is especially important if a patient is known to have chronic renal failure as well. If the cause is not apparent, a large amount of blood tests and examination of a urine specimen is typically performed to elucidate the cause of acute renal failure, medical ultrasonography of the renal tract is essential to rule out obstruction of the urinary tract.

Consensus criteria for the diagnosis of ARF are:

- Risk: serum creatinine increased 1.5 times OR urine production of <0.5 ml/kg body weight for 6 hours.
- Injury: creatinine 2.0 times OR urine production <0.5 ml/kg for 12 h.
- Failure: creatinine 3.0 times OR creatinine >355 mmol/l (with a rise of >44) or urine output below 0.3 ml/kg for 24 h.
- Loss: persistent ARF or more than four weeks complete loss of kidney function.
- Kidney biopsy may be performed in the setting of acute renal failure, to provide a definitive diagnosis and sometimes an idea of the prognosis, unless the cause is clear and appropriate screening investigations are reassuringly negative.

Chronic kidney disease: Stage 5 Chronic Kidney Disease (CKD) can develop slowly and show few initial symptoms, be the long term result of irreversible acute disease or be part of a disease progression. There are many causes of CKD. The most common cause is diabetes mellitus. Stage 1 CKD is mildly diminished renal function, with few overt symptoms. Stage 5 CKD is a severe illness and requires some form of renal replacement therapy (dialysis or kidney transplant).

Signs and symptoms: Initially it is without specific symptoms and can only be detected as an increase in serum creatinine or protein in the urine. As the kidney functions decreases:

- Blood pressure is increased due to fluid overload and production of vasoactive hormones, increasing one's risk of developing hypertension and/or suffering from congestive heart failure.
- Urea accumulates leading to azotemia and ultimately uremia (symptoms ranging from lethargy to pericarditis and encephalopathy). Urea is excreted by sweating and crystallizes on skin ("uremic frost").
- Potassium accumulates in the blood (known as hyperkalemia with a range of symptoms including malaise and potentially fatal cardiac arrhythmias).
- Erythropoietin synthesis is decreased (potentially leading to anemia, which causes fatigue).
- Fluid volume overload–symptoms may range from mild edema to life-threatening pulmonary edema.

- Hyperphosphatemia–due to reduced phosphate excretion, associated with hypocalcemia (due to vitamin D3 deficiency).
- Later this progresses to tertiary hyperparathyroidism, with hypercalcaemia, renal osteodystrophy and vascular calcification that further impairs cardiac function.
- Metabolic acidosis due to accumulation of sulfates, phosphates, uric acid etc. This may cause altered enzyme activity by excess acid acting on enzymes and also increased excitability of cardiac and neuronal membranes by the promotion of hyperkalemia due to excess acid (acidemia).
- People with chronic kidney disease suffer from accelerated atherosclerosis and are more likely to develop cardiovascular disease than the general population. Patients afflicted with chronic kidney disease and cardiovascular disease tend to have significantly worse prognoses than those suffering only from the latter.

Diagnosis: In many CKD patients, previous renal disease or other underlying diseases are already known. A small number presents with CKD of unknown cause. In these patients, a cause is occasionally identified retrospectively.

It is important to differentiate CKD from acute renal failure (ARF) because ARF can be reversible. Abdominal ultrasound is commonly performed, in which the size of the kidneys are measured. Kidneys with CKD are usually smaller (< 9 cm) than normal kidneys with notable exceptions such as in diabetic nephropathy and polycystic kidney disease. Another diagnostic clue that helps differentiate CKD and ARF is a gradual rise in serum creatinine (over several months or years) as opposed to a sudden increase in the serum creatinine (several days to weeks). Additional tests include nuclear medicine MAG3 scan to confirm blood flows and establish the differential function between the two kidneys. DMSA scans are also used in renal imaging; with both MAG3 and DMSA being used chelated with the radioactive element Technetium-99.

Stages: All individuals with a Glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 for 3 months are classified as having chronic kidney disease, irrespective of the presence or absence of kidney damage. The rationale for including these individuals is that reduction in kidney function to this level or lower represents loss of half or more of the adult level of normal kidney function, which may be associated with a number of complications.

Stage 1 CKD: Slightly diminished function; kidney damage with normal or increased GFR (>90 mL/min/1.73 m^2).

Kidney damage is defined as pathologic abnormalities or markers of damage, including abnormalities in blood or urine test or imaging studies.

Stage 2 CKD: Mild reduction in GFR (60-89 mL/min/1.73 m^2) with kidney damage.

Stage 3 CKD: Moderate reduction in GFR (30-59 mL/min/1.73 m^2).

Stage 4 CKD: Severe reduction in GFR (15-29 mL/min/1.73 m^2).

Stage 5 CKD: Established kidney failure (GFR <15 mL/min/1.73 m^2) or permanent renal replacement therapy (RRT).

Causes: The most common causes of CKD are diabetic nephropathy, hypertension, and glomerulonephritis.

Acute on chronic renal failure: Acute renal failure can be present on top of chronic renal failure. This is called acute-on-chronic renal failure (AoCRF). The acute part of AoCRF may be reversible and the aim of treatment, as with ARF, is to return the patient to their baseline renal function, which is typically measured by serum creatinine. AoCRF, like ARF, can be difficult to distinguish from chronic renal failure, if the patient has not been monitored by a physician and no baseline (i.e. past) blood work is available for comparison.

Renal function tests: Renal function, in nephrology, is an indication of the state of the kidney and its role in renal physiology. Kidney function tests, is a collective term for a variety of individual tests and procedures that can be done to evaluate how well the kidneys are functioning.

The kidneys, the body's natural filtration system, perform many vital functions including removing metabolic waste products from the blood stream, regulating the body's water balance and maintaining the pH (acidity/alkalinity) of the body's fluids. Approximately one and a half quarts of blood per minute are circulated through the kidneys, where waste chemicals are filtered out and eliminated from the body (along with excess water) in the form of urine. Kidney function tests help to determine if the kidneys are performing their tasks adequately.

Many conditions can affect the ability of the kidneys to carry out their vital functions. Some lead to a rapid (acute) decline in kidney function; others lead to a gradual (chronic) decline in function. Both result in a build-up of toxic waste substances in the blood.

These tests are done on urine samples, as well as on blood samples.

There are a variety of urine tests that assess kidney function. A simple, inexpensive screening test, called a routine urinalysis, is often the first test administered if kidney problems are suspected. A small, randomly collected urine sample is examined physically for things like color, odor, appearance and concentration (specific gravity); chemically for substances such as protein, glucose, and pH (acidity/alkalinity); and microscopically for the presence of cellular elements (red blood cells, white blood cells, and epithelial cells), bacteria, crystals and casts (structures formed by the deposit of proteins, cells and other substances in the kidneys tubules). If results indicate a possibility of disease or impaired kidney function, one or more of the following additional tests are usually performed to more specifically diagnose the cause and the level of decline in kidney function.

The methods employed for the study of the functional activity of the kidneys include—(1) Concentrating capacity of the kidney (2) Excretion of dyes and (3) Clearances of the substances.

1. **Concentration test:** The ability of the kidney to reabsorb the filtered water resulting in the increased concentration of urine, which is measured as an indication of the functionality of kidneys.
   (a) **Pituitrine (a preparation of ADH):** 10 U.S.P units of pituitrine is injected subcutaneously and the specific gravity (sp. gr.) of the urine is measured by collecting a 24 hour sample. If the sp. gr. is 1.020, the kidneys are functioning normally. If it is lower, then there is some abnormality of the kidneys in concentrating the urine (defect in reabsorption of water by the kidneys).
   (b) **Mosenthal test:** An infrequently used test to evaluate renal concentrating ability by measuring the density of urine every two hours during the ingestion of a controlled diet. Mesenthol is infused in the night and urine is collected every two hours from 8.00 A.M to 8.00 P.M. The total volume of urine in the 12 hours should not be less than 725 ml and it should show a difference in specific gravity of 0.009 between successive samples. The overall specific gravity should be higher than 1.018 in normal healthy persons.
   (c) **Urine osmolality test:** Urine osmolality is a measurement of the number of dissolved particles in urine. It is a more precise measurement than specific gravity for evaluating the ability of the kidneys to concentrate or dilute the urine. Kidneys that are functioning normally will excrete more water into the urine as fluid intake is increased, diluting the urine. If fluid intake is decreased, the kidneys excrete less water and the urine becomes more concentrated. The test is done on a urine sample collected at the first passing in the morning, on multiple timed samples, or on a cumulative sample collected over a twenty-four hour period. The subject is kept on a high-protein diet for several days before the test and is not provided with any fluid in the night before the test. With restricted fluid intake (concentration testing), osmolality should be greater than 800 mOsm/kg of water. With increased fluid intake (dilution testing), osmolality should be less than 100 mOSm/kg in at least one of the specimens collected.

Inability of the kidneys to concentrate the urine in response to restricted fluid intake, or to dilute the urine in response to increased fluid intake during osmolality testing may indicate decreased kidney function.

2. **Excretory function:** 6 mg of the dye bromsulphalein dissolved in 480 ml of water is injected intravenously. 50 ml of urine is collected every half an hour. The excretion of the dye is as under:

35 %	is excreted	in ½ an hour
40-60%	is excreted	in 1 hour
60-80 %	is excreted	in 2 hours

If the dye excretion by two hours time is less than 50% then the kidney is abnormal.

3. **Clearance test:** Clearance refers to the volume of plasma in milliliters which contains the amount of a particular substance removed per minute by renal excretion.

Clearance can be measured as:

$$C = \frac{UV}{B}$$

Where 'U' is concentration of substance in urine

'B' is its concentration in the blood plasma

'V' is the volume of urine excreted per minute

When urine volumes are large, the rate of elimination of a substance is directly proportional to the plasma concentration. This is termed as maximal clearance.

In maximal clearance test the excretion of urine is 2 ml or more/minute.

In standard clearance test the excretion of urine is less than 2 ml/min. In standard clearance test the formula is—

$$\text{Standard clearance} = \frac{U\sqrt{V}}{B}$$

(a) **Creatinine clearance test:** Creatinine clearance is used to measure glomerular filtration rate (GFR) i.e. the volume of fluid filtered from the renal (kidney) glomerular capillaries into the Bowman's capsule per unit time. Creatinine is produced naturally by the body (creatinine is a metabolite of creatine, which is found in muscle). It is freely filtered by the glomerulus, and also actively secreted by the renal tubules in very small amounts. Creatinine clearance (C_{Cr}) can be calculated if values for creatinine's concentration in urine (U), urine flow rate (V) and creatinine's concentration (B) in plasma are known. The formulae is—

$$C_{Cr} = \frac{U \times V}{B}$$

To allow comparison of results between people of different sizes, the C_{Cr} is often corrected for the body surface area (BSA) and expressed compared to the average sized man as ml/min/1.73 m^2.

$$C_{Cr-corrected} = \frac{C_{Cr} \times 1.73}{BSA}$$

BSA = body surface area which averages to 1.7 $(0.007184 \times W^{0.425} \times H^{0\,725})$

For a 24 hour urine collection, normal creatinine clearance values are 90–139 ml/min for adult males less than 40 years old, and 80–125ml/min for adult females less than 40 years old. For people over 40, values decrease by 6.5 ml/min for each decade of life.

(b) **Urea clearance test:** The volume of plasma (or blood) that would be completely cleared of urea by one minute's excretion of urine. It is calculated as urine flow (V) multiplied by urine

urea concentration (U) divided by concentration of urea in whole blood (B). 75 ml/min (64-99 ml/min). It is also expressed in percentage of normal clearance of urine.

75-100%	is in the normal individuals
50% and less	is present in acute nephritis
16% and less	is present in early glomerular nephritis
5% or less	is present in severe renal damage

(c) **Inulin clearance test:** Inulin is a polysaccharide. A known amount of inulin is infused into the blood at a constant rate. The inulin passes freely through the glomerular membranes, so that its concentration in the glomerular filtrate equals that of the plasma. In the renal tubule, inulin is not reabsorbed to any significant degree, nor is it secreted. Consequently, the rate at which it appears in the urine can be used to calculate the glomerular filtration rate (GFR).

$$C_{in} = \frac{UV}{B}$$

Where 'U' is urinary inulin in mg/dl

'B' represents blood or plasma inulin in mg/dl

'V' represents volume of urine in ml/min

Mannitol can also be used.

(d) **Renal plasma flow:** Renal plasma flow is measured using para-aminohippuric acid (PAH), a substance that filters freely through the glomerular membranes. However, unlike inulin, any PAH remaining in the peritubular capillary plasma after filtration is secreted into the proximal convoluted tubules. Therefore, essentially all PAH passing through the kidneys appears in the urine. For this reason, the rate of PAH clearance can be used to calculate the rate of plasma flow through the kidneys. Then, if the hematocrit is known, the rate of total blood flow through the kidneys can be calculated.

4. **Filtration Fraction (FF):** Filtration fraction is the glomerular filtration rate (GFR) to the renal plasma flow (RPF).

$$\text{Filtration fraction} = \frac{GFR}{RPF}$$

GFR corresponds to 120

RPF corresponds to 574

Therefore FF = 0.217 (21.7%) – in a normal healthy person.

Under normal conditions this represents about 20% of the plasma volume passing through the kidneys or approximately 180 L/day and is calculated as the ratio of C_{inulin} to C_{PAH}. The filtration fraction therefore represents the proportion of the fluid reaching the kidneys which passes into the renal tubules. The GFR on its own is the most common and important measure of renal function. However, in a condition such as renal stenosis, the blood flow to the kidneys is reduced. The filtration must therefore be increased in order to perform the normal tasks of the kidney in balancing fluid and electrolytes in the body. This would be reflected by a high filtration fraction, showing that the kidneys have to do more work with the fluid they are receiving.

Catecholamines (Norepinephrine and Epinephrine) increase the filtration fraction by vasoconstriction of afferent and efferent arterioles which possess alpha 1 adrenergic receptors.

In early essential hypertension, FF is normal, as it progresses FF increases. In glomerular nephritis, FF is less. Under renal clearance test measurement of tubular secreting mass is done using PAH at 150 mg/100 ml of plasma. 80 mg/min/1.73 sqm body surface is normal in measurement of above for tubular filtration mass.

Blood tests to assess renal function: Estimation of blood urea nitrogen (8-20 mg/dl) or blood urea (15-40 mg/dl) and plasma creatinine (0.8-1.2 mg/dl for males and 0.6-0.9 mg/dl for females) is taken up to assess the renal functions.

DIAGNOSTIC IMPORTANCE OF NPN SUBSTANCES

The non-protein nitrogenous substances (NPN) include urea, uric acid and creatinine. Estimation of NPN is done in order to access the functioning of the kidneys. In renal dysfunction there will be a moderate increase in the concentration of these compounds in the blood. In total renal failure there will be a large increase in the blood concentration of NPN substances.

Role of blood urea as an aid to diagnosis: Urea is synthesized in the liver from ammonia released by the oxidation of amino acids. Thus urea is a water soluble, non toxic, excretable form of toxic ammonia, released into the blood and is maintained at the concentration of 15-40 mg/100 ml of the blood under normal conditions. Simultaneously it is excreted in urine varying from 15-40 gms /day.

Methods of estimation: The following methods are available for estimation of blood urea – (1) Diacetyl Monooxime (DAM) method (2) Colorimetric method by the use of urease enzyme and (3) Autoanalyser method.

Conditions causing variation in the blood urea level: A decrease in blood urea level indicates liver disorders/dysfunction, whereas an increase in the blood urea level, termed as uremia may be classified into three major classes—(1) Pre-renal (2) Renal and (3) Post-renal.

Pre-renal uremia: The increase in the blood urea upto 300 mg/dl may be due to the following reasons prior to the kidney discerders.

- Dehydration due to vomiting (pyloric obstruction or chronic intestinal obstruction). During dehydration the blood volume decreases leading to hemoconcentration as a result of which the blood urea values seem to be elevated. Due to low blood pressure the Glomerular filtration rate will be lowered leading to increased blood urea levels. Diarrhoea can also cause ureamia.
- Ulcerative colitis (there will be Cl^- loss).
- Diabetic coma.
- Addison's disease (Hypoadrenalism).
- Haematuresis.
- Shock due to burns.
- Post operatively.
- Heart attacks.

Renal uremia: An increase in the blood urea level above 300 mg/dl due to kidney disorders is termed as renal uremia. This may be due to—

- Nephritis.
- Acute glomerulonephritis.
- Total renal failure.

Post-renal uremia: Causes beyond kidney leading to increased blood urea level are mainly due to obstruction in the urinary tract arising from—

- ✓ Urolithiasis.
- ✓ Urethral tumors.
- ✓ Urinary bladder tumors.
- ✓ Enlargement of the prostrate gland.
- ✓ Stricture of urethra.

Role of uric acid as an aid to diagnosis: Nucleic acids are hydrolysed in the liver to purines and pyramidines. Purines are further oxidized to hypoxanthine and then to xanthine. Xanthine is finally oxidized by the enzyme xanthine oxidase to uric acid. The normal concentration of uric acid in the blood is 2.4 to 7.0 mg/dl and is excreted in urine @ 2.5 to 7.5 g/day.

Methods of estimation: The following methods are used for estimation of blood uric acid.

1. Phosphotungstic acid method.
2. Fehling's method.
3. Autoanalyser.

Conditions varying the blood level of uric acids. Whenever there is cell death, the nucleic acids are released and converted to purines and finally uric acid is formed. The conditions where uric acid level increases in the blood are—

In excessive tissue destruction as seen in

- Old age
- Febrile diseases
- Hypoxia
- Trauma
- Kidney dysfunction or total renal failure
- High non-vegetarian diets

High levels of uric acids lead to formation of sodium ureate crystals which get deposited into the joints causing inflammation of the joints, a condition referred to as Gouty arthritis. Gout can be prevented by competitively inhibiting the enzyme xanthine oxidase with the drug allopurinol.

Role of blood creatinine as an aid to diagnosis: Muscle needs a high energy compound higher than ATP as it performs heavy works. Hence a higher energy compound namely creatine phosphate is present in the muscle.

One ATP yields 7.3 kcal of energy.

One Creatine phosphate yields 10.4 kcal of energy.

Creatine in the muscle is phosphorylated to creatine phosphate which upon hydrolysis gets converted to creatinine. Creatinine is an excretable product and cannot be reused. The normal concentration of creatinine in the blood is 0.8–1.2 mg/dl for male; 0.5–1.0 mg/dl for female. Excreted in urine @ 0.8–1.2 g/day in male and 0.5–1.0 g/day in female. Measurement of creatinine coefficient is more accurate than estimation of blood creatinine. Creatinine coefficient is the miligrams of creatinine excreted per kg body weight per day. For males it is 6–8 and for females it is 5–7.

Methods of estimation: Jaffe's picric acid method is the commonly used method for the estimation of blood creatinine levels.

Conditions in which blood creatinine or creatinine coefficient is increased:

1. Kidney failure (coefficient is low)
2. Muscle wasting disease (coefficient is high)
3. Febrile disease (coefficient is high)
4. Heavy exercise

URINE

Urine is a complex fluid excreted by the kidneys, composed of many toxic waste substances suspended in water.

Volume of urine: The normal volume of urine excreted/day is 600 ml to 2500 ml/day, average being 1500 ml/day. In some pathological conditions:—

(a) The excretion of urine may be less than 600 ml/day. This condition is known as oliguria. It is seen in the following conditions—(1) Fever (2) Shock (3) Vomiting (4) Diarrhoea.

(b) If the excretion is below 10 ml/day or if there is no excretion then it is known as anuria. The conditions in which anuria is observed are—(1) Acute renal failure and (2) Nephritis.

(c) If the excretion of urine is more than 2500 ml/day then it is known as polyuria. Condition in which polyuria is observed are—(1) Diabetes insipidus–where ADH is deficient and the reabsorption of water is not complete, resulting in polyuria. (2) Diabetes mellitus. (3) Disease of central nervous system. (4) Amyloid degeneration of kidney (i.e. breakdown of kidney cells by its own enzymes).

Colour of urine: Amber yellow/pale yellow. The substance responsible for the color of urine is urobilin or urochrome. A change in color of urine indicates pathological conditions—

Change in color	Cause of coloration	Pathological conditions
Dark yellow or Reddish brown	Increased physiological constituents	Acute febrile disease i.e. fever causing disease
Milky	Chylomicron	Chyluria
Red	Hb, Uroerythin	Haemoglobinuria
Greenish Yellow	Bile pigments	Jaundice

Transparency: The normal urine is perfectly, clear and transparent.

(a) If mucoid whitish sediments are seen, pus may be present.

(b) If brownish red sediments are seen, blood may be present.

(c) If uniform cloud is seen, bacteria may be present.

Odour: Normal urine exhibits an aromatic smell (odour).

In diabetic patient, the urine gives fruity odour because the cells cannot utilize glucose, therefore the production of ketone bodies will be more than they can be utilized by the cells and so their excess is excreted in the urine and gives a fruity odour due to presence of acetone or volatile substances.

Reaction of litmus or pH of urine: 4–8.8, average is 6. Hence urine is acidic to litmus. The substances responsible for pH of urine are:

Monobasic and dibasic sodium and potassium phosphates. Presence of monobasic phosphates makes the urine more alkaline in nature, pH of urine is also affected by the time of intake of food. The urine collected immediately after food shows an alkaline nature because of the liberation of HCO_3 ions into the blood from the oxyntic cells during the formation of HCl and this is known as alkaline tide. Acid forming diets (non-vegetarian diets with protein rich food) makes the plasma acidic and this makes urine acidic. Base forming diets (vegetarian diets) make the urine alkaline.

Specific Gravity of Urine: The specific gravity of urine is 1.015 (at 15°C–1.012 and at 30°C–1.017). Specific gravity is less in (a) Diabetes insipidus and (b) Amyloid degeneration.

In general, specific gravity is inversely proportional to the volume of urine excreted. Diabetes Mellitus is an exception, wherein both the volume excreted and specific gravity increase. Specific gravity of urine is measured using urinometer. This is standardized at 15°C. For every 3°C rise of temperature add 1 digit to the last digit of the specific gravity noted.

Total solids in the urine: The total solids present in urine are calculated by using Long's Coefficient i.e. 2.6. To calculate the total solids multiply the last 2 digits of the specific gravity with the long coefficient. The normal value of total solids in the urine is 40-60 gms/liter (average 44.2 gr/litre).

Normal constituents of urine: The normal constituents of urine are—

1. Chloride	10–15 gms/day	5. Urea	15–40 gms/day
2. Calcium	0.2 gm/day	6. Uric Acid	2.5–7.5 gm/day
3. Phosphate	1.1 gm/day	7. Creatinine	0.5–1.2 gm/day
4. Sulphur	1 gm/day	8. Ammonia	0.5–1.2 gm/day

Sulphur is clinically important as it takes part in detoxification reactions of toxic substances like phenol, cresol that are water insoluble are made water soluble by conjugation with sulphur and this sulphur is known as ethereal sulphate.

Source of urinary ammonia: The urinary ammonia originates in kidney tubules from the amino acid glutamine by the action of enzyme glutaminase. The purpose of formation of ammonia is to conserve the fixed bases like Na^+ and K^+. The reaction is an example for deamination. It is important to fix pH of blood.

Pathological constituents of urine: Some substances are present in the urine in trace amounts, which cannot be detected by the routine qualitative tests. During pathological conditions, their concentration in the urine increases and they can be easily detected by the qualitative tests. Such substances are called pathological constituents of urine. The pathological constituents of urine are:—

1. Carbohydrates.
2. Ketone bodies.
3. Proteins.
4. Blood.
5. Bile salts and bile pigments.

1. **Carbohydrates:** Only reducing sugars are present in the urine. Excretion of excess amounts of carbohydrates in the urine is known as glycosuria. Benedict's test is most commonly used to identify sugars in urine. The various causes for glycosuria are—
   (a) **Alimentary Glycosuria:** This occurs when excess of carbohydrates is consumed through the diet. This is specially seen in persons who have undergone partial gastrectomy.
   (b) **Renal Glycosuria:** If renal threshold for glucose is reduced, excretion of glucose occurs. Normal value of Renal threshold = 180 mg/100ml (or 350 mg/mt). Renal Glycosuria occurs due to drugs like phlorizin. Hence also known as Phlorizine Glycosuria.
   (c) **Nervous Glycosuria:** This occurs due to nervous tension. It is observed in persons following anesthesia.

Glucosuria: When only glucose is present in the urine it is known as glucosuria. It is observed in diabetes mellitus in which the concentration of sugar in urine ranges from 0.5% to 2%. Insulin helps in uptake of glucose by the cells. In diabetes mellitus:—

i. There is defect in uptake of glucose by the cells, due to insulin deficiency.
ii. Defective insulin receptors on the cell, which bind glucose and help in their uptake.
iii. Anti-insulin factors like epinephrine, glucagon.

Glucosuria is also seen in pregnant female, due to pressure on abdomen.

Fructosuria: Presence of fructose in urine. It is due to defective fructokinase enzyme.

Pentosuria: Generally the pentose, xylulose is present in the urine due to deficiency of the enzyme xylulose dehydrogenase.

Lactosuria: Presence of lactose in the urine is known as lactosuria. This is observed in late pregnancy, lactation and soon after weaning.

Galactosuria: Presence of galactose in the urine due to the deficiency of enzyme Galactose-1-phosphate uridyl transferase. Because of deficiency of galactose-1-phosphate uridyl transferase, there is intolerance to milk. Therefore child vomits out milk. Lactose free milk should be given.

2. **Ketone bodies:** The ketone bodies are acetone, acetoacetic acid, β-hydroxy butyric acid. Presence of ketone bodies in the urine is known as ketonuria. Acetone is the ketone body present in the highest amount in ketonuria because it is not utilized by the body and also because the acetoacetic acid gets converted to acetone on exposure to air.

Ketoacidosis or ketosis: This is a condition wherein there is an excess accumulation of ketone bodies in the blood (ketonemia) followed by excess excretion of ketone bodies in urine (ketonuria). Ketoacidosis is observed in Diabetes mellitus, fever, frequent pregnancies, starvation, vomiting. If glucose and ketone

bodies are present in the urine the condition is known as diabetic ketoacidosis, if only ketone bodies are present then it is starvation, fever etc. Normal concentration of ketone bodies in blood is < 1 mg/100 ml. Rothera's test is used to identify ketone bodies in the urine.

3. **Proteins:** Presence of protein in urine is known as proteinuria. Sulpho salicylic acid test, Heller's test and heat coagulation are used to detect the presence of proteins in the urine. Albumin is the protein found in large amounts among all plasma proteins because of its low molecular weight and its highest concentration in the blood. As albumin is the major protein in proteinuria it is also known as albuminuria. There are two types of albuminuria—
   (a) **Physiological:** There is protein in urine but person is normal i.e. there is no abnormality or disease in the person. This is seen in—
      i. Pregnancy
      ii. Vigorous Exercises
      iii. Orthostatic Proteinuria: Urine formed in lying position is free of albumin whereas urine formed in standing position has albumin (due to pressure on kidney tubule), such condition is known as orthostatic proteinuria.
   (b) **Pathological:** Divided into 3 groups.
      i. **Pre-renal:** Here the kidney is not defective but due to secondary effects like intra-abdominal pressure, congestive cardiac failure, the protein is excreted in urine.
      ii. **Renal:** Proteinuria occurring due to the diseases of kidney like nephritis, nephritic syndrome, Hyaline degeneration of kidney.
      iii. **Post-renal proteinuria:** In this the protein is added some where in the urinary tract. The causes for this are:—
         Trauma of the lower urinary tract.
         Vaginal secretions.
         Seminal secretions.
   (c) **Bence Jones proteinuria:** Multiple myeloma is a condition, wherein there is excessive division of lymphocytes producing immunoglobulins. In patients of multiple myeloma, leukemia and Hodgkin's disease there is increased excretion of special type of proteins known as Bence Jones proteins. They are different from the plasma proteins as they contain abnormal light chains of the immunoglobulin molecules. They can be identified by heating the urine to 60 °C, wherein a coagulum appears and on further heating it dissolves.
4. **Blood:** Presence of blood in the urine, detected by benzedine test is classified as:
   (a) **Haematuria:** Wherein on microscopic examination of urine both RBC and WBC are found. This condition is observed in nephritis, trauma, tuberculosis of kidney and kidney stones.
   (b) **Hemoglobinuria:** Presence of only Hb, observed in malaria, kala-azar, typhoid.
5. **Bile salts and bile pigments:** These are present when a patient is suffering from jaundice. Hay's test is performed to detect the presence of bile salts in the urine. Bile pigments are identified by Gmellin's test and Fouchet's test.

RENAL CALCULI

Renal calculi or kidney stones are solid concretions (crystal aggregations) of dissolved minerals in urine. If calculi are formed inside the kidneys it is termed as nephrolithiasis and if it is present in the bladder it is called urolithiasis.

Some of the substances found in urine have the capability to crystalize and in a concentrated form these compounds can precipitate into a solid deposit attached to the kidney walls. These crystals can grow through a process of accretion to form a kidney stone.

Renal calculi can vary in size from as small as grains of sand to as large as a golf ball. Kidney stones typically leave the body by passage in the urine stream, and many stones are formed and passed without causing symptoms. If stones grow to sufficient size before passage (of at least 2–3 millimeters), they can cause obstruction of the ureter. The resulting obstruction with dilation or stretching of the upper ureter and renal pelvis as well as spasm of muscle, trying to move the stone, can cause severe episodic pain most commonly felt in the flank, lower abdomen and groin (a condition called renal colic). Renal colic can be associated with nausea and vomiting due to the embryological association of the kidneys with the intestinal tract. Hematuria (bloody urine) is commonly present due to damage to the lining of the urinary tract.

Causes: Kidney stones can be due to underlying metabolic conditions, such as renal tubular acidosis, Dent's disease and medullary sponge kidney. Kidney stones are also more common in patients with Crohn's disease. There has been some evidence that water fluoridation may increase the risk of kidney stone formation. However, fluoride may also be an inhibitor of urinary stone formation. Excess vitamin C may cause kidney stones, though there is no clear relationship between excess ascorbic acid intake and kidney stone formation.

Classes of renal calculi: There are five (5) major types of renal calculi viz.—

1. **Calcium oxalate stones:** The most common type of kidney stone is composed of calcium oxalate crystals and the factors that promote the precipitation of crystals in the urine are associated with the development of these stones. Consumption of low-calcium diets is associated with a higher risk for the development of kidney stones. This is related to the role of calcium in binding ingested oxalate in the gastrointestinal tract. As the amount of calcium intake decreases, the amount of oxalate absorbed into the blood-stream increases; this oxalate is then excreted in greater amounts into the urine by the kidneys. In the urine, oxalate is a very strong promoter of calcium oxalate precipitation.
2. **Struvite stones:** The formation of struvite (magnesium, ammonium and phosphate) stones is associated with the presence of urea-splitting bacteria, most commonly Proteus mirabilis (but also Klebsiella, Serratia, Providencia species). These organisms are capable of splitting urea into ammonia, decreasing the acidity of the urine and resulting in favorable conditions for the formation of struvite stones. Struvite stones are always associated with urinary tract infections.
3. **Uric acid stones:** The formation of uric acid stones is associated with conditions that cause high blood uric acid levels, such as gout, leukemias/lymphomas treated by chemotherapy (secondary gout from the death of leukemic cells), and acid/base metabolism disorders where the urine is excessively acidic resulting in uric acid precipitation. 5–10% of all stones are formed from Uric acid.
4. **Calcium phosphate stones:** The formation of calcium phosphate stones is associated with conditions such as hyperparathyroidism and renal tubular acidosis.
5. **Cystine stones:** The formation of cystine stones is uniquely associated with people suffering from cystinuria, who accumulate cystine in their urine.

Symptoms: Symptoms of kidney stones include—

- Colicky pain: 'Loin to groin'. Often described as 'the worst pain ever experienced'.
- Hematuria: Blood in the urine, due to minor damage to inside wall of kidney, ureter and/or urethra.

- Pyuria: Pus in the urine.
- Dysuria: Burning on urination when passing stones (rare). More typical of infection.
- Oliguria: Reduced urinary volume caused by obstruction of the bladder or urethra by stone or extremely rarely, simultaneous obstruction of both ureters by a stone.
- Abdominal distention.
- Nausea/vomiting: Embryological link with intestine–stimulates the vomiting center.
- Fever and chills.

Diagnosis: X-ray is the usual method for the diagnosis of renal calculi. Computed tomography (CT or CAT scan), a specialized X-ray, is considered the gold-standard diagnostic test for the detection of kidney stones.

Other investigations typically carried out include—

- Microscopic study of urine which may show proteins, red blood cells, pus cells, bacteria, cellular casts and crystals.
- Culture of a urine sample to exclude urine infection (either as a differential cause of the patient's pain, or secondary to the presence of a stone)
- Blood tests: Full blood count for the presence of a raised white cell count (Neutrophilia) suggestive of infection, a check of renal function and to look for abnormally high blood calcium levels (hypercalcaemia).
- 24 hour urine collection to measure total daily urinary volume, magnesium, sodium, uric acid, calcium, citrate, oxalate and phosphate.
- Catching of passed stones at home (usually by urinating through a tea strainer) for later examination and evaluation by a doctor.

Prevention: Preventive strategies include dietary modifications and sometimes also taking drugs with the goal of reducing excretory load on the kidneys.

- ✓ Drinking enough water to make 2 to 2.5 liters of urine per day.
- ✓ A diet low in protein, nitrogen and sodium intake.
- ✓ Restriction of oxalate-rich foods, such as chocolate, nuts, soybeans, rhubarb and spinach, plus maintenance of an adequate intake of dietary calcium.
- ✓ Taking drugs such as thiazides, potassium citrate, magnesium citrate and allopurinol, depending on the cause of stone formation.
- ✓ Some fruit juices such as orange, blackcurrant and cranberry may be useful for lowering the risk factors for specific types of stones.
- ✓ Avoidance of cola beverages.
- ✓ Avoiding large doses of vitamin C.

GASTRIC FUNCTION TESTS

GI tract function test: The secretory products of gastric juice are—hydrochloric acid secreted by the parietal cells, enzymes (pepsin from pepsinogen secreted by chief cells, rennin and lipase), haemopoietic factor, absence of which is responsible for the development of pernicious anemia and mucus. Gastric function test is done by the following methods—

1. **Gastric secretory activity:** It is undertaken when suspected for duodenal ulcer, where hypersecretion or hyperacidity occurs, gastric cancer wherein hyposecretion or hypoacidity is seen and pernicious anemia in which achlorhydria or absence of secretion is prevalent.

Tubeless gastric function test is based on the principle that when a quininium resin indicator is given orally, then hydrogen ions from the hydrochloric acid in the stomach can liberate quinine ions (AH + cation) at pH below 3. Quinine hydrochloride is formed and is absorbed in the intestine and begins to be excreted in the urine about 15 minutes after the administration of resin. The quinine can be extracted from the urine and determined by measuring the fluorescence in ultraviolet light.

Diagnex blue is one such resin used in the gastric function assessment. The test is carried out in the fasting state. The bladder is emptied and the urine discarded. The tablets of caffeine sodium benzoate are given orally with a glass of water. Urine is passed an hour later and kept as the control specimen. The Diagnex blue granules are then administered orally suspended in a quarter of a glass of water. The bladder is emptied after two hours which gives the test urine from which Azure A (a component used in the preparation of Diagnex blue) is extracted in its blue form. Then the urine is diluted to a standard volume and reduced with ascorbic acid to the colourless form. The colour intensity of the test urine is compared with standards representing 0.6 mg and 0.3 mg of azure A respectively. If the colour intensity of the test urine is equal to or exceeds the 0.6 mg standard, it means that free HCl is present in the stomach. If the colour is between that of the two standards it indicates hypochlorhydria and if it is less than that of the 0.3 mg standard it is evidence of achlorhydria.

2. **Stimulation tests:** The secretory and motor activity of the stomach is studied by stimulation with the agents like pentagastrin, alcohol, caffeine, histamine and insulin. The gastric contents are aspirated at regular intervals pre and post stimulation and estimated for free and total acids in order to conclude hyperchlorhydria, hypochlorhydria or achlorhydria.
3. **Measurement of gastric components:** Total chloride, occult blood, free HCl, lactic acid, bile pigments and peptic activity in the gastric juice are measured to access the normal functions of the stomach.

Pancreas function tests: Pancreas function tests are taken up for diagnosing early chronic pancreatitis, pancreatic calcification and pancreatic cancer as pancreatic function may decrease in pancreatic diseases. A number of tests are used to diagnose pancreas problems, including the following:—

Blood tests: Blood tests can evaluate the function of the gallbladder, liver and pancreas. Levels of the pancreatic enzymes amylase and lipase can be measured. Blood tests can also check for signs of related conditions including infection, anemia (low blood count) and dehydration.

Secretin stimulation test: Secretin is a hormone made by the small intestines. Secretin stimulates the pancreas to release a fluid that neutralizes stomach acid and aids in digestion. The secretin stimulation test measures the ability of the pancreas to respond to secretin. This test may be performed to determine the activity of the pancreas in people with diseases that affect the pancreas (for example, cystic fibrosis or pancreatic cancer).

A tube is pushed down the throat, into the stomach, then into the upper part of the small intestine. Secretin is administered and the contents of the duodenal secretions are aspirated (removed with suction) and analyzed over a period of about two hours.

Fecal elastase test: The fecal elastase test is another test of pancreas function. The test measures the levels of elastase, an enzyme found in fluids produced by the pancreas. Elastase digests (breaks down) proteins. In this test, a patient's stool sample is analyzed for the presence of elastase.

Secretin-pancreozymin test: Pancreatic exocrine function (standard pancreatic function test) is tested by analysing duodenal contents for volume, mean and maximal bicarbonate output, amylase, trypsin, chymotrypsin and lipase concentration following an intravenous injection of secretin and pancreozymin. After an overnight fast two Salem Sump tubes (Argyle) are passed nasogastrically. Under fluoroscopic control, one tube is positioned in the distal second part of the duodenum and the other in the dependent

part of the stomach for continual aspiration of gastric contents. After a 10 minute basal collection which is discarded, an intravenous bolus of secretin 2 U/kg is given, and the duodenal contents aspirated for six consecutive 10 minute periods. After one hour an intravenous bolus of pancreozymin 1.5 U/kg is given and a further two 10 minute collections are made. The volumes of each sample are noted and the bicarbonate, trypsin, chymotrypsin, amylase and lipase concentrations and output analysed on the same day as the test. Trypsin is determined by using benzoyl-arginine amide hydrochloride as the substrate. Chymotrypsin is measured using N-acetyl-L-tyrosine ethyl ester as substrate. Amylase is determined using starch digestion and the result expressed as milligrams of starch digested by one millilitre of pancreatic juice in 30 minutes at 37°C. Lipase is measured by using alpha-naphthyl laurate as substrate and a diazonium salt as the colour reagent. The result is expressed as the number of micromoles of alpha naphthol liberated by one ml of pancreatic juice in three hours at 37°C. The results for volume, mean and maximal bicarbonate output, amylase and lipase are scored from 0 to 2 and the total is used as an arbitrary value for function on a scale from 10 to 0, where 10/10 represents normal function and increasingly abnormal function ranges from 9/10 to 0/10.

Direct test of exocrine pancreatic function: This uses a specific substrate for chymotrypsin. Chymotrypsin hydrolyses the p-aminobenzoic acid (PABA) moiety of an ingested synthetic peptide, N-benzoyl-L-tyrosyl-p-aminobenzoic acid (Bz Ty PABA). This moiety is then rapidly absorbed across the small intestinal mucosa, conjugated in the liver and excreted in the urine. Assuming normal small bowel and hepatic function the percentage recovery of PABA in the urine is proportional to chymotrypsin activity in the upper small bowel, although this is influenced by intraluminal pH.

Pancreolauryl test (PLT): Is one of the pancreatic exocrine function test that distinguishes among all degrees of and helps in detecting patients with moderate pancreatic insufficiency. The basis of this test is that fluorescein dilaurate, after ingestion with a standard breakfast, is hydrolysed by pancreatic cholesterol esterase, releasing fluorescein, which is readily absorbed by the gut, conjugated by the liver, released into the circulation, and excreted into the urine. Most commonly, serum concentrations are measured, which can be done within several hours thereby avoiding prolonged collection of urine and has the same sensitivity and specificity as the urine collection.

The other tests for pancreatic function are Lundh test, faecal chymotrypsin activity, faecal pancreatic elastase and faecal fat excretion test. Amino acid consumption test (AACT) is a non-invasive tubeless test of exocrine pancreatic function.

Computed tomography (CT) scan with contrast dye: This imaging test can help assess the health of the pancreas. A CT scan can identify complications of pancreatic disease such as fluid around the pancreas, an enclosed infection (abscess), or a collection of tissue fluid and pancreatic enzymes (pancreatic pseudocyst).

Abdominal ultrasound: An abdominal ultrasound can detect gall stones that might block the outflow of fluid from the pancreas. It also can show an abscess or a pancreatic pseudocyst.

Endoscopic retrograde cholangiopancreatography (ERCP): In an ERCP, a health care professional places a tube down the throat, into the stomach, then into the small intestine. Dye is used to help the doctor see the structure of the common bile duct, other bile ducts, and the pancreatic duct on an X-ray.

Endoscopic ultrasound: In this test, a probe attached to a lighted scope is placed down the throat and into the stomach. Sound waves show images of organs in the abdomen. Endoscopic ultrasound may reveal gall stones and can be helpful in diagnosing severe pancreatitis when an invasive test such as ERCP might make the condition worse.

Magnetic resonance cholangiopancreatography: This kind of magnetic resonance imaging (MRI) can be used to look at the bile ducts and the pancreatic duct.

Gallbladder function tests: Gallbladder problems are diagnosed using various tests. These might include—

- Liver function tests (LFTs): These are blood tests that can show evidence of gallbladder disease.
- Check of the blood's amylase or lipase levels to look for pancreatic inflammation; amylase and lipase are enzymes produced in the pancreas.
- Complete blood count (CBC): A CBC looks at levels of different types of blood cells, such as white blood cells. A high white blood cell count might indicate infection.
- Ultrasound: Ultrasound testing uses sound waves to image the intra-abdominal organs, including the gallbladder.
- Abdominal X-ray: This test might show evidence of gallbladder disease, such as gallstones.
- Computed tomography (CT): This scan constructs detailed X-ray images of the abdominal organs.
- HIDA scan: During this test, a radioactive material, called hydroxy iminodiacetic acid (HIDA), is injected into the patient. The radioactive material is taken up by the gallbladder which helps to measure gallbladder function. This test also is referred to as cholescintigraphy.
- Magnetic resonance cholangiopancreatography (MRCP): This test uses magnetic resonance imaging (MRI) to produce detailed pictures.
- Endoscopic retrograde cholangiopancreatography (ERCP): This is a procedure during which a tube is placed down the patient's throat into the stomach, then into the small intestine. Dye is injected and the ducts of the gallbladder, liver and pancreas can be seen on X-ray.

Gall bladder scan: A gallbladder scan is a nuclear scanning test that is done to evaluate gallbladder function. It can detect blockage in the tubes (bile ducts) that lead from the liver to the gallbladder and small intestine (duodenum). During a gallbladder scan, a radioactive tracer substance is injected into a vein in the arm. The liver removes the tracer from the bloodstream and adds it to the bile that normally flows through the bile ducts to the gallbladder. The gallbladder then releases the tracer into the beginning of the small intestine. A special camera (gamma) takes pictures of the tracer as it moves through the liver, bile ducts, gallbladder, and small intestine.

HYPERLIPIDEMIA

Hyperlipidemia, hyperlipoproteinemia or dyslipidemia is the presence of raised or abnormal levels of lipids and/or lipoproteins in the blood. Lipids (fatty molecules) are transported in a protein capsule, and the density of the lipids and type of protein determine the fate of the particle and its influence on metabolism.

Plasma lipids are mainly composed of cholesterol, triglycerides, phospholipids and free fatty acids. These are insoluble in water and are carried in the blood stream in combination with a protein. The lipid protein complex is called lipoprotein. Four major classes of lipoproteins have been found in blood, these are (1) Very low density lipoproteins (VLDL), (2) Low density lipoprotein (LDL), (3) High density lipoproteins (HDL) and (4) Chylomicrons. The different chemical and physical characteristics of these lipid-protein aggregates (described in chapter No. 5) allow classification of hyperlipoproteinemias.

Classification: Hyperlipidemias are classified according to the Fredrickson classification which is based on the pattern of lipoproteins on electrophoresis or ultracentrifugation. It was later adopted by the World Health Organization (WHO).

Fredrickson classification of Hyperlipidemias

Hyperlipoproteinemia	Synonyms	Problems	Labs description	Treatment
Type I	Buerger-Gruetz syndrome, Primary hyperlipoproteinaemia, or Familial hyperchylomicronemia	Decreased lipoprotein lipase (LPL) or altered ApoC2	Elevated Chylomicrons	Diet control
Type IIa	Polygenic hypercholesterolaemia or Familial hypercholesterolemia	LDL receptor deficiency	Elevated LDL only	Bile Acid Sequestrants, Statins, Niacin
Type IIb	Combined hyperlipidemia	Decreased LDL receptor and increased ApoB	Elevated LDL, VLDL and Triglycerides	Statins, Niacin, Fibrate
Type III	Familial dysbetalipoproteinemia	Defect in ApoE synthesis	Increased IDL	Drug of choice: Fibrate
Type IV	Endogenous hyperlipemia	Increased VLDL production and decreased elimination	Increased VLDL	Drug of choice: Fibrate, Niacin
Type V	Familial hypertriglyceridemia	Increased VLDL production and decreased LPL	Increased VLDL and Chylomicrons	Niacin, Fibrate

Hyperlipidemia may be attributed to excess cholesterol or triglycerides or both in the plasma, whereas hyperlipoproteinemia (HLP) may be designated to those conditions in which there are excess amounts of one or more of the lipoproteins carrying cholesterol and triglycerides in the blood plasma. HLP can be primary or secondary.

Primary HLP: When the exhausting elevated serum levels of lipoproteins are not due to one of the many known causes. These are generally determined defects in lipid or lipoprotein metabolism which are exaggerated by environmental factors through poorly understood mechanisms.

Secondary HLP: When excess of lipoproteins and their components are present in the plasma due some other disease or disorder. Several such diseases have been identified and included viz. uncontrolled diabetes mellitus, hypothyroidism, nephrotic syndrome, inappropriate dietary habits, biliary tract obstructive disease, pancreatisis etc.

Hyperlipoproteinemia type I: This very rare form (also known as Buerger-Gruetz syndrome, primary hyperlipoproteinaemia, or familial hyperchylomicronemia) is due to a deficiency of lipoprotein lipase (LPL) or altered apolipoprotein C2, resulting in elevated chylomicrons, the particles that transfer fatty acids from the digestive tract to the liver. Lipoprotein lipase is also responsible for the initial breakdown of endogenously made triacylglycerides in the form of very low density lipoprotein (VLDL). As such, one would expect a defect in LPL to also result in elevated VLDL. Its prevalence is 0.1% of the population.

Hyperlipoproteinemia type II: It is further classified into type IIa and type IIb, depending mainly on whether there is elevation in the triglyceride level in addition to LDL cholesterol.

Type IIa Familial hypercholesterolemia: It is characterised by hyperbetalipoproteinemia, which is associated with increased plasma total cholesterol. This may be sporadic (due to dietary factors), polygen-

ic, or truly familial as a result of a mutation either in the LDL receptor gene on chromosome 19 (0.2% of the population) or the ApoB gene (0.2%). The familial form is characterized by tendon xanthoma, xanthelasma and premature cardiovascular disease.

Type IIb (combined hyperlipoproteinemia): The high VLDL levels are due to overproduction of substrates, including triglycerides, acetyl CoA, and an increase in B-100 synthesis. They may also be caused by the decreased clearance of LDL. Prevalence in the population is 10%.

Treatment: Dietary modification is the initial approach, many patients require treatment with statins (HMG-CoA reductase inhibitors) to reduce cardiovascular risk. If the triglyceride level is markedly raised, fibrates may be preferable due to their beneficial effects. Combination treatment of statins and fibrates, while highly effective, causes a markedly increased risk of myopathy and rhabdomyolysis and is therefore only done under close supervision. Other agents commonly added to statins are ezetimibe, niacin and bile acid sequestrants. There is some evidence for benefit of plant sterol-containing products and fatty acids.

Hyperlipoproteinemia type III: This form is due to high chylomicrons and IDL (intermediate density lipoprotein). Also known as broad beta disease or dysbetalipoproteinemia, the most common cause for this form is the presence of ApoE E2/E2 genotype. It is due to cholesterol-rich VLDL (β-VLDL). Prevalence is 0.02% of the population.

Hyperlipoproteinemia type IV: This form is due to high triglycerides. It is also known as hypertriglyceridemia (or pure hypertriglyceridemia). According to the NCEP-ATPIII definition of high triglycerides (>200 mg/dl), prevalence is about 16% of adult population.

Hyperlipoproteinemia type V: This type is very similar to type I, but with high VLDL in addition to chylomicrons. It is also associated with glucose intolerance and hyperuricemia.

Diagnosis:

1. Observation of the serum obtained after overnight fasting and kept at 4°C. This is checked for the presence of supernatant layer of chylomicrons. The intermittent layer is observed for opalescence which indicates the presence of significant amounts of VLDL.
2. Estimation of serum cholesterol and triglycerides in an overnight fasting specimen.
3. Electrophoresis of serum lipoproteins may sometime be required.

Management of hyperlipoproteinemia: Since HLP is frequently associated with disease and is an important risk factor in promoting diseases like ischemic heart disease, its correction seems desirable. Several methods have been tried, these are:—

(a) The treatment of the underlying disease process in secondary HLP.
(b) Dictous treatment with the specific diet to correct the underlying abnormalities specially if these are diet related or aggravated by improper diet.
(c) Treatment with drugs. The most commonly used drugs are—(1) Chlorophenoxy butyrate (clofibrate) and related compounds (2) Nicotinic acid and its esters (3) Bile sequestrants and
(d) Thyroid active substances.

Hyperlipoproteinemia is monitored by regular blood lipid profile tests, wherein the following lipid levels (mg/100 of blood) are indicated.

Element	Optimal	Borderline	High Risk
LDL cholesterol	< 100	130-159	160+
HDL cholesterol	> 60	35-45	< 35
Triglycerides	< 150	150-199	> 200
Total Cholesterol	< 200	200-239	> 240
Cholesterol to HDL ratio	< 4	5	> 6

ATHEROSCLEROSIS

Atherosclerosis is characterised by the deposition of cholesterylesters and other lipids in the connective tissues of the arterial walls. Disease induction is due to prolonged elevated levels of low and very low density lipoproteins in the blood (due to diabetes mellitus, lipid nephrosis, hypothyroidism) and often accompanied by premature or more severe atherosclerosis.

Experiments on the induction of atherosclerosis in animals indicates a wide species variation in susceptibility. In human, atherosclerosis can be induced by feeding cholesterol. Thyroidectomy and treatment with thermical drugs will allow induction of atherosclerosis in some species. Low blood cholesterol is a characteristic of hyperthyroidism.

Natural occurring oils which are beneficial in lowering plasma cholesterol include peanut, cotton seed oil, corn and soya bean oil, where as both fat and coconut oil raise the level. Linoleic acid is absent in butter fat and coconut oil. Glucose and fructose have a greater effect in raising blood lipid than other carbohydrates. A correlation is seen in the increased consumption of sucrose and other sclerosis has been claimed.

Additional factors considered to play a part in atherosclerosis include high blood pressure, obesity, lack of exercise and consumption of soft water as compared to hard drinking water.

Elevation of plasma free fatty acids will also lead to increased VLDL secretion by the liver, involving extra triglycerol and cholesterol output into the circulation.

Several drugs are known which block the formation of cholesterol at the various stages in the biosynthesis pathways. Many of these drugs have harmful effects and it is now considered that direct interference with cholesterol synthesis is to be avoided. Sitosterol is a hypocholestrolemic agent that acts by blocking the absorption of cholesterol in the GIT. Drugs which are considered to increase the fecel excretion of cholesterol and bile acids include dextrothyroxine, neomycin and possibly clofibrate and nicolmic acid.

CANCER

Cancer is disease in which a there is uncontrolled cell growth (growth and division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood).

Medical term for cancer is malignant neoplasm. There are some terms related to abnormal cell growths viz.—

1. **Tumor:** Any abnormal swelling, lump or mass. Some neoplasms, such as leukemia, do not form tumors.
2. **Neoplasm:** It is an abnormal proliferation of genetically altered cells. Neoplasms can be benign or malignant:
   - ✓ Malignant neoplasm or malignant tumor: it is the actual cancer.
   - ✓ Benign neoplasm or benign tumor: a tumor (solid neoplasm) that stops growing by itself, does not invade other tissues and does not form metastases.
3. **Invasive tumor:** Cancer that invades the surrounding tissues.
4. **Pre-malignancy, pre-cancer or non-invasive tumor:** A neoplasm that is not invasive but has the potential to progress to cancer (become invasive) if left untreated. These lesions are, in order of increasing potential for cancer, atypia, dysplasia and carcinoma in situ.

Classification: Cancers are classified by the type of cell that resembles the tumor and therefore, the tissue presumed to be the origin of the tumor. Carcinoma (epithelial cells–breast, prostate, lung and colon

cancer), sarcoma (connective tissue, or mesenchymal cells) Lymphoma and leukemia (Malignancies of hematopoietic i.e. blood-forming cells), Germ cell tumor (totipotent cells–testicle and ovary in adults and in fetuses, babies, and young children most often found on the body midline, particularly at the tip of the tail-bone) Blastic tumor (resembles an immature or embryonic tissue).

