

Veterinary Biochemistry & Biotechnology

Mohd. Amanullah

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Preface

Undergraduate Veterinary students assume Biochemistry to be a terror among all the subjects. The fear in this aspect is just because of their sudden exposure to a vast knowledge available in basic sciences, especially in Biochemistry. In order to overcome this nightmare, there was an urgent need to prepare a text book for Veterinary Sciences which put forth a brief description of Biochemistry, just enough to understand the other subjects required to complete the B.V.Sc. & A.H course. Hence this book "*Veterinary Biochemistry & Biotechnology*" is prepared keeping in view the immature minds of the students who just entered a professional course. No doubt that there are a number of books, available in the market embedding very many details of each and every aspect of Biochemistry, but this books gives only the basic information in the form of short notes in a very simple language. Understanding a glossary of terminologies in English itself becomes a great task for the students. Had this course been designed in the individual's mother tongue then, more than half of the course would have been easier. Hence care has been taken to explain various topic in as simple language as possible. This book is prepared as per the syllabus prescribed by the Veterinary Council of India (V.C.I) for the undergraduate B.V.Sc. & A.H course. Biochemistry is prescribed to B.V.Sc & A.H students in both semesters of the first year, first semester of 4th year and a laboratory diagnostic course in the final year of study. Hence this book is divided into five different sections each grouping the topics under various courses. This book will serve as a handy material for the students as well as teachers, taking undergraduate and postgraduate courses in Veterinary sciences, Biological sciences, Genetics, Dental, Ayurvedic, Medical, Microbiology, Molecular Biology, Biotechnology and allied subjects. Students, who are appearing for various competitive exams, will find this book equally helpful.

This book is not an original piece of work. The concepts and ideas framed in this book have been developed by me out of my vast knowledge gained in the 22 years of my teaching and from the available literature in the scientific world. The text book is profusely illustrated with figures and table. A text book cannot be complete without a subject index, which is also included at the end of the book. Mistakes for correction and suggestions for improvement are invited. I acknowledge my wife with thanks for helping me in preparing this book and developing the computer graphics of all the figures included in this book.

Mohammed Amanullah

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SECTION – I
General Veterinary Biochemistry – VBC -111
Ist YEAR/ Ist SEMESTER - FIRST COURSE
<i>This course deals with the basic concepts of biochemistry viz. chemistry of biomolecules and their properties. Hence the student should pay attention to only the introductory aspects of these molecules without going into their details.</i>

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Scope and Importance in Veterinary 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.

What is life?

Ans: Life is an organized state of few elements.

What is Death?

Ans: Death is a state of disorganization of these few 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, 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 which 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 co-operation 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 LDH₁ and LDH₂ 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 Benedicts test. Similarly investigation of other disorders like albuminuria, lactosuria etc. 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 complementarily 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 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.

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Chapter 2

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 from where 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 of these particles viz. electrons, protons and neutrons. On gradual cooling of the 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:

¹ H 1 HYDROGEN	<div>Atomic Number ← ⁶C</div> <div>12 → Atomic Weight</div> <div>CARBON</div>																⁵ B 11 BORON	⁶ C 12 CARBON	⁷ N 14 NITROGEN	⁸ O 16 OXYGEN	⁹ F 19 FLUORINE	
³ Li 7 LITHIUM	⁴ Be 9 BERYLLIUM																	¹³ Al 27 ALUMINUM	¹⁴ Si 28 SILICON	¹⁵ P 31 PHOSPHORUS	¹⁶ S 32 SULFUR	¹⁷ Cl 35 CHLORINE
¹¹ Na 23 SODIUM	¹² Mg 24 MAGNESIUM	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 COBALT	²⁸ Ni 59 NICKEL	²⁹ Cu 64 COPPER	³⁰ Zn 65 ZINC	³¹ Ga 70 GALLIUM	³² Ge 73 GERMANIUM	³³ As 75 ARSENIC	³⁴ Se 79 SELENIUM	³⁵ Br 80 BROMINE						
¹⁹ K 39 POTASSIUM	²⁰ Ca 40 CALCIUM	³⁷ Rb 85 RUBIDIUM	³⁸ Sr 88 STRONTIUM	³⁹ Y 89 YTTRIUM	⁴⁰ Zr 91 ZIRCONIUM	⁴¹ Nb 93 NI OBIUM	⁴² Mo 96 MOLYBDENUM	⁴³ Tc 98 TECHNETIUM	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				
⁵⁵ Cs 133 CESIUM	⁵⁶ Ba 137 BARIUM	⁷³ Hf 178 HAFNIUM	⁷⁴ Ta 181 TANTALUM	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 ASTATINE								
⁸⁷ Fr 223 FRANCIUM	⁸⁸ Ra 226 RADIUM	<div>Lanthanides</div> <div>Actinides</div>																				

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 that - all the atoms except ^2He , ^{10}Ne , ^{18}Ar , ^{36}Kr , ^{54}Xe and ^{86}Rn (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, lose 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 ($\text{C} - \text{C}$).

b) Double bond: If two electrons from each of the sharing atoms are contributed for bond formation then a double bond is formed ($\text{C} = \text{C}$).

c) Triple bond: If three electrons are shared ($\text{C} \equiv \text{C}$).

2. Unequal sharing of electrons or co-ordinate 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 co-ordinate bond wherein the sharing electrons are pulled more towards one of the atoms. For example, in water molecule $\text{H}-\text{O}-\text{H}$, 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) contain 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 hydrogens is known as **"hydrocarbons"** (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).

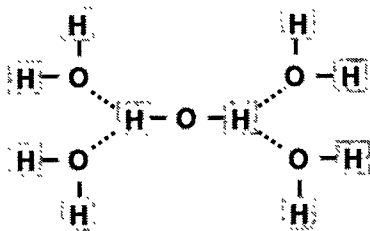
These molecules along with few other elements combine to give rise to carbohydrates, proteins, lipids, nucleic acids etc etc. which in turn contributes to the structure and life of an organism.

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.

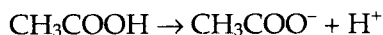
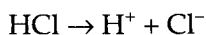
Specific heat of water is one calorie, it is therefore best suited to maintain constant temperature of the body with varying environmental temperature.

The heat of vaporization of 540 cal/gram at 100° 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

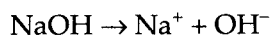
An acid is a proton donor and a base is a proton acceptor (Bronsted-Lowry theory).

Acids:



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

Bases:



There are strong and weak bases similar to that of acids. Ex. 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.

$$\text{pH} = -\text{Log}_{(10)} [\text{H}^+]$$

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 7.0 is neutral and pH range from 0 to 6.9 is acidic and 7.1 to 14 is basic or alkaline. The water molecule dissociates as -



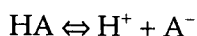
The hydrogen ion concentration in pure water was found to be 0.0000001 moles/litre.

This can also be written as 1×10^{-7} or 1×10^{-7} moles/litre

Taking log of the above number we get = -7

Negative logarithm of the above number = 7

This 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 -



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

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

$$\text{pH} = \text{pK}_a + \text{Log} \frac{[\text{A}^-]}{[\text{HA}]}$$

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 -

- Weak acid and its salt (or its conjugate base).
- Weak base and its salt (or its conjugate acid).
- Weak acid and weak base. Ex. Weak acid CH_3COOH and its base CH_3COONa .

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

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

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

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. Ex. 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 some times 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. Ex. In the erythrocytes, sodium ion is transported out of the cell and potassium ion into the cell.

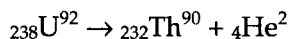
Mechanism of transport: Specific carrier protein (P) contains specific sites for specific substance (S). The 'P' is positioned on one side of the membrane that can take up 'S' to form 'P-S' complex. The 'P' diffuses across the membrane to the other surface or undergoes rotation or conformation change, 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 off 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 (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.



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

2) Beta rays: These are composed of electrons.

3) Gamma rays: Almost similar to Alfa 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 earth's atmosphere by the ozone layer (O_3). Some of the cosmic rays that escape the ozone layer and enter 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.

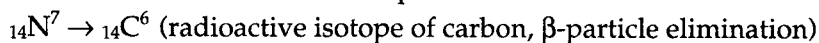
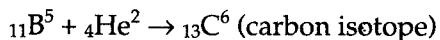
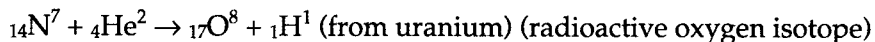
$$\text{U} = 1700 \text{ years and C} = 5570 \text{ Years.}$$

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.



Uses:

- (1) ${}^{131}_{53}\text{I}$ (Normal is ${}^{127}_{53}\text{I}$) 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 ${}^{131}\text{I}$ 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 ${}^{131}\text{I}$ uptake is by scintillation counter.
- (2) Radioactive ${}^{32}_{15}\text{P}$ (normal is ${}^{31}_{15}\text{P}$) 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 (${}^{59}_{26}\text{Fe}$), and vitamin B_{12} ($\text{Co}^{57, 58 \text{ \& } 60}$) are given orally and blood samples are analyzed at regular intervals.
- (4) They are also used to trace the metabolic intermediates. ${}^{14}_6\text{C}$ 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 ${}^{14}_6\text{C}$ is for the detection of genetically defective enzymes.

The use of radioactive substances is not restricted only to 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, reduces cytochromes, causes pyknosis, vacuolization of cells, giant cell formation, mitotic delay, chromosomal breaks and altered permeability is also observed.

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Chapter 3

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 Ex. <i>Borrelia burgdorferi</i> .	Size ranges from tiny yeasts to giant sequoias, dinosaurs etc.

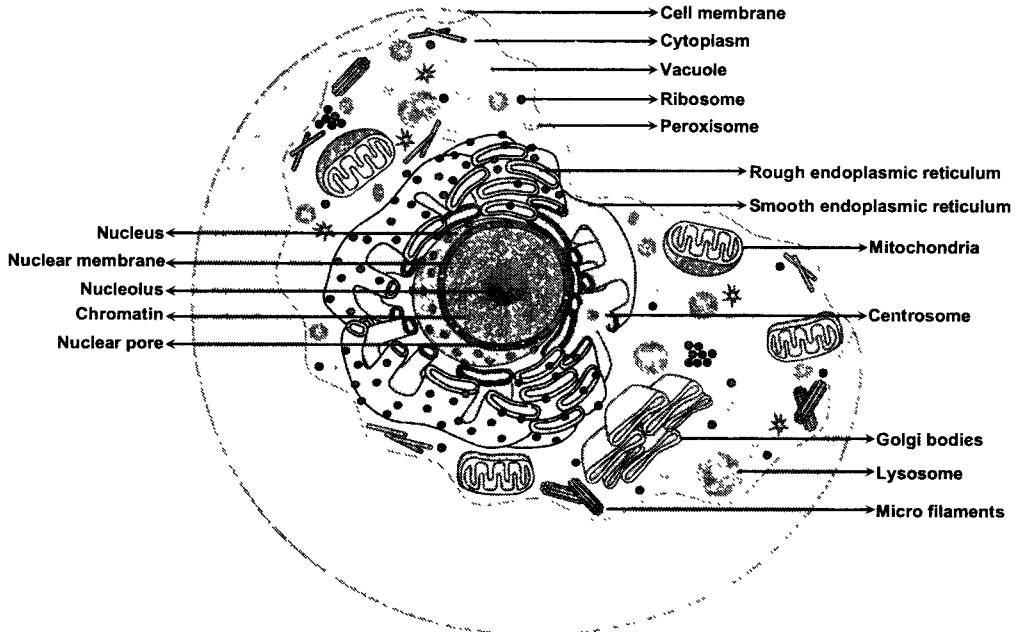
A group of cells forms tissue, various tissues forms an organ and different organs make up the body. The structure and components of a human cell are given below -

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 fiber of the cytoskeletal system, which organize 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 known 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 30 S and 50 S subunits, assemble into 70 S ribosome whereas in eukaryotes ribosomes are made of 40 S and 60 S subunits, assemble into 80 S 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 up 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 center’. 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 center 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 (rough ER). 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 fibers 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 fibers found in the cytoplasm of all eukaryotes. It involves many structures: Cilia, flagella, spindle fibers 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 don't have cell walls, but have ECM i.e. a meshwork of macromolecules outside plasma membrane. 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.
- 3) **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).

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Chapter 4

Carbohydrates

Carbohydrates are the polyhydroxy aldehydes or ketones or their derivatives.

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

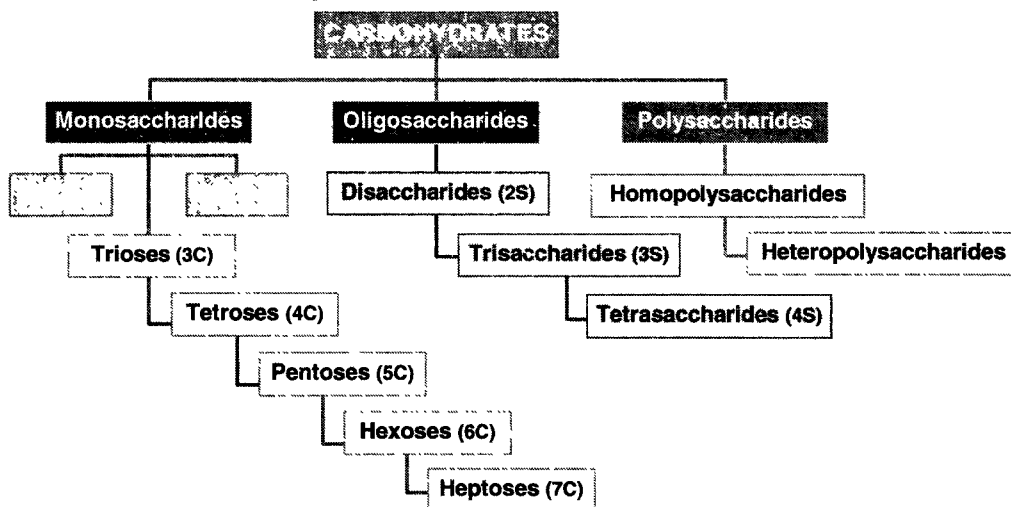
Their general formula is $C_n(H_2O)_n$ [n = 3 (minimum)]

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:

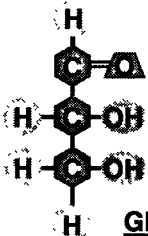
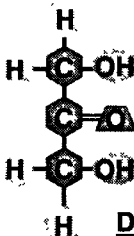
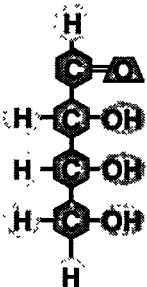
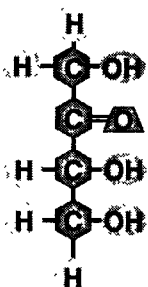
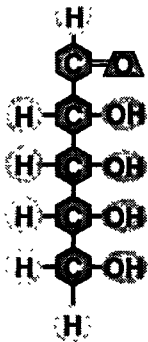
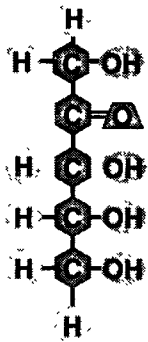


MONOSACCHARIDES

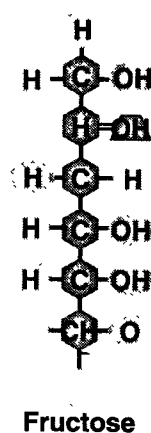
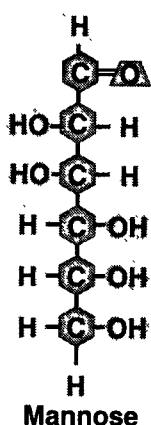
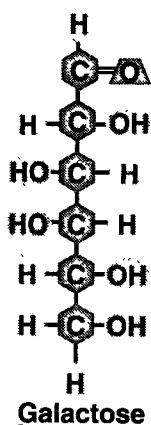
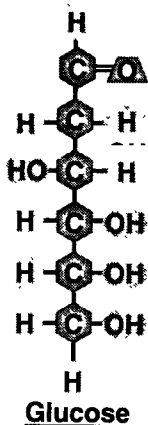
They are the sugar units that cannot be further hydrolyzed into simpler units. There are two major classes of monosaccharides.

1. **Aldoses:** Sugars containing an aldehydic group are known as aldoses. Ex. Glucose, galactose, mannose, ribose and glyceroose.
2. **Ketoses:** Sugars containing a ketonic group are known as ketoses. Ex. Dihydroxyacetone, fructose and seduloheptulose.

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	
 <p><u>Glyceraldehyde</u></p>	 <p><u>Dihydroxyacetone</u></p>
b) Tetroses: Sugars containing four carbon atoms	
 <p><u>Erythrose</u></p>	 <p><u>Threose</u></p>
c) Pentoses: Sugars containing five carbon atoms	
 <p><u>Ribose</u></p>	<div> <p><u>Others</u></p> <p>2-Deoxyribose</p> <p>Arabinose</p> <p>Xylose</p> <p>Lyxose</p> </div>  <p><u>Ribulose</u></p>

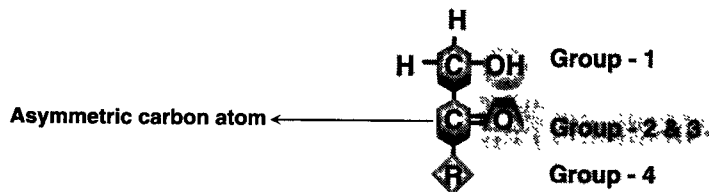
d) Hexoses: Sugars containing six carbon atoms



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

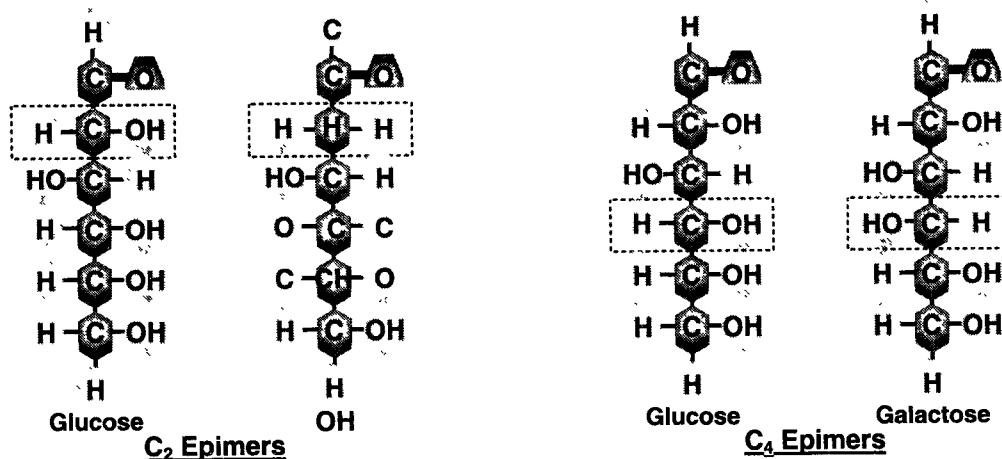
Physical characters of monosaccharides:

- 1) **Asymmetric carbon atom / chiral center:** 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. Ex. Glucose and mannose are epimers at C_2 whereas glucose and galactose are epimers at C_4 .

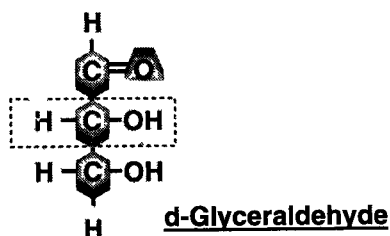
(b)



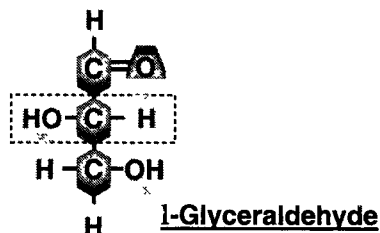
(c) **Enantiomers:** Non super-imposable mirror images are known as enantiomers. Ex. D and L sugars.

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.

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).



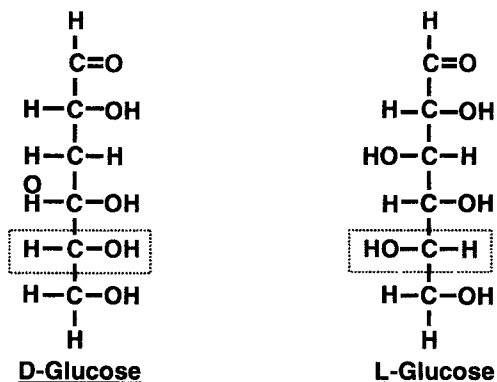
When the solution containing glyceraldehyde with the following configuration around the chiral centre is taken, wherein the -OH group on the asymmetric carbon atom is towards left, when written on paper in the straight line projection form, then the plane polarized light is rotated towards left. Hence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



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 further most 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.

"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.

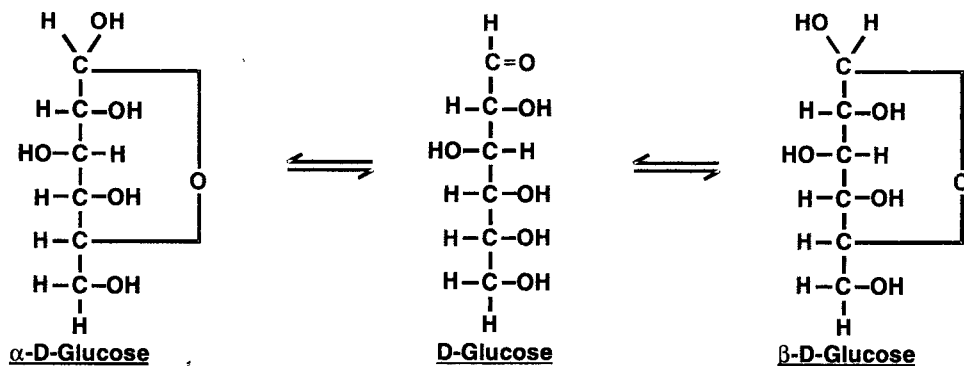


Recimic mixture: A solution containing equal number of d (+) & l (-) forms of a sugar is known as a recimic mixture.

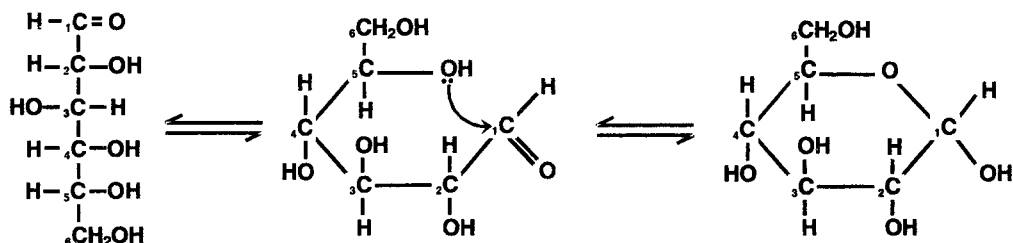
(d) Anomers: Sugars differing at the anomeric carbon atom are known as anomers.

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.

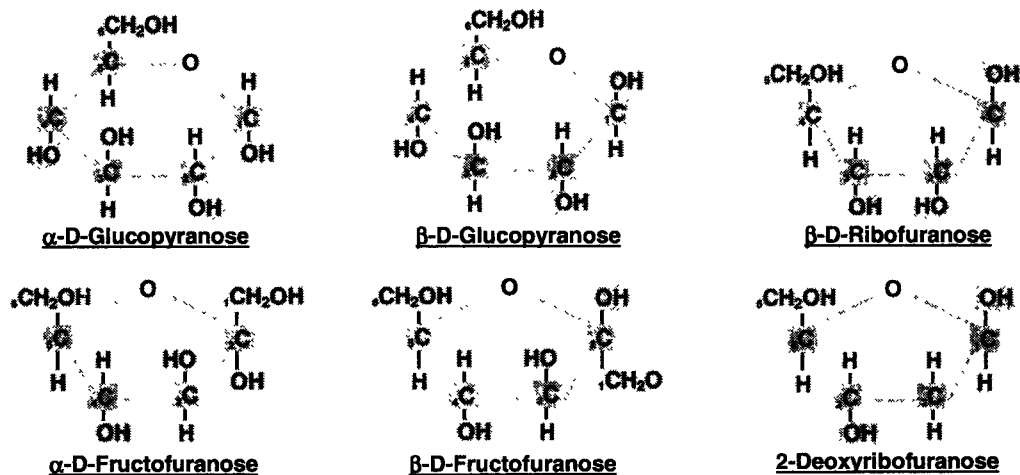


- 3) **Ring structures of carbohydrates:** Aldehydic group on 1st carbon atom of sugars can react with the alcoholic group on 4th carbon atom in pentoses and 4th or 5th carbon atoms in hexoses, forming an hemiacetal (as explained under anomers). This results in the formation of a cyclic ring structure. 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 six 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.



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

Explanation: Glucose crystallized from cold water is α -D-glucose and it shows a specific rotation of $\{\alpha\}_D^{20} = +112.2^\circ$. 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 $1/3^{\text{rd}}$ of α -D-glucose, $2/3^{\text{rds}}$ 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 $\{\alpha\}_D^{20} = +19^\circ$. When this is dissolved in water its rotation gradually changes and finally to 52.7° . This is again due to mutarotation and formation of α , β and straight chain forms of glucose in an equilibrium of $1/3 : 2/3 : 0/1(n)$.

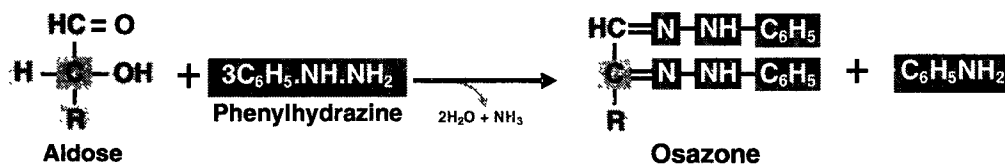
Chemical reactions of carbohydrates:

- 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. Ex. Glucose, fructose, galactose and all other monosaccharides. Among disaccharides maltose and lactose are reducing sugars.
- (b) **Non-reducing sugars:** Sugars that do not have a free aldehydic or ketonic group are called as non-reducing sugars. Ex. 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.

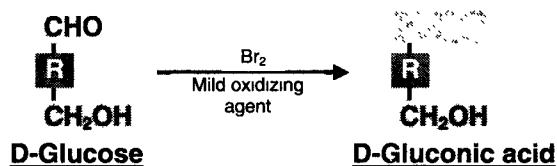
Carbohydrates



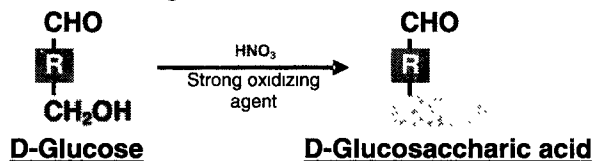
Fructose and glucose forms a broom stick shaped crystal in 3 and 5 minutes respectively. Maltose forms star shaped crystals in 20 minutes whereas lactose forms puff shaped crystals in 30 minutes time.

3) Oxidation of sugars:

(a) **Mild oxidizing agents:** Like bromine oxidizes the aldehydic group of carbohydrates converting it to an acid group.

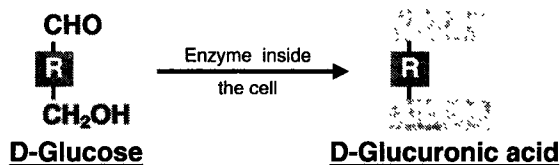


(b) **Strong oxidizing agents:** Like nitric acid oxidizes the primary alcohol of the carbohydrates forming saccharic acids.



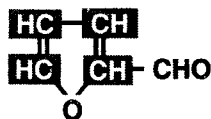
Galactose forms mucic acid, which is insoluble in water. This forms an identification test for galactose known as mucic acid test.

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

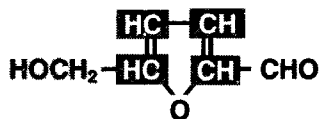


D-glucuronic acid is a component of structural materials like chondroitin sulphate, mucoitin sulphate and glycoproteins (proteoglycons). It plays an important role in detoxification of bile pigments. L-glucose forms iduronic acid.

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



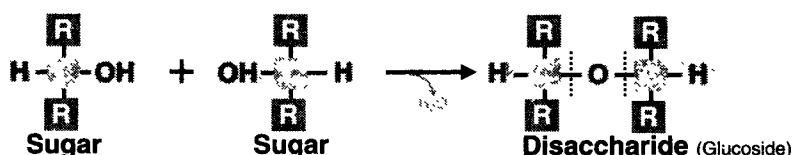
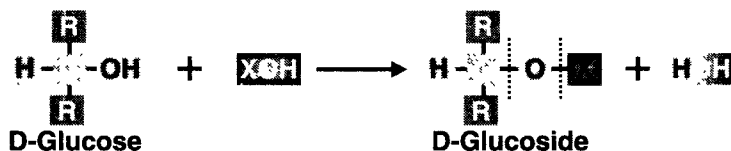
Furfural



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.

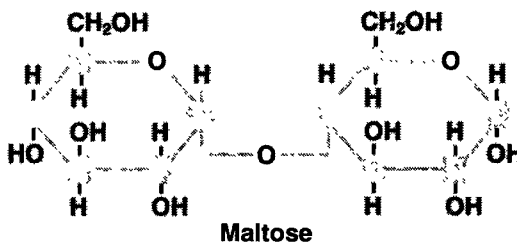
- 5) **Derived sugars:** Substances formed from sugars on oxidation, reduction or addition/replacement of any group is called derived sugars.
- (a) **Amino sugars:** The hydroxyl group at the second carbon of a sugar is replaced by an amino group to form an amino sugar. Ex. Glucosamine, galactosamine.
- (b) **Deoxy sugars:** These sugars are formed due to removal of one of the oxygen from the alcoholic group. Ex. 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 trisacchride and many is a polysaccharide.



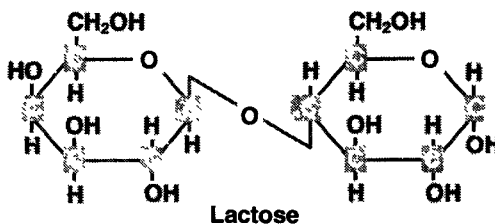
DISACCHARIDES

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

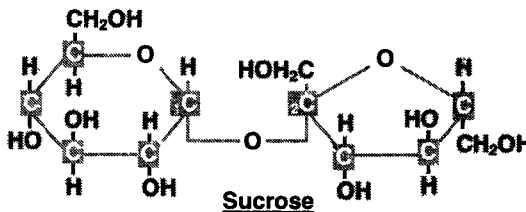
1. **Maltose:** Contains two α -D-glucose units linked by α -1 \rightarrow 4 glycosidic linkage. Chemically it is named as α -D-glucopyranosyl-(α -1 \rightarrow 4)- α -D-glucopyranose. It is also known as malt sugar. It is the product of starch hydrolysis. It is a reducing sugar and forms star shaped osazone crystals.



2. **Lactose:** Made up of β -D-galactopyranose and α -D-glucopyranose linked through β -1 \rightarrow 4 glycosidic linkage. Chemically it is called as β -D-galactopyranosyl-(β -1 \rightarrow 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.



3. **Sucrose:** Contains α -D-glucopyranose and β -D-fructofuranose linked through α -1 \rightarrow 2 glycosidic linkage. Its chemical name is α -D-glucopyranosyl-(α -1 \rightarrow 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.



Invert sugar: Sucrose is known as invert sugar because sucrose is dextrorotatory with a specific rotation of $+62.5^\circ$. 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.

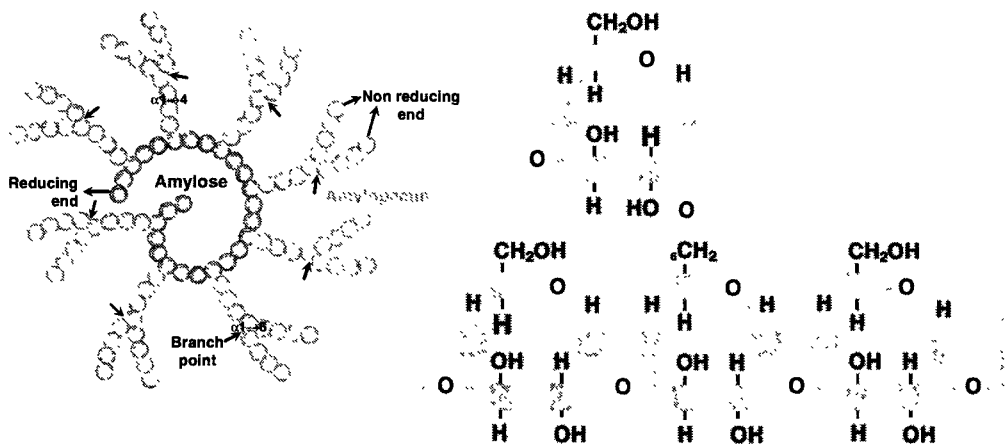
Chocolate companies use invertase enzyme in the preparation of toffees in order to increase the taste and commercial value. In the preparation of these toffees solid sucrose caramel is made and coated with the enzyme-sucrase or invertase over which cocoa is engorged. The enzyme hydrolyses solid sucrose 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. Ex. Starch, glycogen, cellulose, dextran, inulin, agar, chitin etc.

- 1) **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 \rightarrow 4 linkages in the linear chain and α -1 \rightarrow 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.



- 2) **Glycogen:** It is known as animal starch. It is made up of α -D-glucose units linked by α -1 \rightarrow 4 linkages in the linear and α -1 \rightarrow 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.
- 3) **Cellulose:** Made up of β -D-glucose units linked by β -1 \rightarrow 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 \rightarrow 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 sulfated galactose. 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 \rightarrow 4 linkages. Present in the exoskeleton of invertebrates like cockroach and crab.

Heteropolysaccharides: Polysaccharides made up of two or more kinds of monosaccharide units. Ex. Pectins and mucopolysaccharides.

Most of them are branched and exists in conjugation with proteins and hence called proteoglycans. The carbohydrate part is called glycosaminoglycan. They have a repeating disaccharide unit which is acetylated or sulfated. 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 sulfate	Component of cartilage, tendons and skin (connective tissue).

Mucopolysaccharide	Composition	Importance
3. Dermatan sulphate	1. L-Ioduronic acid 2. N-Acetyl-D-galactosamine sulfate	Present in skin.
4. Keratan sulphate	1. D-galactose 2. N-Acetyl-D-glucosamine sulfate	Component of cartilage and cornea.
5. Heparin	1. D- Ioduronic acid (sulfated) 2. N-Acetyl-D-glucosamine (sulfated)	Present in most cells especially in liver, lungs and arterial walls.

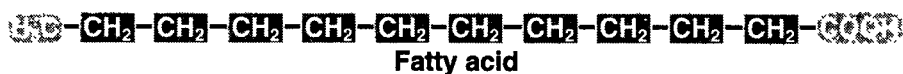
Chapter 5

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.



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

- 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.
Buteric acid	4	Palmitic acid	16
Lauric acid	12	Stearic acid	18
Myristic acid	14	Arachidic acid	20

- 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 -

- 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.

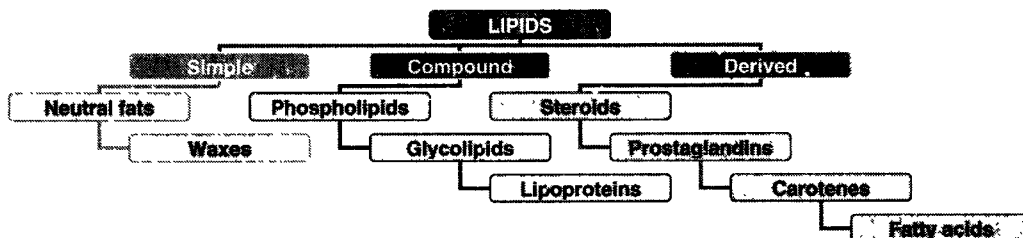
Name of the fatty acid		No. of carbon atoms	No. of double bonds	Position of the double bond
1.	Linoleic acid	18	2	Cis $\Delta^{9, 12}$
2.	Linolenic acid	18	3	Cis $\Delta^{9, 12, 15}$
3.	Arachidonic acid	20	4	Cis $\Delta^{5, 8, 11, 14}$

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

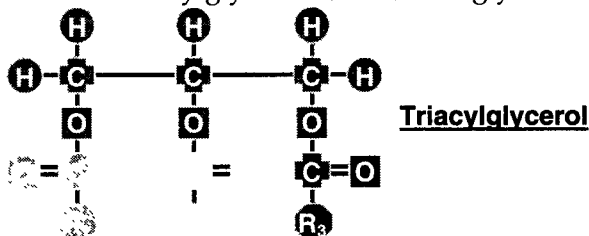
Classification of lipids:



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.



Lipids

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.

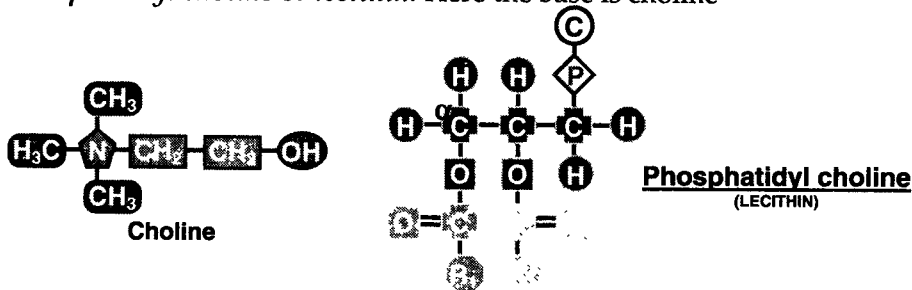
If the ' R ' groups are different then it is spelled out as Palmeto-stearo-olein indicating that glycerol is esterified with palmitic acid, stearic acid and oleic acid.

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

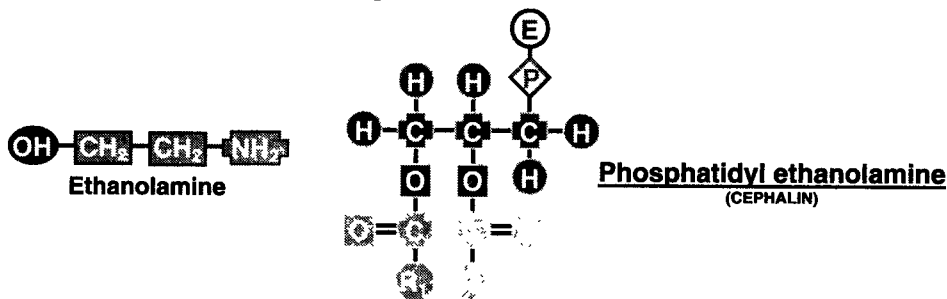
COMPOUND LIPIDS

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 -

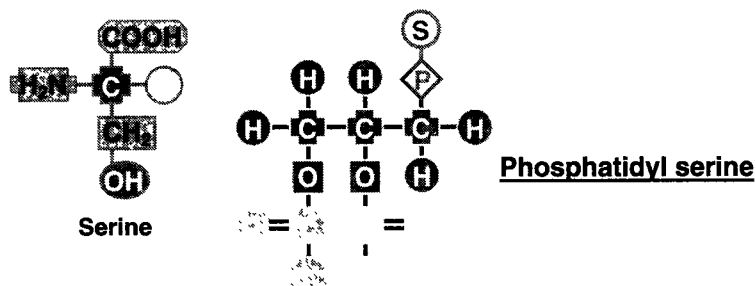
1. **Phospholipids:** They contain a phosphoric acid as the prosthetic group. Depending upon the alcohol present they are further classified as -
 - a) **Glycerophospholipids:** They contain the alcohol-glycerol. The components of glycerophospholipids are glycerol, two fatty acids (the one at α position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



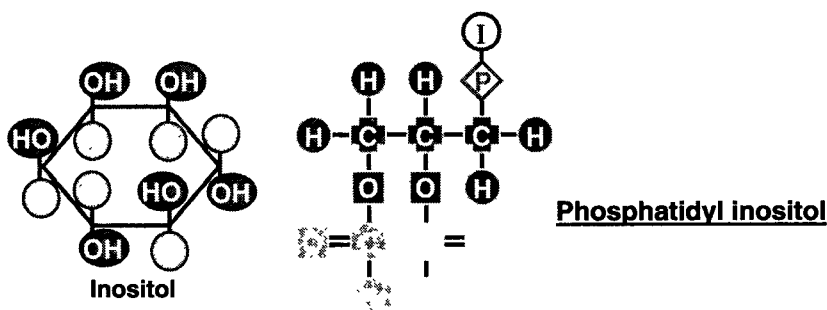
- **Phosphatidyl ethanolamine or cephalin:** Here the base is ethanol amine, attached through - OH group.



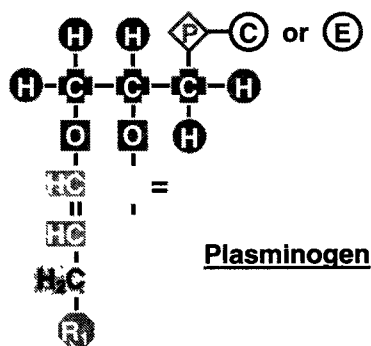
- **Phosphatidyl serine:** Here the base is the amino acid serine.



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

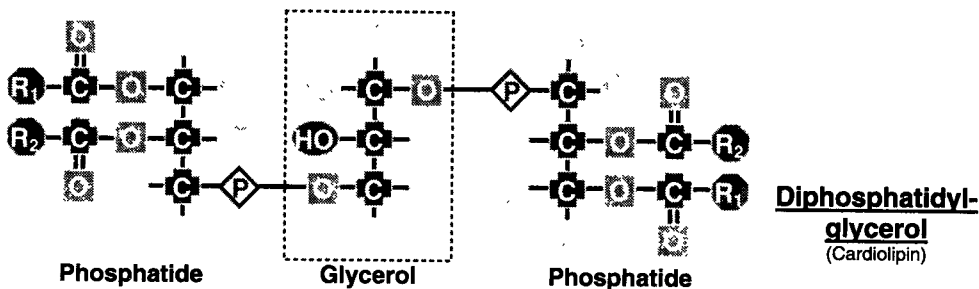


- **Plasminogen:** Here one of the fatty acids of the phosphatide is replaced by a long chain aldehyde which is in an enolic form. The base may be choline or ethanolamine.

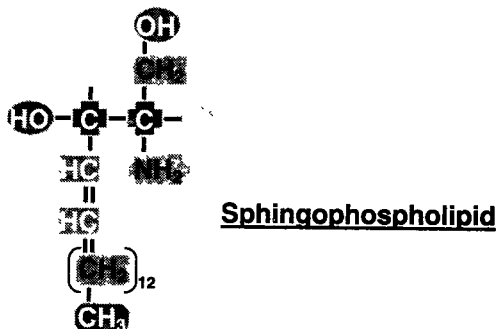


- **Cardiolipin or diphosphatidyl glycerol:** Here two phosphatide groups are linked together through a glycerol.

Lipids



- b) **Spingophospholipids:** 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.



2. **Glycolipids:** These lipids contain a carbohydrate attached to the sphingol at the primary alcohol. They are also known as glycosphingosides or cerebroside.
- 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 galatocerebrosides.
- c) **Gangliosides:** These are complex sphigolipids 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		Triacylglycerol (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	16	22	Transport TAG from liver to adipose tissue
Low Density lipoprotein (LDL)	Apo B	23	10	22	45	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.

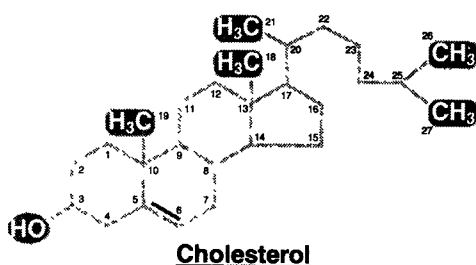
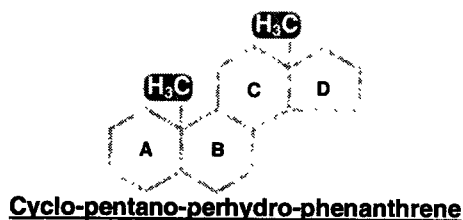
DERIVED LIPIDS

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 fattyacids, steroids, eicosanoids (prostanoids-prostaglandins, prostacyclins, thromboxanes-leukotrienes and lipoxins) carotenoids etc.

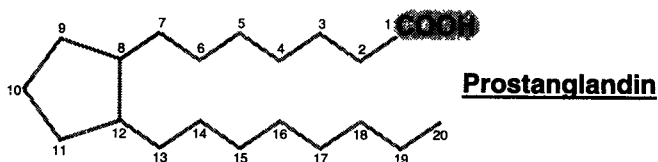
Steroids: All compounds containing the cyclopentanoperhydrophenanthrene ring are called steroids. The most abundant steroids in the human body are the sterols i.e. an alcohol (–OH) group is attached to the steroid nucleus. Ex. Cholesterol, ergosterol, bile acids, sex hormones, adrenal cortical hormones and vitamin D₃.

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.

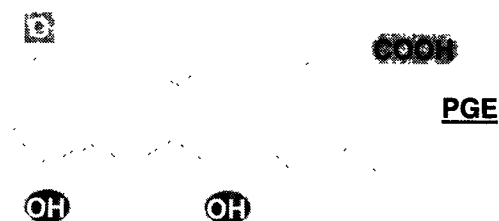
Lipids



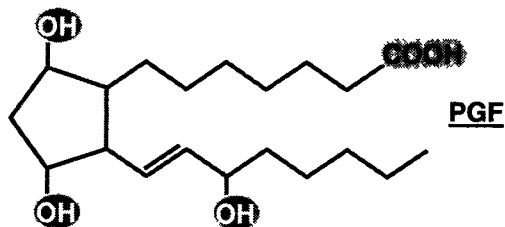
Prostaglandins: They are the derivatives of polyunsaturated fatty acids, mainly the arachidonic acid (C_{20}) or even linoleic acid (C_{18}). They are 20 carbon fatty acids with a 5 membered ring.



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 .



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 sub types there are two more sub-sub types in each of the prostaglandins i.e. α and β .