Causes: The common cause in all cancers is the acquisition of abnormalities in the genetic material of the cancer cell and its progeny. A substance causing abnormality in the cell leading to cancer is known as carcinogen. The various carcinogens are:—

Chemical carcinogens: Depending upon the type of effect that a chemical compound exerts on a cell the chemical carcinogens are grouped under the following categories—

***Mutagens*:** Substances that cause DNA mutations are known as mutagens. All mutagens do not cause cancers and mutagens that cause cancers are known as carcinogens. Tobacco smoking is associated with lung cancer and bladder cancer. Prolonged exposure to asbestos fibers is associated with mesothelioma.

***Chemicals*:** Alcohol is a chemical carcinogen that promotes cancers through their stimulating effect on the rate of cell mitosis. Faster rates of mitosis leaves less time for repair enzymes to repair damaged DNA during DNA replication, increasing the likelihood of a genetic mistake. A mistake made during mitosis can lead to the daughter cells receiving the wrong number of chromosomes.

***Ionizing radiation*:** Sources of ionizing radiation, such as radon gas, can cause cancer. Prolonged exposure to ultraviolet radiation from the sun can lead to melanoma and other skin malignancies.

***Infectious diseases*:** Many cancers originate from a viral infection. The main viruses associated with human cancers are human papilloma virus, hepatitis B and hepatitis C virus, Epstein-Barr virus, and human T-lymphotropic virus.

The mode of virally-induced tumors can be divided into two, acutely-transforming or slowly-transforming. In acutely transforming viruses, the viral particles carry a gene that encodes for an overactive oncogene called viral-oncogene (v-onc), and the infected cell is transformed as soon as v-onc is expressed. In contrast, in slowly-transforming viruses, the virus genome is inserted, especially as viral genome insertion is an obligatory part of retroviruses, near a proto-oncogene in the host genome. The viral promoter or other transcription regulation elements in turn cause overexpression of that proto-oncogene, which in turn induces uncontrolled cellular proliferation. Because viral genome insertion is not specific to proto-oncogenes and the chance of insertion near that proto-oncogene is low, slowly-transforming viruses have very long tumor latency compared to acutely-transforming viruses, which already carry the viral oncogene.

In addition to viruses, bacteria can also cause cancer for example the chronic infection of the wall of the stomach with Helicobacter pylori causes gastric cancer.

Hormonal imbalances: Some hormones can act in a similar manner to non-mutagenic carcinogens in that they may stimulate excessive cell growth. A well-established example is the role of hyperestrogenic states in promoting endometrial cancer.

Immune system dysfunction: HIV is associated with a number of malignancies, including Kaposi's sarcoma, non-Hodgkin's lymphoma, and HPV-associated malignancies such as anal cancer and cervical cancer. Certain other immune deficiency states ex. common variable immunodeficiency and IgA deficiency are also associated with increased risk of malignancy.

Heredity: A number of cancers are caused due to hereditary causes wherein there is a defective tumor suppressor allele. The examples are breast cancer and ovarian cancer which are due to inherited mutations in the genes BRCA1 and BRCA2.

Other causes: Individuals develop cancer from tumors hiding inside organ transplants.

Pathophysiology: Cancer is a disease of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, genes which regulate cell growth and differentiation must be altered. Genetic

changes can occur at many levels, from gain or loss of entire chromosomes to a mutation affecting a single DNA nucleotide. There are two broad categories of genes which are affected by these changes. Oncogenes may be normal genes which are expressed at inappropriately high levels, or altered genes which have novel properties. In either case, expression of these genes promotes the malignant phenotype of cancer cells. Tumor suppressor genes are genes which inhibit cell division, survival, or other properties of cancer cells. Tumor suppressor genes are often disabled by cancer-promoting genetic changes. Typically, changes in many genes are required to transform a normal cell into a cancer cell.

There is a diverse classification scheme for the various genomic changes which may contribute to the generation of cancer cells. Most of these changes are mutations, or changes in the nucleotide sequence of genomic DNA. Aneuploidy, the presence of an abnormal number of chromosomes, is one genomic change which is not a mutation, and may involve either gain or loss of one or more chromosomes through errors in mitosis.

Large scale mutations involve the deletion or gain of a portion of a chromosome. Genomic amplification occurs when a cell gains many copies (often 20 or more) of a small chromosomal locus, usually containing one or more oncogenes and adjacent genetic material. Translocation occurs when two separate chromosomal regions become abnormally fused, often at a characteristic location. A well known example of this is the Philadelphia chromosome, or translocation of chromosomes 9 and 22, which occurs in chronic myelogenous leukemia, and results in production of the BCR-abl fusion protein, an oncogenic tyrosine kinase.

Small-scale mutations include point mutations, deletions and insertions, which may occur in the promoter of a gene and affect its expression, or may occur in the gene's coding sequence and alter the function or stability of its protein product. Disruption of a single gene may also result from integration of genomic material from a DNA virus or retrovirus, and such an event may also result in the expression of viral oncogenes in the affected cell and its descendants.

Epigenetics: Epigenetics is the study of the regulation of gene expression through chemical, non-mutational changes in DNA structure. The theory of epigenetics in cancer pathogenesis is that non-mutational changes to DNA can lead to alterations in gene expression. Normally oncogenes are silent, for example, because of DNA methylation. Loss of that methylation can induce the aberrant expression of oncogenes, leading to cancer pathogenesis. Known mechanisms of epigenetic change include DNA methylation, and methylation or acetylation of histone proteins bound to chromosomal DNA at specific locations. Classes of medications, known as HDAC inhibitors and DNA methyltransferase inhibitors, can re-regulate the epigenetic signaling in the cancer cell.

Oncogenes: Oncogenes promote cell growth through a variety of ways. Many can produce hormones, a 'chemical messenger' between cells which encourage mitosis, the effect of which depends on the signal transduction of the receiving tissue or cells. In other words, when a hormone receptor on a recipient cell is stimulated, the signal is conducted from the surface of the cell to the cell nucleus to effect some change in gene transcription regulation at the nuclear level. Some oncogenes are part of the signal transduction system itself, or the signal receptors in cells and tissues themselves, thus controlling the sensitivity to such hormones. Oncogenes often produce mitogens, or are involved in transcription of DNA in protein synthesis, which creates the proteins and enzymes responsible for producing the products and biochemicals cells use and interact with.

Mutations in proto-oncogenes, which are the normally quiescent counterparts of oncogenes, can modify their expression and function, increasing the amount or activity of the product protein. When this happens, the proto-oncogenes become oncogenes, and this transition upsets the normal balance of cell cycle regulation in the cell, making uncontrolled growth possible.

Tumor suppressor genes: Tumor suppressor genes code for anti-proliferation signals and proteins that suppress mitosis and cell growth. Generally, tumor suppressors are transcription factors that are activated

by cellular stress or DNA damage. Often DNA damage will cause the presence of free-floating genetic material as well as other signs and will trigger enzymes and pathways which lead to the activation of tumor suppressor genes. The functions of such genes is to arrest the progression of the cell cycle in order to carry out DNA repair, preventing mutations from being passed on to daughter cells. The p53 protein, one of the most important studied tumor suppressor genes, is a transcription factor activated by many cellular stressors including hypoxia and ultraviolet radiation damage. p53 has two functions: one a nuclear role as a transcription factor and the other a cytoplasmic role in regulating the cell cycle, cell division and apoptosis. p53 has been shown to regulate the shift from the respiratory to the glycolytic pathway, thus reducing ATP production required for cell proliferation.

A mutation can damage the tumor suppressor gene itself, or the signal pathway which activates it, 'switching it off'. The invariable consequence of this is that DNA repair is hindered or inhibited: DNA damage accumulates without repair, inevitably leading to cancer. Mutations of tumor suppressor genes that occur in germline cells are passed along to offspring, and increase the likelihood for cancer diagnoses in subsequent generations.

Biological properties of cancer cells: The biological properties of malignant tumor cells are summarized as follows:

- Acquisition of self-sufficiency in growth signals, leading to unchecked growth.
- Loss of sensitivity to anti-growth signals, also leading to unchecked growth.
- Loss of capacity for apoptosis, in order to allow growth despite genetic errors and external anti-growth signals.
- Loss of capacity for senescence, leading to limitless replicative potential (immortality)
- Acquisition of sustained angiogenesis, allowing the tumor to grow beyond the limitations of passive nutrient diffusion.
- Acquisition of ability to invade neighbouring tissues, the defining property of invasive carcinoma.
- Acquisition of ability to build metastases at distant sites, the classical property of malignant tumors (carcinomas or others).
- The completion of these multiple steps would be a very rare event without : loss of capacity to repair genetic errors, leading to an increased mutation rate (genomic instability), thus accelerating all the other changes.

These biological changes are classical in carcinomas; other malignant tumors may not need all to achieve them all. For example, tissue invasion and displacement to distant sites are normal properties of leukocytes; these steps are not needed in the development of Leukemia. The different steps do not necessarily represent individual mutations. For example, inactivation of a single gene, coding for the p53 protein, will cause genomic instability, evasion of apoptosis and increased angiogenesis.

Biochemical effects of cancer:

- The tumour cells behave like foetal cells rather than adult cells (hence fast mitosis).
  - ✓ There is re-expression of foetal antigens like α-feto-protein.
  - ✓ Synthesis of isoenzymes which are characteristic of foetal cells and lacking in adult cells. Ex. A placental type of alkaline phosphatase is found in carcinomas.
- Most cancers elaborate protease and glycosidases which contribute to their invasiveness as well as to, many of the alternation in surface properties.
- There is an increase in cellular respiration mechanism (ETC).
- There is an increased rate of glycolysis.
- Some tumour inducing viruses act as ATPase hydrolyzing ATP to ADP and Pi, thereby lowering the ATP/ADP ratio leading to increased glycolysis and more production of lactic acid.

- The circulating levels of cAMP are decreased whereas the cAMP phosphodiesterase activity is increased.
- Excretion of adrenaline and nor-adrenaline increases.
- A number of polypeptide hormones are produced by tumour tissues.
- Fibro-sarcomas synthesize insulin.
- A primary liver carcinoma produces an aldolase isoenzyme characteristics of muscle.
- There are abnormally high levels of the enzyme "terminal deoxy nucleotidyl transferase (TDT)" in leukemia.
- The levels of acetylcholine, cortisol, histamine and the metabolizing enzymes of catecholamines i.e. plasma dopamine –β-hydroxylase (DβH) are increased in cancers.
- The levels of acetylcholine esterase and catecholamine oxidase are lowered in blood.
- A defective LDH_4 isoenzyme is formed in WBC malignancies.
- Bence Jones proteins are excreted in urine during multiple myeloma.
- There is a remarkable increase in urinary polyamines. They are essential growth factors for human liver. Polyamines interact with nucleic acids and enhance the DNA, RNA and protein synthesis.
- Cancer cells produce excessive mucins (membrane surface glycoproteins), which mask surface antigens on cells and protect the cells from immune surveillance.
- They produce abnormal cell surface glycoproteins which leads to their separation from their parent tissue and migrate to another part of the body (metastasis).

Signs and symptoms: Unusual lumps or swelling (tumor), hemorrhage (bleeding), pain and/or ulceration, enlarged lymph nodes, cough, hemoptysis, hepatomegaly (enlarged liver), bone pain, fracture of affected bones and neurological symptoms, weight loss, poor appetite, fatigue and cachexia (wasting), excessive sweating (night sweats), anemia and specific paraneoplastic phenomena, i.e. specific conditions that are due to an active cancer, such as thrombosis or hormonal changes.

Diagnosis: People with suspected cancer are investigated with medical tests. These commonly include blood tests, X-rays, CT scans and endoscopy.

Treatment: Cancer can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods.

Tumor marker: A tumor marker is a substance found in the blood, urine, or body tissues that can be elevated in cancer, among other tissue types. There are many different tumor markers, each indicative of a particular disease process, and they are used in oncology to help detect the presence of cancer. An elevated level of a tumor marker can indicate cancer; however, there can also be other causes of the elevation.

Description: Tumor markers can be produced directly by the tumor or by non-tumor cells as a response to the presence of a tumor. They can be identified by—

- Screening for common cancers on a population basis, ex. elevated prostate specific antigen suggests prostate cancer.
- Monitoring of cancer survivors after treatment, ex. elevated AFP in a child previously treated for teratoma suggests relapse with endodermal sinus tumor.
- Diagnosis of specific tumor types, particularly in certain brain tumors and other instances where biopsy is not feasible.
- The term tumor antigen is sometimes interchangeably used for tumor marker.

Classification: Tumor markers can be classified in two groups: cancer-specific markers and tissue-specific markers.

Cancer-specific markers: Cancer-specific markers are related to the presence of certain cancerous tissue. Because there is a large overlapping between the many different tumor tissue types and the markers produced by these cancer tissues might not be specific in making a diagnosis. They can however, be useful in the follow-up of treated patients to describe progress of the disease or response to treatment. A few examples of these markers are CEA, CA19-9, CA125.

An example of a cancer-specific marker, CEA, or carcinoembryonic antigen, is a blood-borne protein, first noted to be produced by tumors of the gastrointestinal system. Further investigation showed that it was produced by the occasional lung and breast cancer case, meaning that an elevated level does not mean a bowel cancer. However, in a patient with a history of a treated bowel cancer, a rising CEA level can be an early sign of bowel cancer return. This usually occurs before the site of return can be identified on imaging or examination and so many oncologists question the wisdom of doing a blood test for CEA when the end result is bad news that alarms the patient. Nevertheless, a sequence of steady low CEA readings can provide much needed reassurance to the post-operative patient. Also, a rising sequence of CEA readings should alert the physician to the need for diagnostic tests such as PET scans.

Tissue-specific markers: Tissue-specific markers are related to specific tissues which have developed cancer. Generally, these substances are not specifically related to the tumor, and may be present at elevated levels when no cancer is present. But unlike the previous group, elevated levels point to a specific tissue being at fault. Examples include PSA, beta-HCG–(human chorionic gonadotropin), AFP–(alpha-fetoprotein), AFP-L3–(a lectin-reactive AFP) and thyroglobulin. For example, if man has an elevated PSA, a search for prostate cancer will be undertaken. If an individual has an elevated level of beta-HCG, AFP or AFP-L3%, a search for a testicular or liver cancer respectively, will be made.

PSA (prostate specific antigen): Is produced by the normal prostate. It is a protein enzyme called a serine protease that usually acts as an anticoagulant to keep semen liquid. Only small amounts leak into the circulation in normal circumstances. Enlarged prostates leak more substantial amounts, and cancerous prostates also leak substantial amounts.

β-hCG: Elevated levels cannot prove the presence of a tumor, and low levels do not rule it out (an exception is in males who do not naturally produce β-hCG). Nevertheless, elevated βhCG levels fall after successful treatment (e.g. surgical intervention or chemotherapy), and a recurrence can often be detected by the finding of rising levels.

20

TISSUE AND ORGAN BIOCHEMISTRY

Biological tissue is a collection of interconnected cells that perform a similar function within an organism. In other words it is a group of cells working together mainly inside an organ.

The development of a fertilized egg into a newborn child requires an average of 41 rounds of mitosis ($2^{41} = 2.2 \times 10^{12}$). During this period, the cells produced by mitosis enter different pathways of differentiation; some becoming blood cells, some muscle cells and so on.

There are more than 100 visibly-distinguishable kinds of differentiated cells in the body. These are organized into tissues and the tissues into the organs. Groups of organs make up the various systems of the body namely digestive, excretory, etc.

There are four basic types of tissue in the body of all animals, including the human body and lower multicellular organisms such as insects. These compose all the organs, structures and other contents.

EPITHELIAL TISSUE

Epithelium is a tissue composed of layers of cells that line the cavities and surfaces of structures throughout the body. It is also the type of tissue from which many glands are formed. Epithelium lines both the outside (skin) and the inside cavities and lumen of bodies. The outermost layer of our skin is composed of dead stratified squamous, keratinized epithelial cells.

The epithelial tissue is made of closely packed cells arranged in flat sheets. Epithelia form the surface of the skin, line the various cavities and tubes of the body, and cover the internal organs.

Subsets of epithelial tissue: Mucous membranes lining the inside of the mouth, the oesophagus and part of the rectum are lined by non-keratinized stratified squamous epithelium. Other, open to outside body cavities are lined by simple squamous or columnar epithelial cells.

Other epithelial cells line the insides of the lungs, the gastrointestinal tract, the reproductive and urinary tracts, and make up the exocrine and endocrine glands. The outer surface of the cornea is covered with fast-growing, easily regenerated epithelial cells. Functions of epithelial cells include secretion, absorption, protection, transcellular transport, sensation detection, and selective permeability. Endothelium (the inner lining of blood vessels, the heart and lymphatic vessels) is a specialized form of epithelium. Another type the mesothelium forms the walls of the pericardium, pleurae, and peritoneum.

Function: The function of epithelia always reflects the fact that they are boundaries between masses of cells and a cavity or space.

The epithelium of the skin protects the underlying tissues from—

- Mechanical damage
- Ultraviolet light
- Dehydration
- Invasion by bacteria

The columnar epithelium of the intestine—

- Secretes digestive enzymes into the intestine
- Absorbs the products of digestion from the intestine.

Classification: In humans, epithelium is classified as a primary body tissue, the other ones being connective tissue, muscle tissue and nervous tissue. Epithelium is often defined by the expression of the adhesion molecule e-cadherin, as opposed to n-cadherin, which is used by cells of the connective tissue. Epithelial cells are classified by the following three factors—

Shape (of most superficial cells):

1. **Squamous:** All squamous cells are cells with irregular flattened shape. A one-cell layer of simple squamous epithelium forms the alveoli of the respiratory membrane and the endothelium of capillaries and is a minimal barrier to diffusion. Squamous cells can be found included in the filtration tubules of the kidneys and the major cavities of the body. These cells are relatively inactive metabolically and are associated with the diffusion of water, electrolytes and other substances.
2. **Cuboidal:** As the name suggests, these cells have a shape similar to a cube, meaning its width is of the same size as its height. The nuclei of these cells are usually located in the center. The cuboidal epithelium forms the smallest duct glands and many kidney tubules.
3. **Columnar:** These cells are taller than they are wide. Simple columnar epithelium is made up of a single layer of such cells. The nucleus is also closer to the base of the cell. The small intestine is a tubular organ lined with this type of tissue. Unicellular glands called goblet cells are scattered throughout the simple columnar epithelial cells and secrete mucus. The free surface of the columnar cells have tiny hair-like projections called microvilli. They increase the surface area for absorption.
4. **Transitional:** This is a specialized type of epithelium found lining the organs that can stretch, such as the urothelium that lines the bladder and ureter of mammals. Since the cells can slide over each other, the appearance of this epithelium depends on whether the organ is distended or contracted: if distended, it appears as if there are only a few layers; when contracted, it appears as if there are several layers.

Stratification:

1. **Simple:** There is a single layer of cells.
2. **Stratified:** More than one layer of cells. The superficial layer is used to classify the layer. Only one layer touches the basal lamina. Stratified cells can usually withstand large amounts of stress.
3. **Pseudostratified with cilia:** There is only a single layer of cells, but the position of the nuclei gives the impression that it is stratified.

Specializations:

1. **Keratinized cells:** Contain keratin (a cytoskeletal protein). While keratinized epithelium occurs mainly in the skin, it is also found in the mouth and nose, providing a tough, impermeable barrier.
2. **Ciliated cells:** Have apical plasma membrane extensions composed of microtubules capable of beating rhythmically to move mucus or other substances through a duct. Cilia are common in the respiratory system and the lining of the oviduct.

3. **Mesothelia:** These are derived from mesoderm.
   - ✓ **Pleura:** The outer covering of the lungs and the inner lining of the thoracic cavity.
   - ✓ **Peritoneum:** The outer covering of all the abdominal organs and the inner lining of the abdominal cavity.
   - ✓ **Pericardium:** The outer lining of the heart.
4. **Endothelia:** The inner lining of the heart, all blood and lymphatic vessels are derived from mesoderm.

The basolateral surface of all epithelia is exposed to the internal environment (ECF). The entire sheet of epithelial cells is attached to a layer of extracellular matrix that is called the basement membrane or, better (because it is not a membrane in the biological sense), the basal lamina.

An epithelium also lines our air passages and the alveoli of the lungs. It secretes mucus which keeps it from drying out and traps inhaled dust particles. Most of its cells have cilia on their apical surface that propel the mucus with its load of foreign matter back up to the throat.

System	Tissue	Epithelium
Circulatory	Blood vessels	Simple squamous
Digestive	Ducts of submandibular glands	Stratified columnar
Digestive	Dorsum of tongue	Stratified squamous, keratinized
Digestive	Oesophagus	Stratified squamous, non-keratinised
Digestive	Stomach	Simple columnar, non-ciliated
Digestive	Rectum	Stratified squamous, non-keratinised
Digestive	Anus	Stratified squamous, keratinised
Endocrine	Thyroid follicles	Simple cuboidal
Nervous	Ependyma	Simple cuboidal
Lymphatic	Lymph vessel	Simple squamous
Integumentary	Skin–dead superficial layer	Stratified squamous, keratinized
Integumentary	Sweat gland ducts	Stratified cuboidal
Reproductive—female	Ovaries	Simple cuboidal
Reproductive—female	Fallopian tubes	Simple columnar, ciliated
Reproductive—female	Uterus	Simple columnar, ciliated
Reproductive—female	Vagina	Stratified squamous, non-keratinised
Reproductive—female	Labia majora	Stratified squamous, keratinised
Reproductive—male	Rete testis	Simple cuboidal
Reproductive—male	Epididymis	Pseudostratified columnar
Reproductive—male	Vas deferens	Pseudostratified columnar
Reproductive—male	Ejaculatory duct	Simple columnar
Reproductive—male	Seminal vesicle	Pseudostratified columnar
Respiratory	Larynx–true vocal cords	Stratified squamous, non-keratinised
Respiratory	Trachea	Pseudostratified columnar, ciliated
Respiratory	Respiratory bronchioles	Simple cuboidal
Sensory	Cornea	Stratified squamous, non-keratinised

Sensory	Nose	Pseudostratified columnar
Urinary	Kidney–proximal convoluted tubule	Simple columnar, ciliated
Urinary	Kidney–ascending thin limb	Simple squamous
Urinary	Kidney–distal convoluted tubule	Simple columnar, non-ciliated
Urinary	Kidney–collecting duct	Simple cuboidal
Urinary	Urinary bladder	Transitional
Urinary	Penile urethra	Pseudostratified columnar, non-ciliated
Urinary	External urethral orifice	Stratified squamous

CONNECTIVE TISSUE

As the name suggests, connective tissue holds everything together. It is characterized by the separation of the cells by non-living material, which is called extracellular matrix. Connective tissue is derived from mesoderm and is involved in structure and support.

The cells of connective tissue are embedded in a great amount of extracellular material. This matrix is secreted by the cells. It consists of protein fibers embedded in an amorphous mixture of protein-polysaccharide ('proteoglycan') molecules. Collagen is the main protein of connective tissue in animals and the most abundant protein in mammals, making about 25% of the total protein content.

Blood, cartilage, and bone are usually considered connective tissue, but because they differ so substantially from the other tissues in this class, the phrase 'connective tissue proper' is commonly used to exclude those three. There is also variation in the classification of embryonic connective tissues.

Connective tissue proper:

Supporting connective tissue: Gives strength, support, and protection to the soft parts of the body. This includes:

Cartilage: Ex. The outer ear.

Bone: The human body contains 206 bones of different shapes and sizes. It is a very hard extra cellular matrix and a dynamic tissue. The functions of bone include:

- Maintenance of external form
- Weight bearing support
- Site for attachment of muscles
- Protection of internal organs
- Stores calcium and phosphorus
- Site for hematopoietic system (in the bone marrow)
- Facilitates movements

Composition of bone: Bone is a mineralized connective tissue. Its matrix contains both organic (35%) and inorganic (65%) material. The organic matter is mainly proteins. The inorganic or mineral component is mainly crystalline **hydroxyapatite**, $Ca_{10}(PO_4)_6(OH)_2$ along with sodium, magnesium, carbonate and fluoride ions. 99% body's calcium is in the bone which gives the strength of the bone. The principle proteins found in bone are collagen type I and V. The non collagen proteins include CS-PG I, II and III (chondroitin sulfate—proteoglycan), osteonectin, osteocalcin, osteopontin, bone sialoprotein and bone morphogenetic proteins.

Component of bone are marrow, periosteum, endosteum and bone cells in various stages of development. The cells in the bone tissue are osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts.

Osteoblasts: They are mono-nucleated cells that synthesize most of the proteins (Vitamin D is essential). They are responsible for the deposition of new bone matrix (osteoid) and subsequent mineralization. They control mineralization by the passage of calcium and phosphate ions. The menbranes contain the enzyme ***alkaline phosphatase***, that hydrolyzes organic phosphates to release phosphate ions. During bone formation, the osteoblasts secrete collagen and non-collagen proteins to form the organic matrix. The matrix proteins become insoluble and deposition of calcium and phosphate occurs (mineralization) and become hydroxyapatite. As mineralization proceeds the osteoblasts are surrounded by bone tissue and differentiate into osteocytes.

Osteocytes: They are star shaped cells (most abundant in bone). These cells are networked to each other via long processes that occupy tiny canals called **canaliculi**. These canaliculi exchange nutrients and wastes and maintain bone tissue. Hydroxyapatite, calcium carbonate and calcium phosphate are deposited around these cells.

Osteoclasts: They are multinucleated cells. The osteoclasts play a key role in bone resorption (dissolution of bone). Lysosomal enzymes digest bone matrix proteins at pH 4, and result in dissolution of bone matrix. Hydrogen ions are produced by the action of carbonic anhydrase II. The products of bone resorption, mainly calcium and phosphorus inos are transferred to blood capillaries. Bone formation (Osteoblasts) is stimulated by parathyroid hormones (PTH), vitamin D androgens, growth hormone, insulin and inhibited by cortisol. Bone resorption (Osteoclasts) is stimulated also by parathyroid hormone, thyroid hormones (T3), Cortisol, vitamin D and inhibited by calcitonin estrogens.

Bone disorders

Osteoporosis: There is progressive reduction in bone tissue per unit volume (bone density) causing skeletal weakness and fractures of various bones. Some factors like estrogens and interleukins-1 and 6 are involved in stimulating osteoclasts. Genetic and environmental factors are involved in bone resorption, including poor diet (calcium and vitamin D), smoking, alcohol consumption and lack of exercise.

Osteopetrosis (marble bone disease): Increased bone density due to inability to resorb bone. It is due to the mutation in the gene coding for carbonic anhydrase II (CA II). Thus if CA II is deficient in activity in osteoclasts, normal bone resorption does not occur.

Rickets: Rickets is a childhood disorder characterized by bone deformities due to defective mineralization of bone. Most commonly due to deficiency of vitamin D which stimulates the intestinal absorption of calcium and phosphate.

Osteomalacia: Is seen in adults that results from, demineralization of bone especially in women who have little exposure to sunlight often after several pregnancies. Dietary supplementation with egg, cod liver oil, liver can help recover this problem.

Binding connective tissue: It binds body parts together. This includes:

Tendons: Connect muscle to bone. The matrix is principally collagen, and the fibers are all oriented parallel to each other. Tendons are strong but not elastic.

Ligaments: Attach one bone to another. They contain both collagen and the protein elastin. Elastin permits ligaments to be stretched.

Fibrous connective tissue: It is distributed throughout the body. It serves as a packing and binding material for most of our organs. Collagen, elastin and other proteins are found in the matrix. Fascia is

fibrous connective tissue that binds muscle together and binds the skin to the underlying structures. Adipose tissue is fibrous connective tissue in which the cells have become almost filled with oil. The oil is confined within membrane-bound droplets. The cells of adipose tissue, called adipocytes, secrete several hormones, including leptin and adiponectin. All forms of connective tissue are derived from cells called fibroblasts, which secrete the extracellular matrix.

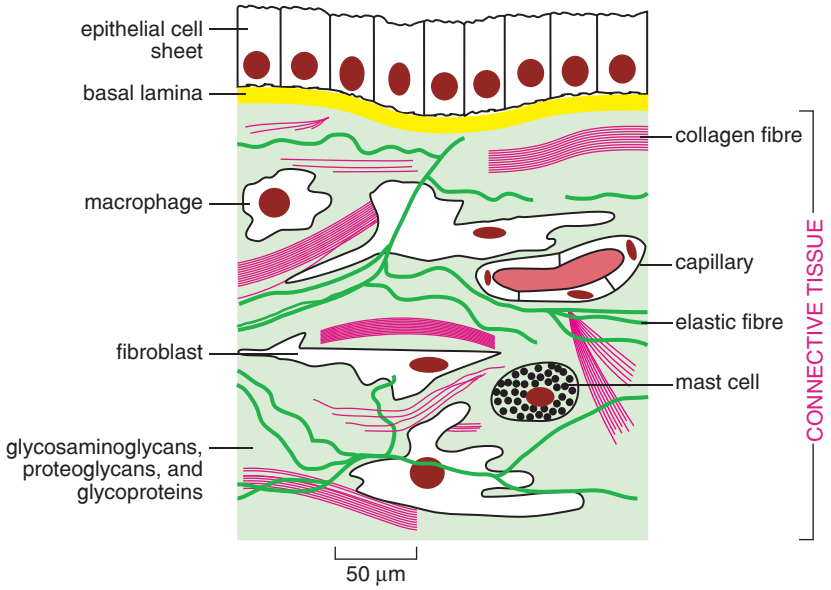
Embryonic connective tissues: There are two types of embryonic connective tissues viz. Mesenchymal connective tissue and mucous connective tissue.

Disorders of connective tissue: The various disorders of the connective tissue which can be both inherited and environmental are—

- **Marfan syndrome:** A genetic disease causing abnormal fibrillin.
- **Loeys-Dietz syndrome:** A genetic disease related to Marfan syndrome, with an emphasis on vascular deterioration.
- **Pseudoxanthoma elasticum:** An autosomal recessive hereditary disease, caused by calcification and fragmentation of elastic fibres, affecting the skin, the eyes and the cardiovascular system.
- **Systemic lupus erythematosus:** A chronic, multisystem, inflammatory disorder of probable autoimmune etiology, occurring predominantly in young women.
- **Fibrodysplasia ossificans progressiva:** Disease of the connective tissue, caused by a defective gene which turns connective tissue into bone.
- **Spontaneous pneumothorax:** Collapsed lung, believed to be related to subtle abnormalities in connective tissue.
- **Sarcoma:** A neoplastic process originating within connective tissue.

Extra Cellular matrix (ECM): The cells of connective tissue are embedded in a great amount of extracellular material (ECM). This matrix is secreted by the cells. The main components of ECM are:

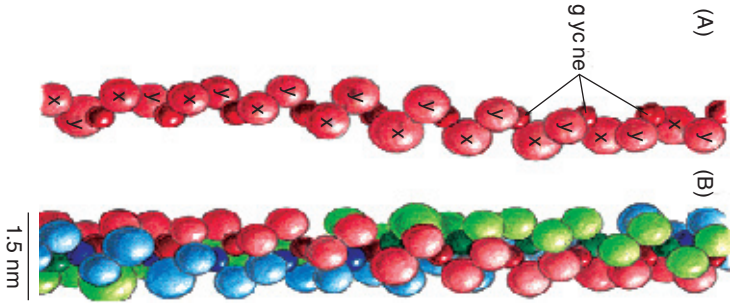
1. Proteins—This includes
   (a) Structural fibrous proteins (collagen and elastin)
   (b) Specialized proteins for cell adhesion (fibrillin, fibronectin & laminin)
2. Proteoglycans—This includes, polysaccharide chains, called glycosaminoglycans (GAGs), often found covalently linked to proteins.



Connective tissue underlying an epithelial cell sheet

Collagens: These are the major component of ECM, constituting 25% of all proteins in mammals. They are distributed in various tissues such as skin, bone, tendon, blood vellels, cornea, cartilage, intervertebral disks and vitreous body as fibril-forming. As a network forming, it is present in basement membranes and beneath squamous equithelium.

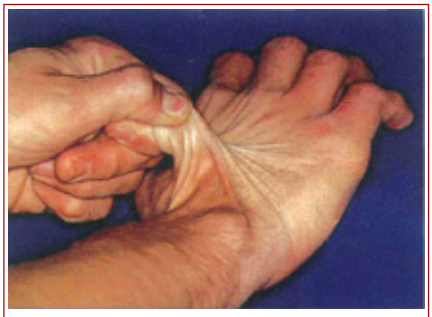
Collagens-Structure: Most collagens are triple helical (three α chains) that are coiled in one another to form a thread like structure (fibril). Each chain is made of 1000 amino acids, rich in glycine and proline. Glycine, the smallest amino acid is found in every third position of the polypeptide chain which enables to form the triple helix. The repeating sequence of the helix is –Gly-X-Y where X is often proline Y is often hydroxy proline or hydroxy lysine (about 100 of each). Proline and hydroxy proline confer the rigidity (strength) on the collagen molecule. Hydroxy proline and hydroxy lysine are formed by posttranslational hydroxylation catalyzed by prolyl hydroxylase and lysyl hydroxylase respectively. Ascorbic acid and α ketoglutarate are cofactors for the enzymes. Some of the hydroxy lysyl residues are modified by the addition of galactose or galactosyl-glucose through O-glycosidic linkage. Collagens are synthesized as pre collagens, then to pro collagen and finally converted to collagens.



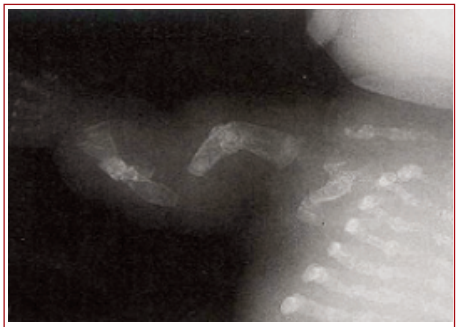
Structure of a typical collagen molecule

Collagen diseases: Some of the common diseases related to collagen of ECM are:

- **Ehlers-Danlos syndrome:** It is a genetic disorder in collagen molecule wherein type-III collagen is deficient. This can result from the deficiency of the enzyme hydroxylase or mutations in the amino acid sequences in collagen synthesis. It results in progressive deterioration of collagens, with different EDS types affecting different sites in the body, such as joints, heart valves, organ walls, arterial walls, etc. Skin and vascular problems are the major symptoms that are expressed.
- **Osteogenesis imperfecta (brittle bone disease):** It is also a group of inherited disorders, caused by insufficient production of good quality collagen to produce healthy, strong bones. It is characterized by bones that easily bend or fracture. Type-I disease presents in the early infancy with fractures and Type-II, is more severe and patients *die* <u>*in utero*</u>. Most patients have mutations in the gene which substitutes bulky side chain amino acids for glycine.



Stretchy skin EDS



Radiograph of a still born fetus with OI type II, in which fractures appear in utero

Elastin: It is a connective tissue protein with rubber-like properties present in the lungs, the walls of large arteries, bladder and elastic ligaments. They can be stretched to several times their normal length, but recoil to their original shape when the stretching force is relaxed.

Elastin is an insoluble protein synthesized from the precursor, tropo elastin which is a single gene. The polypeptide forms a random coil conformation. Pro elastin contains 700 amino acids, primarily glycine, alanine and valine, which are small and nonpolar. It is also rich in proline and lysine but contains little hydroxy proline but no hydroxy lysine. Some of the lysyl residues are oxidatively deaminated to form aldehyde containing allysine residues. Three allysyl residues and one unaltered lysyl form the network called desmosine cross-link and this produces the rubber like elasticity.

Emphysema and α_1 antitrypsin: In the normal lung, the alveoli are exposed to low levels of neutrophil elastase, released from activated and degenerating neutrophils. This proteolytic activity can destry the elastin in the walls of the alveoli of lungs. The most important inhibitor of this elastase is α_1 antitrypsin whose deficiency leads to the destruction of the elastin, caused by the action of elastase and results in a disease called emphysema.

Fibrillin, Fibronectin and Laminin: Fibrillin is a large glycoprotein which is a structural component of microfibrils. **Marfan syndrome** is due to mutation in the gene for fibrillin, which affects lens, skeletal system and cardiovascular system. Fibronectin is an important glycoprotein involved in cell adhesion and cell migration. Laminin is major protein component of renal glomerular and other basal laminas.

Proteoglycans and Glycosaminoglycans (GACs): Glycosaminoglycans are usually linked to proteins to from proteoglycans. GACs occupy large amounts of space and form hydrated gels and resists compression. Their main functions are:

- Act as structural components of the ECM
- Have specific interaction with collagen, elastin, fibronectin, laminin and growth factors
- As polyanions bind to polycations
- Contribute to the characteristic turgor of various tissues
- Act as sieves in the ECM
- Facilitate cell migration
- Have role in compressibility of cartilage in weight-bearing (Hyalyronic acid and Chondroitin sulfate)
- Play role in corneal transparency (Keratin sulfate I and Dermatan sulfate)
- Have structural role in sclera (Dermatan sulfate)
- Act as anticoagulant (Heparin)
- Are components of plasma membrane and act as receptors and participate in cell adhesion and cell to cell interactions (Heparin sulfate)
- Determine charge-selectiveness of renal glomerulus (Heparin sulfate)
- Are components of synaptic and other vesicles

Mucopolysaccharidoses: These are hereditary disorders characterized by the accumulation of GAGs in various tissues, causing skeletal and extra cellular matrix deformities and mental retardation. They are caused due to the deficiency of one of the enzymes in the lysosomal degradation of heparin sulphate and Dermatan sulphate. Examples are Hynter syndrome, Hurler syndrome and Sanfilippo syndrome.

MUSCLE TISSUE

Muscle (from Latin *musculus*, diminutive of *mus* 'mouse') is contractile tissue of the body and is derived from the mesodermal layer of embryonic germ cells. Muscle cells contain contractile filaments that move past each other and change the size of the cell. They are classified as skeletal, cardiac, or smooth muscles. Its function is to produce force and cause motion, either locomotion or movement within internal organs. Cardiac and smooth muscle contraction occurs without conscious thought and is necessary for survival.

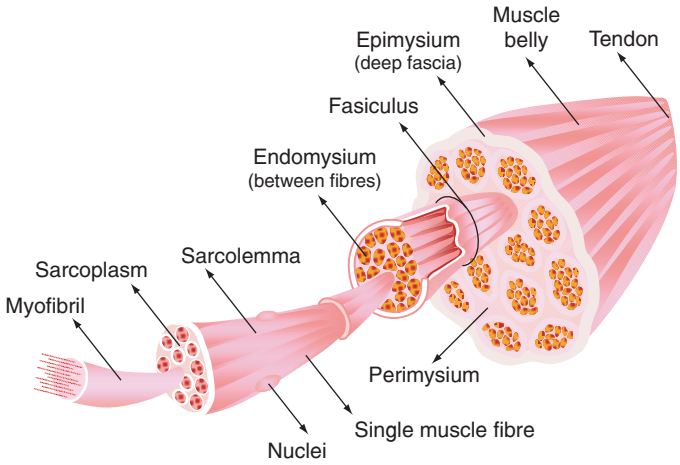
The examples are the contraction of the heart and peristalsis which pushes food through the digestive system. Voluntary contraction of the skeletal muscles is used to move the body and can be finely controlled. The examples are movements of the eye, or gross movements like the quadriceps muscle of the thigh. There are two broad types of voluntary muscle fibers: slow twitch and fast twitch. Slow twitch fibers contract for long periods of time but with little force while fast twitch fibers contract quickly and powerfully but fatigue very rapidly.

Types of muscle: There are three types of muscle—

1. **Smooth muscle or involuntary muscle:** Is found within the walls of organs and structures such as the oesophagus, stomach, intestines, bronchi, uterus, urethra, bladder, blood vessels, and even the skin (in which it controls erection of body hair). Unlike skeletal muscle, smooth muscle is not under conscious control.
2. **Cardiac muscle or involuntary muscle:** It is more akin in structure to skeletal muscle, and is found only in the heart. Cardiac and skeletal muscles are 'striated' i.e. they contain sarcomeres and are packed into highly regular arrangements of bundles; smooth muscle has neither. While skeletal muscles are arranged in regular, parallel bundles, cardiac muscle connects at branching, forming irregular angles (called intercalated discs). Striated muscle contracts and relaxes in short, intense bursts, whereas smooth muscle sustains longer or even near-permanent contractions.
3. **Skeletal muscle or voluntary muscle:** It is anchored by tendons to bone and is used to affect skeletal movement such as locomotion and in maintaining posture. Though this postural control is generally maintained as a subconscious reflex, the muscles responsible react to conscious control like non-postural muscles. The body of an average adult male is made up of 40–50% of skeletal muscle and that of an average adult female is made up of 30-40% (as a percentage of body mass). Skeletal muscle is further divided into several subtypes—
   (1) **Type-I:** Slow oxidative, slow twitch, or 'red' muscle is dense with capillaries and is rich in mitochondria and myoglobin, giving the muscle tissue its characteristic red color. It can carry more oxygen and sustain aerobic activity.
   (2) **Type-II:** Fast twitch muscle, has three major kinds that are, in order of increasing contractile speed:
      (a) **Type-IIa:** Like slow muscle, is aerobic, rich in mitochondria and capillaries and appears red.
      (b) **Type-IIx:** (also known as type-IId)**:** This is less dense in mitochondria and myoglobin. This is the fastest muscle type in humans. It can contract more quickly and with a greater amount of force than oxidative muscle, but can sustain only short, anaerobic bursts of activity before muscle contraction becomes painful (often incorrectly attributed to a build-up of lactic acid).
      (c) **Type-IIb:** This is anaerobically, glycolytic, 'white' muscle that is even less dense in mitochondria and myoglobin. In small animals like rodents this is the major fast muscle type, explaining the pale color of their flesh.

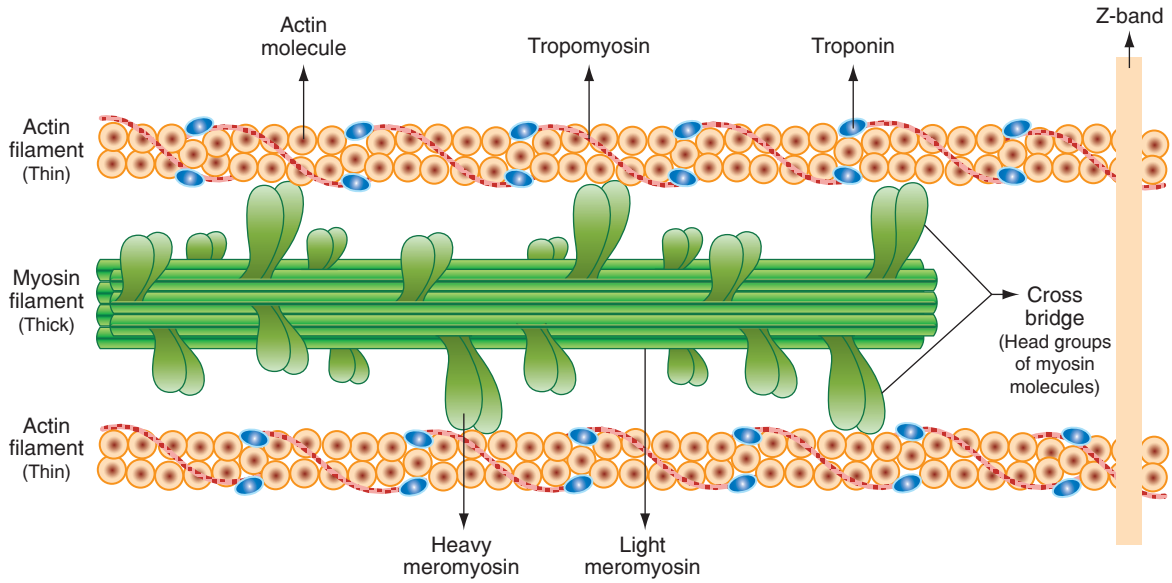
Structure of muscle: Muscle is mainly composed of muscle cells. Within the cells are myofibrils; myofibrils contain sarcomeres, which are composed of actin and myosin. Individual muscle fibres are surrounded by endomysium. Muscle fibers are bound together by perimysium into bundles called fascicles; the bundles are then grouped together to form muscle, which is enclosed in a sheath of epimysium. Muscle spindles are distributed throughout the muscles and provide sensory feedback information to the central nervous system. Skeletal muscle is arranged in discrete muscles, an example of which is the biceps brachii. It is connected by tendons to processes of the skeleton. Cardiac muscle is similar to skeletal muscle in both composition and action, being comprised of myofibrils of sarcomeres, but anatomically

different in that the muscle fibers are typically branched like a tree and connect to other cardiac muscle fibers through intercalated discs, and form the appearance of a syncytium.



Components of muscle

Muscle contraction: The three (skeletal, cardiac and smooth) types of muscle have significant differences. However, all three use the movement of actin against myosin to create contraction. In skeletal muscle, contraction is stimulated by electrical impulses transmitted by the nerves, the motor nerves and motoneurons in particular. Cardiac and smooth muscle contractions are stimulated by internal pacemaker cells which regularly contract and propagate contractions to other muscle cells they are in contact with. All skeletal muscle and many smooth muscle contractions are facilitated by the neurotransmitter acetylcholine.



Diseases of the muscle:

Neuromuscular disease: Symptoms of muscle diseases may include weakness, spasticity, myoclonus and myalgia. Diagnostic procedures that may reveal muscular disorders include testing creatine kinase levels in the blood and electromyography (measuring electrical activity in muscles). In some cases, muscle biopsy may be done to identify a myopathy, as well as genetic testing to identify DNA abnormalities

associated with specific myopathies and dystrophies. Neuromuscular diseases are those that affect the muscles and/or their nervous control. In general, problems with nervous control can cause spasticity or paralysis, depending on the location and nature of the problem. A large proportion of neurological disorders lead to problems with movement, ranging from cerebrovascular accident (stroke) and Parkinson's disease to Creutzfeldt-Jakob disease.

A non-invasive elastography technique that measures muscle noise is undergoing experimentation to provide a way of monitoring neuromuscular disease. The sound produced by a muscle comes from the shortening of actomyosin filaments along the axis of the muscle. During contraction, the muscle shortens along its longitudinal axis and expands across the transverse axis, producing vibrations at the surface.

Atrophy: There are many diseases and conditions which cause a decrease in muscle mass, known as muscle atrophy, ex. Cancer and AIDS, which induce a body wasting syndrome called cachexia. Other syndromes or conditions which can induce skeletal muscle atrophy are congestive heart disease and some diseases of the liver.

During ageing: There is a gradual decrease in the ability to maintain skeletal muscle function and mass, known as sarcopenia. The exact cause of sarcopenia is unknown, but it may be due to a combination of the gradual failure in the 'satellite cells' which help to regenerate skeletal muscle fibers, and a decrease in sensitivity to or the availability of critical secreted growth factors which are necessary to maintain muscle mass and satellite cell survival. Sarcopenia is a normal aspect of ageing, and is not actually a disease state.

Physical inactivity and atrophy: Inactivity and starvation in rodents and mammals lead to atrophy of skeletal muscle, accompanied by a smaller number and size of the muscle cells as well as lower protein content. In humans, prolonged periods of immobilization, as in the cases of bed rest or astronauts flying in space, are known to result in muscle weakening and atrophy. Such consequences are also noted in small hibernating mammals like the golden-mantled ground squirrels and brown bats.

NERVOUS TISSUE

The function of the nervous tissue is to communicate between parts of the body. It is composed of neurons, which transmit impulses, and the neuroglia cells, which assist propagation of the nerve impulse as well as provide nutrients to the neuron. All the nervous tissues constitute the nervous system, which includes the brain, spinal cord and nerves. Nervous tissue is specialized to react to stimuli and to conduct impulses to various organs in the body which bring about response to the stimuli. Nerve tissues (as in the brain, spinal cord and peripheral nerves that branch throughout the body) are all made up of specialized nerve cells called **'neurons'**, bound together by connective tissue. A sheath of dense connective tissue, the epineurium surrounds the nerve. This sheath penetrates the nerve to form the perineurium which surrounds bundles of nerve fibers. Blood vessels of various sizes can be seen in the epineurium. The endoneurium, which consists of a thin layer of loose connective tissue, surrounds the individual nerve fibers. Glial cells are also part of nerve.

Neurons: They function for the conduction of nerve impulses. A typical neuron consists of—

- A cell body which contains the nucleus
- A number of short fibers termed dendrites, extending from the cell body
- A single long fiber, the axon

Classification of neurons: The neurons are classified—

Based upon function as:

(1) **Sensory (or afferent) neurons:** Those that conduct impulses from the sensory organs to the central nervous system (brain and spinal cord) are called sensory (or afferent) neurons.

(2) **Motor (or efferent) neurons:** These conduct impulses from the central nervous system to the effector organs (such as muscles and glands).

(3) **Interneurons:** Those that connect sensory neurons to motor neurons are called interneurons (also known as connector neurons or association neurons).

Based upon structure as:

(1) **Unipolar neurons:** Sensory neurons have only a single process or fibre which divides close to the cell body into two main branches (axon and dendrite). Because of their structure they are often referred to as unipolar neurons.

(2) **Multipolar neurons:** Motor neurons, which have numerous cell processes (an axon and many dendrites), are often referred to as multipolar neurons. Interneurons are also multipolar.

(3) **Bipolar neurons:** Bipolar neurons are spindle-shaped, with a dendrite at one end and an axon at the other. An example can be found in the light-sensitive retina of the eye.

Structure of a motor neuron: A motor neuron has many processes (cytoplasmic extensions) called 'dendrites', which enter a large, grey cell body at one end. A single process, the 'axon', leaves at the other end, extending towards the dendrites of the next neuron or to form a motor endplate in a muscle. Dendrites are usually short and divided while the axons are very long and do not branch freely. The impulses are transmitted through the motor neuron in one direction, i.e. into the cell body by the dendrites and away from the cell body by the axon. The cell body is enclosed by a cell (plasma) membrane and has a central nucleus. Granules called Nissl bodies are found in the cytoplasm of the cell body. Within the cell body, extremely fine neurofibrils extend from the dendrites into the axon. The axon is surrounded by the myelin sheath, which forms a whitish non-cellular fatty layer around the axon. Outside the myelin sheath is a cellular layer called the neurilemma or sheath of schwann cells. The myelin sheath together with the neurilemma is also known as the medullary sheath. This medullary sheath is interrupted at intervals by the nodes of Ranvier.

Neuronal communication: Nerve cells are functionally connected to each other at a junction known as synapse, where the terminal branches of an axon and the dendrites of another neuron lie in close proximity to each other but normally without direct contact. Information is transmitted across the gap by chemical secretions called neurotransmitters. It causes activation in the post-synaptic cell.

The nerve impulse is conducted along the axon. The tips of axons meet other neurons at junctions called synapses, muscles (called neuromuscular junctions) and glands.

Glia: Glial cells surround neurons. Once thought to be simply support for neurons (glia = glue), they turn out to serve several important functions. There are three types—

1. **Schwann cells:** These produce the myelin sheaths that surround many axons in the peripheral nervous system.
2. **Oligodendrocytes:** These produce the myelin sheaths that surround many axons in the central nervous system (brain and spinal cord).
3. **Astrocytes:** These are often star-shaped and the cells are clustered around synapses and the nodes of Ranvier where they perform a variety of functions viz. stimulating the formation of new synapses, modulating the activity of neurons, repairing damage and supplying neurons with materials secured from the blood. [It is primarily the metabolic activity of astrocytes that is being measured in brain imaging by positron-emission tomography (PET) and functional magnetic resonance imaging (fMRI)].

In addition, the central nervous system contains many microglia i.e. mobile cells that respond to damage (e.g. from an infection) by engulfing cell debris.

ORGAN BIOCHEMISTRY

LIVER

The liver is the largest internal organ in the human body. It lies below the diaphragm in the thoracic region of the abdomen. The liver is the largest gland in the body. It plays a major role in metabolism and has a number of functions in the body. Medical terms related to the liver often start with hepato or hepatic from the Greek word for liver, hepar.

Functions of liver: The various functions of the liver are carried out by the liver cells or hepatocytes.

1. The liver performs several roles in carbohydrate metabolism—
   (a) Gluconeogenesis (the synthesis of glucose from certain amino acids, lactate or glycerol).
   (b) Glycogenolysis (the breakdown of glycogen into glucose).
   (c) Glycogenesis (the formation of glycogen from glucose).
   (d) The breakdown of insulin and other hormones.
2. The liver is responsible for the mainstay of protein metabolism, ex. lactic acid is converted to alanine.
3. The liver also performs several roles in lipid metabolism:
   (a) Cholesterol synthesis.
   (b) The production of triglycerides (fats).
4. The liver produces coagulation factors I (fibrinogen), II (prothrombin), V, VII, IX, X and XI, as well as protein C, protein S and antithrombin.
5. The liver breaks down haemoglobin, creating metabolites that are added to bile as pigment (bilirubin and biliverdin).
6. The liver produces and excretes bile (a greenish liquid) required for emulsifying fats. Some of the bile drains directly into the duodenum, and some is stored in the gallbladder.
7. The liver breaks down toxic substances and most medicinal products into less toxic and excretable forms.
8. Ammonia is converted to urea by the liver.
9. The liver stores a multitude of substances, including glucose (in the form of glycogen), vitamin B_{12}, iron, and copper.
10. In the first trimester of pregnancy, the fetal liver is the main site of RBC production. By the 32^{nd} week of gestation, the bone marrow completely takes over this task.
11. The liver is responsible for immunological effects — the reticuloendothelial system of the liver contains many immunologically active cells, acting as a 'sieve' for antigens carried to it via the portal system.
12. The liver produces albumin, the major osmolar component of blood serum.
13. Currently, there is no artificial organ or device capable of emulating all the functions of the liver. Some functions can be emulated by liver dialysis, an experimental treatment for liver failure.