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 -

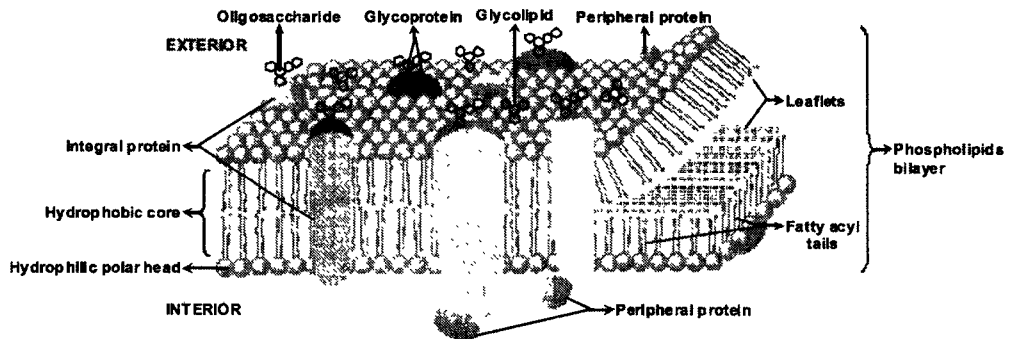
- (1) They act as vasopressors and hence lower the blood pressure.
- (2) They are used to prevent inflammation.
- (3) They promote platelet aggregation (after conversion to thromboxanes).
- (4) PGE₂ acts as a messenger between hormone receptor and adenylate cyclase enzyme.

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. 20 to 80 percent 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.

Lipids



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.

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Chapter 6

Proteins

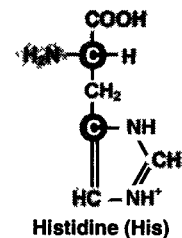
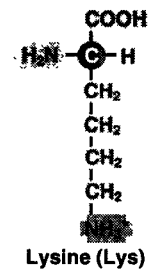
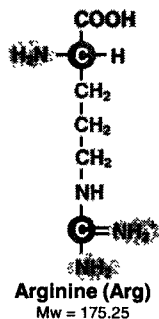
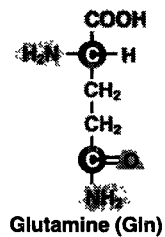
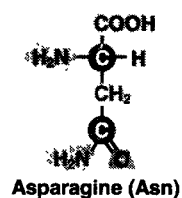
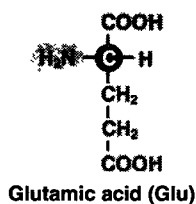
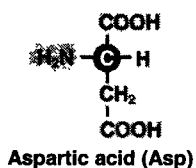
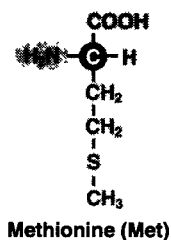
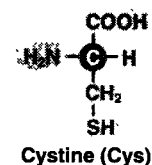
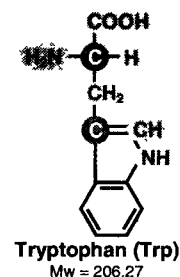
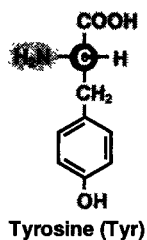
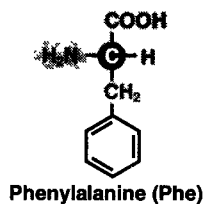
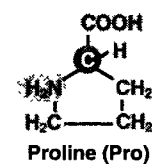
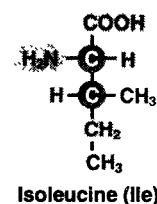
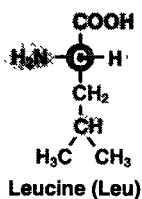
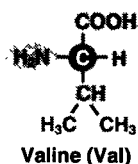
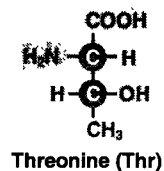
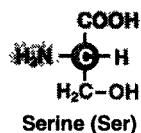
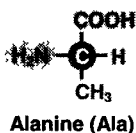
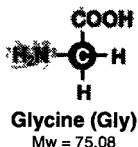
Proteins are nitrogenous organic compounds of high molecular weight which play a vital or prime role in living organisms. They are made up of 20 standard α -amino acids. The main functions of proteins in human body are -

- 1) They serve as body building units. Ex. Muscle proteins.
- 2) They provide support and protection to various tissues. Ex. Collagen and keratin.
- 3) All chemical reactions in the body are catalyzed by proteinaceous enzymes. Ex. Trypsin.
- 4) They transport various molecules and ions from one organ to the other. Ex. Haemoglobin, serum albumin.
- 5) They store and provide nutrients. Milk casein, ovalbumin.
- 6) They defend the body from harmful foreign organisms. Ex. Immunoglobulins, fibrinogen.
- 7) They help to regulate cellular or physiological activity. Ex. 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. $20 \times 19 \times 18 \times 17 \times 16 \times \dots \times 2 \times 1 = 2.4 \times 10^{18}$. 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 heterocyclic 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:** Are those amino acids that contain a positive charge or a basic group – Arg, Lys and 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.

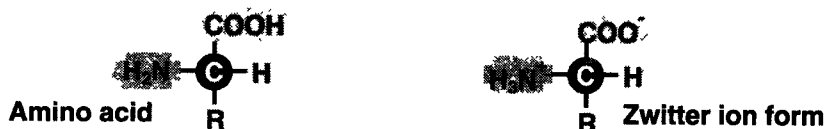
M	–	Methionine		V	–	Valine		P	–	Phenylalanine
A	–	Arginine		I	–	Isoleucine		H	–	Histidine
T	–	Threonine		L	–	Leucine		Ly	–	Lysine
T	–	Tryptophan								

- 2) **Non essential amino acids:** 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.

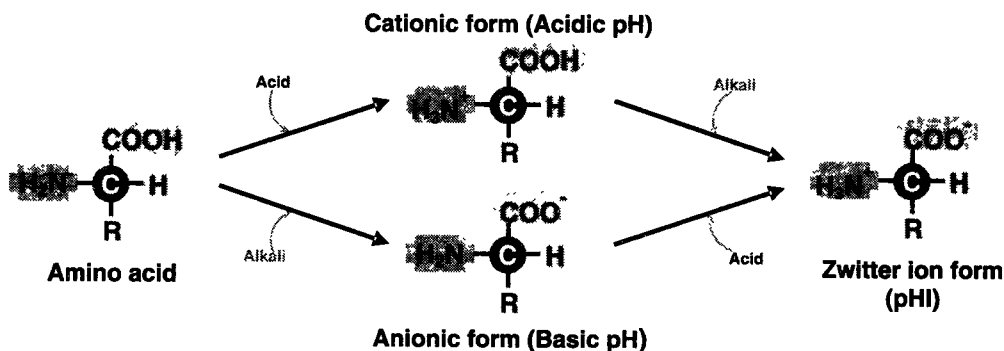
REACTIONS OF AMINO ACIDS

Physical characters of amino acids:

- 1) **Zwitter ions:** Amino acids have an acidic group ($-\text{COOH}$ group) i.e. a proton donor. They also have a basic group ($-\text{NH}_2$ 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.



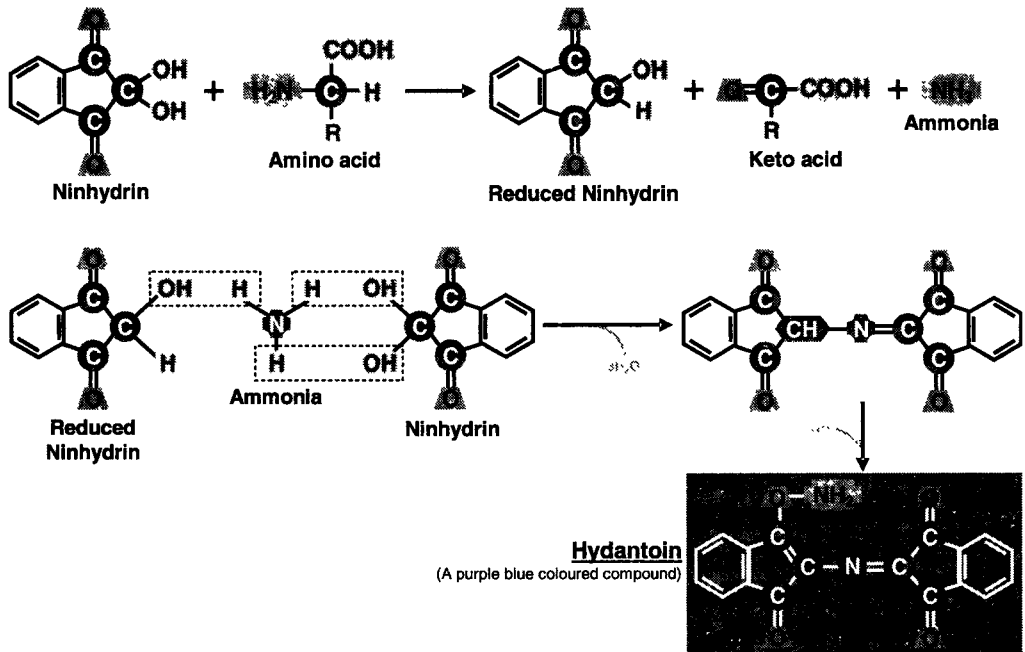
- 2) **Isoelectric pH (pH^I):** The pH at which the positive charge on the amino acid (or any other molecule) is equal to the negative charges, is known as isoelectric pH. At this pH the net charge will be zero and hence it does not move either to positive (anode) or to negative (cathode) electrode,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



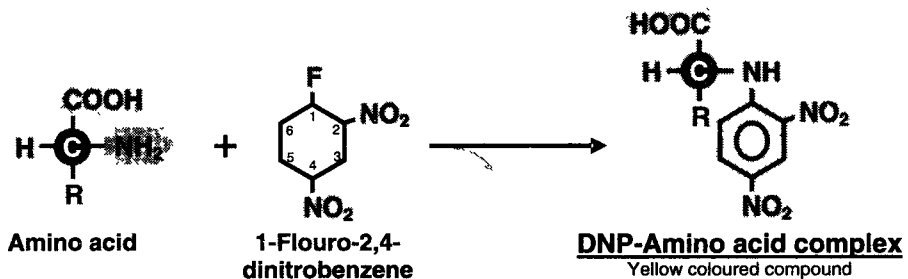
Chemical properties:

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.

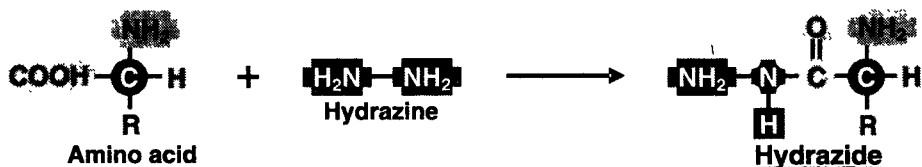


- (b) **Reaction with Sanger's reagent:** Amino acids react with Sanger's reagent i.e. 1-fluoro-2,4-dinitrobenzene, forming a yellow coloured complex. This reagent is used to detect the N-terminal amino acid in the proteins.

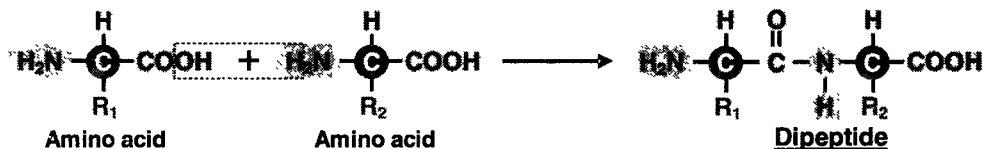


2) Reactions due to carboxylic group:

- (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.



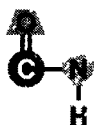
- 3) **Reaction due to both amino and carboxylic group:** Due to the presence of both amino (basic) and carboxylic (acid) groups in amino acids, the amino group of one amino acid reacts with the carboxylic group of another amino acid to form a peptide bond.



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.

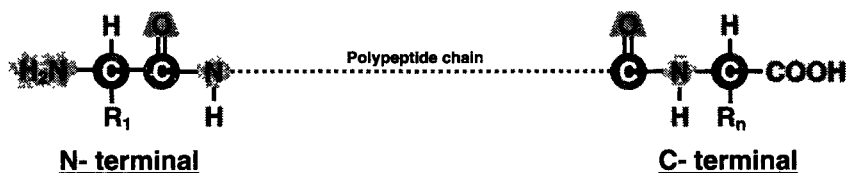


Peptide group

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.

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 is 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.

N-Terminal **Met** **Arg** **Leu** **Ala** **Val** **Phe** C-Terminal

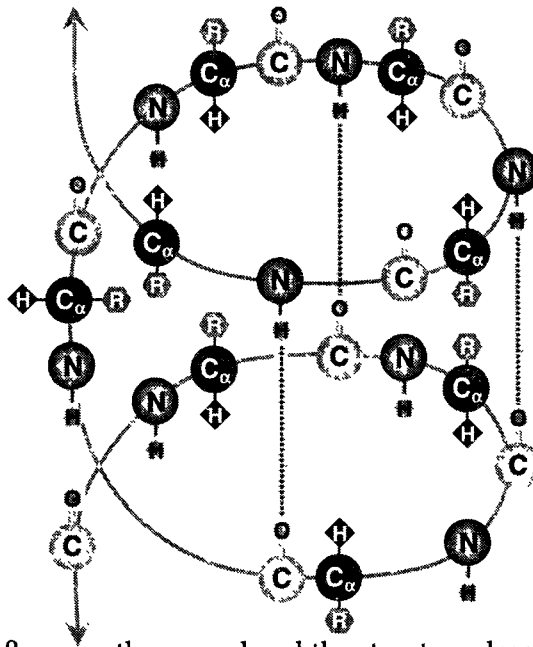
A fixed number of amino acids are arranged in a particular sequence. The sequence of amino acids in the protein determines its biological role. Different proteins have different sequences. Therefore the study of total number and sequence of amino acids in a protein is the study of its primary structure. 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 have 141 amino acids and β -chain has 146 amino acids arranged in a specific sequence. Any change in the sequence results in an abnormal hemoglobin. 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 -

- 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 \AA .
- 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 $-\text{NH}_2$ group combines with oxygen of $-\text{CO}$ 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.

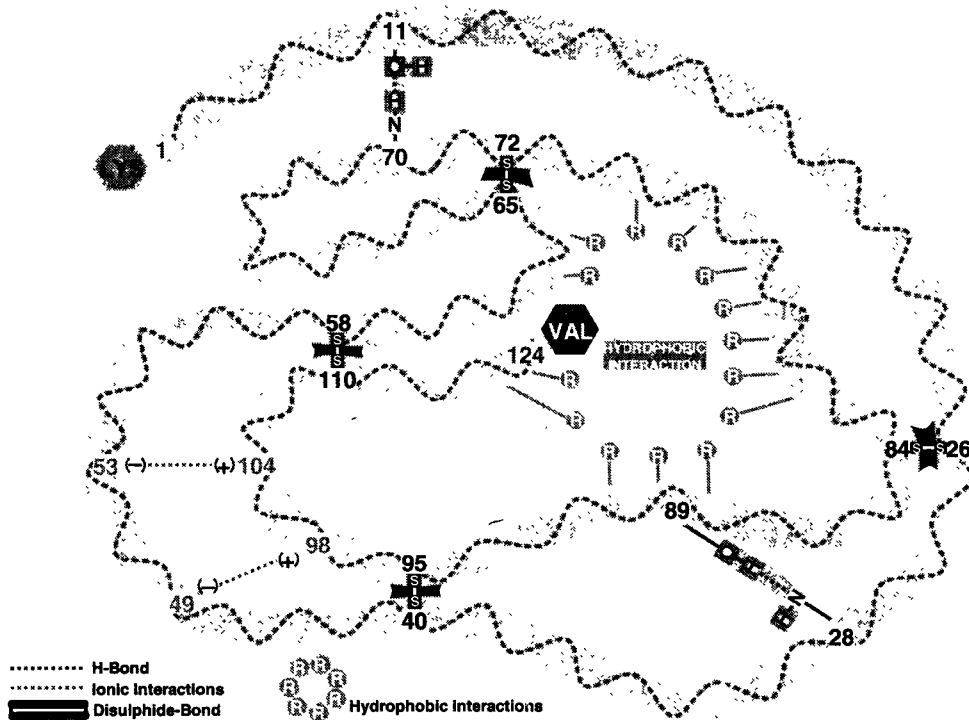


2. **Beta pleated:** β means the second and the structure described below was the second discovery after α helix. The salient features of this structure are -
 - ✧ Here the chain is not helical but zig-zag.
 - ✧ The distance between each turn is 7 \AA .
 - ✧ Polypeptide chains are arranged side by side in the form of pleats.
 - ✧ There is inter-chain hydrogen bonding between the chains (All peptide groups have it).
 - ✧ 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. Ex. 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.

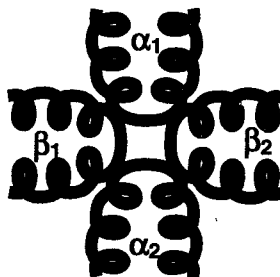


QUATERNARY STRUCTURE OF PROTEIN

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-Waal's forces. Ex. 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.



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 (pHI):** 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 pHI and get precipitated. The pHI 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 (Ex. Collagen) and/or protective function (Ex. Hair keratin and fibrin).
 - 2) **Globular proteins:** They are soluble in water. They include the functional proteins. Ex. 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. Ex. 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. Ex. 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. Ex. Glutelin of wheat.
 - (d) **Prolamines:** Water insoluble but soluble in 70% alcohol. Ex. Glaidin 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. Ex. Globin of haemoglobin.

- (h) **Albuminoids or scleroproteins:** Insoluble in all neutral solvents, dilute acids or alkalies. Ex. 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 prosthetic group does not form any chemical bond with the protein instead are present in conjugation with them, hence 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. Ex. Chromatin (histones + nucleic acids).
- (c) **Glycoproteins:** When a small amount of carbohydrate is attached to a protein it is known as glycoproteins. Ex. 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. Ex. LDL, HDL.
- (f) **Metalloproteins:** They contain metal ion in addition to the amino acids. Ex. Haemoglobin (iron), seruloplasmin (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

Chapter 7

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.

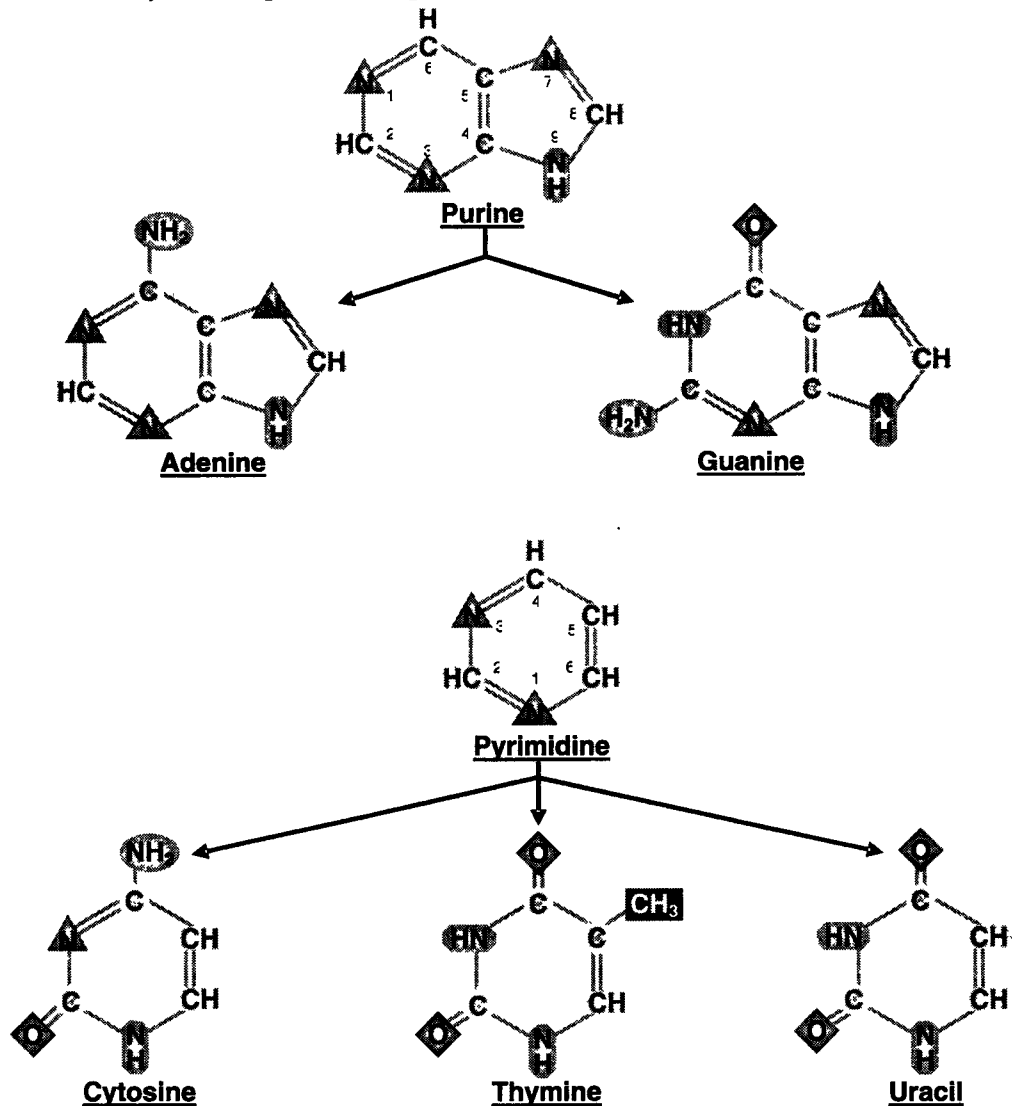
Knowledge of the structural organization of nucleic acids enables the understanding of genetic combinations, gene regulation, genetic improvement of species, genetic diseases, their diagnosis and treatment, modern biotechnological aspects like polymerase chain reaction, genetic engineering, DNA fingerprinting 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

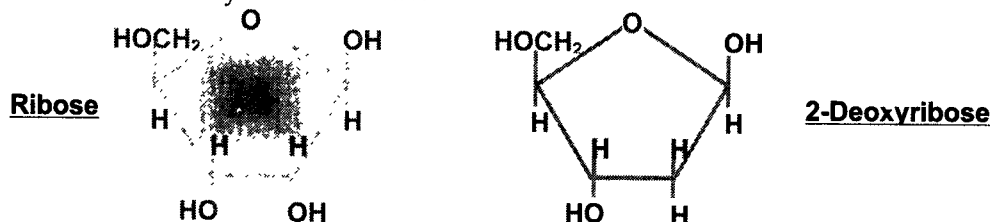
A nucleoside is composed of two components - a) nitrogenous base and b) five carbon sugar (pentose).

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



Nucleic Acids

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

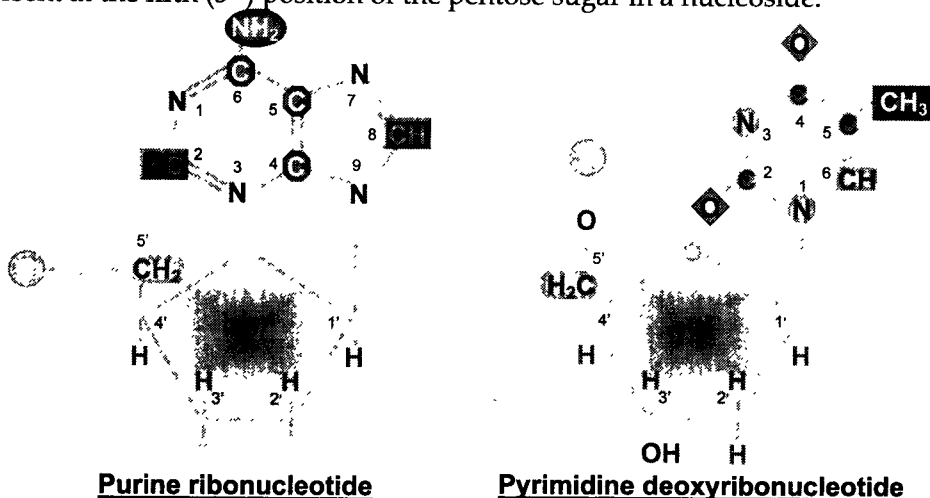


A nucleoside is formed by the linkage of the nitrogenous base to the -OH group at the first carbon of the pentose sugar through an N-glycosyl linkage. The binding of the two nitrogenous bases to the -OH group at the first position in ribose sugar differs. The purine bases are linked through the nitrogen 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	Pyrimidines
Adenine + Pentose = Adenosine	Cytosine + Pentose = Cytidine
Guanine + Pentose = Guanosine	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.



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)

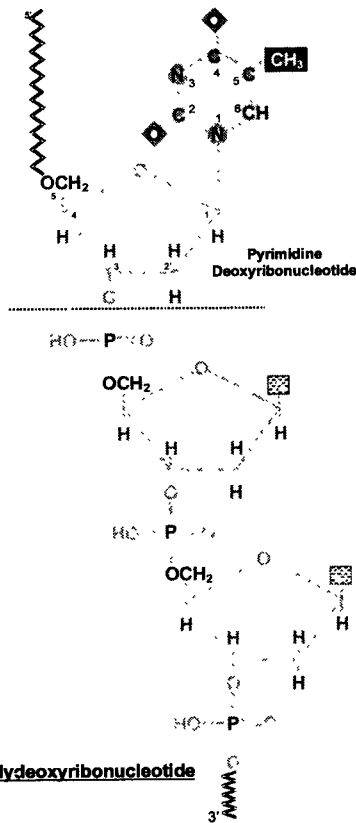
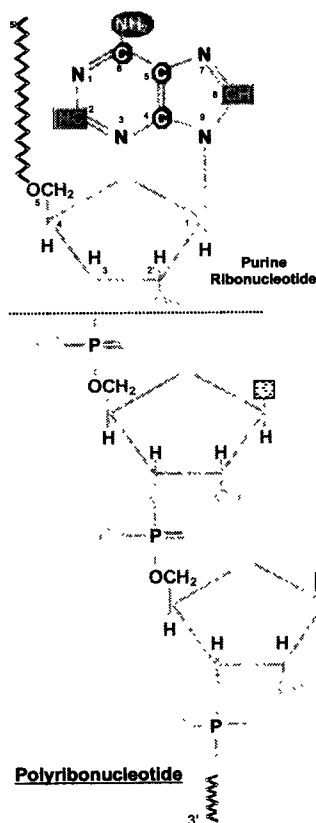
Pyrimidines: Cytosine + Ribose + Phosphoric acid → Cytidylate (present in RNA)

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 -



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.

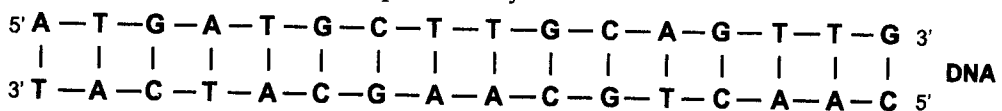
DEOXYRIBONUCLEIC ACID (DNA)

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 -

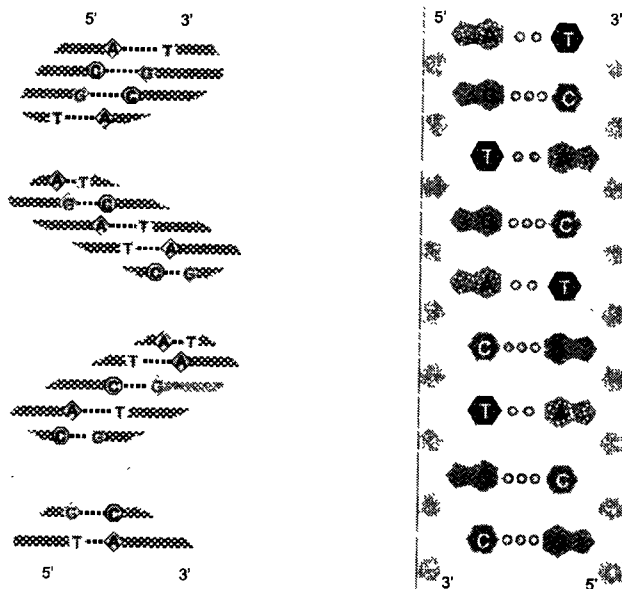
- 1) DNA is made of two helical chains coiled around the same axis, to form a **"right-handed double"** helix.
- 2) The two chains in the helix are **"anti-parallel"** to each other i.e. the 5'-end of one polynucleotide chain and the 3'-end of the other polynucleotide chain is on the same side and close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



Double helical structure of DNA

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.

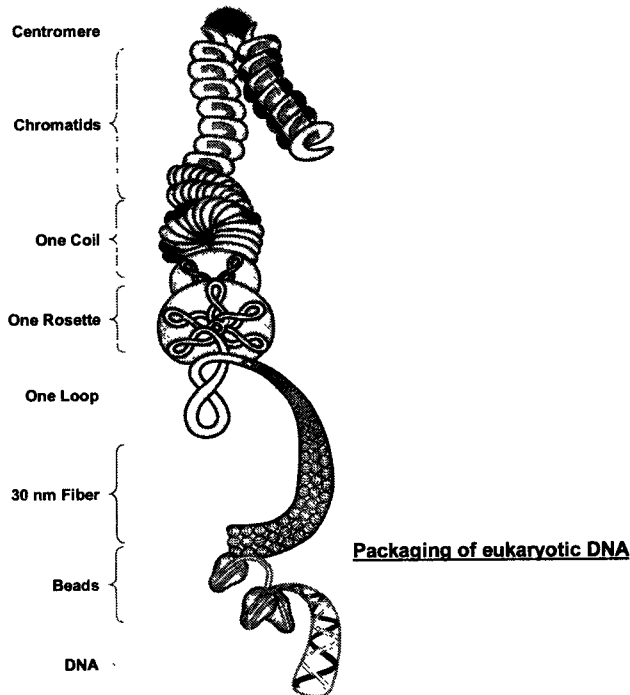
- DNA stores the complete genetic information required to specify (form) the structure of all the proteins and RNA's of each organism.
- DNA is the source of information for the synthesis of all cellular I body proteins. Some of the proteins are structural proteins and some are enzymes. These enzymes 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 inturn 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 cell 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 ϕ X174 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 meters 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 differs 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, it's 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 DNA's respectively in nanometers is T2-210/65520; λ phage-190/17460; T₄-78/14376 and ϕ X174 (in duplex form) - 25/1938. In order to get themselves packed within the particle, most of these viral DNA's are linked covalently by the ends and therefore form an endless belt and thus become circular. Some of the single stranded DNA (ϕ X174) becomes double stranded and circular.

- b) **Packaging of bacterial DNA:** The length of *E.coli* cell is 2 micrometers 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 ($M_w = 10,000$). 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 net-work 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.

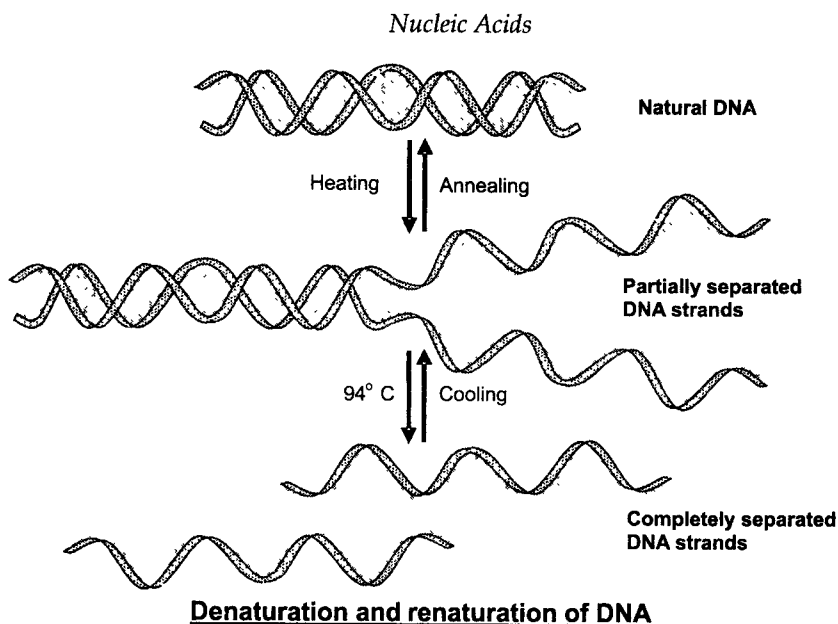


The 2 meter long eukaryotic human cell DNA is to be packed in the cell of about 5-10 micrometer in diameter. In order to facilitate its package, the helical DNA molecule is bound, tightly around beads of basic proteins called "**histones**", which are spaced at regular intervals. The complexes of histones and DNA are called nucleosomes. Each nucleosome contains eight histone molecules, two copies each of H₂A, H₂B, H₃ and H₄ winding 146 base pairs of DNA. Between the two nucleosomes there is a spacer DNA of 54 base pairs with a single histone molecule (H₁).

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 fiber. This provides 100-fold compaction of DNA. The 30 nm fiber then forms plectonemic pleats called loops. Six such loops are bounded by scaffold attachment to proteins (histone-H₁ / 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.

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 (Melting temperature). 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.
- b) **Buoyant density:** When DNA is centrifuged at high speeds in a concentrated solution of cesium chloride - (CsCL), 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 CsCL at that point. Different DNA will have different densities, which again depends upon the G≡C content of that DNA. Higher the G≡C content, higher is the buoyant density of that DNA and vice-versa.



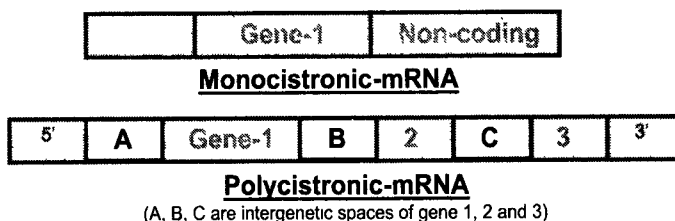
Measurement of these two characters viz. melting temperature and buoyant density will enable us to calculate the proportions of G≡C and A=T pairs in that DNA, which indirectly helps in deducing the gene sequence.

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.

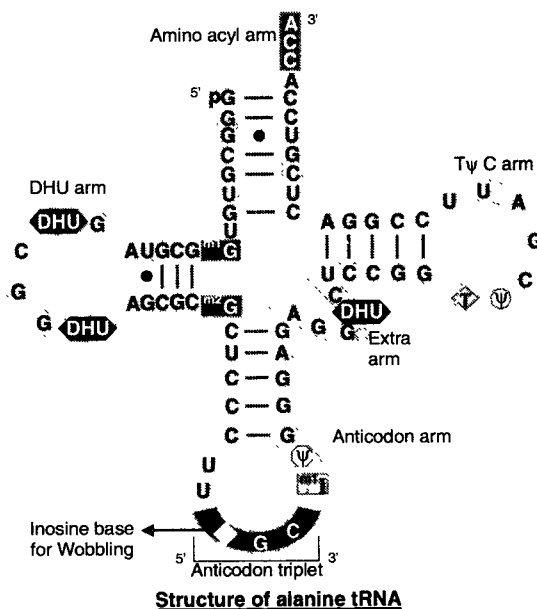
1. **Messenger RNA (mRNA):** It carries the genetic message from the chromosomes (DNA) to the ribosome (site of protein synthesis). This RNA is formed in the nucleus (or nuclear zone in prokaryotes) containing

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.



2. Transfer RNA (tRNA): It is

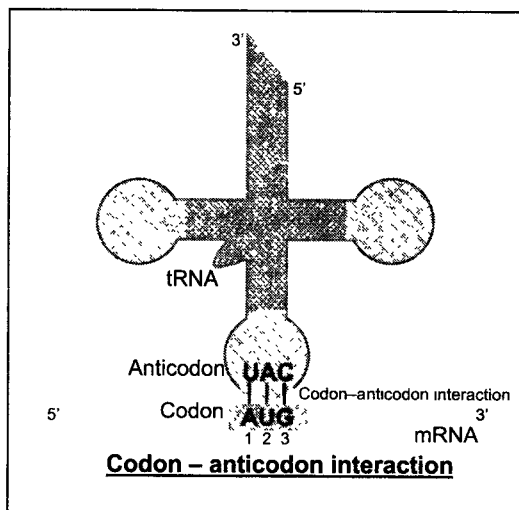
an adapter molecule that reads the information encoded in the mRNA and accordingly transfers the appropriate amino acid to the growing polypeptide chain during protein synthesis. There is at least one kind of tRNA for each amino acid. Some amino acids have two or more specific tRNA's. At least 32 tRNA's are required to recognize all the amino acid codons ($64-3=61$). Most cells have about 40-50 distinct tRNA's. Some tRNA's can recognize more than one



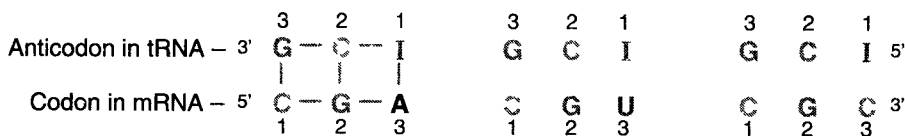
Structure of alanine tRNA

codon (due to wobbling). tRNA's 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).

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 RNA's 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.



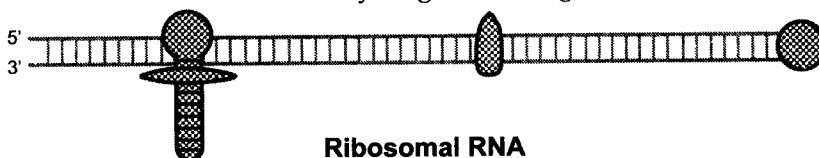
There are totally 64 codons formed by multiple combinations of any three nucleotide bases out of the four nucleotides forming the nucleic acids. At least one codon specifies one amino acid and some amino acid have more than one codon, but the number of different tRNA's for each amino acid is not the same as the number of its codons. This is because some tRNA's contain the first anticodon nucleotide inosinate (I), which can base pair by hydrogen bonds with three nucleotides viz., U, C & A. Therefore all those amino acid codons which differ at the third nucleotide will have the same tRNA. This is known as "**wobbling**". Ex. 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 RNA's are designated depending upon their sedimentation coefficients. The rRNA's 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.



Difference 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, & 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.

SECTION - II
Physiological Chemistry - VBC -121
Ist YEAR / IIInd SEMESTER - FIRST COURSE
<i>This course deals with the biochemical reaction mechanisms, requirements of vitamins and minerals in the diet, energy expenditure, blood and other body fluids and tissue biochemistry. However, as a Veterinary students has to take up a total of six courses in the first year of B.V.Sc & A.H, the above topics are limited and are not comparable to the syllabus prescribed for medical students.</i>

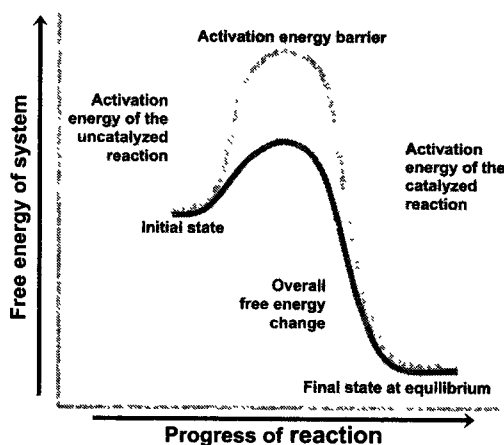
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Chapter 8

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

Enzymes are classified (1) based upon the reaction catalyzed, (2) based upon the presence or absence at a given time, (3) based upon the regulation of action, (4) based upon the place of action and (5) based upon 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 + NH₃ + NADH

- (b) **Transferases:** Enzymes which catalyze transfer of groups from one substrate to another, other than hydrogen. Ex. Transaminase catalyses transfer of amino group from amino acid to a keto acid to form a new keto acid and a new amino acid.

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 catalyses 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-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 + CO₂ + ATP — PYRUVATE CARBOXYLASE → Oxaloacetate + ADP + Pi

2. **Classification based upon the presence or absence at a given time:** Two types are identified -

Enzymes

- a) **Inducible enzymes:** Are 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 TCA cycle.

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 "**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 acts 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 acts 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 considerable high amounts and are functional in the plasma due to the presence of their substrate in the plasma. Ex. Serum lipase, blood clotting enzymes.

- b) **Non-functional plasma enzymes:** Enzymes present in the plasma in negligible amounts and has no function in the plasma due to the absence of their substrate in the plasma. 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

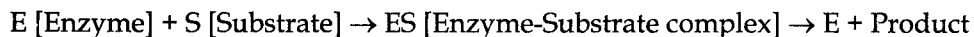
4th digit refers to that particular enzyme

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

MECHANISM OF ENZYME ACTION

Enzyme (or proteins) 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.



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 result 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

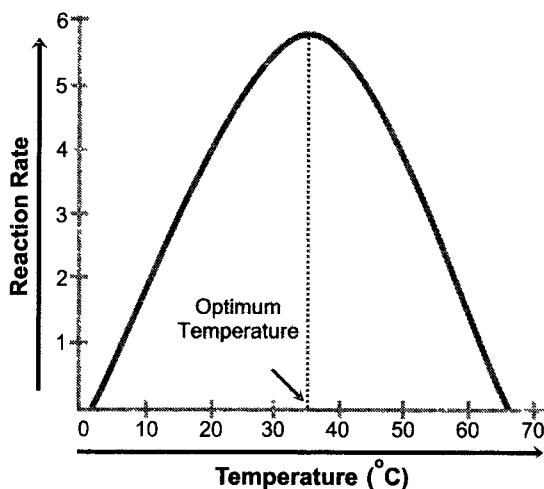
The factors influencing the rate of the reaction are -Temperature

- 1) pH
- 2) Substrate concentration
- 3) Enzyme concentration
- 4) Concentration of products
- 5) Light
- 6) 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 in 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.

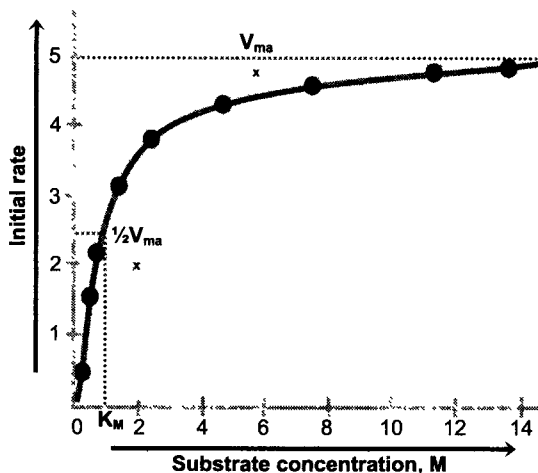
Most of the enzymes are totally inactive at 0°C to 4°C , their activity starts at 10°C and slowly increases and reaches its maximum capacity at its optimum temperature. Majority of the enzymes in the human body have their optimum temperatures between 37°C and 40°C . Beyond this temperature the enzymes become less active and may lose their activity completely at higher temperatures. In fever, increase 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 temperature at extremes of temperatures. Thus refrigeration bacteria exists with the optimum temperature of their 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 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 decrease 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,

$$V = \frac{1}{2}V_{max}$$

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

Dividing by V_{max} we get

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

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

$$\text{or } K_m = [2S - S]$$

$$K_m = [S]$$

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.
- 4. **Effect of enzyme concentration:** As the enzyme concentration increases, the rate of reaction increases steadily in presence of an excess amount of substrate and other factors are kept constant. A linear curve is produced.
- 5. **Effect of products:** When the product is more in the reaction mixture, than the rate of reaction decreases due to feedback inhibition.
- 6. **Effect of light:** Various enzymes act with speed 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 acts 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 to 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 acid to a keto acid and decarboxylase that removes carbon dioxide.
2. **Substrate specificity:** These enzymes are specific about the substrate upon which they act. This is further classified as under:
 - 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 to basic amino acids. Hence cleaves peptide bonds contributed by lysine and arginine.
 - *Aminopeptidase:* Acts on peptide bond near the free amino end.
 - *Carboxypeptidase:* Specific to free carboxylic group.
 - *Amylase:* Specific to α -1 \rightarrow 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 co-enzymes. Ex. Biotin, pyridoxine etc. Enzyme along with a co-enzyme is known as "**holoenzyme**" and that without a co-enzyme is an "**apoenzyme**".

Enzymes

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 enzyme 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 co-enzyme 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 to 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).

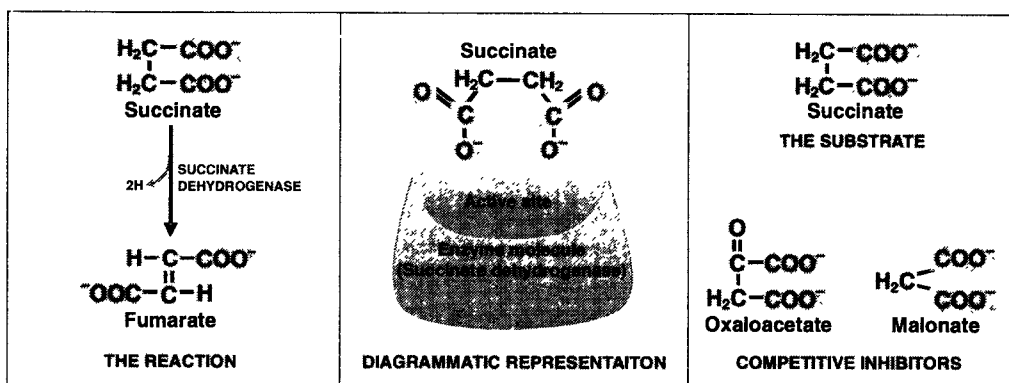
Zymogen: The inactive form of an enzyme is called as zymogen or proenzyme. Pepsinogen and trypsinogen are the zymogens of pepsin and trypsin respectively.

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

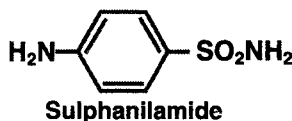
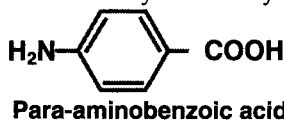
Alteration in the enzyme activity by specific substances other than non-specific substances like pH, temperature etc. is called as enzyme inhibition. There are two types of enzyme inhibitions - (a) irreversible enzyme inhibition and (b) reversible enzyme inhibition.

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-isopropylfluorophosphate (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 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 substrate is succinic acid and the competitive inhibitors are oxalic acid, malonic acid and glutaric acid. Among these, malonic 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 micro-organisms 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 micro-organisms. 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 is 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 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

Anti enzymes: These are substances (generally proteinaceous in nature) that inhibit most of the digestive enzymes. Ex. Certain roundworms and hookworm 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.

Biological Oxidation

This chapter gives the answer to the question as to “How do the food we take in and the oxygen we respire, produces 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 FADH₂ are produced which are electrons 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 to 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).

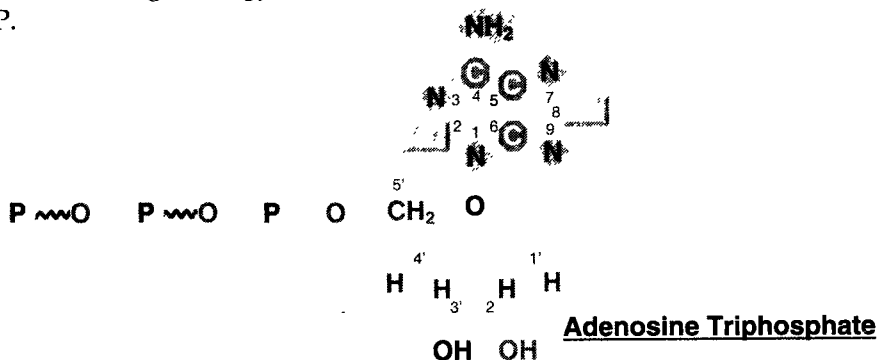
- A compound having a negative value of E_0 is a better electron donor than hydrogen.
- A compound having a positive value of E_0 is a poor electron donor than hydrogen.

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 build into it, which is the energy of the chemical bonds holding the atoms together. This is the free energy.

High energy bond and energy rich compounds (-): 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 yield only 3 Kcal/mol of energy, hence it is not a high energy bond. On hydrolysis ATP is converted to ADP and to AMP.



Biological oxidation: Biological oxidation is catalyzed by enzymes which functions 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', 'c₁', 'c', 'a' and 'a₃'.
- 6) **Hydroperoxidases:** These enzymes have either hydrogen peroxide (H₂O₂) or organic peroxide as their substrate. There are two types of hydroperoxidases - (1) Peroxidase and (2) Catalase. Their prime function is destruction of H₂O₂.

ELECTRON TRANSPORT CHAIN

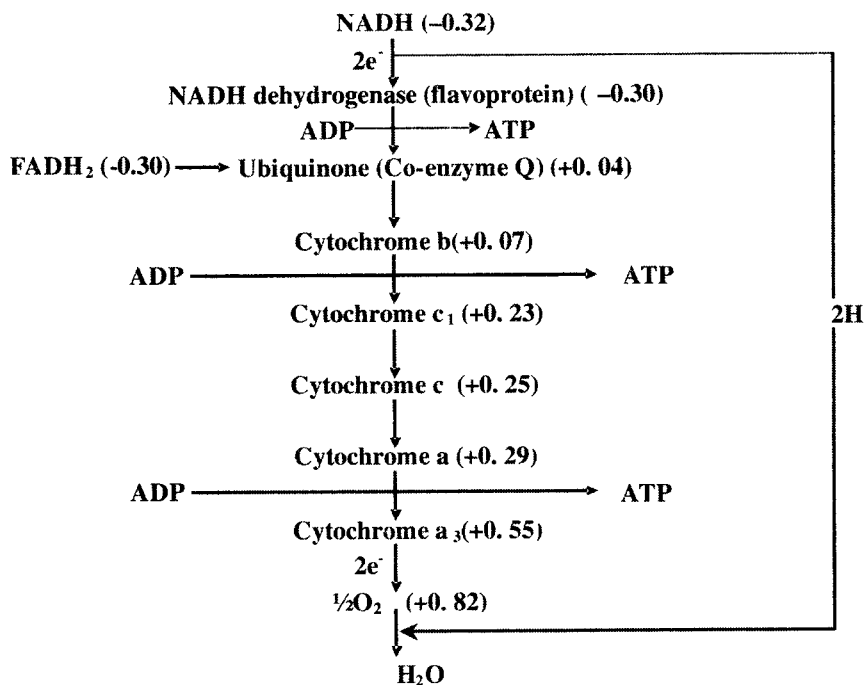
When electrons are transferred from the most electronegative system [(NADH or FADH₂) (-0.32)] to the most electropositive system (+0.82) (Oxygen), there will be liberation of all the energy at one time in an explosive manner. But, if they are transferred in a step wise manner through some intermediate 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 contain a series of catalysts called 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 than there ATP formation takes place. NADH forms 3 ATP's 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 (co-enzyme Q).
- (2) Between cytochrome-b and cytochrome-c₁.
- (3) Between cytochrome-a and cytochrome-a₃ (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 (P_i) with any other compound through high energy bond is known as phosphorylation. 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.
- 2) **Oxidative phosphorylation:** The enzymatic phosphorylation of ADP to ATP coupled to 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_1 ATPase, 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 which is then transferred to F_0F_1 ATPase thereby it 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_0F_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 products 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 -



H_2O_2 is detoxified as -



Cytochrome-aa₃: Cytochrome-aa₃ 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** inhibits 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 **H₂S** gas inhibits.

Uncouplers of oxidative phosphorylation: Uncouplers are those substances which prevent oxidative phosphorylation (formation of ATP) though ETC in 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 $\text{F}_0\text{F}_1\text{ATPase}$.
- 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 $\text{F}_0\text{F}_1\text{ATPase}$.
- 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 mechanisms 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.

Carbohydrate Metabolism

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

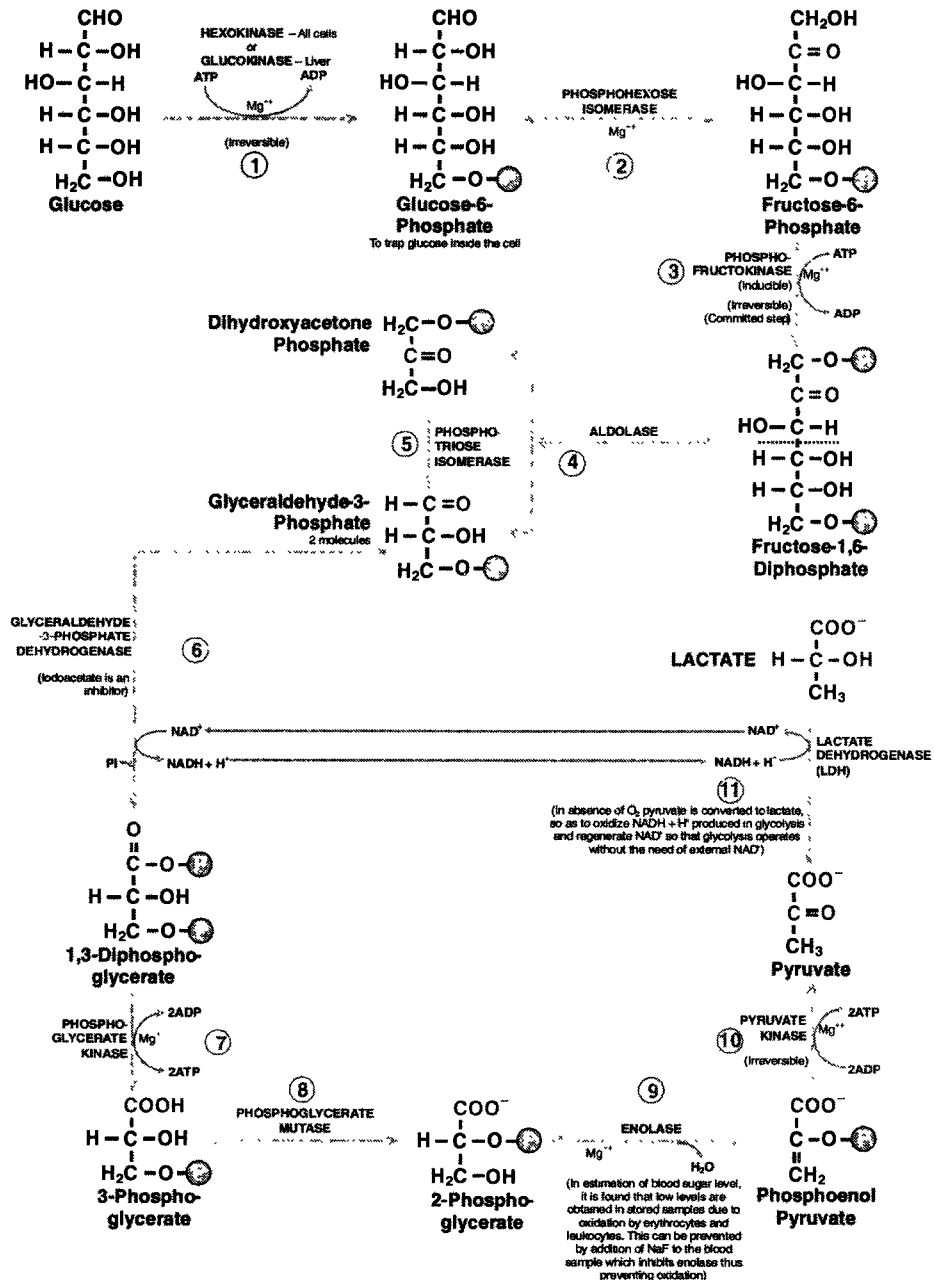
Glycolysis is the breakdown (lysis) of glucose to pyruvic acid under aerobic conditions and to lactic acid under anaerobic conditions.

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 is 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 ATP. In the next four reactions, four additional ATP's are synthesized (two each from both the

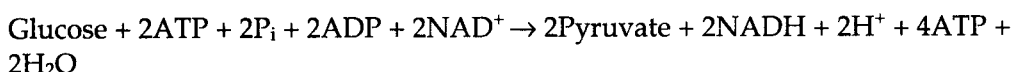
three carbon compounds), before the final product of glycolysis i.e. pyruvate is formed. The three-carbon structure of pyruvate has several fates depending upon the energy state of the cell.



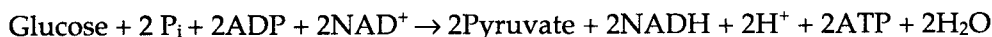
Mechanism of glycolysis

Overall reaction stoichiometry/chemical summary of reaction:

• **Overall reaction**



• **Net reaction**



Under anaerobic conditions:



(Regenerates NAD^+ allowing reaction to continue in the absence of oxygen)

Calculation of ATP's produced per glucose molecule in glycolysis:

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

ATP's utilized in glycolysis

Hexokinase	ATP	1	-	-	1	1 st step
Phosphofructokinase	ATP	1	-	-	1	3 rd step
Total utilized					2 ATP's	

Net gain

ATP's produced	10
ATP's utilized	2
Net gain	8 ATP's

Total 8 ATP'S are produced in aerobic glycolysis

In anaerobic glycolysis, $\text{NADH} + \text{H}^+$ is not oxidized through the electron transport chain, instead is oxidized by lactate dehydrogenase, hence no production of 6 ATP's i.e. 6 ATP's are produced less in number.

ATP's produced in anaerobic glycolysis = 4 (7th & 10th step)

ATP's utilized in anaerobic glycolysis = 2 (1st & 3rd step)

Net gain of ATP's = 2 ATP's

Therefore, only two (2) ATP's are produced in anaerobic glycolysis of glucose.

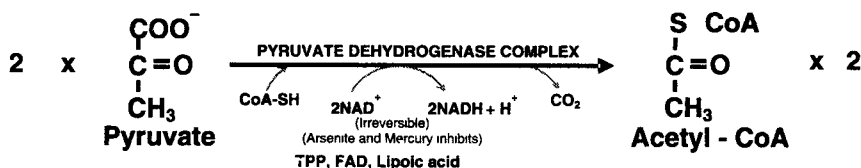
Salient features of glycolysis: It is the main route for glucose metabolism. It occurs in all the cells of the body. Brain and RBC depend only on glucose for oxidation and production of energy. In brain aerobic glycolysis occurs whereas in

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 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 break down 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.

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 regulation of glycolysis is the enzyme phosphofructokinase, 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 via the lactate dehydrogenase enzyme. Finally, pyruvate can be made 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) Co-enzyme-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., 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

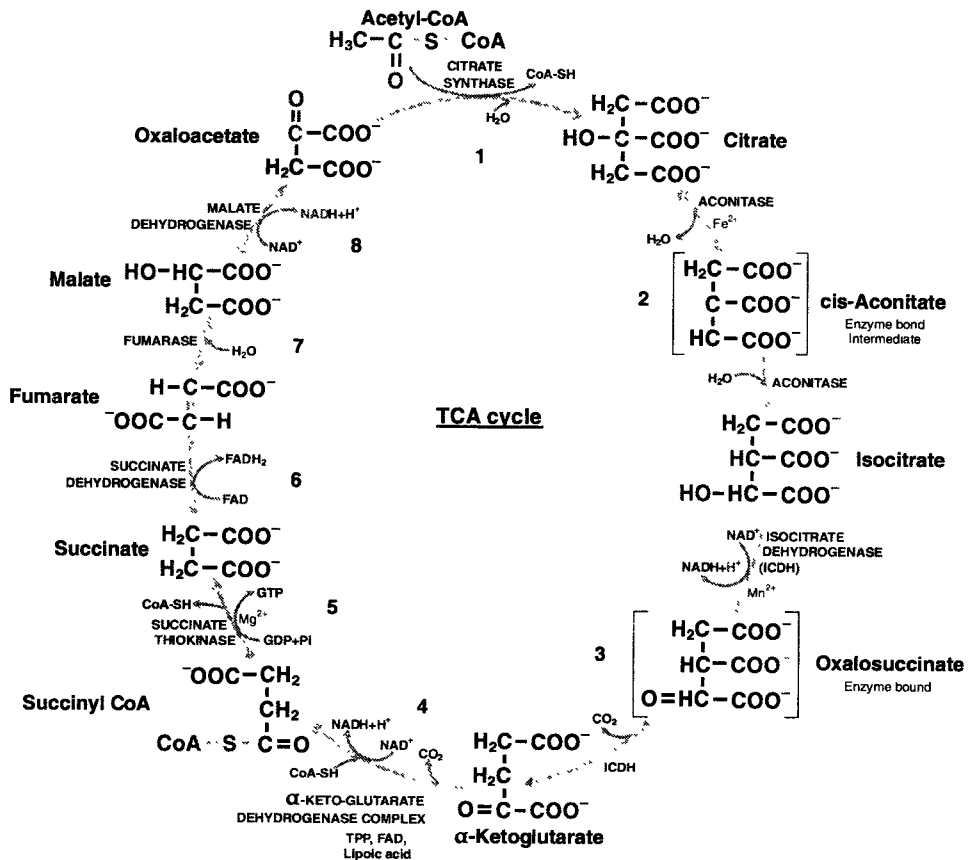
Citric acid cycle also known as tricarboxylic acid (TCA) cycle is named after the scientist Sir Hans Krebs (1900-1981) who discovered it. He proposed the key elements of this pathway in 1937 and was awarded the Nobel Prize in Medicine for the discovery in 1953. Krebs' cycle is a set of continuous reactions (8 steps) occurring in a cyclic manner in the mitochondrial 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 . This 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.

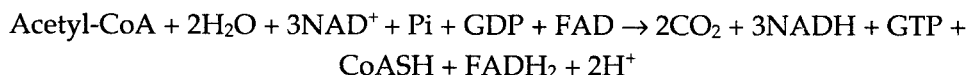
Step 6 - Flavin-dependent dehydrogenation: The succinate produced by succinyl CoA-synthetase in the prior reaction needs to be converted to oxaloacetate to complete the Krebs's cycle. The first step in the conversion is the dehydrogenation of succinate to yield fumarate facilitated by the enzyme **succinate 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 ATP's.

Step 7 - Hydration of a carbon-carbon double bond: Fumarate undergoes a stereo-specific hydration of the $\text{C}=\text{C}$ 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 $\text{NADH} + \text{H}^+$.

The formation of oxaloacetate completes the Krebs's cycle.

The sum of all reactions in the citric acid cycle is:



Number of ATP's produced in one TCA cycle: The TCA cycle produces 3 $\text{NADH} + \text{H}^+$ 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 ATP's and oxidation of FADH_2 through ETC produces 2 ATP's.

Calculation of ATP's produced in each round of citric acid cycle

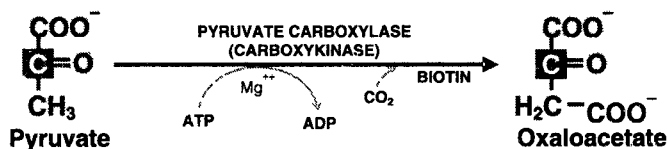
Enzyme involved	Equivalent produced	Fate of the equivalent	No. of ATP's produced	The step in TCA cycle
Isocitrate dehydrogenase	NADH	Oxidized through ETC	3	3 rd step
α -Ketoglutarate dehydrogenase	NADH	- Do -	3	4 th step
Succinate thiokinase	GTP	Converted to ATP	1	5 th step
Succinate dehydrogenase	FADH_2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

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 ATP's produced when glucose is completely oxidized to CO₂ and H₂O:

- (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**

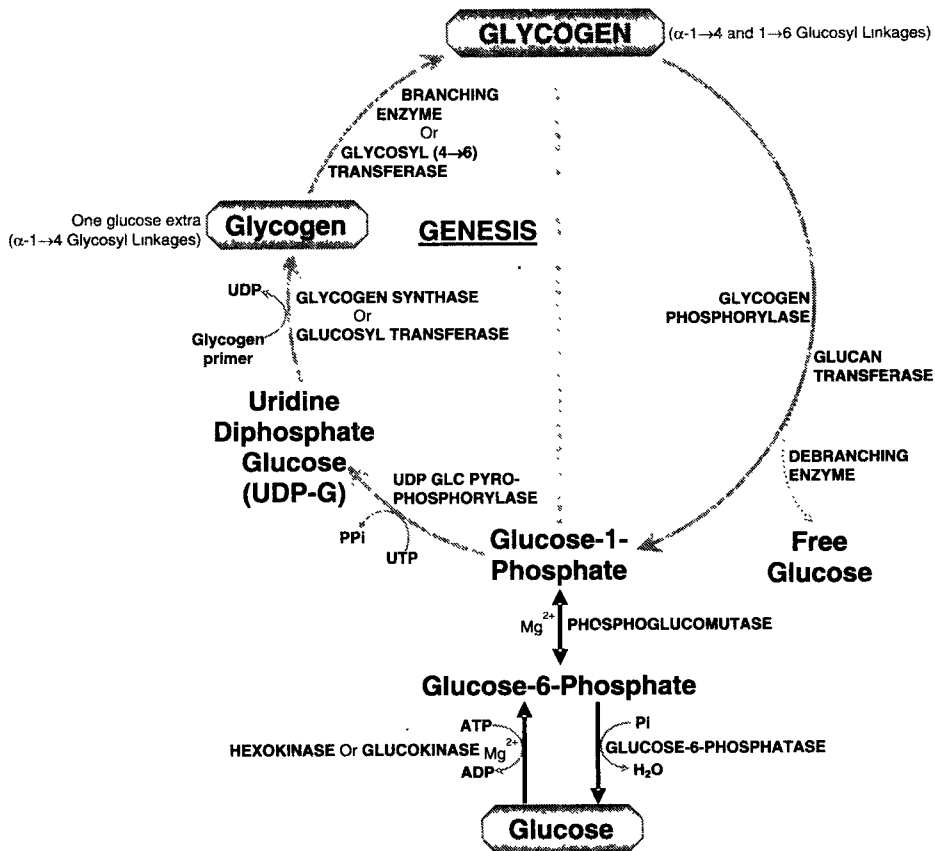
- Total 38 ATP's are formed when one molecule of glucose is completely oxidized to CO₂ and H₂O.
- Net gain of 36 ATP'S is seen when NADH produced in glycolysis in the step catalysed by glyceraldehyde-3-phosphate dehydrogenase in the cytosol is transported to the mitochondria for oxidation in ETC, facilitated 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.

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.

Synthesis of glycogen from glucose is known as glycogenesis.

Breakdown of glycogen to glucose is known as glycogenolysis.



Gluconeogenesis: It 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 isoleucine) 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 from proteins in the diet and during starvation, from the breakdown of proteins in skeletal muscle. Glycerol is derived from 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

Hexose monophosphate shunt 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 $\text{NADPH} + \text{H}^+$, for the synthesis of lipids (fatty acids and steroids) and to keep 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.

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).

Lipid Metabolism

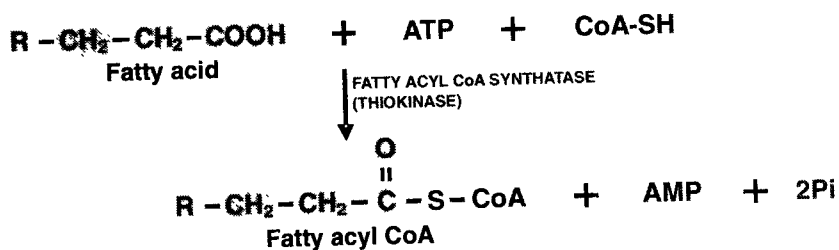
LIPOLYSIS

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 breakdown 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 rise the blood glucose level. Ex. Glucagon, growth hormone, adrenaline, nonadrenaline, 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 tissue. 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

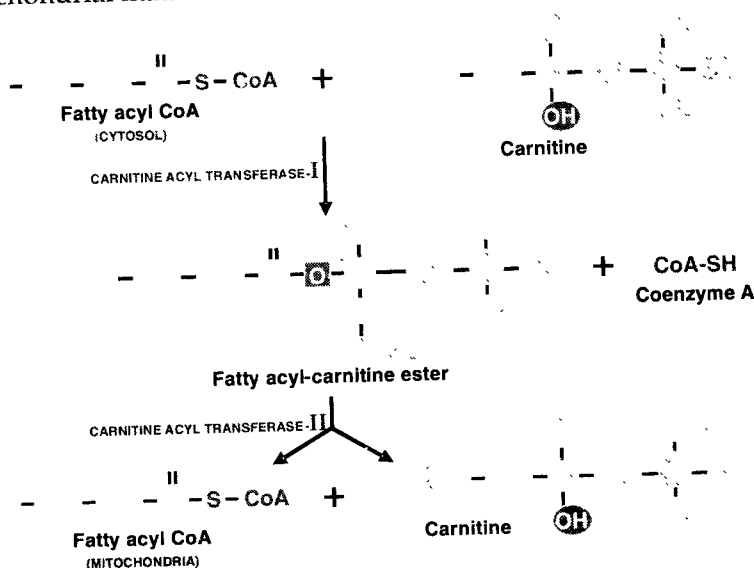
1) Activation of fatty acids: Oxidation of fatty acids can take place only if they are activated. The fatty acids are activated in the cytosol of the cell wherein the enzyme fatty acyl CoA synthetase (thiokinase) condenses the fatty acids with coenzyme-A by esterification which requires two high energy bonds.

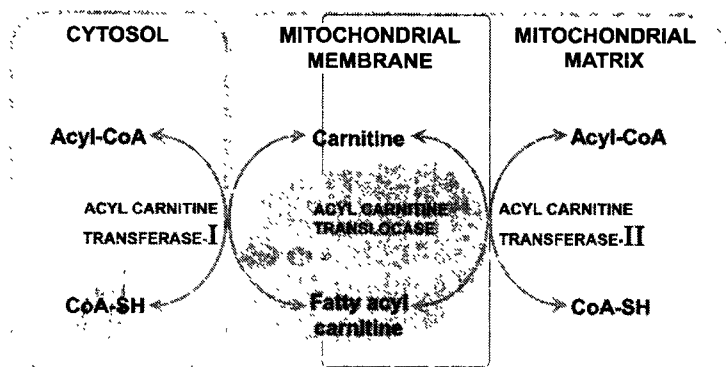


Further oxidation of fatty acids takes place in the mitochondrial matrix. The mitochondrial membrane is impermeable to the acylated fatty acids of the cytosol. Hence these are transported by carnitine mechanism after activation.

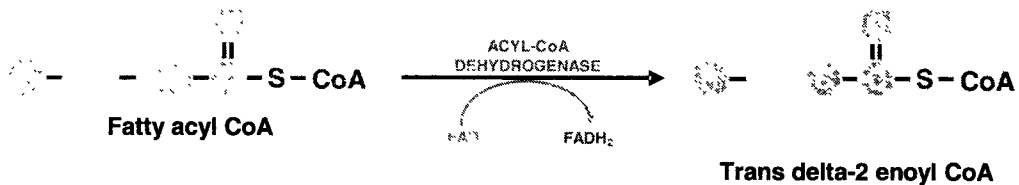
Carnitine mechanism: Carnitine (beta-hydroxyl-gamma-trimethyl-ammonium butyrate) $[(\text{CH}_3)_3\text{N}^+-\text{CH}_2-\text{CH}(\text{OH})-\text{CH}_2-\text{COOH}]$, is present in the inner mitochondrial membrane and it helps in the transport of fatty acids both from the cytosol to mitochondria and 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.

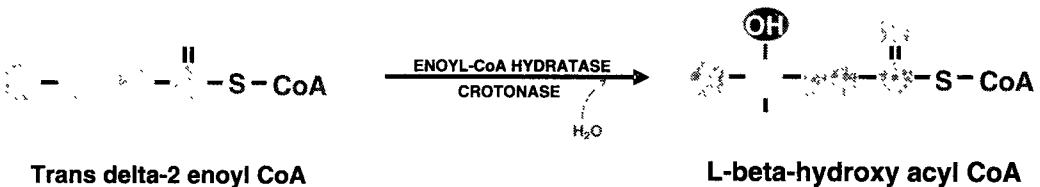




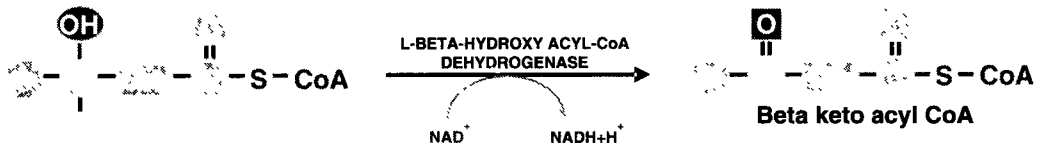
- 2) **Formation of α - β unsaturated acyl-CoA (Enoyl-CoA):** The fatty acyl-CoA undergoes dehydrogenation at the α and β carbon atoms forming trans alpha-beta unsaturated acyl-CoA. These hydrogens are taken up by the coenzyme, Flavin Adenine Dinucleotide (FAD) which gets converted to FADH₂. Oxidation of FADH₂ through electron transport chain produces two ATP's. The enzyme is fatty acyl CoA dehydrogenase.



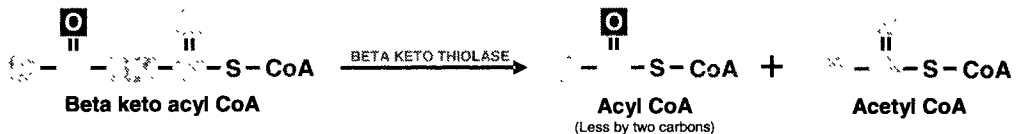
- 3) **Formation of β -hydroxy acyl-CoA:** Enoyl-CoA is then hydrated by the enzyme crotonase (Enoyl-CoA hydratase) which adds water across the double bond. The product formed is β -hydroxy acyl-CoA.



- 4) **Formation of β -keto acyl-CoA:** β -Hydroxy acyl-CoA undergoes another dehydrogenation of the β -oxidation 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 ATP 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 inturn oxidized through TCA cycle and the reducing equivalents produced are oxidized through ETC.

The number of ATP's produced by palmatic acid

Name of the enzyme / process	Number of reducing equivalents / ATP produced	Total number of ATP
Fatty acyl CoA dehydrogenase	7 $\text{FADH}_2 \times 2$	14
β -hydroxyacyl CoA dehydrogenase	7 $\text{NADH} + \text{H}^+ \times 3$	21
Total ATP's 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
ATP's utilized	Activation step $\text{ATP} \rightarrow \text{AMP}$ 	2
NET GAIN OF ATP's		129

Total 129 ATP's are produced when palmatic acid is oxidized completely

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. Their concentration under normal conditions in blood plasma (per 100 ml) and urine (per day) is less than 1 mg. 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 increases the capacity of the peripheral tissue 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**”.

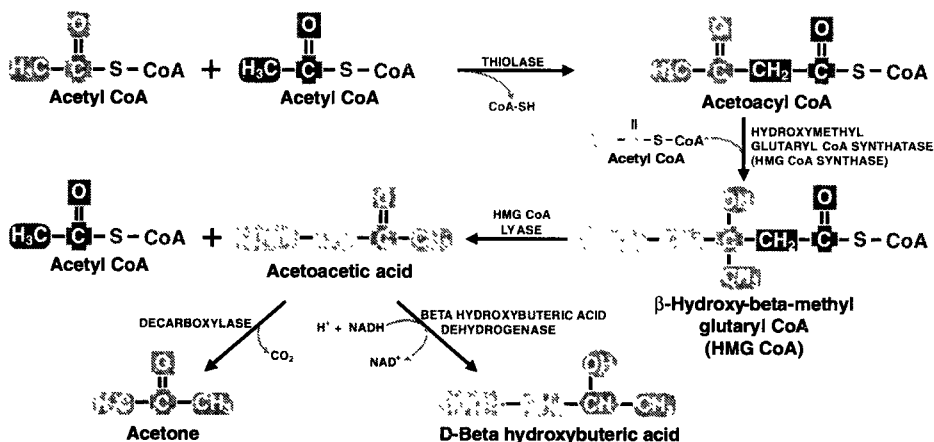
Ketosis: Ketosis is a condition in which there is an increased accumulation of ketone bodies in blood (ketonemia) and consequently increased excretion in urine (ketonuria).

Biochemical changes in ketosis:

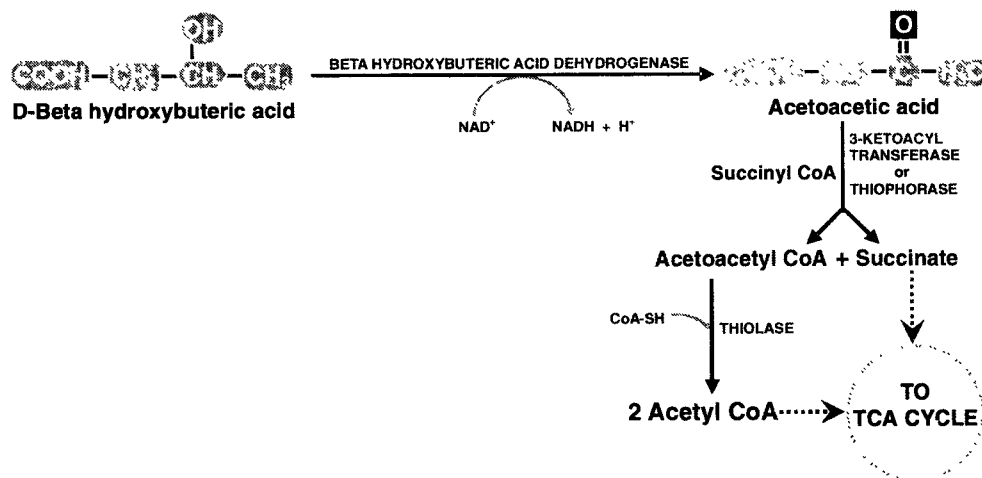
- (1) Acetoacetic acid and β -hydroxy butyric acid are strong acids, their accumulation causes ketoacidosis, thereby lowering the pH of blood.
- (2) Buffering capacity is disrupted because bicarbonate of the blood decreases.
- (3) Along with ketone bodies, large amounts of H_2O and Na^+ ions are lost leading to electrolyte imbalance and dehydration.

Symptoms: Depression, thirst, fatigue and coma.

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 tissue the ketone bodies are utilized in the following manner -



Only acetoacetic acid and beta hydroxybuteric 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.

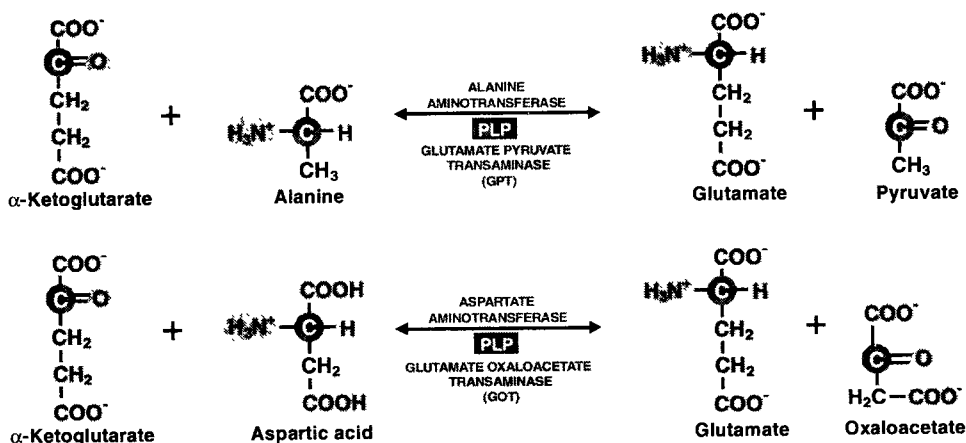
Clinical conditions in which ketosis occur: Starvation, diabetes mellitus, pregnancy, lactation, febrile diseases, and heavy exercise.

Protein Metabolism

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.

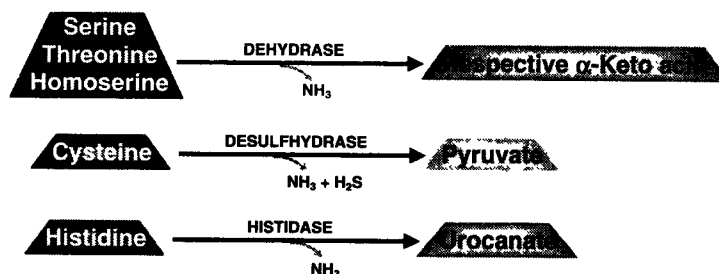


Generally three keto acids participate in this reaction. They are - (1) Pyruvate (2) α -keto glutarate and (3) Oxalo acetic acid. Transamination reaction mainly serves two roles in the amino acid metabolism - (a) Inter conversion of amino acids and (b) To channel the amino group of amino acids ultimately to glutamate and aspartate.

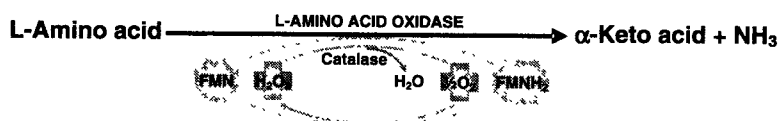
If these two amino acids are in excess than the requirement then the amino group is removed by deamination forming ammonium ion (NH_4^+) or urea which are excreted. All the amino nitrogen is ultimately concentrated on glutamate.

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 hydroxyl amino acids like serine and threonine.

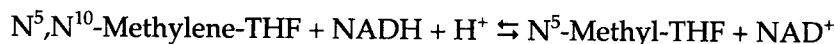
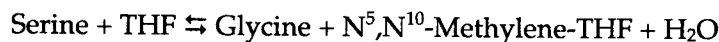


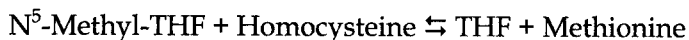
- (b) **Oxidative deamination:** Removal of amino group following its oxidation is known as oxidative deamination. It takes place both in the liver and kidney. The coenzymes involved are FMN and FAD which are reduced to FMNH_2 and FADH_2 . The enzyme is amino acid oxidase which is auto oxidizable i.e. 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.

- 3) **Transmethylation:** It is a process in which there is the transfer of methyl group from a donor to the acceptor.

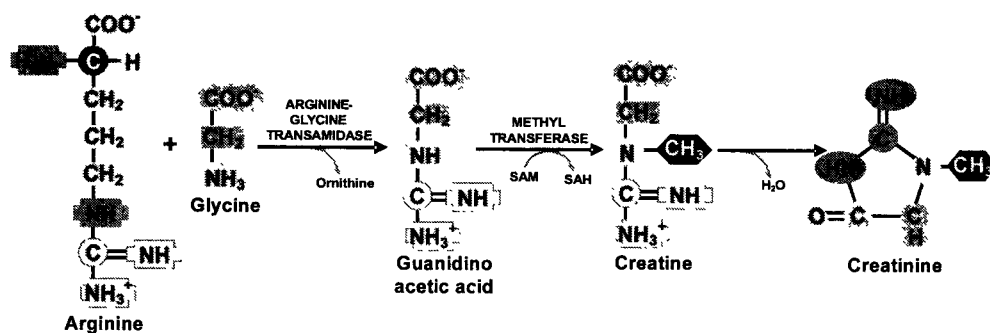




- 4) **Decarboxylation:** Reactions in which removal of CO_2 takes place from the carboxylic group of amino acids. Enzyme catalyzing the reaction is decarboxylase. Cofactor is PLP. An amine is formed due to decarboxylation. The amines so formed are known as '**biogenic amines**'. This is mainly confined to G-I tract where due to the putrefaction in intestines toxic amines like tyramine, tryptamine, putrescine and cadaverine are formed. However some amines are useful.

Amino acids	Amine	Occurrence and significance
Histidine	Histamine	Effects 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.



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 $\mu\text{g/dl}$. 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.

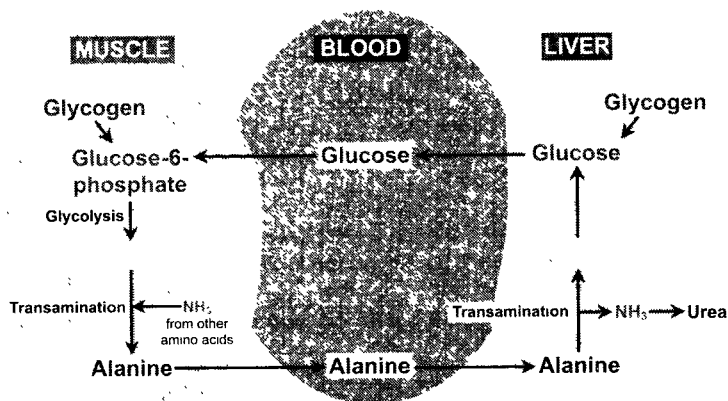
Transport of ammonia: Removal of ammonia takes place by the following mechanisms -

- (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.

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, asparagine and serine.

Excretion of ammonia: Ammonia is excreted in three different forms in various species, they are -

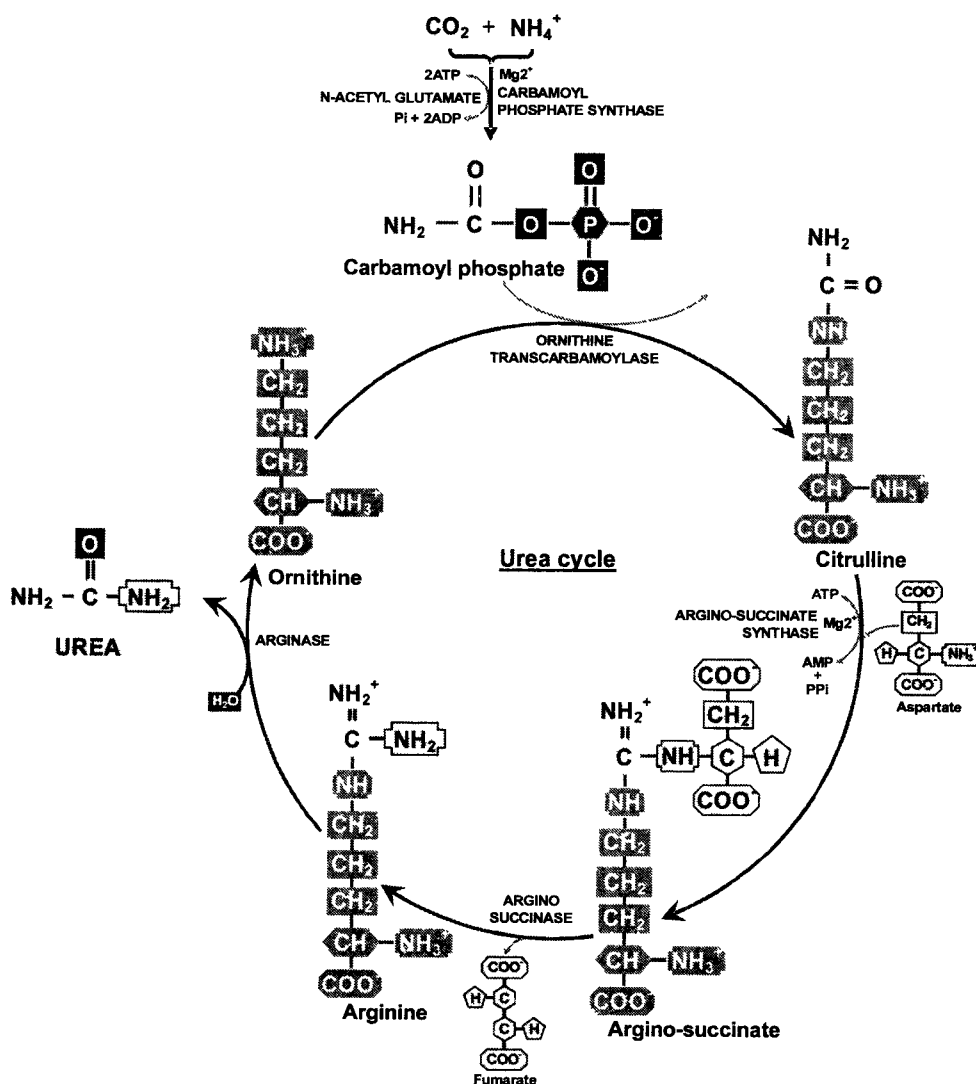
- (1) In fishes it is excreted as ammonia hence they are known as ammonotelic animals.
- (2) In uricotelic animals like birds and lizards, ammonia is excreted as uric acid.
- (3) In ureotelic animals including humans, ammonia is excreted as urea.

UREA CYCLE

Urea cycle was discovered by Kreb and a medical student Henseliet of Germany in 1932. Hence it is also known as Kreb's 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 and released into the blood and cleared by the kidney.

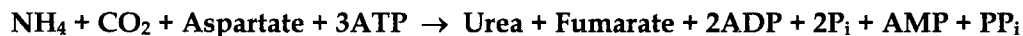
Steps of urea cycle:

- 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 ATP.
- 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+} is a 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 Kreb's 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 acids 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 -



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 $\text{HCO}_3^-/\text{H}_2\text{CO}_3$. Though 3 ATP's are utilized, the ultimate cost of making a molecule of urea is 4 ATP's (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.
- 4) **Argino-succinic aciduria:** It is a rare recessive disease. Higher level of argino-succinic acid in plasma and CSF. Usually present in the early age. Feeding arginine and benzoate promotes nitrogen excretion in these patients.
- 5) **Hyper-arginemia:** High level of arginine due to lack of arginase enzyme.

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Chapter 13

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

B - Complex group of vitamins and their coenzyme forms

Vitamin/ chemical name	Active coenzyme form	Biochemical role/ function	Reaction participating
B₁ Thiamine	Thiamine Pyrophosphate (TPP)	Oxidative decarboxylation reactions Transketolation reactions	<p><u>Pyruvate dehydrogenase complex</u></p> <p>Pyruvate $\xrightarrow[\text{NAD}^+ \rightarrow \text{NADH}+\text{H}^+]{\text{PYRUVATE DEHYDROGENASE}}$ Acetyl - CoA</p> <p><u>Transketolase</u></p> <p>Xylulose-5-P \longrightarrow Glyceraldehyde + Ribose-5-P \longrightarrow -3-P + Sedo- heptulose-5-P</p>
B₂ Riboflavin	Flavin Mononucleotide (FMN) Flavin Adenine Dinucleotide (FAD)	Oxidation reduction reactions	<p>L-Amino acid $\xrightarrow[\text{FMN} \rightarrow \text{FMNH}_2]{\text{L-AMINO ACID OXIDASE}}$ L-Imino acid</p> <p>Succinic acid $\xrightarrow[\text{FAD} \rightarrow \text{FADH}_2]{\text{SUCCINATE DEHYDROGENASE}}$ Fumaric acid</p> <p>Fatty acyl-CoA $\xrightarrow[\text{FAD} \rightarrow \text{FADH}_2]{\text{FATTY ACYL-COA DEHYDROGENASE}}$ Enoyl CoA</p>

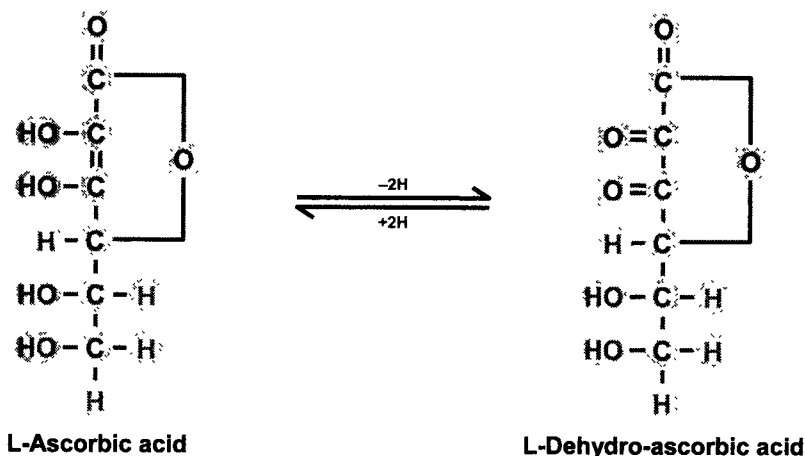
B₃ Niacin (Nicotinic acid)	Nicotinamide Adenine Dinucleotide (NAD ⁺) Nicotinamide Adenine Dinucleotide Phosphate (NADP ⁺)	Oxidation reduction reactions	<p> Malate $\xrightarrow[\text{NAD}^+ \rightarrow \text{NADH} + \text{H}^+]{\text{MALATE DEHYDROGENASE}}$ Oxaloacetate </p> <p> Glucose-6-phosphate $\xrightarrow[\text{NADP}^+ \rightarrow \text{NADPH} + \text{H}^+]{\text{GLUCOSE-6-PHOSPHATE DEHYDROGENASE}}$ 6-Phospho gluconolactone </p> <p> Mevalonate $\xrightarrow{\text{HMG CoA REDUCTASE}}$ HMG CoA </p>
B₄ Biotin	Biocytin	CO ₂ Transfer and CO ₂ fixation	<p> Pyruvate $\xrightarrow{\text{PYRUVATE CARBOXYLASE}}$ Oxaloacetate </p>
B₅ Pantothenic acid	Coenzyme-A	Acyl group transfer	<p> Pyruvate $\xrightarrow{\text{PYRUVATE DEHYDROGENASE}}$ Acetyl CoA </p> <p> Fatty acid $\xrightarrow{\text{FATTY ACYL-CoA SYNTHETASE}}$ Fatty acyl CoA </p>
B₆ Pyridoxine	Pyridoxal Phosphate (PLP) Pyridoxamine phosphate	Transamination reactions -Takes up amino group to form Pyridoxamine phosphate	<p> α-Keto glutarate + Alanine $\xrightarrow[\text{(GPT)}]{\text{ALANINE TRANSAMINASE}}$ Glutamic acid + Pyruvate </p> <p> Pyruvate $\xrightarrow{\text{PYRUVATE DEHYDROGENASE}}$ Acetyl CoA </p>
B₇ Folic Acid	Tetrahydrofolic acid (THF)	Transfer of one carbon units	<p> Deoxyuridyate $\xrightarrow{\text{TRYMIDILATE SYNTHETASE}}$ Deoxythymidylate </p>
B₈ Lipoic acid	Dihydro lipoic acid (DHL)	Acyl group transfer and hydrogen carrier	
B₁₂ Cobalamine Or Cyano-cobalamine	Cobamide coenzyme (5' Deoxy adenosyl cobalamine)	1,2-Hydrogen shift	<p> Succinyl-CoA $\xrightarrow{\text{MUTASE}}$ Methyl malonyl-CoA </p>

VITAMIN - C

ASCORBIC ACID

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

Vitamins



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.