Diseases of the liver: Many diseases of the liver are accompanied by jaundice caused by increased levels of bilirubin in the system. The bilirubin results from the breakdown of the hemoglobin of dead red blood cells; normally, the liver removes bilirubin from the blood and excretes it through bile.

1. **Hepatitis:** Inflammation of the liver, caused mainly by various viruses but also by some poisons, autoimmunity or hereditary conditions.

2. **Cirrhosis:** Is the formation of fibrous tissue in the liver, replacing dead liver cells. The death of the liver cells can for example be caused by viral hepatitis, alcoholism or contact with other liver-toxic chemicals.
3. **Haemochromatosis:** Hereditary disease causing accumulation of iron in the body, eventually leading to liver damage.
4. **Cancer of the liver:** Primary hepatocellular carcinoma or cholangiocarcinoma and metastatic cancers, usually from other parts of the gastrointestinal tract.
5. **Wilson's disease:** Hereditary disease which causes the body to retain copper.
6. **Primary sclerosing cholangitis:** Inflammatory disease of bile duct, likely autoimmune in nature.
7. **Primary biliary cirrhosis:** Autoimmune disease of small bile ducts.
8. **Budd-Chiari syndrome:** Obstruction of the hepatic vein.
9. **Gilbert's syndrome:** A genetic disorder of bilirubin metabolism, found in about 5% of the population.
10. **Glycogen storage disease type-II:** The build-up of glycogen causes progressive muscle weakness (myopathy) throughout the body and affects various body tissues, particularly in the heart, skeletal muscles, liver and nervous system.

There are also many pediatric liver diseases, including biliary atresia, alpha-1 antitrypsin deficiency, alagille syndrome and progressive familial intrahepatic cholestasis.

KIDNEY

The medical field that studies the kidneys and diseases of the kidney is called nephrology. The kidneys are located in the posterior part of the abdomen. There is one on each side of the spine; the right kidney sits just below the liver, the left below the diaphragm and adjacent to the spleen. Above each kidney an adrenal gland (also called the 'suprarenal gland') is present. The asymmetry within the abdominal cavity caused by the liver results in the right kidney being slightly lower in position than the left one while the left kidney is located slightly more medially.

The kidneys are retroperitoneal and range from 9 to 13 cm in diameter; the left being slightly larger than the right. They are approximately at the vertebral level T12 to L3. The upper parts of the kidneys are partially protected by the eleventh and twelfth ribs and each kidney and adrenal gland are surrounded by two layers of fat (the perirenal and pararenal fat) and the renal fascia which help to cushion it. Congenital absence of one or both kidneys, known as unilateral or bilateral renal agenesis, can occur.

Functions of kidney: The kidneys are complex organs that have numerous biological roles. Their primary role is to maintain the homeostatic balance of bodily fluids by filtering and secreting metabolites (such as urea) and minerals from the blood and excreting them, along with water, as urine. Because the kidneys are poised to sense plasma concentrations of ions such as sodium, potassium, hydrogen, oxygen and compounds such as amino acids, creatine, bicarbonate and glucose, they are important regulators of blood pressure, glucose metabolism and erythropoiesis (the process by which red blood cells i.e. erythrocytes are produced).

Excretion of waste products: The kidneys excrete a variety of waste products produced by metabolism, including the nitrogenous wastes – urea (from protein catabolism) and uric acid (from nucleic acid metabolism) and water.

Homeostasis: The kidney is one of the major organs involved in whole-body homeostasis. Among its homeostatic functions are acid-base balance, regulation of electrolyte concentrations, control of blood

volume and regulation of blood pressure. The kidneys accomplish these homeostatic functions independently and through coordination with other organs, particularly those of the endocrine system. The kidney communicates with these organs through hormones secreted into the blood stream.

Acid-base balance: The kidneys regulate the pH of blood by adjusting H^+ ion levels, referred as augmentation of mineral ion concentration and water composition of the blood.

Blood pressure: Sodium ions are controlled in a homeostatic process involving aldosterone which increases sodium ion reabsorption in the distal convoluted tubules.

Plasma volume: Any significant rise or drop in plasma osmolality is detected by the hypothalamus, which communicates directly with the posterior pituitary gland. A rise in osmolality causes the gland to secrete antidiuretic hormone, resulting in water reabsorption by the kidney and an increase in urine concentration and volume. The two factors work together to return the plasma osmolality to its normal levels.

Hormone secretion: The kidneys secrete a variety of hormones, including erythropoietin, urodilatin, renin and vitamin D.

Diseases and disorders:

Congenital: Congenital hydronephrosis, congenital obstruction of urinary tract, duplicated ureter, horse-shoe kidney, polycystic kidney disease, renal dysplasia, unilateral small kidney, multicystic dysplastic kidney.

Acquired: Diabetic nephropathy, glomerulonephritis, hydronephrosis is the enlargement of one or both of the kidneys caused by obstruction of the flow of urine. Interstitial nephritis, kidney stones are relatively common and particularly painful disorders. Kidney tumors (Wilm's tumor, renal cell carcinoma), lupus nephritis, minimal change disease.

- ❖ ***Nephrotic syndrome*:** In this syndrome the glomerulus has been damaged so that a large amount of protein in the blood enters the urine. Other frequent features of the nephrotic syndrome include swelling, low serum albumin and high cholesterol.
- ❖ ***Pyelonephritis*:** It is infection of the kidneys and is frequently caused by complication of an urinary tract infection.
- ❖ ***Renal failure***
- ✓ Acute renal failure
- ✓ Stage 5 chronic kidney disease

BRAIN

The brain controls the central nervous system (CNS), by way of the cranial nerves and spinal cord, the peripheral nervous system (PNS) and regulates virtually all human activity. Involuntary, or 'lower', actions, such as heart rate, respiration and digestion, are unconsciously governed by the brain, specifically through the autonomic nervous system. Complex or 'higher' mental activity, such as thought, reason and abstraction, is consciously controlled.

Anatomically, the brain can be divided into three parts: the forebrain, midbrain and hindbrain; the forebrain includes the several lobes of the cerebral cortex that control higher functions, while the mid and hindbrain are more involved with unconscious, autonomic functions. During encephalization, human brain mass increased beyond that of other species relative to body mass. This process was especially pronounced in the neocortex, a section of the brain involved with language and consciousness. The neocortex accounts for about 76% of the mass of the human brain; with a neocortex much larger than that of other animals,

humans enjoy unique mental capacities despite having a neuroarchitecture similar to that of more primitive species. Basic systems that alert humans to stimuli, sense events in the environment and maintain homeostasis are similar to those of basic vertebrates. Human consciousness is founded upon the extended capacity of the modern neocortex, as well as the greatly developed structures of the brain stem.

Functions of brain: Brain receives signals through nerves arriving from the sensors of the body. These signals are then processed throughout the central nervous system; reactions are formulated based upon reflex and learned experiences. A similarly extensive nerve network delivers signals from a brain to control important muscles throughout the body. Anatomically, the majority of afferent and efferent nerves (with the exception of the cranial nerves) are connected to the spinal cord, which then transfers the signals to and from the brain.

Sensory input is processed by the brain to recognize danger, find food, identify potential mates and perform more sophisticated functions. Visual, touch and auditory sensory pathways are routed to specific nuclei of the thalamus and then to regions of the cerebral cortex that are specific to each sensory system, the visual system, the auditory system and the somatosensory system. Olfactory pathways are routed to the olfactory bulb, then to various parts of the olfactory system. Taste is routed through the brain stem and then to other portions of the gustatory system.

To control movement the brain has several parallel systems of muscle control. The motor system controls voluntary muscle movement, aided by the motor cortex, cerebellum and the basal ganglia. The system eventually projects to the spinal cord and then out to the muscle effectors. Nuclei in the brain stem control many involuntary muscle functions such as heart rate and breathing. In addition, many automatic acts (simple reflexes, locomotion) can be controlled by the spinal cord alone.

Brain also produces a portion of the body's hormones that can influence organs and glands elsewhere in the body; conversely, brain also reacts to hormones produced elsewhere in the body. The hormones that regulate hormone production throughout the body are produced in the brain by the structure called the pituitary gland.

Hormones, incoming sensory information and cognitive processing performed by the brain determine the brain state. Stimulus from any source can trigger a general arousal process that focuses cortical operations to processing of the new information. This focusing of cognition is known as attention. Cognitive priorities are constantly shifted by a variety of factors such as hunger, fatigue, belief, unfamiliar information, or threat. The simplest dichotomy related to the processing of threats is the fight-or-flight response mediated by the amygdala and other limbic structures.

The brain is the source of the conscious, cognitive mind. The mind is the set of cognitive processes related to perception, interpretation, imagination, memories and crucially language, of which a person may or may not be aware. Beyond cognitive functions, the brain regulates autonomic processes related to essential body functions such as respiration and heart beat. The brain controls all movement from lifting a pencil to building a superstructure.

Extended neocortical capacity allows humans some control over emotional behavior, but neural pathways between emotive centers of the brain stem and cerebral motor control areas are shorter than those connecting complex cognitive areas in the neocortex with incoming sensory information from the brain stem. Powerful emotional pathways can modulate spontaneous emotive expression regardless of attempts at cerebral self control.

21 MOLECULAR BIOLOGY

MENDELIAN GENETICS AND INHERITANCE

Biological inheritance is the process by which an offspring cell or organism acquires or becomes predisposed to characteristics of its parent cell or organism. Through inheritance, variations exhibited by individuals can accumulate and cause a species to evolve.

The study of biological inheritance is called genetics. The description of a mode of biological inheritance consists of three main categories—

1. Number of involved loci:

- ➢ Monogenetic (also called 'simple')—one locus
- ➢ Oligogenetic—few loci
- ➢ Polygenetic—many loci

2. Involved chromosomes:

- ❖ Autosomal—Loci are not situated on a sex chromosome
- ❖ Gonosomal—Loci are situated on a sex chromosome
- ❖ X-chromosomal—Loci are situated on the X chromosome (the more common case)
- ❖ Y-chromosomal—Loci are situated on the Y chromosome
- ❖ Mitochondrial—Loci are situated on the mitochondrial DNA

3. Correlation genotype-phenotype:

- ✓ Dominant
- ✓ Recessive
- ✓ Intermediate (also called 'codominant')

These three categories are part of every exact description of a mode of inheritance in the above order. Additionally, more specifications may be added as follows—

1. Coincidental and environmental interactions:

- ✪ Penetrance
- ✪ Incomplete (percentual number)
- ✪ Invariable
- ✪ Heritability (in polygenetic and sometimes also in oligogenetic modes of inheritance)
- ✪ Complete
- ✪ Expressivity
- ✪ Variable
- ✪ Maternal or paternal imprinting phenomena

2. Sex-linked interactions:

- ✧ Sex-linked inheritance (gonosomal loci)
- ✧ Sex-limited phenotype expression (ex. Cryptorchism)
- ✧ Inheritance through the maternal line (in case of mitochondrial DNA loci)
- ✧ Inheritance through the paternal line (in case of Y-chromosomal loci)

3. Locus-locus-interactions:

- ❑ Epistasis with other loci (ex. Overdominance)
- ❑ Gene coupling with other loci
- ❑ Homozygotous lethal factors
- ❑ Semi-lethal factors

Determination and description of a mode of inheritance is primarily achieved through statistical analysis of pedigree data. In case the involved loci are known, methods of molecular genetics can also be employed.

Mendelian inheritance (or Mendelian genetics or Mendelism) is a set of primary tenets relating to the transmission of hereditary characteristics from parent organisms to their children.

Mendel's laws:

Law of segregation: The "Law of Segregation", also known as Mendel's first Law essentially has three parts—

1. **Alternative versions of genes account for variations in inherited characteristics:** This is the concept of alleles. Alleles are different versions of genes that impart the same characteristic. For example, each human has a gene that controls eye color, but there are variations among these genes in accordance with the specific color for which the gene 'codes'.
2. **For each characteristic, an organism inherits two alleles, one from each parent:** This means that when somatic cells are produced from two alleles, one allele comes from the mother and one from the father. These alleles may be the same (true-breeding organisms/homozygous, ex. 'ww' and 'rr' in the figure 3 below), or different (hybrids/heterozygous, ex. 'wr' in figure 3 below).
3. **The two alleles for each characteristic segregate during gamete production:** This means that each gamete will contain only one allele for each gene. This allows the maternal and paternal alleles to be combined in the offspring, ensuring variation.

It is often misconstrued that the gene itself is dominant, recessive, codominant, or incompletely dominant. It is however, the trait or gene product that the allele encodes that is dominant, etc.

Law of independent assortment: The law of independent assortment, also known as "Law of Inheritance" or Mendel's Second Law, states that the inheritance pattern of one trait will not affect the inheritance pattern of another. While his experiments with mixing one trait always resulted in a 3:1 ratio (As shown in figure 1 below) between dominant and recessive phenotypes, his experiments with mixing two traits (dihybrid cross) showed 9:3:3:1 ratios (As shown in figure 2 below). But the 9:3:3:1 table shows that each of the two genes are independently inherited with a 3:1 ratio. Mendel concluded that different traits are inherited independently of each other, so that there is no relation, for example, between a cat's color and tail length. This is actually only true for genes that are not linked to each other.

Independent assortment occurs during meiosis-I in eukaryotic organisms, specifically anaphase-I of meiosis, to produce a gamete with a mixture of the organism's maternal and paternal chromosomes. Along with chromosomal cross-over, this process aids in increasing genetic diversity by producing novel genetic combinations.

Of the 46 chromosomes in a normal diploid human cell, half are maternally derived (from the mother's egg) and half are paternally derived (from the father's sperm). This occurs as sexual reproduction involves the fusion of two haploid gametes (the egg and sperm) to produce a new organism having the full

complement of chromosomes. During gametogenesis, the production of new gametes by an adult the normal complement of 46 chromosomes needs to be halved to 23 to ensure that the resulting haploid gamete can join with another gamete to produce a diploid organism. An error in the number of chromosomes, such as those caused by a diploid gamete joining with a haploid gamete, is termed aneuploidy.

In independent assortment the chromosomes that end up in a newly-formed gamete are randomly sorted from all possible combinations of maternal and paternal chromosomes. Because gametes end up with a random mix instead of a pre-defined "set" from either parent, gametes are therefore considered assorted independently. As such, the gamete can end up with any combination of paternal or maternal chromosomes. Any of the possible combinations of gametes formed from maternal and paternal chromosomes will occur with equal frequency. For human gametes, with 23 pairs of chromosomes, the number of possibilities is 2:23 or 8,388,608 possible combinations. The gametes will normally end up with 23 chromosomes, but the origin of any particular one will be randomly selected from paternal or maternal chromosomes. This contributes to the genetic variability of progeny.

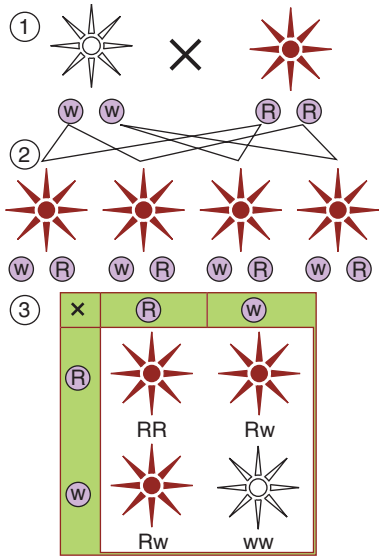


Figure1: Dominant and recessive phenotypes.
(1) Parental generation.
(2) F_1 generation.
(3) F_2 generation. Dominant (red) and recessive (white) phenotype look alike in the F_1 (first) generation and show a 3:1 ratio in the F_2 (second) generation

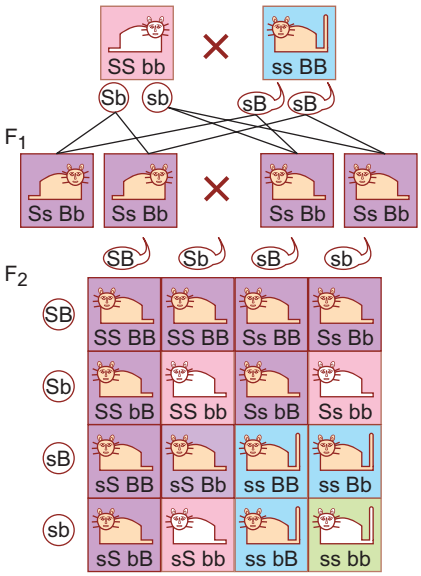


Figure2: The genotypes of two independent traits show a 9:3:1 ratio in the F_2 generation. In this example, coat color is indicated by 'B' (brown, dominant) or 'b' (white) while tail length are homozygous for each trait ('SSbb' and 'ssBB'), their children in the F_1 generation are heterozygous at both loci and only show the dominant phenotypes. If the children mate with each other, in the F_2 generation all combination of coat color and tail length occur: 9 are brown/short (purple boxes), 3 are white/short (pink boxes), 3 are brown/long (blue boxes) and 1 is white/long (green box).

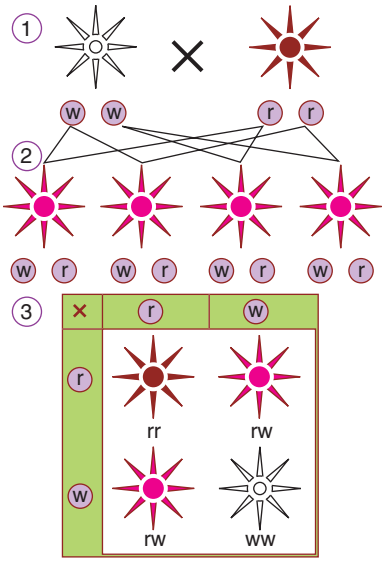


Figure3: The color alleles of *Mirabilis jalapa* are not dominant or recessive
(1) Parental generation.
(2) F_1 generation.
(3) F_2 generation. The 'red' and 'white' allele together make a 'pink' phenotype, resulting in a 1:2:1 ratio of red:pink:white in the F_2 generation.

The reason for these laws is found in the nature of cell nucleus. It is made up of several chromosomes carrying the genetic traits. In a normal cell, each of these chromosomes has two parts, the chromatids. A reproductive cell, which is created in meiosis, usually contains only one of those chromatids of each

chromosome. By merging two of these cells (usually one male and one female), the full set is restored and the genes are mixed. The resulting cell becomes a new embryo. The fact that this new life has half the genes of each parent (23 from mother, 23 from father for total of 46) is one reason for the Mendelian laws. The second most important reason is the varying dominance of different genes, causing some traits to appear unevenly instead of averaging out (whereby dominant doesn't mean more likely to reproduce; recessive genes can become the most common, too).

There are several advantages of this method (sexual reproduction) over reproduction without genetic exchange (asexual reproduction)—

1. Instead of nearly identical copies of an organism, a broad range of offspring develops, allowing more different abilities and evolutionary strategies.
2. There are usually some errors in every cell nucleus. Copying the genes usually adds more of them. By distributing them randomly over different chromosomes and mixing the genes, such errors will be distributed unevenly over the different children. Some of them will therefore have only very few such problems. This somewhat helps reduce problems with copying errors.
3. Genes can spread faster from one part of a population to another. This is for instance useful if there's a temporary isolation of two groups. New genes developing in each of the populations don't get reduced to half when one side replaces the other, they mix and form a population with the advantages of both sides.
4. Sometimes, a mutation (ex. Sickle cell anemia) can have positive side effects (in this case malaria resistance). The mechanism behind the Mendelian laws can make it possible for some offspring to carry the advantages without the disadvantages until further mutations solve the problems.

Mendelian trait: A Mendelian trait is one that is controlled by a single locus and shows a simple Mendelian inheritance pattern. In such cases, a mutation in a single gene can cause a disease that is inherited according to Mendel's laws. Examples include Sickle-cell anemia, Tay-Sachs disease, cystic fibrosis and xeroderma pigmentosa. A disease controlled by a single gene contrasts with a multi-factorial disease, like arthritis, which is affected by several loci (and the environment) as well as those diseases inherited in a non-Mendelian fashion. The Mendelian inheritance in man data-base is a catalog of, among other things, genes in which Mendelian mutants causes disease.

In genetics, dominance describes a specific relationship between the effects of different versions of a gene (alleles) on a trait (phenotype). Animals (including humans) and plants are mostly diploid, with two copies of each gene, one inherited from each parent. If the two copies are not identical (not the same allele), their combined effect may be different than the effect of having two identical copies of a single allele. But if the combined effect is the same as the effect of having two copies of one of the alleles, we say that allele's effect is dominant over the other.

For example, having two copies of one allele of the EYCL3 gene causes the eye's iris to be brown, and having two copies of another allele causes the iris to be blue. But having one copy of each allele leads to a brown iris. Thus the brown allele is said to be dominant over the blue allele (and the blue allele is said to be recessive to the brown allele).

We now know that in most cases a dominance relationship is seen when the recessive allele is defective. In these cases a single copy of the normal allele produces enough of the gene's product to give the same effect as two normal copies, and so the normal allele is described as being dominant to the defective allele. This is the case for the eye color alleles described above, where a single functional copy of the 'brown' allele causes enough melanin to be made in the iris that the eyes appear brown even when paired with the non-melanin-producing 'blue' allele.

Dominance was discovered by Mendel, who introduced the use of uppercase letters to denote dominant alleles and lowercase to denote recessive alleles, as is still commonly used in introductory genetics courses (e.g. 'Bb' for alleles causing brown and blue eyes). Although this usage is convenient it is misleading, because dominance is not a property of an allele considered in isolation but of a relationship between the

effects of two alleles. When geneticists loosely refer to a dominant allele or a recessive allele, they mean that the allele is dominant or recessive to the standard allele.

Geneticists often use the term dominance in other contexts, distinguishing between simple or complete dominance as described above, and other relationships. Relationships described as incomplete or partial dominance are usually more accurately described as giving an intermediate or blended phenotype. The relationship described as codominance describes a relationship where the distinct phenotypes caused by each allele are both seen when both alleles are present.

Nomenclature: Genes are indicated in short-hand by a combination of one or a few letters–for example, in cat coat genetics the alleles Mc and mc (for 'mackerel tabby') play a prominent role. Alleles producing dominant traits are denoted by initial capital letters; those that confer recessive traits are written with lowercase letters. The alleles present in a locus are usually separated by a slash '/'; in the 'Mc' vs 'mc' case, the dominant trait is the 'mackerel-stripe' pattern, and the recessive one the 'classic' or 'oyster tabby' pattern, and thus a classical-pattern tabby cat would carry the alleles 'mc/mc', whereas a mackerel-stripe tabby would be either 'Mc/mc' or 'Mc/Mc'.

Relationship to other genetics concepts: Humans have 23 homologous chromosome pairs (22 pairs of autosomal chromosomes and two distinct sex chromosomes, X and Y). It is estimated that the human genome contains 20,000-25,000 genes. Each chromosomal pair has the same genes, although it is generally unlikely that homologous genes from each parent will be identical in sequence. The specific variations possible for a single gene are called alleles–for a single eye-color gene, there may be a blue eye allele, a brown eye allele, a green eye allele, etc. Consequently, a child may inherit a blue eye allele from the mother and a brown eye allele from the father. The dominance relationships between the alleles control which traits are and are not expressed.

An example of an autosomal dominant human disorder is Huntington's disease, which is a neurological disorder resulting in impaired motor function. The mutant allele results in an abnormal protein, containing large repeats of the amino acid glutamine. This defective protein is toxic to neural tissue, resulting in the characteristic symptoms of the disease. Hence, one copy suffices to confer the disorder. A list of human traits that follow a simple inheritance pattern can be found in human genetics. Humans have several genetic diseases, often but not always caused by recessive alleles.

Punnett square: The genetic combinations possible with simple dominance can be expressed by a diagram called a **'Punnett square'**. One parent's alleles are listed across the top and the other parent's alleles are listed down the left side. The interior squares represent possible offspring, in the ratio of their statistical probability. In an example of flower color, 'P' represents the 'dominant purple-colored allele' and 'p' the 'recessive white-colored allele'. If both parents are purple-colored and heterozygous (Pp), the Punnett square for their offspring would be—

	P	p
P	PP	Pp
p	Pp	pp

In the PP and Pp cases, the offspring is purple colored due to the dominant P. Only in the pp case is there expression of the recessive white-colored phenotype. Therefore, the phenotypic ratio in this case is 3:1, meaning that F2 generation offspring will be purple-colored three times out of four, on average. Dominant alleles are capitalized.

Dominant allele: Dominant trait refers to a genetic feature that hides the recessive trait in the phenotype of an individual. A dominant trait is a phenotype that is seen in both the homozygous 'AA' and heterozygous 'Aa' genotypes. Many traits are determined by pairs of complementary genes, each inherited from a single parent. Often when these are paired and compared, one allele (the dominant) will be found to effectively shut out the instructions from the other, the recessive allele. For example, if a person has one allele for blood type A and one for blood type O, that person will always have blood type A. For a person to have blood type O, both their alleles must be O (recessive).

When an individual has two dominant alleles (AA), the individual is referred to as homozygous dominant; an individual with two recessive alleles (aa) is called homozygous recessive. An individual carrying one dominant and one recessive allele is referred to as heterozygous. A dominant trait when written in a genotype is always written before the recessive gene in a heterozygous pair. A heterozygous genotype is written Aa, not aA.

Types of dominances:

Simple dominance or complete dominance: Consider the simple example of flower color in peas. The dominant allele is purple and the recessive allele is white. In a given individual, the two corresponding alleles of the chromosome pair fall into one of the three patterns—

- Both alleles purple (PP)
- Both alleles white (pp)
- One allele purple and one allele white (Pp)

If the two alleles are the same (homozygous), the trait they represent will be expressed. But if the individual carries one of each allele (heterozygous), only the dominant one will be expressed. The recessive allele will simply be suppressed.

Simple dominance in pedigrees: Dominant traits are recognizable by the fact that they do not skip generations, as recessive traits do. It is therefore quite possible for two parents with purple flowers to have white flowers among their progeny, but two such white offsprings could not have purple offspring (although very rarely, one might be produced by mutation). In this situation, the purple individuals in the first generation must have both been heterozygous (carrying one copy of each allele).

Incomplete dominance: Discovered by Karl Correns, incomplete dominance (sometimes called partial dominance) is a heterozygous genotype that creates an intermediate phenotype. In this case, only one allele (usually the wild type) at the single locus is expressed in a dosage dependent manner, which results in an intermediate phenotype. A cross of two intermediate phenotypes (monohybrid heterozygotes) will result in the reappearance of both parent phenotypes and the intermediate phenotype. There is a 1:2:1 phenotype ratio instead of the 3:1 phenotype ratio found when one allele is dominant and the other is recessive. This lets an organism's genotype be diagnosed from its phenotype without time-consuming breeding tests. The classic example of this is the color of carnations.

	R	R′
R	RR	RR′
R′	RR′	R′R′

R is the allele for red pigment. R' is the allele for no pigment. Thus, RR offspring make a lot of red pigment and appear red. R'R' offspring make no red pigment and appear white. Both RR' and R'R offspring make some pigment and therefore appear pink.

Codominance: In codominance, neither phenotype is recessive. Instead, the heterozygous individual expresses both phenotypes. A common example is the ABO blood group system. The gene for blood types has three alleles: A, B, and i. i causes O type and is recessive to both A and B. The A and B alleles are codominant with each other. When a person has both an A and a B allele, the person has type AB blood.

When two persons with AB blood type have children, the children can be type A, type B, or type AB. There is a 1A:2AB:1B phenotype ratio instead of the 3:1 phenotype ratio found when one allele is dominant and the other is recessive. This is the same phenotype ratio found in matings of two organisms that are heterozygous for incomplete dominant alleles.

Ex. Punnett square for a father with A and i, and a mother with B and i—

	A	i
B	AB	B
i	A	O

Dominant negative: Some gain-of-function mutations are dominant and are called 'dominant negative' or antimorphic mutations. Typically, a dominant negative mutation occurs when the gene product adversely affects the normal, wild-type gene product within the same cell. This usually occurs if the product can still interact with the same elements as the wild-type product, but block some aspect of its function. Such proteins may be competitive inhibitors of the normal protein functions.

Types:

1. A mutation in a transcription factor that removes the activation domain, but still contains the DNA binding domain. This product can then block the wild-type transcription factor from binding the DNA site leading to reduced levels of gene activation.
2. A protein that is functional as a dimer. A mutation that removes the functional domain, but retains the dimerization domain would cause a dominant negative phenotype, because some fraction of protein dimers would be missing one of the functional domains.

Autosomal dominant gene:

Autosomal dominant pedigree chart: An autosomal dominant gene is one that occurs on an autosomal (non-sex determining) chromosome. As it is dominant, the phenotype it gives will be expressed even if the gene is heterozygous. This contrasts with recessive genes, which need to be homozygous to be expressed. The chances of an autosomal dominant disorder being inherited are 50% if one parent is heterozygous for the mutant gene and the other is homozygous for the normal, or 'wild-type', gene. This is because the offspring will always inherit a normal gene from the parent carrying the wild-type genes, and will have a 50% chance of inheriting the mutant gene from the other parent. If the mutant gene is inherited, the offspring will be heterozygous for the mutant gene, and will suffer from the disorder. If the parent with the disorder is homozygous for the gene, the offspring produced from mating with an unaffected parent will always have the disorder.

The term vertical transmission refers to the concept that autosomal dominant disorders are inherited through generations. This is obvious when you examine the pedigree chart of a family for a particular trait. Because males and females are equally affected, they are equally likely to have affected the children. Although the mutated gene should be present in successive generations in which there are more than one or two offspring, it may appear that a generation is skipped if there is reduced penetrance.

Autosomal dominant disorders:

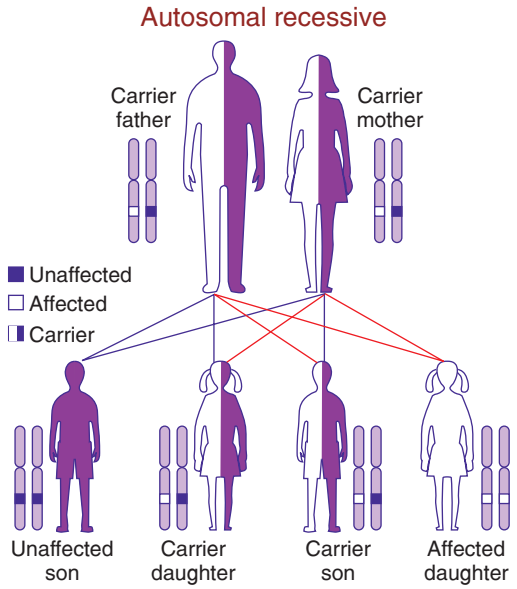
1. Achondroplasia
2. Antithrombin deficiency
3. Autosomal dominant polycystic kidney disease, ADPKD (Adult-onset)
4. BRCA1 and BRCA2 mutations (Hereditary breast ovarian cancer syndrome)
5. Brugada syndrome
6. Charcot-Marie-Tooth syndrome
7. Ectrodactyly
8. Cleft Chin
9. Ehlers-Danlos syndrome
10. Familial hypercholesterolemia
11. Familial adenomatous polyposis
12. Facioscapulohumeral muscular dystrophy
13. Fatal familial insomnia
14. Fibrodysplasia ossificans progressiva
15. Hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu syndrome)
16. Hereditary spherocytosis

17. Huntington's disease
18. Hypertrophic cardiomyopathy
19. Kabuki syndrome (potentially)
20. Lactase persistence
21. Malignant hyperthermia
22. Mandibulofacial dysostosis
23. Marfan syndrome
24. Mowat-Wilson syndrome
25. Multiple endocrine neoplasia
26. Noonan syndrome
27. Neurofibromatosis
28. Osteogenesis imperfecta
29. Pfeiffer syndrome
30. Tuberous sclerosis

Recessive allele: The term 'recessive allele' refers to an allele that causes a phenotype (visible or detectable characteristic) that is only seen in homozygous genotypes (organisms that have two copies of the same allele) and never in heterozygous genotypes. Every diploid organism, including humans, has two copies of every gene on autosomal chromosomes, one from the mother and one from the father. The dominant allele of a gene will always be expressed while the recessive allele of a gene will be expressed only if the organism has two recessive forms. Thus, if both parents are carriers of a recessive trait, there is a 25% chance with each child to show the recessive trait.

The term 'recessive allele' is part of the laws of Mendelian inheritance formulated by Gregor Mendel. Examples of recessive traits in Mendel's famous pea plant experiments include the color and shape of seed pods and plant height.

Autosomal recessive allele: Autosomal recessive is a mode of inheritance of genetic traits located on the autosomes (the pairs of non-sex determining chromosomes—22 in humans).



Relationship between two carrier parents and probabilities of children being unaffected, carriers, or affected

In opposition to autosomal dominant trait, a recessive trait only becomes phenotypically apparent when two similar alleles of a gene are present. In other words, the subject is homozygous for the trait. The frequency of the carrier state can be calculated by the Hardy-Weinberg formula: $p2 + 2pq + q2 = 1$ (p is the frequency of one pair of alleles, and $q = 1 - p$ is the frequency of the other pair of alleles).

Recessive genetic disorders occur when both parents are carriers and each contributes an allele to the embryo, meaning these are not dominant genes. As both parents are heterozygous for the disorder, the chance of two disease alleles landing in one of their offspring is 25% (in autosomal dominant traits this

is higher). 50% of the children (or 2/3 of the remaining ones) are carriers. When one of the parents is homozygous, the trait will only show in his/her offspring if the other parent is also a carrier. In that case, the chance of disease in the offspring is 50%.

Nomenclature of recessiveness: Technically, the term 'recessive gene' is imprecise because it is not the gene that is recessive but the phenotype (or trait). It should also be noted that the concepts of recessiveness and dominance were developed before a molecular understanding of DNA and before development of molecular biology, thus mapping many newer concepts to 'dominant' or 'recessive' phenotypes is problematic.

Many traits previously thought to be recessive have mild forms or biochemical abnormalities that arise from the presence of one copy of the allele. This suggests that the dominant phenotype is dependent upon having two dominant alleles, and the presence of one dominant and one recessive allele creates some blending of both dominant and recessive traits.

Mendel performed many experiments on pea plant (*Pisum sativum*) while researching traits, chosen because of the simple and low variety of characteristics, as well as the short period of germination. He experimented with color ('green' vs. 'yellow'), size ('short' vs. 'tall'), pea texture ('smooth' vs. 'wrinkled'), and many others. By good fortune, the characteristics displayed by these plants clearly exhibited a dominant and a recessive form. This is not true for many organisms.

For example, when testing the color of the pea plants, he chose two yellow plants, since yellow was more common than green. He mated them, and examined the offspring. He continued to mate only those that appeared yellow, and eventually, the green ones would stop being produced. He also mated the green ones together and determined that only green ones were produced.

Mendel determined that this was because green was a recessive trait which only appeared when yellow, the dominant trait, was not present. Also, he determined that the dominant trait would be displayed whether or not the recessive trait was there.

Autosomal recessive disorders: Dominance/recessiveness refers to phenotype, not genotype. An example to prove the point is sickle cell anemia. The sickle cell genotype is caused by a single base pair change in the beta-globin gene, normal is GAG (glu) and sickle is GTG (val).

There are several phenotypes associated with the sickle genotype—

1. Anemia (a recessive trait)
2. Blood cell sickling (co-dominant)
3. Altered beta-globin electrophoretic mobility (co-dominant)
4. Resistance to malaria (dominant)

This example demonstrates that one can only refer to dominance/recessiveness with respect to individual phenotypes.

Other recessive disorders:

1. Albinism
2. Alpha 1-antitrypsin deficiency
3. Autosomal Recessive Polycystic Kidney Disease–ARPKD (Child-onset)
4. Bloom's syndrome
5. Certain forms of spinal muscular atrophy
6. Chronic granulomatous disease
7. Congenital adrenal hyperplasia
8. Cystic fibrosis
9. Dry (also known as 'rice-bran') earwax
10. Dubin-Johnson syndrome
11. Familial Mediterranean fever
12. Fanconi anemia
13. Friedreich's ataxia
14. Galactosemia
15. Glucose-6-phosphate dehydrogenase deficiency
16. Glycogen storage diseases

17. Haemochromatosis types 1-3
18. Homocystinuria
19. Mucopolysaccharidoses
20. Pendred syndrome
21. Phenylketonuria
22. Rotor syndrome
23. Tay-Sach's disease
24. Thalassemia
25. Wilson's disease
26. Xeroderma pigmentosum

Mechanisms of dominance: Many genes code for enzymes. Consider the case where someone is homozygous for some trait. Both alleles code for the same enzyme, which causes a trait. Only a small amount of that enzyme may be necessary for a given phenotype. The individual therefore has a surplus of the necessary enzyme. Let's call this case 'normal'. Individuals without any functional copies cannot produce the enzyme at all, and their phenotype reflects that. Consider a heterozygous individual. Since only a small amount of the normal enzyme is needed, there is still enough enzyme to show the phenotype. This is why some alleles are dominant over others.

In the case of incomplete dominance, the single dominant allele does not produce enough enzymes, so the heterozygotes show some different phenotype. For example, fruit color in eggplants is inherited in this manner. A purple color is caused by two functional copies of the enzyme, with a white color resulting from two non-functional copies. With only one functional copy, there is not enough purple pigment, and the color of the fruit is a lighter shade, called violet.

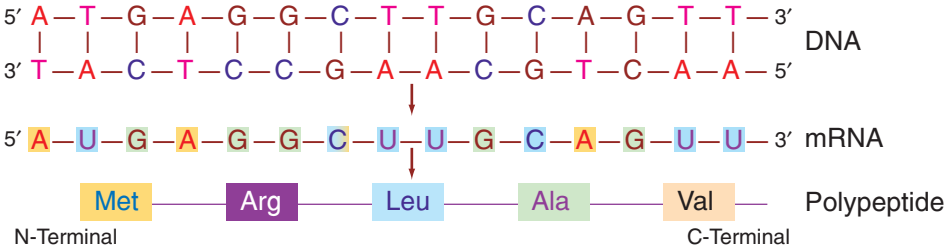
Some non-normal alleles can be dominant. The mechanisms for this are varied, but one simple example is when the functional enzyme E is composed of several subunits where each Ei is made of several alleles Ei = ai1ai2, making them either functional or not functional according to one of the schemes described above. For example one could have the rule that if any of the Ei subunits are nonfunctional, the entire enzyme E is nonfunctional in the sense that the phenotype is not displayed. In the case of a single subunit say E1 is E1 = F where F has a functional and nonfunctional allele (heterozygous individual) (F = a1A1), the concentration of functional enzyme determined by E could be 50% of normal. If the enzyme has two identical subunits (the concentration of functional enzyme is 25% of normal). For four subunits, the concentration of functional enzyme is about 6% of normal (roughly scaling slower than 1/2c where c is the number of copies of the allele-1/24 is about 51% percent). This may not be enough to produce the wild type phenotype. There are other mechanisms for dominant mutants.

Sex-limited genes: Sex-limited genes are genes which are present in both sexes of sexually reproducing species but turned on in only one sex. In other words, sex-limited genes cause the two sexes to show different traits or phenotypes. An example of sex-limited genes are genes which instructs male elephant seal to grow big and fight, at the same time instructing female seals to grow small and avoid fights. These genes are responsible for sexual dimorphism.

INFORMATIONAL MACROMOLECULES

Macromolecule of the cell can, conveniently be defined as, polymers of high molecular weight, assembled from relatively simple precursors. Many of the molecules found within cells, like the polysaccharides, proteins and nucleic acids are macromolecules. Macromolecules have molecular weights from tens of thousands to billions. Proteins have molecular weights ranging from 5000 to over 1 million; the nucleic acids have molecular weights upto several billions; and polysaccharides have molecular weights upto the millions. Macromolecules are produced by the polymerization of relatively small subunits with molecular weights of 500 or less. Macromolecules themselves may be further assembled into supramolecular complexes, membranes and organelles. Out of the three macromolecules, the polysaccharides i.e. polymers made up of a single kind of unit or two different alternating sugar units, serve as energy-yielding fuel stores and as extracellular structural elements, hence are not informational macromolecules. On the other hand the major tools of molecular biology and biotechnology are the proteins and nucleic acids and are known as the informational macromolecules.

Gene: A chromosomal (DNA or RNA in some) segment that codes for a single polypeptide chain or RNA molecule is known as a gene.



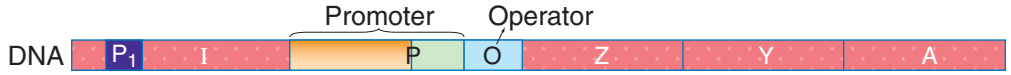
Representation of a gene translated into a polypeptide

One amino acid in a polypeptide chain is coded by a sequence of three consecutive nucleotides of a single strand of DNA (or RNA) and this sequence of three nucleotides (in mRNA) is called the genetic code. Thus the number of nucleotides in a gene for a particular polypeptide will be three times the number of the amino acids present in it. In prokaryotes there are two types of genes—

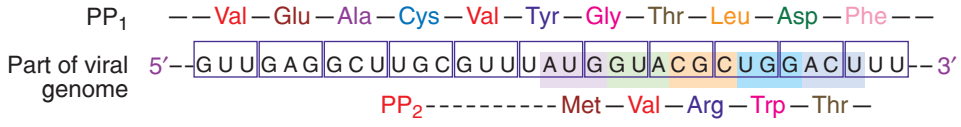
(a) **Structural genes:** Are those which specify the final gene product i.e. a protein or RNA.

(b) **Regulatory genes:** Are those genes which control the structural genes.

All the proteins/RNA are not always required by the cell. Some of the proteins/RNA are required at one time and yet some others at other times. At a given time some are required in lesser quantities and at other times in larger quantities. In order to control the level of these proteins/RNA in the cell, the functioning of structural genes is controlled by the regulatory genes, which composed of three regions called (i) operator site–O, (ii) promoter site–P and (iii) repressor/inhibitor/initiator site–I. Further, a gene, in biological sense is a portion of a chromosome that determines or affects a single character or phenotype.

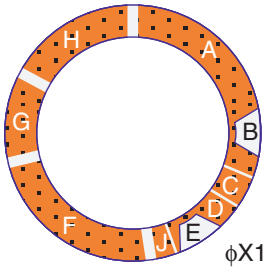


Genome: All the genes and intergenic DNA of an organism or an individual are collectively known as **'genome'**. The genome constitutes the complete chromosomes of an organism. Many virus and bacterial cells have a single chromosome and eukaryotes have many chromosomes. The number of genes in a viral genome like ϕX174 is 9 genes. The *E. Coli* genome (chromosome) is about 4,400 genes and the human genome consists of about 1,00,000 different genes. There is a difference between the viral (particulate) and cellular genome. In virus the genome has genes within genes or overlapping genes i.e. part of one gene will also be a part of another gene.



Gene within gene

Likewise many genes in viral genome share the bases. ϕX174 genome has nine genes (A to J). Gene B is within the sequence of gene A and Gene E is within gene D.



ϕX174 Overlapping genes

In eukaryotes many genes contain intervening non-translated sequences called introns. The translated sequences are called exons. Due to these alternating introns and exons, the co-linear relationship between the nucleotide sequence of the gene and the amino acid sequence of polypeptide it codes, is lost. Introns vary in number, position and fraction of the total length of the gene they occupy. Generally, the introns of a gene are much longer (60-80%) than the exons (20-40%). There are 50 introns in the collagen gene. Likewise ovalbumin has 7 introns and cytochrome-b gene has 4 introns.

Palindromes: A segment of duplex DNA in which the base sequence of the two strands exhibit two fold rotational symmetry about an axis.

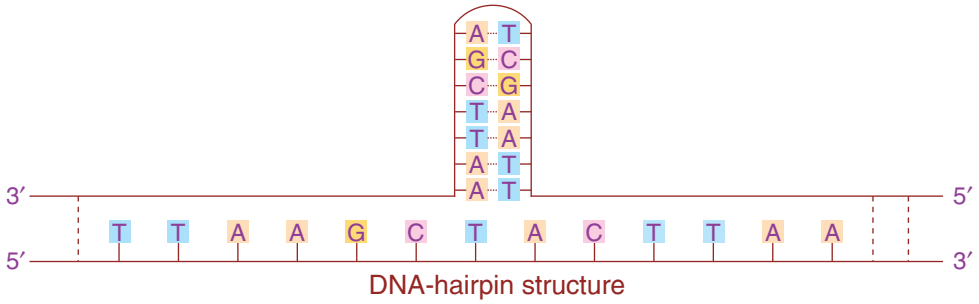
5′ A – A – T – T – C – G – A – T – C – G – A – A – T – T 3′
*
3′ T – T – A – A – G – C – T – A – G – C – T – T – A – A 5′

This is just like a sentence which is spelled the same way, when read forward or backward.

For example—

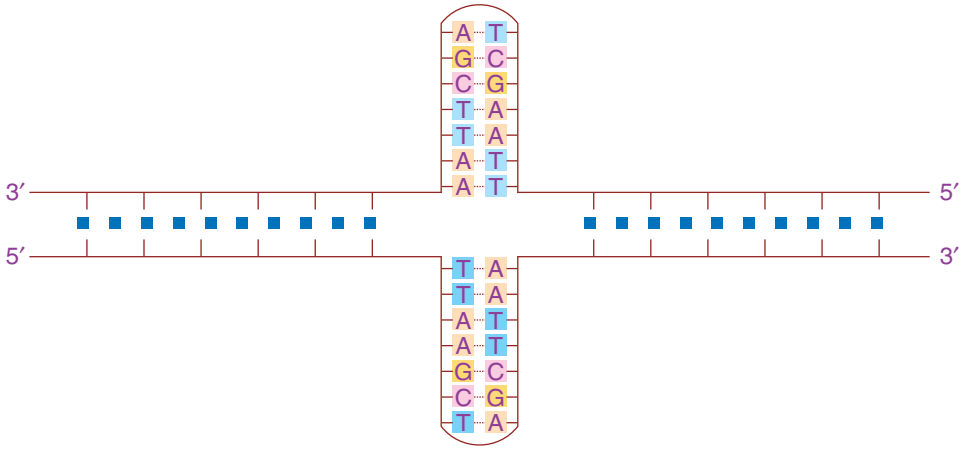
RADAR RADAR	NURSES RUN NURSES RUN	REFER REFER

The palindromic sequences are self-complementary within each of the strands. Due to this complementarity, intra-chain base pairing is possible giving rise to a hairpin or cruciform structure.



DNA-hairpin structure

The same type of hairpin is possible in the other strand also and at the same point of rotational symmetry. This results in a cruciform structure. Many such palindromes occur in each genome. The hairpin and cruciform structures help in functioning of DNA like termination of transcription.

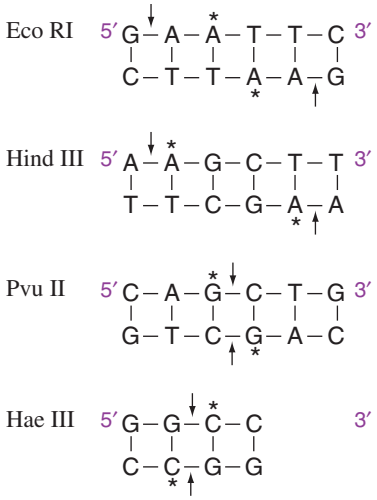


DNA-cruciform structure

Restriction modification/restriction endonucleases: Most of the bacteria have a special type of mechanism which protects its own DNA, whereas destroys foreign DNA i.e. the DNA of invading viruses. This is brought about by a system of complex enzymes called restriction modification system. This system con-

tains two types of enzymes—(a) DNA methylase and (b) restriction endonucleases. Some of the bases in the genome are methylated by the enzyme DNA methylase and thus modifies the nucleotides at specific sequences in a particular bacterium. These sequences are short and palindromic. The restriction endonucleases recognise these sequences and cleave at that very point of those DNA which are not methylated. Thus the cell's own DNA is not cleaved, whereas it recognises and cleaves the foreign DNA. This cleaved DNA is called **"restricted DNA"**.

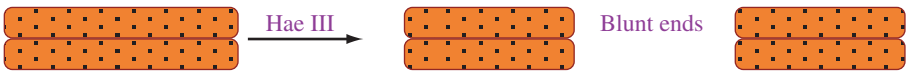
More than 800 restriction endonucleases are discovered so far, in different bacterial species. Each enzyme (in some cases more) can recognise 100 different specific sequences. There are three types of restriction endonucleases designated as type–I, II & III. The name of each enzyme consists of a three letter abbreviation of the bacterial species from which it is derived. The following are the recognition sequences for some type–II restriction endonucleases showing methylated bases (*) and the site of cleavage (↑).



Restriction endonucleases and their recognition sites
Modified bases = * Point of cleavage = ↑

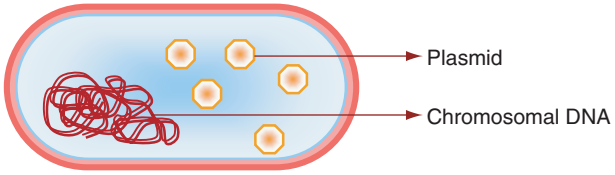
Note that by the action of *Eco*RI and *Hin*dIII, the resulting DNA will be in two pieces, such that there are 2 to 4 nucleotides of one strand unpaired at each resulting end. These are referred to as cohesive ends or sticky ends, because they can base pair with each other or with complementary sticky ends of other DNA fragments.

On the other hand the action of PvuII and HaeIII, cleaves both strands of DNA resulting in no unpaired bases. These are called blunt ends.



Restriction endonucleases are the key tools of recombinant DNA technology and thereby of Biotechnology.

Plasmids: Plasmids are extrachromosomal, independently replicating small circular DNA molecules. Plasmids are found in yeast, other fungi and in bacteria.



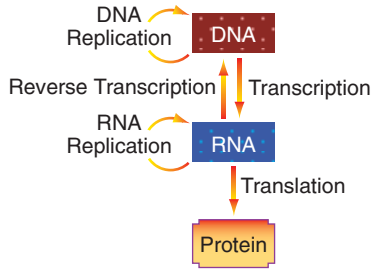
Bacterial cell showing plasmids

Naturally occurring bacterial plasmids range in size from 5000 to 400000 base pairs. The number of plasmid in each cell may be one or more than one (10 to 50 copies/cell). Generally, the plasmids remain

separately and detached from the chromosomal DNA. Plasmids carry genetic information and undergo replication to yield daughter plasmids, which pass into the daughter cells at cell division. Plasmids do not have any role in the physiological functions of the organism in which they exist. Plasmids carry the gene for making the bacterium resistant to antibacterial agents (antibiotics), ex. plasmids carry gene for the enzyme beta-lactamase and thus makes that bacterium resistant to beta lactum antibiotics like amoxicillin and penicillin. These plasmids can also pass from an antibiotic resistant cell to an antibiotic sensitive cell of the same or another bacterial species, thus making the other cell also resistant. Plasmids are very useful tools of modern biotechnology.

CENTRAL DOGMA OF MOLECULAR BIOLOGY

The organizing principle of molecular biology is known as **'central dogma'**. The expression of the template (message) upon which life is based is that of assembling the nucleic acids with correct sequences of nucleotides and proteins with that of amino acids. The central dogma constitutes the flow of genetic information from the DNA to RNA to protein.



Flow of genetic information

The central dogma of molecular genetics defines five processes in the various cellular mechanisms of utilization of genetic information.

DNA replication: The copying of parental DNA to form daughter DNA molecules having dentical nucleotide sequences is known as DNA replication.

Transcription: Parts of the genetic message in DNA is copied in the form of RNA by the process called transcription.

Translation: The genetic message coded in mRNA is translated to the language of proteins with a specific sequence of amino acids.

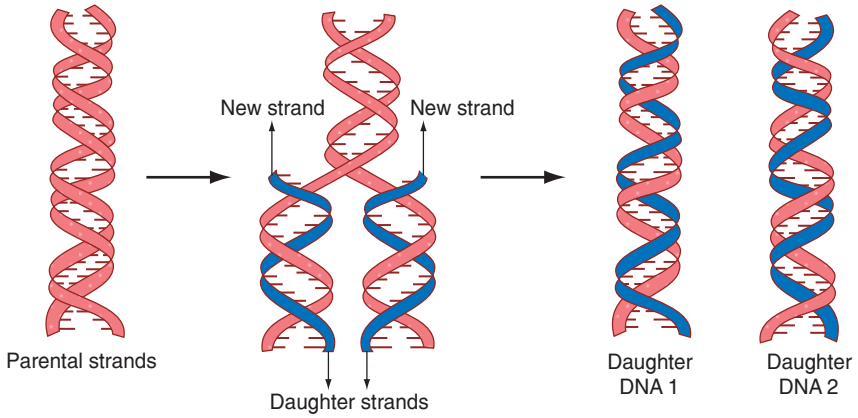
RNA replication: Some *E.Coli* bacteriophages like f_2, MS_2, R_{17} and Qb have RNA genomes and these RNA are replicated forming identical daughter RNA by the enzyme RNA-directed-RNA-polymerase or RNA replicase.

Reverse transcription: In certain RNA viruses, there is an enzyme called reverse transcriptase, which in the host cell synthesises a copy of DNA complementary to its RNA. This is known as reverse transcription. The DNA so formed gets incorporated into the host cell DNA and is transcribed and replicated with the host cell DNA.

DNA REPLICATION

Synthesis of a daughter duplex DNA molecule, identical to the parental duplex DNA is known as replication.

DNA replication is semi-conservative i.e. one of the each parental strand of DNA is present in each of the two daughter DNAs.



Semi-conservative DNA replication

DNA replication requires more than 20 enzymes and factors, which are collectively called DNA replicase system or **'replisome'**.

Steps of DNA replication: The various steps of DNA replication are—

(a) Recognition of the origin
(b) Unwinding of the parental duplex
(c) Holding the template strands apart
(d) Initiation of the new daughter strands
(e) Elongation
(f) Rewinding

Chromosomes contain an origin of nucleotide sequence of about 100 to 300 (10-20 in viral DNA) base pairs, where replication begins and which is recognised by specific cell proteins. There are many such origins in each DNA.

Enzymes known as helicase unwind DNA just at the origin of replication fork. This requires energy in the form of ATP. As soon as a short sequence has been unwound, some DNA binding proteins (DBP or SSP) bind to the strands, thereby keeping them apart. Further in order to prevent the complete duplex DNA from unwinding an enzyme known as 'DNA gyrase' (topoisomerase) puts a knot a little away from the replication fork.

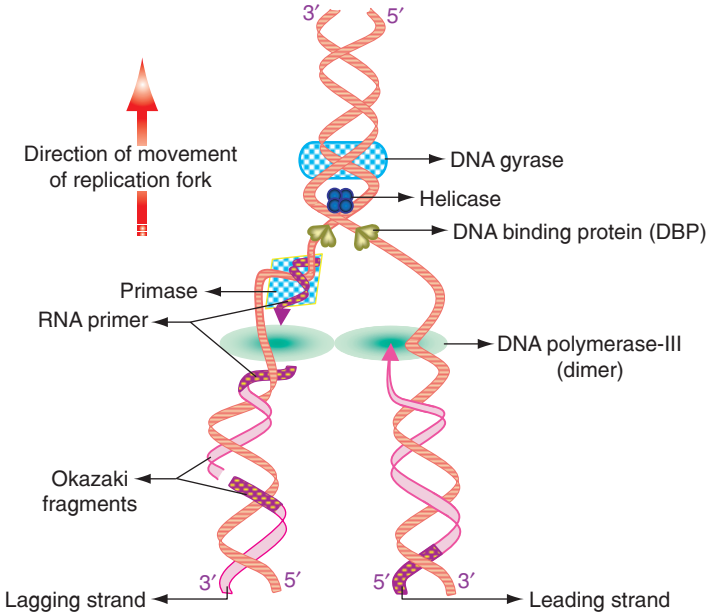
The initiation of replication requires a short length of RNA as a primer, which is complementary to DNA short length. This RNA is made in the 5′→3′ direction by an enzyme primase, contained in a complex called 'primosome'. To the 3′ end of this short single stranded RNA primer, the deoxyribonucleotides, complementary to the other DNA strand, known as 'template strand', are added by the enzyme DNA polymerase-III.

Both the strands of DNA are elongated at the same time. But because of the specificity of DNA polymerase-III, which can add nucleotides only to the free 3′ end of the chain (i.e. 5′→3′ direction elongation), only one of the two strands of DNA can be continuously elongated and this strand is known as leading strand, (in which 5′→3′ synthesis proceeds in the direction as replication fork moves). The other strand is made in small pieces known as 'okazaki fragments' and this strand is known as lagging strand (in which 5′→3′ synthesis proceeds in the direction opposite to the direction of fork movement.) A travelling protein machine called primosome, moves along the lagging strand in the direction of the movement of the replication fork. Primosome contains 7 different proteins, one of which is the enzyme primase, which synthesises the RNA primer of each Okazaki fragment in the 3′→5′ direction to which deoxyribonucleotides are added at the 3′ end by DNA polymerase-III in the 5′→3′ direction. The synthesis of leading and lagging strands is actually coupled i.e. synthesis is carried out concurrently on both the leading and lagging strands by a single dimeric DNA polymerase-III.

Then the RNA primer is removed, nucleotide by nucleotide by an enzyme DNA polymerase-I, due to which ribonucleotide units are removed and they are replaced with complementary deoxyribonucle-

otides, using the 3' end of the preceding Okazaki fragment as primer. The various Okazaki fragments are then joined together at the nick, by the enzyme DNA ligase.

When both the strands are completely replicated then the DNA binding proteins (SSP) are released and each of the two strands rewinds separately, each having one new strand and one parental strand (semi-conservative). Thus this terminates the process of replication.



Mechanism of DNA replication

More about DNA replication:

1. Replication begins at an origin and proceeds bi-directionally.
2. There is a single origin in the prokaryotes (circular) and many origins in eukaryotic DNA.
3. The point of origin called ori C is 245 base pairs in prokaryotes and 300 base pairs in eukaryotes. These sequences are highly conserved bases particularly rich in A=T base pairs.
4. In prokaryotes there are three enzymes for DNA replication namely DNA polymerase-I, DNA polymerase-II and DNA polymerase-III.
5. DNA polymerase-III is the main enzyme for DNA replication. DNA polymerase-II repairs DNA damaged by ultraviolet rays and chemicals. DNA polymerase-I is involved in removing the RNA primer and replacing deoxyribonucleotides in its place. It also proof reads the bases added in the new strand and if any wrong base is added, it removes it and replaces it by the proper base.
6. Chemically, DNA polymerase II and III are oligomeric proteins whereas DNA polymerase-I is monomeric, but by mild protease treatment a particular structural domain can be removed and this domain possesses the 5′→3′ exonuclease activity (which removes RNA primer at the terminal end) and the remaining other large fragment called the Klenow fragment has got the polymerization and proof reading activity.
7. In eukaryotes the enzymes of replication are known as DNA polymerase alpha (which is similar in structure and function to the DNA polymerase-I of the prokaryotes), DNA polymerase delta (similar to DNA polymerase-III) which works in association with a protein called proliferating cell nuclear antigen (PCNAP) and DNA polymerase epsilon (similar to DNA polymerase-II).
8. DNA replication in some viral circular DNA is by rolling circular model and hence is unidirectional.
9. All the enzymes and proteins involved in DNA replication are known as DNA replicase system or replisome.

Enzymes and proteins contained in a replisome

Protein	Mol. wt.	No. of subunits	Function
1. DNA A protein	50,000	1	Opens duplex at specific origin sites
2. DNA B protein (helicase)	300,000	6	Unwinds DNA–primosome constituent
3. Rep (helicase)	65,000	1	Unwinds DNA–primosome constituent
4. DNA helicase-II	75,000	1	Unwinds DNA–primosome constituent
5. DNA C protein	29,000	1	Required for DNA 'B' binding at origin–primosome constituent
6. Hu	19,000	2	Histone like protein; stimulates initiation
7. SSB (RFA & RFC in eukaryotes)	75,000	4	Binds single stranded DNA
8. DNA T proteins	66,000	3	Primosome constituent
9. Protein *n*	28,000	2	Primosome assembly and function
10. Protein *n*′	76,000	1	Primosome constituent
11. Protein *n*″	17,000	1	Primosome constituent
12. Primase (DNA G protein)	60,000	1	Synthesizes RNA primers–primosome constituent
13. RNA polymerase	454,000	6	Facilitates DNA 'A' activity
14. DNA topoisomerase-II (Gyrase)	400,000	4	Supercoiling (relieves torsional strain generated by DNA unwinding)
15. DNA topoisomerase-I	100,000	4	Relaxing negative supercoils
16. DNA polymerase-III (DNA polymerase-δ)	900,000	2 × 10	Chain elongation
17. DNA polymerase-I	103,000	1	Excision of primers, filling of gaps, proof reading

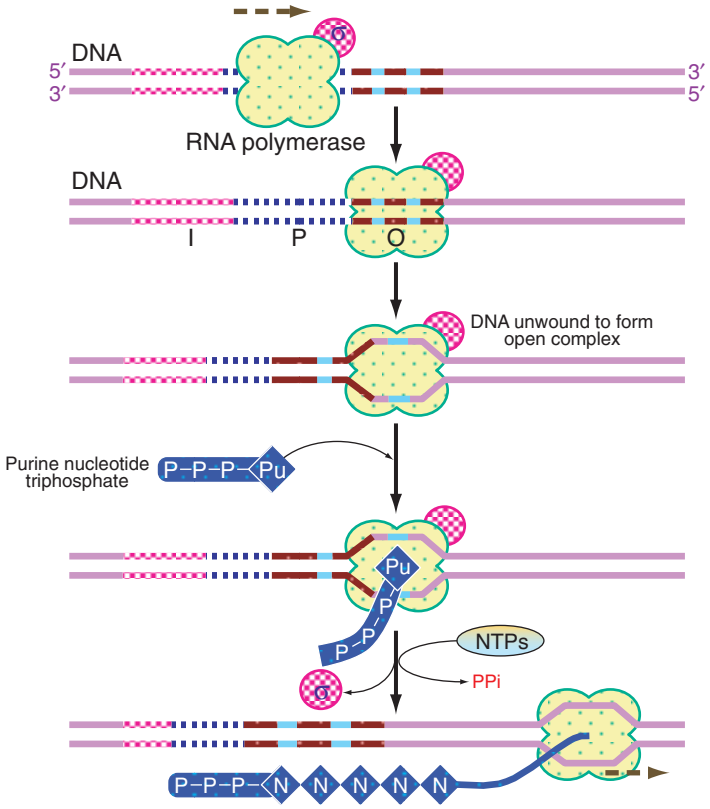
TRANSCRIPTION

Transcription is a process of enzymatic synthesis of RNA strand having a base sequence complementary to a part of one of the strands of DNA. Three types of RNA are formed by transcription—(a) mRNA (b) tRNA and (c) rRNA. During transcription, the complete DNA strand is not copied but only a part of it called the gene or group of related genes are transcribed.

The genes consist of mRNA, tRNA, rRNA, regulatory sequences, leaders, spacers and tails. Messenger RNAs code for polypeptide chains. If a single mRNA molecule codes for a single polypeptide, then it is known as monogenic or monocistronic and if it codes for two or more different polypeptides then it is known as polygenic or polycistronic mRNA. One amino acid in a polypeptide is coded by three nucleotide bases. The DNA sequence is colinear to the amino acid sequence in the virus and some bacteria, whereas in the eukaryotes there is no co-linearity between the DNA sequences in the gene to the amino acid sequence in a protein, as the mRNA transcribed contains the needed bases, some leader sequences at the 5′ end and some intergenic spacers in polygenic mRNA.

RNA synthesis is initiated at specific sequence in the DNA called promoters, which directs the transcription of adjacent segments of DNA (gene). These sequences are 10 to 30 base pairs away from the point where RNA synthesis actually begins. The enzyme for transcription i.e. capable of forming RNA polymer is called DNA-directed-RNA-polymerase and this enzyme binds to the promoter site, forming the 'closed complex'. The DNA is then unwound to about 17 base pairs, exposing the template strand at the initiation site. This enables the RNA polymerase to bind more tightly forming an 'open complex'. The RNA

polymerase moves along the helix during which it unwinds the DNA ahead and rewinds it behind. Some DNA binding proteins prevent complete unwinding of the DNA helix.



Mechanism of transcription

RNA polymerase requires DNA template, all four ribonucleotide 5′ triphosphates (ATP, UTP, CTP, GTP), Mg^{2+} and contains Zn^{2+}. It does not need any primer and the elongation is in the 5′→3′ direction i.e. RNA is copied in the 3′→5′ direction of one of the two DNA strands (template). Each nucleotide in the newly formed RNA is selected by Watson-Crick base pairing interactions i.e. uridylate (U) is added in RNA opposite to adenylate (A) in DNA template, A opposite to T, G opposite to C and C opposite to G.

RNA synthesis usually starts with a GTP or ATP residue, whose 5′-triphosphate group is not cleaved but remains intact. During transcription the new RNA strand base pairs temporarily with the DNA template to form a short length of hybrid RNA-DNA double helix, which 'peels off' immediately after completion of transcription. RNA synthesis proceeds until the RNA polymerase meets a specific self complementary sequence in template DNA which forms a hairpin due to which DNA-RNA hybrid formation is not possible resulting in the release of the RNA. The other signal for termination is a protein called ρ (rho) which interrupts transcription by preventing the further movement of the enzyme and this terminates transcription.

In eukaryotes there are three RNA polymerases; namely I, II and III. Each synthesizes a different type of RNA viz.—

RNA polymerase-I synthesizes 18S, 5.8S and 28S rRNAs

RNA polymerase-II synthesizes mRNAs

RNA polymerase-III synthesizes tRNAs and 5S rRNAs

Inhibition of transcription: RNA polymerase is inhibited by—

(a) Actinomycin-D, which intercalates into DNA double-helix between successive G ≡ C base pairs that affect the movement of the enzyme along the template and thus jams and zipper.

(b) Acridine also inhibits like actinomycin-D.

(c) Rifampicin binds to the beta-subunit of RNA polymerase (RNA polymerase is an oligomeric protein with 6 subunits viz. two alpha, two beta, one delta and one omega subunit) and thus prevents initiation.

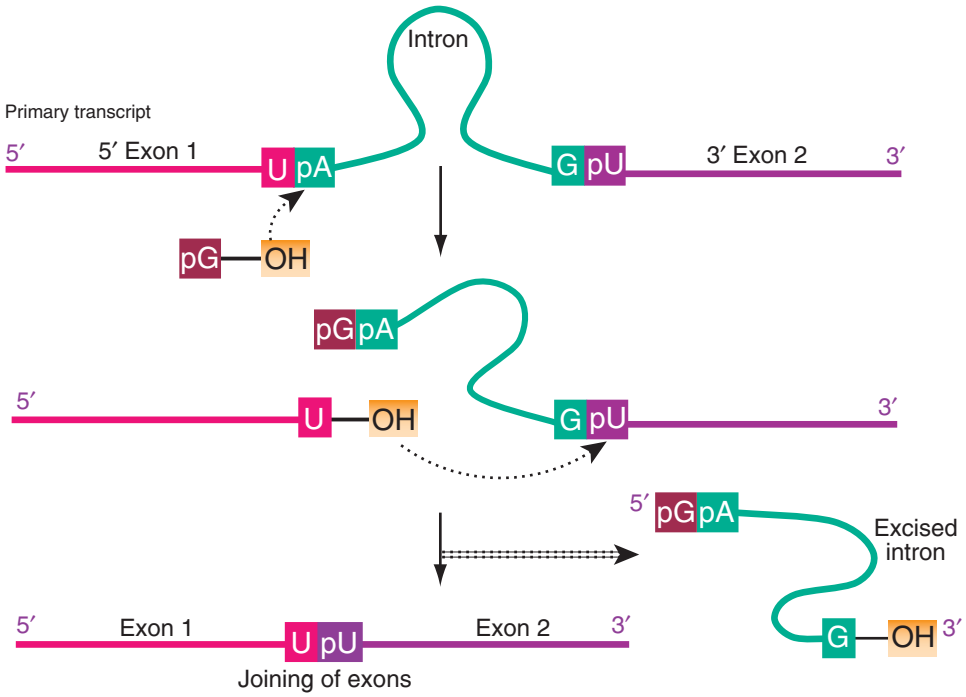
(d) Alpha-amanitin inhibits RNA synthesis in animals, which is a toxic component of the poisonous mushroom *Amanita phalloides*.

Post-transcriptional modification: The enzymatic processing of the primary RNA transcript, producing functional mRNA, tRNA and/or rRNA molecules is known as post-transcriptional modification.

A newly synthesised RNA molecule is called as primary transcript. The principal products of transcription are mRNAs, tRNAs and rRNAs, which are synthesised as inactive preribonucleotides. Therefore these ribonucleotides are processed into functional ribonucleotides by various mechanisms, as described below—

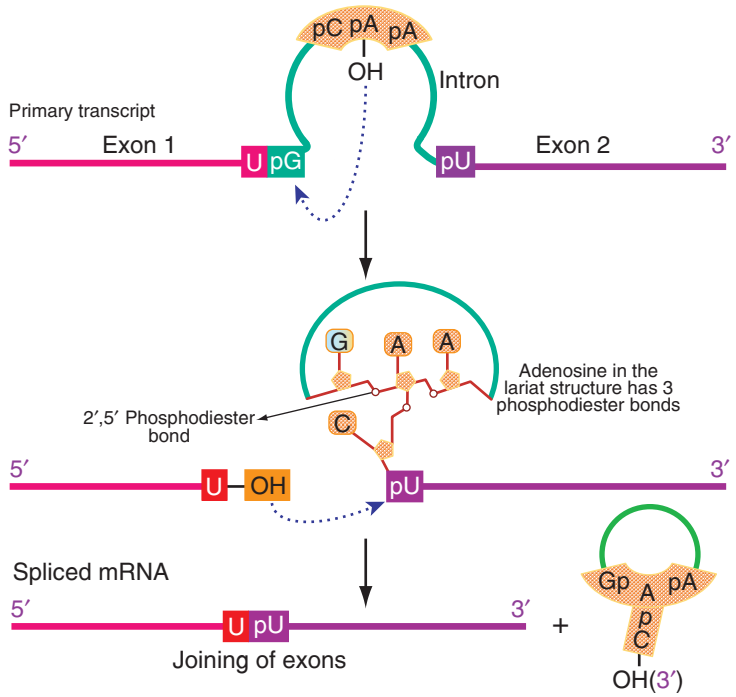
(a) Primary transcript mRNAs: Primary transcript mRNAs of few prokaryotes and all the eukaryotes are made up of some non-coding sequences called introns and some of the coding sequences called the exons. The non-coding introns are removed from the primary transcript mRNAs, to form the mature mRNA. There are three groups of introns found in mRNAs–group-I, group-II and group-III. The introns present in each group of mRNA are excised and the two exons joined together.

The removal of group-I introns requires a guanine nucleotide cofactor whose 3′-hydroxyl group forms a 3′5′-phosphodiester bond with the 5′ end of the intron. Due to this the 3′-hydroxyl group of the exon becomes free and attacks (nucleophilic) the 3′ end of the intron resulting in splicing (excision) of the intron and joining of the exons.



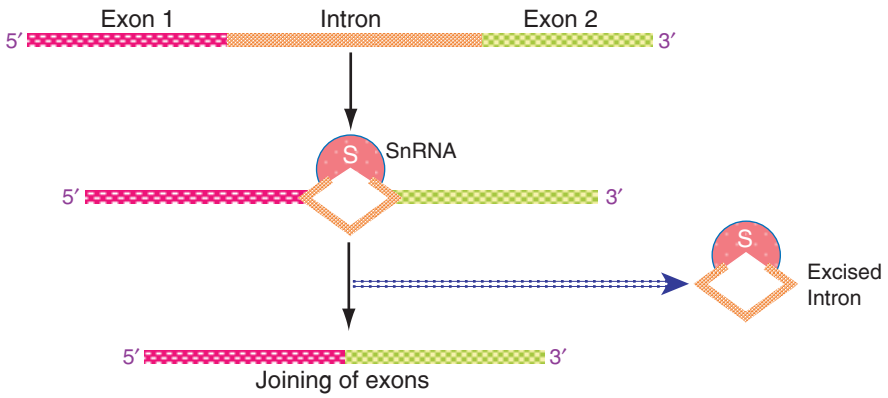
Splicing of group-I introns and joining of exons

The removal of group-II introns does not require any external cofactor, instead the 2′-hydroxyl group of an adenylate residue within the intron attacks the 3′ end of the exon forming an unusual branched lariat structure having 2′,5′-phosphodiester bond. This is a self splicing mechanism.



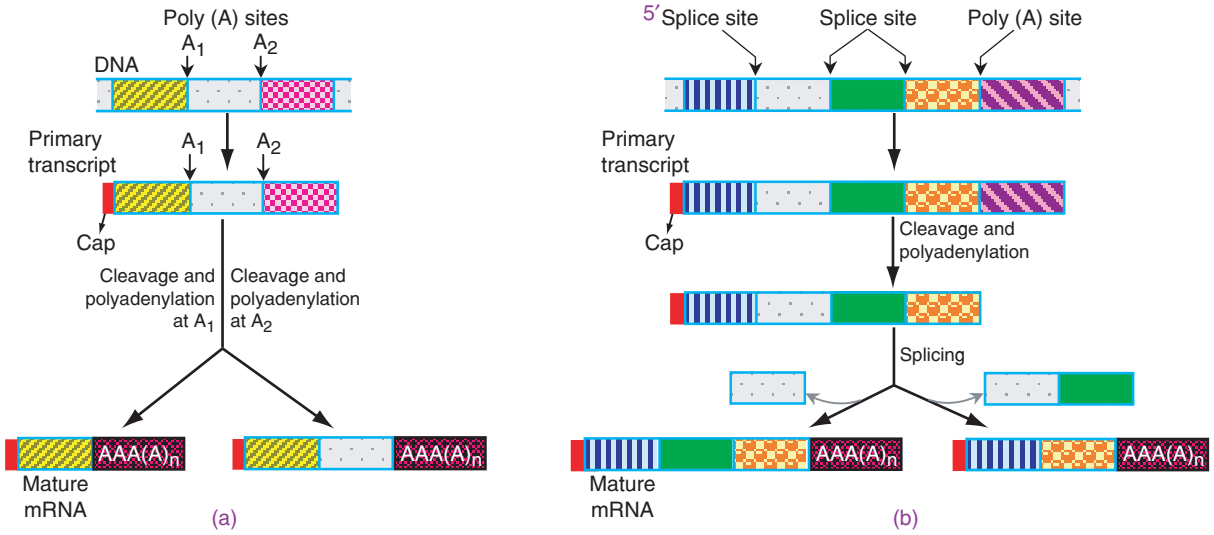
Splicing of group-II introns and joining of exons

The removal of group-III introns needs an RNA type called 'small nuclear RNA' (SnRNA), which has a sequence complementary to the sequences at the two terminal ends of the introns. Hence the SnRNA base pairs with these ends and thus helps in removing the introns and joining the exons of the heteronuclear RNAs (hnRNA) i.e. the primary transcript RNA.



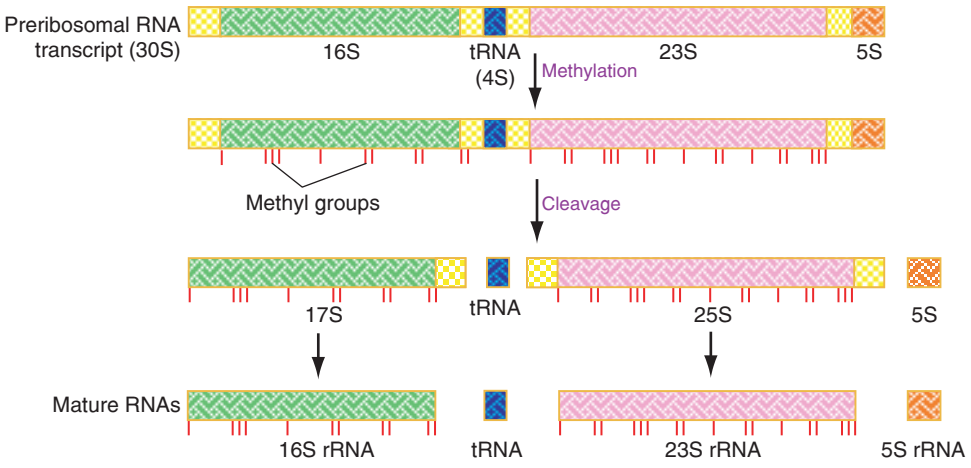
Splicing of group-III introns and joining of exons

The mRNAs in eukaryotes are added with a 5′ cap of 7-methyl guanosine, which is linked by an unusual 5′,5′- triphosphate linkage to the 5′ residue, this remains in mature RNA. Some of the nucleotides from the 3′ end are removed and immediately an enzyme called polyadenylate polymerase adds about 20-25 adenylate residues, called poly A tail, at this free 3′ end.

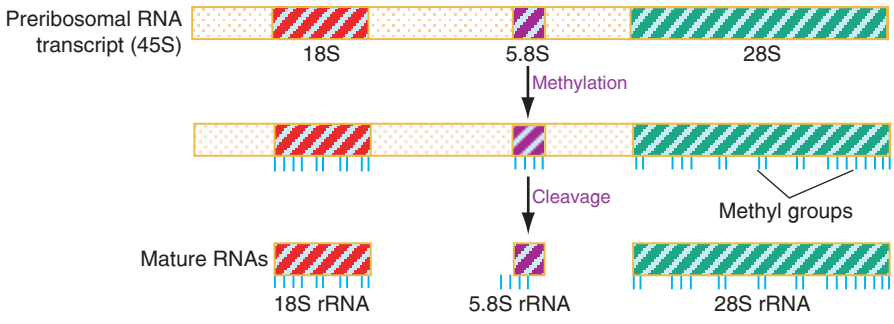


Eukaryotic mRNA splicing

rRNAs in prokaryotes and eukaryotes are made from precursors called preribosomal RNAs. In prokaryotes a 30S RNA having 65000 nucleotides gives rise to the 16S, 23S and 5S rRNAs and a tRNA. In eukaryotes a 45S rRNA gives rise to 18S, 28S and 5.8S rRNA.



Processing of prokaryotic rRNA



Processing of eukaryotic rRNA

In eukaryotes most of the cells have 40 to 50 types of tRNAs in multiple copies. These tRNAs are formed from longer RNA precursors by enzymatic removal of extra nucleotide units from the 5′ and 3′

ends. The 3′ terminal trinucleotide C-C-A (3′) is added by the enzyme tRNA nucleotidyl transferase. The tRNA is finally processed to mature tRNA by methylation, deamination or reduction of some of its bases.

TRANSLATION

(PROTEIN SYNTHESIS)

The genetic information stored in the DNA in the form of nucleotide base sequence is transcribed into the mRNA, which is again specified in the form of nucleotide base sequence, but complementary to the sequence of that part of DNA from which it is formed. The message present in the mRNA is then translated into the language of protein, which is made up of amino acids, whose sequence is specified by the sequence of nucleotide bases in the mRNA.

Genetic code: One amino acid is specified by or incorporated in place of three consecutive bases of mRNA. The set of triplet code words (bases) in mRNA (or its complementary bases in DNA) coding for the amino acids of proteins is known as 'genetic code'.

The genetic code is the natural code word of three nucleotide bases, specifying an amino acid, just like we (humans) have formulated the international subscribers dial (ISD) phone code. The ISD code 1 stands for USA, 91 stands for India, 966 stands for Saudi Arabia etc. Similarly the three letter code of the nucleotide bases in sequence forms the genetic codes for an amino acid; for example AUG stands for methionine, GCA for alanine and GGC for glycine etc.

Now let us examine as to how many genetic codes are possible from the four nucleotide base letters of RNA i.e. A, U, G, C. So the number of genetic code words (three letter bases) possible from the four letters (nucleotide bases) is given by $4^3 = 4 \times 4 \times 4 = 64$. This amino acid code words (genetic code) dictionary as it occurs in mRNA can be written as under, in the 5′→3′ direction of mRNA—

GENETIC CODE

	U		C		A		G		
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys	C
	UUA	Leu	UCA	Ser	UAA	STOP	UGA	STOP	A
	UUG	Leu	UCG	Ser	UAG	STOP	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg	C
	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg	A
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg	G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUG	Ile	ACC	Thr	AAC	Asn	AGC	Ser	C
	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg	A
	AUG	Met	ACG	Thr	AAG	Lys	AGG	Arg	G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly	C
	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly	A
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly	G

Some important features of genetic code or codon:

(1) The genetic code words or codons are three consecutive bases in mRNA, which specifies a particular amino acid.
(2) The codons do not overlap i.e. any of the three bases which has specified for one amino acid in a mRNA, does not share with any other codon nucleotides.
(3) There is no punctuation between codons for successive amino acid residues i.e. once a set of three nucleotides specifies an amino acid, the subsequent set of three nucleotides specifies amino acids one after another without any single base being missed in between them. Therefore the amino acid sequence of a protein is co-linear to the sequence of continuous triplet codons.
(4) Out of the 64 codons, AUG is known as the initiation codon which always initiates protein synthesis both in prokaryotes and eukaryotes.
(5) Three codons do not specify any amino acid hence they are known as non-sense codons or termination codons or stop codons as protein synthesis stops whenever these codons appear in mRNA. They are UAA, UAG & UGA, also termed as Amber, Ochre and Opal, respectively.
(6) The genetic code is degenerate i.e. there are more than one codon for a given amino acid, except for methionine and tryptophan, which have single codon each. The following are the number of codons for the 20 standard amino acids—
Gly-4; Ala-4; Ser-6; Thr-4; Val-4; Leu-6; Ile-3; Pro-4; Cys-2; Met-1; Phe-2; Tyr-2; Trp-1; Glu-2; Gln-2; Asp-2; Asn-2; Lys-2; Arg-6; His-2.
(7) The genetic code is unambiguous i.e. no codon specifies more than one amino acid.
(8) The genetic code is universal i.e. the 64 codons for the 20 standard amino acids are the same in virus, bacteria, plants, animals and humans.

Mechanism of translation: The process by which the genetic information present in a mRNA molecule is converted into protein with specific amino acid sequence is known as translation or protein synthesis.

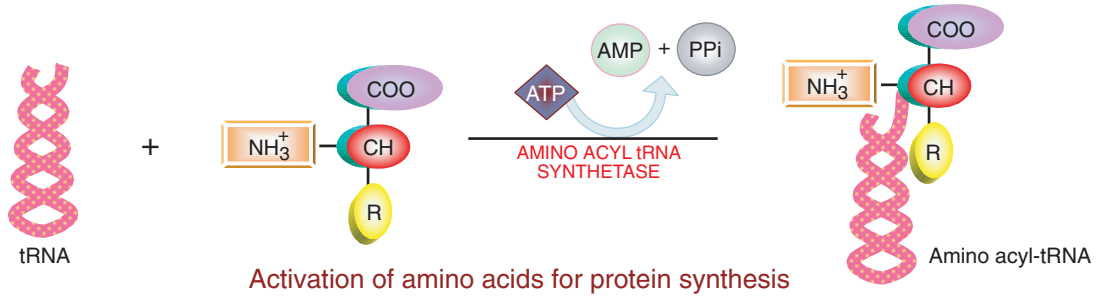
There are five (5) steps involved in the process of protein synthesis, they are—

Name of the process	Components required
1. Activation of amino acids	20 amino acids, 20 aminoacyl-tRNA synthetases, 32 or more transfer-RNAs, ATP and Mg^{2+}
2. Initiation of polypeptide chain	mRNA, N-formyl methionyl-tRNA, initiation codon (AUG), 30S and 50S ribosomal subunits, initiation factors–IF_1, IF_2 & IF_3, GTP and Mg^{2+}
3. Elongation	Initiation complex-70S subunit. Elongation factors–Tu, Ts and G, aminoacyl-tRNAs, peptidyl transferase, GTP and Mg^{2+}
4. Termination	Termination codon in mRNA i.e. UAA, UAG and UGA, also known as amber, ochre and opal. Peptide releasing factors–R_1, R_2 & S, ATP and Mg^{2+}
5. Folding and processing i.e. post translational modification	Varies

1. **Activation of amino acids:** Amino acids present in the cytosol are transported to the site of protein synthesis with the help of tRNAs. Each amino acid has got a specific tRNA, some amino acids have got more than one tRNA. A minimum of 32 tRNAs are required to read all the 64 codons for the 20 amino acids. There may be 54 to 60 types of tRNAs in different cells. The particular tRNA attaches with the respective amino acid at the 3' end with the help of the enzyme amino acyl-tRNA synthetase forming amino acyl-$tRNA^{AA}$. ATP is required for this reaction which gets hydrolysed to AMP and 2Pi.

2. Initiation of the polypeptide chain:

(a) IF_3 binds to 30S ribosomal subunit and prevents its union with 50S subunit.



Activation of amino acids for protein synthesis

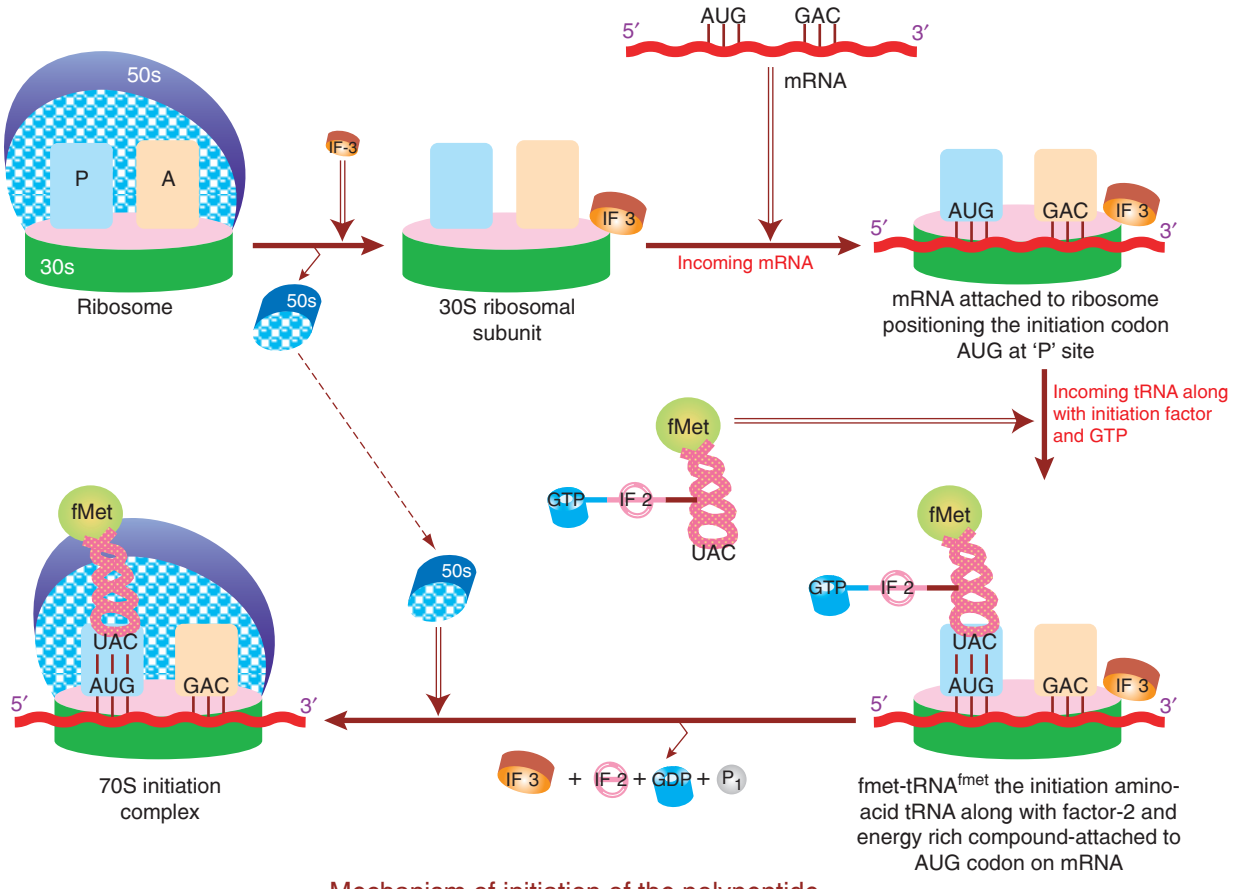
(b) mRNA binds to the 30S subunit by an initiation signal on mRNA made up of A and G residues, which base pairs with rRNA.

(c) AUG-the initiation codon is identified by FMet-$tRNA^{fmet}$ (Met-$tRNA^{met}$ in eukaryotes) containing IF_2 & GTP and binds there by base pairing.

(d) Then the 30S subunit combines with the 50S subunit to form the 70S ribosomal subunit called **'initiation complex'**.

(e) The initiation complex 70S has two sites (i) P-site and (ii) A-site.

(f) Finally IF_3 and IF_2 are released by the hydrolysis of GTP to GDP and Pi.



Mechanism of initiation of the polypeptide

3. Elongation:

(a) The next aminoacyl-tRNA is bound to Tu and GTP and this binds to the A site on the 70S initiation complex, which is directed by the next 3 bases in the mRNA i.e. next codon.

(b) Simultaneously Tu-GTP is released with the hydrolysis of GTP to GDP.

(c) Tu-GDP is converted to Tu-GTP with the help of Ts and GTP.

(d) A peptide bond is formed between the carboxylic group of amino acid at P-site (i.e. fmet initiation amino acid) and amino group of amino acid at A-site with the help of the enzyme peptidyl transferase. This results in the shift of amino acid from P-site to A-site. Thus a dipeptide is formed at A-site.

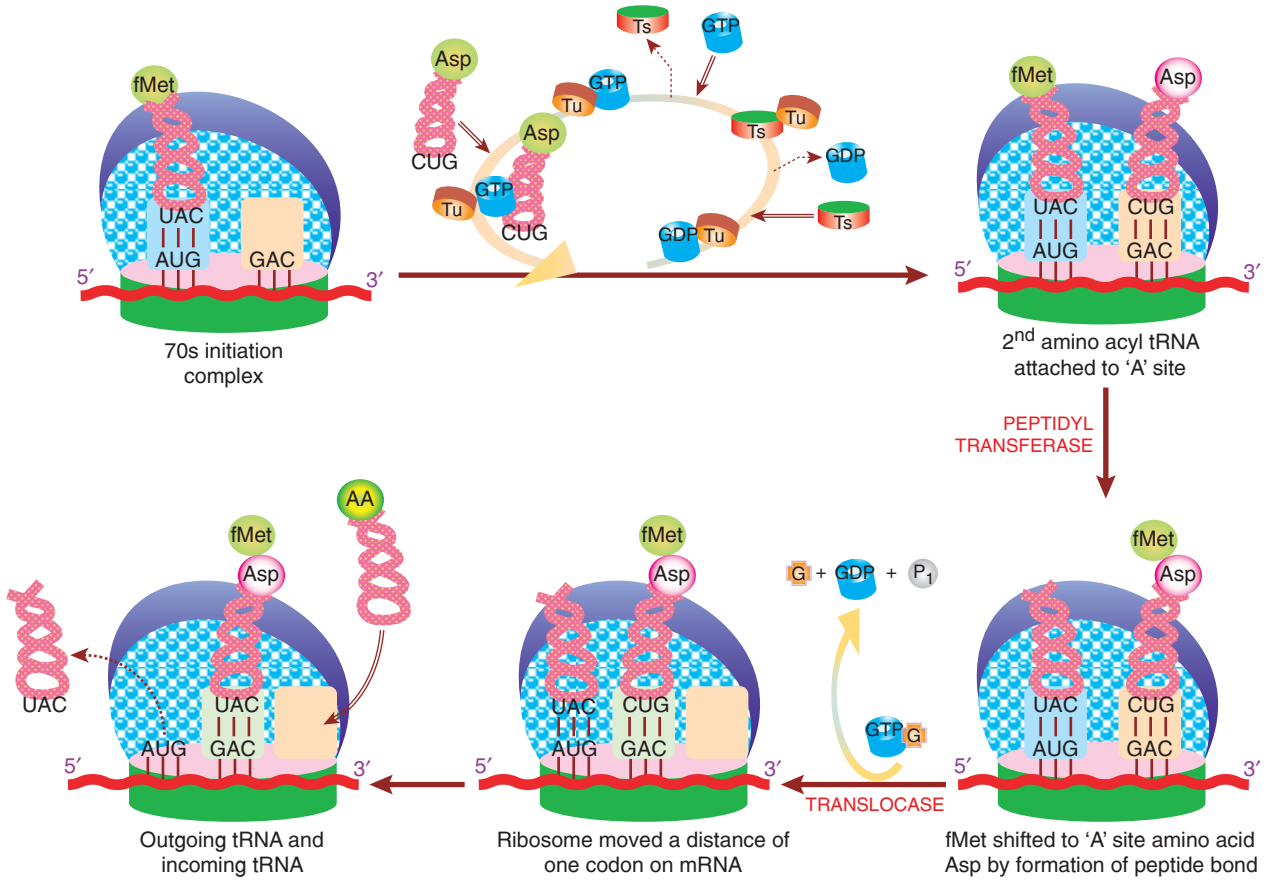
(e) The ribosome moves along the mRNA towards the 3' end by a distance of a codon (3 bases) with the help of G protein and GTP. This is known as translocation.

(f) The translocation requires elongation factor-G (EF-G), also called translocase.

(g) As a result of translocation the dipeptide is shifted from A-site to P-site along with its tRNA and the deacylated tRNA (i.e. the first tRNA) is released into the cytosol.

(h) The third codon of mRNA is now at the A site and the second codon attached to tRNA with peptide is in the P-site.

(i) The ribosome, with its attached dipeptidyl-tRNA and mRNA is now ready for another elongation cycle to attach the third amino acid residue. This process occurs in the same way as the addition of second amino acid. For each amino acid residue added to the chain, two GTPs are hydrolyzed to GDP and Pi. Ribosome moves from codon to codon along the mRNA towards 3' end, adding one amino acid residue at a time to the growing chain.



Mechanism of elogation of the polypeptide

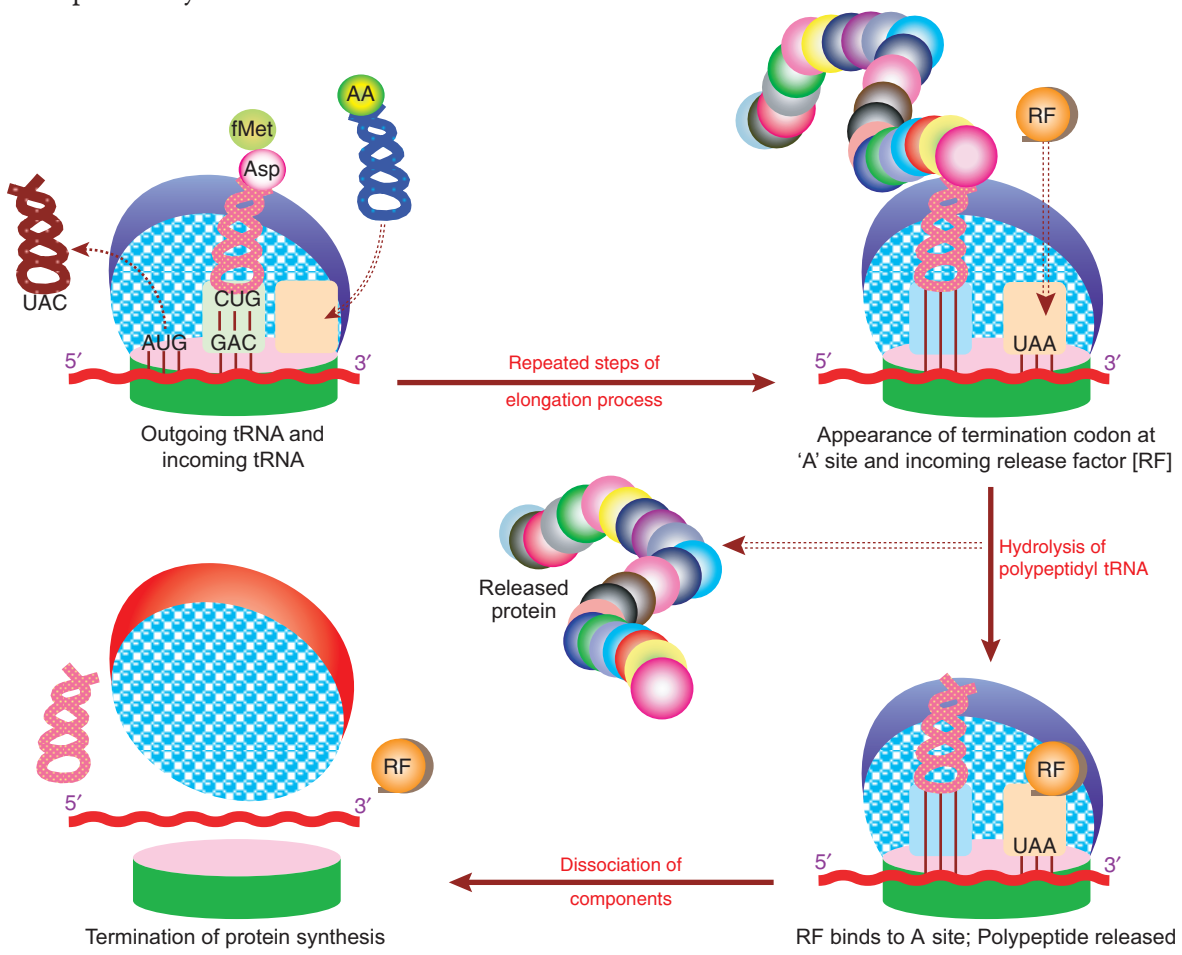
4. Termination: The elongation continues until the ribosome adds all the amino acids and it reaches the termination signal in mRNA specified by one of the termination codons or non-sense codons i.e. UAA, UAG & UGA also called Amber, Ochre and Opal respectively.

Once a termination codon occupies the ribosomal A-site, three termination or release factors i.e. RF_1, RF_2 and RF_3 will contribute to—

(1) The hydrolysis of the terminal peptidyl tRNA

(2) Release of the free polypeptide and the last tRNA from the P site and

(3) The dissociation of the 70S complex into 30S and 50S subunits. This terminates the process of protein synthesis.



5. Post-translational modification: The enzymatic processing of a polypeptide chain after translation from its mRNA is known as post-translational modification.

The nascent polypeptide chain formed from the mRNA on the ribosome is folded and processed into its biologically active form in the following manner—

(a) The N-formyl methionine (in prokaryotes) or methionine (in eukaryotes) and some more N-terminal and some C-terminal amino acids are removed enzymatically. In 50% of the eukaryotic proteins, the amino group of amino terminal residue is acetylated.

(b) The signal sequence, which directs the proteins to its destination, which are 10-30 amino acid residues at N-terminal end are also removed.

(c) The hydroxyl groups of certain Ser, Tyr and Thr residues are enzymatically phosphorylated by ATP, ex. milk protein casein has phosphoserine groups which bind Ca^{2+}.

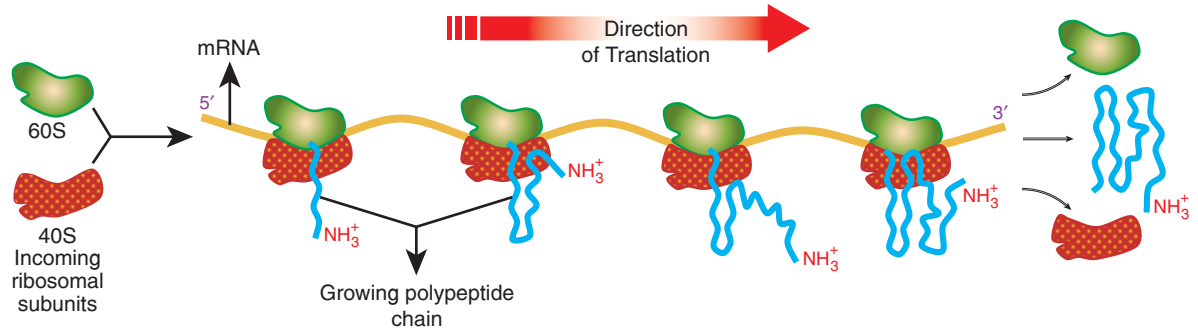
(d) Extra carboxyl groups are added to Asp and Glu of some proteins, ex. blood clotting protein, prothrombin, has many gamma-carboxyglutamic acid residues in its N-terminal region.
(e) The lysine residue of some proteins is methylated, ex. dimethyl-lysine in cytochrome-c.
(f) To Asn, Ser or Thr of some extracellular lubricating glycoproteins, carbohydrate side chains are attached enzymatically.
(g) To some proteins, isoprene group is attached.
(h) To some proteins, a prosthetic group is attached covalently, ex. heme to hemoglobin.
(i) Many proteins like insulin, trypsin and chymotrypsin are synthesised as longer inactive proteins called the zymogen. These are enzymatically cleaved to smaller active proteins.
(j) The proteins undergo spontaneous folding into their native globular or ellipsoidal conformations, which permit the maximum number of hydrogen bonds, Van-der-waals, ionic and hydrophobic interactions and also the intra-chain and inter-chain disulphide bonds between two cysteine molecules.

Thus a complete biologically active protein is released in the media i.e. either in the cell or extracellular. Some of these proteins are structural proteins and yet some others are enzymes. These enzymes now carry the various functions which depend upon their structure, which in turn depends upon the amino acid sequence, which in turn has been specified by the mRNA and which has been formed from the base sequence contained in the segment of DNA called the gene. This is how the DNA controls all the activities of the cell and hence called the holder and transferor of genetic information.

Inhibitors of protein synthesis: There are some drugs and antibiotics which inhibit protein synthesis. Some of these are specific to the prokaryotes, where as others are inhibitors only in eukaryotes and yet some others in both.

1. **Puromycin:** It binds to A-site and terminates protein synthesis.
2. **Tetracycline:** Blocks A-site and inhibits protein synthesis in bacteria.
3. **Chloramphenicol:** Blocks peptidyl transfer in prokaryotes, mitochondrial and chloroplast ribosomes but not in eukaryotic cytosolic ribosomes.
4. **Cycloheximide:** Blocks peptidyl transferase of eukaryotic ribosomes but not of prokaryotic ribosomes.
5. **Streptomycin:** Causes misreading of genetic code in bacteria and inhibits initiation.
6. **Diphtheria toxin:** Inactivates EF-2 in human cells.
7. **Ricin:** Inactivates 60S/28S ribosomal subunit in eukaryotes.

Polysomes: The single stranded mRNA, is always translated simultaneously by many ribosomes (10 to 100 ribosomes) and spaced close together, called polysomes. In bacteria the transcription and translation are coupled, wherein the mRNAs synthesised in the 5′→3′ direction are translated by ribosomes beginning from the 5′ end of the mRNA before transcription is complete. In eukaryotes the newly transcribed mRNAs first get transferred out of the nucleus and are then translated. The life of mRNA is very short (only a few minutes) and hence the polysomes maintain a high rate of protein synthesis. Further the reason for life span of mRNA being short is that the protein synthesis can cease when it is not needed by the cell.



Translation of eukaryotic mRNA (Polysomes)

REGULATION OF GENE EXPRESSION

Gene is a part of DNA that specifies a protein/RNA. All the proteins/RNA are not required by the cell all the time. Some proteins are required at some time and yet other proteins are required at another time. Moreover these proteins are required in lesser quantities at one time, yet at other times they may be required in higher quantities. There are yet another class of proteins which are constantly (always) present in the cell, like the enzymes of the TCA cycle. Therefore genes can be conveniently grouped under two classes—

1. **Constitutive genes:** Those genes whose products are constantly present in the cell are called constitutive genes or housekeeping genes.
2. **Inducible genes:** Those genes whose products vary with time and need, both in their presence and concentration are called inducible genes or those genes whose products (proteins/RNA) are induced by some inducer molecule.

The activity of the constitutive genes is not regulated as their products don't vary much with time, where as the activity of inducible genes are always regulated. The regulation is primarily at the level of transcription. The gene or a set of related genes are switched on or off as per the need of the cell. These changes are brought about by some proteins or modulator. If a particular protein/compound puts a gene into operation then that protein is called stimulatory protein/compound and the process is called positive regulation. If a protein/compound stops the operation of a gene then it is called the repressor protein/compound and this process is referred to as negative regulation, ex. a steroid hormone acts as a positive modulator, wherein its presence enhances the rate of gene expression. As soon as the hormone is destroyed the gene expression diminishes.

The mechanism of regulation, though similar in the prokaryotes and eukaryotes, it differs in some aspects. Hence regulation of gene expression in prokaryotes and eukaryotes will be taken separately.

Regulation of gene expression in prokaryotes: Many prokaryotic genes are regulated in units called operons. Operon is unit of genetic expression consisting of one or more related genes and sequences (gene) controlling them, which includes the operator and promoter sequences that regulate their transcription.

The LAC operon: It is the operon for utilization and metabolism of lactose in bacteria. It consists of the following set of genes—

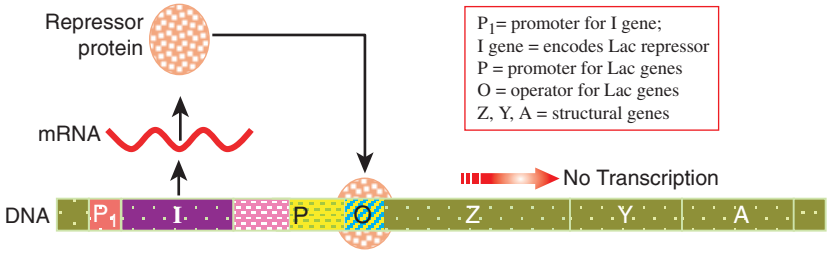
P_I = The promoter gene for regulatory genes
I = The gene for regulatory gene
P = The promoter sequence for the related protein (repressor protein)
O = Operator sequence for these genes
Z = The first gene for utilization of lactose, which forms the enzyme beta-galactosidase
Y = The second gene for the membrane protein galactoside permease
A = The third gene for the enzyme thiogalactoside transacetylase

This complete set of sequences (i.e. the operon) helps in switching on/off, the machinery for the utilization of the carbohydrate–lactose by the bacteria *E. coli.* When glucose is present in the media where the cell is growing, then the lac operon is switched off and when the medium is devoid of glucose, and instead lactose is present as the sole source of carbon, then the Lac operon becomes operational.

The transcription by RNA polymerase begins at the promoter site i.e. the enzyme binds to the promoter and moves along the DNA towards the structural genes of the operon to transcribe the mRNA for these genes and in this process it passes through the operator region of the operon.