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

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

Deficiency disease: 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 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.

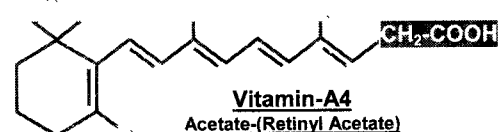
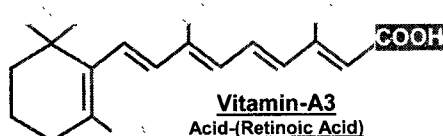
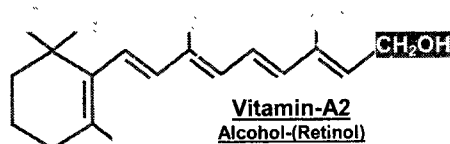
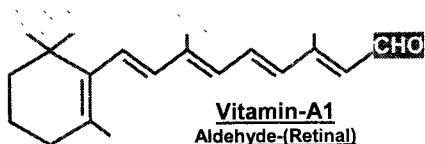
Fat soluble vitamins: Vitamins insoluble in water but soluble in fats or fat solvents are known as fat soluble vitamins. The fat soluble vitamins are vitamin A, D, E & K.

VITAMIN - A

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

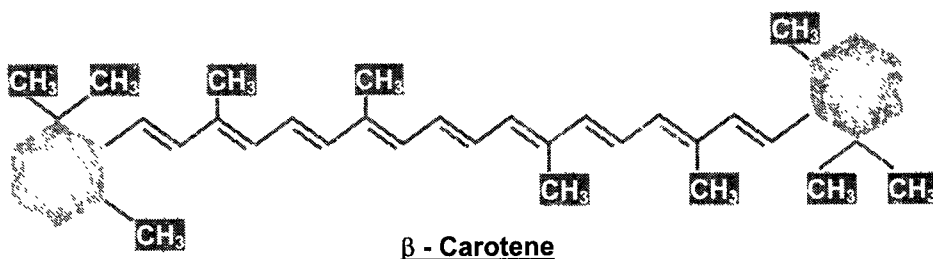
- (1) Vitamin A₁ known as Retinal - the aldehydic form

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



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 the central double bond of beta-carotene to yield 2 molecules of vitamin A aldehyde (retinal). Vitamin A alcohol is then produced by reduction of the aldehyde in an NADH-dependent reaction catalyzed by retinene reductase.

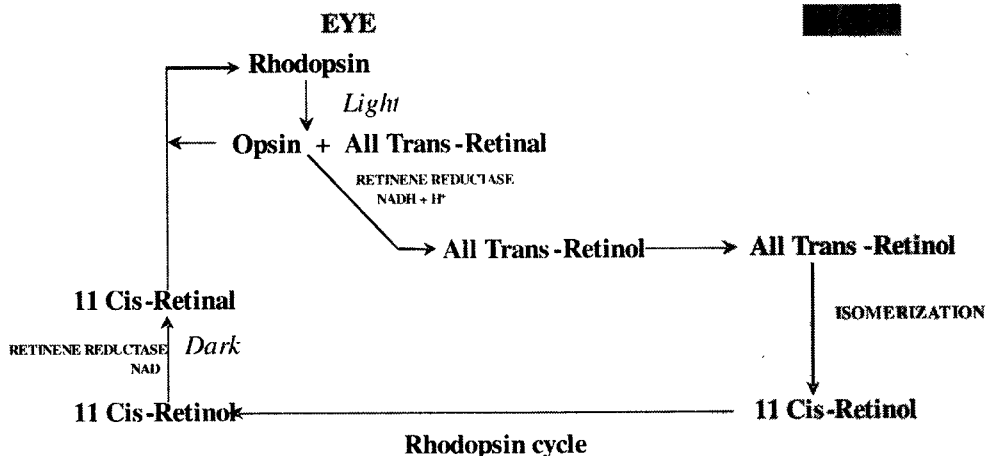
Absorption and transport: The dietary sources of vitamin A to the humans and animals are via the conversion of beta-carotenes (plant sources) to vitamin A and hydrolysis of retinyl esters (animal sources) to retinol in the intestine. Retinol is absorbed in the cell membrane of the intestine, re-esterified 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 forms 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) bound to retinol-binding protein (RBP) which has a molecular weight of about 20,000 Da. It is also transported 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 developments 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.

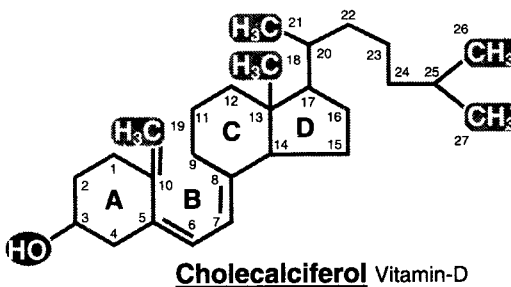
Vitamin – A and vision (Rhodopsin cycle): There are two types of cells in the retina of the eye i.e. rods (for dim light vision) and cones (for bright light vision). The rod cells contain rhodopsin (retinal pigment or visual purple). When light strikes the retina, rhodopsin is split into its protein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



Deficiency diseases: (1) Nyctalopia – night blindness (2) Xerophthalmia – complete blindness (3) Colour blindness (4) Keratomalacia – Dryness of eye, skin and keratinization of respiratory, intestinal, urinary tracts, salivary glands and the genital system.

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 D₂ (ergocalciferol) and (2) vitamin D₃ (cholecalciferol). Cholecalciferol is the most prominent form of vitamin D in humans. Vitamin D is similar to the classic steroid hormones. It is derived from cholesterol.



Daily requirements and sources: The daily requirement of vitamin D is 100 units. Lactating women needs 400 units/day. The rich dietary sources of vitamin D are cod liver oil, fish liver oil, egg yolk, milk and animal liver. Vitamin D₃ 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 D₃ 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 7th position to 7-dehydrocholesterol. In the skin, photoconversion of 7-dehydrocholesterol to cholecalciferol (vitamin D₃) takes place. Cholecalciferol is metabolised with the help of the enzyme D₃-25-hydroxylase in the liver to 25-hydroxycholecalciferol (25(OH)D₃), 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)D₃-1-hydroxylase (mediated by parathormone) to 1,25-Dihydroxycholecalciferol, this is the active form of vitamin D. Plasma vitamin D binding protein (DBP) carries vitamin D₃ and all of its metabolites to their various target organs through the blood. The production of 1,25(OH)₂D₃ is modulated according to the calcium and other endocrine needs of the body. The chief regulatory factors are 1,25(OH)₂D₃ 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)₂D₃ are low, production of 1,25(OH)₂D₃ by the kidney is high, and when circulating concentrations of 1,25(OH)₂D₃ are high, the output of 1,25(OH)₂D₃ 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 D₃ to 25(OH)D₃ and then to 1,25(OH)₂D₃.
- Variations in end organ responsiveness to 1,25(OH)₂D₃ or possibly 25(OH)₂D₃.

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

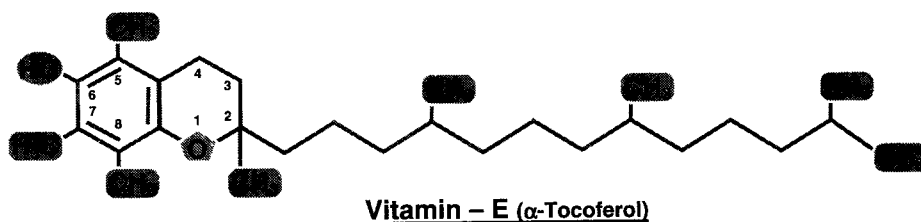
- (1) **Rickets:** Bowed legs.
- (2) **Osteomalacia:** Soft bones with tendency to break easily.

Hypervitaminosis: Nausea, vomiting 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 different substituents possible 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 chromanol ring.



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 of surface tension and 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.

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), ubiquone, vitamin A and carotenes act as antioxidants thus 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 needs this vitamin.
- (7) Requirement of vitamin E depends 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 on 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:

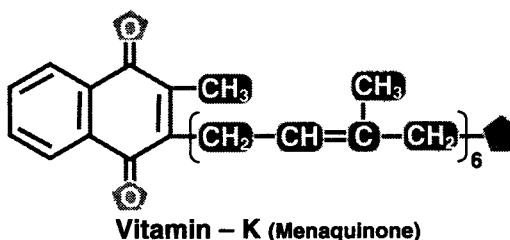
- (1) It causes discolouration of the enamel of the teeth due to oxidation of unsaturated fatty acids present in these structures to peroxide.
- (2) Anaemia is caused in monkeys due to lack of hemophysins in bone marrow, rather than destruction of RBC.
- (3) Increased fragility of RBC.
- (4) Thrombocytosis and oedema.
- (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. K₃) whereas K₁ and K₂ are naphthaquinones (present in plants). Menaquinone is an anti-haemorrhagic vitamin i.e. it prevents haemorrhages by activating the process of blood clotting. Menadione is a synthetic analogue of vitamin K.



Sources: Green leafy vegetables like cabbage, spinach etc. predominantly contain vitamin K₁ and are the best sources. Cauliflower, soyabean, wheatgerm etc. proves 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 K₂ 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 electron 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.

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Chapter 14

Minerals

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 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 classified 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, e.g. 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 as 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.

CALCIUM

Absorption: Absorption of calcium by the intestine is never complete. Ca is absorbed by an active transport process occurring mainly in the upper small intestine. The process is regulated by $1, 25 \text{ D}_3$. 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 water and thus absorption of calcium is increased in presence of amino acids. Calcium salts particularly carbonates and phosphates are quite soluble in acid solution and are relatively insoluble in alkaline solution. Hence an increase in acidophilic flora, e.g. lactobacilli is recommended to lower pH which favours the absorption of Calcium.

If calcium, phosphorus ratio is much high, $\text{Ca}_3(\text{PO}_4)_2$ 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. 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 lost in faeces. 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.

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. 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 phosphorus are markedly improved by administration of estrogen or by androgens such as testosterone. A combination of estrogen and androgen is more effective. 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. Oxalate in certain foods precipitate calcium in the intestine as the insoluble calcium oxalate. The phytic acids of food form insoluble salt with calcium and reduce calcium absorption.

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 absorbed 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 or 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 - (a) Isocitric dehydrogenase, phosphorylase and (b) 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.

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.

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. Rickets is characterized by faulty calcification of bones due to low vitamin D content of the body, a deficiency of calcium and phosphorus in diet or a combination of both.

PHOSPHORUS

Absorption: Like calcium phosphorus is also absorbed by upper small intestine and factors influence 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 up till 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 thus significant in (RNA and DNA) protein synthesis and genetics point of view.
- (4) Formation of organic phosphates as intermediate in metabolic processes. Ex. In glycolysis $\text{Glucose} + \text{ATP} \rightarrow \text{G-6-P} + \text{ADP}$.
- (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.

Hemostasis: 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 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 hemostasis and seems to be secondarily involved in certain endocrinopathies, e.g. involving parathormone, growth hormone and corticosteroids.

SODIUM, POTASSIUM, CHLORIDE

Substances whose solutions conduct an electric current are called 'electrolytes'. They are about 11 electrolytes in general. Na, K, Ca and Mg are cations whereas Cl, HCO₃, HPO₄, SO₄, organic acids and proteins are anions. Among which sodium, potassium and chloride are important in the distribution and the retention of body water. Na, K and Cl, all the three elements have close relationship among them. Thus it is practical to discuss the metabolic and nutritional aspect of these three elements together.

Absorption: Normally Na, K and Cl are completely absorbed by the gastrointestinal tract. About 95% of sodium which leaves the body is excreted in the urine.

Distribution: In the tissues both Na and K occur in relatively large amount as chloride and other inorganic salts as well as protein and organic salts. Sodium is present in extra cellular fluid and very little inside the cells whereas potassium is mainly found inside the cells and very little in the extracellular fluid.

Functions: These electrolytes maintain normal osmotic pressure in the body and protect the body against excessive loss of fluid.

- (1) They maintain 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) Sodium helps in the formation of the gastric juice. NaCl takes part in the series of reaction as a result of which HCl is manufactured by the stomach.
- (7) Potassium of KHb in the red cells helps carbondioxide transport.
- (8) Potassium ions inhibit cardiac contraction and prolong relaxation.
- (9) Potassium ions exert important effects on the function of nervous system.

Functions of chloride:

- (1) It provides 2/3rd of the anion of plasma and is main factor for regulating body reactions.
- (2) Sodium chloride (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) Chloride ions are essential in acid-base regulation. Chloride plays a role in the body by chloride shift.

Metabolism: The metabolism of these elements is influenced by 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 whereas excretion increases.

Temperature: When atmospheric temperature is high as in summer, large amounts of sodium and chloride are lost in respiration (sweating) and this loss is 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.

Disorders:

Hyponatraemia: On sodium deficient diet, young ones grow slowly, lack fat deposit, 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 hypokalaemia in young ones as 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 become 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 not to increase 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.

IRON METABOLISM

Iron is present in all organisms and in all cells. It does not exist in the free state, but is always present in organic combination, usually with proteins. It exists in two states 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 charged with the function of oxygen transport such as haemoglobin and cytochromes.

Metabolism:

- (1) **Distribution:** The total amount of iron in the body is 3-5 g out of which haemoglobin has 1.5-3 g, storage iron is about 0.6-1.5 g, myoglobin and iron in enzymes constitute to 0.3 g and plasma iron ranges from 3-4 mg.
- (2) **Absorption:** Absorption mainly occurs in the duodenum and the proximal jejunum. The amount absorbed depends principally on the body needs. The absorption of iron is affected by a number of factors -
 - (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 the 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.
- (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.

Ferrous ion on entering the mucosal cells is oxidized to ferric state and then combined with apoferritin forms 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.

- (3) **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 irons 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.
- (4) **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.
- (5) **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 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 $\mu\text{g}/100\text{ ml}$; in females it is 90-120 $\mu\text{g}/100\text{ml}$. However, the total iron binding capacity (TIBC) is about the same in both sexes i.e. 300-360 $\mu\text{g}/100\text{ ml}$.

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 is 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 burned to CO₂ 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

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 -

$$RQ = \frac{V_c}{V_o}$$

This quotient is useful because the volumes of CO₂ and O₂ 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, comfortable humidity, 10-12 hours after the last meal, awake and sitting in a reclining position. 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. Large sized persons have higher BMRs, but the BMR per unit body weight is higher in the smaller individuals. Ex. Although the BMR of a man as given above is higher than that of a 15 kg boy that expends 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/m² 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 metabolisms and some with slower metabolisms. 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. This means they have a higher basal metabolic rate as compared to women.
- 3) **Age:** BMR reduces with age. 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 the metabolic rate 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%.
- 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 (produced by the thyroid gland) 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 (a condition known as thyrotoxicosis) BMR can actually double. If too little thyroxine is produced (myxoedema) BMR may shrink to 30-40 percent of normal. 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 burn even when sleeping.

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.

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Blood and Body Fluids

Almost $3/4^{\text{th}}$ 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 foetus | (5) Interstitial fluid |
| (2) Aqueous humour or CSF | (6) Lymph |
| (3) Bile | (7) Semen |
| (4) Blood and blood plasma | (8) Urine |

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 (cytoplasm) is 7.2.

Composition of blood: Blood is composed of -

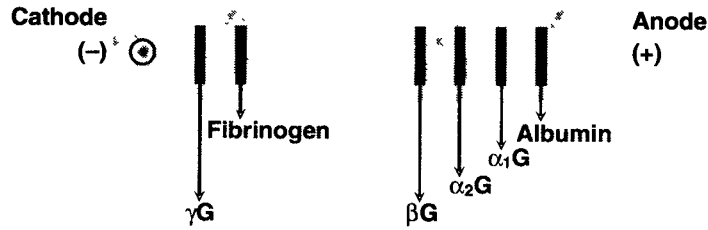
- (a) Blood cells which includes - (i) Erythrocytes (ii) Leukocytes and (iii) Platelets.
(b) Blood plasma.

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 constituents (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. The amount of plasma proteins ranges from 6.5 to 7.5 gms/dl.



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 retinol binding protein	-	-
2. Albumin: synthesized in the liver-contains large amounts of essential amino acids- freely soluble in water – coagulate on heating and precipitated 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 causes 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) Oros mucoid (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 sulphosalicylic acid.	0.1	Transport of hexoseamines	Cancer, acute myocardial infarction and rheumatoid arthritis	Infective hepatitis, portal cirrhosis

Blood and Body Fluids

Protein component	Conc. gms/dl	Functions	Increased in	Decreased in
(ii) α_1 -glycoprotein	0.03	Inhibitor of trypsin	-	-
(iii) α_1 -lipoproteins (HDL)	0.25 - 0.4	Transport of phospholipids and cholesterol	-	-
(iv) Thyroxine binding globin	0.01 - 0.02	Transport of thyroid hormones	-	-
(v) Transcortin	0.03 - 0.035	Transport of cortisol and corticosterol	-	-
(vi) Retinol binding globin	0.03 - 0.06	Retinol transport	-	-
B. α_2-globulins	0.4 - 0.9			
(i) α_2 -glycoprotein	0.1	Inactivator of progesterone	-	-
(ii) α_2 -macroglobulin	0.2	Anti trypsin and anti plasmin	-	-
(iii) Hepatoglobulin	0.1 - 0.26	Binds free hemoglobin and prevent 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 transport iron	Chronic iron deficiency, pregnancy	Pernicious anemia, liver disease, chronic infections

Protein component	Conc. gms/dl	Functions	Increased in	Decreased in
(iii) Hemopexin	0.05 - 0.1	Binds and aids disposal of heme		
(iv) Plasminogen (Profibrinolysis)	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 - 1.7	Functions as antibodies	Cirrhosis of liver, multiple myeloma, bacterial, viral and protozoal infections	Agammaglobulinemia, nephrosis and agranulocytosis
E. Fibrinogen: It is a glycoprotein having a Mw. of 340,000. pHI 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, abruptio placenta and abortions

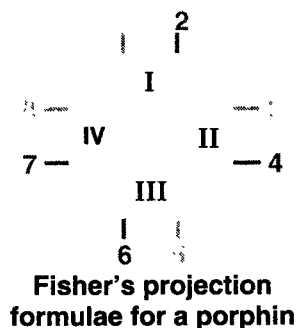
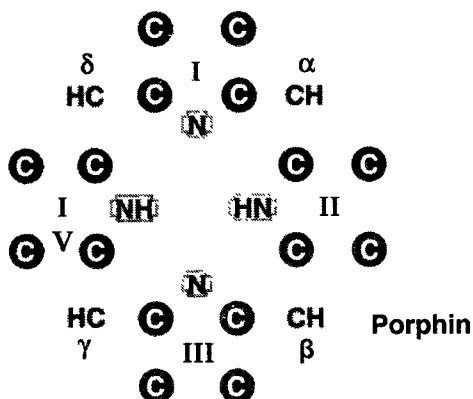
HAEMOGLOBIN

It is an oxygen/CO₂ carrier protein present in the red blood corpuscles of blood. Haemoglobin is a conjugated chromoprotein having heme as its prosthetic group. Haeme is the prosthetic group, not only of hemoglobin but it is a prosthetic group of myoglobin, cytochromes etc.

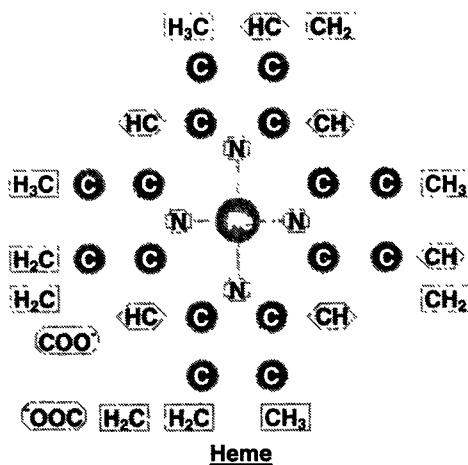
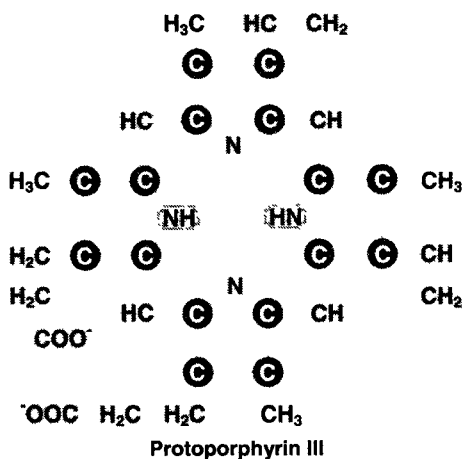
Haemoglobin is formed by the combination of heme with globin (protein). Globin is made up of four polypeptide chains (an oligomeric protein). Two of these polypeptides are known as alpha (α) and the other two are known as beta (β). Each alpha chain has 141 amino acids and each beta chain has 146 amino 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 position 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.

Haeme: It is an iron-porphyrin compound. It is the prosthetic group embedded in the packet like structure formed by hemoglobin folding of the tertiary structure.

Porphyrin: Porphyrin is a complex compound with a tetrapyrrole ring structure. Pyrrole is a heterocyclic compound having the following structure. 4 pyrrole rings join together through methylenedene bridges ($-\text{CH}=\text{CH}-$) to form a porphyrin.



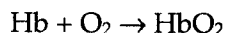
This porphin is substituted by different groups at positions numbered from 1-8 to form the porphyrin. Depending upon the groups (methyl, acetyl, propyl, butyl or vinyl) present on these positions different types of porphyrins are identified. The type of porphyrin present in heme is protoporphyrin-III (also known as No. IX).



Haemoglobin derivatives: There are some derivatives of normal Hb that arise due to metabolic changes in the RBC. The various haemoglobin derivatives are:-

- 1) **Oxyhaemoglobin (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 haemoglobin is 95-96% saturated with oxygen. On binding with O₂ in the lungs haemoglobin is converted to oxyhaemoglobin (HbO₂). O₂ is bound to haeme iron.



- 2) **Reduced haemoglobin (HHb):** Oxyhemoglobin moves to the tissue where the partial pressure of O₂ 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.
 $\text{HbO}_2 + \text{H}^+ \rightarrow \text{HHb} + \text{O}_2$
- 3) **Carbaminohaemoglobin:** Hemoglobin also binds CO₂ in the tissues. CO₂ is bound to the α-amino group at the N-terminal end of each of the four polypeptide chains of hemoglobin to form carbamino haemoglobin. As one CO₂ binds O₂ is released.

Abnormal haemoglobins: There are three types of haemoglobins that are normally found in human beings, they are -

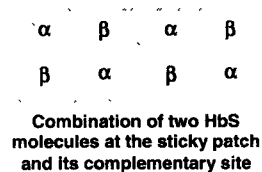
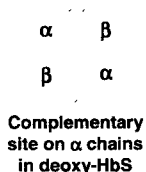
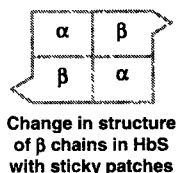
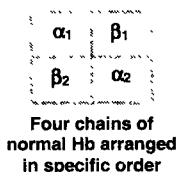
- 1) **HbA:** Found in normal adult human beings-contains 2α and 2β chains.
- 2) **HbA₂:** Found in some human beings and is considered normal-contains 2α and 2δ chains.
- 3) **HbF:** Foetal haemoglobin - found in growing foetus - contains 2α and 2γ chains.

Each chain is synthesized by the information obtained from the gene for haemoglobin. α chain is synthesized from α genes of haemoglobin, β chain from β genes of haemoglobin 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 haemoglobins arise due to mutation in the gene for the haemoglobin synthesis. There are about 300 abnormal haemoglobins. Some of them are those which have defect in α genes and some are with defective β chains.

Biochemistry of abnormality in the haemoglobins:

HbS or sickle cell haemoglobin: Sickle cell haemoglobin (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 residue 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 -



When haemoglobin 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 unsatable, 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 methaemoglobin: 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 oxygen carrying capacity is disrupted leading to anemia and hypoxia (low O_2 to tissues).

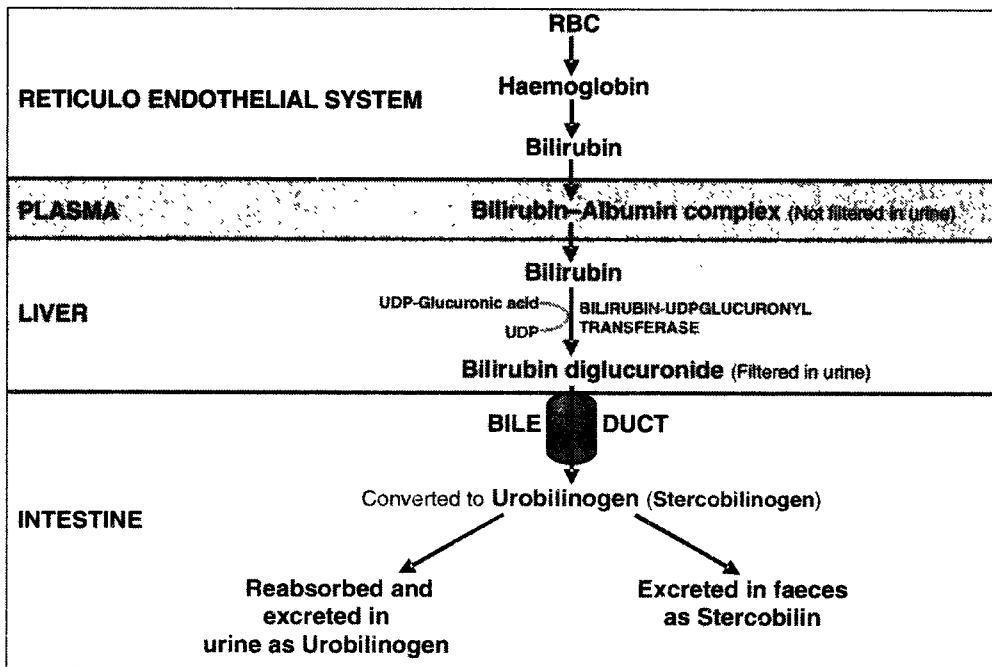
Thalassemias: The defect in thalassemias is the decreased rate of synthesis of one of the polypeptide chains of the globin molecule. One of the chains is synthesized in less amounts than the other due to the defect in DNA. There are two types of thalassemias -

- (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 $\beta_2\gamma_2$.

Degradation of haemoglobin: The life span of hemoglobin is about the same as that of the erythrocytes i.e. 120 days. Red blood cells (RBC) are phagocytized by macrophages in the reticuloendothelial system, mainly 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 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 thereby it ruptures releasing the hemoglobin. Haemoglobin undergoes cleavage of the methene bridge to give choleglobin, an iron pyrrole complex. This reaction is catalysed by methene oxygenase requiring NADPH and Fe^{2+} . Then globin is removed forming verdohaemochrome. Finally iron is removed by a mixed function oxidase called haem 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 to 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.

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Tissue 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 - digestive, excretory, etc of the body.

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 of 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.

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 it.

Classification: 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 flat cells with an 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 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 cells that are longer than they are wide. 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 cell has tiny hairlike projections called microvilli. They increase the surface area for absorption.
- 4) **Transitional:** This is a specialized type of epithelium found lining 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.

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.

Cartilage: Ex. The outer ear.

Bone: The matrix of bone contains collagen fibers and mineral deposits. The most abundant mineral is calcium phosphate, although magnesium, carbonate, and fluoride ions are also present.

Binding connective tissue: It binds body parts together.

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 also 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.

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. 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. 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, irregular angles (called intercalated discs). Striated muscle contracts and relaxes in short, intense bursts, whereas smooth muscle sustains longer or even near-permanent contractions.

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. An average adult male is made up of 40–50% of skeletal muscle and an average adult female is made up of 30-40%.

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, which assists propagation of the nerve impulse as well as provide nutrients to the neuron. All nervous tissue constitutes 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 a response to the stimulus. 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 does 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 a 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.

SECTION – III

**Molecular Biology and
Biotechnology – VBC -122**

**Ist YEAR / IInd SEMESTER - SECOND
COURSE**

This course deals with details of modern aspects of molecular biology and its application in biotechnology. This course is designed with an intention to expose the student to the basic concepts of molecular biology and biotechnology. Hence the teacher and students need not go deep into the subject but take a superficial survey of the subject.

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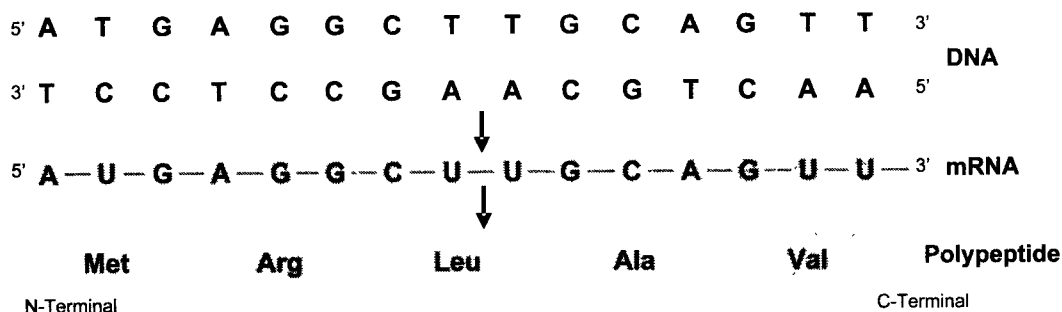
Chapter 18

Molecular Biology

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 weight ranging from 5000 to over 1 millions; the nucleic acids have molecular weights upto several billions; and polysaccharides have molecular weight 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) segments 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 as 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 -

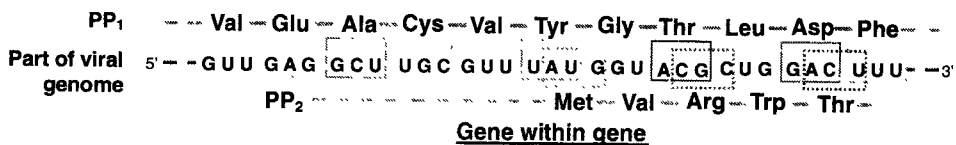
- a) **Structural genes:** Are those which specify the final gene product i.e. a protein or RNA.
- b) **Regulatory genes:** Are those genes which control the structural genes.

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 is 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.

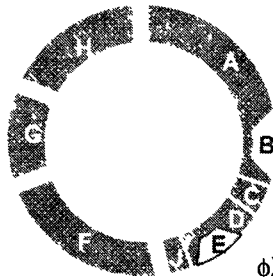


Genome:

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 cell have a single chromosome and eukaryotes have many chromosomes. The number of genes in a viral genome like ϕ X174 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 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.



Likewise many genes in viral genome share the bases. ϕ X174 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'

*

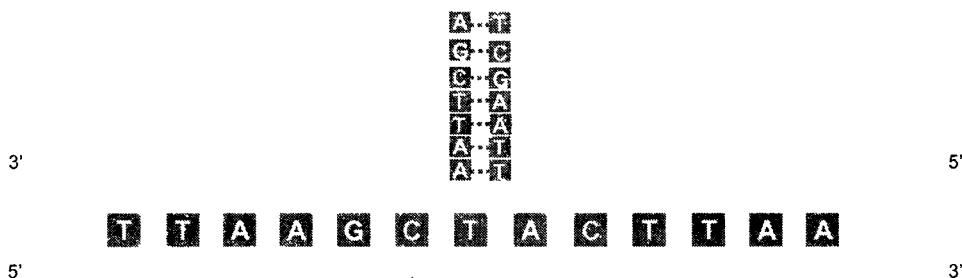
3' T - T - A - A - G - C - T - A - G - C - T - T - A - A 5'

This is just like a sentence which is spelled the same way, when read forward or backward.

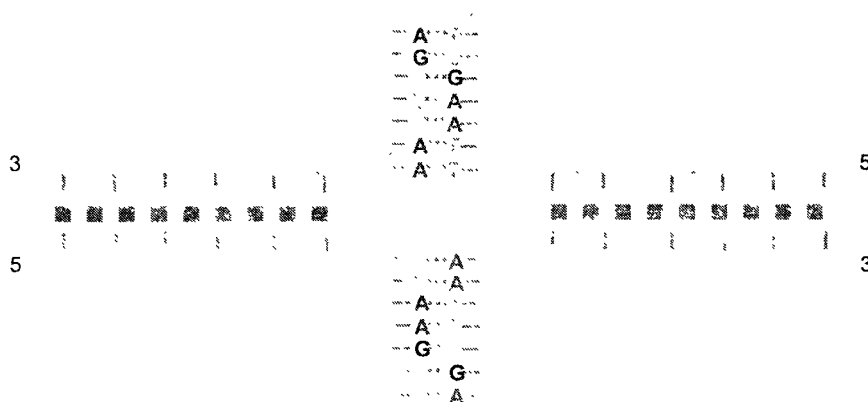
For example -

RADAR	⋮	NURSES RUN	⋮	REFER
RADAR	⋮	NURSES RUN	⋮	REFER

The palindromic sequences are self-complementary within each of the strands. Due to this, complementarily intra-chain base pairing is possible giving rise to a hairpin or cruciform structure.



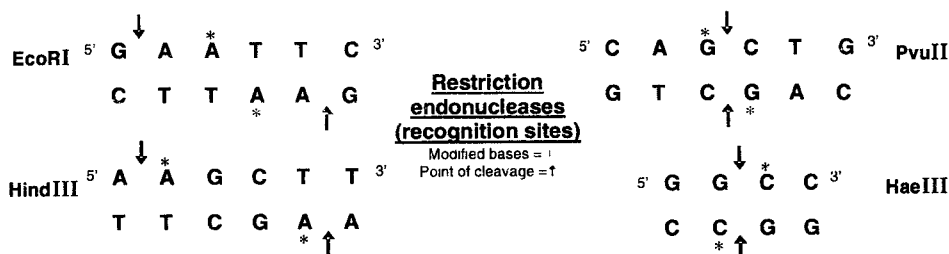
The same type of hairpin is possible in the other strand also and at the same point of rotational symmetry. This results in a cruciform structure. Many such palindromes occur in each genome. The hairpin and cruciform structures help in functioning of DNA like termination of transcription.



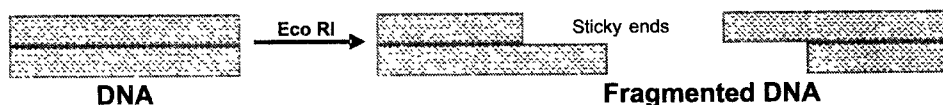
Restriction modification/restriction endonucleases: Most of the bacteria have a special type of mechanism which protects its own DNA, whereas destroys foreign DNA i.e. the DNA of invading viruses. This is brought about by a system of complex enzymes called restriction modification system. This system contains 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 (↑).



Note that by the action of *EcoRI* and *HindIII*, 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.

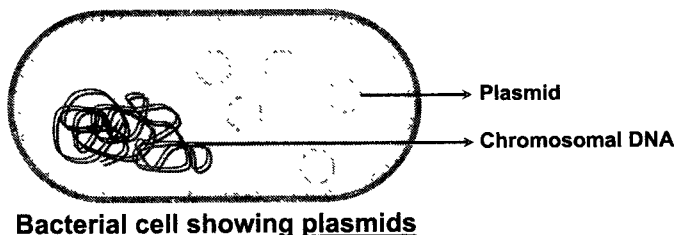


On the other hand the action of *PvuII* and *HaeIII*, cleaves both strands of DNA resulting in no unpaired bases. These are called blunt ends.



Restriction endonucleases are the key tools of recombinant DNA technology and thereby of Biotechnology.

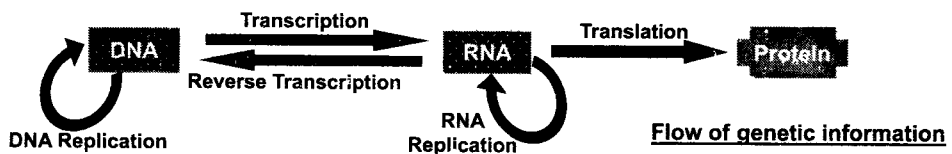
Plasmids: Plasmids are extrachromosomal, independently replicating small circular DNA molecules. Plasmids are found in yeast, other fungi and in bacteria.



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.



The central dogma of molecular genetics defines five processes in the various cellular mechanisms of the utilization of genetic information.

DNA replication: The copying of parental DNA to form daughter DNA molecules having identical 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 ϕ_2 , MS₂, R₁₇ and Q β have RNA genomes and these RNA are replicated forming identical daughter RNA by the enzyme RNA-directed-RNA- polymerase or RNA replicase.

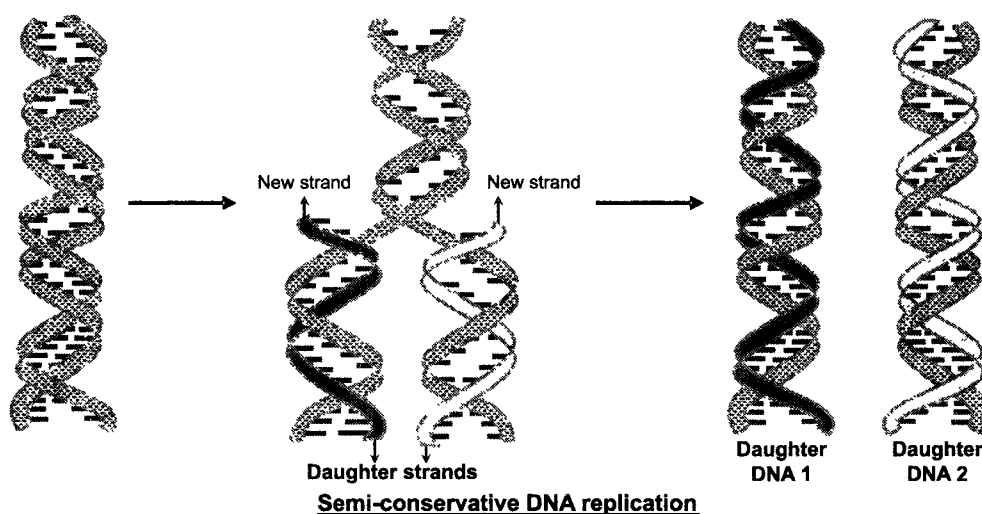
Reverse transcription: In certain RNA viruses, there is an enzyme called reverse transcriptase, which in the host cell synthesises a copy of DNA complementary to

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.



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.

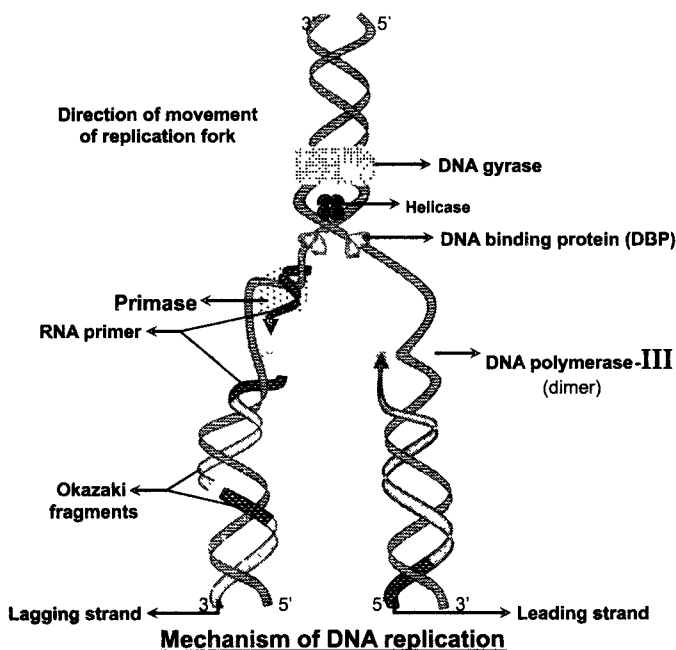
Enzymes known as helicase unwind DNA just at the origin of replication fork. This requires energy in the form of ATP. As soon as a short sequence has been unwound, some DNA binding proteins (DBP or SSP) bind to the strands, thereby keeping them apart. Further in order to prevent the complete duplex DNA 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 traveling 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 deoxyribonucleotides, 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.



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 proofreads 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 is removed and this domain has got 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 proofreading activity.

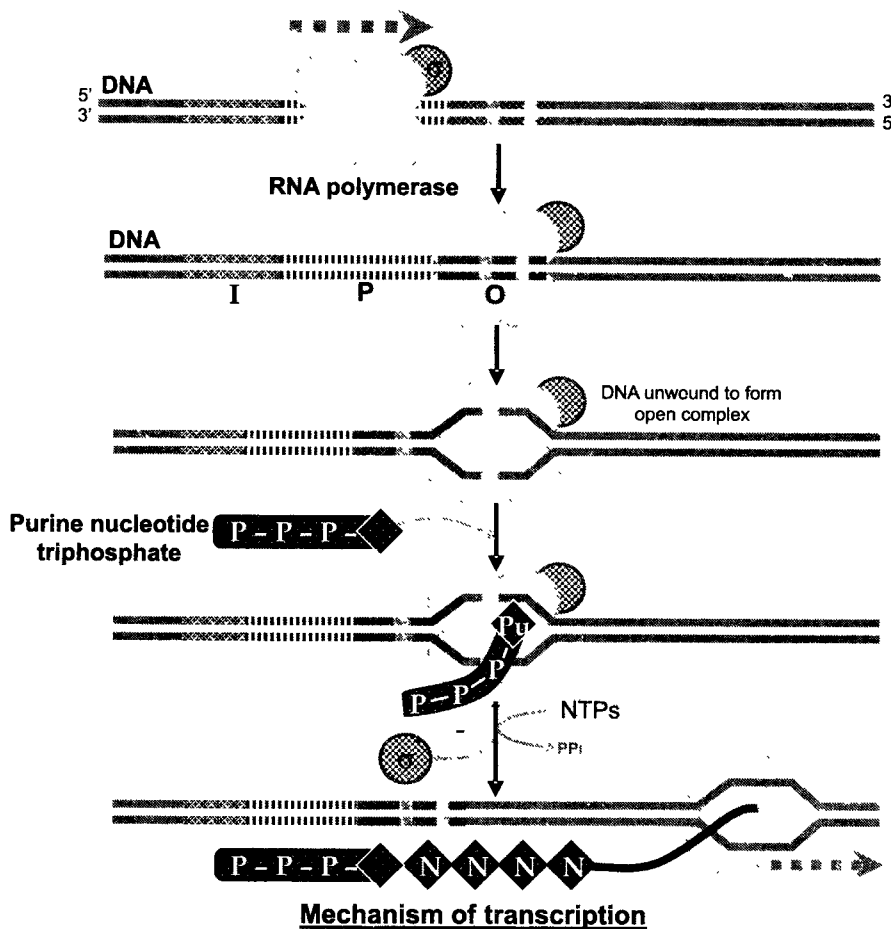
- (7) In eukaryotes the enzymes of replication are known as DNA polymerase alpha (which is similar in structure and function to the DNA polymerase-I of the prokaryotes) DNA polymerase delta (similar to DNA polymerase-III) which works in association with a protein called proliferating cell nuclear antigen (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.

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. 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 prevents complete unwinding of the DNA helix.



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. uridylylate (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 \equiv 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 products of transcription are synthesised as inactive preribonucleotides, these ribonucleotides are processed into functional ribonucleotides by various mechanisms.

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, 96 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		
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC	Phe	UCC	Se	UAC	Tyr	UGC	Cys	C
	UUA	Leu	UCA	Sec	UAA	STOP	UGA	STOP	A
	UUG	Leu	UCG	Sec	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	His	CGA	Arg	A
	CUG	Leu	CCG	Pro	CAG	His	CGG	Arg	G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser	C
	AUA	Ile	ACA	Thr	AAA	Asn	AGA	Arg	A
	AUG	Met	ACG	Thr	AAG	Asn	AGG	Arg	G
G	GUU	Val	GCU	Ala	GAU	Gly	GGU	Gly	U
	GUC	Val	GCC	Ala	GAC	Gly	GGC	Gly	C
	GUA	Val	GCA	Ala	GAA	Gly	GGA	Gly	A
	GUG	Val	GCG	Ala	GAG	Gly	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.

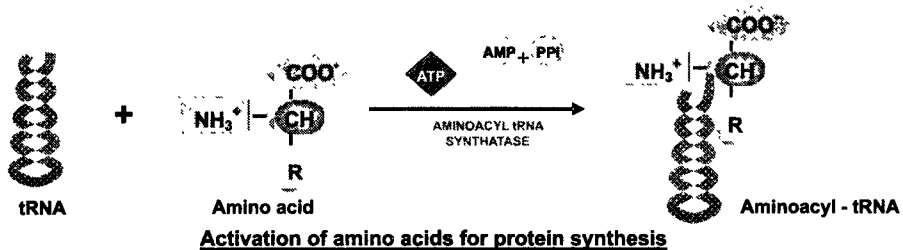
- (4) Out of the 64 codons, AUG is known as the initiation codon which always initiates protein synthesis both in prokaryotes and eukaryotes.
- (5) Three codons does not specify any amino acid hence they are known as non-sense codons or termination codons or stop codons as protein synthesis stops whenever 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 an mRNA molecule is converted into protein with specific amino acid sequence is known as translation or protein synthesis.

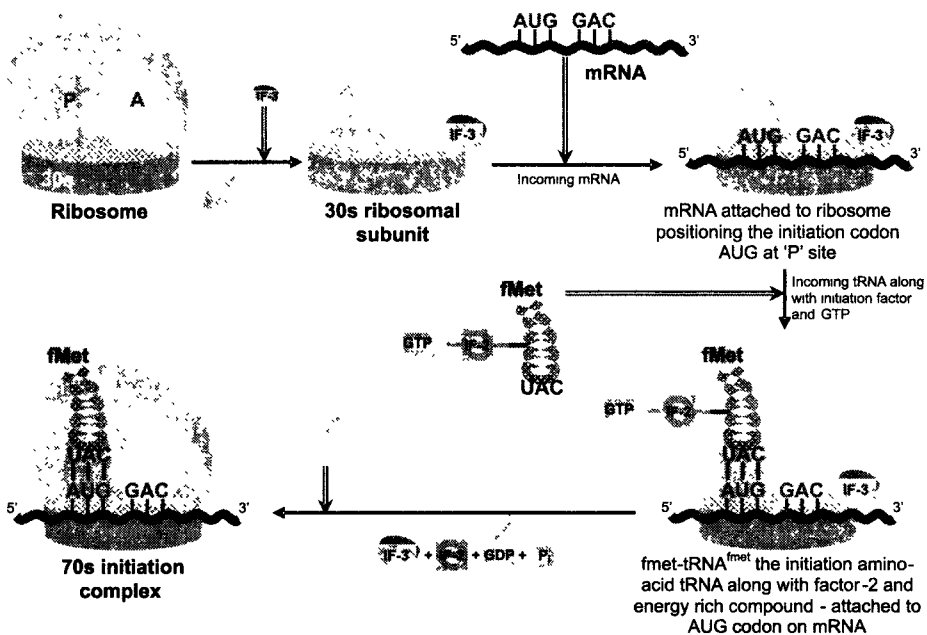
There are five (5) steps involved in the process of protein synthesis, they are -

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-formylmethionyl-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 aminoacyl-tRNA^{AA}. ATP is required for this reaction which gets hydrolysed to AMP and 2Pi.



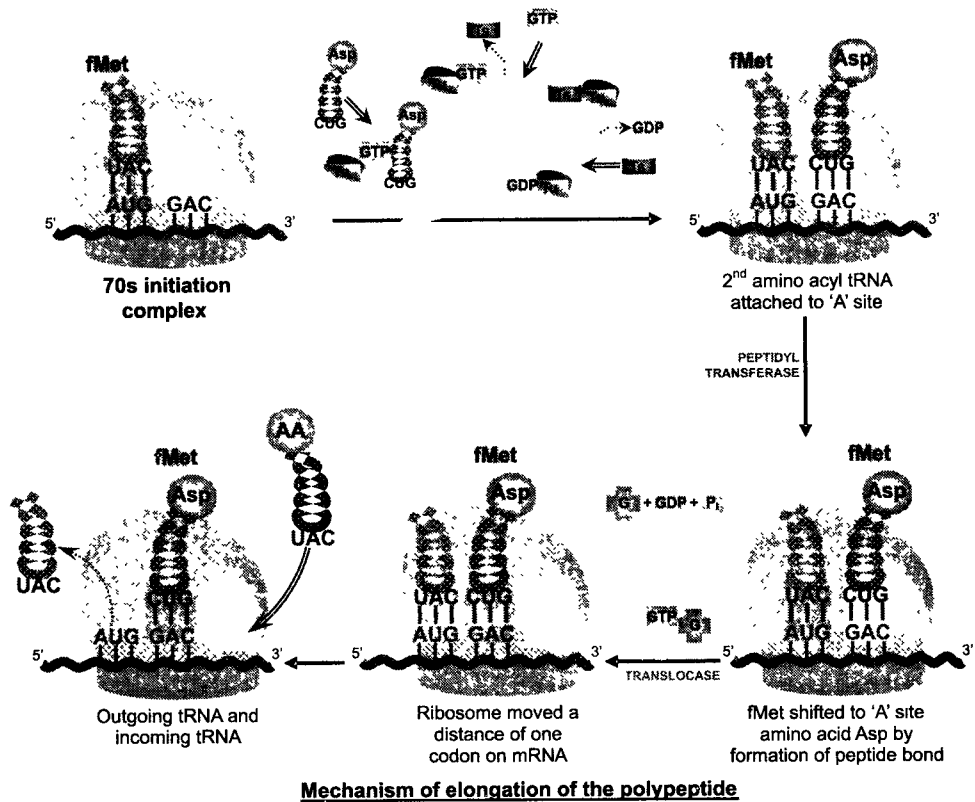
- 2) **Initiation of the polypeptide chain:**
 - a) IF₃ binds to 30S ribosomal subunit and prevents its union with 50S subunit.



- b) mRNA binds to the 30S subunit by an initiation signal on mRNA made up of A & G residues, which base pairs with rRNA.
- c) AUG-the initiation codon is identified by FMet-tRNA^{fmet} (Met-tRNA^{met} in eukaryotes) containing IF₂ & 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 IF₃ and IF₂ are released by the hydrolysis of GTP to GDP and Pi.

3) Elongation:

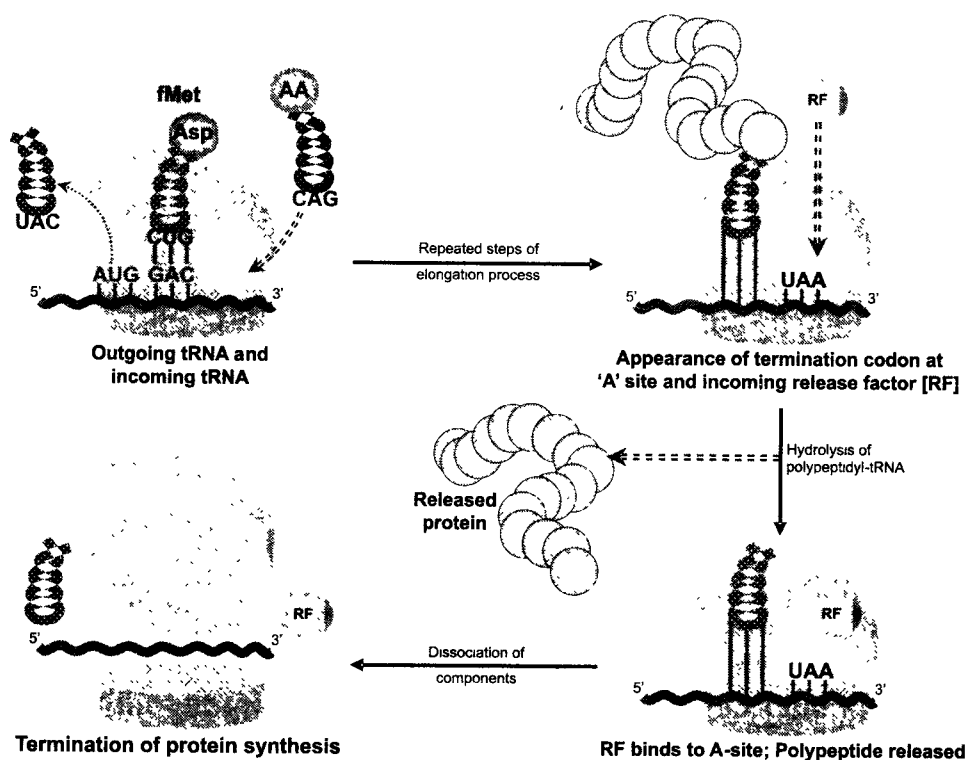
- a) The next aminoacyl-tRNA is bound to Tu and GTP and this binds to the A site on the 70S initiation complex, which is directed by the next 3 bases in the mRNA i.e. next codon.
- b) Simultaneously Tu-GTP is released with the hydrolysis of GTP to GDP.
- c) Tu-GDP is converted to Tu-GTP with the help 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.



- 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, Ochra and Opal respectively.

Once a termination codon occupies the ribosomal A-site, three termination or release factors i.e. RF₁, RF₂ and RF₃ 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 -

- The N-formylmethionine (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.
- The signal sequence, which directs the proteins to its destination, which are 10-30 amino acid residues at N-terminal end are also removed.
- The hydroxyl groups of certain Ser, Tyr and Thr residues are enzymatically phosphorylated by ATP. Ex. Milk protein casein has phosphoserine groups which binds Ca^{2+} .
- 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.
- To Asn, Ser or Thr of some extracellular lubricating glycoproteins, carbohydrate side chains are attached enzymatically.

- f) To some proteins, a prosthetic group is attached covalently. Ex. Heme to haemoglobin.
- g) Many proteins like insulin, trypsin and chymotrypsin are synthesised as longer inactive proteins called the zymogen. These are enzymatically cleaved to smaller active proteins.
- h) 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.

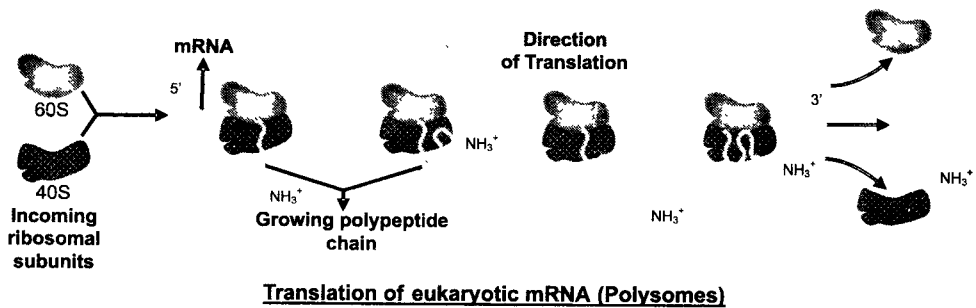
Thus a complete biologically active protein is released in the media i.e. either in the cell or extracellular. Some of these proteins are structural proteins and yet some others are enzymes. These enzymes now carry the various functions which depend upon their structure, which in turn depends upon the 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 inhibits 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.

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 gets 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.



Chapter 19

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 binds if a sample of nucleic acid containing a base sequence complementary to that of the probe, is present.

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 deducing 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 method.

- 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 only at G, A and G, C and T or only C, by adopting different chemical procedures.

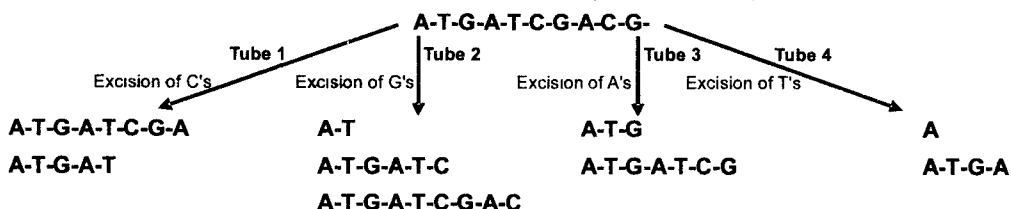
If DNA is heated at neutral pH, then the glycosidic bond (the bond between the sugar and base) of a methylated purine is broken off, when the heating is continued after adding alkali, the backbone of the fragment at G is broken. If this heating is done in dilute acid, the backbone at both A & G is broken. Treatment of DNA with hydrazine in 2M NaCl and then with piperidine cleaves at C only. Treatment only with 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



First of all this piece is labeled with radioactive ^{32}P at the 5' end.

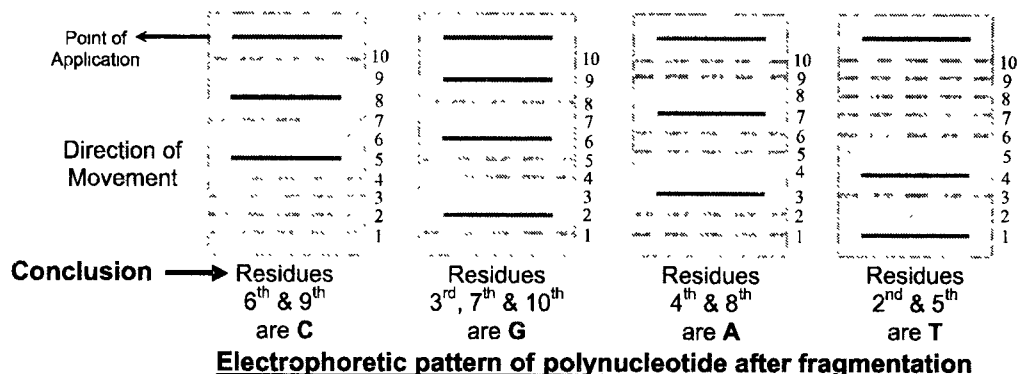


This labeled molecule is taken into four different tubes. Each tube is treated with different chemical procedures, so as to cleave either at A, T, G, or C, as described above. This results in the DNA molecule breaking into the following smaller fragments. (Fragments having the label are only considered)



Each of these four mixtures is separately subjected to electrophoresis on polyacryl amide 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 labeled (^{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 -



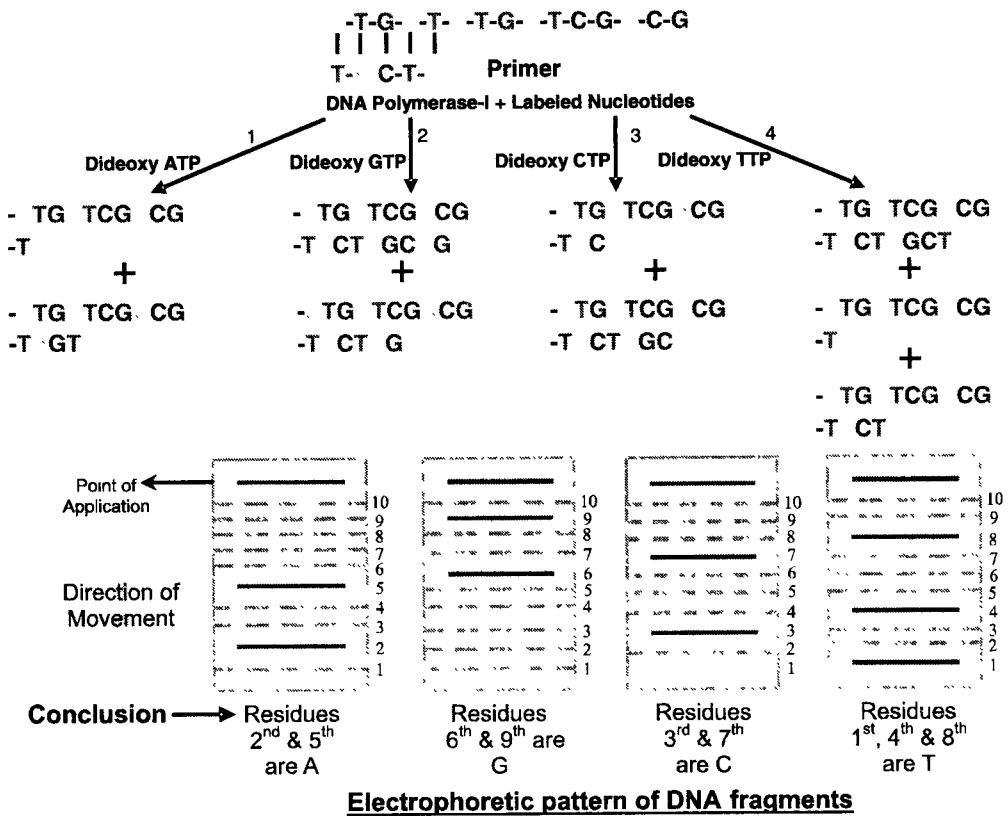
It is seen that the labeled fragments obtained by deletion of C residues moved at rates indicate that they had 5 and 8 nucleotide units. Thus residues 6th and 9th of the original polynucleotide must have been C. Likewise tallying the number of nucleotides in each set of the chemically reacted fragments derive that the sequence of the polynucleotide is -



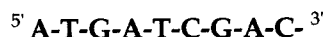
To depict the sequence of the complete DNA molecule, it is fragmented by different restriction endonucleases and each of the resulting fragment is sequenced as above and the overlaps in the fragments helps in arranging the sequence of the complete DNA.

- 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 labeled 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 di-deoxy 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.



The sequence is TACTAGCTG, which is the complementary strand. Hence the original strand will contain the sequence -



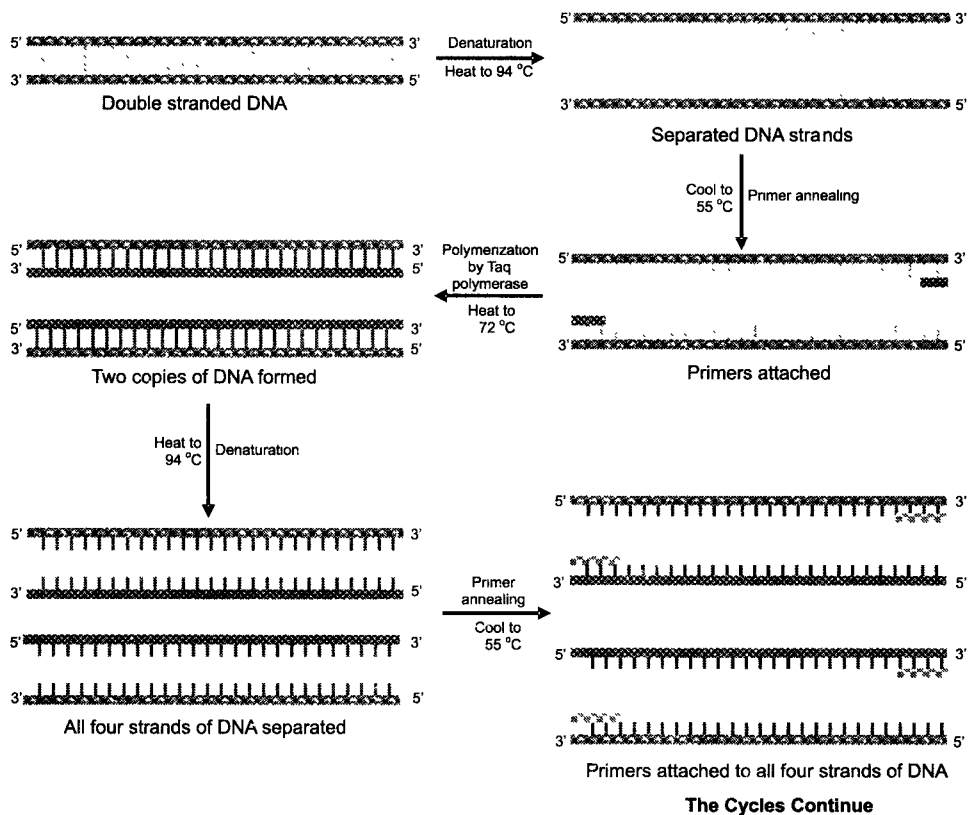
POLYMERASE CHAIN REACTION

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. MgCl₂ (1.5 mM) and 100 µg of gelatin is an additional component 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.

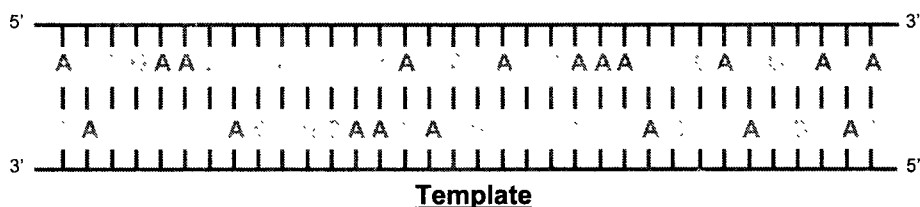


Mechanism of P.C.R. cycles

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 the double stranded template DNA strands separation. 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 and 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 naive 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 95 °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), dNTP's (0.7 to 2.4 mM), KCl (50 mM) and an optimum pH of 8.4 for its activity. Higher concentration of magnesium, dNTP's (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 does 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.

SUBUNIT VACCINES

Modern Vaccines or Sub-Unit Vaccines: Sub-Unit vaccines are specific cell (viral or bacterial) surface antigens, injected into the animal, which produces immunity against this virus and other related viruses.

Sub-unit vaccines are made from one or a few components of the organism, instead of injecting 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, rec DNA technology, gene cloning and the selection of antigens by the expression of the gene product. Animal diseases for which biotechnology offers new or improved vaccines are - for viral diseases like Foot and Mouth disease, Rabies, Parvovirus, African Swine Fever, Blue Tongue and New Castle Disease; for bacterial diseases like Neonatal Scours and Clostridial Toxicoses; for parasitic diseases like Trypanosomiasis and Helminthic diseases.

Design of Various Modern Sub-Unit Vaccines:

- a) **Vaccine for Foot and Mouth Disease:** Foot and mouth disease - FMD - is a viral disease caused by the FMD virus in farm animals. This virus is an RNA virus, with a diameter of 30 nM. It has four (4) structural glycosylated proteins designated as Vp1, Vp2, Vp3 & Vp4 on the capsid, which is a lipid bilayer. Out of these proteins, the main immunogenic site is the viral capsid protein Vp1. The genes for these structural proteins are located near the 5' end of the viral genome. A sub-unit vaccine for FMD virus is produced by developing a pure Vp1 protein.

There are two methods adopted for the production of a sub-unit vaccine for FMD virus.

1. **Protein Extraction from Whole Virus Culture:** The FMD virus is cultured in In-Vitro culture media in large amounts and the protein Vp1, is extracted from it and is injected into the animal to elicit an immunological response and thus generate memory cells to Whole FMD virus, which neutralizes the viral infection.
2. **Use of recDNA Technology for Production of Vp1 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 recDNA and is cloned in yeast cell as the host. Cloning of the recDNA in yeast cell produces glycosylated structural proteins but not in *E. coli*, which gives unglycosylated proteins (Vp1). Thus large amounts of Vp1 protein is obtained by gene cloning, which is injected to give protection to the animals against FMD disease.

The pure individual protein (Vp1) injected, though succeeded in eliciting an immune response in the vaccinated animal, 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 (Vp1 in this case) a mixture of plant glycoside saponin, cholesterol, and phosphatidylcholine is added to many copies of the protein Vp1 to form a cage-like structure, mimicking the natural micro-organism. These complexes known as immunostimulating complexes have antigenic activity equivalent to the viral vector.

There is further development in the preparation of sub-unit vaccine for FMD virus. In the FMD virus capsid protein Vp1, only 20 amino acids are truly antigenic/immunogenic present at two regions in the protein and these regions are termed as "EPITOPE". Therefore synthetic peptide is made with 20 amino acids in the same sequence as Vp1 epitope. Eight such peptides were linked together by lysine residues to form a frame work (immunostimulating complex)

and this sub-unit vaccine was equally good as an immunogen as that of the whole viral particle itself.

Further a synthetic DNA was prepared which can code for these 20 amino acids and was joined to the gene coding for hepatitis B core protein. This recDNA produced a Chimeric protein which takes a spherical shape of about 22 nm in diameter. This protein, obtained by cloning in the virus *Autographa californica* as the host, acted as a sub-unit vaccine and protected the animal from FMD, *on par* with the whole viral particle vaccination.

b) The 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 typhimurium*, *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 invitation by the virus of another strain causing the same disease. Therefore an effective sub-unit vaccine can be prepared by recDNA 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 avirulent 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 the animal becoming immune 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 its strains. Such recombinant vaccines are prepared for Rabies and rinderpest. Immunogenic genes from rabies virus have been cloned in vaccinia virus, which produced antibodies against rabies in animals.

c) Other Sub-Unit Vaccines: Sub-unit vaccines based on specific viral surface antigens are made for new castle disease, vesicular stomatitis, infectious bovine rhinotracheitis, gastroenteritis and rift valley fever. Bacterial vaccines are developed against neonatal scours. 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 prevents adherence of natural virulent bacteria thus preventing the disease.

A 68 Kd outer membrane proteins 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 an animal. 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.

- d) DNA Vaccines:** The most recent approach in the vaccine design is the use of synthetic DNA for the epitope of an antigen and produces a recombinant DNA, insert it into a non-virulent viral vector and inject it into the animal. This virus Proliferates in the animal 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.

ANIMAL 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.

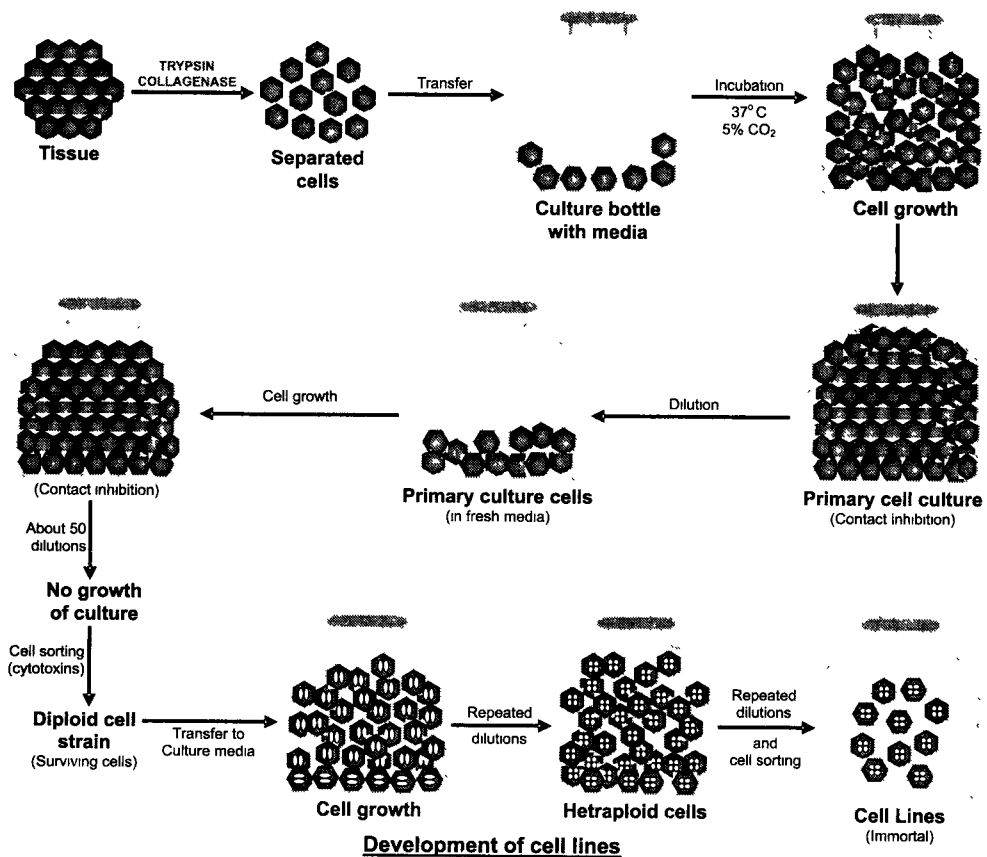
Those cells which can be grown *in vitro* are neither suitable for industrial use nor for scientific purpose because many cells die during the course of time releasing toxic substances which inhibit the activity of other live cells. Hence, in order to avoid this problem and to achieve an exponential 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 contain nutrient solution, known as culture media (made up of appropriate salts and nutrients). These cells are incubated in 5-10% CO₂ 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 looks like the tissue from which they are derived. This culture is referred 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 build-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, making them effectively immortal. These survivors are known as "**cell line**". They are said to have undergone transformation. Transformed cell lines grow indefinitely, exhibits heterogeneity, lose contact inhibition and does 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.



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, MgSO₄, CaCl₂, NaHCO₃, KH₂PO₄, gassed with O₂-CO₂ 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). Tyrpan 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).

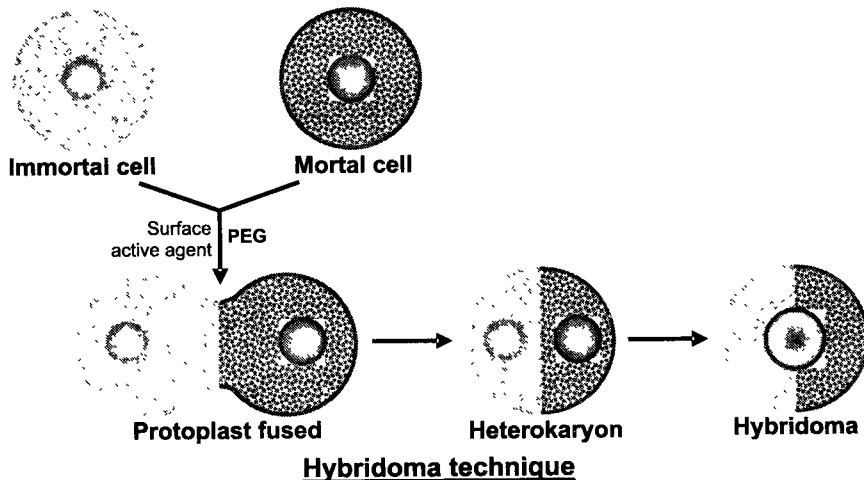
Hybridoma technique is the process of producing a hybridoma cell. Antibody raised to a particular antigen in the serum is used for many purposes like the detection of presence of a toxic substance in clinical samples and also for its quantization, localization and purification. Any antigen will contain 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 differs 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

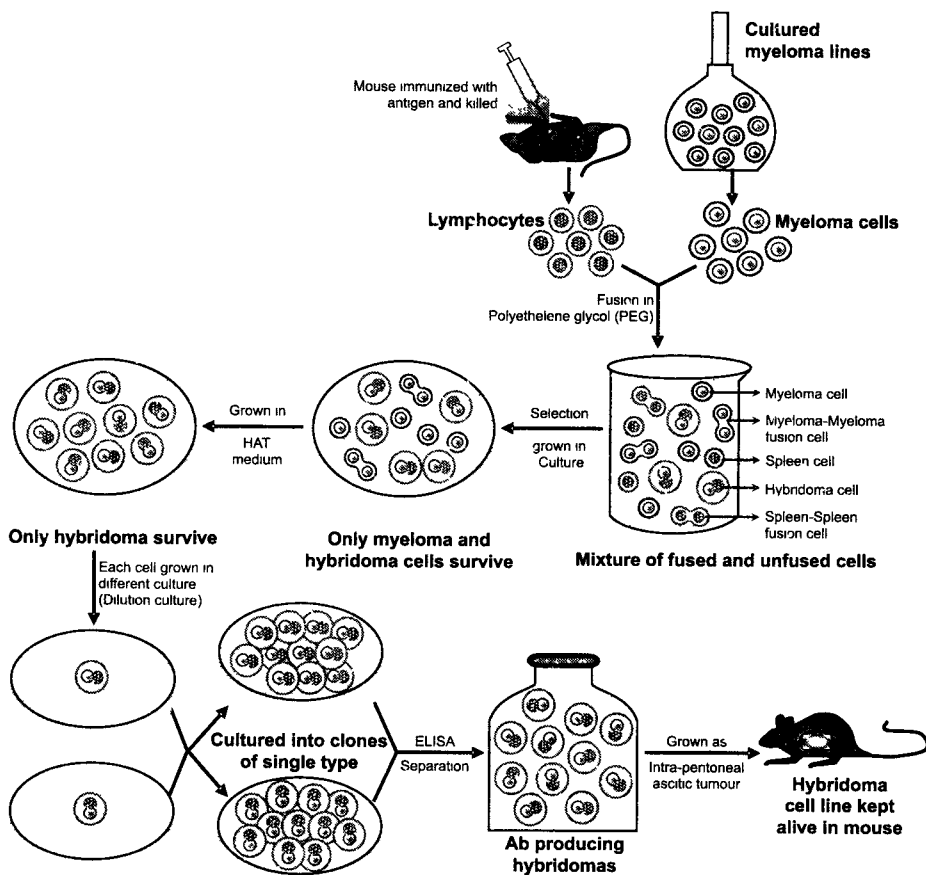
components for producing this specific antibody and the cell division functions of a similar type of cell so as to enable the hybrid cells to grow in culture. Some B-lymphocytes, naturally become cancerous (myeloma) and hence become able to grow in culture. So these myeloma cells were used to fuse with 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.



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 hybridomas 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 Immunosorbent Assay (ELISA) or solid phase radio-labeling. 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-labeling and the hybridoma cell 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 secretes 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 intra-peritoneal 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) Analyze mixtures of complex antigens.
- (4) Determination of the structure of cell membranes and membrane proteins.
- (5) Labeling 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.

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.

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 just above is replaced by carbon dioxide released.

- f) 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 cooking steel 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, where as 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. Further more the metabolizing microbes replace the oxygen with carbondioxide released.

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 ($1500/\text{m}^3$). 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 common foods are usually lacking or not in sufficient quantities of lysine and methionine amino acids. Single cell protein produced by different sources is

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.

Lysine-rich SCP (7g lysine/16 g N) producing organisms are *Chlorella sorokiniana*, *Cellulomonas alkaligenes*, *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 of a carbohydrate (like hexose sugar) by various microbes are outlined below -

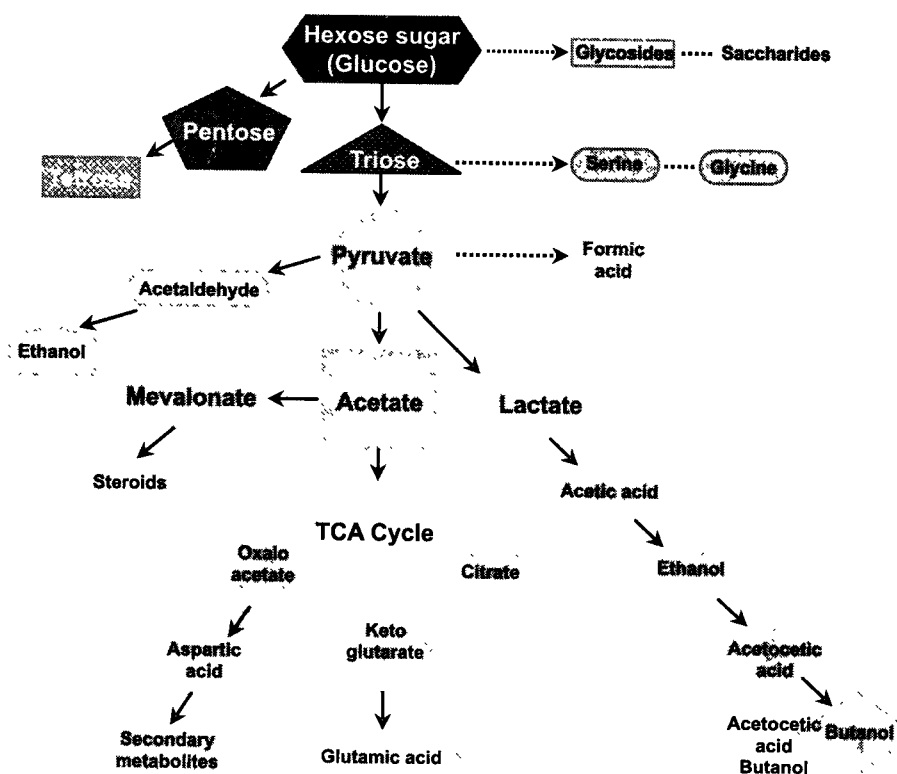
The metabolites produced by the microbes can be grouped into two categories - (a) Primary metabolites and (b) Secondary metabolites.

(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



Pathways showing production of primary metabolites

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*

Chloramphenicol-*Streptomyces venezuelae*

Erythromycin-*S. erythraeus*

Gentamycin-*Micromonospora purpurea*

Gramicidin-*Bacillus brevis*

Penicillin-*Penicillium chrysogenum*

Streptomycin-*S. griseus*

Tetracycline-*S. aureofaciens*

These microbes are grown under suitable fermentation conditions so as to get the desired antibiotic. Mutant microbes are used for industrial production of antibiotics.

- 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 cell so as to induce them to produce enzymes like renin by *E. coli* and amylases by *Bacillus stearothermophilis*. These enzymes are generally bound to matrix or in some manner retained in a 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 thermophilic 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 stearothermophilis thermosia*. Further, genes for thermophilic enzymes are introduced into *E. coli*, which is cultured 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 was 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 constituent for which techniques like sedimentation, floatation, filtration, ultra-filtration, centrifugation and micro-filtration are applied.

Solid State Fermentation: Fermentation carried out on a solid matrix containing absorbed moisture is called solid state fermentation (SSF). The normal fermentation is carried out in dilute liquid (water) media. Examples of SSF are rotting of wood, ripening of cheese, composting of wastes. Some enzymes like cellulase, protease and amylases are being produced by solid state fermentation. S.S.F can be carried out in less space, without sterilization, and aeration and gives high and pure products not requiring purification.

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Biotechnology in Reproduction

INDUCTION AND SYNCHRONIZATION OF ESTRUS AND OVULATION

Biotechnological methods enable reproductive rates of dairy animals to be maximized by breeding females for the first time at the earliest opportunity. They are then rebred promptly after each parturition, thus overcoming delayed onset of puberty or post partum anoestrus. In order to induce ovulation in anoestrus females, a single follicle or group of follicles are stimulated to a state of maturity so that a natural surge or Luteinizing Hormone (LH) will cause ovulation.

Ovulation in normal cycling animals can be manipulated artificially by controlling the functioning of Corpus Luteum (CL) through either its luteolysis by prostaglandins or mimicking its function by administering progesterone exogenously. The detailed methodology used to induce and synchronize estrus in different animals is as follows -

1. Progesterone as an ear implant (norgestomet) or as an intravaginal pessary is given for 7-12 days and then withdrawn, which leads to ovulation in 24 hours after withdrawal.
2. Prostaglandin single injection and double injection is another method to induce ovulation, provided there is presence of a mature CL (which occurs between day 6 and day 16 of the estrus cycle). Lutalyze injection (composed of PGF₂-Alpha) is injected 25 mg IM and the animal will come to heat in 2-4 days after the injection. Those animals which do not show estrus conditions after this treatment are separated and another dose of the same is injected on the 11th day (higher conception rate is seen, if injected on 14th day) after the first injection to make all the animals ovulate.
3. Ovulation can also be induced in postpartum milking cows by injection of GnRH on day 14 postpartum, wherein they ovulate one day after treatment.
4. Injection of 5 mg of estradiol valerate (estrogen conjugate) brings the animal immediately to heat, but there is no guarantee that the animal will ovulate.

SUPEROVULATAION

Superovulation is a process of artificial induction of increased ovulation, by external agents, over and above the natural level of ovulation expected to occur for a given animal.

In dairy cattle only one follicle is natured at a time (i.e., per cycle) releasing a single ovum, resulting in production of a single offspring in a year i.e., to say that normal breeding in cows gives one calf in a year and 5-6 calves in whole life time. Biotechnology has evolved methods to extract increased number of embryos from animals of superior genetic merit by superovulation and develop in some other recipient animal (of the same species) by a process called Embryo Transfer Technology (E.T.T.).

Super-ovulation is induced by subcutaneous or intramuscular injections of pregnant mare serum gonadotrophin (PMSG), recently called equine chorionic gonadotrophin (eCG), or Follicle stimulating hormone (FSH), in order to stimulate additional follicular growth. In Cows, Goats and Sheep, once the follicles grow and mature, they ovulate spontaneously under the influence of internal estrogen. However other animals are required to be injected intravenously with Leutinizing hormone or Human chorionic Gonadotrophin (hcG). Recently superovulation is created by the use of prostaglandin F₂-alpha analogous, such as cloprostenoc. These treatments are given at different days of estrus cycle depending upon the animal.

Commonly available hormones for Superovulation

Hormone	Commercial Name	IU Present in the Vial
PMSG	Folligon	1000 or 5000 IU
PMSG	Trophovet	1000
FSH- P	FSH- P	50
Purified FSH	Super - Ov	75
Purified FSH	Folltropin	-

The ovulatory response is quite variable and is dependent on various factors. Follicles on each ovary grow in a fashion of waves. There are usually three waves in each estrous cycle and the recruitment of growing follicles is maximum in second wave which commences on 9th day of the cycle. Therefore, superovulatory treatment starting on day 9 or 10 of the cycle leads to more ovulations as compared to treatments started very early or late in the cycle. The presence of dominant follicle at the time of superovulatory treatment results in less number of transferable embryos, since a single large follicle inhibits the growth of other smaller follicles. However, the growth of this dominant follicle can be

suppressed with oestradiol treatment on day 1 or 3 of the cycle. The dominant follicle can also be removed by unilateral ovariectomy or by electrocautery.

ARTIFICIAL INSEMINATION (A.I.)

Introduction of the semen into the female reproductive track, by methods (manually) other than copulation by a male is known as Artificial Insemination (A.I.).

Artificial insemination enables the genetic improvement of animals. Spermatozoa produced by any male (especially of selected high genetic merit) are sufficient to inseminate thousands of females per year by A.I. whereas natural mating produces only a few progeny per selected female, even by superovulation and embryo transfer technology. Hence Biotechnology has developed methods by which semen is artificially collected from superior males, concentrated, X & Y spermatozoa separated, preserved in cold for longer & wider use and finally inseminated artificially.

The uses or advantages of A.I. are:

1. Genetic improvement of animals.
2. It is required after superovulation and synchronization of estrus in large group of animals, so as to produce more embryos, thus facilitating E.T.T.
3. It controls spreading of sexually transmitted diseases.
4. Accelerates the introduction of new genetic material intercontinentally.
5. Provides a good tool for the study of male and female reproductive physiology.

Methods of Artificial Insemination: The semen is either collected freshly or the cryopreserved semen is used for A.I. The cryopreserved semen stored in appropriate vials is thawed to an appropriate temperature and inseminated artificially. A.I. is done within 12-14 hours of onset of heat or estrus, which is identified by cows mounting other cows, swollen vulva, mucus discharge from vagina, frequent micturition and restlessness.

There are two main methods of artificial insemination

- a) **Recto-Vaginal Method:** A special equipment is made for this method. It contains a glass catheter and a 2 ml syringe to draw or inject semen. The catheter contains 40-45 cm long glass pipetter. Semen is taken into the syringe and the animal is made to stand in the stall. The external genitalia are cleaned and one hand of the inseminator is lubricated and inserted through the rectum and the cervix is located. Then the sterilized glass catheter is introduced through the vulva and vagina into the OS of the cervix and the tip of the pipette is passed into the length of the cervix. The semen is deposited in this region by pushing the syringe piston.

This is the more commonly used method.

b) **Vaginal Speculum Method:** This method of A.I. is rarely used.

EMBRYO TRANSFER TECHNOLOGY

Transfer of embryos from a valuable donor (superior over others in genetics and characters) to recipients, thereby producing increased number of valuable off springs in a short duration of time is known as Embryo Transfer Technology (E.T.T.).

Embryo Transfer Technology is an animal improvement programme which increases productivity through faster multiplication of superior females (donors) reducing generation intervals, exploitation of female gene pool, faster evaluation of sire, sexing of embryos, cloning and genetic manipulation of embryos.

Normal breeding in cows gives one calf in a year and 6-7 calves in whole life. E.T.T. produces 4-5 calves yearly and over 25 calves in whole life span.

A sexually mature female, referred to as the donor, is injected with external hormones to produce more ova, which are fertilized inside her preferably by artificial insemination. The embryos are then removed prior to their implantation and transferred to the reproductive tracts of synchronized substitutive mothers of the same species referred to as the recipients. The fertilized ova i.e., embryos are thus developed to its full term in the recipients body and the resulting offspring derives their genes from the donor and from the male to which the donor was bred.

The steps involved in E.T.T. are:

1. Selection and management of donors and recipients.
2. Superovulation and estrus synchronization of the donor and the recipients.
3. Insemination of the donor.
4. Collection of embryos.
5. Characterization and storage of embryos.
6. Transfer of embryos into the recipients.

Procedure for Embryo Transfer Technology: Embryo Transfer technology (E.T.T.) in cows is carried by selecting high yielding donor fertile cows, with normal sized healthy and functional reproductive organs free from any uterine infection. The recipients are also selected who are fertile with normal sized healthy and functional reproductive organs in their 2nd to 6th stage of lactation.

The selected animals are screened for negative brucellosis, tuberculosis and tumours and vaccinated against common diseases. The ration supplied has all of the 20 essential nutrients in adequate levels, meeting the minimum requirements of crude protein, total digestible nutrients and vitamins & minerals.

Both the donors and recipients estrous cycles are made to run on the same periods. The recipient's estrus is synchronized (periods made the same) with that of the donor by any one of these method is:

- 1) **Natural heat selection method:** 5% of animals in a herd of cycling recipients can be expected to show heat on any particular day. These recipients showing heat on the same day are selected as the donor for E.T.T.
- 2) **Prostaglandin single and double injection method:** Synchromate - B as an ear implant (kept under the skin of the ear) containing norgestomet and 2 ml injection of norgestomet / estradiol valerate. The implant is removed on 10th day and the animals' shows heat in 35 hours after removal of the implant. PRID is progesterone releasing intra vaginal device, which is placed in the vagina of the recipient and removed at 10th day showing estrus in 2-4 days. Prostaglandin F₂-alpha is injected 25 mg IM and estrus exhibited in 2-4 days after injection. Another injection of the PGF₂-alpha can also be given 11 days later and heat is exhibited 2-4 days after 2nd injection.

The donors wave of follicles are rescued from degeneration resulting in maturation of multiple follicles thereby producing superovulation, for which any one of the following protocols can be followed -

Species : Buffalo
 Drug : FSH-P
 Dose : 35 - 50 mg NIH
 Schedule : 4 or 5 days : Descending/constant
 Day of start : 9 to 12 day of estrus cycle

Example - 1: 50 mg : 1 mg/ml : Descending

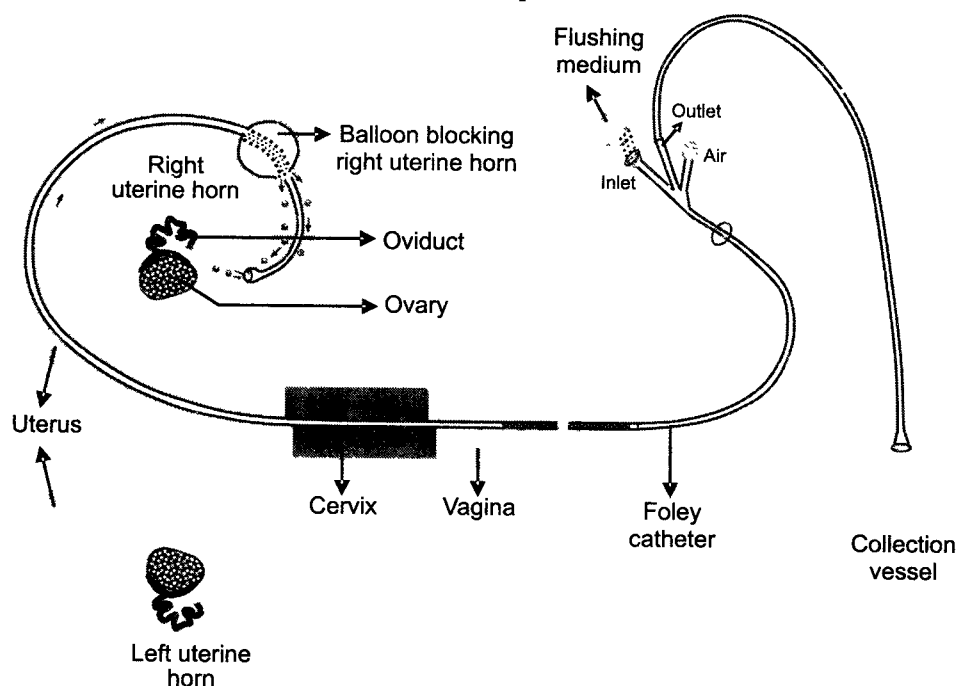
Day of Injection of Drug	Morning (MI)	Evening (MI)
1	8	8
2	7	7
3	6 + PGF (30mg)	6 + PGF (20mg)
4	4	4
5	A.I	A.I
6	A.I	-

Example - 2: 36 mg : 1 mg/ml : Constant

Day of Injection of Drug	Morning (MI)	Evening (MI)
1	4.5	4.5
2	4.5	4.5
3	4.5 + PGF (30mg)	4.5 + PGF (20mg)
4	4.5	4.5
5	A.I	A.I
6	A.I	-

A.I. in the above protocol table indicates Artificial Insemination, which is one of the factors determining the success rate of E.T.T. A.I. is done after the onset of heat is detected in the donors. Then semen of high pedigree is selected from the cryopreserved semen bank. The semen is thawed at 350 C and the straw is inserted into pre-warmed insemination rod. The semen is deposited in OS-uterus. Two to three inseminations at 12 hours interval are made so as to fertilize the wave of follicles ovulating over a wide time span.