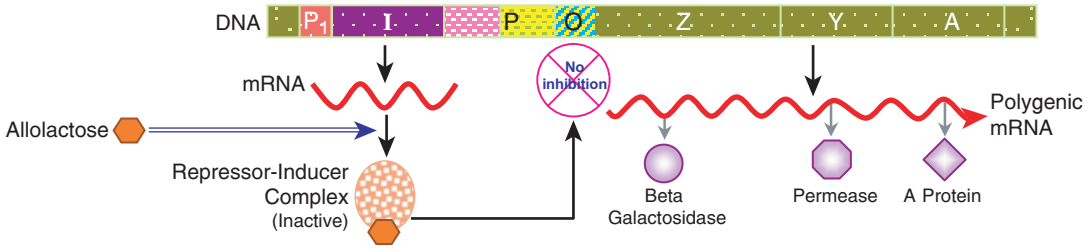
Under all circumstances i.e. whether glucose or lactose is to be utilized by the cell, the I gene of the lac operon synthesizes a protein called repressor protein. This protein binds to the operator site in the

DNA and thus prevents the movement of the RNA polymerase beyond this point (site), which results in the inhibition of the synthesis of the structural genes Z,Y and A.



Lac operon in repressed state

Thus, when the cell is utilizing glucose as the only carbon source, the lac operon is switched off. Then, if the cell shifts over to the utilization of lactose as the carbon source then lactose is first converted to allolactose by the enzyme beta-galactosidase (which is always present in the cell in a few copies, irrespective of glucose or lactose is being utilized), and this allolactose acts as a positive modulator or inducer for the lac operon. Here the allolactose binds to the repressor protein present at the operator site resulting in the release of the repressor protein from the operator site thereby permitting the enzyme RNA polymerase to pass freely through this operator site from the promoter site and thus transcribe all three structural genes Z, Y, & A.

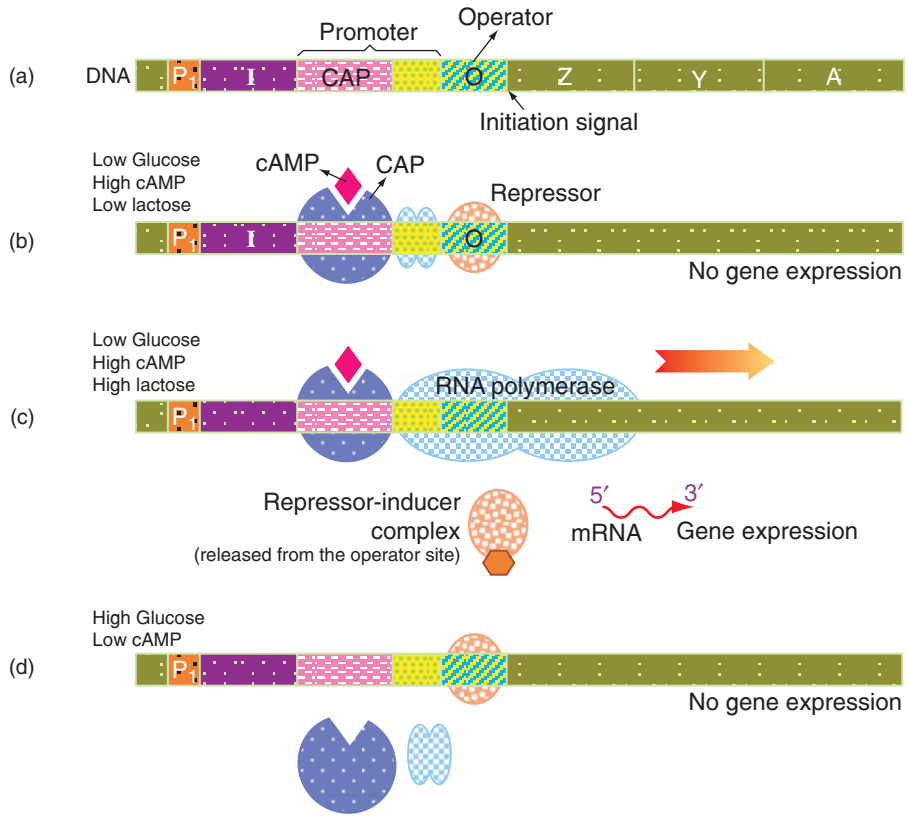


Induction of the Lac operon in response to a molecular signal

The activity of the lac operon is not only dependent upon the binding and release of repressor molecule (with modulator) but it is also cAMP dependent. When glucose is low in the media/cell, then the cellular cAMP concentration increases. This increased amount of cAMP results in its binding at a particular site (sequences) on the promoter. The promoter site can be divided into two parts—(1) The site for the binding of RNA polymerase (2) The site for a protein called catabolite gene activator protein (CAP).

The RNA polymerase can bind to the promoter site only if the CAP is bound to the promoter sequence and CAP can bind to the promoter only if cAMP is bound to it and cAMP binds to CAP only when its cellular concentration increases, which occurs when the cell is devoid of glucose and hence this facilitates the utilization of these sugars and the presence of lactose converts it to allolactose. This acts as a positive modulator for switching on the lac operon genes by releasing the repressor protein from the operator site and producing the products of the three structural genes which produces—(1) The membrane protein β-galactoside permease, that enhances the uptake of lactose by the cells (2) β-galactosidase which hydrolysis lactose to allolactose and then to glucose and galactose (3) The enzyme thiogalacatosidase–transacetylase, whose function is unknown. When glucose is again available to the cell the cAMP concentration decreases in the cytosol, resulting in its release from the CAP, this in turn results in the release of CAP from promoter site, which in turn results in release of the enzyme RNA polymerase from the promoter site and further prevents its binding to promoter. This again results in the diminished synthesis of the structural genes, one of which is beta-galactosidase, that results in low production of allolactose (or no synthesis of allolactose), this is turn results in the repressor protein (formed from I gene) being devoid

of the modulator and thus is free to bind at the operator site thereby prevent the movement of RNA polymerase and thus resulting in the inhibition of lac operon.



Combined effect of glucose and lactose on expression of the lac operon

Each and every metabolite has got its own operon, with different number of structural genes and whenever the genes for that metabolite are required it is switched on by a similar mechanism as that of the lac operon and switched off whenever not required. The other operons and their details are as under—

Operon for	No. of structural genes	Function
His operon	9	Enzymes required in synthesis of histidine
Leu operon	4	Conversion of alpha-keto-isovalerate to leucine
Ara operon	4	Transport and utilization of the carbohydrate arabinose

All of the operons found in the bacteria do not function only by completely switching on or off their genes. Some operons function at differential rates depending upon the need of the cell by a mechanism called the transcription attenuation i.e. slowing down of the rate of synthesis of enzymes, ex. those enzymes involved in the synthesis of amino acids (His).

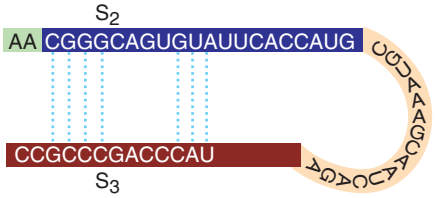
Attenuation: Transcription attenuation is a process in which transcription is initiated normally but is abruptly halted before the complete operon genes are transcribed. The frequency with which transcription is attenuated depends upon the cellular concentration of that particular amino acid for which the operon is meant for.

Attenuation of His operon: In bacteria, transcription and translation are closely coupled. The rate at which RNA is transcribed and the rate at which that protein is translated is almost the same. Most of the transcribed RNAs for amino acid metabolism in the cell contain various complementary intra base pairing sequences. For example the following is the part of RNA that is being transcribed for His operon, which is also simultaneously being translated.

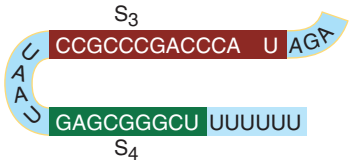
AA CGGGCAGUGUAUUCACCAUG CGUAAAGCAAUCAGA UACCCAGCCCGCC UAAU GAGCGGGCU UUUUUU
S_2 S_3 S_4

Complementary intra-base pairing sequences of His operon (shown in dark)

The sequence 2 and 3 are complementary and can base pair with each other. Likewise sequences 3 and 4 are also complementary and can also base pair with each other. If 2 and 3 bases pair, then transcription can proceed normally and if 3 and 4 bases pair the transcription is terminated, just like the termination of transcription due to appearance of a hair pin structure in DNA. The base pairing between the sequences 2 & 3 or 3 & 4 is dependent upon the rate of translation of the mRNA, which in turn is dependent upon the concentration of His-tRNA^{His} that reflects the concentration of histidine in the cell. If the concentration of His-tRNA^{His} is more and the rate of translation is very fast such that it passes the 2nd site before site 3 is transcribed, then this results in the site 3 base pairing with site 4 as soon as it is transcribed resulting in the termination of transcription.



Intrachain base pairing between S_2 and S_3 in mRNA do not affect translation

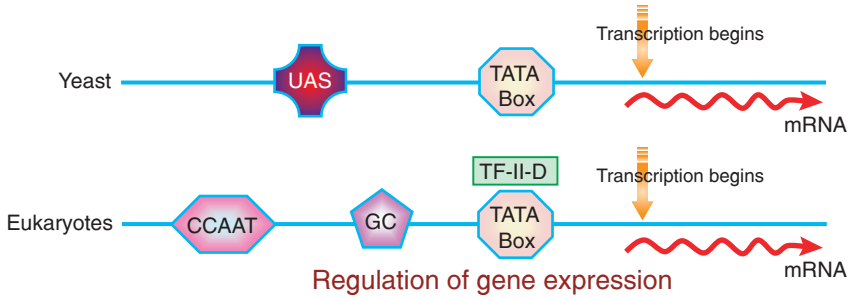


Intrachain base pairing between S_3 and S_4 in mRNA attenuating translation

On the other hand when the His-tRNA^{His} concentration is low, the rate of translation is very slow and thus the process of translation does not pass the 2nd site on mRNA by the time site (sequences) 3rd is transcribed then this result in the continuation of transcription because this will result in the 2 & 3 sites base pairing and so site 3 is not free for base pairing with site 4. Thus this results in a continuous operation of His operon.

Regulation of gene expression in eukaryotes: The genes in eukaryotes are also regulated in more or less the same manner as that of prokaryotes, but the regulation is mostly positive and very rarely negative regulation is seen. In higher eukaryotes the regulation of gene expression is solely by positive modulation and negative inhibition of the genes/operon is totally absent. However in yeast some genes are regulated by negative modulation. Further, there is a physical separation between the process of transcription and translation is eukaryotes as transcription takes place in the nucleus and translation occurs in the cytosol.

Mechanism: The gene regulation is only by positive regulation. Most of the genes are normally inactive in eukaryotes i.e. RNA polymerases cannot bind to the promoters. The cells synthesize only the selected group of activator proteins needed to activate transcription of the small subset of genes required in that cell. There are at least five regulatory sites for RNA polymerase promoter sites in higher eukaryotes designated as (a) TATA box (b) GC box and (c) CAT box. In yeast there are two types of promoter sequences i.e. TATA box and UAS i.e. upstream activator sequence. These sequences are the binding sites for the transcription factors called TF-II-D that is required for RNA polymerase binding. Each of these sequences are recognised and bound specifically by one or more regulatory proteins called transcription factors. These regulatory sequences are about 1000 bases away form the main gene, thus to activate the main gene a protein-protein interaction is required which can reach the main gene sequence.



Regulation of gene expression

MUTATIONS, DNA DAMAGE AND REPAIR

Mutations are changes in the nucleotide sequence of the genetic material of an organism. Mutations can be caused by copying errors in the genetic material during cell division, by exposure to ultraviolet or ionizing radiation, chemical mutagens, or viruses, or can occur deliberately under cellular control during processes such as hypermutation. In multicellular organisms, mutations can be subdivided into germ line mutations, which can be passed on to descendants and somatic mutations, which are not transmitted to descendants.

Causes of mutation: Mutations are caused by molecular decay resulting in spontaneous mutations whereas induced mutations are caused by artificial mutagens.

Spontaneous mutations: Spontaneous mutations at the molecular level include—

1. **Tautomerism:** A base is changed by the repositioning of a hydrogen atom.
2. **Depurination:** Loss of a purine base (A or G).
3. **Deamination:** Changes a normal base to an atypical base; C→U (corrected by DNA repair mechanisms), or spontaneous deamination of 5-methylcytosine (irreparable), or A→HX (hypoxanthine).
4. **Transition:** A purine changes to another purine, or a pyrimidine to another pyrimidine.
5. **Transversion:** A purine becomes a pyrimidine, or vice versa.

Induced mutations: Induced mutations on the molecular level can be caused by—Chemicals ex. Nitrosoguanidine (NTG), Hydroxylamine (NH_2OH), Base analogs (BrdU), Simple chemicals (Acids), Alkylating agents {N-ethyl-N-nitrosourea (ENU)}.

These agents can mutate both replicating and non-replicating DNA. In contrast, a base analog can only mutate the DNA when the analog is incorporated in the replicating DNA. Each class of chemical mutagens has certain effects that lead to transitions, transversions, or deletions. A few of them are—

- ✧ Methylating agents {Ethyl methane sulfonate (EMS)}
- ✧ Polycyclic hydrocarbons (Benzopyrenes found in internal combustion engine exhaust)
- ✧ DNA intercalating agents (Ethidium bromide)
- ✧ DNA crosslinker (Platinum)
- ✧ Oxidative damage caused by oxygen ($O\cdot$) radicals
- ✧ Radiations
- ✧ Ionizing radiation
- ✧ Ultraviolet radiation (non-ionizing radiation) excites electrons to a higher energy level. Cytosine and thymine are the two nucleotide bases in DNA that are most vulnerable to excitation which can change base-pairing properties. UV light can induce adjacent thymine bases in a DNA strand to pair with each other, as a bulky dimer.

Nomenclature: Nomenclature of mutations specifies the type of mutation and base or amino acid changes. Two types of mutations exists viz. amino acid substitution and deletion. The amino acid substitution/deletion may either be harmful or beneficial as detailed under.

- ➢ **Harmful mutations:** Changes in DNA caused by mutation can cause errors in protein sequence, creating partially or completely non-functional proteins. To function correctly, each cell depends on thousands of proteins to function in the right places at the right times. When a mutation alters a protein that plays a critical role in the body, a medical condition can result.
- ✧ If a mutation is present in a germ cell, it can give rise to offspring that carries the mutation in all of its cells. This is the case in hereditary diseases. On the other hand, a mutation can occur in a somatic cell of an organism. Such mutations will be present in all descendants of this cell, and certain mutations can cause the cell to become malignant and thus cause cancer.

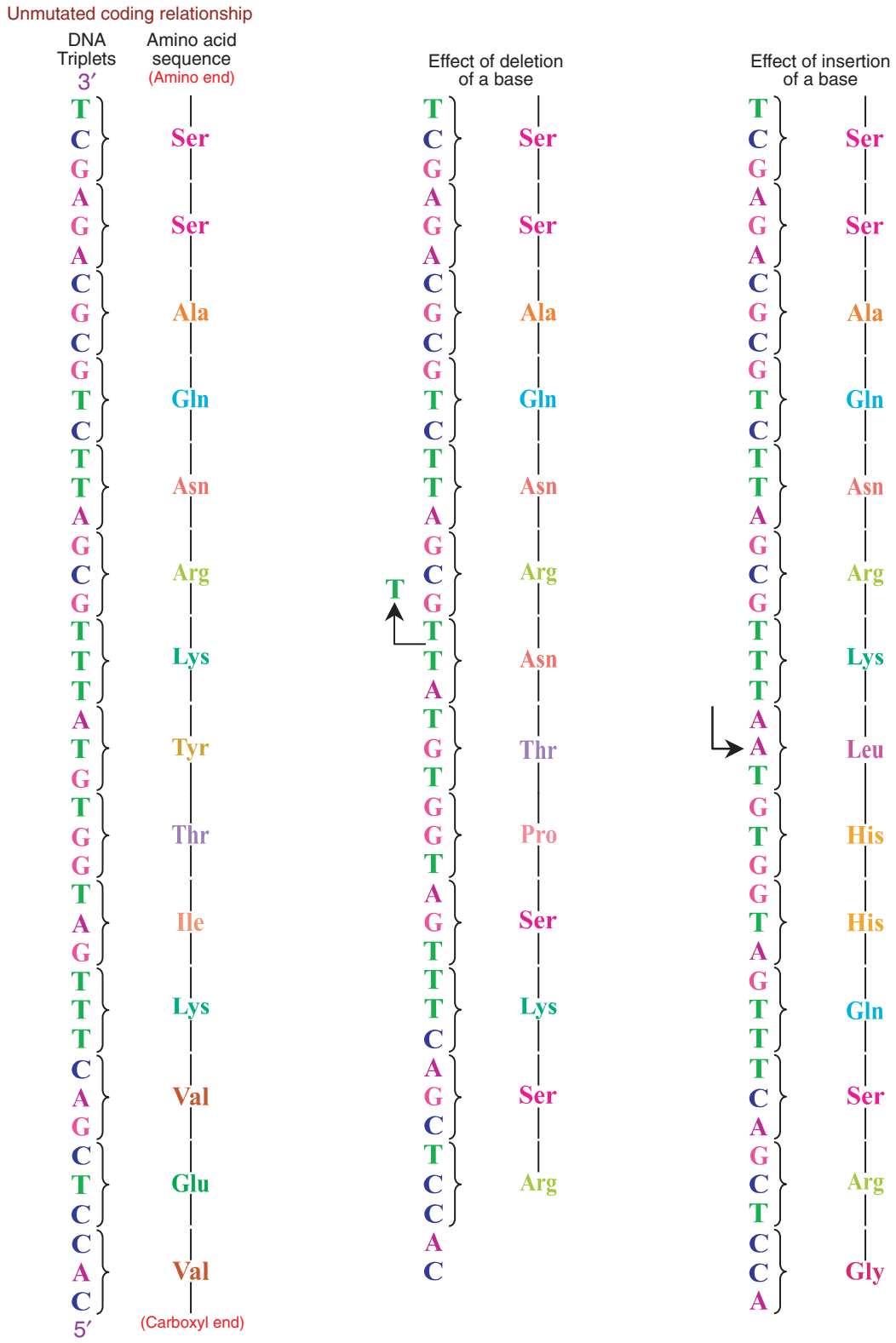
➢ **Beneficial mutations:** A very small percentage of all mutations actually have a positive effect. These mutations lead to new versions of proteins that help an organism and its future generations better adapt to changes in their environment, for example, a specific 32 base pair deletion in human CCR5 (CCR5-Δ^{32}) confers HIV resistance to homozygotes and delays AIDS onset in heterozygotes. The CCR5 mutation is more common in those of European descent.

Types of mutations:

1. **Adaptive mutation:** Mutations occurring in order to adopt the extremes of stressful conditions, ex. In order to get adapted to cold temperatures organisms undergo such mutations.
2. **Point mutation:** A point mutation or substitution is a type of mutation that causes the replacement of a single base nucleotide with another nucleotide. Often the term point mutation also includes insertions or deletions of a single base pair (which have more of an adverse effect on the synthesized protein due to nucleotides still being read in triplets, but in different frames a mutation called frame-shift mutation).

3. **Back mutation:** Back mutation is a change in a nucleotide pair of a point-mutated DNA sequence that restores the original sequence and hence the original phenotype.
4. **Missense mutation:** Missense mutations or non-synonymous mutations are types of point mutations where a single nucleotide is changed to cause substitution of a different amino acid. This in turn can render the resulting protein non-functional. Such mutations are responsible for diseases such as sickle-cell anemia.
5. **Silent mutation:** Silent mutations are DNA mutations that do not result in a change to the amino acid sequence of a protein. They may occur in a non-coding region (outside of a gene or within an intron), or they may occur within an exon in a manner that does not alter the final amino acid sequence.
6. **Neutral mutation:** A neutral mutation is a mutation that occurs in an amino acid codon (presumably within a mRNA molecule) which results in the use of a different, but chemically similar amino acid. This is similar to a silent mutation, where a codon mutation may encode the same amino acid; for example, a change from CCA to CCU will still encode proline, so no discernible change occurs (a silent mutation).

7. **Frame shift mutation:** A frame-shift mutation is a mutation caused by indels, i.e. inserts or deletes a number of nucleotides that is not evenly divisible by three from a DNA sequence. Due to the triplet nature of gene expression by codons, the insertion or deletion can disrupt the reading frame, or the grouping of the codons, resulting in a completely different translation from the original. The earlier in the sequence the deletion or insertion occurs, the more altered the protein produced is.



Frame shift mutations caused by deletion or insertion of a base

8. **Nonsense mutation:** A nonsense mutation is a frameshift mutation in a sequence of DNA that results in a premature stop codon or a nonsense codon in the transcribed mRNA, and possibly a truncated and often non-functional protein product.

DNA damage: DNA damage due to environmental factors and normal metabolic processes inside the cell, occurs at a rate of 1,000 to 1,000,000 molecular lesions per cell per day. While this constitutes only 0.000165% of the human genome's approximately 6 billion bases (3 billion base pairs), unrepaired lesions in critical genes (such as tumor suppressor genes) can impede a cell's ability to carry out its function and appreciably increase the likelihood of tumor formation.

The vast majority of DNA damage affects the primary structure of the double helix; that is, the bases themselves are chemically modified. These modifications can in turn disrupt the molecule's regular helical structure by introducing non-native chemical bonds or bulky adducts that do not fit in the standard double helix. Unlike proteins and RNA, DNA usually lacks tertiary structure and therefore damage or disturbance does not occur at that level. DNA is however supercoiled and wound around 'packaging' proteins called histones (in eukaryotes), and both superstructures are vulnerable to the effects of DNA damage.

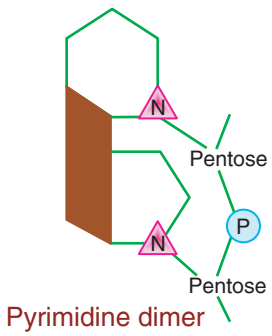
Sources of DNA damage: DNA damage can be subdivided into two main types—

1. **Endogenous damage:** Endogenous damage such as attack by reactive oxygen species produced from normal metabolic byproducts (spontaneous mutation), especially the process of oxidative deamination. There are four main types of damage to DNA due to endogenous cellular processes—
   (a) ***Oxidation of bases*:** [Ex. 8-oxo-7,8-dihydroguanine (8-oxoG)] and generation of DNA strand interruptions from reactive oxygen species.
   (b) ***Alkylation of bases*:** (Usually methylation), such as formation of 7-methylguanine, 1-methyladenine, 6-methylguanine.
   (c) ***Hydrolysis of bases*:** Such as deamination, depurination and depyrimidination.
   (d) ***Mismatch of bases*:** Due to errors in DNA replication, in which the wrong DNA base is stitched into place in a newly forming DNA strand, or a DNA base is skipped over or mistakenly inserted.
2. **Exogenous damage:** Caused by external agents such as—
   - Ultraviolet [UV 200-300 nm] radiation from the sun.
   - Other radiation frequencies, including x-rays and gamma rays.
   - Hydrolysis or thermal disruption.
   - Certain plant toxins.
   - Man-made mutagenic chemicals, especially aromatic compounds that act as DNA intercalating agents.
   - Cancer chemotherapy and radiotherapy.

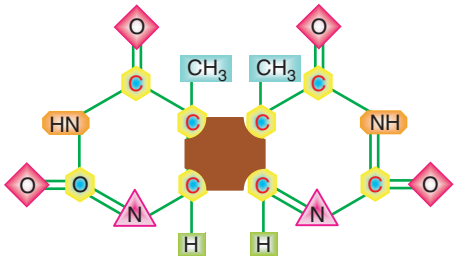
The replication of damaged DNA before cell division can lead to the incorporation of wrong bases opposite damaged ones. Daughter cells that inherit these wrong bases carry mutations from which the original DNA sequence is unrecoverable (except in the rare case of a back mutation, for example, through gene conversion).

Damage caused by exogenous agents comes in many forms. Some examples are—

- UV-B light causes crosslinking between adjacent cytosine and thymine bases creating pyrimidine dimers. This is called direct DNA damage.



Pyrimidine dimer



Thymine dimer

- UV-A light creates mostly free radicals, especially if sunscreen penetrated into the skin. The damage caused by free radicals is called indirect DNA damage.
- Ionizing radiation such as that created by radioactive decay or in cosmic rays causes breaks in DNA strands.
- Thermal disruption at elevated temperature increases the rate of depurination (loss of purine bases from the DNA backbone) and single strand breaks, for example, hydrolytic depurination is seen in the thermophilic bacteria, which grow in hot springs at 85-250°C. The rate of depurination (300 purine residues per genome per generation) is too high in these species to be repaired by normal repair machinery; hence a possibility of an adaptive response cannot be ruled out.
- Industrial chemicals such as vinyl chloride and hydrogen peroxide, and environmental chemicals such as polycyclic hydrocarbons found in smoke, soot and tar create a huge diversity of DNA adducts-ethenobases, oxidized bases, alkylated phosphotriesters and cross-linking of DNA just to name a few.

UV damage, alkylation/methylation, X-ray damage and oxidative damage are examples of induced damage. Spontaneous damage can include the loss of a base, deamination, sugar ring puckering and tautomeric shift.

DNA repair: DNA repair refers to a collection of processes by which a cell identifies and corrects damage to the DNA molecules that encode its genome. DNA damage causes structural damage to the DNA molecule and can alter or eliminate the cell's ability to transcribe the gene that the affected DNA encodes. Other lesions induce potentially harmful mutations in the cell's genome, which affect the survival of its daughter cells after it undergoes mitosis. Consequently, the DNA repair process is constantly active as it responds to damage in the DNA structure.

The rate of DNA repair is dependent on many factors, including the cell type, age of the cell, and the extracellular environment. A cell that has accumulated large amount of DNA damage, or one that no longer effectively repairs damage incurred to its DNA, can enter one of three possible states—

1. An irreversible state of dormancy, known as senescence.
2. Cell suicide, also known as apoptosis or programmed cell death.
3. Unregulated cell division, which can lead to the formation of a tumor that is cancerous.

The DNA repair ability of a cell is vital to the integrity of its genome and thus to its normal functioning and that of the organism. Many genes that were initially shown to influence life span have turned out to be involved in DNA damage repair and protection. Failure to correct molecular lesions in cells that form gametes can introduce mutations into the genomes of the offspring and thus influence the rate of evolution.

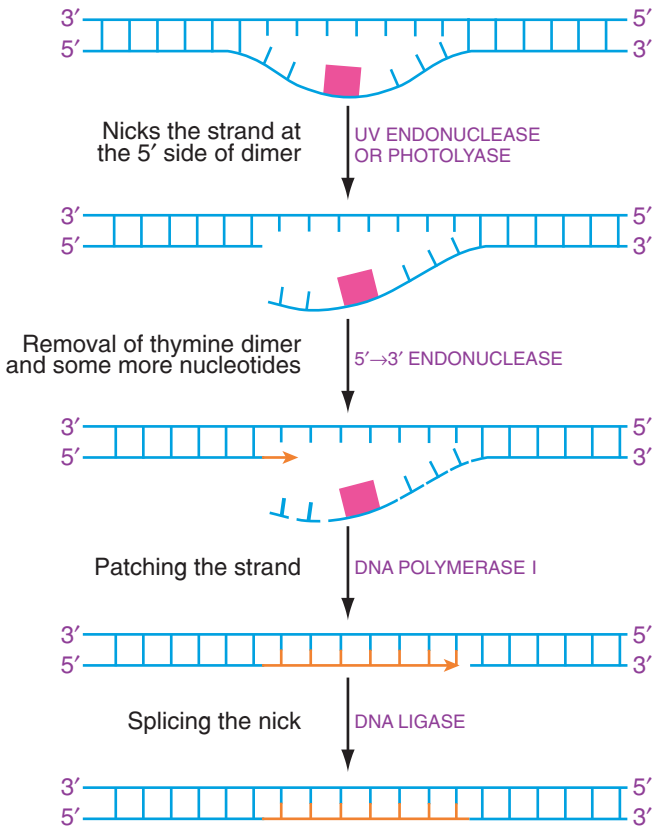
DNA repair mechanisms:

Single strand and double strand DNA damage: Cells cannot function if DNA damage corrupts the integrity and accessibility of essential information in the genome (but cells remain superficially functional when so-called 'non-essential' genes are missing or damaged). Depending on the type of damage inflicted on the DNA's double helical structure, a variety of repair strategies have evolved to restore lost information. If possible, cells use the unmodified complementary strand of the DNA or the sister chromatid as a template to loosely recover the original information. Without access to a template, cells use an error-prone recovery mechanism known as translesion synthesis as a last resort.

Damage to DNA alters the spatial configuration of the helix and such alterations can be detected by the cell. Once damage is localized, specific DNA repair molecules bind at or near the site of damage, inducing other molecules to bind and form a complex that enables the actual repair to take place. The

types of molecules involved and the mechanism of repair that is mobilized depend on the type of damage that has occurred and the phase of the cell cycle that the cell is in.

Direct reversal: Cells are known to eliminate three types of damage to their DNA by chemically reversing it. These mechanisms do not require a template, since the types of damage they counteract can only occur in one of the four bases. Such direct reversal mechanisms are specific to the type of damage incurred and do not involve breakage of the phosphodiester backbone. The formation of thymine dimers (a common type of cyclobutyl dimer) upon irradiation with UV light results in an abnormal covalent bond between adjacent thymidine bases. The photoreactivation process directly reverses this damage by the action of the enzyme photolyase, whose activation is obligately dependent on energy absorbed from blue/UV light (300–500 nm wavelength) to promote catalysis.

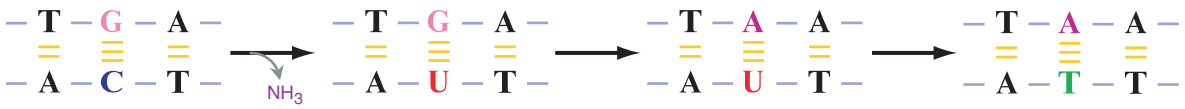


Another type of damage, methylation of guanine bases, is directly reversed by the protein methyl guanine methyl transferase (MGMT), the bacterial equivalent of which is called OGT. This is an expensive process because each MGMT molecule can only be used once; that is, the reaction is stoichiometric rather than catalytic. The third type of DNA damage reversed by cells is certain methylation of the bases cytosine and adenine.

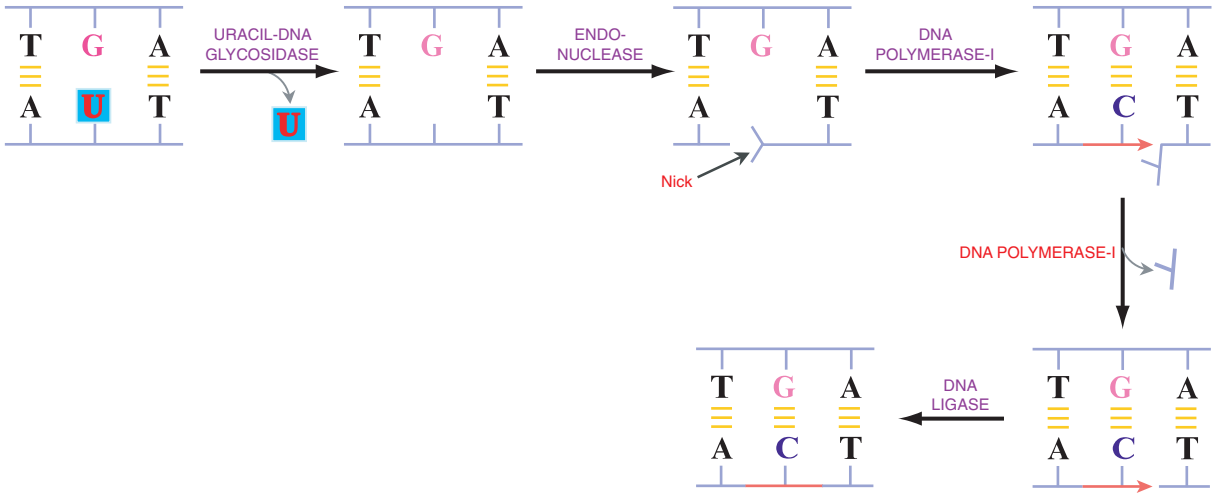
Single strand damage: When only one of the two strands of a double helix has a defect, the other strand can be used as a template to guide the correction of the damaged strand. In order to repair damage to one of the two paired molecules of DNA, there exists a number of excision repair mechanisms that remove the damaged nucleotide and replace it with an undamaged nucleotide complementary to that found in the undamaged DNA strand.

1. **Base excision repair (BER):** This repairs damage to a single nucleotide caused by oxidation, alkylation, hydrolysis, or deamination. The base is removed with glycosylase and ultimately replaced by repair synthesis with DNA ligase.

Spontaneous deamination of cytosine converts it to uracil. Replication of the altered strand results in replacement of G with A in the new complementary strand. Replication of the new strand with the incorrect A residue results in replacement of U with T. The end result of the spontaneous deamination of cytosine to uracil is the replacement of a G-C base pair with an A-T pair.



Spontaneous deamination of cytosine residue of DNA to yield uracil can cause a mutation unless repaired. Hence a well developed repair mechanism exists in the system wherein uracil can be removed enzymatically and the empty deoxyribose phosphate replaced with correct deoxycytidine phosphate residue.



2. **Nucleotide excision repair (NER):** It repairs damage affecting longer strands of 2–30 bases. This process recognizes bulky, helix-distorting changes such as thymine dimers as well as single-strand breaks (repaired with enzymes such as UvrABC endonuclease). A specialized form of NER known as Transcription-Coupled Repair (TCR) deploys high-priority NER repair enzymes to genes that are being actively transcribed.
3. **Mismatch repair (MMR):** It corrects errors of DNA replication and recombination that result in mispaired (but normal, that is non-damaged) nucleotides following DNA replication.

22 BIOTECHNOLOGY

Biotechnology can, conveniently be defined as the manipulation of biological materials and their processes, for serving human beings in a better way.

Some of the biological techniques are described here in detail.

NUCLEIC ACID PROBES

Nucleic acid (NA) probes are pieces of nucleic acid (DNA or RNA) that can be used to identify the presence of a gene of interest. This probe is linked either to a radioactive substance, fluorescent compound, or an enzyme that gives a coloured product in order to be detected. The probe is bound if a sample of nucleic acid contains a base sequence complementary to that of the probe.

Applications of DNA probes: DNA probes are being used—

(a) To identify genes of interest to differentiate normal and mutated genes.
(b) To identify oncogens in biopsy sample.
(c) To detect DNA polymorphism due to variation in a single locus caused by point mutation.
(d) Detection of gene causing genetic diseases and screen foetus for genetic disorders.
(e) To detect presence of pathogenic organisms in blood or tissue samples.
(f) As primers for PCR.
(g) Pathogens can be identified by detecting specific RNA base sequences with probes.

Methods for preparation of DNA probes: Probes are synthesized by three methods viz. (1) Synthetic oligonucleotide probe (2) PCR generated probes and (3) *In vivo* cloning. These methods are based upon the knowledge of the nucleotide sequence of many genes. Hence first of all the method for gene sequencing will be described hereunder and later methods for preparation of DNA probes will be taken up in detail.

GENE SEQUENCING

The method for deducting the sequence of nucleotide bases in a gene or DNA is known as gene sequencing. There are three methods for gene sequencing (a) Chemical cleavage method (b) Enzymatic method and (c) Oligonucleotide hybridization.

1. **Chemical cleavage method or Maxam-Gilbert method:** A DNA molecule can be cleaved chemically at a particular nucleotide base into small fragments. DNA can be cleaved specifically at G, A and G, C and T or only C, by adopting different chemical procedures.

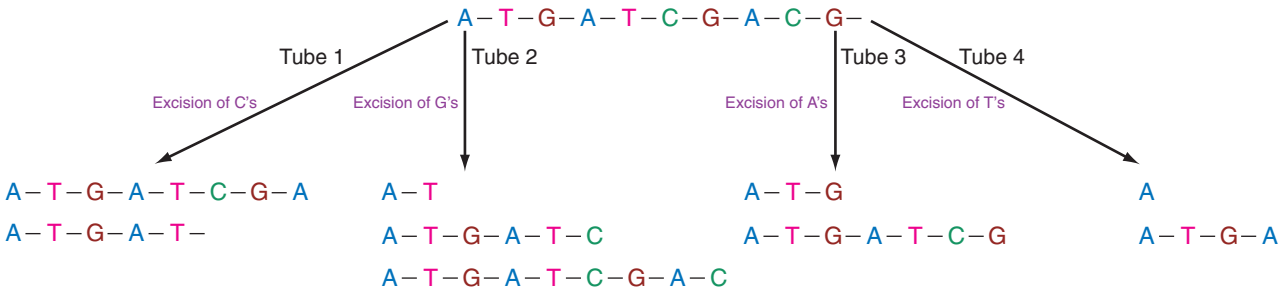
If DNA is heated at neutral pH, then the glycosidic bond (the bond between the sugar and base) of a methylated purine is broken off, when the heating is continued after adding alkali, the backbone of the fragment at G is broken. If this heating is done in dilute acid, the backbone at both A & G is broken. Treatment of DNA with hydrazine in 2M NaCl and then with piperidine cleaves at C only. Treatment with only piperidine cleaves both at C & T. Using any one of these treatments, DNA is cleaved at a specific point. Supposing the following is the sequence of DNA whose sequencing is to be done

$^{5'}$ A–T–G–A–T–C–G–A–C–G– $^{3'}$

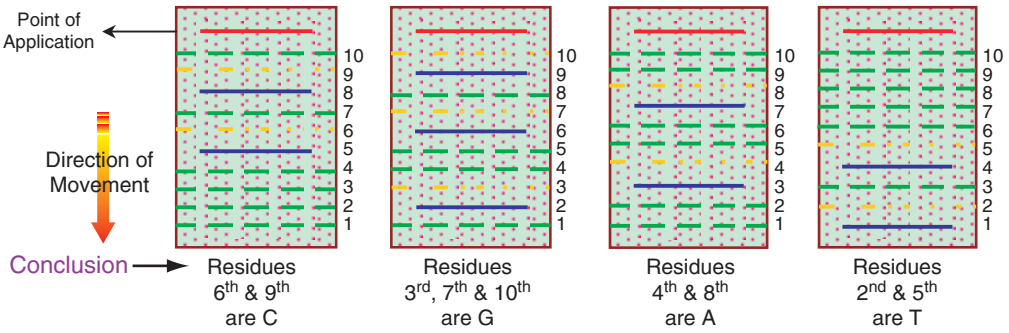
First of all this piece is labelled with radioactive ^{32}P at the 5′ end.

$^{5'}$ ^{32}P–A–T–G–A–T–C–G–A–C–G– $^{3'}$

This labeled molecule is taken into four different tubes. Each tube is treated with different chemical procedures, so as to cleave either at A, T, G, or C, as described above. This results in the DNA molecule breaking into the following smaller fragments. (Fragments having the label are only considered)



Each of these four mixtures is separately subjected to electrophoresis on polyacrylamide gel (PAGE). In this electrophoresis the polynucleotide moves according to the number of nucleotide residues they contain, wherein the smallest fragment will move faster. Difference in a single nucleotide from one fragment to the other results in a great difference in their migration fronts. Thus these fragments can be separated depending upon their number of nucleotides. The exact position of only the labelled (^{32}P) fragments in the gel can be determined by autoradiography on a photographic film. The results of these procedures are compared with the calibrated electrophoretic pattern corresponding to nucleotides having from 1 to 10 bases. Thus the electrophoretic pattern for the above polynucleotide after fragmentation with different chemical methods will be as follows—



Electrophoretic pattern of polynucleotide after fragmentation

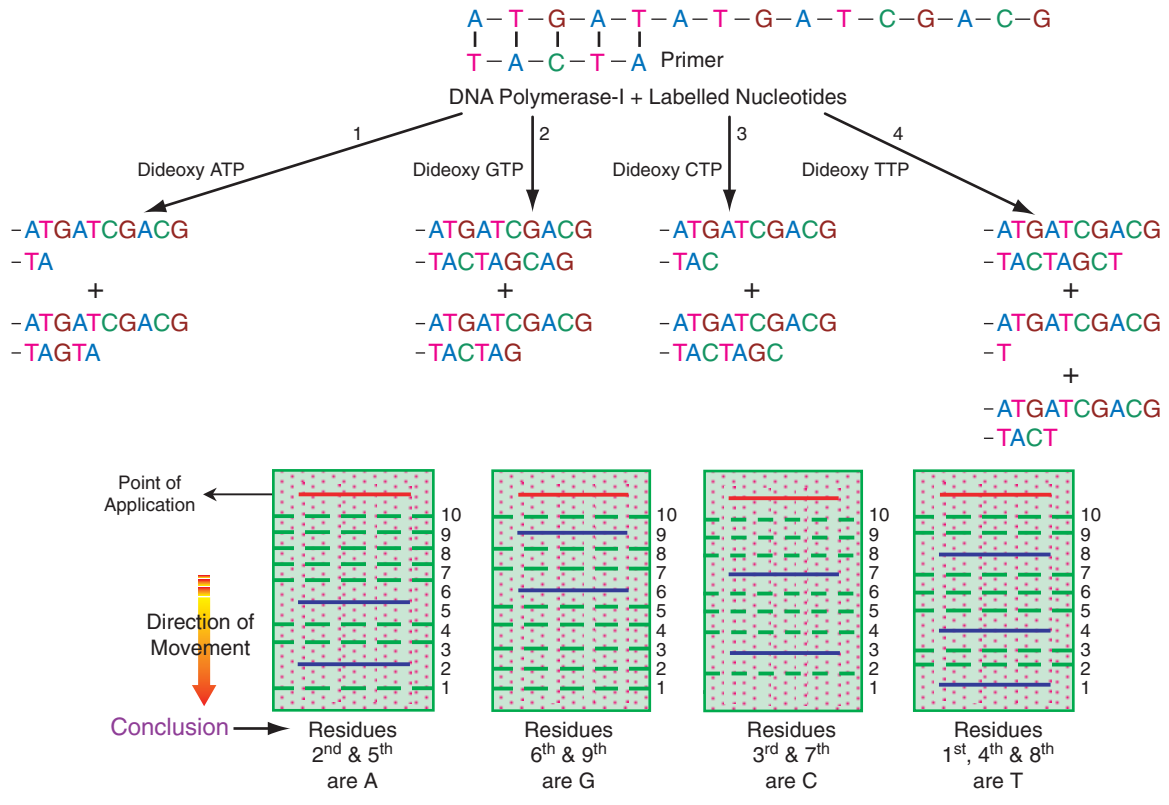
It is seen that the labelled fragments obtained by deletion of C residues moved at rates indicate that they had 5 and 8 nucleotide units. Thus residues 6th and 9th of the original polynucleotide must have been C. Likewise tallying the number of nucleotides in each set of the chemically reacted fragments derive that the sequence of the polynucleotide is—

$^{5'}$ A–T–G–A–T–C–G–A–C–G– $^{3'}$

To depict the sequence of the complete DNA molecule, it is fragmented by different restriction endonucleases and each of the resulting fragment is sequenced as above and the overlaps in the fragments helps in arranging the sequence of the complete DNA.

1. **Enzymatic method or Frederick Sanger method:** Biologically DNA is duplicated by enzymes using each of the strands as a template i.e. a complementary strand is synthesized to each of the strands. The enzyme for replication is known as DNA polymerase (I, III).

The single stranded DNA fragment, whose sequence is to be determined is taken in a tube and to it is added a short length of complimentary primer and the enzyme DNA polymerase-I (Klenow fragment), radioactively labelled four deoxyribonucleotides and any one of the 2′,3′-dideoxy nucleotide analog is taken. The enzyme adds on nucleotide by nucleotide and whenever the analog is added, growth of the new chain is blocked because it lacks the 3′-hydroxyl end needed to form the next phosphodiester bond. Hence, fragments of various lengths are produced in which the dideoxy analog is at the 3′ end. Four such sets of chain terminated fragments (one of each dideoxy analog - A,T,G,C) are then electrophorised and the base sequence of the new DNA is read from the autoradiogram of the four lines.



Electrophoretic pattern of DNA fragments

The sequence is TACTAGCTG, which is the complementary strand. Hence the original strand will contain the sequence—

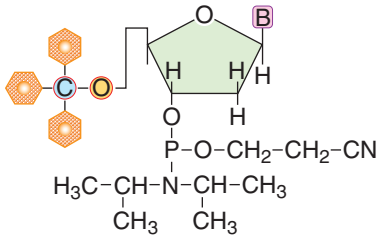
$^{5'}$ A–T–G–A–T–C–G–A–C– $^{3'}$

ARTIFICIAL SYNTHESIS OF DNA

DNA can be synthesized with a desired sequence of nucleotides, by the use of chemical reactions in the laboratory, which are fast, easy and inexpensive.

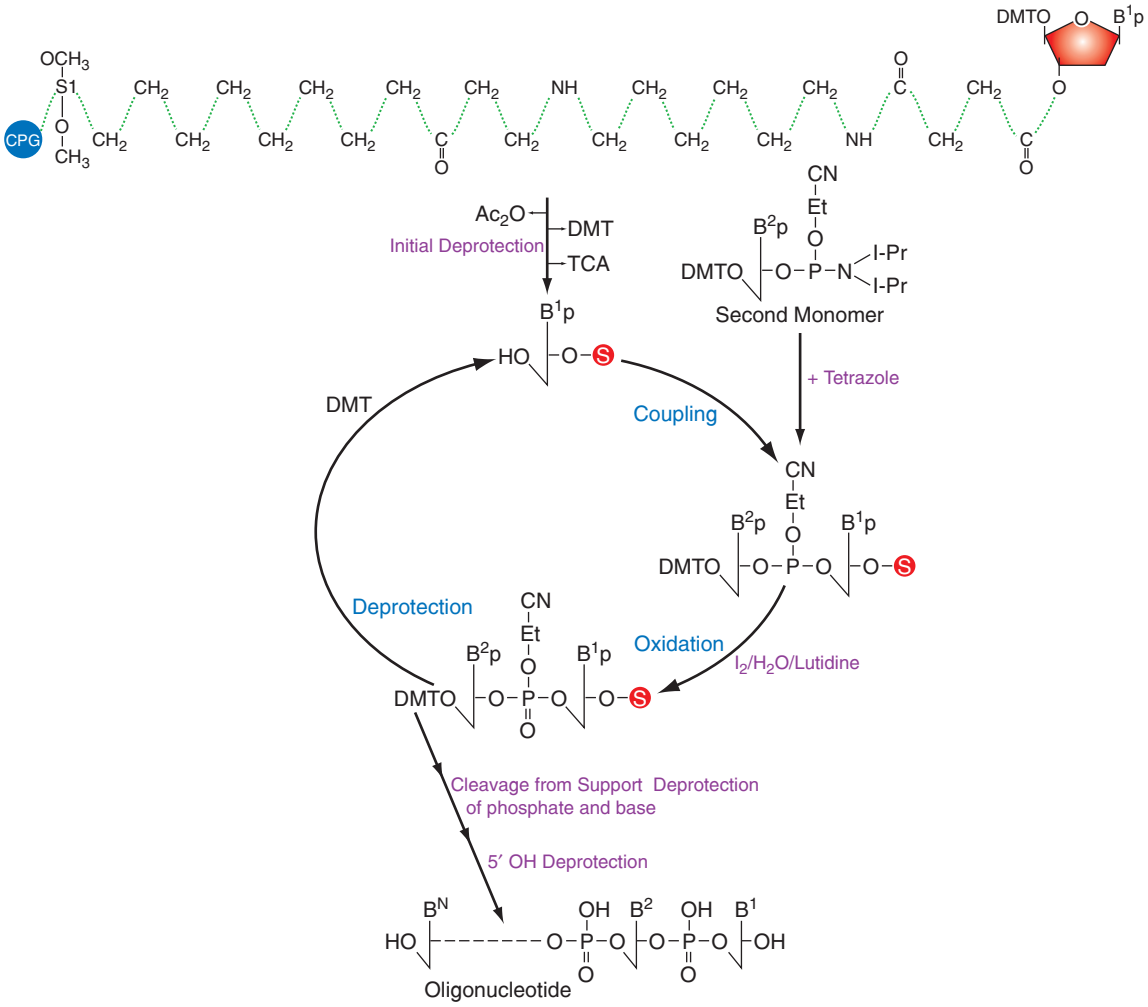
Procedure: A chromatographic column is filled with controlled pore glass (CPG) beads, which acts as an inert solid support. The first nucleoside of the 3′-end is attached to a long chain alkylamine-succinate

which is the spacer linked to the glass bead. The 5′-OH group is protected with dimethoxy trityl (DMT) group. Further every nucleotide being added has a 5′-DMT group and a di-isopropylamine group at 3′-phosphite group with a methyl group. This structure is called phosphoramidite.



Phosphoramidite structure

Further the amino groups of all the bases are protected from cross reaction with benzoyl and isobutyl groups. The nucleotide chain is elongated on the first nucleoside attached to glass beads through the spacer, in a cyclic manner as follows—



Schematic representation of chemical synthesis of DNA

(a) The column is washed with acetonitrile to remove water and any nucleophiles present.
(b) Acetonitrile is then removed from the column by flushing it with argon.
(c) TCA is added to remove the 5′-DMT (detritylation step) and thus produce a reactive 5′-OH group.
(d) To remove TCA, the column is washed with acetonitrile which is in turn flushed out with argon.

(e) The next base (phosphoramidite) and tetrazole are introduced into the column.
(f) Tetrazole activates the phosphoramidite to form a covalent bond between its 3′-phosphite and 5′-OH group of the first nucleoside (coupling step).
(g) Argon is flushed to remove unused phosphoramidite and tetrazole.
(h) The inter-nucleotide linkage in the form of phosphite triester bond is unstable and hence it is oxidized with iodine mixture to form phosphate triester bond (oxidation).
(i) Those support-bound nucleosides which are not coupled to the second nucleotide added are inactivated for further reaction by acetic anhydride and dimethyl-amino pyridine (capping step).
(j) The column is washed again to remove all the unwanted material.
(k) Then the cycles of detritylation, phosphoramidite activation, coupling, capping and oxidation are repeated till all the nucleotides are added.
(l) When all the cycles are completed, the methyl groups of phosphate triester are removed with triethyl ammonium thiophenate in dioxane.
(m) The oligonucleotide is cleaved from the support by concentrated ammonia and eluted out of the column.
(n) The bases are deprotected (benzyl and isobutyl groups removed) by heating to 50°C in concentrated ammonia solution for 6 hours.
(o) The 5′-DMT is removed with 80% acetic acid.
(p) The 5′-terminal end of the artificially synthesized DNA strand is phosphorylated by an enzyme T_4 polynucleotide kinase using ATP.
(q) The synthesized DNA is finally purified by HPLC.

Uses of artificially synthesized DNA by chemical method:

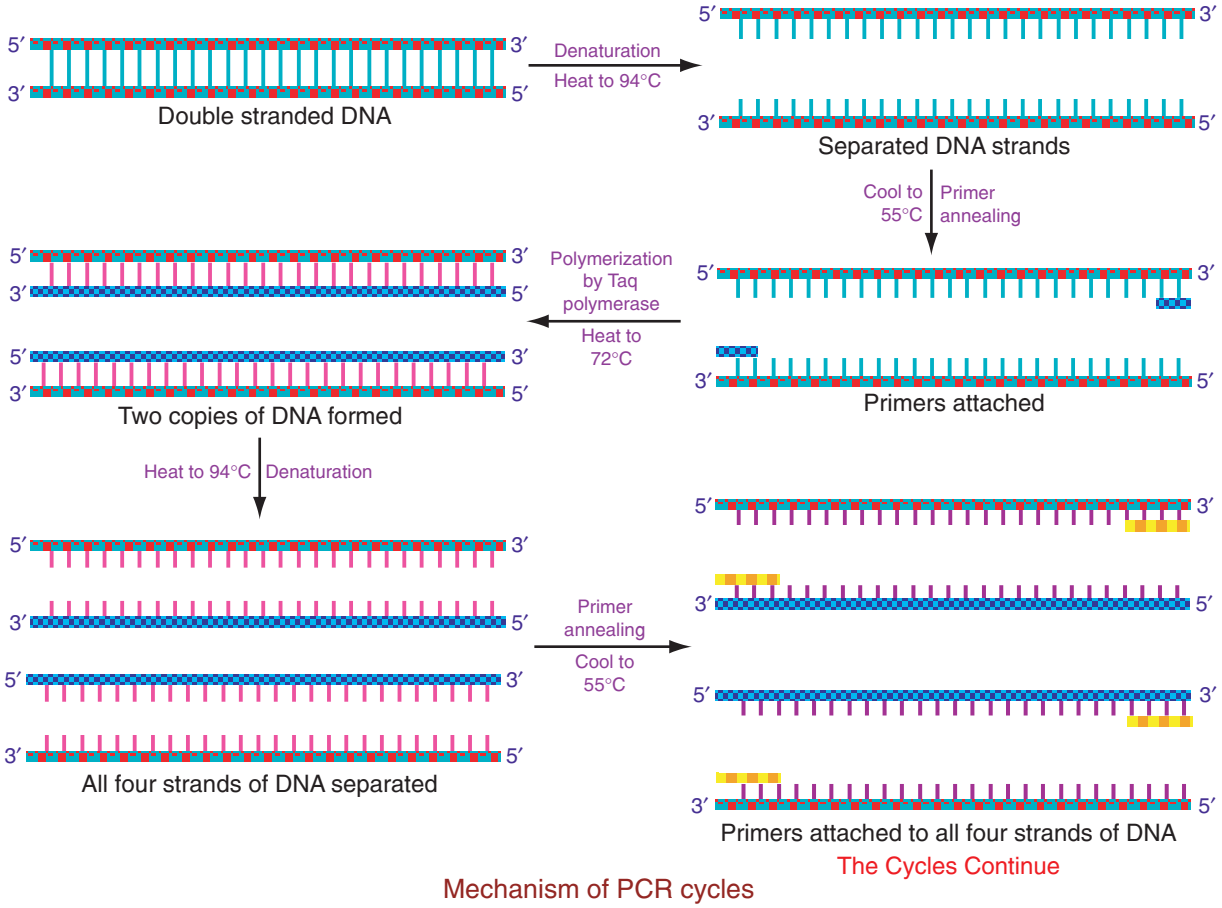
(1) Chemically synthesised DNA, with a specific sequence are used as primers for polymerase chain reaction.
(2) They are also used in DNA sequencing–as a short length of primer, complementary to the sequencing DNA fragment, in the enzymatic method of DNA sequencing.
(3) Oligonucleotides are used for creating mutations i.e. an oligonucleotide is synthesized with an altered base and is introduced in place of the original gene. This improves the performance of many bacteria or various human/animal/plant genetic diseases can be treated.
(4) If the amino acid sequence is known, then a DNA strand is synthesized using the codons for these amino acids. The gene synthesized for this protein is used for gene cloning.
(5) Double stranded DNA strands, called linkers can be synthesized containing restriction endonuclease recognition site, which helps in the cloning of DNA fragments (after breaking and joining).
(6) DNA sequences called adaptor sequences can be synthesized, which contains sites for two different restriction endonucleases–one used for insertion of the DNA and the other for excision at a later time.
(7) DNA fragments synthesized are used as DNA probes for diagnosis of various molecular diseases.
(8) Chemically synthesized DNA fragments are also used in the treatment of genetic disorders.