Embryos are recovered non-surgically. The estimation of the number of corpora lutea is made by palpating the ovaries per rectum. A cervix dilator is passed through the cervix and the correct gauge catheter is inserted, which is placed in the right or left uterine horn depending upon the site of ovulation such that the balloon of the catheter is ahead of the bifurcation of the horns. A syringe is attached and air is blown to make the balloon tight, in order to seal the horn. Now the catheter is plugged. The system is now flushed with Dulbecco Phosphate buffered saline with 0.4 % lyophilized BSA, at body temperature. Approximately 500 to 700 ml of the flushing medium is used and the cuff is deflated. The contents of the embryo filter are poured in a plastic dish with grid and searched under a zoom stereomicroscope.



Technique of Embryo Transfer Technology

Depending upon the morphology of the embryos they are grouped into various classes as given under -

Quality of Embryo	Characters
Excellent	Perfect embryo for its stage. The blastomeres are of similar size with even colour and texture. They are neither very light nor very dark. The cytoplasm is not granular or unevenly distributed and contains some moderate sized vesicles. The perivitelline space is empty and of regular diameter and the zona pellucida is even and neither wrinkled nor collapsed.
Good	Trivial imperfections such as an oval zona, a few small excluded blastomeres and slightly asymmetrical.
Fair	Definite but not severe problems such as moderate numbers excluded blastomeres, small size and small amount of degeneration.
Poor	Partly degenerate, vesiculated cells, greatly varying cell size, very small or similar problems.
Very Poor	Severely degenerate, probably not worth transferring, unfertilized, zona only ghost like, 3-cell, debris, bacteriologic contamination.

Only the transferable (good to fair) embryos are transferred in the epilateral horn of the synchronized recipients, by non surgical means, which involves loading of the inseminating straw with the evaluated transferable embryos the straw is then loaded into the inseminating gun and the embryo is deposited deep into the uterine horn as far as possible without causing trauma to the sensitive uterine wall.

Summary of Events during E.T.T.

Day of Estrus Cycle	Event
-3	Induction of estrus (injection of PGF ₂ - alpha)
0	Induced estrus/natural estrus (confirmation through bull parading or per rectum)
9	CL confirmation of abnormal animals
10	Start superovulation of donors
11	Induction of estrus in recipients (PGF ₂)
12	Induction of super estrus in donors (PGF ₂)
14	Donors / recipients in estrus, confirmation, breeding of donors
19 & 20	Flushing of buffalo donors
20 & 21	Flushing of cattle donors
22 & 23	PGF injection

PREGNANCY DIAGNOSIS

The main purpose of pregnancy diagnosis is to enable the early identification of the non-pregnant females and to reduce the chance of infertility in females. Clinically pregnancy is diagnosed by rectal palpation at 45 ± 5 days after A.I., ultrasonography at 28 ± 2 days after A.I., immunological methods and also by chemical tests.

Chemical Tests:

- 1) An orange colour is obtained when mucus from pregnant animals is heated with 10% sodium hydroxide.
- 2) Quantitative estimation of mucus shows 110 to 130 mg % Fructose in pregnant animals, whereas in non pregnant it is 35-65%.
- 3) Mucus turns to rubber like substance when mixed with saturated solution of copper sulphate, in pregnant animals.
- 4) Milk of pregnant animals coagulate when 1 ml is mixed with 10 ml of 3 % copper sulphate and heated.
- 5) Urine of pregnant animals does not precipitate when added to 1 % barium chloride solution, where as the urine of non-pregnant animals precipitate.

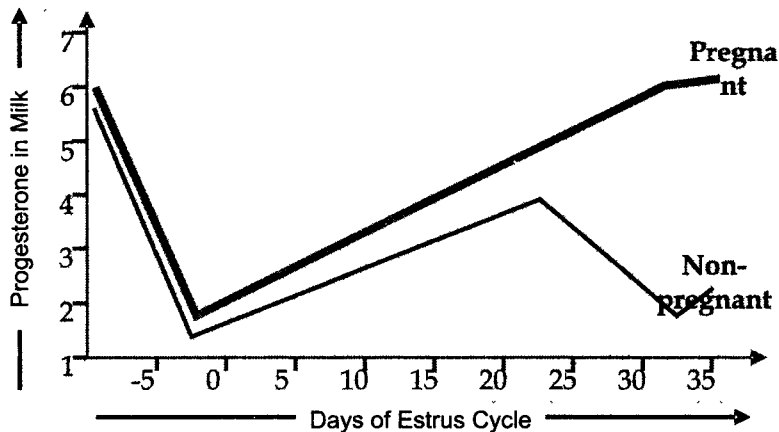
Ultrasonic Techniques: It is based on the principle that sound waves striking a moving object are reflected to the transmitting source at a slightly altered frequency. Pregnant and non-pregnant animals give different reflections of the sound waves, which forms the basis of pregnancy diagnosis.

There are two methods of ultrasonography by which pregnancy diagnosis is performed:

- 1) **Doppler Phenomenon:** This technique detects sounds from foetal movements, foetal heart, uterine arterial circulation and the umbilical vessels. The detector consists of an amplifier and a transducer, which is applied as a probe to the animal's abdomen or inserted into the rectum. The probe emits a narrow beam of low energy that is reflected from tissues to the probe where it is converted into sound.
- 2) **Pulse-echo Ultrasound:** Pulses of ultrasound are produced by a transducer. When the ultrasound contacts tissues of varying transducer and converted into electrical energy and displayed on a cathodes ray oscilloscope in various ways.

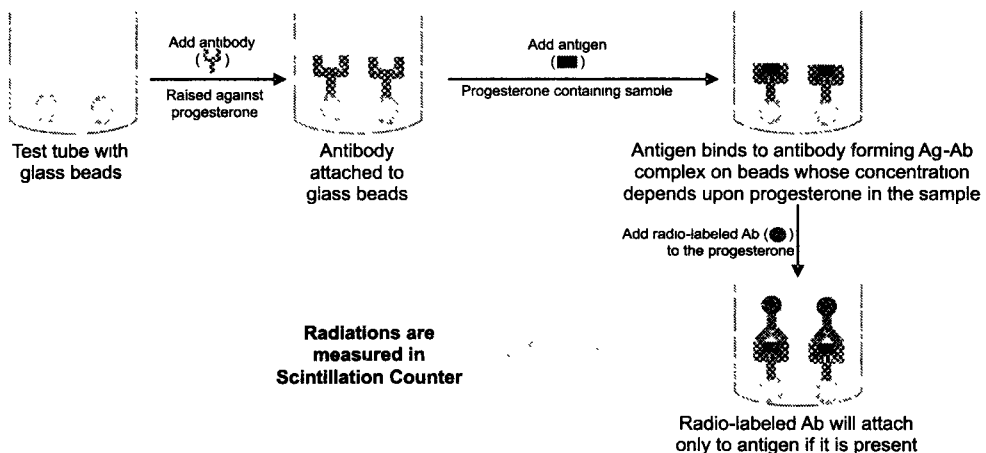
Immunological Methods: Pregnancy diagnosis is also done by assaying milk or blood samples for progesterone by Radio-Immunoassay (RIA) or Enzyme-Immunoassay (EIA), (ELISA) Enzyme Linked Immunosorbent Assay. Milk samples or blood samples are collected at regular intervals of 5, 10, 15, 20,

22, 24 days after A.I. and progesterone is estimated quantitatively. The progesterone level in milk / blood is elevated in pregnancy, whereas it is low in non-pregnant animals. Curve below shows the progesterone levels in milk during the various days of estrus cycle in pregnant and non-pregnant animals.



Progesterone Levels in Milk during Days of Estrus cycle in Pregnant and Non-Pregnant Animals

Early pregnancy factors (E.P.F.) is found in the circulation of pregnant animals, which has immunosuppressive properties and can be detected in the serum owing to its property of reducing the number of rosettes formed in the rosette-inhibition test.



Radio-Immuno-Assay (RIA) Technique for Progesterone Estimation

IN VITRO FERTILIZATION

Fertilization of an Oocyte by a sperm in a test tube, outside the animal body is known as *in-vitro* fertilization (I.V.F.). I.V.F. is of practical significance to obtain large numbers of embryos for scientific investigation or for subsequent transfer to appropriate recipients.

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 fertilization. This is known as capacitation of sperm.

For the *in vitro* fertility (i.e. fertility in glass) the spermatozoa from the artificially collected semen 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.

Oocytes for I.V.F. are obtained from the oviducts, follicles and surface of the ovary, shortly after ovulation, either from live animals by flushing some medium, as described in Embryo Transfer Technology, or from slaughtered animals. 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. Spermatozoa readily become attached to the surface of zona pellucida and passes through this membrane, affecting the fertilization.

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 sperm with vigorous motility and a fertilizable ovum with a first polar body.

TRANSGENIC ANIMALS

Transgenic animals are those animals containing incorporated extra-parental DNA sequences. They are produced by microinjection of genomic DNA into the male pronucleus of recently fertilized ova.

The livestock companies or research organizations produce transgenic animals by incorporating their own "hall - mark - DNA" sequences into the pronucleus of the recently fertilized ova of a particular animal. For example - the gene for good quality meat production from goat is incorporated into sheep with superior quality wool, thereby producing a transgenic animal giving both wool and meat of high commercial value. Similarly genes for medicinal proteins in milk are being incorporated.

The desired gene is joined (spliced) to a promoter sequence of bacterial origin producing a recDNA or chimeric gene. Numerous copies of this chimeric gene are made either by cloning in a host cell or by Polymerase Chain Reaction technology. The DNA copies so obtained are injected into the fertilized ova. Several copies of the gene are incorporated in random into any chromosome, where there happens to be a break at that particular time (during natural crossing - over process). Therefore the expression of extra-parental gene is unpredictable. Hence the Noval gene sequence is detected by carrying out the DNA finger printing of the entire genomic DNA. The detection of the inserted gene is also done by making a cDNA probe from mRNA of the affected tissue and gene library is thus constructed.

It has been established that genes from different species can be integrated into various species and thus expressed as a mixed transgenic animal.

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SECTION - IV
Clinical Biochemistry - VBC -411
IVth YEAR/ Ist SEMESTER - FIRST COURSE
<i>This course is designed to expose the students to various clinical and applied aspects of biochemistry. They are expected to be well versed with the biochemical changes in conditions of health and disease, diagnostic importance of various biochemical constituents, biochemical role of hormones and immunoglobulins. There can be no book, good enough to include all these topics in depth. This book gives only the outlines, just sufficient for the examination point of view. Hence the student is required to refer to standard books like Clinical Biochemistry of Domestic Animals edited by J.J. Kaneko, J.W. Harvey and M.L. Bruss of Academic Press.</i>

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Clinical Biochemistry

Biochemistry is applied in each and every field of veterinary sciences. This is the basic subject which supports and enables the student to understand all the other disciplines of veterinary viz. physiology, microbiology, pathology, pharmacology, medicine and even gynaecology and obstetrics.

ACID BASE BALANCE

Respiration is a process of exchange of gases (O_2 & CO_2) in the alveoli. 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 releasing 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 favors 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.

Physiology of acid-base balance: pH is defined as the negative log of hydrogen ion concentration. The normal pH of blood is 7.35 to 7.45 (average is 7.4). The pH is always maintained within this normal range because :

- (a) Most of the proteins are very sensitive to hydrogen ion concentration; any change in the $\{H^+\}$ disrupts the activity of the protein.
- (b) Many enzymes have an optimum pH in this range and a change in pH results in the change in the rates of reaction of these enzymes.

pH varies inversely with hydrogen ion concentration $\{H^+\}$. When $\{H^+\}$ in blood increases, pH decreases and the animal develops acidosis. When the $\{H^+\}$ decreases, pH rises and the animal develops alkalosis. The acid-base balance in the body involves the following mechanisms.

- (1) Extracellular and intracellular buffering.
- (2) Regulation of the rate of alveolar ventilation

(3) Regulation of renal hydrogen excretion.

Buffers: They are substances that resist the change in pH. They are able to take up or release hydrogen ion. So that changes in hydrogen ion concentration are minimized. A buffer system consists of a weakly dissociated acid and the salt of that acid. The buffer systems of the body are (1) The extracellular buffers (2) The intracellular buffers (3) The bone

The extracellular buffers are (a) Bicarbonate buffer ($\text{HCO}_3^-/\text{H}_2\text{CO}_3$) (b) Phosphate buffer ($\text{HPO}_4^-/\text{H}_2\text{PO}_4$) (c) Plasma proteins (Albumin or Hb)

The intracellular buffers are (a) Proteins (amino acids) (b) Organic and inorganic phosphates (ATP, H_3PO_4) (c) Hemoglobin in RBC.

Cation exchange involving the intracellular movement of hydrogen ion in exchange for potassium (or sodium) is another cellular mechanism of buffering system. The carbonate in the bone contributes upto 40% of the buffering capacity of the body.

Acidosis: It is lowering of the blood pH. Animal can bear pH of 7.0 to 7.8. pH below 7 or above 7.8 leads to the death of the animal. Alkalosis is more fatal than acidosis because the animal has well developed mechanisms to counteract acidosis. The reasons for acidosis are-

- (1) Irregular metabolism – Metabolic acidosis
- (2) Disturbances in respiration – Respiratory acidosis
- (3) Disturbances in kidney –Renal acidosis
- (4) Miscellaneous disturbances.

Respiratory acid-base balance: Lungs play a crucial role in the acid base balance of the blood. In the event of acidosis the lungs undertake hyperventilation (increased rate of respiration) and when alkalosis occurs there will be hypoventilation (decreased respiratory rate). During hyperventilation (acidosis) there is excessive elimination of CO_2 thereby reducing the H_2CO_3 concentration and thus decreasing the $\{\text{H}^+\}$ concentration and resulting in increase in the pH. Conversely in alkalosis there will be hypoventilation preserving H_2CO_3 and thus increasing the $\{\text{H}^+\}$ concentration and resulting in decrease in the pH of blood.

Respiratory acidosis:

Identification: Increase in the level of CO_2 and H_2CO_3 in blood

Reasons and biochemical changes: Hypoventilation resulting in accumulation of CO_2 and increased H_2CO_3 .

Clinical causes: Pulmonary diseases interfering ventilation like acute upper respiratory obstruction, pneumonia, chronic obstructive lung diseases.

Medicines like salicylate, methanol, ethylene glycol etc. Drugs affecting CNS that inhibit the medullary respiratory centre like general anesthesia using volatile agents viz. chloroform, ether, opium, barbiturates. Congestive heart failure.

Symptoms: Depression of CNS, reclamation, coma and death in sever cases.

Physiological compensation Hyperventilation, more of CO_2 is released to decrease the acidity. Renal excretion of H^+ increases and retention of HCO_3^- by kidney is seen.

Buffers: Only proteins and phosphates can act as buffers but not carbonate.

Treatment: High pressure ventilation apparatus to be used.

Diagnosis: Arterial blood with heparin anticoagulant is collected anaerobically for estimation of gasses, bicarbonate and titratetable acids.

Respiratory alkalosis: Increase in the blood pH, above 7.45, upto a maximum of 7.8 is termed as alkalosis. It is not common and also not easily regulated by the body hence more fatal.

Identification: Decrease in CO_2 in blood

Reasons and biochemical changes Hypoventilation (strangulation)

Clinical causes: Pulmonary disease, congestive heart failure, severe anaemia, pain, psychological stress, non-sweating animal like dogs show hyperventilation, especially in summer.

Physiological compensation: Blood bicarbonates are buffered by the intracellular buffers like proteins. Kidneys excrete more of bicarbonates and retain more of H^+ .

Symptoms: Non symptomatic fatality.

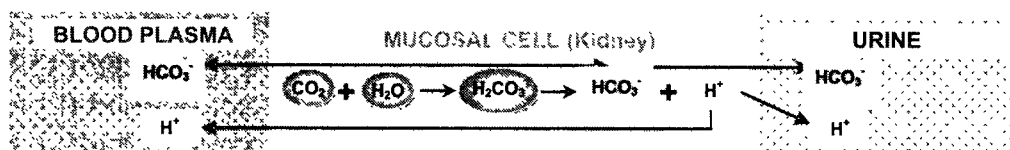
Renal acid-base balance: In total renal failure the animal dies within a week due to imbalance in acid-base regulation. In renal dysfunction, "azotemia" occurs due to accumulation of nitrogenous substance in blood like urea, uric acid and creatinine etc. Kidney regulates the acid-base balance by two mechanisms.

(1) Through bicarbonate i.e. the extracellular buffers.

(2) Through phosphates and ammonia i.e. kidney's intracellular buffers.

Regulation of acid-base balance by kidney through bicarbonates: Carbon dioxide (CO_2) released by the metabolic process combines with water (H_2O) forming carbonic acid (H_2CO_3) with the help of the enzyme carbonic anhydrase. At the normal pH of the blood, carbonic acid dissociates into bicarbonate HCO_3^- and protons (H^+). Blood bicarbonate is easily filtered by glomerular filtration and

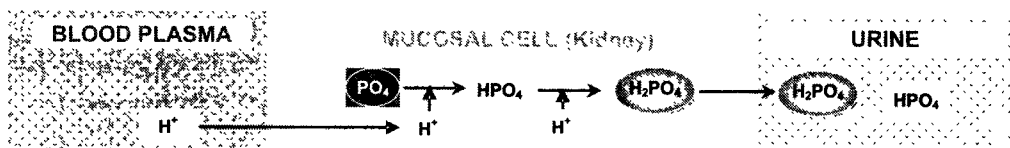
is totally reabsorbed back into the circulation by the proximal tubules. Metabolism by the kidney cells also produces H_2CO_3 which releases HCO_3^- & H^+ .



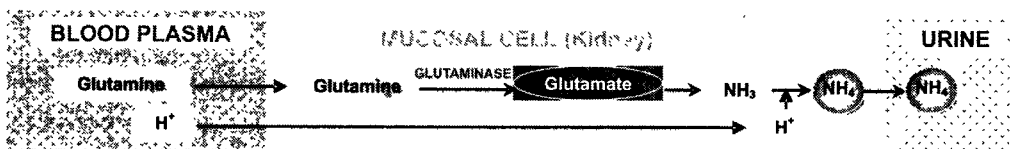
In acidosis HCO_3^- is totally reabsorbed without elimination of any bicarbonates in the urine. Further the production of HCO_3^- by the kidney increases 10 folds which enters the blood stream aiding in increasing the pH of the blood. All the H^+ produced is excreted in the urine.

In alkalosis, there is increase in the excretion of HCO_3^- by the kidney and reduced or negligible reabsorption into the blood. Whereas H^+ is completely absorbed into the blood stream with any H^+ being excreted in the urine.

Regulation of acid-base balance by kidney through Phosphates: Hydrogen ions present in the blood diffuses into the kidney tubules and combines with inorganic phosphate producing HPO_4^- which again combines with another H^+ ion forming H_2PO_4 . Both HPO_4^- and H_2PO_4 are excreted in the urine thereby overcoming the acidic pH of the blood.



Regulation of acid-base balance by kidney through Ammonia: Most of the ammonia is converted to urea in the liver. The remaining ammonia from all the tissues is concentrated on the amino acid, glutamic acid. In the tissue ammonia combines with glutamic acid with the help of the enzyme glutamine synthase and forms the amino acid glutamine. It is released into the blood circulation and enters the kidney where it is acted upon by the enzyme glutaminase to release free ammonia and glutamic acid. Ammonia (NH_3) combines with hydrogen ions in the kidney and gets converted to ammonium ion (NH_4^+). This is water soluble and is excreted in the urine, carrying H^+ ions with it, thereby combating acidosis.



BIOCHEMISTRY OF STRESS AND SHOCK

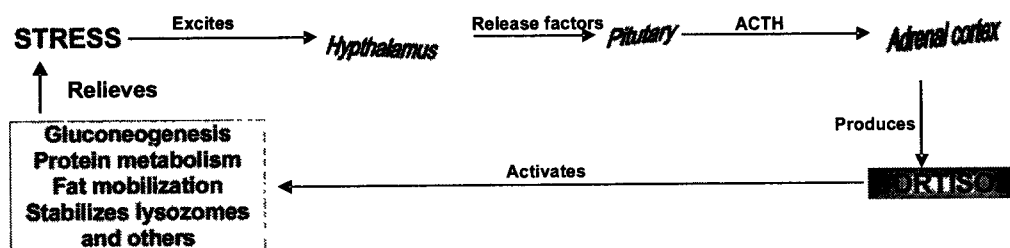
Stress: It can be defined as a continuous pressure upon the animal body. Stress can be classified into :

- (a) **Physical stress:** Heavy exercise, dog tied tightly, intense heat or cold, fracture, length of the day, heavy duty etc. are the categories of physical stress.
- (b) **Neurogenic (nervous) stress:** Infection, trauma, surgery rage and fright are the reasons that cause nervous stress.

Many biochemical changes occur, in-order to combat/overcome this stressful condition. The main reaction is that a large portion of the sympathetic nervous system discharge at the same time termed as Mass discharge, which increases the ability of the body to perform vigorous muscle activity due to which / or the changes that are seen during stress are-

1. Increased arterial pressure.
2. Increased blood flow to active muscles, with decreased blood flow to organs such as the G.I. tract and kidneys.
3. Increased rates of cellular metabolism like protein mobilization, fat mobilization, stabilization of lysosomes.
4. Increased blood glucose due to increased glycogenolysis and gluconeogenesis.
5. Increased muscle activity
6. Increased mental activity
7. Increased rate of blood coagulation.
8. Increased rate of hormonal secretion like epinephrine, ACTH, cortisol, thyroxine and gonadal hormones.

The sum of these effects permits a person to perform far more strenuous physical activity than normal. This is called as sympathetic stress response. Stress initially reduces gastric acid secretion but prolonged stress increases acid secretion leading to ulcers.



Shock: It can be defined as a sudden pressure upon the animal. This is also physical and neurological.

Causes of shock:

- (1) **Inadequate cardiac output:** Resulting from hypovolemia (whole blood loss or plasma loss). This can be due to decreased ability of the heart to pump blood, decrease in venous return (low blood volume, decreased vascular tone, obstruction to blood flow).

Hypovolemia due to haemorrhage, plasma loss due to vomiting, diarrhoea, excessive copulation which leads to vasomotor failure. There will be blockage in minute vessels (blood). Increased capillary permeability, release of toxins by cells and generalized cellular deterioration. There will also be tissue necrosis, acidosis, anaesthesia, muscle weakness, decreased body temperature, depressed mental function, reduced renal function and renal deterioration.

When blood is lost from the animal body it passes into a condition of shock, this results in increase in the heart rate to 150 to 180 beats/minute, due to the sympathetic nervous system exiting the heart.

Treatment: Fluid therapy wherein dextran as plasma substitute can be given or blood and plasma transfusion can also be used in case of hypovolemia. Drugs like epinephrine, norepinephrine, and histamine help in relieving shock. The best life saving treatment for shock is treatment with glucocorticoids (produced by adrenal cortex). Oxygen therapy (infusion) or standing on head down position relieves shock.

- (2) **Hypoglycemic shock:** When blood glucose levels falls below 20-50 mg/dl then hypoglycemic shock develops due to which there is nervous irritability that leads to profuse sweating, fainting, seizures and even coma and death.

Insulin shock occurs due to hyperinsulinism caused by Islet tumours over producing insulin or over dose of insulin in treatment of diabetes.

Treatment: Immediate treatment of hypoglycemic shock is intravenous glucose or glycogen infusion.

Other causes of Chock (circulatory) are (a) excessive metabolism of the body (b) Abnormal tissue perfusion. Tissue deterioration is the end stage of shock.

There are three stages of shock viz.

1. **Non progressive stage:** Wherein the body can cope up with the shock and overcome it.
2. **Progressive estate:** Wherein the body cannot overcome the shock all by itself but needs treatment. If untreated, it can lead to coma and death.

3. **Irreversible stage:** wherein the shock condition has prolonged beyond control and no treatment can reverse this condition.

DIGESTIVE DISORDERS

Digestive disorders in ruminants differs from those in non-ruminants, hence both will be discussed separately.

Digestive disorders in ruminants:

(1) Acute rumen indigestion (Rumen overload/lactic acidosis): It occurs mostly in domestic cattle and goats due to consumption of large amounts of grains and apples as they contain readily fermentable carbohydrates. *Streptococcus bovis*, a bacteria present in the rumen utilize these carbohydrates and convert them into lactic acid (by anaerobic glycolysis). As the utilization of carbohydrates increases the bacteria multiplies and there results in overproduction of lactic acid more than the rumen can absorb it i.e. the rate of production is more than the rate of absorption resulting in accumulation of large quantity of lactic acid in the rumen. There is production of both 'D' and 'L' forms of lactic acid, but only 'L' form is metabolizable (absorbed and forms glucose). Accumulation of lactic acid in the rumen results in lowering the ruminal pH leading to decreased rumen microflora causing indigestion. Ruminal acidity extends to the blood causing metabolic acidosis, resulting in reduced blood bicarbonate and finally a fall in urine pH. Rumen indigestion leads to change in the osmotality, hence there will be pouring of fluid from the blood into the rumen causing haemoconcentration, resulting in hypovolumic shock and death.

(2) Bloat or acute rumen tympany: Accumulation of gas in the rumen is termed as bloat. Regular fermentation by the rumen produces about 1.2 to 2.0 liters of gas per day (average is 1.5), which include CO₂ and CH₄ and these gases are continuously removed by eructation. Any disruption in the eructation process leads to the accumulation of gas in the rumen. The reasons for interruption in the normal release of gas may either be (1) Physiological (i.e. mechanical obstruction of the oesophagus or (2) Neurological (i.e. interruption of eructation reflex). This results in acute tympany of the rumen (bloat). There are two types of bloat.

1. Dry bloat or free gas bloat or temporary bloat: This occurs due to mechanical obstruction of the oesophagus resulting in the accumulation of dry gas in the rumen which is free flowing and is temporary because as soon as the oesophageal obstruction is relieved the gasses are erupted out. But this needs surgical intervention because the accumulation of gas is so fast that before the oesophageal obstruction is relieved there will be rapid death.

(2) Wet bloat or frothy bloat or stable bloat: this is caused due to consumption of large amount of leguminous plants by the ruminant, which contains considerable levels of the polysaccharide pectin and its hydrolytic enzyme pectin methyl

esterase. The enzyme pectin methyl esterase hydrolyses pectin into pectic acid and galactouronic acid (a mucopolysaccharide-sticky in nature). Due to sticky nature of galactouronic acid it greatly increases the viscosity of rumen fluid and adheres the food particles in the rumen. This results in increasing the surface tension (i.e. decrease in intramolecular space) which will not allow free flow of gasses, leading to entrapping the gas produced in normal amounts. Further production of gas bubbles the rumen contents forming froth, which further exaggerates the condition forming highly stable foam. Some bacteria overproduce mucopolysaccharides causing bloat. Another enzyme present in the feed of the animal is ribulose diphosphate carboxylase which also produces mucopolysaccharides forming stable bloat.

Treatment:

- a. Oral infusion of non-ionic detergents with surfactant properties that break up or prevent formation foam.
- b. Treatment with sodium alkyl sulfonate which inhibits the enzyme pectin methyl esterase.
- c. Antifoaming agents like poloxalene or silicone can be used.
- d. Genetic selection of cattle that are less prone to bloat is the best remedy.

(3) Urea poisoning: Urea is a normal component of animal feed. Ruminal microflora contains the enzyme urease which acts upon urea splitting it into CO_2 and NH_3 . Ammonia is utilized by the microbes in the rumen for their protein synthesis. If more than 3% of urea is consumed in the diet by the ruminant, then it leads to toxicity. Excess amount of urea produces more amount of ammonia resulting in its accumulation in the rumen and its subsequent entry into the blood stream. From the blood, ammonia enters the brain and gets protonated to ammonium ion which cannot be released back into circulation. Accumulation of ammonia in the brain disrupts the functioning of the brain leading to nervousness, depression, slurring of the voice, blurring of vision, tremors, finally to coma and if situation continues it leads to death.

Biochemical control: Ammonia exists in two forms depending upon the pH i.e. NH_3 and NH_4^+ . NH_3 is hydrophobic and so is easily diffusible through the cell membrane. NH_4^+ is more hydrophilic and so cannot easily diffuse across the cell membrane. Hence in order to inhibit the absorption of ammonia from the rumen cells it should be converted to NH_4^+ form for which the $\{\text{H}^+\}$ concentration in the rumen should be increased. So oral administration of acetic acid is taken up without altering the ruminal pH, which decreases the absorption of free NH_3 . Addition of acetic acid in the feed itself, depending upon the urea content is recommended to avoid ammonia intoxication or urea poisoning.

Digestive disorders in non-ruminants:

(1) Vomition: It is the forceful expulsion of gastric contents from the mouth. This is caused due to gastric irritation by toxins, infectious agents, foreign bodies, gastric tumors and obstruction of the pyloric canal etc. Due to vomition there is loss of large quantities of water, H^+ , Cl^- and K^+ ions. This results in dehydration, metabolic alkalosis (increased HCO_3^-), hypochloremia, hypokalemia, hypovolemia resulting in renal tubular damage and renal failure. This is seen in dogs, cats and pigs but never in horses, occasionally seen in cattle and goats.

(2) Diarrhoea: Passage of abnormally fluid faeces with increased frequency and volume is termed a diarrhoea. The reasons for diarrhoea are increase in the rate of intestinal transit (hyper motility), decreased intestinal assimilation of nutrients (maldigestion, malabsorption) and production of enterotoxins by *E. coli*.

Vibrio cholerae (a strain of *E. coli*) produces two proteins (a) High molecular weight heat labile toxin (CT one subunit is LT) and (b) low molecular weight heat stable toxin (ST). Cholera toxin (CT) catalyzes the transfer of ADP-ribose from NAD^+ to the α -subunit of G_s , blocking its GTPase activity and thereby rendering it permanently activated. This results in continuous activation of the adenylate cyclase of intestinal epithelial cells and the resultant high concentration of cAMP triggers (through activation of protein kinase cascade) continual secretion of water, Cl^- and HCO_3^- into the intestinal lumen, resulting in hypermotility of the intestinal mucosa causing diarrhea. The resulting dehydration and electrolyte loss are the major pathologies in cholera.

Biochemical changes resulting from this acute enteritis are; dehydration, hemoconcentration, hypovolumic shock and death. There is metabolic acidosis due to decreased excretion of H^+ ions, increased production of organic acids by anaerobic glycolysis, hyperkalemia due to movement of K^+ from cells to blood which leads to cardiac arrest and death. Hypoglycemia is also seen due to improper carbohydrate metabolism.

Treatment: Inhibitors of protein synthesis like cycloheximide when given orally inhibits the production of CT, LT and ST by the enteropathogenic strains of *E. coli*, thereby by overcoming the effect of diarrhoea. Acetazolamide inhibits intestinal fluid secretion. Ethacrynic acid acts as diuretic thereby dragging water from the intestine to the blood stream. Others are Indomethacin and aspirin (inhibits intestinal secretions), diltiazem (calcium channel blockers), Idoxamide & tromethamine (inhibits PG synthesis), Chlorpromazine & propranolol (Adrenergic blockers), pepto-bismol (intestinal adsorbent) and attapulgate (anti-entero-toxic effects).

(3) Gastric dilatation or Volvulus / Ischemia and tissue reperfusion injury:

Gastric dilatation-volvulus is seen in deep chested dogs. There is accumulation of gas and fluid in the stomach due to pyloric obstruction. Due to accumulation of gastric contents, the stomach distends and rotates about the distal oesophagus. This causes obstruction of the caudal vena cava and portal vein resulting in venous stasis. Hence blood enters the splanchnic, renal and posterior muscular capillary beds with a net result in decrease in circulating blood volume. There is decrease in cardiac output and low arterial blood pressure. This is one of the causes for tissue perfusion or ischemia wherein there is decreased blood and O₂ supply to organs resulting in the attenuation of electron transport chain and decreased production of ATP. Therefore the cells switch over to anaerobic glycolysis producing lactic acid. The acid produced decreases the pH i.e. acidosis resulting in precipitation of the cellular proteins. Cellular acidosis causes damage to the cell membrane allowing water to flow inside the cell causing cellular oedema. Increased intracellular concentrations of Ca²⁺ are also seen. Lactic acid damages the lysosomal membrane releasing hydrolytic enzymes causing destruction of cell organelles and cell death.

The picture in the blood is that due to leakage of cellular contents there is hyperkalemia, hyperphosphatemia, increased BUN, creatinine, ALT, AST & CK. Due to hypovolemia there will be hypovolumic shock and death.

Reperfusion injury: when the pyloric or intestinal obstruction is relieved there is a sudden gush of blood to the tissues and an increased supply of O₂ (tissue reperfusion). This aggravates the ischemic induced tissue injury. ETC was in an attenuated state due to GDV and now when it is relieved and the oxygen supply is normal, ETC cannot utilize the O₂ being supplied in normal amount as it is attenuated. This results in improper reduction of oxygen producing oxygen free radicals i.e. super oxide (O₂⁻), hydroxyl free radicals (OH⁻) and hydrogen peroxide (H₂O₂) leading to cellular membrane damage due to oxidation of the unsaturated fatty acids of the membrane phospholipids at the double bonds (lipid peroxidation). The end result is auto destruction of the cells due to leakage of lysosomal enzymes. Tissue reperfusion injury is most common in horses with strangulating intestinal obstruction. Hypoxanthine is formed during GDV which is converted by xanthine oxidase to uric acid and O₂⁻ upon reperfusion.

Pharmacological agents used to prevent reperfusion injury are allopurinol (xanthine oxidase inhibitor), superoxide dismutase, catalase, glutathione peroxidase are free radical scavenging enzymes. Mannitol, albumin and dimethyl sulfoxide (DMSO) are non enzymatic free-radical scavengers.

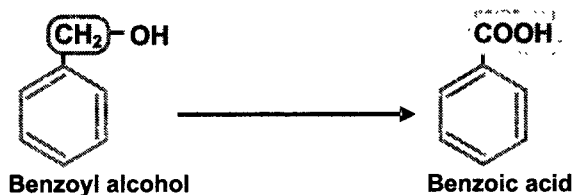
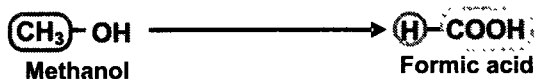
DETOXIFICATION

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.

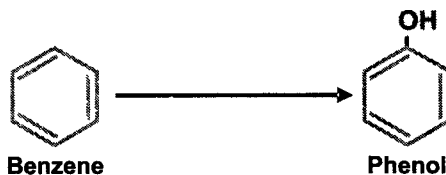
Several unwanted and harmful substances get entry into the body either by absorption from the gastrointestinal tract (e.g. drugs) or by parenteral route. Some of the physiological substances normally produced in the body (e.g. hormones) also require to be eliminated regularly to prevent accumulation 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 render it to a form suitable for excretion. The following are some of the sample reactions for each of the class of reaction -

1. Oxidation:

i) **Alcohols:** Are oxidized to corresponding acids.



ii) **Aromatic hydrocarbons:** Are oxidized to phenols.

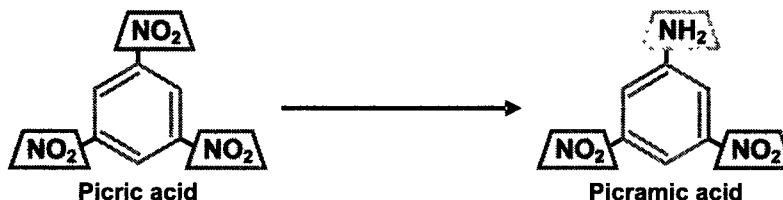


2. Reduction:

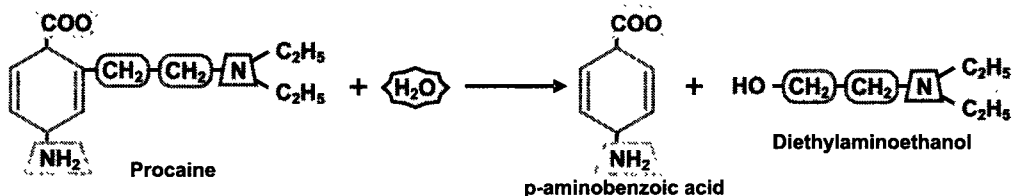
i) Aldehydes: Are reduced to alcohols.



ii) **Aromatic nitro compounds:** Are reduced to corresponding amines.

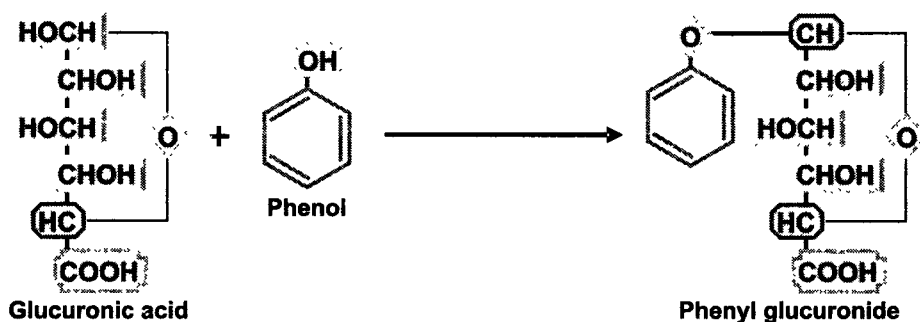
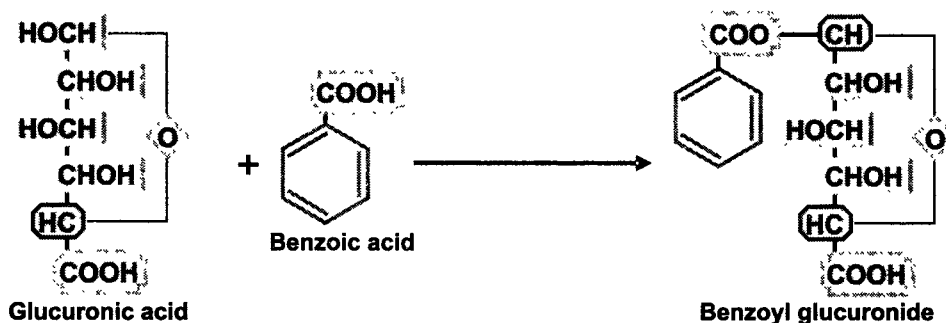


3. **Hydrolysis:** Drugs like procaine and acetyl salicylic acid and cardiac glycosides like digitalin undergo hydrolysis.

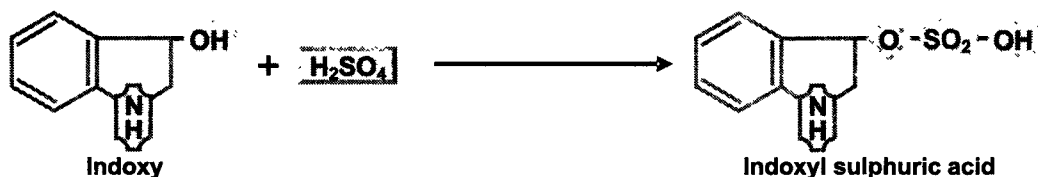
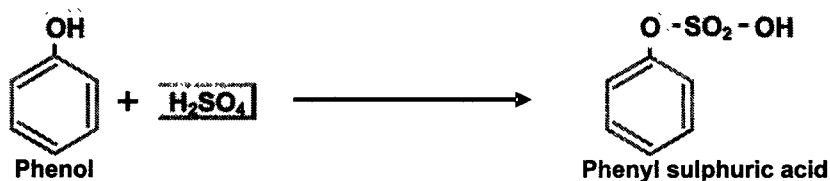


4. **Conjugation:** The foreign substances as such or after suitable preparation by one or other of the process of oxidation, reduction and hydrolysis are usually conjugated with another substance before they are excreted. The following are some examples -

i) **Glucouronic acids:** Aromatic acids (e.g. benzoic acid) and phenols are conjugated with glucuronic acid. The glucuronic acid is derived from uridine diphosphate glucuronic acid. The drug chloramphenicol and the bile pigments are among the important substances conjugated with glucuronic acid. Derivative of steroid hormones also are conjugated with glucuronic acid before excretion.

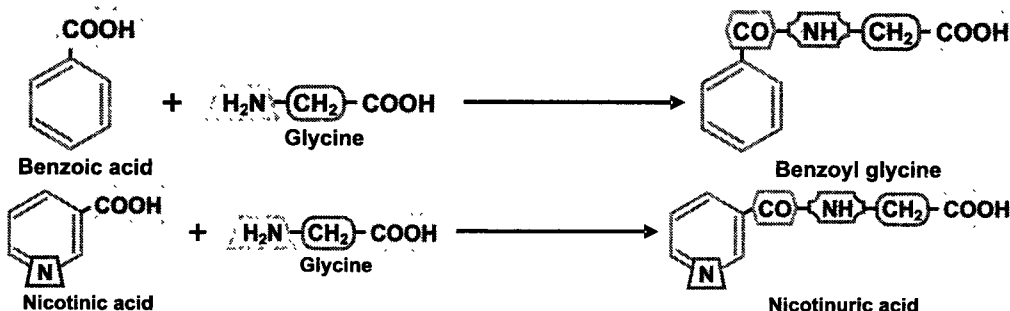


- ii) **Active sulfate:** Is used to conjugate phenolic compounds. The derivatives are called as ethereal sulfates. An increase in their amount in urine signifies excessive intestinal putrefaction or stasis. Adrenal cortical hormones are also excreted after conjugation with sulfuric acid.

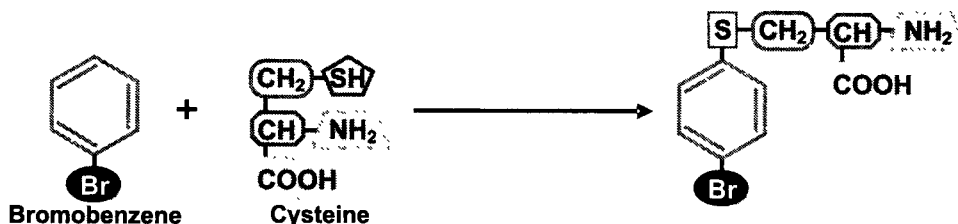


Yeast and mammalian liver contain enzymes that can activate inorganic sulfate by adding it to 3-phosphoadenosine-5-phosphate. Active sulfate is, adenine-3-phosphoribose-5-phosphosulfate. Incorporation of sulfate into sulfated mucopolysaccharides and conjugation of steroid hormones and others with sulfate 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.

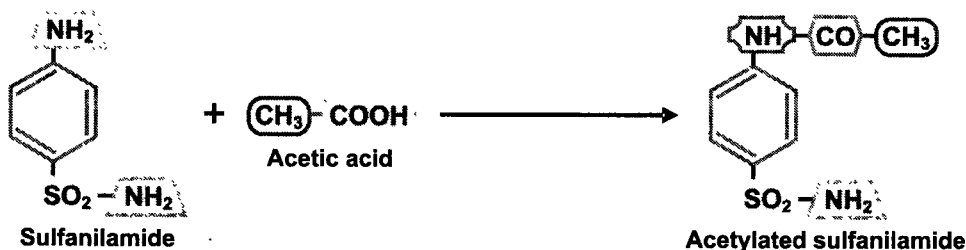


- iv) **Cysteine:** Is used in the conjugation of certain Aromatic compounds like benzene and halogenated ring compounds like bromobenzene.

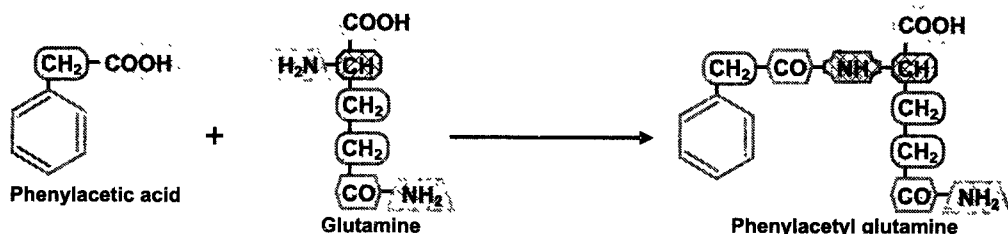


The amino group is later acetylated to form what are known as mercapturic acids.

- 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'.



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Chapter 22

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).