POLYMERASE CHAIN REACTION

Polymerase chain reaction is abbreviated as P.C.R. technology and termed as *in vitro* enzymatic gene amplification or *in vitro* gene cloning without its expression. It resembles the electronic xeroxing of pages, hence this process can also be termed as **'DNA xeroxing'**.

P.C.R. technology is the amplification/cloning of DNA (or a gene) in a test-tube, using a mixture of template + primers + enzyme + bases (dNTPs) + buffer, by cycling the temperature within the reaction tube.

Procedure: A micro test tube is taken with the DNA template (the sample), that is to be amplified, along with tris buffer (50 mM KCl + 10 mM tris HCl) to get a pH of 8.4. $MgCl_2$ (1.5 mM) and 100 μg of gelatin are additional components required to stabilize the reaction mixture. Sufficient concentration (200 µM each) of deoxy-ribonucleotide triphosphates (dATP, dCTP, dTTP, dGTP) and 2.5 units of the enzyme, Taq polymerase (obtained from a thermophilic Bacterium *Thermus aquaticus*–Taq) is added to it. Two primers of about 20 to 30 bases long, complementary to the 3′ ends of the two chains of the template are also added to about 0.25 M of each primer. A few drops of mineral oil are added to seal the reaction and condensation prevention. This total reaction mixture is made to a volume of 100 μl.

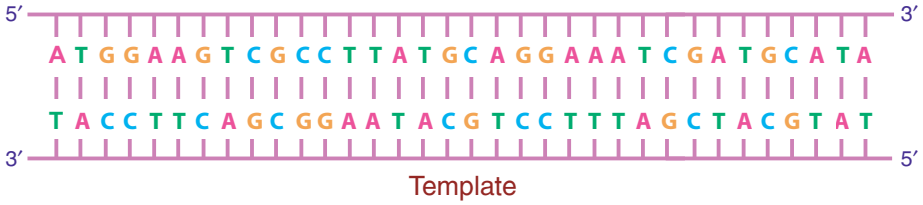


Mechanism of PCR cycles

The P.C.R is carried out with the above mixture in the DNA thermal cycler which cycles between three temperatures at regular time intervals, automatically. The first temperature in the cycle is maintained at 94°C for 20 sec, which causes separation of the double stranded template DNA strands. Then the temperature is changed to 55°C for 20 sec, due to which the primers are attached (annealed) with each of the complementary template strands. Then finally the temperature is maintained at 72°C for 30 sec, which is the optimum temperature for the enzyme Taq polymerase that facilitates the enzyme to extend the polynucleotides on the primers and completes the polymerization. This cycling of temperature then continues for 20-30 cycles, thereby causing a polymerase chain reaction and thus producing one million copies in 20 cycles which needs about 40 minutes of time.

The Template: Template is a double stranded DNA fragment which is to be amplified by PCR. It can be any DNA fragment (or gene) of interest, for instance the beta-globin gene fragment, albumin gene or any one DNA selected for the purpose. This DNA fragment can either be in a pure form or in a homogenous mixture of two or more DNA fragments (i.e. crude form) or may be a part of the complete genomic DNA (chromosome). It can either be synthetic DNA or native DNA (a natural one). It is very

much necessary to know the complete DNA sequence of the DNA fragment that is to be amplified by PCR, because a primer of 10-20 bases has to be added which will be complementary to the 3′ end bases of both the strands and this primer can be prepared only when the base sequence of the DNA template is known. So upon heating to 94°C both the strands separate and the primers attach to both the ends and the enzyme adds bases, nucleotide by nucleotide complementary to that DNA strand (template) to the free 3' end of the primer. The minimum number of the template DNA required for P.C.R. is 10^2 (i.e. = 0.1 µg of human genomic DNA). It can be up to a maximum of 10^5 DNA molecules (= 2.6 µg). In each cycle of P.C.R., the template doubles exponentially, thereby doubling the rate of P.C.R in each and every cycle.



The Primer: The primer is a small DNA fragment of 10-20 bases long, synthesized chemically and is complementary to the base sequence of both the template DNA strands at the 3'-ends. To synthesize this primer the sequence of DNA that has to be amplified should be known, so that complementary primers can be synthesized. The concept is that, a particular gene (DNA fragment) will have a specific sequence, so preparation of the primer complementary to that specific sequence results in amplification of that particular DNA (gene) whether it is in a crude mixture or as a part of DNA within the genomic DNA.

When heated to 94°C the two template strands are separated and on cooling to 55°C the primers, being more in concentration and shorter in length will anneal to complementary template strands at 3' ends. The concentration of primer in the P.C.R ranges between 0.05µM to 0.1µM of each oligonucleotide primer.

The Enzyme: The enzyme used in polymerase chain reaction is a special type of DNA polymerase known as Taq polymerase. This enzyme does not denature even at high temperatures of 98 °C and thus is said to be **'thermo stable enzyme'**. This enzyme is found (synthesized) in thermophilic (growing in hot springs) bacteria —*Thermus aquaticus* abbreviated as Taq. Its molecular weight is 94 KDa and its optimum temperature is 72°C. It is also active in the temperature range of 22°C to 89°C.

Separation of the two DNA strands is carried out by heating the reaction mixture to 94 °C which does not denature the enzyme Taq polymerase, whereas its activity is retained and upon cooling, the enzyme starts polymerization of nucleotides and when its optimum temperature (72°C) is kept, the enzyme would have acted totally for about 3 minutes and by this time it will add 200 to 20000 Kbp. Hence the time given for each step is very short i.e. sufficient for complete polymerization. This enzyme needs magnesium ions (2.0 mM), dNTPs (0.7 to 2.4 mM), KCl (50 mM) and an optimum pH of 8.4 for its activity. Higher concentration of magnesium, dNTPs (4.6 mM) and KCl (200 µM) will inhibit the activity of Taq polymerase. The conventional protein denaturing agents like urea, ethanol, formamide, at lower concentrations do not have any effect of denaturation on Taq polymerase.

The DNA thermal cycler: The polymerase chain reaction needs three different temperatures—(1) 92-97°C for 20 sec, for denaturation of DNA and strand separation, (2) 40-60°C for 20 sec, for primer annealing and (3) 65-80°C for 30 sec, for primer extension or polymerization by Taq polymerase. The total time per cycle of P.C.R. comes to about 3.75 minutes, which includes the time needed to reach each and every changed temperature and the time interval for each of the temperatures. In this short time interval three

different temperatures have to be changed and maintained for a specified time. Therefore an automatic DNA thermal cycler is prepared which heats up water by electric current resistance and then the cooling is effected by fluid flow in the cycler (refrigeration). To this is attached a temperature sensitive knob which automatically switches on/off in a cyclic manner in the specified time limit, for that particular temperature.

Uses of P.C.R.:

(1) It is used to alter a particular template sequence for production of newer and desired DNA, obviously by getting ample number of DNA copies.
(2) A particular DNA sample can be isolated (by amplification) from a crude sample of DNA which has got great research applications, medical diagnostic applications and forensics.
(3) To detect any defect in DNA sequence either hereditary or infected by virus/bacteria. PCR has been used to detect sickle cell anemia, mutation, HIV genomic sequence infection, and altered sperm genetics by DNA amplification.
(4) PCR is an effective procedure for detecting the presence of a known DNA sequence in very small, crude samples, without purification. Due to this, PCR can be used to determine whether a particular illness is due to a viral infection. If the sequence of the viral DNA is known previously, then a pair of primers that anneal to sites in the targeted viral DNA can be synthesized. After PCR cycling a DNA fragment of a specific size will be amplified only if the viral DNA is in the sample, if not no amplification.
(5) PCR is used to detect naturally occurring mutations.
(6) It is also used to produce mutations artificially.
(7) To assemble whole genes from synthetic DNA oligonucleotides, PCR is used.
(8) PCR is also used for DNA sequencing.
(9) PCR is used in DNA finger printing technique.

TB-PCR-RNA: The bacterium *Mycobacterium tuberculosis* (TB) has been found only in humans. The disease spreads directly between people through the air. Every second, someone somewhere in the world gets infected, and each year TB kills about 2 million people. The ancestor of contemporary *Mycobacterium tuberculosis* originated from a 3 million years old species. *Mycobacterium tuberculosis* bacilli are inhaled through the lungs to the alveoli, where they are phagocytosed by polymorphonuclear leukocytes and macrophages. Although most bacilli are initially contained, some are carried to the region's lymph nodes. Eventually, the thoracic duct may deliver mycobacteria to the venous blood; this may result in seeding of different organs, including the kidneys. The genitourinary system is a common site of extrapulmonary tuberculosis (TB). Genitourinary tuberculosis (GUTB) may involve the kidneys, ureter, bladder or genital organs. Clinical symptoms usually develop 10-15 years after the primary infection. Only about a quarter of patients with GUTB have a known history of TB; about half of these patients have normal chest radiography findings.

PCR is a very sensitive diagnostic tool for TB. As the TB is widespread over the complete human body, sample from any part of the body can be used to conduct PCR. PCR-RNA Enzyme Immunoassay may detect mycobacteria. However, polymerase chain reaction DNA enzyme immunoassay of blood may be a sensitive means of diagnosing mycobacterial infection. The following PCR tests are available with near-equivalent quality—

- ✓ Genus-specific 16S rRNA PCR test
- ✓ Species-specific IS6110 PCR test
- ✓ Roche amplicor MTB PCR test
- ✓ Amplified *Mycobacterium tuberculosis* direct detection test (AMDT)

RNA extraction and reverse transcriptase (RT) PCR: The mRNA extracted from the sample is used as a template for reverse transcription followed by PCR. RNA is extracted from the strain infecting the

patient, for instance H37Rv cells, cultured in vitro in Middlebrook 7H9 medium supplemented with albumin-dextrose complex and dissolved in 50 µl of nuclease-free water. The first-strand synthesis is carried out using avian myeloblastosis virus reverse transcriptase. This is followed by heat denaturation to inactivate the enzyme. Subsequent second-strand synthesis is done using Tfl polymerase. The upstream primers for DNA of H37Rv are—

$^{5'}$–GGATCCATGCATTTCGAAGCGTAC–$^{3'}$

The downstream primers for DNA of H37Rv are—

$^{5'}$–AAGCTTCTAAGTGTCTGTACGCGATGA–$^{3'}$

The PCR product is visualized by electrophoresis in a 1% agarose gel. A 597-bp RT-PCR product is observed upon staining with ethidium bromide. A 600-bp DNA molecular size marker is run alongside.

RECOMBINANT DNA TECHNOLOGY

It is abbreviated as recDNA/rDNA technology. It is also called genetic engineering, gene cloning, molecular cloning and gene transfer technology.

Recombinant DNA technology is the process of formation of an altered DNA (chromosome) by artificial addition of exogenous DNA (gene) which can be replicated, transcribed and translated.

It is possible to isolate genes specifying two different proteins from two different species and join them together in a test tube, to form a new combination of gene/DNA/protein which is known as chimeric molecule.

Various steps involved in the preparation of a recombinant DNA are:

(1) Selection of a DNA of interest which is called foreign DNA, target DNA, passenger DNA, cloned DNA or insert DNA (ex. gene for insulin, GH, albumin or β-globin etc).

(2) Selection of a suitable cloning vector DNA or vehicle DNA, into which the target DNA can be inserted or loaded (ex. plasmid or lambda phage DNA).

(3) Selection of a specific restriction endonuclease, which can make suitable internal cuts at specific sites in both the target DNA and vector DNA, at the same point.

(4) The target DNA from a donor organism is extracted and cleaved enzymatically by the selected restriction endonuclease and joined (ligated) by the enzyme ligase, to the vector DNA to form a new recombinant DNA molecule called cloning vector-insert DNA construct.

(5) Then a host cell is selected to replicate, transcribe and translate the recombinant DNA, which may be a prokaryotic cell or an eukaryotic cell (ex. *E. coli* cell, lambda phage particle, yeast or fungi). The introduction of the rDNA into a host cell is called transformation.

(6) The transformed cells i.e. those host cells which have taken up the rDNA are identified and selected (separated/isolated) from the non-transformed cells i.e. those that do not have the rDNA.

(7) Finally it has to be ensured that the DNA of interest inserted into the vector DNA is producing the protein product continuously, if not, the rDNA has to be manipulated again, so as to ensure continuous production of the desired protein.

Details of recombinant DNA technology:

1. **Selection of target DNA:** Target DNA is selected considering the following points—
   (a) It should be easily extractable from its source of natural existence.
   (b) It should be able to be incorporated in the vector at such a place where it can be replicated, transcribed and translated as desired.
   (c) The gene product (protein) produced should either be commercially important or important for research purpose.
   (d) The foreign DNA (gene) of interest may be viral, bacterial, of plant or animal origin.

The genes for the following proteins are generally cloned i.e. inserted into the vector DNA and the recombinant DNAs produced and the protein extracted for human use.

Gene cloned for protein	Use
Human insulin	Hormone that controls blood glucose levels
Human somatostatin	Hormone that regulates growth
Human somatotropin	Growth hormone that acts in conjunction with somatostatin
Human interferon	Antiviral agent
Hepatitis B core antigen	Diagnosis of hepatitis B virus
Beta-Endorphin	Analgesic
Interleukin-2	Cancer chemotherapy
Relaxin	Facilitates child birth
Calcitonin	Increases blood calcium level
Lymphotoxin	Kills tumour cells
Tumour necrosis factor	Kills tumour cells
Ricin	Immunotoxin
Chymosin	Cheese production
Thaumatin	Sweetner
Polyaspartyl phenylalanine	Preparation of aspartame sweetener
Insulin like growth factor	Stimulating growth hormone release

2. **Cloning vectors:** The cloning vector is the DNA molecule into which the target DNA is introduced producing the recombinant DNA molecule. A good cloning vehicle is one which has only a single site for cutting by a particular restriction endonuclease. There are different types of vectors which can be used to clone fragments of foreign DNA and propagate (clone) them in a suitable host.

Some vectors and their respective hosts

Vector	Host cell
1. Plasmids	*E. Coli*
2. Bacteriophage - lambda	*E. Coli*
3 Bacteriophage M13	*E. Coli*
4. Cosmids	*E. Coli*
5. Bacteriophage QC31	Yeast
6. YE_p	Yeast
7. yIpS	Yeast and fungi

Vector	Host cell
8. yRpS	Yeast and fungi
9. Ti plasmid	Plants (dicotyledons)
10. CaMv (Cauliflower Mosaic virus)	Cauliflower
11. pBR322 plasmid	Plants
12. Simian virus - 40 (SV40)	Mammalian cells
13. Bovine papilloma virus (BPV)	Mammalian cells

Plasmid: These are extra-chromosomal genetic elements found in a variety of bacterial species. They are double stranded, closed circular DNA molecules that range in size from 1 kb to 200 kb. Often, plasmids contain genes coding for enzymes that under certain circumstances are advantageous to the bacterial host. The phenotypes conferred by different plasmids are—

- Resistance to antibiotics.
- Degradation of complex organic compounds.
- Production of enterotoxins.
- Production of antibiotics.
- Production of colicins.
- Production of restriction and modification enzymes.

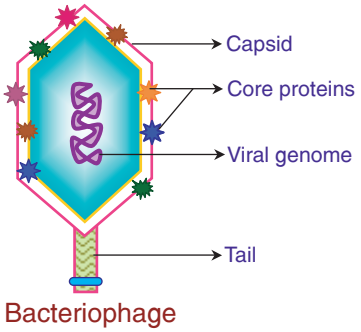
To be useful as cloning vector, the plasmid should possess several properties, like—

- It should carry one or more selectable markers to allow identification of transformants and to maintain plasmid in bacterial population.
- It should contain single recognition site for one or more restriction enzymes in the regions of plasmid that are not essential for replication.
- It should be relatively small and should replicate in a relaxed fashion.
- Preferably these restriction sites, into which foreign DNA can be inserted, should be located within the genes coding for selective markers, so that insertion of a foreign DNA fragment inactivates the gene.

Some commonly used plasmids as vectors for the preparation of recombinant DNA are–pMB-9, pBR-322, pBR-325, pKC-7, pAC-4, C-184, pAC-105, pMK-16, pMF-3, pBRH1, pUB-110, and pCB-16.

Bacteriophages: Bacteriophages are commonly known as phages. They are the viruses that infect bacteria. Similar to other viruses, the phages are very simple containing a protein coat called capsid, enclosing DNA or RNA molecules as the genome. The genes in this genome include the replication gene for DNA/RNA of the phage and the gene for protein coat.

Bacteriophage lambda (λ): The infection is caused by the phage particle by attacking to the outside of the bacterium and injecting its DNA chromosome into the cell. This DNA, about 50 kb, linear-double stranded, with single stranded complementary ends of 12 nucleotides in length (cohesive ends), gets arranged in circle in the host cell through pairing of cohesive ends and is transcribed as circular molecule, during early phase of infection. During this phase the phage may either adopt lytic phase or lysogenic phase.



Bacteriophage

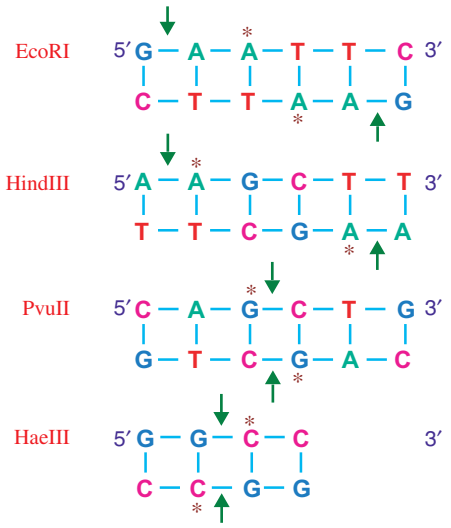
In the course of lytic phase the phage DNA remains independent in the cell, replicates, codes for capsid proteins and forms large number of phage particles within the cell, resulting in the lysis (breakage) of the bacterial cell and thus releasing the phage particle. In the course of lysogenic phase the phage DNA gets incorporated into the bacterial chromosome and exists so for numerous cell divisions, meanwhile synthesizing capsid components and releasing phage particles occasionally. This may convert to lytic phase at any time.

Bacteriophage M13: It is a very small, single stranded DNA phage of 10 Kb. The injection of an M13 DNA molecule into an *E. coli* occurs via the pilus i.e. the structure that connects two cells during sexual conjugation. Inside the cell the DNA synthesizes a complementary strand and becomes the double stranded intermediate known as the replicative form (RF). The original strand is (+) strand and the complementary strand is the (–) strand made in the bacteria. Only the (+) strand is packaged into the new phage coats. RF of M13 acts like a plasmid, which can be easily obtained from *E. coli* cells and used for rDNA technology. Some of the M13 vectors are M13 mp2, M13 mp5, fd101, fd107, fd-tet.

Cosmids: Cosmid is a cloning vector consisting of the lambda cos site (single stranded cohesive extensions at the ends of the DNA molecule) inserted into a plasmid, used to clone DNA fragments upto 40 Kb in size. The maximum size of the DNA that can be introduced into any plasmid is 5 kb and that for bacteriophage M13 is less than 3 Kb. Hence it will become difficult to insert and clone large genes, especially those of eukaryotic DNA. Ex. The alpha-2 collagen gene of chicken is 38 Kb. Hence the bacteriophage lambda genome was modified by Collins and John in 1978 by deleting some bases and introducing single stranded complementary extension sequences at the ends of its DNA molecule called the Lambda Cos site, in order to facilitate insertion and cloning of large DNA molecules. Due to their capacity to hold large sized DNAs, cosmids are used to construct genomic library i.e. a set of recombinant genes that contains the entire DNA present in an individual organism. The construction of libraries in bacteriophage λ vectors has proven to be an affective means of isolating segments of DNA from complex eukaryotic genomes. Cosmids are identified by—

1. A drug resistance marker and a plasmid origin of replication.
2. One or more unique restriction sites for cloning.
3. A DNA fragment that carries the ligated cohesive ends (cos site) for bacteriophage λ.
4. Small size so as to accommodate the eukaryotic DNA fragments of 40-45 kb in length.

3. **Selection of restriction endonucleases:** They are endonucleases (enzymes) that cut DNA molecules only at a limited number of specific nucleotide sequences which are unmethylated palin-



Restriction endonucleases and their recognition sites
Modified bases = * Point of cleavage = ↑

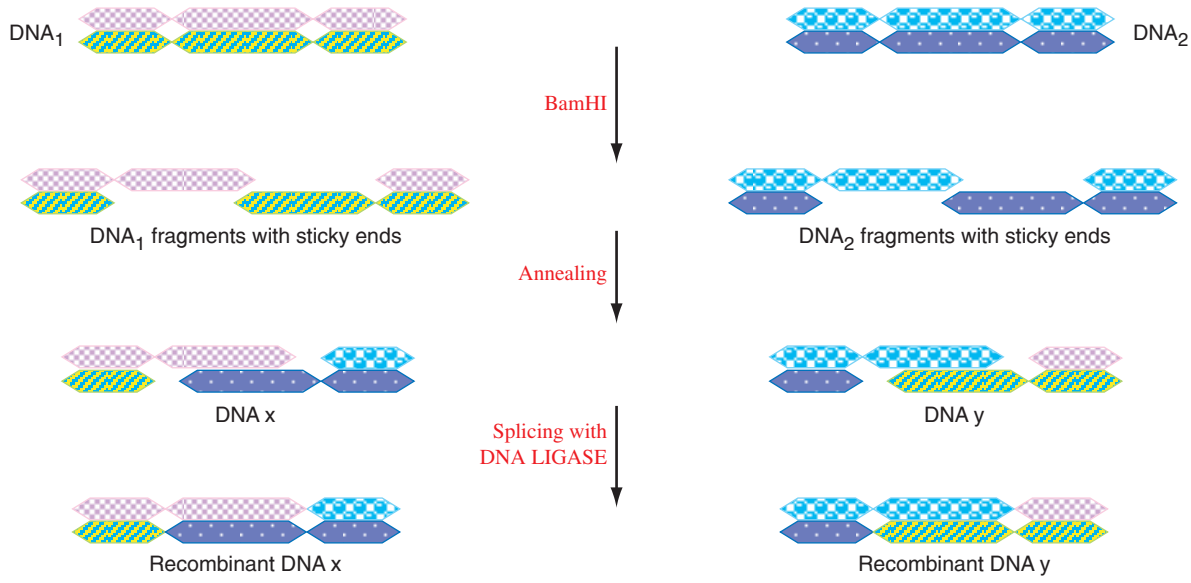
dromes. The action of some endonucleases gives DNA fragments with sticky/cohesive ends whereas others give blunt ended DNA fragments. A few restriction endonucleases are shown in the figure:

4. Procedure for production of recombinant DNA (rDNA):

(a) *Preparation of cloning vector-insert DNA construct*: The target DNA is extracted from source organism which can either be a bacterium, fungi, plant or an animal cell DNA, by various analytical methods for which the cells are first broken open to release the contents either by mechanical disruption (grinding frozen material) or by the use of chemicals like lysozyme, EDTA, the detergent–sodium dodecyl sulphate (SDS) etc, either solely or in combination with one or more chemicals. Then the DNA is purified from the cell extract for which the extract is treated with proteases and endonucleases and then the proteins precipitated with phenol and chloroform and finally centrifuged. The DNA will be measured in a spectrophotometer at 260 nm, at this wavelength the absorbance (A_{260}) of 1.0 corresponds to 50µg of double-stranded DNA/ml. This ultraviolet absorbance can also be used to check the purity of a DNA wherein the ratio of DNA absorbance at 260 nm and 280 nm (A_{260} / A_{280}) is 1.8. The ratio less than 1.8 indicates that the preparation is contaminated, either with protein or phenol.

Similar procedure is followed to extract the DNA from the vectors. The target DNA is then inserted into the vector DNA, by various procedures. Two of them are described below—

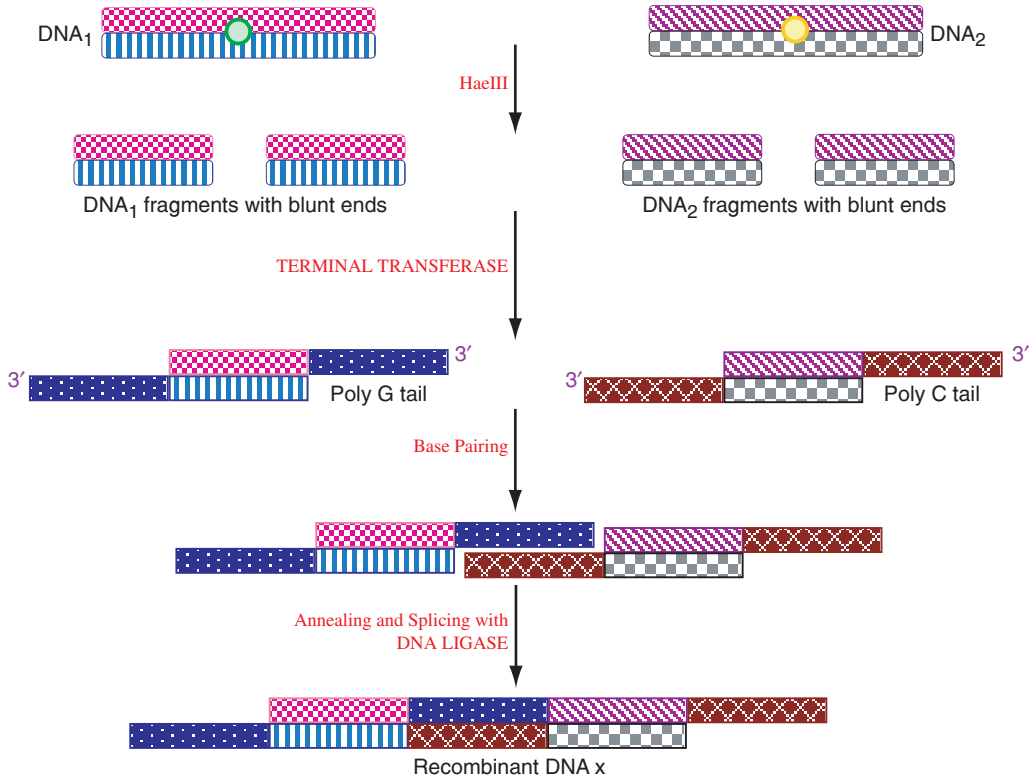
(i) *rDNA formation by the use of restriction endonuclease creating sticky ends*: To join together two duplex DNAs from different species, the two DNAs are separately acted upon by the same restriction endonuclease (BamHI) giving staggered (cohesive/sticky) two stranded cut. Therefore the staggered ends of the two DNAs will be complementary in sequence. Then the two cut DNAs are heated, mixed and cooled, so the sticky ends will base-pair to produce a new kind of recombinant DNA which is joined by DNA ligase.



Formation of recDNA when sticky ends are produced

(ii) *rDNA formation by use of restriction endonuclease creating blunt ends*: Both the target DNA and the vector DNA are acted upon by the same restriction endonuclease (HaeIII) producing blunt ends. Poly 'G' tails are added at the 3′ ends on both strands of the target duplex DNA and poly 'C' tails at the 3′ end of the vector DNA by the use of enzyme terminal transferase. Since these two added tails are

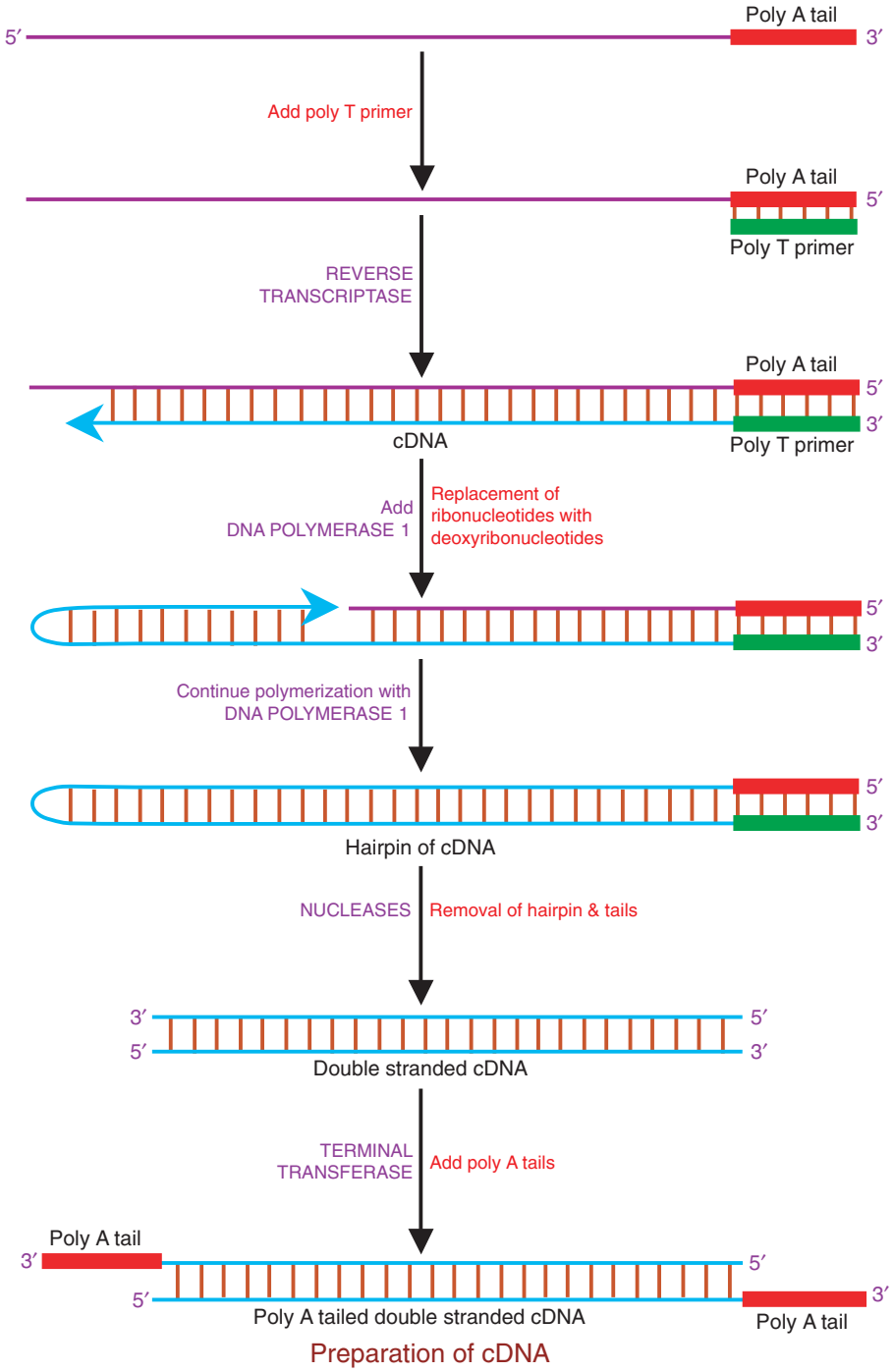
complementary to each other, they will enable the two DNAs to be paired with the created cohesive ends upon heating and cooling. The nicks are joined by the enzyme ligase.



Formation of rDNA when blunt ends are produced

(b) *Preparation of complementary DNA (cDNA)*: It is a cloning technique which involves the conversion of purified mRNA to DNA, prior to its insertion into a vector. Depending upon the source of mRNA its purification procedure varies and in fact many methods for purification of mRNA are available. Here the mRNA specifying the protein beta-globin gene will be described in detail. The mRNA of interest i.e. beta-globin gene mRNA is obtained by lysing the reticulocytes and the polyribosomes are collected by centrifugation and treated with antibody specific for this protein. The antibody will attach to the just complete protein on the polyribosome and precipitate it. From this precipitate the mRNA is obtained by affinity column chromatography. The mRNA obtained so, is used as a template and a complementary DNA (cDNA) is made with the enzyme reverse transcriptase. All eukaryotic mRNAs contain poly 'A' tails at the 3' end. Therefore a poly T nucleotide is added, which base pairs with the poly 'A' tail of mRNA. This serves as the primer for the enzyme reverse transcriptase that now transcribes the mRNA to make a new complementary strand of cDNA. The mRNA is then removed and the single stranded cDNA is now replicated by DNA polymerase-I to yield a 'hairpin' double stranded

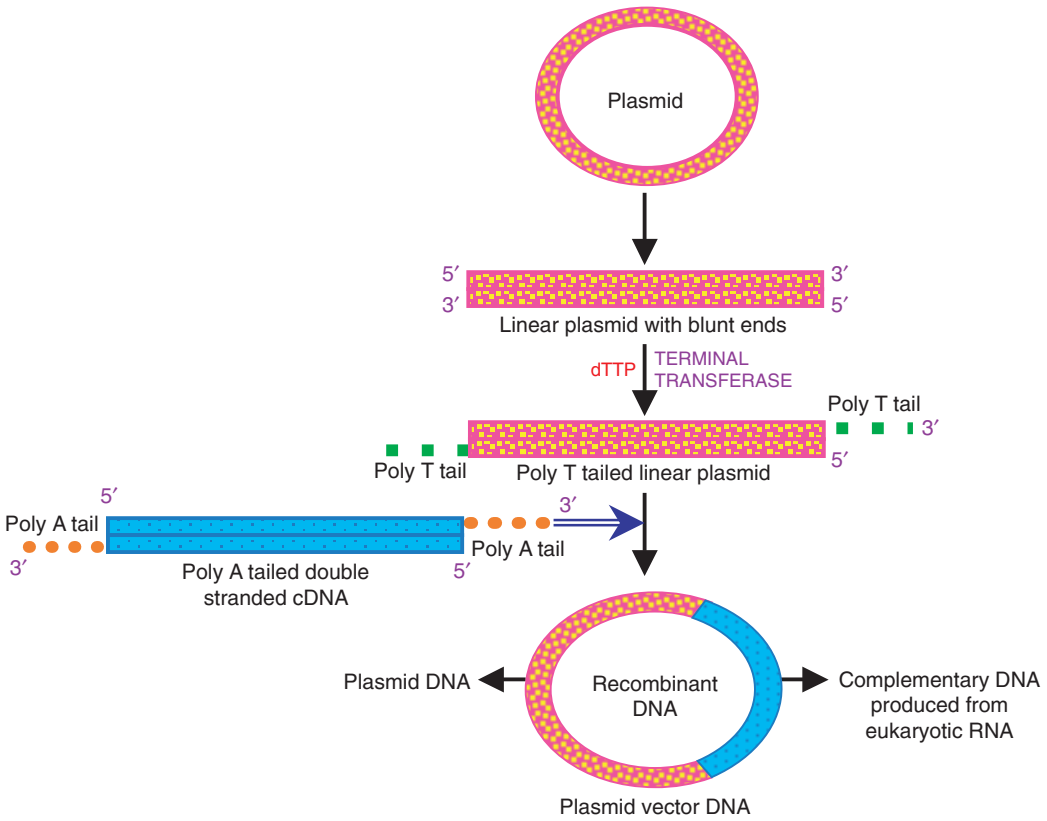
DNA. The hair pin and poly A & T tails are cleaved by nucleases. Thus a synthetic double stranded cDNA, specific for the protein beta-globin is produced.



Preparation of cDNA

The complimentary DNA prepared above is inserted into a vector (plasmid, phage-DNA etc). For insertion, a poly 'A' tail is added to the opposite 3′ ends of the two strands of the duplex cDNA by terminal transferase. Then the plasmid or vector is opened at a single point to yield the linear DNA by the action of restriction endonuclease producing flush/blunt ends. Conversely, poly 'T' tails are attached to the two 3′ ends of the linear plasmid vector. The tailed linear plasmid and the tailed DNA are allowed

to undergo base pairing by heating, mixing and cooling, thereby forming an enlarged circular plasmid containing the new gene i.e. the rDNA. The ends are joined by ligase.



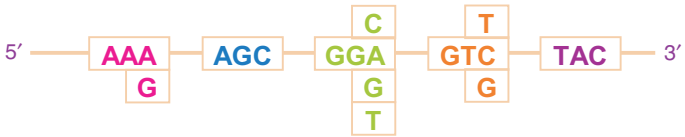
cDNA incorporated into plasmid to form recombinant vector DNA

(c) *Construction of a DNA probe*: If the amino acid sequence of a protein/peptide is known then a DNA probe can be chemically synthesized and used to prepare a rDNA and thus clone it in a suitable host. This DNA probe can also be used to screen the gene for this particular protein/peptide in the genomic library. The DNA probe obtained by this method is not the exact sequence as present naturally in the genomic DNA, because the genetic code is degenerate i.e. more than one codon exists for some of the amino acids. Hence all the possible coding sequences for a given amino acid sequence have to be prepared.

Supposing the following is the amino acid sequence of a part of a peptide—

—— **Phe-Trp-Pro-His-Met** ———

Then the possible nucleotide sequences in the DNA sense strand will be—



The nucleotide sequence obtained in any one of the mentioned sequences above, will serve the purpose of formation of rDNA and it's cloning, resulting in the production of the same desired protein/peptide.

5. Introduction of the rDNA into a host cell: The rDNA produced in the 4th step above can be cloned (made into many copies) and/or its gene product expressed by introducing it into a suitable host. Various methods are adopted for introduction of the different vectors into different hosts. Any DNA molecule can be introduced into any living cell and the process is called transformation. Transfection is a process of introduction of purified phage DNA.

(a) When the DNA molecule is kept in close proximity of bacterial cells, most species of bacteria are able to take up DNA molecules from the medium without any difficulty.

(b) Some species of bacteria cannot take DNA easily hence they have to be treated physically and/or chemically inorder to make them competent to take up DNA molecules.

(c) *E. coli* cells incubated with ice-cold solution of 50 mM calcium chloride at 4°C for 30 minutes, become competent to take up DNA molecules, wherein the DNA molecules get attached to the cell exterior. Later the DNA molecules enter the cell upon incubation at 42°C for 2 minutes.

(d) Even after the above mentioned physical/and/or chemical treatment, only 0.01% of the bacterial cells in the same culture gets transformed. Hence the transformed cells have to be selected from non-transformed cells.

(e) Transfection of phage DNA into bacterial cells is also done by the same procedure as that of plasmid introduction into *E. coli* cells, described above (a) to (d).

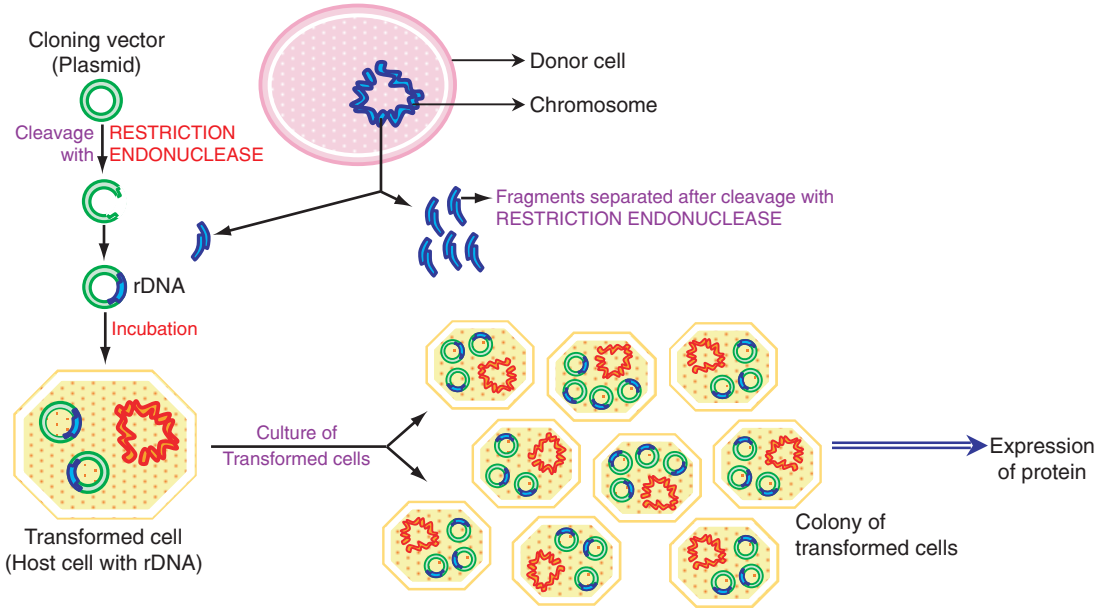
(f) Transfection can also be done by packaging the phage DNA into the mature lambda phage particle and then infecting the bacterial cells with it.

(g) The yeast cells of *Saccharomyces cerevisiae* are transformed by treatment with lithium chloride or lithium acetate.

(h) Calcium phosphate, DEAE dextran and protoplast fusion are used to introduce DNA into the animal cells.

(i) The cell wall of fungal and plant cells are broken by enzymes to get the intact protoplast, which can easily take up DNA. In some cases, the transformation of protoplast is stimulated by a special technique called electroporation. The transformed protoplast reforms the cell wall, divide and regenerate a transformed organism.

(j) An alternative method of introducing any DNA into any of the living cells is by microinjection, using a fine syringe, DNA molecules are directly injected into the nucleus of the cell to be transformed.



Mechanism of introduction of the rDNA into a cell

6. Selection of the transformed/transfected cells: All the cells, incubated in the same culture with the rDNA do not take up the DNA even after physical and/or chemical treatment. Only 0.01% of the total cells incubated becomes competent and takes up DNA to be inserted. Rest of the cells remain without

taking up the DNA. Therefore there should be some procedure by which the cells that have taken up the external DNA can be sorted out from those cells which have not taken up the external DNA.

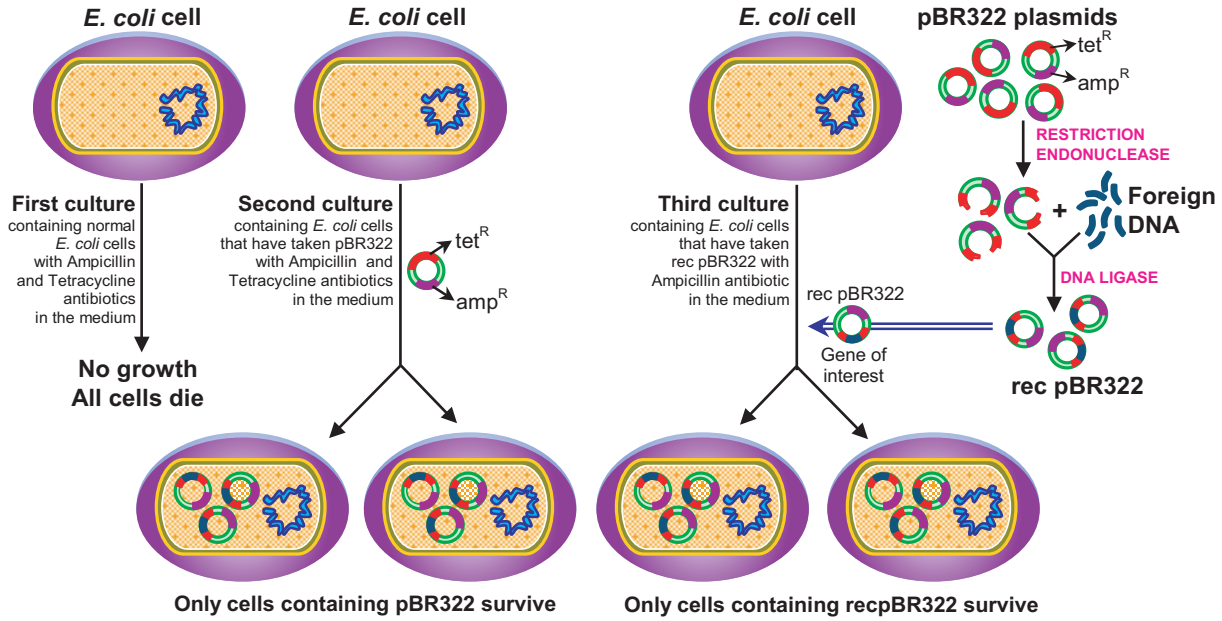
There are various methods for distinguishing the transformed cells from the non-transformed cells, which depends upon the type of vector used and the type of the cell transformed. The basic principle in all these methods is the use of a 'marker' i.e. either the vector or the transformed cell will either be resistant or sensitive to an antibiotic and/or will synthesize or be devoid of an enzyme acting on a metabolite and converting it into some recognizable product.

Thus the 'markers' are the antibiotics and/or enzymes to which the transformed cells respond.

Some of the markers found on some of the vectors

Vector	Marker
pBR322 plasmid	Ampicillin and tetracyline resistant
pVC 8 plasmid	Ampicillin resistant and lac 'Z' gene for betagalactosidase (which is lost in the rDNA)
Lambda phage and M13 phage	Lac 'Z' gene
pBR325 plasmid	Chloramphenicol acetyl transferase (CAT) resistant gene
PAT153	Ampicillin resistant and tetracycline resistant (amp^R-tet^R)
2 µm plasmid in Yeast	Leu 2 gene for beta-isopropyl malate dehydrogenase and amp^R-tet^R
YRp7	Trp1 gene for tryptophan synthesis and amp^R-tet^R.

One of the example based on which all the above phenotypic markers are selected, is that of the plasmid cloning vector pBR322. *E. coli* cells are normally sensitive to the antibiotic ampicillin and tetracycline i.e. they die if the medium in which they are grown is supplied with these antibiotics.



Selection of transformed *E. coli* cells

E. coli cells which take up the plasmid vector pBR 322 become resistant to ampicillin and tetracycline antibiotics because pBR 322 has two sets of genes, one set of gene that codes for β-lactamase enzyme that modifies ampicillin into a form that is non-toxic to the bacterium and a different set of genes that codes for enzymes that detoxify tetracycline. So, those *E. coli* cells which have taken pBR322 (i.e. transformed

cells) can be sorted out from the cells which have not taken pBR322 (i.e non-transformed cells) by growing all the cells in medium containing ampicillin and tetracycline wherein the cells containing the plasmid pBR322 survive, whereas the cells devoid of pBR322 die. This is to say that the *E. coli* cells have transformed from ampicillin and tetracycline sensitive (amp^S-tet^S) to ampicillin and tetracycline resistant (amp^R-tet^R) cells. rDNA prepared using the plasmid pBR322, when entered into an *E. coli* cell exhibits only ampicillin resistance but not tetracycline resistance, because the restriction endonuclease (Bam-II) used cleaves pBR322 in the gene for tetracycline resistance, hence this gene gets inactivated.

7. Isolation of the cell containing insert-vector rDNA holding the gene of interest: In the 6^{th} step the transformed cells i.e. those cells that have taken up the rDNA are sorted out. Now from these cells the cell containing the gene of interest has to be isolated.

The complete human genomic DNA is cleaved into about 7,00,000 pieces by the action of the restriction endonuclease and all of these DNA fragments can be inserted into a cosmid and such an insert containing all the genomic DNA of an organism is called genomic library. An *E. coli* genomic library contains all *E. coli* genes and likewise human genomic library contains all human genes, so on and so forth. From this gene library any one gene, which is commercially important i.e. for example insulin gene, beta-globin gene, growth hormone gene etc, has to be isolated. For this each cell is cloned separately and different procedures are adopted to select the desired clone. All these procedures are based on two basic themes—

1. ***Direct selection of the desired gene*:** This is just like marker selection procedure, described above, where selection occurs directly in the culture plate itself by the detection of the presence of the protein product of that gene.
2. ***Identification of the clone from a genomic library*:** In this an initial "shot gun" cloning is done to clone all the genes in the genomic library and each gene clone is identified separately by one of the following methods—
   (a) By analyzing the expression of the gene product.
   (b) A DNA probe can be synthesized chemically with a complementary sequence to the desired gene and used to hybridize the DNA from each clone.
   (c) A complementary DNA (cDNA) can be prepared from mRNA for a particular gene and then hybridized with the DNA insert.

Hybridization of DNA with another DNA can be done or DNA with RNA or RNA can be hybridized with another RNA, for which different methods are developed. A few among them are—

- When DNA-DNA hybridization is made then it is known as southern blotting.
- When DNA-RNA hybridization occurs, it is known as northern blot.
- When RNA-RNA hybridization occurs, it can be called eastern blot. Actually eastern blot is named for hybridization between protein and ginseng (plant glycoside) so as to identify small molecules like cholesterol, phospholipids etc.
- When protein-protein hybridization takes place, it is known as western blotting.

Southern and northern blot hybridization: DNA is fragmented by restriction endonuclease and then separated by electrophoresis. The DNA is then denatured and filtered on nitrocellulose. To this filter is added radio labelled ^{32}P-DNA probe, with base sequence complementary to the DNA (gene) of interest, which will hybridize (stick/base pair/anneal) to the complementary DNA. The position of hybridization is detected by autoradiography. The radiolabelled probe specific for gene under study can be purified RNA, a cDNA, or a segment of DNA cloned in *E. coli*.

Western blotting: In AIDS diagnosis, the component protein of HIV-virus are separated in polyacrylamide gel electrophoresis (PAGE) and transblotted onto nitrocellulose strips, which are then incubated with individual's serum. Any HIV antibodies present, bind to viral proteins contained on test strip. The bound antibody visualized by using a conjugate enzyme and chromogen.

Dot-blot hybridization: The procedure is the same as southern/northern blotting, but the only difference is that the DNA fragments are not separated by electrophoresis, instead they are directly applied as a dot on nitrocellulose and hence it is known as dot-blot hybridization.

Uses of rDNA technology/gene cloning: The uses of rDNA technology/gene cloning are enormous and wide spread in all the fields of biological sciences. rDNA technology is being implemented in newer and newer fields of application. To quote a few uses of rDNA in use at present are—

(1) By the use of rDNA technology, genes of interest can be inserted, so as to treat various diseases.
(2) Hereditary diseases can be diagnosed in the fetus itself.
(3) rDNA and gene cloning helps in the sequencing of DNA/gene.
(4) Various genes in the chromosomes can be located and the integrated viral genes can be traced out in it.
(5) Mechanism of gene regulation can be studied.
(6) Desired proteins like insulin, hormones, interferons, vitamins can be manufactured in bacteria on an industrial basis.
(7) Improved antibiotics can be produced from mixtures of genes from different bacteria.
(8) Recombinant vaccines can be produced by rDNA technology.

Synthesis of human insulin (humulin) using rDNA technology: Diabetic patients, whose elevated sugar levels are due to impaired insulin production, have been treated with insulin derived from the pancreatic glands of abattoir animals. Though bovine and porcine insulin are similar to human insulin, their composition is slightly different. Consequently, a number of patients' immune systems produce antibodies against it, neutralizing its actions and resulting in inflammatory responses at injection sites. This led to synthesizing human insulin i.e. '***humulin***' by inserting the insulin gene into a suitable vector, the *E. coli* bacterial cell, to produce insulin that is chemically identical to its naturally produced counterpart. The genetic code for insulin is found in the DNA at the top of the short arm of the eleventh chromosome. It contains 153 nitrogen bases (63 in the A chain and 90 in the B chain).

Manufacturing humulin: DNA with specific nucleotide sequences for A and B polypeptide chains of insulin are synthesized chemically. 63 nucleotides are required for synthesizing the A chain and 90 for the B chain, plus a codon at the end of each chain, signalling the termination of protein synthesis. An anticodon, incorporating the amino acid methionine, is then placed at the beginning of each chain which allows the removal of the insulin protein from the bacterial cell's amino acids. The synthetic A and B chain 'genes' are then separately inserted into the gene for a bacterial enzyme β-galactosidase, which is carried in the vector's plasmid. Insertion of the A gene is carried out by use of the restriction enzyme EcoR1 and for B gene Hind-III is used. The recombinant plasmids are then introduced into *E. coli* cells. The insulin gene is expressed as it replicates with the β-galactosidase as the cell multiplies.

The protein which is formed consists partly of β-galactosidase, joined to both the A and B chains of insulin. The A and B chains are then extracted from the β-galactosidase fragment and purified. The two chains are mixed and reconnected in a reaction that forms the disulfide cross bridges, resulting in pure ***humulin*** i.e. synthetic human insulin.