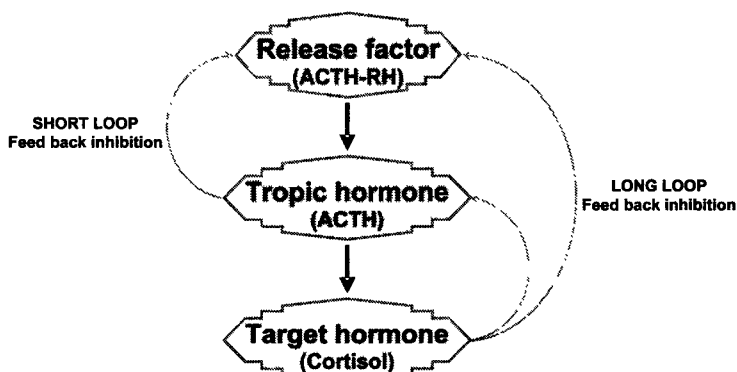
Endocrine glands and the hormones produced

Endocrine gland	Hormone produced
1. Pituitary gland	
a) Anterior pituitary (Adenohypophysis)	1. Growth hormone (GH)
	2. Thyrotropic or Thyroid Stimulating Hormone (TSH)
	3. Adrenocorticotrophic Hormone (ACTH)
	4. Pancreotropic Hormone
	5. Metabolic Hormone
	6. Intestinal Cell Stimulating Hormone (ICSH) or Leutinisising Hormone (LH)
	7. Follicle Stimulating Hormone (FSH)
	8. Prolactin or Leutotropic (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-cell 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) Pancreozimin
	4) Cholesystokinin
	5) Enterogastron
	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 (Non-Adrenaline)
7. Gonads	
a) Testis	1. Testosterone
b) Ovaries	1) Estrogen
	2) Progesterone

In addition to these major glands, there are other few 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 passes through the hypophysial-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 of 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 ↓	Thyrotrophic release factor ↓
Pituitary gland ↓	Pituitary gland ↓	Pituitary gland ↓
ACTH ↓	Tropic hormone ↓	Thyrotrophic hormone (TSH) ↓
Adrenal gland ↓	Respective endocrine gland ↓	Thyroid gland ↓
Epinephrine ↓	Specific hormone ↓	Thyroxine ↓
Muscles ↓	Target tissues ↓	Various tissues ↓
Glycogenolysis	Metabolic action	Increased BMR

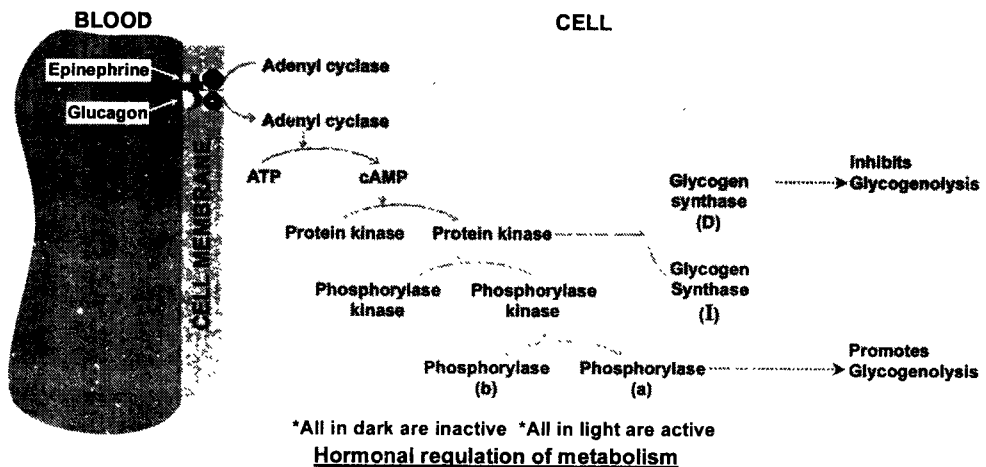
Classification of hormones: The hormones are classified into four classes -

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. **Catacholamines:** 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 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.



Feed back regulation of hormone production: The production of hormones is regulated by feedback mechanism. There are two types of feedback regulation (1) Negative feed back 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 factor. 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.

Hormones

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 called 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
	Adrenocorticotrophic Hormone (ACTH)	Adrenals
	Pancreotropic Hormone	Pancreas
	Metabolic Hormone	Liver
	Intestitial Cell Stimulating Hormone (ICSH) or Leutinisig Hormone (LH)	Gonads
	Follicle Stimulating Hormone (FSH)	Gonads
	Prolactin or Leutotropic 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 result 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 osteogenesis.

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.

Stimulus: 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) **Amenorrhoeic effect:** It has an amenorrhoeic 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 PITUITARY

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 hypertonic plasma is there 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 later 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 raises 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 the either side of the trachea. The development and secretion of the thyroid gland is controlled by TSH.

THYROID HORMONES

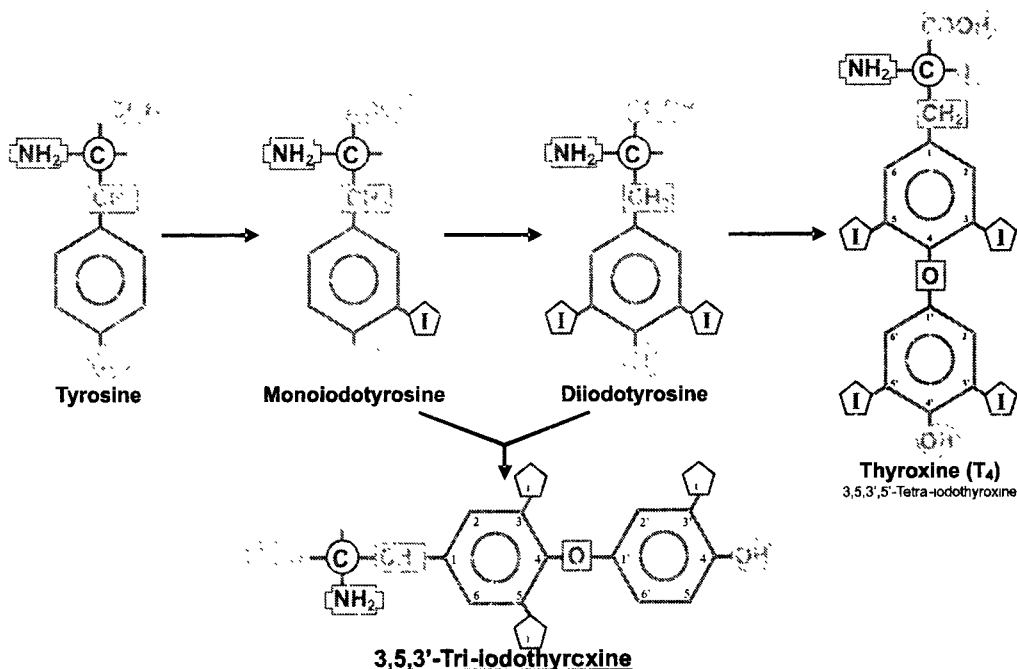
The hormones secreted by thyroid are (1) Thyroxine (T_4) & (2) Triiodothyronine (T_3).

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 tissue.

- (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 T₃ & T₄. 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, tongue and skin are thick, mentally retarded and sexually undeveloped.

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 hypercholesterolemia is seen. T₃ and T₄ administration is the only remedy.

2) **Hyperthyroidism or thyrotoxicosis:** It is due to excessive production of thyroid hormones. Main cause is tumors of the gland.

Grave's disease: This is due to long acting thyroid stimulator (LATS) and LATS protector, which acts similar to that of TSH resulting in the 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 graves 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) Rises serum calcium by -
 - (a) Formation of calcitriol in the kidney, which helps in the absorption of Ca²⁺ from the intestine.
 - (b) Mobilization of Ca²⁺ 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.

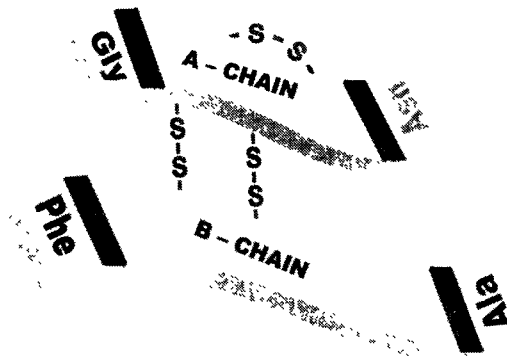
Hyperparathyroidism: Occurs mainly due to tumors of the gland. It results in high serum calcium, low serum phosphate, bone destruction and kidney stones.

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, A chain having 21 amino acids and B chain having 30 amino acids.



There are two interchain disulphide bonds (1) between A 7 & B 7 and (2) between A 20 & B 19. There is one intrachain disulphide bond in A chain between 6th and 11th 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 adenylcyclase 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.

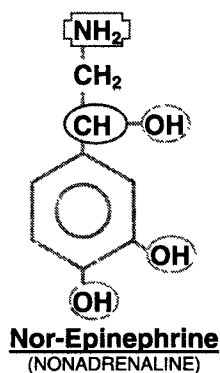
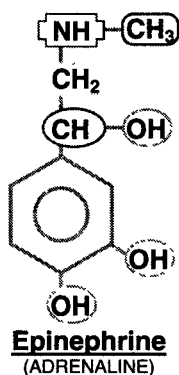
ADRENAL MEDULLA

They arise from the autonomic nervous system. The cells of the adrenals are called phenochromocytes 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 (non-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.



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). Epinephrine 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) 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 the other tissues). This is known as fight or flight mechanism.

Metabolism and excretion: The life span of catecholamines is 10-30 sec. 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. Vanilmandelic acid - VMA which is the major excretory product of catecholamines).

Pathophysiology: Tumors of the adrenal medulla causes excessive production of catecholamines resulting in hypertension. The condition is known as pheochromocytomas.

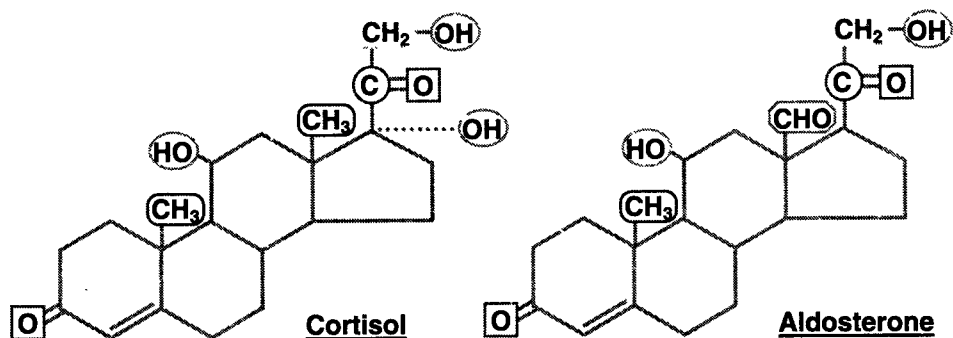
ADRENAL CORTEX

It surrounds the adrenal medulla.

CORTICOSTEROIDS

The hormones produced by adrenal medulla are known as steroids or steroid hormones, as they contain the parent ring the cyclo-pentano-perhydro-phenanthrene ring. It produces -

- | | | |
|------------------------|---|---------------------------|
| (1) Glucocorticoids | → | Cortisol, Corticosteroids |
| (2) Mineralocorticoids | → | Aldosterone |
| (3) Sex Hormones | → | Androgen |



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 converted to pregnenolone. The synthesis of mineralocorticoids is regulated by renin-angiotensin system which varies 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 immunonologically mediated disorders. However, the response to infection is also reduced and this represents a significant drawback to their use.

Minerelocorticoids: 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 result in -

- (1) Hyperglycemia and glycosuria.
- (2) Retention of sodium and water resulting in oedema and hypertension.
- (3) Negative nitrogen balance.
- (4) Hypokalemia, and
- (5) Hirsutism (Opposite sex characters).
- (6) **Cushing's syndrome:** Tumors of pituitary or adrenal results 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

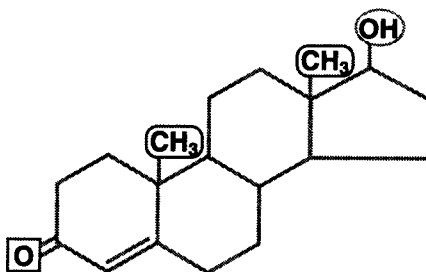
The gonads constitute the testis in males and in females, the ovaries, corpus luteum and placenta.

TESTIS

Testosterone is the major hormone produced by the testis.

TESTOSTERONE

It is a 19 carbon compound. It is synthesized from cholesterol and progesterone 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 form 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 favors development of secondary sexual features.
- (6) It is responsible for the male pattern behavior.
- (7) It increases the secretions 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.

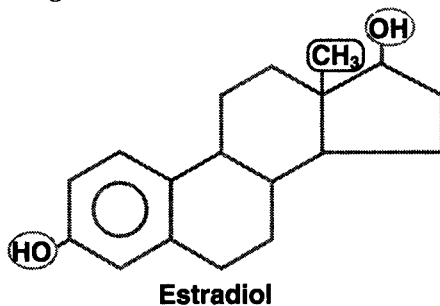
Lack of male hormones: Primary (testis defective) or it can be secondary (pituitary defect) results in failure to develop secondary sex characters, lack of masculinization, atrophy of sex organs in later life.

OVARIES

They produce estrogens and progesterone.

ESTROGENS

The most active estrogen is 17-beta-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:

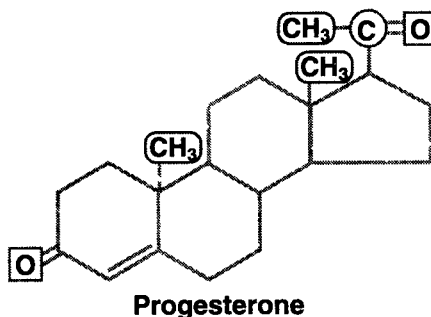
- (1) Growth and maturation of female sex organs and maintenance of the reproductive capacity.
- (2) Stimulate the growth of follicles.
- (3) Increases the blood flow and transudation of water.
- (4) Increased cell division.
- (5) Regulates bone development (i.e. it stops bone development after a certain 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.



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)	Causes 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.

Hormones

	Luteinizing hormone (LH)	Stimulates ovulation and corpus luteum function. Secretion of progesterone, 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 edometrial secretions, maintains pregnancy, stimulates mammary alveolar growth and control gonadotrophin secretion.
	Relaxin	Loosening of symphysis pubis and sacroshiatic 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.

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Diagnostic Biochemistry

Diagnosis is the basic aspect of any clinical treatment. Modern science has evolved various methods and devices that enable diagnosis of various diseases and disorders. Biochemistry plays a vital role in the estimation of various constituents in the biological fluids like Blood, Urine, Cerebrospinal fluid, Amniotic fluid etc. The role of following biochemical parameters will be discussed here in detail.

- (1) Role of Blood sugar as an aid to diagnosis.
- (2) Role of Ketone Bodies as an aid to diagnosis.
- (3) Role of Non-Protein nitrogenous (NPN) substances as an aid to diagnosis.
- (4) Role of Plasma enzymes as an aid to diagnosis (Clinical enzymology).

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 → (1) Enzymatic method (2) Complex formation (3) Reduction methods

- (1) **Enzymatic method:** The enzyme glucose oxidase is used in the estimation of blood glucose. Glucose oxidase oxidizes glucose releasing hydrogen peroxide. The amount of hydrogen peroxide released is measured as an index of the blood glucose. This enzyme is very specific for glucose hence the value obtained is true glucose value in the blood. But it is more expensive. The electronic gadgets available for the estimation of glucose use this method.
- (2) **Complex formation:** The carbonyl carbon is reacted with reagents like ortho-toulidine 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.
- (3) **Reduction methods:** 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.

The 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:** i.e., after meals (1-2 hours after the first meal) blood glucose level ranges between 100-140 mg/100 ml.
- (3) **Random Blood glucose level:** It is measured any time after 1st, 2nd or 3rd meal that ranges between 100-180 mg/100 ml.

The following the fasting blood glucose level (true glucose in mgs) in various species.

Human	Cow	Sheep	Goat	Horse	Dog	Cat	Pig	Chicken
60 – 90	45 – 75	50 – 80	50 – 75	75–115	65–118	73–134	80–150	175-300

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). Continues glycosylation of the proteins disrupts the membranes and accumulation of blood clots and cholesterol, a condition known as atherosclerosis/arteriosclerosis. Glycosylation of the membranes also leads to reduced life of RBC, cell necrosis, kidney failure etc.

Blood Glucose Haemostasis: 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. 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 is saturated in the liver, muscle and other tissues then, the adipose tissue converts the excess of glucose to fat by lipogenesis and is stored 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 tissue viz., liver, brain, intestine, RBC and retina of the eye (hence causes cataract).

If the glucose level in the blood increases further, more than 180 mg/ 100 ml as it 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), 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 increases 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 down, as seen in fasting, starvation, shock, severe exercise, febrile disease, lactation, multiple pregnancies etc., the hormone glucagon is released from 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 and 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 from 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 about 30-40 days of starvation. After which 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
	Renal excretion
	Amino acid synthesis

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 – 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 leads 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
- (2) Polydipsia: Frequent intake of water due to frequent urination leading to dehydration.
- (3) Polyphagia: Frequent intake of food due the starving condition of the cells.

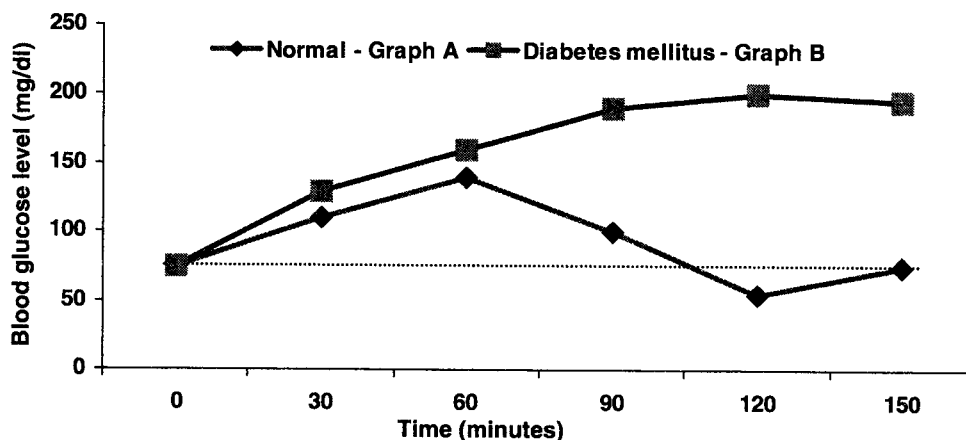
Classification of diabetes mellitus:

- (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 measurement 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 it is positive then blood sugar is estimated and if it is showing a fluctuation then glucose tolerance test is done to confirm it.
- (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 the subject is given oral dose of 50-60 grams of glucose in 300 ml of water (or 0.75 grams per kg body weight). 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:



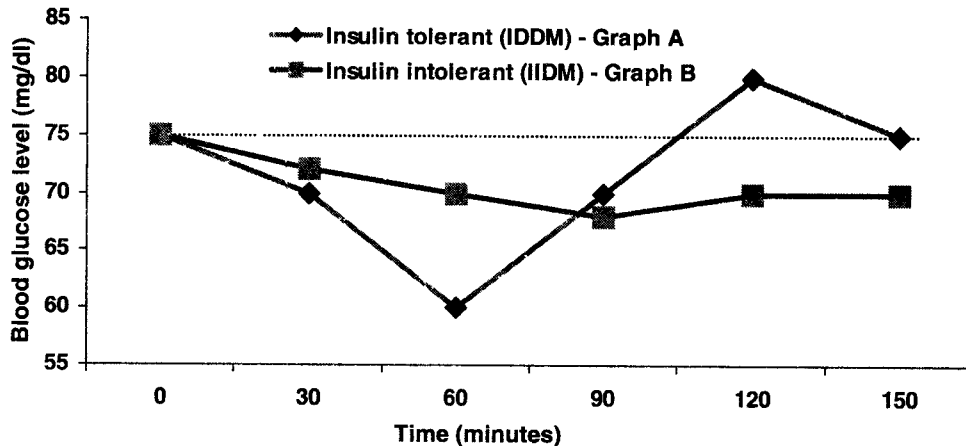
Graph – A: In normal persons the fasting blood glucose level will be at the lower limit i.e., 75 mg/100 ml. Upon providing oral glucose the blood glucose rises in one hour's time and falls below the fasting level in another half to one hour due to the secretion of insulin. Then, in about a time of 2 ½ 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 give 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 units 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 or not (IDDM). The result of insulin tolerance is plotted in a graph as follows:

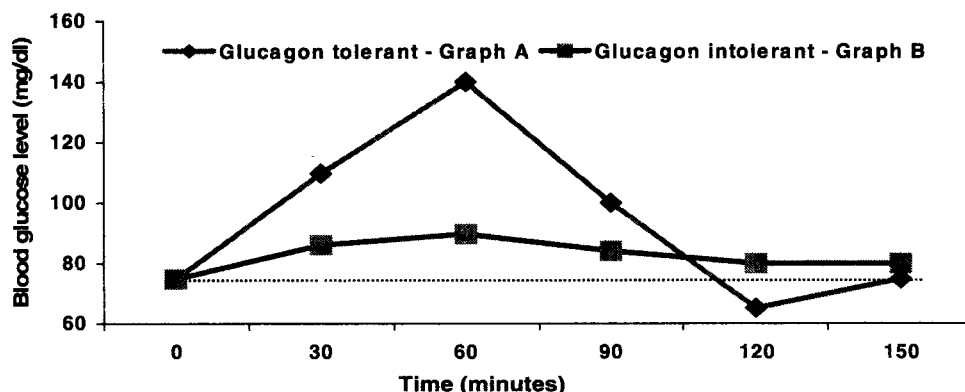


Graph – A: Injection of insulin in normal persons or insulin responsive diabetes mellitus (IDDM) persons will lead to fall a in the blood glucose level in one hour's time due to utilization of glucose by the cells aided by insulin and slowly increases and rises above the fasting level (base line) in 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 Langerhan's 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. His blood glucose is measured in fasting condition (12-14 hours). Then he is given intravenous dose of 30 μ g 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 glucagons (upon degradation) or in 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).

The following are the 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 reabsorbing 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 pancreas.
- (14) To assess the intestinal status of carbohydrates in non ruminants – 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.

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. Intestinal wall and even the rumen can synthesise the ketone bodies in the ruminants. 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:

Category	Blood (mg/dl)	Urine (gms/day)
Non Ruminants	< 1	< 1
Ruminants	< 10	< 10

Methods of estimation of Ketone Bodies: In non ruminants 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

In ruminants, basically the ketone bodies concentration is very high; hence estimation of the exact amount is necessary. 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 increases the capacity of the peripheral tissue 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:

- (4) Acetoacetic acid and Beta hydroxyl butyric acid are strong acids; their accumulation causes ketoacidosis, thereby lowering the pH of blood.
- (5) Buffering capacity is disrupted because bicarbonate of the blood decreases.
- (6) 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 occur:

- (1) **Starvation:** Includes both fasting period of 12-24 hours or continuous starvation for days together, specially seen in stray dogs and cats. During this condition there be lack of glucose leading to non entry of glucose into adipose resulting in lowered glycolysis, low intermediates of glycolytic pathway. Low concentration of glyceraldehyde-3-phosphate, an intermediate of glycolysis cannot be converted to glycerol phosphate therefore there will be no reesterification of fatty acids resulting in the release of fatty acids 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 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 fasting, brain also shifts to the utilization of ketone bodies which leads to the destruction of brain cell due to ketosis.

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 hydrolysed 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 animal.

- (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 resulting in ketoacidosis.
- (3) **Pregnancy:** During the third stage of pregnancy, the demand for glucose is doubled and hence there will be overproduction of ketone bodies leading to ketoacidosis. Especially in sheep the condition is severe because of presence of multiple foetus and is termed as pregnancy toxemia. This can lead to the death of foetus.

- (4) **Lactation:** this is seen in so high yielding breeds of bovine where production of milk is more and hence more energy is required mainly glucose is required for
- i. Production of lactose.
 - ii. Formation of milk fat.
 - 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 is referred to as milk fever. This is developed within hours in lactating animals that is characterized by sudden fall in blood pH and decrease in milk production. The animal lies in a recumbent position because of muscular weakness. If not treated, the animal may die within 12 to 14 hours.

- (5) **Febrile diseases:** In fever causing disease there is a demand for glucose for the formation of antibodies thereby depleting glucose to adipose leading to ketosis.
- (6) **Heavy Exercise:** Heavy physical exercise suddenly raises the level of ketone bodies and if the exercise continues with intake of glucose then it may result in ketosis.
- (7) **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 and serine etc.

Only acetoacetic acid and beta hydroxybuteric 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 sweat and lungs, hence starvation and diabetic patients show an alcoholic smell in their breath.

DIAGNOSTIC IMPORTANCE OF NON – PROTEIN NITROGEN SUBSTANCES

The non-protein nitrogenous substances (NPN) include urea, uric acid and creatinine. Estimation of NPN is done in order to assess 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

that is 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

1. Diacetyl Monooxime (DAM) method.
2. Calorimetric method by the use of Urease enzyme.
3. Autanalyser method.

Conditions causing variation in the blood urea level

A decrease in the blood urea level indicates liver disorders/dysfunction. Whereas an increase in the blood urea level, termed as ureamia may be classified into three major classes.

1. Pre-renal
2. Renal
3. Post-renal.

Pre-renal ureamia: The increase in the blood urea upto 300 mg/dl may be due to the following reasons prior to the kidney.

- Dehydration due to vomiting (pyloric obstruction) or chronic intestinal obstruction. During dehydration the blood volume decreases leading to haemoconcentration as a result of which the blood urea values seems to be elevated. Due to low blood pressure the glomerular filtration rate will be lowered leading to increased urea levels. Diarrhoea can cause ureamia.
- Ulcerative Colitis (There will be Cl^- loss).
- Diabetic coma
- Addison's disease (Hypoadrenalism)
- Haemateuresis.
- Shock due to burns
- Post operatively
- Heart attacks.

Renal ureamia:- the increase in the blood urea level above 300 mg/dl due to kidney disorders is termed as renal ureamia. This may be due to :

- Nephritis
- Acute glomerulonephritis.
- total renal failure

Post-renal ureamia:- 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 prostate gland.
- Stricture of urethra.

Role of Uric acid as an aid to diagnosis.

In ureotelic animals only the estimation of uric acid is of diagnostic importance. Nucleic acids are hydrolysed in the liver to purines and pyrimidines which 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 0.2 to 0.8 mg/dl and is excreted in urine @ 0.2 to 0.8 g/day.

Methods of estimation:

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 urate 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 Allantoin.

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 1.2 – 1.8 mg/dl for male; 0.8 – 1.5 mg/dl for female. Excreted in urine @ 1.20-1.8 g/day in male and 0.8-1.5 g/day in female. Measurement of creatinine coefficient is more accurate than estimation of blood creatinine. Creatinine coefficient is the grams of creatinine excreted per kg body weight per day. For male it is 6 – 8 and for females it is 5 – 7.

Methods of estimation: Jaffe's picric acid method.

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.

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 considerable high amounts and are functional in the plasma due to the presence of their substrate in the plasma. 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 has no function in the plasma due to the absence of their substrate in the plasma. 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 exists. 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 kinase (CPK), alanine amino transferase (ALT) or serum glutamate pyruvate transaminase (SGPT), aspartate transaminase (AST) or serum glutamate oxaloacetate transaminase (SGOT), sorbitol dehydrogenase, alkaline phosphate, acid phosphatase, amylase, pancreatic lipase etc.

However functional plasma enzymes are already higher in 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:

Enzyme 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 (γ -GT)	Liver damage (indicator of alcoholism)

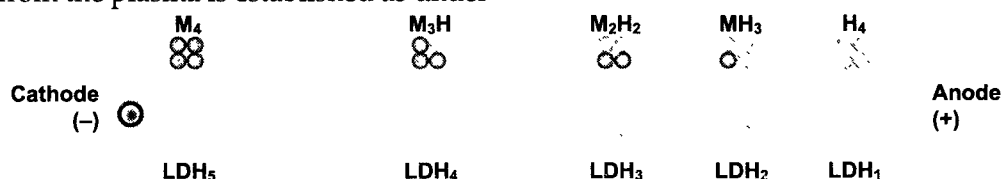
Diagnosis of myocardial infarction using enzyme assay: There are three main enzymes that are used in the diagnosis of myocardial infarction (1) Lactate dehydrogenase (LDH) (2) Creatine phospho kinase (CPK) - marker enzyme and (3) Transaminase (AST or SGOT).

- 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 at some time 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 been leaked.

However LDH exists in five isoenzyme forms i.e. multiple forms of the same enzyme (These enzymes bring about the same reaction but exhibits 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 K_m values and K_m is indirectly proportional to affinity ($K_m \propto 1/\text{affinity}$). 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 phospho kinase (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 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 forms dimmers in the following combinations to give rise to three isoenzymes of CPK.

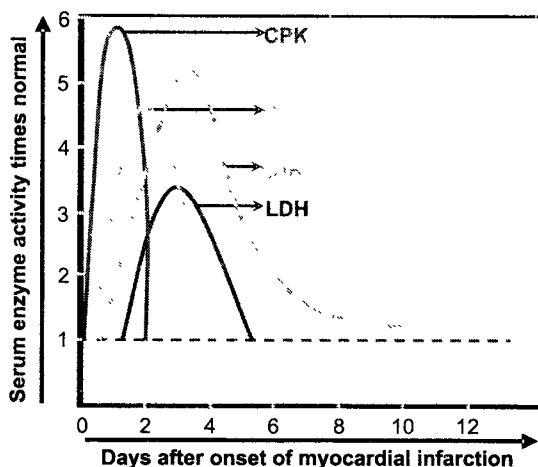
BM – Predominant in cardiac muscle

B2 – Predominant in brain

M2 – Predominant in skeletal muscle

Thus estimation of the isoenzyme BM 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 rises 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.



Chapter 24

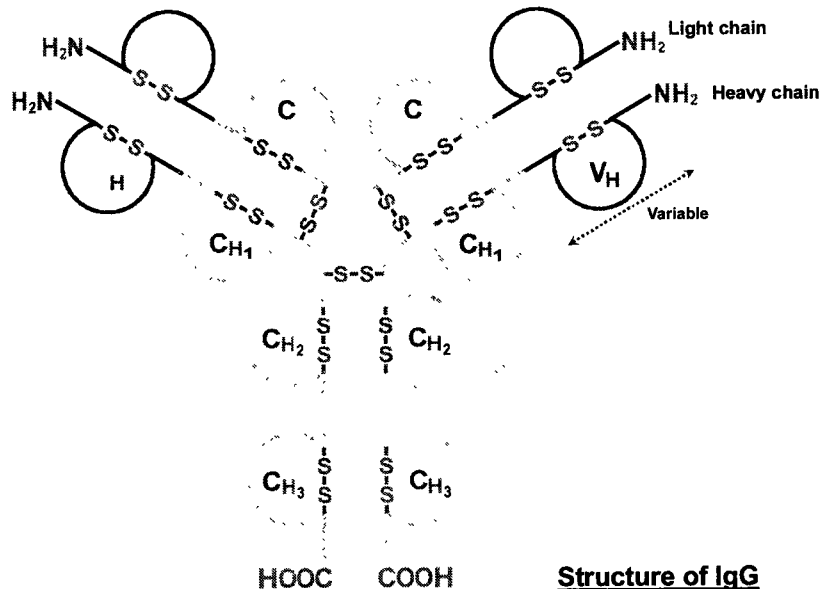
Immunology

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 a 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. The antigen 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 a 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-third ($1/3$) 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 binds 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 regions 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 it is 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), mu (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 AA's.

VL region is from 1-108 amino acids from N-terminal.

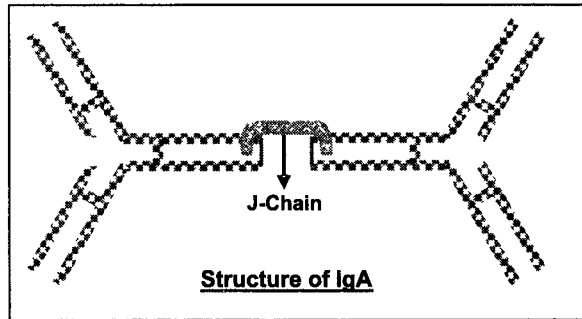
CL region is from 109-214 amino acids from the N-terminal.

VH region is from 1 to 118 amino acids from the N-terminal.

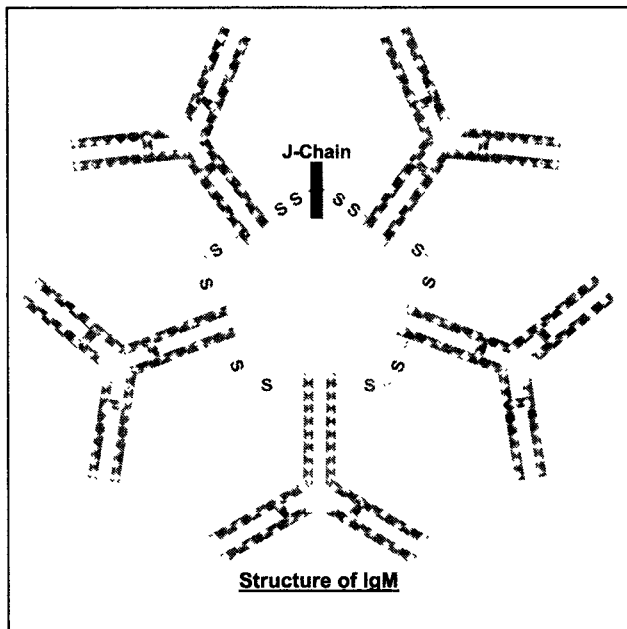
CH region is from 119 to 446 amino acids from the N-terminal.

There are 4 subclasses of IgG viz. IgG₁, IgG₂, IgG₃, IgG₄, 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 1,60,00 daltons. IgA is the important immunoglobulin in the seromucous secretions such as the saliva, secretions of respiratory and gastro-intestinal tract, tears, colosturm etc., and prevents the entry of micro-organisms into the body.



IgM: IgM is also called as macroglobulin because it is the biggest molecule among all the immunoglobulins. It has a molecular weight of 9,00,000 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.



IgD: It has a molecular weight of 1,85,000 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 2,00,000 Daltons and is present in very small amounts in blood to about 17-450 ng/ml ($1 \text{ ng} = 10^{-9} \text{ g}$). 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.

Immunopotency: The ability of the antigen molecule to act as an antigenic determinant and to induce the formation of specific antibodies is called immunopotency. Factors which influence immunopotency are:

- (1) Genetic factors play an important role in the ability of different animals to produce antibodies of different specificities against the same antigen. The dominant component of the antigenic determinant is termed as immunodominant.
- (2) Exposure to aqueous environment is very important in determination of immunopotency of an antigen.
- (3) Charge residues contribute to the specificity of antigens. Any charged group is hydrophilic and is in close contact with the environment of the blood plasma.

SECTION - V

**Veterinary Laboratory
Diagnosis – VLD – 421 & 511**

**IVth Year / IInd Semester and Vth Year /
Ist Semester**

*This course is designed to expose the student to various diagnostic techniques,
their clinical importance and their correlation to the health and disease
condition of the animal.*

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Collection of Biological Fluids

BLOOD COLLECTION

Anti coagulated venous blood is the most common specimen submitted to the laboratory for biochemical analysis. Arterial blood is recommended for blood gas analysis. Further the specimen is to be heparinized and scaled in order to avoid gaseous exchange between the specimen and its environment. Blood is mostly collected after over night fasting during which water intake may not be restricted. Post-prandial (after food intake) specimen show high glucose, low inorganic phosphate, increased triglycerides and lipids. In case of lipaemic specimens lipid can be removed by centrifugation, placing in refrigerator overnight or by ether extractor.

A. Procedure: Blood is collected by venipuncture, for haematological work, the needle and syringe must be dry, since the presence of water haemolysis the red blood cells. The needle should in addition be sterile.

1. The site from where the blood is to be drawn is clipped.
2. Tincture of iodine is applied as an antiseptic and the site is allowed to dry.
3. The vein is raised by pressure.
4. Large bore needle, which is sterilized and dried, is used for insertion into the vein.
5. Dry syringe is connected to the needle and required amount of blood is drawn in to the syringe.
6. The needle is disengaged, syringe is then inserted into the specimen tube (containing anti coagulant) to very near its bottom and blood is allowed to flow out without exerting excess pressure on the piston. Immediately the rubber stopper is put, the tube is placed on the palm of one hand and with the other; rotatory movements are made, so that blood mixes with the anti-coagulant.
7. Care is taken to avoid vigorous shaking, which otherwise leads to froth formation, haemolysis of blood cells and in turn erroneous results may be obtained.

SITES OF COLLECTIONS

	SITE	ANIMALS
1.	Jugular vein	Cattle, Horse, Sheep, Goat and large wild mammals. Occasionally used in the dog, cat, rabbit, rat, mouse, Hamster G. pig and bird.
2.	Cephalic vein	Commonly used for collecting small amounts of blood in the dog.
3.	Ear vein	Can be used in the cat, small dog, pig, rabbit, G. pig, monkey & chinchilla.
4.	Toe or Toe Nail	Small dog, puppy, G. pig and small wild mammal.
5.	Tail amputation	Rat and mouse
6.	Heart	May be used in any animal, fish, reptile, bird
7.	Femoral, saphenous, tibial vessels	Dog, cat, primate rat & small mammal
8.	Mammary vain	Dairy cattle
9.	Anterior venacava	Pig
10.	Retro-orbital venous plexus	Rat, mouse, G. pig hamster
11.	Wing vein and comb	Bird
12.	Vein in the web between toes or foot pad	Mink, G. pig, and small wild mammals

For larger animals, 5 ml of blood is sufficient for routine examination. However for small dogs and cats 2 ml of blood is manageable.

Errors commonly made in blood collection:

A. Haemolysis:

1. Use of wet syringe or failure to remove the needle before filling collection tubes, haemolysis RBC.
2. Serum, rather than plasma is preferred for most chemical analysis, because of the possible interference by various anti-coagulants. However plasma that has been carefully separated from cellular mass will have less haemoglobin than serum and where anti coagulants will not interfere. Plasma may be the specimen of choice.
3. When the concentration of haemoglobin exceeds 0.02 gm/dl. serum appears grossly haemolysed, although in ecteric serum much higher levels may be detected.

4. Chemical components of blood are often present in varying concentration between the plasma and RBC. If the concentration in RBC is less than plasma, haemolysis will produce dilution effect. If the concentration is greater in RBC than plasma then haemolysis will cause concentrating effect. Haemolysis may directly interfere with urea nitrogen, inorganic phosphate, serum potassium, alkaline phosphatase.
5. During blood collection, collection tube has to be slanted. So that blood is collected along sides. Vigorous shaking to be avoided. During collection through syringe, frothing is to be avoided. Sudden unloading of blood should be avoided.
6. During separation of serum, ringing of the blood clot is to be done gently which otherwise leads to haemolysis. For serum separation, blood should not be placed immediately into refrigerator or before good clot formation and retraction occurs, since this retards serum collection.
- B. Lipemia may occur if the patient has not been fasted for adequate time before blood collection.
- C. Fibrin clot occurs when the blood is centrifuged too soon after the sample is taken or if the speed of centrifuge is fast.
- D. Blood should be collected when the animal is at rest and without undue excitement.
- E. The longer whole blood is held before examination, the greater will be the deterioration.
- F. When tubes contain concentrated anticoagulant solution, care should be taken to add an adequate amount of blood to minimize the dilution factor.

Preparation of serum specimen for biochemical analysis:

- 1) Blood is allowed to clot for 15 mts. at room temperature. If clotting time is prolonged (1 hour) it yields greater quantity of serum and minimizes haemolysis. Tube is kept in slight slanting position.
- 2) The clot adhering to the wall of the centrifuge tube is removed by "ringing" which refers to the gentle sweep around inside walls of the tube with a glass rod to dislodge the clot from the wall.
- 3) Later centrifuged at 2500 rpm for 5 minutes.
- 4) The supernatant (serum) is removed within two hours from the time of specimen collection. If serum is not promptly removed or separated, intracellular fluid is excreted which yield, erroneous results/findings for such as analysis of potassium.

Serum glucose level is also found to fall more rapidly due to glycolysis, if the cells are in contact with the serum.

- 5) Serum is kept in labeled tubes at room temperature. If delay is expected, refrigeration is done and refrigerated specimen can be analysed within 24 hours.

Preparation of plasma specimens:

- 1) Adequate amount of anti coagulant is added to collected blood sample to get suspended blood fluid.
- 2) Later the suspended blood fluid is centrifuged to get plasma (supernatant fluid).

For estimation of glucose, plasma specimens are used. For glucose estimation NaF is the anti coagulant of choice, because it neutralises phospho-fructo-kinase and thereby glycolysis is prevented. Hence, glucose utilization by RBC, WBC and bacteria (anaerobic organism) is prevented. It also controls bacterial growth. Besides this NaF is a weak anti-coagulant which cannot complete coagulation. Hence to avoid ambiguity glucose is estimated from plasma sample only.

URINE COLLECTION

Urine specimens are routinely analysed for the diagnosis of renal and metabolic disorders. They can also be used in toxicology investigations. Qualitative screening of a single discharge can provide an overall picture of renal disorder & diabetic condition (glucosuria), but quantitative biochemical analysis of a urine specimen is done for hormonal assays and diagnosis of endocrine related problems.

A composite sample of the urine discharged through the 24 hours period is necessary because many of the constituents exhibit diurnal variation. The 24 hours urine gives a total picture, which is fairly reproducible. A morning specimen of urine, collected before breakfast, is considered to be ideal for the chemical screening of a "Single specimen". For urobilinogen analysis with a single specimen, an afternoon specimen is preferred. The specimen should be collected between 2 P.M and 4 P.M. The lab should provide labeled clean containers for collection. For collecting a 24 hours specimen the container should be about 4 liters capacity with appropriate preservative. The bottle must have a proper label with the name of patient, hospital, date of collection, starting time and finishing time, written in bold letters: **DO NOT DISCARD** (24 hour urine collection, refrigerate promptly). Inadequate amount of preservative, loss of voided specimens or inclusion of two morning specimens in a 24 hour period are common sources of error in specimen collection.

The 24 - hours creatinine excretion is the same for an individual and can be used as a guide to the adequacy of the 24 hour collection, This is particularly useful if several 24 hour urine specimens are collected from the same person.

Procedure:

- 1) Take a clean urine collection bottle with preservative and label. Discard the first morning specimen and fill the information on label. Note that from this point urine will be collected for a 24-hour period. Record the starting time on the label.
- 2) Collect all the urine discharged from the recorded time until the following 24-hour period. At the end of the 24 hours collection period the bladder is emptied and this urine specimen is added to those already collected. Refrigerate the specimen.
- 3) Measure the total volume and take a portion of well-mixed urine for biochemical analysis. Volume can be measured either from the marking on the bottle (if provided) or by pouring into a graduated cylinder. An easy way is by weight method.
 - a) Weigh the container (WC). Write the wt.
 - b) Weight the container with the 24-hour urine specimen (WUC)
 - c) Volume of the urine is (WUC-WC). This method considers the specific gravity of urine to be 1, instead of greater than 1 (1.0/0 or higher), but the error is not very significant.

COLLECTION OF CEREBROSPINAL FLUID (CSF)

Chemical analysis of CSF is needed to diagnose maningeal problems like infection or development of tumor. Routine chemical analysis of CSF is usually restricted to protein (globulin and total) and glucose. Glucose analysis must be done promptly in order to avoid loss of glucose due to glycolysis. Add sodium fluoride (0.5 mg/ml) to a portion of CSF and then refrigerate. Use 1 ml of diluted specimen (dilute 1:10 with distilled water) for glucose assay.

Two sites are available for collection of CSF. One is the puncture of cisterinomagna (Sub-occipital). This site is most useful for horses, cattle, sheep, dogs and cats. The second is sublumbar puncture. This is an alternative site useful in cattle, sheep and goats.

Procedure:

Sub-occipital puncture: Cast the animal on the right side, flex the head to the left so that its longitudinal axis forms a right angle with the longitudinal axis of the neck, Shave, clean and sterilize the area. Take a 3-4 inches long, 16 gauge, sterile, needle with a stylet & insert it at the cervical midline at the level of cranial edge

of the wings of the atlas. The direction of needle should be parallel to the long axis of the head. When the needle enters the subarchnoid space, we suddenly feel no resistance. On removal of stylet CSF will flow out. If ready flow does not occur, withdraw 5 ml of the fluid by means of a sterile syringe.

Sub-lumbar puncture: Here CSF can be collected while the animal is standing. The site is a depression found in the midline between dorsal spinous processes of the last lumbar vertebra and cranial edge, the median saronal crest. The area is shaved, cleaned and sterilised. A long 5-inch, 14-16 gauge needle is inserted in the midline. On entering into the subarachnoid space, CSF will flow out when style is withdrawn, if the fluid does not flow out, 5 ml. of it may be drawn with a sterile syringe.

COLLECTION OF SYNOVIAL FLUID

Synovial fluid is found around the joint. The Chemical composition of synovial fluid resembles that of other body fluids except that it has hyluronic acid. Hyluronic acid is a mucopolysaccharide that acts as a binding and protective agent for the connective tissue.

Specimen: The synovial fluid is obtained by aspiration of joint. About 1 ml is present in each joint such as the knee, ankle, hip, elbow, wrist or shoulder. A trained nurse obtains the specimen or the attending physician does dispatch of the specimen in the same way as CSF.

EDTA is added to the specimen, which will be used for cell counting while fluoride is added to the specimen to be used for glucose analysis.

Chapter 26

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
 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 cell by its own enzymes).

Colour of urine: Amber yellow/pale yellow. The substance responsible for the color of urine is 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>i.e.</i> fever causing disease.
Milky	Chylomicron	Chyluria
Red	Hb, Uroerythin	Haemoglobinuria
Greenish Yellow	Bile pigments	Jaundice

Transparency: The normal urine is perfect, 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 shows 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 oxentia cells during the formation of HCl and this is known as alkaline tide. Acid forming diets (non-vegetarian diets is protein rich food) makes the plasma acidic and this makes urine acidic. Base forming diets (vegetarian diets) makes 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 increases. Specific gravity of urine is measured using urinometer. This is standardized at 15 °C. For every 3° 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. 2.5. 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 0.2-0.8 gm/day |
| 3. Phosphate 1.1 gm/day | 7. Creatinine 1.2-1.8 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 by 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 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 as 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. Benedicts 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 the 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 the pressure on abdomen.

Lactosuria: Presence of lactose in the urine is known as lactosuria. This is observed in the 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 $< 1 \text{ mg}/100 \text{ ml}$. Rothera's test is used to identify ketone bodies in the urine.

3. Proteins: Presence of protein in urine is known as proteinuria. Sulpho salicyclic 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 type 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 pregnancy, vigorous exercises and orthostatic Proteinuria i.e. the urine formed in lying position is free of albumin whereas urine formed while standing position has albumin (due to pressure on kidney tubule), such condition is known as orthostatic protienuria.

b. Pathological: Divided in to 3 groups.

i. Prerenal: 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, Nephrotic syndrome, Hyaline degeneration of kidney.

iii. **Post renal proteinuria:** In this the protein is added some where in the urinary tract. The cause for this are; Trauma of the lower urinary tract, vaginal secretions, seminal secretions.

Bence Jones Proteinuria: Multiple myeloma is a condition, where in 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 immunoglobulins molecules. Heating the urine to 60°C, where in a coagulum appears and on further heating it dissolves can identify them.

4. **Blood:** Presence of blood in the urine detected by Benzedine Test is classified as:

a. **Haematuria:** Where in on microscopic examination of urine both RBC and WBC are present. 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. Haye's test is performed to detect the presence of bile salts in the urine. Bile pigments are identified by Gmellins test and Fouchet's test.