SUBUNIT VACCINES

Subunit vaccines are the specific cell surface antigens, injected into the body, which produce immunity against this infectious agent and its other related species.

"Prevention is better than cure"—hence medical sciences has coined the term **'prophylaxis'** which underlines the methods for prevention of diseases from emergence and spread. Vaccination is one among them. Its concept and mechanism is described hereunder.

Immunological system: All the components present inside the body, specially the proteins and oligosaccharides attached to it, are recognized as **'self'** by the internal environment of the body. Any compound entering inside an individual from outside is recognized as **'foreign body'** by the internal mechanism of the person. This foreign body can either be a protein, oligosaccharide or any other compound, preferably with a molecular weight more than 5000 kDa. This foreign body is immediately destroyed by the natural biochemical mechanism of the body, which is known as bodies' defense mechanism or immune system. Failure to modify, destroy and detoxify the foreign body especially those of viruses and bacteria leads to various infections, which may either be hazardous to health or lethal.

Natural defense mechanism by the body: The foreign substance termed as 'antigen' entering into the circulation is first recognized by the plasma Antibody Presenting Cell (APC) named as macrophage, which takes up the antigen by phagocytosis, then hydrolyzes it and either kills it, or processes it by cleaving it at all the points except at a particular sequence, recognizable by other cellular receptors, which is a particular pattern of amino acids (or sugars) called epitope. This epitope is presented on its cell surface, in combination with some proteins called 'Major Histocompatibility Complex (MHC)'. The antigen engulfed macrophages also produce some protein messengers called interleukins or lymphokines.

Further action on the antigen is carried out by specialized cell called lymphocytes. The lymphocytes present in the blood circulation are of two types (1) B-lymphocytes (B-cells) and (2) T-lymphocytes (T-cells). Once the interleukins and MHC-epitope complex on macrophages are found in the circulation, the T-cells start responding to it and differentiate themselves into four types—

1. **T-helper cells (T_h-cells):** These cells help in differentiation of B-cells specific to this antigen.
2. **T-suppressor cells- (T_s-cells):** These are the cells which suppress the activity of B-cells.
3. **T-cytotoxic cells (T_c-cells):** They kill or lyse the infected cell.
4. **T-delayed type hypersensitive cells (T_d-cells):** These cells are involved in delayed hypersensitive reaction.

The differentiation of a T-cell in response to a particular antigen, into the above mentioned T-cells takes place by DNA rearrangements and the differentiated cells help in clearing that particular antigen/infection, either directly or indirectly. Once the infection is cleared these specific T-cells degenerate except some cells which are stored as **'Memory T-cells'** for that particular antigen.

Simultaneously, the antigen is also specifically recognized by the B-lymphocytes by their cell surface receptors called immunoglobulins (Ig). These immunoglobulins are also secreted into the blood by the B-cells. There are five types of immunoglobulins–(a) IgG (b) IgM (c) IgA (d) IgD and (e) IgE.

The IgM receptors on the B-cells, present non-specifically, first recognize any antigen, bind to it and takes it inside the cell by phagocytosis, degrades it into small pieces (or peptides) and presents it on its cell surface in the processed form in combination with major histocompatibility complex-II. This complex is recognized by the T_h-cells (produced in response to the same antigen) which induce (through interleukins secretion) the differentiation of the B-cell into a mature antibody secreting plasma cells. This differentiation of B-cell takes place by the rearrangement of the cell DNA by a process called 'transposition', which arranges the genes in such a way that the antibody gene product thus produced i.e. the protein immunoglobulin, is complementary to the antigen, which results in antibody binding specifically to the antigen thereby precipitating it, thus resulting in the inactivation of the antigen.

Most of these plasma secreting B-cells degenerate excepting few cells which are stored as **'Memory B-cells'** for that particular antigen.

Thus B-cells inactivate an infection (antigen) and T-cells clear an infection (antigen). Both types of cells are generated in response to an infection (antigen). After the infection is over, both the B and T-cells are stored as memory cells containing a number of Immunoglobulin receptors on B-cells and marker recognition receptors on T-cells, both specific and of high affinity to that particular antigen, as compared to the undifferentiated cells. When the same infection (antigen) enters the body for the second time, these memory

cells come into play and the immune response is elicited at a faster rate and in a shorter time interval, than that of the first instance of infection, because the T and B cells are already differentiated and their DNA rearranged for this specific antigen. This is known as an immune response by the individual to a foreign body or an infection. All these processes i.e.—

- Identification of the antigen by the macrophages
- Endocytosis of the antigen by the macrophages
- Processing of the antigen by the macrophages
- Presentation of the antigen epitope-MHC on the surface of macrophage
- Production of interleukins by macrophages
- Interleukin response by T-cells
- Epitope-MHC recognition by T-cells
- Differentiation of T-cells into T_h, T_c, T_s, T_d cells
- Killing and clearing of antigen by T-cells
- Recognition of the antigen by B-cells
- Processing of the antigen by B-cells
- Presentation of the antigen epitope-MHC-II receptor on B-cell surface
- Recognition of epitope-MHC-II B-cells receptors by T_h cells
- Differentiation of B-cells into mature immunoglobulin secreting plasma cells
- Inactivation of the antigen by formation of antigen-antibody complex

take a considerable duration of time interval (from 2-4 days). If the rate of multiplication of the infection (virus or bacteria)/antigen is faster than the rate of growth of the immune response, then it results in the development of the disease. On the other hand if the immune response is faster than the multiplication of the virus or bacteria, then it results in the prevention of the disease by the immune / natural biochemical mechanism of the body.

In the case of the antigen (virus or bacteria) entering the body for the first time, the time required is very high to elicit any immune response. So depending upon the infected antigen, i.e. whether it is fast growing or slow growing, the person either becomes resistant to it or succumbs to its disease condition. The disease may be hazardous to heath or lethal. In the case of a second infection by the same virus / bacteria / antigen, the immune response is much faster than the first infection, due to the presence of memory cells specific for this antigen. These memory cells can make the person immune within few hours or in a day or two, depending upon the infective agent, thereby preventing the emergence of the disease.

Among the various viruses/bacteria infecting the human population, some are very wild and vigorously multiplying in the body, such that the immune response becomes incapable of controlling it, leading to ill health, permanent disability or loss of life. On the other hand, some others are slow growing and are easily controlled by the immune system of the body, hence it essential to vaccinate against fast growing microbes.

Concept of vaccination: A vaccine is an inactivated infectious agent (virus or bacteria)–whole vaccine or a segment (epitope) of the infectious agent–subunit vaccine, injected in small amounts into the person to elicit an immune response, create and store memory cells specific for this particular antigen.

Vaccination is the set of complete schedule prepared in respect of the procedure for inactivation of the infective agent, preparation of epitope and its delivery system, dosage, number of doses and interval of each dose, so as to store a good number of memory cells for longer periods of time in the human body and thus provide it with a safe prevention from that particular infectious disease.

For the preparation of a vaccine it is important to know about the process of infection of an agent and the immune response to that agent.

A vaccine is a live or dead viral particle or bacteria, which is inactivated or given in so small quantity that it does not cause any disease in the person, instead will develop resistance or immunity to this virus or bacteria by producing and storing memory cells specific for this virus or bacteria. Later when the active

natural form of the same virus/bacteria enters the body, the immune response will be faster than the growth of the virus/bacteria to cause infection, resulting in the prevention of the disease. That means, the antigen is being pre-exposed to the body before the virulent (disease causing) agent attacks the person. This is known as **'whole vaccine'**.

In some cases even if the whole vaccine agent is inactivated/or injected in small quantities, it will cause some side effects i.e. create infection. Hence one or a few components of the infectious organism are extracted and injected along with some high molecular weight compound called adjuvant, so that this will create memory cells safely as would have been created by the whole organism and are equally good in defending the person from the act of that particular infective agent. This is known as **'subunit vaccine'**.

However the type of immunity required is not the same for all diseases. The infection may either be systemic infection or localized mucosal infection. Therefore a particular type of vaccine preventing or modifying one type of infection may not be effective against the other type of infection. Therefore different types of vaccines are developed for different infections.

The existing vaccines: The infective agent is grown in large amounts in tissue culture and then inactivated with formalin, beta-propinolactone or an imine, under such a condition that it does not cause any infection instead can create a good immune response. Alternatively, the infective agent is obtained from the infected host and is grown in an unnatural host (attenuated vaccine) i.e. in such a condition that this product does not cause any disease in the natural host, but it proliferates and elicits an immune response.

Drawbacks of existing vaccines:

(1) The rate of production is less than the requirement.
(2) Cost of production is very high.
(3) They may contain some live and active organism and can cause infection, instead of immunization.
(4) Culturing of large numbers of virulent organisms (infectious agents) is hazardous to the person handling and to the environment.
(5) The existing vaccines are not efficient and specific for the disease used, because a particular virus may have a number of different strains and each strain will have extreme antigenic variability.
(6) The attenuated vaccines may reverse to virulence.

Hence the existing vaccines have been less effective in case of some diseases and there are no vaccines at all for some other diseases.

Modern vaccines or sub-unit vaccines: Sub-unit vaccines are specific cell (viral or bacterial) surface antigens, when injected into a person, will produce immunity against this agent and other related agents.

Sub-unit vaccines are made from one or a few components of the organism, instead of the whole organism. The modern vaccines or subunit vaccines are safe, efficient and cost effective, which are a result of the scientific knowledge relating to the structure of the genomes of many organisms, structure and functions of antigens for the causative viral disease, rDNA technology, gene cloning and the selection of antigens by the expression of the gene product. Human diseases for which biotechnology offers new or improved vaccines are—

- For viral diseases like measles, mumps, rubella (MMR), polio, tuberculosis, hepatitis, pneumonia, influenza, rabies.
- For bacterial diseases like enteritis, neonatal scours and clostridial toxicoses.
- For parasitic diseases like malaria, leishmaniasis.

Design of various modern sub-unit vaccines: Sub unit vaccines are produced by selecting a particular viral capsid protein/polysaccharide which is antigenic. The immunogenic components for a few of the disease causing organisms are—

- ✓ HBsAg for hepatitis B
- ✓ Capsid protein VP3 for hepatitis A
- ✓ Pneumococcal capsular polysaccharide for pneumonia
- ✓ Haemagglutinin and neuraminidase for influenza

The following methods are adopted for the production of a sub-unit vaccine—

1. **Protein extraction from whole virus culture:** The virus is cultured in *in-vitro* culture media in large amounts and the protein is extracted from it and is injected into the person to elicit an immunological response and thus generate memory cells to whole virus, which neutralize the viral infection.
2. **Use of rDNA technology for production of capsid protein:** The viral genomic RNA is transcribed into DNA, cleaved by a restriction endonuclease, inserted into a plasmid vector, which is also cleaved by the same restriction endonuclease, so as to produce the rDNA and is cloned in yeast cell as the host. Cloning of the rDNA in yeast cell produces glycosilated structural proteins but not in *E. coli*, which gives unglycosilated proteins. Thus large amounts of immunogenic proteins are obtained by gene cloning, which is injected to give protection to the human beings against the disease.

The pure individual protein injected, though succeeded in eliciting an immune response in the vaccinated individual, it was much less immunogenic than the intact whole particle, because the pure protein created B-memory cells but not the T-cells. In order to enhance the immunogenic activity of the separated proteins, a mixture of plant glycoside saponin, cholesterol, and phosphatidyl choline is added to many copies of the protein to form a cage-like structure, mimicking the natural microorganism. These complexes known as immuno-stimulating complexes have antigenic activity equivalent to the viral vector.

There is further development in the preparation of sub-unit vaccine for virus. In the virus capsid protein only few amino acids are truly antigenic / immunogenic present at few regions in the protein and these regions are termed as **'epitope'**. Therefore synthetic peptide is made with these amino acids in the same sequence as that of the epitope. Eight such peptides when linked together by lysine residues to form a frame work (immunostimulating complex) will be equally good as an immunogen as that of the whole viral particle itself.

Further a synthetic DNA can be prepared which can code for these amino acids and joined to the gene coding for hepatitis B core protein. This rDNA produced a chimeric protein which takes a spherical shape of about 22 nM in diameter. This protein, obtained by cloning in *Autographa californica* as the host, acted as a sub-unit vaccine and protected at par with the whole viral particle vaccination.

3. **Recombinant virus as a sub-unit vaccine for different diseases:** Vaccinia virus exists in two forms or in other words it has got two different strains, one of them is infectious and hence is termed as virulent form of vaccinia virus and the other one is non-infectious, which is termed as non-virulent vaccinia virus. The non-virulent form is extensively used as a vehicle or agent to introduce recombinant antigenic epitopes for different diseases. The other agents used are 17D strain of yellow fever virus, *Salmonella typhi murium*, *Bacillus Calmetta Guerin* (BCG) and poliovirus. All these are attenuated vaccine vectors used as delivery vehicles for various sub-unit vaccines.

Viruses causing a particular disease will exist in a number of different strains. Therefore vaccination for a disease by one strain results in invasion by the virus of another strain causing the same disease. Therefore an effective sub-unit vaccine can be prepared by rDNA technology wherein antigenic epitopes from all the strains are mixed with a plasmid vector and joined into a single recombinant DNA and then transferred into any of the agents mentioned above–for instance a virulent vaccinia virus, which will produce the antigenic epitopes for all the strains of a particular disease causing virus or bacteria, on its capsid coat. This is known as recombinant vaccine.

Therefore, vaccination with this recombinant vaccine agent (vaccinia virus with various antigenic epitopes) results in development of immunity to a disease (or many diseases) for all the strains causing that disease. Non-virulent vaccinia virus can also be used as an agent to introduce epitopes for two or more disease causing viruses with epitopes for all of the strains. Such recombinant vaccines are prepared for Rabies.

4. **Other sub-unit vaccines:** Virulence genes of enteric pathogens have been excised or modified and this recombinant organism colonizes in the intestinal mucosa and will express surface antigens which prevent adherence of natural virulent bacteria thus preventing the disease.

A 68 kDa outer membrane protein is present in virulent strain of *Bordetella bronchi septica*, but not in avirulent strain, which is the main immunogenic site. This protein is cloned in the avirulent strain or some other host, thus producing a cost effective subunit vaccine. For any disease causing antigen–the epitope is chemically synthesized as a peptide and injected into the body. The antibodies produced to this are Ab1, which are used to produce anti-Ab1 antibodies i.e. Ab2 or anti-idiotypes. Ab2 will be structurally similar to the epitope of the original antigen. Therefore this anti-idiotype Ab2 is produced in large quantities and used as sub-unit vaccine against that particular disease. Vaccines are more commonly produced by this method, as monoclonal antibodies by hybridoma technique.

5. **DNA vaccines:** The most recent approach in the vaccine design is the use of synthetic DNA for the epitope of an antigen and produce a recombinant DNA, insert it into a non-virulent viral vector and inject it into the person. This virus proliferates in the body expressing the epitope of interest, thereby producing immunity to the disease for which the epitope is designed. This reduces the cost and labour of producing the antigenic protein/epitope peptide, its extraction, purification and characterization.

Subunit hepatitis B vaccine for human use: The surface antigen of hepatitis B virus (HBsAg) is a protein consisting of 226 amino acids with a mol. weight of 25,398. A synthetic nucleotide sequence of 892-base pair is prepared and inserted into a plasmid vector. The portion of the gene coding for this protein does not contain any intervening sequences. This recombinant DNA is introduced into the yeast *Saccharomyces cerevisiae*. The antigen is harvested and purified from fermentation cultures of a recombinant strain of the yeast containing the gene for the adw subtype of HBsAg. The fermentation process involves growth of *Saccharomyces cerevisiae* on a complex fermentation medium which consists of an extract of yeast, soy peptone, dextrose, amino acids and mineral salts. The HBsAg protein is released from the yeast cells by cell disruption and purified by a series of physical and chemical methods. The purified protein is treated in phosphate buffer with formaldehyde and then coprecipitated with alum (potassium aluminum sulfate) to form bulk vaccine adjuvanted with amorphous aluminum hydroxyphosphate sulfate.

Hepatitis B vaccine (Recombinant) is a non-infectious subunit viral vaccine derived from hepatitis B surface antigen (HBsAg) produced in yeast cells.

ORGAN, TISSUE, CELL CULTURE

Growing organs, tissue or cells in laboratory dishes, outside the body is known as cell, tissue and organ culture.

If individual cells (bacteria, viral or separated animal / plant cell) are grown in culture media then it is known as cell culture. If a group of cells in a tissue are grown in laboratory glass then it is known as tissue culture. If the whole organ is cultured in culture media/laboratory glass then it is known as organ culture.

Every cell present in the human body is not capable of growing in laboratory, only a few types of cells can grow *in vitro*. Cells that can be grown in culture are tumour cells, steroid producing adrenal cells, ACTH-secreting pituitary cells, insulin secreting pancreatic islet cells, growth hormone and prolactin secreting cells from pituitary tumour, melanocytes, neural cells, epithelial tissues, skeletal tissue etc.

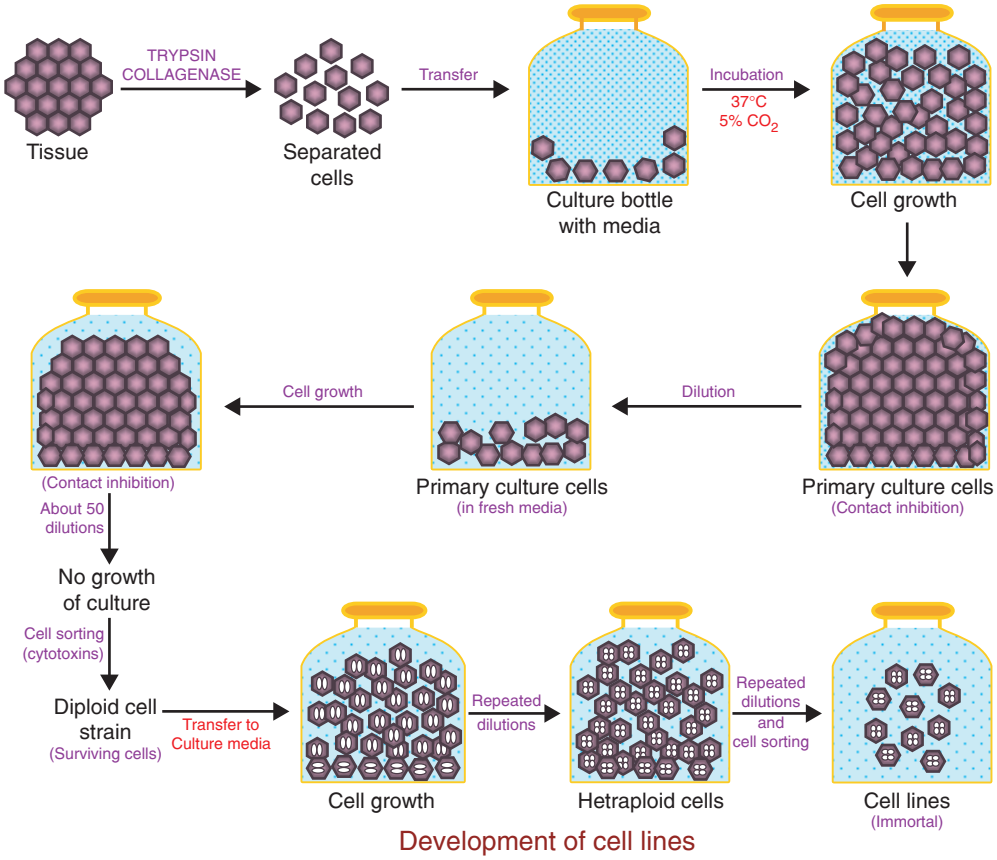
Those cells which can be grown *in vitro* are neither suitable for industrial use nor for scientific purpose because many cells die during the course of time releasing toxic substances which inhibit the activity of other live cells. Hence, in order to avoid this problem and to achieve an exponential cell growth, the cells are converted into immortal cells called **'cell line'**. The following is the procedure for the production of cell lines.

Procedure for production of a cell line: A piece of tissue is removed from an organism. Then the adhesion between cells is broken with enzymes like trypsin or collagenase. These cells are then transferred to a plastic dish or bottle. The dish / bottle contains nutrient solution, known as culture media (made up of appropriate salts and nutrients). Then the cells are incubated at 37 °C in 5-10% CO_2 to aid in pH maintenance and perfused with 90-95% oxygen.

Human or animal cells grow only when they actually touch a solid support, they are not free floating. The solid supports generally used are plastic, glass, teflon, DEAE-Sephadex, etc. all of which are transparent and aid in microscopic observation. Further any manipulation relating to the cells or tissue is done in a laminar flow hood, so as to prevent any contamination with microorganisms. When provided with all the suitable requirements the cells grow, divide and cover the surface of the container and look like the tissue from which they are derived. This culture is referred to as primary cell culture, because all cells touching other cells or the walls of their container will stop dividing due to contact inhibition (which can be overcome by growing cultures on micro-carriers like anion exchange resins). If this primary culture is diluted two fold and the culture transferred to fresh medium, the cells will again start growing. This type of repetitive culturing of the cells is limited because the growth of animal cells ceases after about 50 cell divisions, either due to lack of proper culture media or built-in-senescence mechanism.

However, some among these cells in each culture continue to grow after numerous transformations, but these cells are not normal as they undergo some chromosomal abnormalities and these are termed as diploid cell strains and are not different from cells of the primary culture. After sometime, even the diploid cell strains lose the ability to grow, but once again a few cells among them will survive and are termed as heteroploid cells, because they undergo many chromosomal rearrangements and deletions. These cells will grow in culture indefinitely as long as the medium is replenished, becoming effectively immortal. These survivors are known as **'cell line'**. They are said to have undergone transformation. Transformed cell lines grow indefinitely, exhibit heterogeneity, lose contact inhibition and do not require solid support for growth.

A tumour tissue represents a transformed cell line. The most famous and the oldest cell line is the Hela cell line, derived from human cervical cancer cells, which are growing since decades and creating problems in tissue culture laboratory.



Development of cell lines

Purpose of cell and tissue culture:

(1) Cultured cells are used as substitute hosts to study the pattern of viral infection.
(2) Cell lines are used in the manufacture of vaccines, antibodies, hormones, interferons, urokinase enzyme, vitamins, steroids etc on a large scale industrial basis.
(3) They are good tools for testing the potency of drugs.
(4) These cells also serve as models to study the metabolism of various substances.

Culture media: Culture media is the environment provided for the growth of the cells in laboratory, similar to those conditions that the cell has been exposed to *in vivo* (i.e. inside the animal body). When a cell is removed from its original tissue or organism and placed in the laboratory glass for culture (i.e. multiplication) it will not proliferate, differentiate and divide until and unless it is provided with all those components and substances upon which it was surviving in the tissues or organism. These components and substances, required for the proper growth and maintenance of the culture is known as culture media. The culture media should contain a support or matrix (termed as physical media) and appropriate nutrients, hormones and stromal factors (the chemical media), for survival and growth of the cells *in vitro*.

Serum is the most economical, easily available and most widely used culture media for animal cell culture. It provides all the necessary nutrients and substances to sustain a culture. Serum is an extremely complex mixture of many small and large biomolecules with different, physiologically balanced growth promoting and growth inhibiting activities. Fetal calf serum is the preferred serum as culture media. The major serum components are–glucose, urea nitrogen, proteins including albumin, macroglobulin, fibronectin, uric acid, creatinine, haemoglobin, bilirubin, alkaline phosphatase, LDH, insulin, TSH, FSH, growth hormone, prolactin, T3, cholesterol, cortisone, testosterone, progesterone, PGE & F, vitamin A & E, Na, K, Cl, Fe, Zn, Cu, Mn, Co, V, Mg, Se, Ca, P_i etc.

The major functions of serum as a culture media are–to provide nutrients, hormones, growth factors, attachment and spreading factors, binding proteins, vitamins, minerals, lipids, protease inhibitors and pH buffer. Though serum is widely used culture media, it has some disadvantages like all cells in the animal body do not come in direct contact with blood, some enzymes present in serum can convert the cell secretions into toxic compounds. Serum collected in different batches differs. The constituents present in it may be inadequate for maintenance of the culture and virus, fungi, and bacteria may contaminate the media easily. Hence, recently culture media are developed artificially by mixing appropriate nutrients and chemicals, so as to provide an equivalent environment for culture. There are three types of artificial culture media—

(a) **Serum-free culture media:** i.e. No serum but some proteins extracted from serum are supplemented.
(b) **Protein-free culture media:** i.e. All the constituents of serum, without serum proteins.
(c) **Chemically defined media:** It contains substances of small molecules and genetically engineered proteins or peptides.

The various artificial culture media available commercially are Eagle's minimal essential medium (MEM), Dulbecco's modified enriched medium (DME), Ham's F-12, CMRL 1066, RPMI-1640, McCoy's 5A, IMDM and MCDB-301.

The main components of the chemically prepared artificial culture media are–minerals and trace elements like Cu, Zn, Co, Mn, V, Se, Mg, Al, Ba, Cr, Ge, Ti, Sn, Ni etc, vitamins–C, E, and B-complex. Carbohydrates as energy source like glucose, or pyruvate as a source of acetyl-coA. Lipids like cholesterol, long-chain fatty acids, glycerides and lipoproteins. Amino acid, proteins like albumin, transferrin and synthetic polymers, hormones and growth factors plus extracellular matrix.

Kreb's ringer bicarbonate solution is also used as a culture media which is composed of NaCl, KCl, $MgSO_4$, $CaCl_2$, $NaHCO_3$, KH_2PO_4, gassed with O_2-CO_2 mixture. It also contains balanced salt solutions, vitamins, carbon source like mannitol, amino acids, plasma proteins and antibiotics.

Cell sorting: Cell sorting is the process of separating the cells of interest from the unwanted cells. Cell sorting is done at two levels in developing a cell line, (1) While selecting a cell from a tissue and (2) While shifting the culture from primary cell culture to secondary cell culture (diploid cell) to tertiary cell culture (heteraploid cell) to the immortal cell line (transformed cell).

Cells in a culture die continuously beginning with the primary culture to the diploid culture to heteraploid culture to the immortal cell lines. However some cells remain live in each of the stages of the cell transformation. Hence cell sorting is a process of separating the live cell from the dead cells in each of the culture types. The procedure adopted for cell sorting in the culture medium are–chemicals which can dissolve dead cells easily are used wherein the dead cells dissolve leaving the live cells intact. Other methods used for cell sorting are density gradient centrifugation, electrophoresis, isoelectric focusing sorting or separation of cells can also be carried out using magnetism, complement lysis of specific antibody tagged to unwanted cells, flow cytometry (one of the best methods of cell sorting) and affinity chromatography. Cytofluorimetry is the most recent and widely used cell sorting technique. In this the cells are stained with a fluorescent dye, wherein the dead cells get stained and the live cells are unstained. Depending upon the fluorescence, the cells are separated and hence the method is termed as fluorescence activated cell sorting (FACS).

Cell counting: The counting of the viable (live) cells in a culture is done to determine the number of viable cells/ml of the cell suspension, so that the exact and optimum dilution of cells may be made, which would be suitable for the cell growth. The viable cell count is achieved by staining the living cells by using a vital dye like neutral red (NR). Trypan blue may also be used, which stains only the dead cells. The stained cells are counted by using Neubauer counting chamber. Cell counting is also done by electronic particle counter and Coulter particle counter.

Cryopreservation of cell lines: When dealing with established cell lines, it is essential that a set of cell line be preserved so that if the culture becomes unsuitable for use due to contamination, loss of growth, defects in incubation etc, resulting in loss of the culture, then the preserved cells can be used and thus the cell line is not lost. In addition, freshly trypsinized / collagenized cells, cells of secondary culture, embryonic cells, human placenta or fetus and other such cells which are difficult to procure at will are preserved so that they may be used afterwards. Cells are preserved by two methods:

Freezing with glycerol: The cells at a concentration of 2×10^6/ml are taken in Eagle's medium containing 20% calf serum and 5% glycerol and distributed in aliquots of 2-4 ml in 5 ml vials, which are then frozen so that there is a slow fall in temperature of 10° C/minute till –25° C. The vials are then placed in liquid nitrogen and stored at –196° C.

Freezing with dimethyl sulfoxide: 2×10^6 cells/ml are taken in Eagle's medium containing 15% calf serum and 10% dimethyl sulfoxide and distributed in 3-5 ml vials and stored in liquid nitrogen at –196°C or at –70°C.

HYBRIDOMA TECHNIQUE

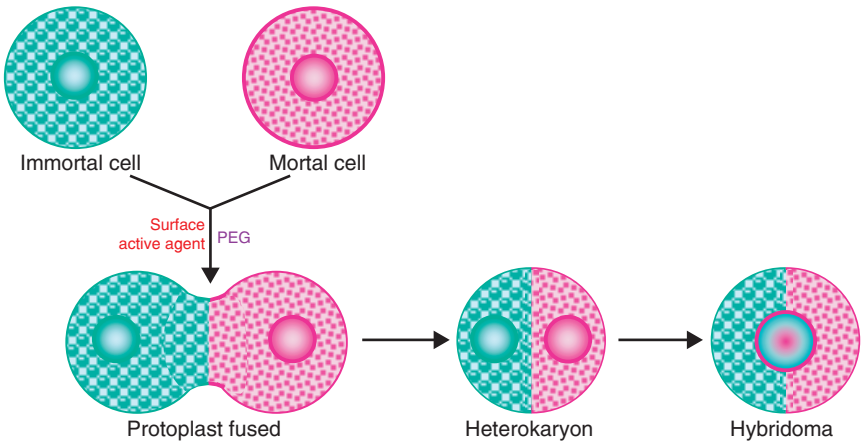
Hybridoma is an immortal cell or cell line, formed by the fusion of a myeloma cell with an antibody secreting plasma cell (lymphocyte).

Hybridoma technique is the process of producing a hybridoma cell. Antibody raised to a particular antigen in the serum is used for many purposes like the detection of presence of a toxic substance in clinical samples and also for its quantization, localization and purification. Any antigen will contain two or more epitopes or antigenic parts and the immune system differentiates in such a way that many plasma cells (B-cells) are differentiated specific to each epitope and each B-cell will produce an antibody having affinity to only one epitope on the antigen. Therefore different B-cells will produce different antibodies specific for the different epitopes on the same antigen. These many different antibodies produced by different B-cells for a single antigen are termed as polyclonal antibodies as they are produced by clones of different cells.

The use of polyclonal antibodies as diagnostic and therapeutic agents was not specific because antibodies produced to the same antigen differ from batch–to–batch due to the difference in the response of the immune system in the body. Therefore Monoclonal Antibodies (MAb) were produced i.e. those antibodies which are identical and specific to a single epitope of the antigen and produced by a single clone of B-cells.

B-lymphocytes producing a specific antibody (MAb) are incapable of growing in culture. Hence a hybrid cell type was created having the B-cell genetic components for producing this specific antibody and the cell division functions of a similar type of cell so as to enable the hybrid cells to grow in culture. Some B-lymphocytes, naturally become cancerous (myeloma) and hence become able to grow in culture. So these myeloma cells were used to fuse with the antibody producing B-cell forming the hybridoma cell capable of growing in culture indefinitely and secreting the specific MAb.

The Technique: When cells from two different sources are mixed and incubated with certain surface-active agents like polyethylene glycol (PEG), sendai virus or high voltage direct current pulses, then the cells fuse to form heterokaryones i.e. a single cell having two nucleus from different cells. The nuclei of some heterokaryones fuse to form hybrids. If one of the cell forming the hybrid is immortal (i.e. capable of growing indefinitely in culture) and the other is mortal (i.e. incapable of growing in culture), then the hybrid will be immortal and can be maintained indefinitely.

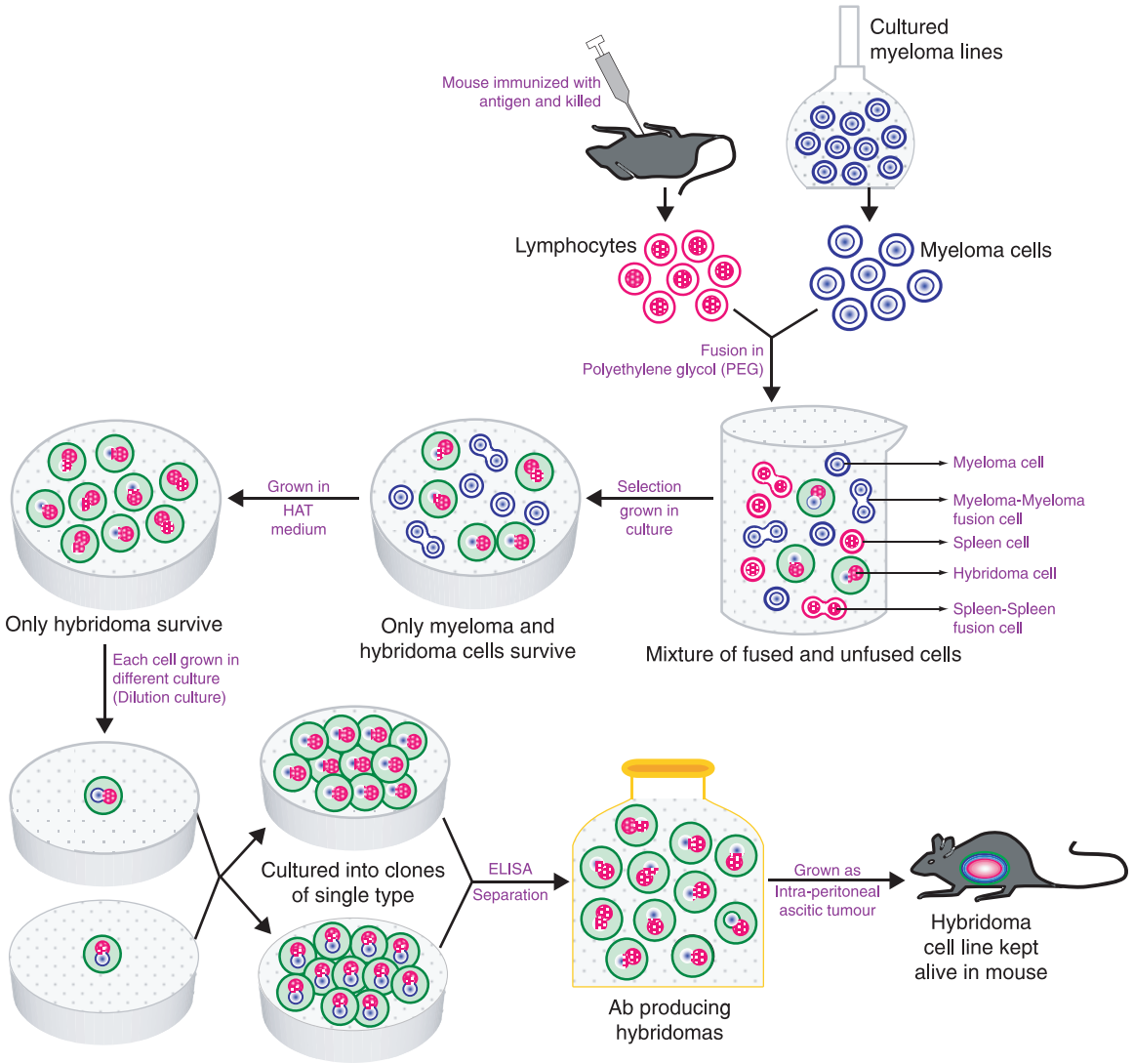


Hybridoma technique

Production of monoclonal antibody (MAb): Monoclonal antibody (MAb) producing immortal hybrid cells (hybridomas) are produced by injecting mice with a particular antigen and an immune response to this antigen is developed in the animal. Then the mouse is killed, spleen is taken and the cells are separated. Only the spleen B-lymphocytes (as source of antibody) of this immunized mouse are mixed with cultured mouse myeloma cells (tumour cells) and incubated with 35% polyethylene glycol for few minutes. This procedure will not fuse all the cells, instead only a few cells are fused. So the incubated mixture is expected to contain myeloma cells, spleen cells, myeloma-myeloma fusion cells, spleen-spleen fusion cells and a few of them will be hybridoma cells i.e. myeloma-spleen fusion cells.

In order to isolate the fused cells (hybridoma cells) producing antibody of interest, first of all the hybridomas are detected and separated from the other unfused cells. The spleen B-cells, however die as they cannot grow in culture, whereas the myeloma cells are selected and separated by growing the cell mixture in a medium containing Hypoxanthine (H), Aminopterin (A), and Thymidine (T), hence known as HAT medium. Myeloma cells are devoid (mutant) of the enzyme–Hypoxanthine Guanine Phosphoribosyl Transferase ($HGPRT^-$), so they cannot naturally utilize bases by scavenging process and the de-novo synthesis of bases is inhibited by aminopterin (in the HAT medium), hence the myeloma cells die, whereas the hybri-

domas survive because they have the enzyme HGPRT$^+$, coming from the B-lymphocytes. Therefore growing cells in HAT medium for 10 to 14 days after fusion results in spleen B-lymphocytes-myeloma hybrid cells to survive and all others die.



Production of monoclonal antibodies using hybridoma technique

Among these hybrid cells, the cells producing antibody against the immunizing antigen are identified and separated by Enzyme Linked Immunosorbant Assay (ELISA) or solid phase radio-labelling. For this, dilution culture is taken up such that each cell is grown in a different culture to form a clone of single type, the secretions of these cells present in the culture media is taken as sample for ELISA or solid phase radio-labelling and the hybridoma cells are selected. After screening and selecting the hybridoma cells producing the antibody of interest, they are cultured by soft agar cloning or dilution cloning, which secrete monoclonal antibodies (MAb) highly specific for the epitope of the injected antigen.

Culturing hybridoma cells producing MAb in laboratory culture dishes produces less number of antibodies, hence in order to get large quantities of antibodies in short time, the hybridoma cell line is grown as intraperitoneal ascitic tumour, induced by mineral oil injection (pristane) into mice. Once cloned in the ascitic tumour, all growing cells will produce antibodies in large amounts (i.e. 1-2 mg/ml).

Uses of monoclonal antibodies:

(1) Diagnosis or treatment of infectious diseases, tissue typing for organ transplantation and blood grouping.
(2) Calculation and differentiation of human lymphocytes at various stages of development.
(3) Analyzing mixtures of complex antigens.
(4) Determination of the structure of cell membranes and membrane proteins.
(5) Labelling and identification of specialized cells.
(6) Tumour and cancer detection and therapy.
(7) Understanding the mechanism of antibody diversity generation at DNA level.
(8) Monoclonal antibody based assay is replacing the immuno-florescence assays.
(9) MAb are used as tools in enzyme purification and genetics.
(10) Used to isolate receptors, non-histone chromosomal proteins, hormones etc.
(11) Used to treat diseases like tetanus, snake bite, rabies, herpes-B, leukemia virus etc.

MOLECULAR BASIS OF DISEASE DIAGNOSIS AND TREATMENT

A disease, in molecular sense, can be defined as any abnormality in the living system. The abnormality can be caused due to infection by virus, bacteria, fungi, parasites, proteins or small molecules in/from humans, animals, plants, water and soil. The abnormality can also arise due to changes in the molecular structure within the cells. As an example, a change in the DNA sequence known as mutation can cause various disorders/diseases.

The prevention and treatment of these diseases is possible only if the causative agent of the disease can be diagnosed at the appropriate time. Hitherto many costly and laborious clinical procedures were used in the diagnosis and treatment of these diseases. With the advancement of Molecular Biotechnology, various molecular diagnostic methods are now applied in the diagnosis and also treatment of these diseases.

Molecular diagnosis: A diagnostic test can be effective only if it is (a) specific for the target molecule (b) sensitive to detect even minute levels of the target and (c) technically simple. There are two classes of molecular diagnostic techniques.

(1) DNA detection methods—which uses nucleic acid hybridization or the polymerase chain reaction to detect a specific nucleic acid sequence.
(2) Immunological methods—are based upon the specificity of an antibody for a particular antigen.

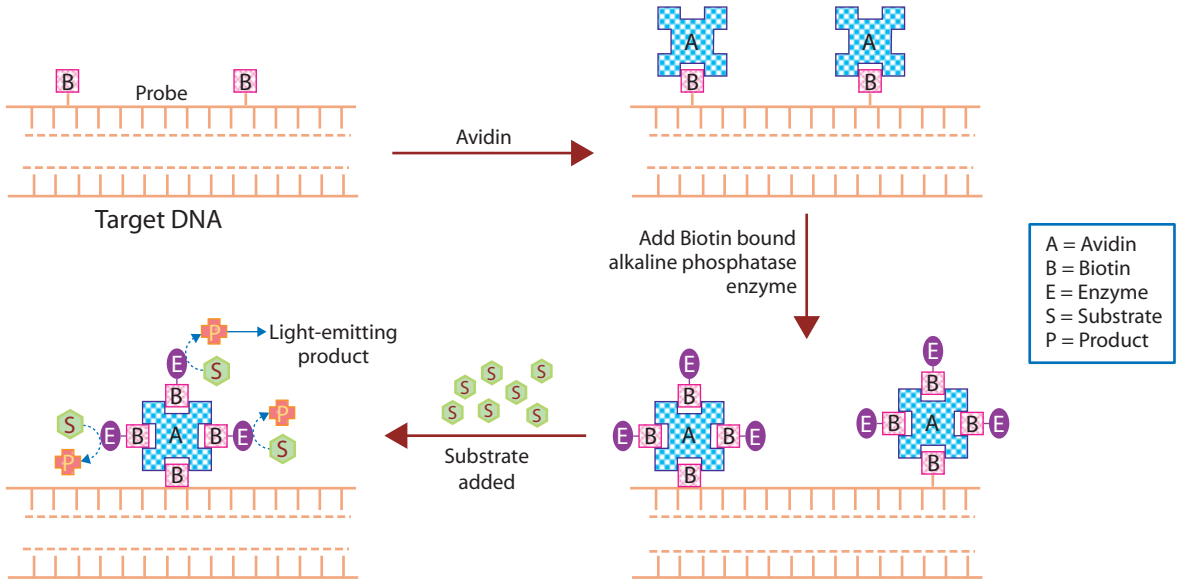
1. **DNA detection methods:** Various methods have been devised for the detection of various diseases, based on the sequence of DNA, built in a specific manner. Some methods discussed here are (a) detection of a pathogenic organism by nucleic acid hybridization (b) diagnosis of genetic disease using restriction endonucleus (c) diagnosis of genetic disease by P.C.R./oligonucleotide ligation assay (PCR/OLA) and (d) detection of mutants at different sites within one gene.

(a) Detection of a pathogenic organism by nucleic acid hybridization: The disease causing (pathogenic) organism can be detected very specifically in biological samples by nucleic acid hybridization i.e. if the nucleic acid sequence of a disease causing organism is present in the blood, urine, faeces, etc, then it can be hybridized with a nucleic acid probe complementary to the sequence of this target nucleic acid. If the pathogenic organism is present in the biological sample, hybridization occurs and if not, there is no hybridization.

The parasite *Plasmodium falciparum* causes malaria in man. A specific gene (thereby its product) is the causative agent in this parasite. A complementary DNA probe to this gene is synthesized chemically with radiolabelled ^{32}P. This probe is bonded to a membrane support. Then the biological sample to be analyzed is added, under appropriate conditions of temperature and ionic strength to promote base pairing between the probe and the target DNA in the sample. It is then washed to remove the excess of the sample and then the

hybridized double stranded DNA is extracted and the hybrid sequences are detected by autoradiography. The specific DNA probe chosen will hybridize only with *P. falciparum* but not with *P. vivax*, *P. cyanomolgi* or human DNA. This probe can detect as little as 10 picogram of purified *P. falciparum* DNA or 1 nanogram of *P. falciparum* DNA in blood samples. If hybridization has occurred then the pathogenic organism is present and if no hybridization occurs (i.e. no radiations) then the pathogenic organism is absent.

The above procedure is adopted for detection of all pathogenic organisms in any biological sample. Here the disadvantage of using the radioactive phosphorus is that it is hazardous, hence now-a-days non-radioactive hybridization procedures are used. In this method all the DNA from the sample is extracted and is bonded to a support, then the biotin-labelled DNA probe complementary to the pathogen DNA is hybridized to the target DNA. Then, either avidin or streptavidin is added, which will bind to the biotin on the hybridized probe-target DNA. Then a biotin labelled enzyme like alkaline phosphatase is added which binds to the avidin bonded on the probe. Then the substrate specific for this enzyme is added, which will convert the colourless substrate into a coloured product. Appearance of the colour indicates the presence of the pathogenic DNA and non-development of the colour is an indication of the absence of the organism.



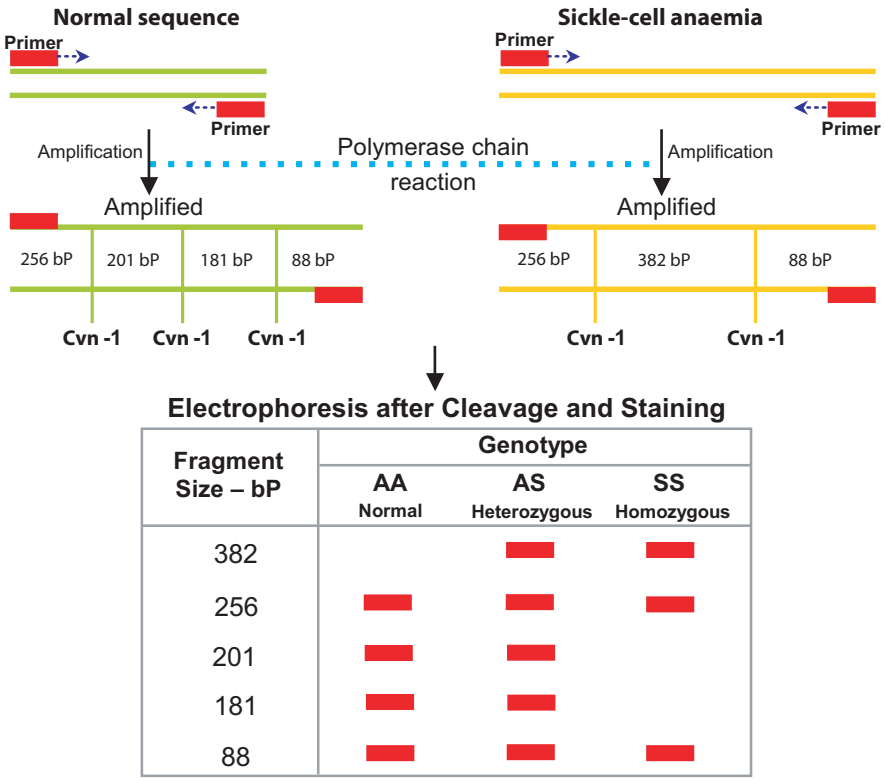
Detection of pathogenic organism by DNA hybridization

(b) Diagnosis of genetic disease using restriction endonuclease: Sickle-cell anemia is a genetic disease due to the change in a single nucleotide in the codon for the sixth amino acid of the beta-chain of the hemoglobin molecule. The gene for beta-globin in normal persons is designated as A/A, in heterozygous individuals as A/a, and in homozygous individuals as S/s. Individuals containing the sickle gene are screened before the expression of the symptoms and for screening the carrier, who are at risk of transmitting this gene to their offspring.

The principle for the detection is that, within the beta-globin gene of a normal individual, there are three sites for the restriction endonuclease Cvn-1, but in sickle-cell gene one of these sites is lost due to replacement of the single nucleotide. In the normal gene, the DNA sequence is CCTGAGG whereas in the sickle-cell anemia gene, the sequence is CCTGTGG. Further the recognition sequence and site of cleavage by Cvn–1 is CCTNAGG. Thus, the difference in sequence of normal and sickle-cell gene in the recognition site of Cvn–1 forms the basis of this DNA diagnosis.

Two primers with sequences that can pair within the Cvn-1 sites in the beta-globin gene are added and this part of DNA is amplified using P.C.R. and then digested with Cvn-1. Finally the cleavage products

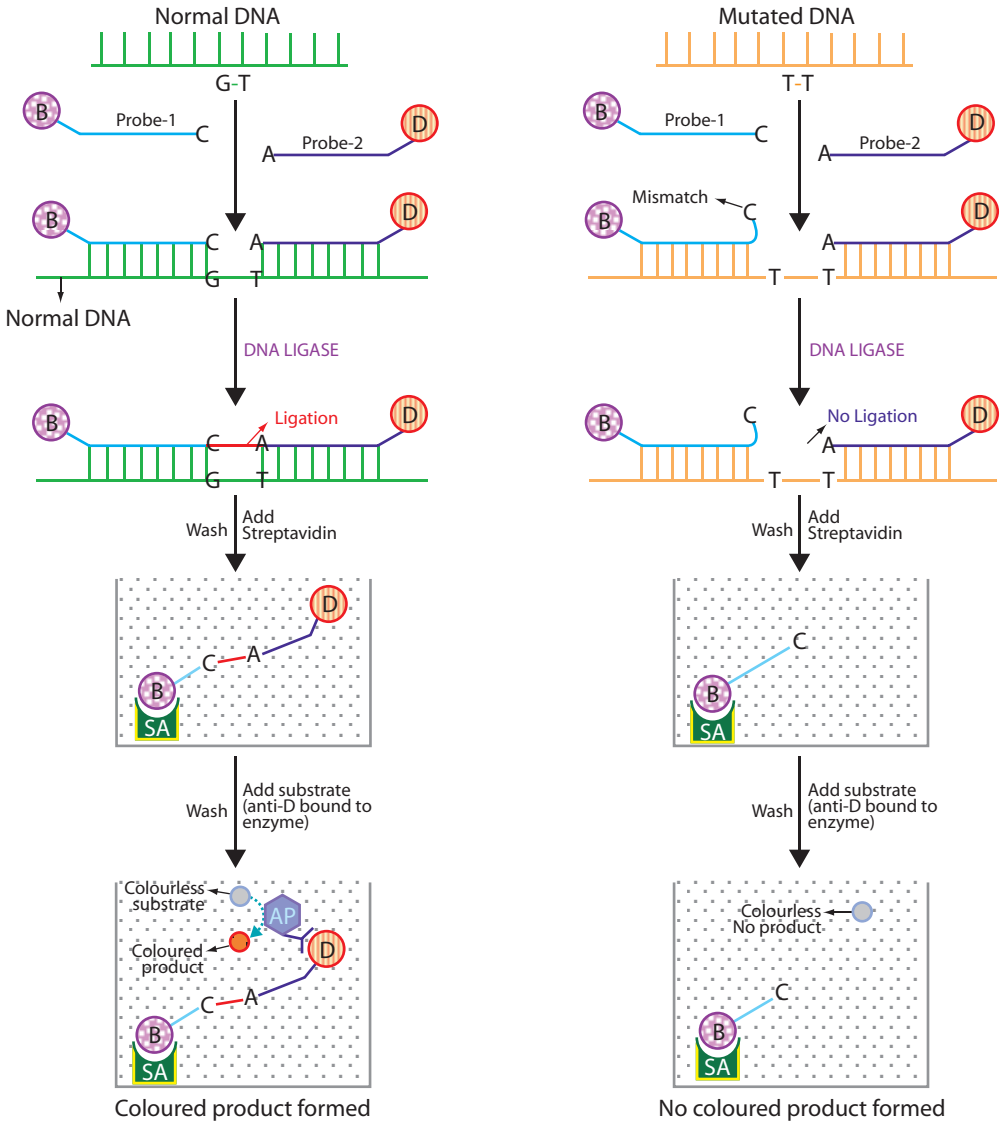
are separated by gel electrophoresis and stained by ethidium bromide. The results indicate that in the normal gene, four DNA fragments are obtained with 88, 181, 201 and 256 base pairs. But in heterozygous individual five DNA fragments are obtained containing 88, 181, 201, 256 and 382 base pairs and in homozygous individuals only three fragments are obtained with 88, 256 and 382 nucleotide base pairs, indicating the loss of one of the recognition site in the sickle cell gene.



Detection of genetic disease by restriction endonuclease

(c) Diagnosis of genetic disease by PCR/OLA procedure: This procedure is applied for those disorders due to genetic mutations which does not affect the restriction endonuclease sites. Let us take an example of a gene, which has undergone mutation at position 98. At this specific site the base pair in the normal gene be C≡G and in the mutant gene let it be A=T. Two oligonucleotides of about 20 nucleotide length each are synthesized with sequence complementary to one of the strands of this gene on either side of position 98. One of these oligonucleotides has biotin at its 5′ end and 'C' as the terminal nucleotide at the 3′ end. The other oligonucleotide probe has 'A' base nucleotide at the 5′ end and digoxigenin (compound 'D') at its 3′ end. The target DNA is amplified by PCR and then is hybridized with the synthesized probes. The two probes base pair such that the 5′ end of the 2^{nd} probe lies next to the 3′ end of the 1^{st} probe. Then DNA ligase is added, which will ligate only the normal DNA fragment but not the mutated fragment hybridized with the probes. This is because of the mismatch between the 2^{nd} probe and the mutated gene, which cannot base pair. Further, in order to determine whether ligation has occurred or not, the hybrid probes are taken into a well containing avidin which binds to biotin. Then it is washed, which removes the unligated probe. Then anti-digoxigenin ('D' compound) antibody–alkaline phophatase conjugate is added and washed in both the wells (the normal and mutated hybridized

probes). It is expected that the antibody enzyme will bind only to the ligated probe well. When substrate is added, the coloured product is produced only in the well where ligation has occurred, whereas no colour is formed where no ligation has taken place.