ANALYSIS OF NORMAL CONSTITUENTS OF URINE

1. Physical properties

a. Colour	Amber yellow	d. Specific gravity	15°C
b. Odour	Aromatic	30°C	1.012
c. Reaction to litmus	Blue to red → acidic Red to blue → alkaline	1.017	
		e. Total solid constituents	44.2 gr/litre

II Chemical Reactions:

	Experiment	Observation	Inference
1	<u>Test for Chloride:</u> Take 2 ml of urine and add 2-3 drops of conc. HNO ₃ to it. Mix well and finally add 1 ml of silver nitrate.	White precipitate of silver chloride	Indicates presence of chloride
2	<u>Test for calcium:</u> Take 2 ml of urine and add 1 ml of Barium chloride to it.	White crystalline precipitate	Calcium is present
3	<u>Test for phosphate:</u> Take 2 ml of urine and add 1 ml of conc. HNO ₃ and 1 ml of Ammonium molybdate+1ml of liquor ammonia	Cannery yellow precipitate	Phosphate is present

4	<u>Test for sulphates:</u> Take 3 ml of urine in a test tube and add 2 ml of Barium chloride to it. Mix it well precipitate and filter the above. Heat the filtrate to boil	White precipitate is formed Turbidity is seen	Total sulphur is present Ethereal sulphate is present
5	<u>Test for ammonia:</u> Take 3 ml of urine in a test tube and add 2-3 drops of Phenolphthalein indicator. Make it alkaline by adding sodium carbonate drop by drop till a pale pink colour is obtained. Heat this solution and hold a rod dipped in HCl at the mouth of the test tube	White fumes (NH_4Cl is formed)	Ammonia is present
6	<u>Test for Urea:</u> a) Sodium Hypobromite test: Take 2 ml of urine. To it add few drops of sodium Hypobromite solution. a. Specific Urease Test: Take 2 ml of urine and add a few drops of urease suspension. Add 2-3 drops of Phenolphthalein	Brisk effervescence is formed Pink color is seen	Indicates presence of urea Indicates presence of urea
7	<u>Test for Uric Acid:</u> a. Phosphotungstic acid test: Take 3 ml of urine solution Add 4-6 drops of phosphotungstic acid reagent to it and 1 ml of Na_2CO_3 . Mix well	Deep blue colour	Indicates presence of uric acid
	b. Fehling's Test: Take 1 ml of Fehling A + 1 ml of Fehling B + 2 ml of urine. Heat it to boil	Red ppt is formed	Indicates presence of uric acid
8.	<u>Test for Creatinine:</u> a. Jaffe's Test: Take 2 ml of urine solution + 2-3 drops of picric acid. Mix well + few drops of 40% NaOH	Ruby red colour changes to yellow	Indicates the presence of creatinine
	b. Wyle's Test: Take 3 ml of urine solution + 3-4 drops of sodium nitroprusside and mix well + 2-3 drops of NaOH	Ruby red colour changes to yellow	Creatinine is present

Conclusion: The normal constituents present in the urine are Chloride, Calcium, Phosphate, Sulphates, Ammonia, Urea, Uric acid and Creatinine.

IDENTIFICATION OF PATHOLOGICAL CONSTITUENTS OF URINE

I. Physical characteristics:

Colour : Colourless

RxN to Litmus : Acidic

Odour : Fruity

Transparency : Turbid

II Chemical Properties:

Experiment	Observation	Inference
I. TEST FOR PROTEIN:		
a. Sulpho salicyclic Acid Test: Take 2 ml of urine + 1 ml of sulpho salicyclic acid solution.	Yellow precipitate	Presence of protein sulphosalicylate
b. Heller's Test: Take 2 ml of conc. HNO_3 ; add 2 ml of urine along side of the test tube.	White ring	Presence of protein
c. Heat coagulation Test: Fill $\frac{3}{4}$ of test tube with urine add 2-3 drops of 1% acetic acid and heat the upper $\frac{1}{3}$. To the same solution add 5-6 drops more of glacial acetic acid	Coagulum is seen Turbidity remains	Protein is present. Turbidity is due to proteins only & not due to phosphates
II. TEST FOR CARBOHYDRATES:		
Benedicts Test: Take 5 ml of Benedicts reagent and add 8 drops of urine, heat it to boil.	Green Precipitate	Presence of reducing sugar
III. TEST FOR KETONE BODIES:		
Rothera's Test: 5 ml of urine in a test tube. To it add ammonium sulphate till it saturates. Then add 2-3 drops of sodium nitroprusside solution. Mix well. Add 5 ml of liquor ammonia along sides of the test tube.	Permanent Permanganate pink colour is seen	Acetone is present
IV. TEST FOR BILE SALTS:		
Haye's Test: Fill $\frac{3}{4}$ of test tube with urine sprinkle sulfur powder on the surface of urine without shaking.	Sulphur settles or sinks down	Bile salts are present
V. TEST FOR BILE PIGMENT:		
a. Gmellins Test: 5 ml of urine is taken in a test tube to this add 5 ml of conc. HNO_3 along sides of test tube	Play of colours (Blue to green to purple to Yellow)	Bile pigments are present

b. Fouchet's Test: 3 ml of urine in a test tube. Add 1 ml of BaCl ₂ . Add 1 ml of MgSO ₄ . Mix well and keep it aside for 1min. filter it. Dry the ppt and add 2-3 drops of Fouchet's reagent on ppt.	Green colour	Bile pigments are present.
VI. TEST FOR BLOOD: Benzedine Test: Take 3 ml of urine in a test tube. Add 2 ml of benzedine reagent to it mix it well and then add 1 ml of H ₂ O ₂ .	Deep blue or green color	Blood is present

Conclusion:

- (1) If reducing sugars and ketone bodies are present, the animal is diabetic.
- (2) If proteins, blood and bile are present then there is some kidney disorder like nephritis or renal damage.

Reagents and principles governing the analysis of pathological constituents of urine:

1) Heat coagulation test for albumin: The addition of 1% acetic acid before heating urine is to bring urine to the isoelectric pH of albumin. If the pH of urine is highly acidic i.e. 5, then avoid adding acetic acid.

2) 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 gms)	Color of the precipitate
0.5 - 0.9	Green
1.0 - 1.4	Yellow
1.5 - 1.9	Orange
2.0 and above	Brick red

Urine

- 3) **Rothera's Test:** The addition of ammonium sulphate to the urine is to precipitate proteins present in the urine.
- 4) **Haye's Test:** Presence of bile salts like sodium glycocholate and sodium taurocholate reduces the surface tension thereby causing sulphur powder to sink down.
- 5) **Gmellin's Test:** The play of colours observed upon the addition of nitric acid to urine is due to the oxidation of Bilirubin to Biliverdin to Bilicyanin to urobilinogen to urobilin.
- 6) **Fouchets Test:** The formation of green color is due to the oxidation of Bilirubin to Biliverdin

Composition of the reagent: Ferric Chloride in trichloroacetic acid (TCA).

- 7) **Benzedine Test:** Hb catalytically decomposes H_2O_2 and liberates nascent O_2 . This oxygen oxidizes Benzedine to a blue or green coloured compound.

Composition of the reagent: Saturated solution of Benzedine in glacial acetic acid.

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Chapter 27

Colorimetry

Colorimetry, literally means, the measurement of colour.

The term colorimetry is now replaced with photometry, wherein the absorbance of light (visible or invisible) of a particular wavelength is measured.

Quantitative estimations of a particular substance present in the biological sample is done by the use of colorimetry. For the calorimetric estimations first of all the interfering substances from the sample are removed, (For example blood cells from blood) or the substance is extracted in pure form, from the sample (For example sugars are extracted from crude feed). Then the sample is suitably diluted (if required) and is reacted with a reagent in order to develop a particular colour. The intensity of the colour developed is proportional to the amount of the substance present. The intensity of the colour is measured by using a calorimeter or spectrophotometer.

Colorimeter and Spectrometer: These are electronic devices that measure the intensity of the colour of a solution. There are two laws, which govern the colorimetric estimations. (1) Beer's Law (2) Lambert's (Bouguer) Law.

According to Beer's law the amount of light transmitted through a coloured solution decreases exponentially with increase in the concentration of the coloured substance. According to Lambert's law the amount of light transmitted decreases exponentially with increase in thickness of the layer of the solution through which the light passes. Thus combination of the two laws gives the principle of colorimetry i.e. the optical density of a solution is directly proportional to the concentration of the substance and the depth of the solution through which the light passes.

Optical density (O.D.) or extinction:

Optical density (O.D): Optical density is the logarithmic ratio of the incident light to that of emergent light.

$$\text{O.D.} = \log I_0/I$$

Transmission (T): Transmission is the ratio of intensity of transmitted light to that of incident light.

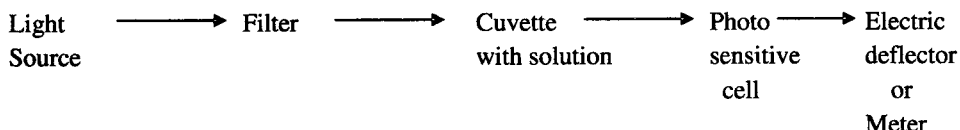
$$T = I/I_0$$

Relationship between optical density and transmission:

$$\text{O.D} = 2 - \log \% T$$

When transmission is 100% the optical density is 0.

COMPONENTS OF A COLORIMETER



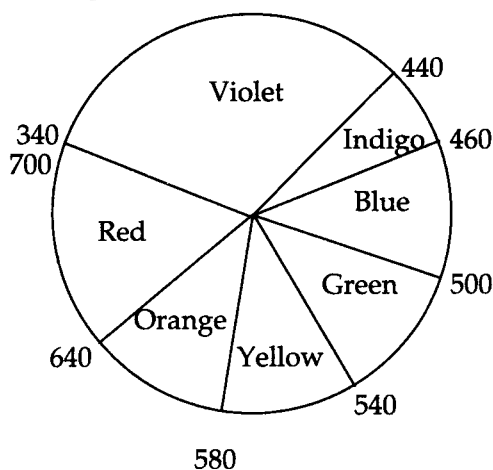
Colorimeter consists of a light source, which is generally a tungsten filament. Light is a composite of 7 colours. So only one colour corresponding to a single wave length is passed, which is complementary to the colour of the solution and all the other colours are filtered out by a filter.

The solution under test, taken in a cuvette is placed in the pathway of this light. Some of the light is absorbed by the solution, which depends upon the intensity of the colour and the remaining light is transmitted. The transmitted light falls on the photosensitive cells, which converts it into electrical impulses, which in turn deflects a pointer on a scale or gives digital reading. The readings can be taken as % transmission or optical density (O.D).

Complementary colours and their wave-lengths: The natural light (solar spectrum) is composed of

Deuterium lamp		Tungsten lamp	Hydrogen Lamp		
Vacuum UV	Ultra violet	Visible	Intra Red	Micro Wave	Radio
110-200 nm	200-400 nm	400-760 nm	760-10 ⁵ nm	10 ⁷ nm	10 ⁸ nm

Cosmic rays, ultraviolet rays, visible range, intra red and X-rays. The visible region is spread over 360 nm. If a circle is drawn with an angle of 360 degrees, each angle of the circle corresponds to one wavelength.



The wavelengths appearing at the two edges of each diameter are the complementary wavelength and the colours at those points are complementary to each other. The following is the list of the colours of the solutions, their corresponding complementary colours and wavelength to be used, in colorimetric estimation.

	Colour solutions	Complementary Colour/Filter colour	Wavelength
1	Purple	Green	520
2	Blue	Red	680
3	Bluish-Green	Red	680
4	Green	Red	710
5	Yellowish-Green	Violet	430
6	Yellow	Violet	420
7	Orange-Red (Red-Orange)	Blue-blue green	450
8	Red	Blue-Green	490

Colorimetry needs three types of solutions (1) Blank (2) Test and (3) Standard

1. **Blank Solution:** The function of the blank solution is to eliminate the effect of light absorption by the reagents. If the reagents used are coloured, they will also absorb light and give rise to high value of optical density and hence gives a false higher value for the concentration of the substance to be analysed.
2. **Test Solution:** This is the solution, which is under test or the sample in which the chemical constituent is to be estimated.
3. **Standard Solution:** A standard solution is a solution, which contains a known quantity of the chemical constituents, being estimated, dissolved in pure distilled water or pure organic solvent. The amount of this constituent present in the solution will be an average value of that constituent suspect to be found in normal amount in the biochemical sample under test.

The following table gives the normal amounts of various chemical constituents found in the blood of an adult cattle and the concentration of the standard solution to be prepared.

Chemical constituent	Blood fraction	Normal value (in cow)	Amount of substance dissolved/ 100 ml of standard
Glucose (mg/100ml)	Whole blood	35-55	45 mg.
Protein (g/100ml)	Plasma	6-8	7 g
Cholesterol (mg/100ml)	Serum	50-230	150 mg.
Phosphorus (mg/100ml)	Serum	4-7	5 mg

The standard solution prepared is subjected to the same conditions as that of the test sample i.e. same reagents are added to the same quantity of the solution and incubated at the same temperature and the optical density is taken. The optical density is taken. The optical density of the standard is compared with the O.D. of the test and is multiplied with the concentration of the standard solution. This gives the exact amount of the chemical constituent present in the biochemical sample under test.

For example the concentration of standard protein is 7 Grams per 100 ml.

O.D. of standard = 0.22

O.D. of test = 0.19

Then the amount of protein in 100 ml of plasma is calculated as under.

0.22 O.D. is given by 7 Grams

0.19 O.D. is for ?

$0.19 \times 7 = 6.05$ Grams/100 ml.

0.22

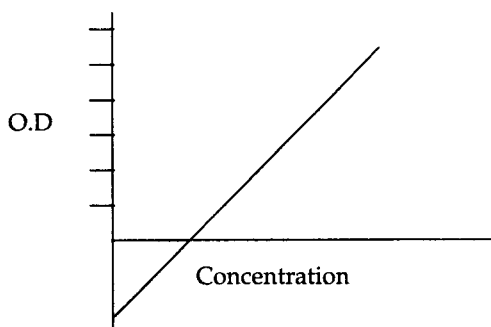
Therefore the general calculation for colorimetric estimation is as under.

O.D. of Test × concentration of standard

O.D. of Standard

Calibration curve: Preparation of a single standard and comparing it with the unknown sample will have a potential for a high degree of error. Hence a range of standards is prepared with an ascending order of concentration, each varying in equal amounts. All these standards are reacted with the same reagent and subjected to the same condition and the O.D. is taken at a particular wavelength. Then a graph is drawn with the concentration of standards on the x-axis and optical densities on y-axis.

Colorimetry



This is known as a calibration curve for the particular constituent at specified conditions. A good calibration curve is that which passes through the origin, maintains an angle of 45° with x/y axis, and cuts maximum number of points along its length. Once a calibration curve is made, the concentration of the unknown is traced out from this graph and reported. The calibration curve should be re-drawn with every new batch of reagent for each individual colorimeter or change of bulb in the same colorimeter.

The following tables give the method of preparation for different biochemical constituents for the preparation of their respective calibration curves (standard curves).

- (a) **Glucose:** Dissolve 1 g of glucose (dextrose) in 100 ml of 0.2 % benzoic acid solution. This solution is diluted as follows:

Sl.No.	Volume of stock standard glucose (ml)	Distilled water (ml)	Glucose concentration in the final solution (mg/dl)
1	5	95	50
2	10	90	100
3	20	80	200
4	30	70	300
5	40	60	400
6	50	50	500

Then react each of the six solutions prepared above with those reagents by which method the blood glucose is being analysed. If that particular method demands dilution of blood sample, the standards prepared above should be diluted accordingly. Then take the O.D. and plot the graph.

- (b) **Protein:** Dissolve 2 gram of bovine serum albumin (egg albumin/Gelatin) in 200 ml water. This standard protein contains 10 gms/dl, diluted to 10 folds.

Sl.No.	Volume of stock standard protein (ml)	Distilled water (ml)	Protein concentration in the final solution with 10 fold dilution (mg/dl)
1	1	9	1
2	2	8	2
3	3	7	3
4	4	6	4
5	5	5	5
6	6	4	6
7	7	3	7
8	8	2	8
9	9	1	9
10	10	0	10

(c) **Cholesterol:** Dissolve 250 mg of cholesterol in 100 ml glacial acid, dilute it to 4 fold.

Sl.No	Cholesterol (ml)	Acetic acid (ml)	Concentration of Cholesterol in final solution
1	0.5	2.0	50
2	1.0	1.5	100
3	1.5	1.0	150
4	2.0	0.5	200
5	2.5	0.0	250

Quantitative Estimations

ESTIMATION OF BLOOD SUGAR

(I) Folin – Wu – Method:

1. **Specimen required:** Whole blood \ Plasma \ Serum: 1 ml.
2. **Principle:** The blood / plasma / serum is first made protein free by precipitating the proteins with acid. The protein – free filtrate is boiled with alkaline copper tartarate solution. The cupric ions are reduced to cuprous ions, which reduces phosphomolybdic acid to phosphomolybdous acid (blue colour).
3. **Procedure:** Take 7 ml of distilled water in a centrifuge tube, add 1 ml of the sample (blood) and 1 ml of 10 % sodium tungstate to it. Mix it well and then add 1ml of 2/3 N H₂SO₄ drop by drop with constant shaking. Let stand for 5 minutes and centrifuge at 1500 rpm for 10 minutes or filter. The supernatant or the filtrate is the protein free blood filtrate that will be used for the estimation.

Take three Folin-Wu tubes; label them as “T” (for test), “S” (for standard), and “B” (for blank). Pipette out 2ml of Protein free blood filtrate to the tube marked “T”, 2 ml of working standard glucose solution to tube “S” and 2 ml of distilled water into tube “B”. Add 2 ml of alkaline copper tartarate in each of the tubes and mix. Keep all the tubes in boiling water bath for 8 minutes. Cool the tubes in running tap water and add 2 ml of phosphomolybdic acid solution to each tube and wait for 2 minutes. Fill the tubes up to the 25 ml mark with water and mix thoroughly by repeatedly inverting the tubes. Take the optical density at 420 nm or use blue filter.

Note: Folin-Wu tube is designed to prevent the oxidation of cuprous ions by atmospheric oxygen. The constricted neck of the tube decreases the surface of the solution exposed to the atmosphere.

4. Calculation:

$$\text{Blood glucose mg /dl} = \frac{\text{O.D of Unknown}}{\text{O.D of Standard}} \times 0.2 \times \frac{100}{0.2}$$

5. Reagents:

- a. **Sodium tungstate 10 %:** Dissolve 10 g of Sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) in 100 ml of water.
- b. **Sulphuric acid 2/3 N:** Dilute 5 ml of Conc. H_2SO_4 to 250 ml with dist. water and standardized against 2/3 N NaOH.
- c. **Alakaline copper tartarate:** Dissolve 40 g of anhydrous sodium carbonate in about 400 ml of water. Add 7.5 g of tartaric acid. Dissolve 4.5 g of crystalline copper sulphate separately and then add to the above mixture. Finally make the volume to 1000 ml with water.
- d. **Phosphomolybdic acid:** Dissolve 35 g of molybdic acid in 200 ml of 10 % NaOH. Add 5 g of sodium tungstate and 200 ml dist. Water and boil the solution for 30 – 40 minutes. Cool the solution and make the volume to about 350 ml with water. Add 125 ml of 85 % phosphoric acid and dilute to 500 ml with water.
- e. **Stock standard glucose solution:** Dissolve 100 mg of glucose in 100 ml of 0.1 % benzoic acid solution. Benzoic acid acts as a preservative.
- f. **Working standard glucose solution:** Dilute 10 ml of the stock standard glucose solution to 100 ml with 0.1 % benzoic acid solution. 1 ml of this solution contains 0.1 mg of glucose.
6. **Merits and Demerits of this method:** Plasma with anti-coagulant is used. Is laborious and needs deproteinisation. The values are 10-20% higher than true glucose value, as other reducing substances like Hexoses, pentoses. Vit. C glutathione, Urea, Uric acid etc. contribute to the reaction.

(II) O-Toulidine method:

1. **Specimen required:** Serum (1 ml)
2. **Procedure:** Take 3 test tubes and label them as T (Test Serum), 'S' (Standard glucose) and 'B' (for Blank). Add 1 ml of deproteinized diluted serum in the tube marked 'T', 1 ml of diluted standard in 'S' and 1 ml of distilled water in 'B'. Then add 7 ml of O-Toluidine reagent to all the three test tube and mix them well. Then cover them with a loose cap, cotton or aluminium foil. Then place them in boiling water bath for 10 minutes and cool them under tap after. Read the absorbance of the colour within 30 minutes at 630 nm.

3. **Calculation:**

$$\% \text{ blood sugar} = \frac{\text{Optical Density of the Test} \times \text{Conc. of std.}}{\text{Optical Density of Standard}}$$

4. **Reagents:**

- a. **O-Toluidine reagent (5 ml/dl):** Dissolve 3 grams of thiourea in 1900 ml of glacial acetic acid and add 100 ml of O-Toluidine solution to it and mix well. Store it in brown bottle at room temperature. It keeps indefinitely.
 - b. **Standard glucose solution (100 mg/100 ml):** Dissolve 1 gram of glucose (dextrose) in 100 ml of 0.2% benzoic acid solution. Then take 10 ml of this solution and dilute it to 100 ml with 0.2% benzoic acid solution. Dilute the standard four fold (1:3:: Standard: H₂O).
5. **Principle:** The aldehydic group of glucose on heating to 100° C condenses with O-Toluidine in acetic acid to form N-glucosylamine giving an Emerald blue-green colour. The intensity of the colour, which is proportional to the glucose concentration, is measured photometrically. Thiourea in the reagent stabilizes the reaction.
7. **Merits and Demerits of this Method:** This method is simple easy and cheaper. In this the value of glucose is much closer to the true blood glucose value. However a fewer substances like fructose and galactose can effect the colour. This method is discouraged because of the carcinogenic effect of O-Toluidine.

III. **Other methods for blood glucose estimation:**

- A) **Nelson-Somogyi method:** It is modified Folin-Wu-Method.
- B. **Glucose oxidise method:** It is very costly, laborious but very accurate for 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.

IV. Interpretation: The normal fasting level of serum glucose determined as "True glucose" is 65 to 95 mg/dl (by glucose Oxidase method) or 80-120 mg/dl as "reducing substance" (by other methods). Every method of glucose estimation has own normal value. The serum glucose is 15% higher than whole blood. Immediately after meals the blood glucose level starts increasing and the increase in the concentration depends upon the type of the meal (break fast, lunch or dinner). In normal persons the maximum concentration of Random blood glucose will not be higher than 200 mg/dl and not below 40 mg/dl.

Hyperglycemia: An increase in the blood glucose level is known as Hyperglycemia. Blood glucose level can rise to 500 mg/dl and above which it will lead to coma and death. The highest values of the fasting blood sugar are seen in diabetes mellitus. The other conditions in which hyperglycemia occurs are pancreatitis and carcinoma of the pancreas. Sepsis, infectious disease, Meningitis, Encephalitis, tumors and Hemorrhage are other conditions of hyperglycemia. Anesthesia also produces hyperglycemia and glycosuria, intensity of which depends upon the degree and duration of anesthesia.

Hypoglycemia: Decrease in blood glucose level is known as Hypoglycemia. Glucose level below 40 mg/dl as true glucose is referred to as Hypoglycemia. Hypoglycemia occurs due to over dosage of insulin in the treatment of diabetes. Insulin secreting tumors of pancreas, hypothyroidism, hypopituitarism and

hypoadrenalism, creates severe hypoglycemia. Steatorrhea, partial gastrectomy and alcoholism are the other causes of Hypoglycemia.

ESTIMATION OF TOTAL SERUM PROTEIN BY BIURET METHOD

1. Specimen required: Serum 0.2 ml

2. Procedure: Take 3 dry test tubes and label them as 'T' (test), 'S' (standard) and 'B' (blank). Add 4 ml of Biuret reagent to each of the tubes, then add 0.2 ml of serum to the test tube marked 'T' and 0.2 ml of standard solution to the tube marked 'S' then add 0.8 ml distilled water to the tube 'T' and 'S'. Finally add 1 ml distilled water to the tube marked 'B' mix the contents thoroughly and leave it for 30 minutes at room temperature, then measure absorbance/(O.D.) optical density at 540 nm.

3. Calculation:

$$\frac{\text{OD of Test}}{\text{OD of standard}} \times \text{Concentration of Standard} = \text{_____ gms/100 ml}$$

4. Reagents

- i. **Biuret diluent:** 5 g of potassium iodide (KI) in 0.25 N sodium hydroxide (=10 grams NaOH/l). The sodium hydroxide solution may be prepared in larger quantities as a stock solution (2.5 N) which is diluted ten fold with distilled water.
- ii. **Stock biuret reagent:**
 1. Dissolve 15 grams of copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) in 70 to 80 ml of water placed in a beaker.
 2. In a 1000 ml volumetric flask, prepare a solution of 45 g. Rochelle salt (Potassium sodium tartarate, tetrahydrate) in 600 to 700 ml of Biuret diluent (solution (i)).
 3. Add slowly the copper sulfate solution (1) into the volumetric flask containing tartarate solution (2).
 4. Add biuret diluent (i) up to the 1000 ml. mark of the volumetric flask. Store in a polyethylene bottle, away from strong, direct light.
 5. Working biuret solution: On the day of use dilute the stock biuret five fold with the biuret diluent (20 ml stock + 80 ml diluent).
- iii. **Standard protein solution:** Dissolve 5 g of bovine albumin in 100 ml of distilled water.
5. **Principle:** Proteins and peptides react with alkaline copper tartarate solution to give a violet coloured complex. The intensity of the final colour

complex is measured, colorimetrically at 540 nm. (range 530 to 560 nm) and is proportional to concentration of total protein in the specimen under test.

6. **Merits and demerits of this method:** This method is still popular, easy to follow and provides accurate results. This method is recommended by the international federation of the clinical chemistry (IFCC). The demerit of this method is that micro quantity of proteins and large quantities cannot be estimated.
8. **Other methods of protein estimation:**
 - a. **Lowry's Method:** Proteins react with folin-ciocalteau reagent and gives a colour complex. The intensity of color depends on the amount of aromatic amino acids. Hence the colour varies for different proteins. This limits the use of this method. 5 ml of alkaline solution (made up of 5 grams CuSO_4 , 10 gms of Na, K, Tartarate, 20 gms Na_2CO_3 in Lit of 0.1 M NaOH) is added to 1 ml of the test solution. After 10 minutes 0.5 ml of diluted folin-ciocalteau reagent is added and the OD taken at 750 nm after 30 minutes and compared with standard.
 - b. **Ultra-violet absorption of protein:** Proteins absorb light at 280 nm, due to tyrosine, tryptophan and phenylalanine and at 210 nm, due to peptide bond. By using a spectrophotometer at these wavelengths, proteins can be estimated. The disadvantage of this method is that many other compounds absorb in this region. Ex. Nucleic acids.
9. **Interpretation:** Serum proteins consist of albumin and globulins. Normal values (adult) total proteins: 6.2 to 8.2 g/dl. Albumin: 3.5 - 5.0 g/dl. Globulin: 2.7 - 3.2 g/dl. Serum protein analysis can diagnose liver disorders, nutritional deficiency of protein, renal failure and others. An increase in the protein concentration in blood is known as Hyperproteinemia which is seen in condition like dehydration, excess issue destruction, cancer, TB., Nephritis, Pregnancy, Myocardial, infraction. Decrease in total protein value is known as hypoproteinemia, associated with cirrhosis of liver, malnutrition, hemodilution hemorrhage, burns, hepatitis, malaria and abortion.

ESTIMATION OF TOTAL SERUM CHOLESTEROL, DIRECTLY USING THE LIEBURMANN BURCHARD REACTION

1. **Specimen required:** Serum 1 ml.
2. **Procedure:** Take 3 test tubes and label them as 'T' for test, 'S' for standard and 'B' for blank. Take 1 ml of serum in 'T', 1 ml of standard cholesterol in 'S' and 1 ml of acetic acid in 'B'. Then add 5 ml of ferric chloride reagent to all the three test tubes. Mix them thoroughly and place them in boiling

water bath for 2 mts. Then cool immediately in running tap water and take optical density at 560 nm.

3. Calculation:

$$\frac{\text{O.D. of test}}{\text{O.D. of Standard}} \times \text{Concentration of standard}$$

4. Reagent:

- a. **Ferric chloride reagent:** Dissolve 2.5 gms of ferric chloride in 100 ml of phosphoric acid. It's stable indefinitely.
- b. **Cholesterol standard:** Dissolve 250 mgs of cholesterol in 100 ml of glacial acetic acid and dilute it to four fold, with acetic acid.
5. **Principle:** The classic Liebermann-Burchard reaction is the basis for this calorimetric determination cholesterol in acetic acid gives a red color with ferric chloride and phosphoric acid (or H₂SO₄).
6. **Other methods of cholesterol estimation:** Modified Leffler method, and Zlatkis, Zak and Boyle methods.
7. **Interpretation:** Cholesterol is a lipid and is classified as a sterol. It is widely distributed in various animal tissues and cells. It is consumed with food, and can also be synthesized in liver, is a normal constituent of bile and principle constituent of gallstones. It is important in metabolism as a precursor of steroid hormones, like sex hormones and adrenal corticoids. The total serum cholesterol includes esterified cholesterol and non-esterified cholesterol. The normal amount of total cholesterol is 50-250 mgs/dl and increases with age and may reach 300 mgs/dl in middle age. Cholesterol level is elevated in coronary artery disease, hyperlipoproteinaemias, hypothyroidism, nephritis, diabetes mellitus and various liver diseases. Low cholesterol in pernicious anemia, hemolytic jaundice, malnutrition, acute infections and hyperthyroidism.

ESTIMATION OF BLOOD UREA BY OXIME METHOD

Principle: Diacetyl monoxime in a hot acid medium in presence of ferric ions reacts with urea producing a specific pink coloured complex. The intensity of the colour developed is proportional to the concentration of urea nitrogen in the sample, measured at 520 nm.

Procedure: Take three test tubes and label them as 'T' (For test) 'S' (For standard) and 'B' (For blank). Pipette 3 ml of Oxime colour reagent and 3 ml of acid reagent in each of the test tubes and mix it well. Then add 1ml of the serum to the tube 'T' 1ml of standard urea to tube 'S' and 1 ml distilled water to 'B'. Mix them

well and keep them in a boiling water bath for 10 minutes. Allow them to cool and read the optical density at 520 nm.

Calculation : Standard urea solution contains 40 mg / 100 ml.

Therefore the amount of urea in the sample = $\frac{\text{OD 'T'}}{\text{OD 'S'}} \times 40$

Reagents :

- 1) Oxime colour reagent contains 1gm of diacetyl monoxime, 0.2gm of thiosemicarbazide and 9 Gms of sodium chloride.
- 2) Acid reagent contains 60 ml of conc. Sulphuric acid, 10ml of phosphoric acid and 0.1 ml of ferric chloride.
- 3) Standard urea contains 40 mgs of urea and 0.2% benzoic acid in 100ml of water.

Interpretation: The normal amount of blood urea is 15 – 40 mg / dl. Its level increases in renal disorders. In pre-renal cause the increase in urea is due to increased protein degradation due to febrile disease or muscle wasting disease. The renal causes are nephritis, nephritic syndrome, or total renal failure. In post-renal causes, obstruction in the urinary tract can cause increase in urea.

DETERMINATION OF BLOOD BILIRUBIN

Principle: The coupling of Bilirubin with O-benzenediazonium sulfonate to form ozobilirubin is used for the estimation. Photometric estimation of the purple colour developed exactly 5 minutes after diazotisation with sulfanilic acid in aqueous solution is a measure of direct Bilirubin. The colour developed after adding methanol at a level that releases bound Bilirubin into solution without precipitating proteins represents total Bilirubin.

Reagents:

1. **Sulfanilic acid solution:** Dissolve 100 mg of sulfanilic acid in 1.5 ml conc. Hydrochloric acid and add water to make 100 ml.
2. **25% sodium nitrite stock solution in water:** Store at 4° C. Prepare just before use by diluting stock sodium nitrite solution 1:50 with water (0.5%).
3. **Diazo reagent:** Prepare just before use by mixing 0.3 ml of 0.5% sodium nitrite solution and 10 ml sulfanilic acid solution.
4. **Diazo blank solution:** Dilute 0.5 ml conc. HCl to 100 ml with water.

Procedure

1. Take in two tubes 0.4 ml serum and dilute with 3.6 ml water.

Quantitative Estimations

2. Add 1 ml diazo blank solution to one (blank) and to the other (test) 1 ml diazo reagent and mix immediately.
3. Read absorbance of test exactly 5 minutes after addition of diazo reagent at 540 nm against blank.
4. Add 5 ml absolute methanol to each tube and mix by gentle inversion.
5. Leave for 30 minutes and read the OD at 540 nm by avoiding formation of air bubble in cuvette.

Calculation: Assuming the absorbability of azobilirubin equivalent to 5 mg Bilirubin / 10 ml at 540 nm to be 0.211; direct Bilirubin (mg/100 ml plasma) = A_{540} at 5 min \times 11.85

Total Bilirubin (mg/100 ml plasma) = A_{540} at 30 min after methanol addition \times 23.70.

ESTIMATION OF SERUM CALCIUM BY CLARK AND COLLIP METHOD

1. **Specimen required:** Serum 2 ml undiluted.
2. **Procedure:** Take 2 ml of serum in a centrifuge tube and add 1ml of 4 N Ammonium Oxalate solution. Mix it well, and leave it for thirty minutes. Then centrifuge it at 1,500 rpm, for 5 minutes. Then pour out the Supernatant completely by inverting the tube. (Magnesium can be estimated with the supernatant). Dry the mouth of the tube with filter paper. Then wash the sides of the tubes with 3 ml of dilute ammonia and stir up the precipitate. Again centrifuge it at 1,500 rpm, and then add 2 ml of 1N H₂SO₄ by blowing it from pipette directly upon the precipitate to break it up. Keep it in boiling water bath. (at 100⁰ C) for 1 minute. Finally titrate it with 0.01N Potassium permanganate to a pink colour at 70⁰ C (The colour lasts only for 1 minute). Simultaneously run a blank with 2 ml 1N H₂SO₄.
3. **Calculation:**
$$\text{Calcium (mg/100ml)} = (X-B) \times 0.0002 \times 100/2 \times 1000$$

Where X is = test reading; B= Blank reading.
4. **Reagents:**
 - a) 4% Ammonium Oxalate Solution.
 - b) 2% Dilute Ammonia Solution.
 - c) 1N Sulphuric acid Solution
 - d) 0.01 N Potassium Permanganate Solution.

5. Principle :

Serum Calcium is precipitated as oxalate and later titrated with standard potassium permanganate for estimation.

6. Other methods of Calcium estimation

- a) EDTA Titration i.e., method of Wilkinson or The method of Baron and Bell.
 - b) Iodometric titrations.
 - c) Determination by flame photometry.
 - d) Auto-Analyser method.
7. **Interpretation:** Normal total Serum calcium ranges from about 9-11 mg/100ml. It is higher in children and it is lower during pregnancy. The level of calcium is affected by deficient calcium absorption from the intestine by alternations in the amount of parathyroid Hormone secreted by changes in serum phosphorus and by alteration in plasma protein. The lowest serum calcium is found in hypo parathroidism due to which symptoms of tetany are exhibited. Serum calcium is low in rickets, oestomalacia, steatorrhoea. High Serum phosphorus due to renal failure results in decrease serum calcium. Serum calcium is low in acute pancreatitis. Increased serum calcium is seen in Hyper-Parathroidism, and Hyper-Vitaminosis-D, resulting from over dose of Vit. D.

ESTIMATION OF INORGANIC PHOSPHORUS IN SERUM BY FISKE & SUBARROW METHOD

1. **Specimen required:** Serum 1ml – Undiluted.
2. **Procedure :** Take 9ml of 10% Trichloroacetic acid in a test tube and add 1ml serum/plasma with constant shaking. After mixing it properly, leave it aside for 5 minutes. Then centrifuge it at 2500 rpm for 5 minutes. Then take 3 test tubes & label them as "T", "S" and "B". Pipette out 5 Ml of supernatant to one tube (marked "T") and 1 ml of molybdate-II reagent. Take 5ml of diluted standard solution in tube "S" & 5ml of H₂O in tube "B" & 1ml molybdate-I reagent. Then add 0.4 ml of aminonaphtho sulphonic acid (ANSA) and 4.6 ml of distilled water to each of the three test tubes. Then take the Optical density at 680 nm. (Red filter) after 10 minutes.

Calculation: 5 ml of standard contains 0.04 mg of phosphorus and 5ml of filtrate is equivalent to 0.5 ml of serum. Therefore mg of inorganic phosphorus / 100 ml of serum.

$$= \frac{\text{O.D of test}}{\text{O.D of std}} \times 0.04 \times 100 / 0.5 \quad \text{OR} \quad \frac{\text{O.D of test}}{\text{O.D of std}} \times 8$$

3. Reagents :-

- a. **10% T.C.A. (W/V):** Dissolve 10 grams of trichloro acetic acid in 100 ml of distilled water.
- b. **Sulphuric acid:** (10N, 5 N and 3 N) Add 450 ml of conc. sulphuric acid to 1300 ml of distilled water. This gives 10 N sulphuric acid.
Take 500 ml of this 10 N sulphuric acid & add 500 ml of water to it, this gives 5N H₂SO₄.
Take 300 ml of 10N sulphuric acid & add 700 ml of distilled water. This gives 3N sulphuric acid.
- c. **Molybdate I:** Dissolve 2.5 grams of Ammonium molybdate in 100 ml of 5N sulphuric acid.
- d. **Molybdate II:** Dissolve 2.5 grams of Ammonium molybdate in 100 ml of 3 N H₂SO₄.
- e. **0.25 % of 1,2,4 – amino naphtho sulphonic acid (ANSA):** - Dissolve 0.5 grams of Amino naphtho sulphonic acid in 195 ml of 15% sodium bisulphite and 5ml of 20% sodium sulphite.
4. **Standard phosphate Solution:** Dissolve 0.351 grams of potassium dihydrogen phosphate in 1 Lit water containing 10 ml of 10 N sulphuric acid, Dilute this 1:10. (1ml contains 0.008 mg of phosphorus).
5. **Principle:** Proteins from the serum are removed by precipitation with trichloroacetic acid, the filtrate is treated with acid molybdate reagent which reacts with inorganic phosphate to form phosphomolybdic acid which is reduced with aminonaphtho sulphonic acid, giving blue compound.
6. **Other method of inorganic phosphorus estimation.**
 - a) COMORRY METHOD.
 - b) KUTTENER AND LICHTENSTEIN METHOD.
7. **Interpretation:** The normal serum Inorganic phosphate is between 2-6-mg/100 ml, It Increases in chronic nephritis, Hypoparathyroidism. It is low in rickets, osteomalacia and Hyperparathyroidism. Injection of insulin reduces serum inorganic phosphate below 1 mg / 100 ml.

METABOLITES DIAGNOSTIC TO DISEASES

Metabolite	Condition	Associated disorders
Glucose	Hypoglycemia	Liver dysfunction, chronic starvation, malabsorption, ketosis, pancreatic tumor or hyperinsulinism.
	Hyperglycemia	Diabetes mellitus, convulsions, following a meal, insulin, overdose, pancreatitis, fear, excitement.
Urea	Increased plasma urea (Hyperureamia)	High protein or urea feeding, carbohydrate deficiency, catabolic drugs or increased catabolism, dehydration, severe haemorrhage, shock, hypoalbuminaemia, intestinal haemorrhage, fever and necrosis, prolonged exercise, hyperthyroidism.

	Decreased plasma urea	Low protein diet, insipidus, liver dysfunction, anabolic steroids, malnutrition, and aflatoxicosis
Creatinine	Increase plasma	Dehydration, primary and chronic renal failure, severe exercise, high NPN feeding, muscular degeneration, lipemia, hemolysis, ketosis.
Protein	Hyperproteinaemia	Dehydration, acute, subacute and chronic inflammation, liver disease, neoplasia, autoimmune disorders, increased globulin level, newborn, haemolysis, glomerular disease, anabolic steroids, uremia.
	Hypoproteinaemia	Protein starvation liver disorders, intestinal malabsorption, haemorrhage, burns, congestive heart failure, wasting disease.
Albumin: Globulin ratio.	Increased	hypogammaglobulinaemia congenital and acquired immuno-deficiency, uraemia
	Decreased	Increased globulin, decreased albumin anaphylaxis, following immunization, protein starvation and conditions of hypoproteinaemia.
Bilirubin	Hyperbilirubinaemia	Severe acute haemolysis, liver dysfunction, cholestasis
Cholesterol	Hypercholesterolaemia	Following a fatty meal, hypothyroidism, diabetes mellitus, starvation, severe trauma, liver dysfunction, nephrotic syndrome, steatitis, cholestasis.
	Hypocholesterolaemia	Hepatitis, hyperthyroidism, low fat diets, malnutrition, severe anaemia, intestinal malabsorption.
Calcium	Hypercalcemia	Hyperparathyroidism, hypervitaminosis D, osteoneoplastic disease, high Ca diet.
	Hypocalcemia	Hypoparathyroidism, milk fever, osteoporosis steatorrhoea, nephritis, hypovitaminosis D, low Ca diet, ricket.
Phosphorous	Hyperphosphataemia	Chronic nephritis, hypoparathyroidism, hypervitaminosis D, high P diet, wheat poisoning.
	Hypophosphataemia	Rickets, hyperparathyroidism, pica, low P diet, periparturient haemoglobinuria, osteoporosis, secondary hyperparathyroidism.
Alkaline Phosphatase	Increased activity	Bone diseases, severe starvation, increased osteoplastic activity, increased bone metabolism, renal diseases, hepato-biliary diseases, osteomalacia, rickets, cholestasis, active growth, last-trimester of pregnancy.

VARIOUS ANTICOAGULANTS USED IN BIOCHEMICAL ESTIMATIONS

PRODUCT	MODE OF ACTION	AMOUNT NEEDED PER 10 ml OF BLOOD	ADVANTAGES	DISADVANTAGES
EDTA (Na/K salt of ethylene diamine tetra acetate)	Forms insoluble calcium salts	10-20 mg (1 ml of 1% solution. Dried at room temp. or in incubator)	Excellent preserving power for 6 hrs. Preserves cellular elements better than heparin or oxalates	Na salt is less soluble than K-EDTA. So dipotassium is recommended excess over 2 mg/ml – shrinks cells.
Heparin	Antithrombin and anti thromboplastin	1-2 mg (0.2 ml of 1% soln. Can be moisten syringe and needle with concentration (10 mg/ml) solution	Least effect on size and hemolysis of RBC used for blood gas analysis	May cause WBC clumping not suited for smears as it affects staining of WBC. Expensive will not prevent clotting longer than 8 hours. not suited for agglutination or prothrombin time test.
Sodium citrate	Combines with Ca^{+} to form insoluble salt of calcium citrate	10-20 gm for some coagulation studies one part of 3.8% solution to 9 parts of blood.	Used for transfusion	Interferes with many chemical tests; prevents clotting for only few hours. Shrinks cells.
Potassium oxalate	Units with calcium to form insoluble calcium oxalate	20 mg/2 drops of 20% soln. Dried in incubator or under 55°C (over heating converts oxalates to carbonates)	Very soluble	Causes 6-8% of shrinkage in cell volume in excess. Interferes with protein precipitation, too low glucose levels, alters electrolyte distribution poisonous.
Sodium Oxalate	Unites with calcium to form calcium oxalate (insoluble)	20 mg for prothrombin time 0.5 ml of 0.1 ml in exactly 4.5 ml of blood	Used mainly for prothrombin time	Same as potassium oxalate shrinks cells.
Ammonium and potassium oxalate (Heller's and Paul's double oxalate)	Combines with calcium to form calcium oxalate (insoluble)	1 ml or 20 mg dried at temperature no higher than 60°C	Used for most hematological work less dissolution and hemolysis of RBC	Potassium oxalate shrinks RBC while ammonium oxalate swells; cannot be used for BUN Estimation

PRODUCT	MODE OF ACTION	AMOUNT NEEDED PER 10 ml OF BLOOD	ADVANTAGES	DISADVANTAGES
Lithium oxalate	Combines with calcium to form calcium oxalate	2 mgs.	More soluble than sodium or potassium oxalate	Same as potassium oxalate. Shrinks cells.
Sodium fluoride and thymol (10:1)	Forms a weakly dissolved calcium component	100 mg NaF and 10 mg thymol	Both anti coagulant and preservative. Excellent preservative for blood glucose as it interferes with glycolysis	Interfere with enzymatic methods for glucose and BUN increases; thymol causes high values in ferricyanide
ACD solution (Acid citrate dextrose).	-	For transfusions use 25 ml ACD for 100 ml blood	-	

NORMAL BIOCHEMICAL VALUES IN BLOOD/PLASMA/SERUM OF VARIOUS SPECIES

Species of Animal	SERUM				BLOOD				PLASMA
	Ca mg/100 ml	P mg/100 ml	Mg mg/100 ml	Total Cholesterol mg/100 ml	Sugar mg/100 ml	NPN mg/100 ml	Creatinine mg/100 ml	Urea nitrogen mg/100 ml	Total plasma protein gms/100 ml
1. Cattle	9.4 - 2.2	4 - 7.12	2.0 - 5.0	50 - 230	35 - 55	20 - 40	1.0 - 2.07	6.0 - 27.0	7.56
2. Sheep	11.4	5.1 - 9.0	2 - 5	64 - 120	35 - 74	20 - 38	1.2 - 1.93	8.0 - 20.0	5.18
3. Goats	10.7	3.0 - 11.0	2 - 3	55 - 200	45 - 60	30 - 44	0.9 - 1.82	13 - 28	6.25
4. Pigs	11.0	4 - 11	2 - 3	152 - 154	65 - 95	20 - 45	1.0 - 2.7	8 - 24	7.4
5. Dogs	9.8 - 11.6	2.2 - 4.0	2.1	125 - 250	70 - 100	17 - 38	1.0 - 1.7	10 - 20	6.1 - 7.8
6. Cats	8.22	5.3 - 7.6	2 - 3	95 - 130	60 - 100	-	1.0 - 2.0	20 - 30	5.4 - 7
7. Elephants	6.90	3.2	2.2	93.6 - 116.7	42 - 73	45 - 46	1 - 2	16 - 19	10.72
8. Horses	12 - 13.4	2.4 - 4.2	2.5	96.8	60 - 100	20 - 40	1.2 - 1.9	10 - 20	6.72
9. Fowl	9 - 12	4 - 8	5 - 9	58 - 94	125 - 275	20 - 35	1 - 2	0.4 - 1	
10. Man	9 - 11	2 - 4	2 - 4	150 - 250	70 - 100	25 - 40	1 - 2	9 - 15	7.5

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