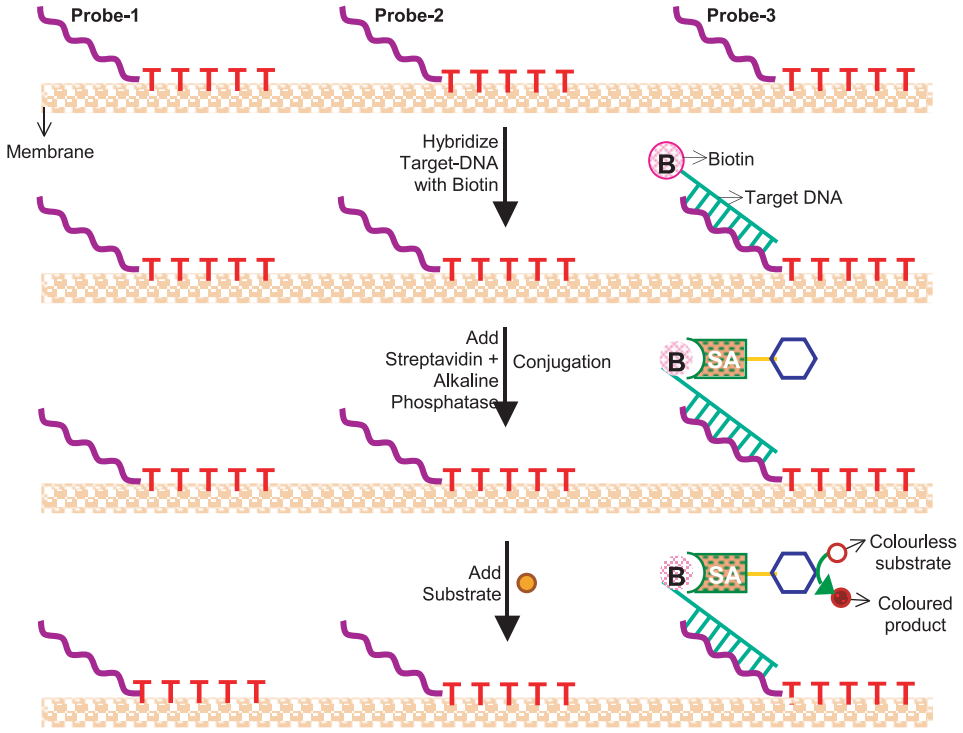


Diagnosis of genetic disease by PCR/OLA procedure

(d) Detection of mutations at different sites within one gene: Beta-thalassemia is a genetic disease that is caused due to mutation in beta-globulin at eight or more sites, thus results in low rate of its synthesis. Hence instead of detecting each mutation separately all the eight sites are scanned at the same time.

DNA probes are synthesized to all these eight sites of beta-globin gene where mutations are expected. Each probe is 20 nucleotide in length with a poly 'T' tail at the 3' end. This is used to attach the probe to a membrane. Segments of the sample DNA (beta-gene) that includes each of the possible mutant sites are amplified by PCR, using primers labeled with biotin at the 5' end. The amplified target DNA is then hybridized to the membrane bound probes under conditions that allow only perfect matches to hybridize. Then streptavidin with attached alkaline phosphatase is added, the membrane washed and a colourless substrate is added. A coloured spot on the membrane appears wherever there is a perfect nucleotide match between the amplified target DNA segment and one of the specific oligoneucleotide probes. Where there is no hybridiza-

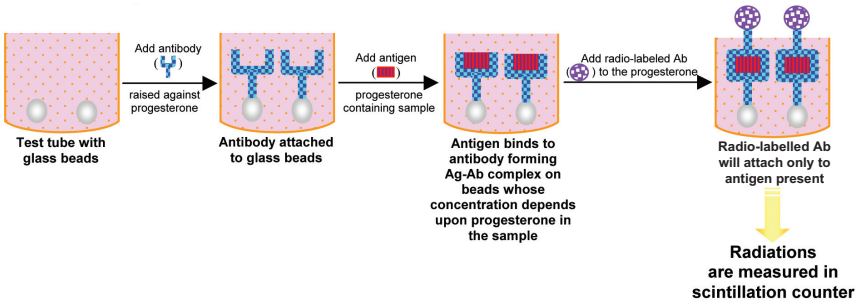
tion (mutant DNA segments) no colour appears. In the illustration given below, gene 1 and 2 are mutated but gene 3 is normal. (Represented as probe 1, 2 and 3 respectively in the figure).



Detection of mutations at different sites within one gene

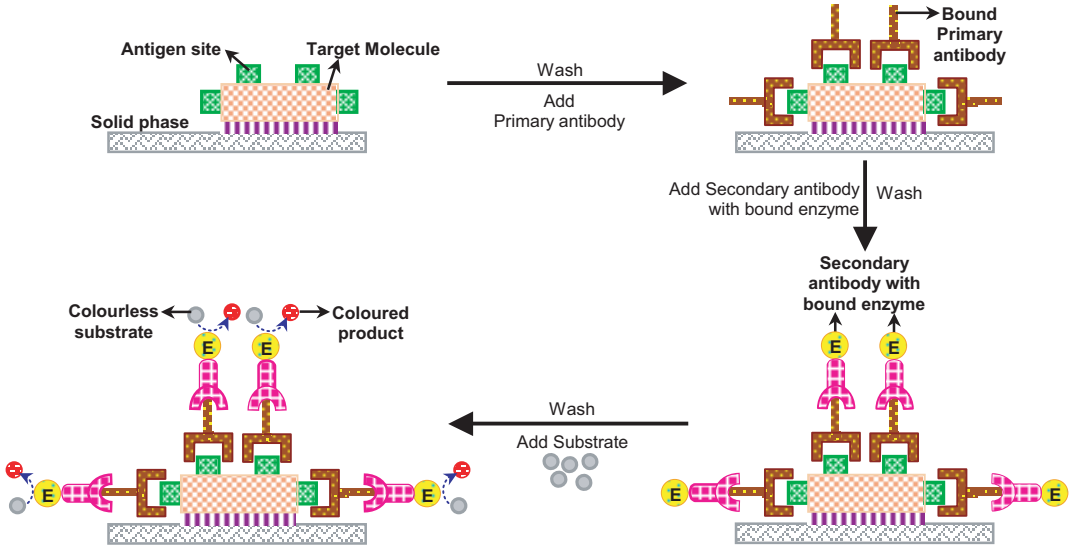
2. Immunological methods: Antibody molecules consist of four chains, two identical light chains and two identical heavy chains. The fv (fragment variable) region of each antibody molecule binds tightly to a specific site (epitope) on an antigen. This specificity is used to identify the presence of a particular epitope of a disease causing molecule or organism in a biological sample. There are two methods by which the antigen-antibody reaction or binding is detected.

(a) Radio-immuno assay (RIA): The concentration of progesterone in blood (for example) is to be determined by RIA. First of all antibodies to progesterone are raised and taken in a test tube containing glass beads. The antibodies get readily attached to the glass beads. Then progesterone containing sample is added to this test tube which binds the antibodies forming antigen-antibody complex, whose concentration depends upon the amount of progesterone in the blood sample. Another test tube is taken and the antibodies are labelled with radioactive compounds like ^{123}I or ^{3}H or ^{14}C. This radio labelled antibody is then added to the first test tube containing progesterone attached to unlabelled antibodies. Radio labelled antibodies will now attach to the progesterone and form labelled antigen-antibody complex which is measured using a scintillation counter.



Radio-immuno-assay (RIA) technique for progesterone estimation

(b) Enzyme linked immunosorbent assay (ELISA): The sample which is to be tested for the presence of a specific molecule or organism is bound to a solid support such as a plastic plate. Then a marker-specific antibody (primary antibody) is added to the bound material and then the support is washed to remove unbound primary antibody. Then a second antibody (secondary antibody) is added, which binds specifically to the primary antibody and not to the target molecule. The secondary antibody contains bound enzyme like alkaline phosphatase which catalyzes the conversion of a colorless substrate into a colored product. The system is washed again to remove any unbound secondary antibody-enzyme conjugate. Then a colorless substrate is added which is converted to a colored product only if the specific antigen is present, if not there is no colour. If there is no antigen (or the causative agent) then the primary antibody will not bind to the target site in the sample, hence the first washing step removes it. Consequently, the secondary antibody–enzymes conjugate will have nothing to bind to and is removed during the second washing step, and the final mixture remains colorless. Conversely, if the antigen (or the causative agent) or the target site is present in the sample, then the primary antibody binds to it, the secondary antibody binds to the primary antibody and the attached enzyme will catalyze the reaction to from a colored product which can be detected colorimetrically.



ELISA tests for detection of antigen-antibody reaction

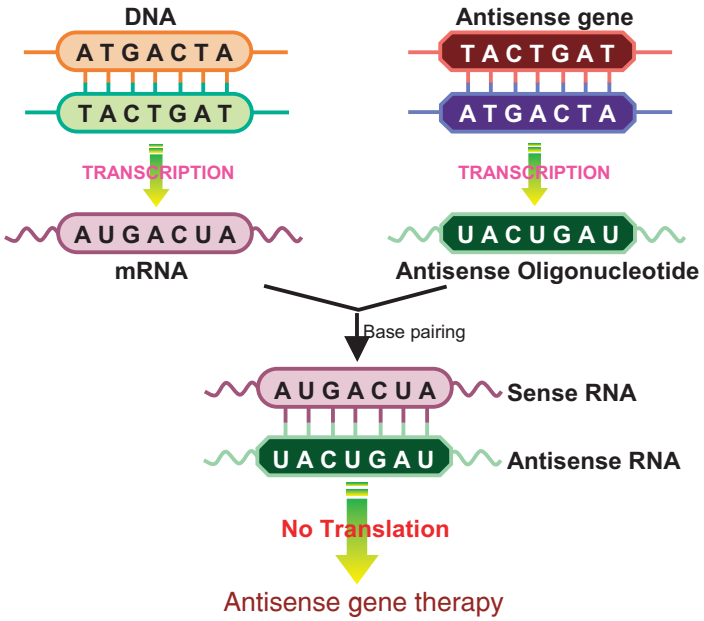
Molecular treatment or gene therapy: In order to treat a genetic disease, the normal gene for that disease has to be sequenced and cloned. This cloned normal gene can be used to correct the defect in individuals who have a mutant form of that gene. Here, the objective is to add a normal functioning gene to defective cells, thereby providing the required protein and correcting the genetic disease. In addition, it will be necessary to prevent the over expression of a deregulated normal gene, in some diseases. There are three methods for the therapy of genetic diseases (1) *Ex vivo* gene therapy (2) *In vivo* gene therapy and (3) Antisense therapy.

(a) *Ex vivo* gene therapy: Somatic cells from an affected individual are collected. The isolated cells are grown in culture. These cells are then transfected by retroviral cloning vectors containing the remedial gene construct. The cells are further grown and those cells which contain the gene of interest are selected and finally transplanted or transfused back into the patient. These transplanted transfected cells will synthesize the gene product i.e. the protein. Examples for this type of treatment include gaucher disease, sickle cell anaemia, thalassemia etc.

(b) *In vivo* gene therapy: In this type of treatment there is the direct delivery of the remedial gene into the cells of a particular tissue of the patient, using retroviral vectors. Even plasmid DNA constructs

are used. This type of treatment is used in case of muscular dystrophy, neuronal degeneration and brain cancer patients.

(c) Antisense therapy: Antisense therapy is designed to prevent or lower the expression of a specific gene. In some type of genetic diseases and cancers, the genes are deregulated or over expressed resulting in the production of excess of the gene product or its continuous presence in the cell will disrupt the normal functioning of the cell. In such type of diseases the addition of normal gene will not solve the problem; instead blocking the synthesis of the gene product (protein) will be helpful. Thus in anti-sense therapy a nucleic acid sequence is introduced into the target cell which is complementary to complete or a part of that specific mRNA. Hence the mRNA produced by the normal transcription of the gene will hybridize with the antisense oligonucleotide by base pairing, thereby preventing the translation of this mRNA, resulting in reduced amount of target protein. The antisense therapy is used in treatment of various cancers, AIDS, atherosclerosis, leukemia and sickle cell anaemia.



FERMENTATION TECHNOLOGY

Fermentation is the process involving the biochemical activity of organisms, during their growth, development, reproduction, even senescence and death. Fermentation technology is the use of organisms to produce food, pharmaceuticals and alcoholic beverages on a large scale industrial basis.

The basic principle involved in the industrial fermentation technology is that organisms are grown under suitable conditions, by providing raw materials meeting all the necessary requirements such as carbon, nitrogen, salts, trace elements and vitamins. The end products formed as a result of their metabolism during their life span are released into the media, which are extracted for use by human being and that have a high commercial value. The major products of fermentation technology produced economically on a large scale industrial basis are wine, beer, cider, vinegar, ethanol, cheese, hormones, antibiotics, complete proteins, enzymes and other useful products.

Fermentation methodology: Fermentation process is carried out in a container called the fermentor or bioreactor. The design and nature of the fermentor varies depending upon the type of fermentation

carried out. Invariably all the fermentors have facilities to measure some of the fermentation parameters like temperature, pressure, pH, elapsed fermentation time, liquid level, mass etc.

The different types of fermentors are—

(1) External recycle airlift fermentor–for producing bacterial biomass, with methanol as substrate.
(2) Internal recycle airlift fermentor–for producing yeast with oil as substrate.
(3) Tubular tower fermentor–Used for making beer, wine, vinegar etc.
(4) Nathan fermentor–used in brewing industry.
(5) Stirred fermentor–used for making antibiotics.

Types of fermentation processes: There are three different process of fermentation viz. (1) Batch fermentation (2) Feb-batch fermentation and (3) Continuous culture.

Batch fermentation: This term is attributed to that type of fermentation wherein there is change in culture medium, number of microorganisms and the amount of the product produced (i.e. the metabolite or target protein). In batch fermentation six phases of the microbial growth are seen.

(a) ***Lag phase*:** Immediately after inoculation, there is no increase in the numbers of the microbial cells for some time and this period is called lag phase. This is in order that the organisms adjust to the new environment they are inoculated into.
(b) ***Acceleration phase*:** The period when the cells just start increasing in numbers is known as acceleration phase.
(c) ***Log phase*:** This is the time period when the cell numbers steadily increase.
(d) ***Deceleration phase*:** The duration when the steady growth declines.
(e) ***Stationary phase*:** The period where there is no change in the microbial cell number is the stationary phase. This phase is attained due to depletion of carbon source or accumulation of the end products.
(f) ***Death phase*:** The period in which the cell numbers decrease steadily is the death phase. This is due to death of the cells because of cessation of metabolic activity and depletion of energy resources.

Depending upon the product required the different phases of the cell growth are maintained. For microbial mass the log phase is preferred. For production of secondary metabolites i.e. antibiotics, the stationary phase is preferred.

Feb-batch fermentation: In this type of fermentation, freshly prepared culture media is added at regular intervals without removing the culture fluid. This increases the volume of the fermentation culture. This type of fermentation is used for production of proteins from recombinant microorganisms.

Continuous fermentation: In this type of fermentation the products are removed continuously along with the cells and the same is replenished with the cell girth and addition of fresh culture media. This results in a steady or constant volume of the contents of the fermentor. This type of fermentation is used for the production of single cell protein (S.S.P), antibiotics and organic solvents.

Procedure:

(a) Depending upon the type of product required, a particular bioreactor is selected.
(b) A suitable substrate in liquid media is added at a specific temperature, pH and then diluted.
(c) The organism (microbe, animal/plant cell, sub-cellular organelle or enzyme) is added to it.
(d) Then it is incubated at a specific temperature for the specified time.
(e) The incubation may either be aerobic or anaerobic.
   - Aerobic conditions are created by bubbling oxygen through the medium.
   - Anaerobic conditions are created by using closed vessels, wherein oxygen cannot diffuse into the media and the oxygen present just above is replaced by carbon dioxide released.

- After the specified time interval, the products are removed, as some of the products are toxic to the growing cell or at least inhibitory to their growth. The organisms are re-circulated. The process of removal of the products is called down stream processing.

Example of batch fermentation technology using tubular tower fermentor: The simplest and the most commonly used fermentation technology is the preparation of curd from milk. The details of the technology are as under—

The fermentor or bioreactor: Mud pot or steel cooking vessel or a dish or a cup

The substrate: Milk

The specific temperature: 37°C

The organism: Preformed curd (containing the microbe *Lactobacillus cecai*)

The incubation: At 37° C for 6-8 hours

The aeration: The process is anaerobic

The process: Preformed curd has microbes that utilize lactose present in the milk. Lactose is hydrolyzed into glucose and galactose. Galactose is converted to glucose. Glucose is broken down to lactic acid by glycolytic pathway. The lactic acid produced, lowers the pH of milk from 6.6 to 4.5. The isoelectric pH of milk protein–casein is 4.5. At this pH, casein precipitates forming fine micelles in the milk thereby curdling it. Milk is warmed before adding it to the fermentor so as to maintain the temperature required for the growth of the microbes. The container/fermentor is not disturbed (no stirring is taken up) so that the precipitation is uniform. The elapsed time i.e. the time required to form the curd is crucial; it depends upon the atmospheric temperature. During summer the curd is formed within 4-6 hours, whereas during rainy season it takes 6-8 hours and in winter it takes almost 8-12 hours. The process is anaerobic hence it is better to keep the vessel closed. Though it cannot be air tight but still the surface of the milk that contains fat prevents air from penetrating in the liquid, furthermore the metabolizing microbes replace the oxygen with carbon dioxide released.

Example of continuous fermentation technology using stirred fermentor: Another common fermentation technology is the process of food digestion. The details of the technology are as under—

The fermentor or bioreactor: Stomach (G.I. tract)

The substrate: Food

The specific temperature: 37°C

The organism: Enzymes, microbes, acids etc

The incubation: At 37°C for 3-4 hours

The aeration: The process is anaerobic

The process: The food eaten by us is stored in the stomach where HCl and some enzymes are secreted that convert food into chyme (semi solid). The food stays for 3-4 hours at 37° C. The digested food is absorbed in the blood and the undigested food is excreted. This is a continuous fermentation because the substrate (food) is continuously added and the products (digested / undigested material) are continuously removed. Stomach–the fermentor is a stirred type i.e. the peristaltic movement of the gastro-intestinal tract mixes the food.

Categories of fermentation technology: Fermentation technology can be grouped into four major categories viz.—

1. Microbial biomass production: Microbial cells (biomass) are grown commercially as continuous culture on a large scale (1500/m^3). The microbial cells including algae, bacteria, yeasts, moulds and mushrooms are dried and used as a good source of a complete protein called **'single cell protein (SCP)'** which serves as human food or animal feed. The incubation of wheat flour for preparation of 'tanduri roti' and

rice flour for 'dosa' are good examples for the production of single cell protein. These two food stuffs or the common food are usually lacking or not having sufficient quantities of lysine and methionine amino acids. Single cell protein produced by different sources is a rich source of different essential amino acids, thereby supplementing the amino acid lacking in a particular food. The incubation of the foods results in the growth of the microbial biomass, producing the SCP. SCP produced by some microorganisms is lysine rich whereas those produced by others are methionine rich. The substrates used by the microbes producing the single cell protein range from carbohydrates to hydrocarbons and petrochemicals. Other organisms use the gases–methane, ethane, propane, n-butane etc as substrate for fermentation.

SCP producing organisms and their substrates

Member	Substrate used
Chlorella pyrenoidesa	CO_2 (0.7% in air), fluorescent light
Scenedesmus acutus	CO_2, sunlight
Spirulina maxima	CO_2 (0.5%), $NaHCO_3$, sunlight
Achromobacter deluacuate	Diesel oil
Nocardia sps.	n-Alkanes
Pseudomonas sps.	Fuel oil
Candida tropicalis	Molasses
Trichoderma sps.	Coffee wastes

Lysine-rich SCP (7g lysine/16 g N) producing organisms are *Chlorella sorokiniana, Cellulomonas alkaligens, Saccharomyces cerevisiae* etc.

Methionine-rich SCP (2g methionine/16 g N) producing organisms are *Methylococcus capsulatus*, *Saccharomyces cerevisiae* and *Aspergillus*.

2. Microbial metabolites: During the metabolism of microbial cells a number of compounds are produced and many are secreted out of the cell, which can be easily extracted and are very useful to man and animals. Therefore fermentation by microbial cells is carried out on an industrial scale, in order to get various metabolites.

The metabolites produced by the microbes can be grouped into two categories—(a) Primary metabolites and (b) Secondary metabolites.

(a) *Primary metabolites*: Metabolites which are produced by the metabolism required for the maintenance of the minimum life process of a microbe are known as primary metabolites. The primary metabolites are produced in abundance at an early stage of growth. Examples of primary metabolites are ethanol, citric, acid, glutamic acid, lactic acid, acetic acid, acetone, formic acid, butanol, propionic acid, dihydroxy-acetone, glycerol etc. These metabolites are produced by fermentation technology applying different microbes under varying conditions of fermentation.

(b) *Secondary metabolites*: Secondary metabolites are those metabolites, which are not produced directly by the metabolism required for the vital life process of microbes, instead are produced by some specialized metabolic process. However most of the secondary metabolites are derived from the primary metabolites. The secondary metabolites include the antibiotics, alkaloids, toxic pigments, vitamins etc.

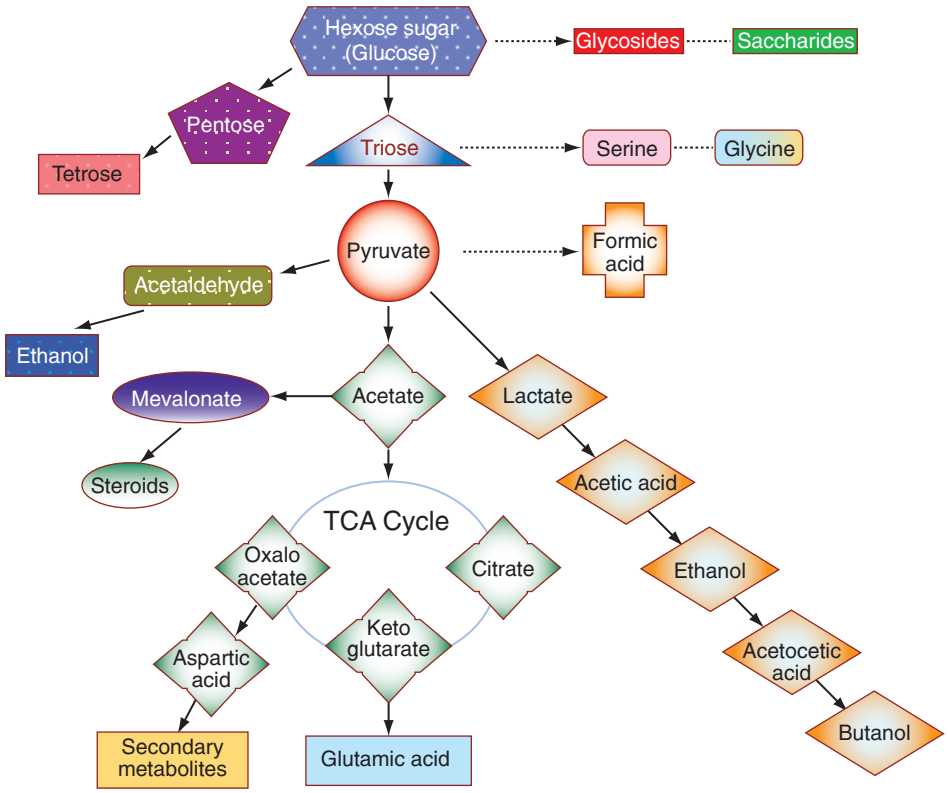
***Antibiotics*:** An antibiotic is a substance produced by a microorganism which can inhibit growth or completely destroy other microorganisms.

The antibiotics are not synthesized by the microorganism by a single gene, instead a set of 10-20 genes takes part in the synthesis of an antibiotic. The genes for antibiotic are mostly located on the plasmid, however in some microbes they are found on the chromosomal DNA. About 45000 different antibiotics

have been discovered so far, but only about 100 are used for human treatment, because only those antibiotics can be used which are toxic to the invading microbe but non-toxic for the human. Some of the antibiotics and the microbes which produce them are—

Amphotericin-B–*Streptomyces nodosus* Chloramaphenicol–*Streptomyces venezuelae*, Erythromycin–*S. eythirus*, Gentamycin–*Micromonospora purpurea*Gramicidin–*Bacillus brevis*, Penicillin–*Penicillum chryosogen* Streptomycin—*S. grises*, Tetracycline–*S. aureofacins*.

These microbes are grown under suitable fermentation conditions so as to get the desired antibiotic. Mutant microbes are used for industrial production of antibiotics.



Pathways showing production of primary metabolites

3. Microbial enzymes: When microbes are cultured, they secrete some enzymes into the media and these enzymes are extracted and widely used in several industries like detergent, food processing, brewing and pharmaceutical. They are also used for diagnostic, scientific and analytical purposes. Biotechnological methods are used to engineer microbial cells so as to induce them to produce enzymes like renin by *E. coli* and amylases by *Bacillus stearothermophillis*. These enzymes are generally bound to matrix or in some manner retained in the reactor to be reused and hence these are called immobilized enzymes. In some cases the microorganism producing enzyme is immobilized. Some of the enzymes produced by fermenting microbes are–Glucose oxidase, protease glucoamylase, amylase, glucose isomerase, rennin, pectinase, superoxide dismutase, cellulase, invertase, lactase and lipase.

Some thermophillic bacteria produce enzymes that are thermostable and which can be used in industrial processes at high temperatures, ex. glyceraldehyde-3-phosphate dehydrogenase, phosphofructo-kinase, alcohol dehydrogenase, superoxide dismutase and restriction endonucleases, which are produced by *Bacillus stearothermophillis thermoysia*. Further, genes for thermophilic enzymes are introduced into *E. coli*, which is cultured for producing the thermophilic enzymes.

4. Bioconversion, biotransformation or modification of the substrate: The fermenting microbes have got the capacity to convert an added substrate into some more valuable product, ex. conversion of ethanol to acetic acid (vinegar), isopropanol to acetone, glucose to gluconic acid, sorbitol to sorbose (this is used in the manufacture of vitamin C), sterols to steroids.

Among all these bioconversions, the production of steroids is the most widely applied fermentation biotechnology for the conversion of sterols into steroids, like cortisone, hydroxycortisone, prednisone, dexamethasone, testosterone, estradiol etc. Hitherto, steroids were produced by chemical methods, which were laborious and costly. For instance the chemical synthesis of cortisone required 37 steps under extreme conditions. One of the steps is introduction of oxygen at position 11 in the steroid nucleus. The microbe *Rhizopus arrhizus* being capable of hydroxylating progesterone (a steroid) at position 11 is used in the fermentation to produce progesterone an intermediate in the synthesis of cortisone. Thus, this reduced the chemical synthesis steps from 37 to 11 and all under normal conditions, thereby making it economical and easy. Steroids are used as anti-inflammatory agents, contraceptives, treating hormonal insufficiency, allergy, skin diseases etc.

Down stream processing (DSP): The method by which the products of fermentation are recovered and separated is known as down stream processing. This forms the major (about 85%) portion of the complete fermentation technology. There are various methods by which DSP is carried out. First of all, the broth is conditioned i.e. the cells are aggregated and form large clumps, which makes the separation easier. The conditioning is done by heating, freezing, pH change, antigen-antibody reactions etc. Then the conditioned broth is used for the separation of the constituents for which techniques like sedimentation, floatation, filtration, ultra-filtration, centrifugation and micro-filtration are applied.

23

BIOTECHNOLOGY IN REPRODUCTION

Reproduction is the sexual union of the sperm and ova to produce the offspring.

Biotechnology has developed methods enabling improved and economical reproduction. Advances in Biotechnology have developed improved reproductive methods for infertile couples.

The broad areas of reproduction in which biotechnology is applied are—

1. Sperm concentration/washing
2. Separation of X and Y spermatozoa for sex selection
3. Cryopreservation of semen, ova and embryo
4. Sperm capacitation
5. Artificial insemination (Intrauterine, intracervical)
6. Induction of ovulation
7. Control of oestrous cycles
8. Superovulation
9. In-vitro fertilization and embryo cloning
10. Micromanipulation of embryos
11. Early pregnancy diagnosis

Ever since the pre-historic age, man has been trying various means to improve reproductive methods. With time and long, methods have been developed that have enabled advances in the fields of reproduction, in order to overcome infertility problems. Description of all these techniques is out of the scope of this book. Anyway a few of them, relevant to biochemistry laboratory diagnostics are described in short here-under.

SEMEN

Semen is a semi-gelatinous or liquid suspension containing the male gametes, called spermatozoa and secretions from the male accessory organ, called seminal plasma. Sperms or spermatozoa are produced in the testis from the seminiferous tubules. The epithelial cells of these tubules differentiate into germ cells, which undergo repeated cell divisions both by mitosis and meiosis, finally producing the spermatozoa. This is known as spermatogenesis and is under the influence of hormones like testosterone. As a result of meiosis all the sperm cells are haploid in number i.e. the number of chromosomes in each sperm is half the number of chromosomes in the somatic cells. A human somatic cell has 46 chromosomes (2N) and the sperm has 23 (N) chromosomes. One of these chromosomes in each sperm is the sex chromosome. There are two types of sex chromosomes viz. X-chromosome and Y-chromosome. Any one of these chromosomes (either

X or Y) may be present in a given spermatozoa. Fertilization of the ova by X chromosomal spermatozoa results in a female and with that of Y chromosomal spermatozoa results in a male offspring. There are specific receptors on the ova for X and Y spermatozoa. An ova will have either X or Y receptors on it and not both. Ova released in a particular month will either have X receptors on it or Y receptors. Hence it is the female who is responsible for the birth of a baby boy or baby girl.

The constituents other than spermatozoa are called the seminal plasma which is a collection of secretions from testis, epididymis and accessory glands of the male (i.e. the prostrate). The sperms, after their production and maturity are stored in the tail of the epididymis of the testis. The seminal plasma is added to the semen at the time of ejaculation i.e. the process of release of the semen through the penis.

Composition of human semen

	Component	Range	Average value
1.	Volume (ml)	2.30 – 4.99	3.65
2.	pH	7.19 – 8.47	7.83
3.	Osmolality (mosm)	254 – 423	339
4.	Fructose (mg/dl)	136 – 628	382
5.	Glucose (mg/dl)	5 – 295	150
6.	Total protein (mg/dl)	3700 – 7460	5580
7.	Albumin (mg/dl)	1100 – 2000	1550
8.	Urea (mg/dl)	13 – 98	45
9.	Lactic acid (mg/dl)	22 – 136	62
10.	Citrate (mg/dl)	304 – 751	528
11.	Ca (mg/dl)	16 – 53	34.5
12.	Cl (mg/dl)	130 – 158	144
13.	K (mg/dl)	50 – 248	149
14.	Mg (mg/dl)	7.89 – 31.8	19.85
15.	Na (mg/dl)	236 – 512	374
16.	Zn (mg/dl)	6.78 – 69.29	38
17.	Sperm concentration	20×10^6 spermatozoa/ml or more	
18.	Total sperm count	40×10^6 spermatozoa per ejaculate or more	
19.	Motility	50% or more with forward progression (categories a and b) or 25% or more with rapid progression (category a) within 60 minutes of ejaculation	
20.	Morphology	30% or more with normal forms	
21.	Vitality	75% or more live	
22.	White blood cells	fewer than $1x10^6$/ml	
23.	Other components include lipids, usually present as 'lipid bodies', choline, sialic acid, inositol, spermine, pyruvate, creatine and ascorbic acid		

There are several factors which affect the quality and quantity of semen produced viz. body and atmospheric temperatures decline semen quality and quantity. Nutrition directly affects semen produc-

tion. Bad nutrition specially lacking in good proteins and vitamins (vitamin A deficient) adversely affects semen production. Younger men give better quality and quantity semen.

Parameters to be considered for semen collection for a semen bank:

- The person selected for semen collection should be well built, well nourished and should be of well established pedigree i.e. having a proven high degree of fertility.
- Before collection of semen the person should be fed well so as to increase the sperm output.
- The person should abstain from sex for a minimum of three days prior to collection of semen, but he should not be under prolonged sexual rest as it results in the presence of high percentage of degenerating or stale spermatozoa.
- The container should be sterilized well.

Methods of semen collection: Mechanical massage technique is the generally used method for the collection of semen. In cases with poor libido and lack of erection due to some other problems the electro ejaculation method is used. A mild alternating current is applied to the sacral and pelvic nerve by electrodes placed in the rectum, which induces ejaculation.

The semen collected by any of the above methods, is evaluated for its quality. Good quality semen should be viscous and cream-white. Discoloured semen, watery or contaminated with blood should be discarded. The sperm motility should be 70-80% and all the chemical constituents should be as mentioned above. Semen collected in presence of a female will be of the best quality, followed by mechanical message and the poorest quality semen is obtained by electro-ejaculation method.

Artificial collection of semen allows sperm donation to oligospermic or azoospermic couples enabling them to have children, sex selection by separation of X and Y bearing spermatozoa, artificial insemination/intra-uterine insemination facilitating good fertility.

Separation of X and Y spermatozoa: The separation of X and Y spermatozoa is based on the difference in their physical properties as given in the table below—

	X – spermatozoa	Y – spermatozoa
1.	Contains more DNA and hence is larger due to which it is more in weight, density and slow in motility	Contains less DNA and hence the chromosome in the cell is smaller, due to which it is less in weight
2.	It is non-fluorescent	It is fluorescent, which is due to its getting stained with quinacrine and orcein dyes
3.	It is positively charged on the surface, migrates towards the cathode upon electrolysis	Has no surface charge
4.	Has no surface antigen	It contains H-Y surface antigen, hence selectively binds to H-Y antibodies in affinity chromatography columns
5.	One type of lactate dehydrogenase (LDH) isoenzyme is present	Another type of lactate dehydrogenase (LDH) isoenzyme is present

Techniques for separation of X and Y spermatozoa: The various techniques for the separation of X and Y spermatozoa are—

(1) *Sedimentation*: Sedimentation of immobilized sperm on medium like skimmed milk powder, glycine, sodium citrate, glycerol and egg yolk.

(2) *Velocity sedimentation*: It is automatic separation due to difference in size, density and shape of spermatozoa, wherein Y-sperms float on the top with X-sperms at the bottom.

(3) *Separation using albumin columns*: Separation is based upon size, shape and motility.
(4) *Density gradient centrifugation*: Separated depending upon their density.
(5) *Electrophoretic separation*: Separated depending upon their charge and density.
(6) *Isoelectric focusing*: The semen is subjected to electrical field in an ascending / descending pH and as the sperms move in this field the charge upon them changes and wherever the positive and negative charges are equal on the spermatozoa it sediments there. As only the X–sperm has surface charge, it precipitates at its isoelectric pH.
(7) *H-Y antisera*: H-Y antigens found only on Y sperms can specifically bind to H-Y antibodies.
(8) *Separation on sephadex columns*: A type of gel filtration in which the semen is layered on the top of a gel column prepared by sephadex and the eluent coming out first contains more of X-spermatozoa, owing to its larger size and hence faster passage.

Theoretically and practically 95-97% separation of X and Y spermatozoa can be achieved by applying combinations of various techniques mentioned above. Out of all these methods, only two methods are used commercially, they are separation on albumin-columns which yields 75-80% Y-spermatozoa and sephadex filtration which gives 70-75% X-spermatozoa.

Sperm washing: A sperm's energy output is 20 times greater once it is removed from the seminal fluid. Methods for washing sperm can have a dramatic effect on the ability of sperm to move towards the egg. The simplest method involves—

- The sperms are mixed with a nutrient-rich fluid (or culture media) in a test tube.
- They are then centrifuged (spun very rapidly) for about 5 minutes.
- The sperms which are heavy, settle on the bottom, forming a dense button of millions of pure sperm. The fluid left on top is siphoned off.
- This procedure may be repeated.

This simple method of sperm washing, however, does not eliminate heavy debris such as dead sperm, white blood cells, or bacteria which may impair fertility. Addition of a substance called platelet-activating factor during the sperm washing process may enhance pregnancy rates.

Swim-up technique: The swim-up technique is not only an useful diagnostic procedure for testing the ability of sperm to escape from the semen into the cervical mucus, but it also achieves the goal of removing sperm from semen.

- A specially prepared semen sample is placed in a tube.
- A culture media (a nutrient-rich substance in which cells thrive) is placed on top of the sample.
- The medium is a hospitable environment for sperm, and those that are healthy will swim up to it.
- After an hour or more, the culture is examined, and the number of sperm that have reached the medium is compared to the number still remaining in the semen. The supernatant is collected.

The result gives a fair estimation of the number of sperm potentially capable of fertilization. It is superior to sperm washing because the live sperm will swim up to the culture media, leaving behind most of the debris, although some may float up into the medium. There is also some evidence that such sperm may have fewer genetic abnormalities than those retrieved through sperm washing. The strongest sperms, which are those at the top of the medium, can be collected for *in vitro* fertilization or artificial insemination. A good swim test yields about half a million of very active sperm.

Cryopreservation of semen: Preservation of semen in cold is known as cryopreservation and is termed as **'frozen semen'**.

The artificially collected semen/concentrated semen/separated X and Y spermatozoa can be preserved separately and insemination can be done by the appropriate spermatozoa, as desired at the specified time.

Frozen semen is the extended semen packaged in a single dose, kept in a state of suspended animation by deep freezing with virtual suspension of its metabolic activities, but without affecting its viability and fertility functions. For storing semen in a frozen state, the followings are to be provided—

(1) Nutrients as a source of energy (usually glucose or fructose).
(2) Protectant to protect against the harmful effect of rapid cooling i.e. prevent cold shock (egg yolk or milk).
(3) Provide a buffer to prevent harmful effects due to change in pH as lactic acid is formed (Tris-hydroxymethyl-aminomethane or phosphate or disodium citrate is used).
(4) Maintain the proper osmotic pressure and electrolyte balance. (All the above constituents from 1 to 3 so as to provide 285 milli osmoles pressure).
(5) Inhibit bacterial growth (penicillin and streptomycin).
(6) Increase volume of semen so that it can be used for multiple inseminations (semen extenders).
(7) Protect the sperms cells during freezing (glycerol).

Semen extender: It is a medium or mixture of solutes and solvents in appropriate amounts which extends (dilutes or increases in volume) the volume of the ejaculate and favours the survival of the spermatozoa in cold condition for long duration of time (unlimited).

About 3-5 ml of semen is generally ejaculated by a male containing about 100-300 million spermatozoa. It has been established that 10-20 million live spermatozoa in one milliliter is sufficient for fertilization by intra uterine insemination/artificial insemination. So semen extenders, dilute the semen appropriately so as to increase the volume of the semen and also keep the spermatozoa alive during cold storage. Extenders can be divided into three broad classes—

(a) **Egg yolk extenders:** Containing 2.9% sodium citrate, 25% egg yolk, 1000 i.u. penicillin, 500 mg streptomycin/ml, fructose and minerals.
(b) **Milk extenders:** These are prepared either from whole milk, skimmed milk or powder milk, heated to 90°C, cooled and the other constituents mixed appropriately.
(c) **Extenders for semen freezing:** The above two extenders or diluents are used for freezing of semen with addition of 3-10% of glycerol, which is a cryoprotective agent for semen. Spermatozoa survival is better if the glycerol is mixed with the semen at 5°C as spermatozoa get damaged if glycerol is added to semen at higher temperatures. Therefore for freezing semen, the total extender is divided into two equal parts. Semen is diluted with one part and in the other part double the amount of glycerol is added to make the optimum concentration of glycerol at final mixing. Both are cooled to 5°C and the glycerolated diluent is added to the 50% diluted semen in 3 to 4 fractions, at 10 minutes intervals.

Method for the cryopreservation of semen: The ejaculated semen is at the body temperature i.e. 37°C. This is kept at 30°C in a water bath and mixed with three to four parts with the extender at the same temperature i.e. 30°C. The extender is prepared by dissolving the following components in one litre of distilled water.

TRIS	—	24.2 gms
Citric acid monohydrate	—	13.4 gms
Glucose	—	10.0 gms
Penicillin	—	1000 IU/ml
Streptomycin	—	1000 μg/ml
Polymyxin-b	—	500 units/ml
Glycerol	—	70 ml
Egg yolk	—	200 ml

The above mixture is cooled gradually to 5°C, allowing one hour time to come down from 30°C to 5°C. The semen-extender mixture is held at 5°C for several hours (4-6 hours) before freezing, to allow sperm cells to equilibrate with the extender. This mixture is then packed into polyvinyl chloride straws, totally filled upto the mouth to avoid excess air and agitation during shifting, however giving room for expansion and contraction of the fluid during thawing and freezing. Then the straw containing the semen is frozen in nitrogen vapour. The freezing is carried out at the rate of 3°C/mt upto –15°C and then 5°C/mt upto –150°C. These frozen straws are then stored in vacuum sealed liquid N_2 refrigerators at –196°C.

Semen thawing: Thawing is melting the frozen semen and bringing it back to normal body temperature. Whenever semen is required for insemination, it should be slowly melted and brought back to the body temperature. The straws, storing the semen are thawed in ice water and slowly heated to about 37°C, by carefully controlling the time of thawing, so as to avoid killing of the sperms.

IN VITRO FERTILIZATION

Fertilization of an oocyte by a sperm in a test tube, outside the human body is known as *in vitro* fertilization (I.V.F.).

In vitro fertilization is adopted in humans under the following conditions—

- ✓ Absence or blocked fallopian tubes preventing the fertilization.
- ✓ Immunologic infertility, due to antibodies to sperm prevent normal motility and function.
- ✓ Other fertility methods, like artificial or intrauterine insemination were not successful.
- ✓ Unexplained infertility.
- ✓ I.V.F is of practical significance to obtain large numbers of embryos for scientific investigation or for subsequent transfer to infertile women.

Transvaginal oocyte retrieval: The technique for recovery of eggs from the ovary in females uses a sonographically-guided needle to recover oocytes (eggs).

In order to prepare a proper environment in the woman and to increase the chances of recovering several healthy and mature eggs, the woman will undergo about two weeks of intensive preparation. This will include hormonal therapy with 'fertility drugs' which include:—

(1) Leuprolide acetate (Lupron), an injectable drug that blocks secretions of the pituitary gland, thereby optimizing the number of oocytes retrieved.

(2) Human menopausal gonadotropin (Pergonal or hMG) or follicle stimulating hormone (Metrodin or FSH), hormones that stimulate ovarian activity, are injected daily for about 6-10 days prior to the procedure.

(3) Human chorionic gonadotropin (hCG), a hormone that mimics the action of the hormone which naturally induces ovulation, is injected 34 to 36 hours before retrieval and may be used after retrieval to supplement natural progesterone production.

Blood tests and ultrasound scans of the ovaries are used to determine the optimal time to retrieve the eggs from the ovary. This optimal time is just before ovulation when the oocytes are almost ready for fertilization. At the proper time, an outpatient procedure under local anesthesia will allow the female's eggs to be visualized by ultrasound and retrieved from the ovary by placing a needle through the vaginal wall. Progesterone, a natural hormone that enables the uterus to support pregnancy, may be used as a daily injection after egg retrieval. The fluid from the follicles is examined under the microscope and the eggs located are kept in the laboratory under physiological conditions.

The spermatozoa do not attain their full capacity to fertilize until they are transported in the female reproductive tract, during which they undergo further physiologic changes and become capable of fertil-

ization. This is known as capacitation of sperm. For the *in vitro* fertility (i.e. fertility in glass) the spermatozoa from the artificially collected semen of the husband is capacitated by subjecting the semen to high speed centrifugation. This method of sperm capacitation is widely used at present. Sperms can also be capacitated by the addition of the sperms to a media of high ionic strength. Unlike the sperm, the oocyte requires no exposure to the reproductive tract following release from the gonads, in order to be fertile.

Special types of culture dishes are used for I.V.F. into which the sperms and oocytes are taken when they are ready for fertilization. Spermatozoa readily become attached to the surface of zona pellucida and pass through this membrane, effecting the fertilization.

Usually the eggs will develop into cleaving pre-embryos, whose cells divide 2 or 3 times to become preimplantation embryos (pre-embryos). They are maintained in laboratory dishes in a nutrient mixture which acts as a substitute for the environment that would otherwise have been provided by the fallopian tubes. Using a special catheter, the couple's pre-embryos will be passed through the vagina and into the uterus at the time the pre-embryos would normally have reached the uterus (2+ days after retrieval). Serophene, a pill used to promote egg development is given.

In vitro fertilization success rate depends upon the nuclear and cytoplasmic maturation of gametes in the gonads, development of the fertilizability of gametes in the male and female reproductive tracts, an optimal number of fertilizable sperms with vigorous motility and a fertilizable ovum with a first polar body. Further there are other factors involved that limit IVF like a very high cost, lack of awareness among people, religious limitations and psychological sentiments.

REFERENCES

Alan Fersht, Structure and Mechanism in Protein Science : A Guide to Enzyme Catalysis and Protein Folding. W.H. Freeman, 1998. ISBN 0-7167-3268-8

Alberts B, Lewis J, Raff M, Roberts K, Walter P (2002). Molecular biology of the cell (4th ed.). New York: Garland Science. ISBN 0-8153-3218-1.

Allison, Lizabeth A. Fundamental Molecular Biology. Blackwell Publishing. 2007. ISBN 978-1-4051-0379-4

Barciszewski J , Frederic B , Clark C (19 99). RNA biochemistry and biotechnology. Springer. ISBN 0792358627.

Berg JM, Tymoczko JL, Stryer L (2002). Biochemistry (5th ed.). WH Freeman and Company. ISBN 0-7167-4684-0.

Bhagavan NV (2002). Medical Biochemistry. San Diego: Harcourt/Academic Press. ISBN 0-12-095440-0.

Branden C, Tooze J (1999). Introduction to Protein Structure. New York: Garland Pub. ISBN 0-8153-2305-0.

Brooks Cole, Introduction to Clinical Biochemistry, 2nd edition, 2011, Dr. Graham Basten ISBN: 978-87-7681-673-5

Bugg, T im (2004). I ntroduction t o Enzyme a nd Coe nzyme Che mistry. Ca mbridge, M A: Blackwell Publishers. ISBN 1-4051-1452-5.

Butler, John M. (2001). Forensic DNA Typing. Elsevier. ISBN 978-0-12-147951-0.

Campbell, Neil A.; Brad Williamson; Robin J. Heyden (2006). Biology: Exploring Life. Boston, Massachusetts: Pearson Prentice Hall. ISBN 0-13-250882-6.

Chaherjea MN and Rana Shinde, Textbook of Medical Biochemistry, 7th edition , 2007

Cornish-Bowden, Athel (2004). Fundamentals of e nzyme ki netics (3rd e d.). London: Portland Press. ISBN 1-85578-158-1.

Devlin TM (1997). Textbook of Biochemistry: With Clinical Correlations (4th ed.). Chichester: John Wiley & Sons. ISBN 0-471-17053-4.

Dobson CM (2000). "The nature and significance of protein folding". In Pain RH (ed.). Mechanisms of Protein Folding. Oxford, Oxfordshire: Oxford University Press. ISBN 0-19-963789-X.

Doolittle, R.F. (1989) Redundancies in protein sequences. In Predictions of Protein Structure and the Principles of Protein Conformation (Fasman, G.D. ed) Plenum Press, New York.

Jeremy M Berg, John L Tymoczko, and Lubert Stryer, Biochemistry, 5th edition, Johns Hopkins University School of Medicine, New York: WH Freeman; 2002.ISBN-10: 0-7167-3051-0

Lane N (2006). Power, Sex, Suicide: Mitochondria and the Meaning of Life (1st ed.). Oxford University Press, USA. ISBN 0-19-920564-7.

Lane, N., Oxygen: The Molecule that Made the World. (Oxford University Press, USA, 2004), ISBN 0-19-860783-0

Laurence A Kaplan, Amadeo J Pesce, Clinical Chemistry: Theory, Analysis, Correlation.

Lehninger's Principles of Biochemistry, Cox and Nelson

Lippincott's Reviews of Biochemistry, 3rd edition by Champe P C, Harvey R A, Ferrier D R, Lippincott William & Wilkins London, 2005

London: Garland Science. ISBN 0-8153-3218-1.

Martin A. Crook, Clinical Medicine and Metabolic Medicine, 7th edition, 2006.

Matthews, van Holde, and Ahern, Biochemistry.

Meierhenrich, U.J.: Amino acids and the asymmetry of life, Springer-Verlag, Berlin, New York, 2008. ISBN 978-3-540-76885-2

Murray R F, Harper H W, Granner D K, Mayes P A, Rodwell V W (2006). Harper's Illustrated Biochemistry. New York: Lange Medical Books/McGraw-Hill. ISBN 0-07-146197-3.

Rajan SS (2003). Introduction to Bioenergetics (1st ed.). Anmol. ISBN 8-126-11364-2.

Reginald Garrett and Charles Grisham, Biochemistry, 2nd edition.

Rose, S. and Mileusnic, R., The Chemistry of Life. (Penguin Press Science, 1999), ISBN 0-14-027273-9

Russell, Peter (2001). Genetics. New York: Benjamin Cummings. ISBN 0-8053-4553-1.

Saenger, Wolfram (1984). Principles of Nucleic Acid Structure. New York: Springer-Verlag. ISBN 0-387-90762-9.

Schneider ED; Sagan D (2006). Into the Cool: Energy Flow, Thermodynamics and Life (1st ed.). University of Chicago Press. ISBN 0-226-73937-6.

Singh, S.P. (1995). MCQs in Biochemistry. CBS Publishers.

Singh, S.P. (1995). Viva Voce and Short Notes in Biochemistry (2nd ed.). CBS Publishers.

Singh, S.P. (2006). Principles of Biochemistry for Home Science and Nursing. CBS Publishers. ISBN 978-81-239-1322-3.

Singh, S.P. (2008). Viva in Biochemistry (4th ed.). CBS Publishers. ISBN 978-81-239-1582-1.

Singh, S.P. (2009). Concepts of Biochemistry for Physiotherapy and Pharmacy. CBS Publishers. ISBN 978-81-239-1391-9.

Singh, S.P. (2009). Practical Manual of Biochemistry (6th ed.). CBS Publishers. ISBN 978-81-239-1395-7.

Singh, S.P. (2009) . Textbook of Biochemistry (4th ed.). CBS Publishers. ISBN 978-81-239-1414-5.

Singh, S.P. (2009). Textbook of Dental Biochemistry (3rd ed.). CBS Publishers. ISBN 978-81-239-1719-1.

Stevens, Lewis; Price, Nicholas C. (1999). Fundamentals of enzymology: the cell and molecular biology of catalytic proteins. Oxford [Oxfordshire]: Oxford University Press. ISBN 0-19-850229-X.

Thomas M Devlin, Textbook of Biochemistry with Clinical Correlations, 6th edition 2006.

Van Holde KE, Mathews CK (1996). Biochemistry (2nd ed.). Menlo Park, Calif: Benjamin/Cummings Pub. Co. ISBN 0-8053-3931-0.

The following is a very selective list of Internet sources on Biochemistry:

- ://databases.biomedcentral.com
- ://mcb.harvard.edu/BioLinks.html
- ://www.ncbi.nlm.nih.gov/
- ://highwire.stanford.edu
- ://www.nlm.nih.gov/
- ://www.biology.arizona.edu/default.html
- ://mcb.harvard.edu/BioLinks.html
- ://archive.uwcm.ac.uk/
- ://www.genome.gov/
- ://www.nih.gov/
- ://rarediseases.info.nih.gov/index_main.html
- ://www.faseb.org/protein/index.html
- ://www.endocrinology.org
- ://www.geocities.com/bioelectrochemistry/sorensen.htm
- ://molbio.info.nih.gov/cgi-bin/pdb/
- ://sickle.bwh.harvard.edu/
- ://ntri.tamuk.edu/cell/kinetics.html
- ://www.cellularsignaling.org
- ://www.pkunetwork.org/

Another tool for finding biochemistry literature is Google Scholar (://scholar.google.com), a free database on the web which provides access to many full-text articles.

BIOCHEMICAL JOURNALS AND REVIEWS

The following is a partial list of biochemistry journals and review series and of some biomedical journals that contain biochemical articles.

- Annual Reviews of Biochemistry, Cell and Developmental
- Biology, Genetics, Genomics and Human Genetics
- Archives of Biochemistry and Biophysics (Arch Biochem Biophys)
- Biochemical and Biophysical Research Communications (Biochem Biophys Res Commun)
- Biochemical Journal (Biochem J)
- Biochemistry (Biochemistry)

- Biochemistry (Moscow) (Biochemistry Mosc)
- Biochimica et Biophysica Acta (Biochim Biophys Acta)
- Biochimie (Biochimie)
- European Journal of Biochemistry (Eur J Biochem)
- Indian Journal of Biochemistry and Biophysics (Indian J Biochem Biophys)
- Journal of Biochemistry (Tokyo) (J Biochem [Tokyo])
- Journal of Biological Chemistry (J Biol Chem)
- Journal of Clinical Investigation (J Clin Invest)
- Nature (Nature)
- Nature Genetics (Nat Genet)
- Proceedings of the National Academy of Sciences USA (Proc Natl Acad Sci USA)
- Science (Science)
- Trends in Biochemical Sciences (Trends Biochem Sci)

Index

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Z

WORLD PUBLIC LIBRARY EDITION

Contemporary Books and Poetry for the Independent Reader

Dr. Amanullah Mohammed

Born at Hyderabad,India, completed his Masters in Biochemistry from Aligarh Muslim University, Aligarh, was awarded a Doctorate in Biotechnology from Jawaharlal Nehru Technological University, Hyderabad. Extensively experienced in teaching undergraduate and postgraduate medical students. Also involved in research with 15 publications to his credit. E-mail: amanullahmohammed@yahoo.com