B.Sc. Ag I Sem

Fundamentals of Plant Biochemistry and Biotechnology

Credit - 3(2+1)

As per ICAR 5TH Dean Syllabus

- 111 Fundamentals of Plant Biochemistry and Biotechnology

A-Theory			
S.No	Topics	No. of Lec.	
1	Importance of Biochemistry; Properties of Water, pH and Buffer	1	
2	Carbohydrate: Importance and classification; Structures of Monosaccharides, Reducing and oxidizing properties of Monosaccharides, Mutarotation	3	
3	Structure of Disaccharides and Polysaccharides	1	
4	Lipid: Importance and classification; Structures and properties of fatty acids	2	
5	Proteins: Importance of proteins and classification; Structures, titration and zwitterions nature of amino acids	2	
6	Structural organization of proteins	1	
7	Enzymes: General properties; Classification; Mechanism of action	2	
8	Michaelis & Menten and Line Weaver Burk equation & plots	1	
9	Nucleic acids: Importance and classification	1	
10	Structure of Nucleotides, A, B & Z DNA	2	
11	RNA: Types and Secondary & Tertiary structure	2	
12	Metabolism of carbohydrates: Glycolysis, TCA cycle, Glyoxylate cycle, Electron transport chain	3	
13	Metabolism of lipids: Beta oxidation, Biosynthesis of fatty acids	2	
14	Concepts and applications of plant biotechnology	1	
15	Nutritional Requirement in Tissue Culture	1	
15	Techniques in *in vitro* culture: Callus culture, Organ culture, cell suspension culture, embryo culture, anther and pollen culture, ovule culture and their applications	2	
16	Micro-propagation methods	1	
17	Organogenesis and somatic embryogenesis	1	
18	Synthetic seeds and their significance	1	
19	Somatic hybridization and cybrids	1	
20	Application in crop improvement of somatic hybrids and cybrids	1	
21	Somaclonal variation and its use in crop improvement; Cryo-preservation	1	
22	Introduction to recombinant DNA methods	1	
23	Physical (Gene gun method), chemical (PEG mediated) and Agrobacterium mediated gene transfer methods	2	
24	Transgenics and its importance in crop improvement	2	
25	PCR techniques and its applications	1	
26	RFLP, RAPD, SSR	2	
27	Marker Assisted Breeding in crop improvement	2	
28	Biotechnology regulations	1	
Total lectures of theory		44	

Introduction of Biochemistry

Biochemistry comprises study of chemical nature of living organisms and their relationship with their environment. It tries to explain life processes at molecular level; the processes by which an exchange of chemical substances takes place between the living organisms and the environment the processes by which the absorbed materials are utilized for synthetic reactions leading to growth and replenishment of tissues and multiplication of the cell and the species the metabolic breakdown of the materials to supply energy for all the above and the mechanisms which regulate with precision all these processes. All these studies come under the purview of biochemistry. Correlation of biological functions and molecular structures is the central theme of biochemistry.

Biochemistry includes various aspects of organic chemistry, physical chemistry, physics, biology and other basic disciplines. It is also interrelated with physiology, microbiology, medicine and agriculture.

Importance of biochemistry

During the early part of the twentieth century, the central theme of biochemistry was the development of the field of intermediary metabolism that is the elucidation of the pathways for the synthesis and degradation of the constituents of living organisms. Although studies concerned with intermediary metabolism continue to be important, at present, biochemical research may be classified into the following major areas:

1. Composition and characteristics of chemical compounds of living organisms.
2. Cell ultra structure.
3. Cellular control mechanisms.
4. Physical chemistry of bio macromolecules.
5. Structure-function, kinetics, regulation and mode of action of enzymes.
6. Intermediary metabolism.
7. Bioenergetics particularly the mechanisms of formation of adenosine triphosphate (ATP) in the process of oxidative phosphorylation.
8. The molecular basis for genetic and developmental phenomena.
9. The molecular basis for physiological phenomena including nerve conduction, muscle contraction, vision and transport across membrane
10. Role, transformation and requirement of nutrients in plants, animals and other organisms.
11. Chemistry of inheritance: structure-function and regulation of gene expression.

Contribution of biochemistry in various field of Biology

Several research done in field of biochemistry, microbiology, molecular genetics, cell biology and recombinant DNA has led to the development of 'biotechnology'. In 1981, the European Federation of Biochemistry defined this branch of science as "the integrated use of biochemistry, microbiology and chemical engineering in order to achieve the technological application of the capacities of microbes and culture cells". A recent off-shoot of biotechnology research is genetic engineering, which involves gene splicing and recombinant DNA cloning.

Some of the applications of genetic engineering and biotechnology in various fields are listed below:

1.	Agriculture	Improvement of crop plants for higher photosynthetic efficiency, nutrient and water uptake, biological nitrogen fixation, nutritional quality of cereals, resistance to pests and diseases, plant cell and tissue culture and improvement of animal stock for desirable characteristics
2.	Chemical industry	Transformation of substances by biocatalysts to products such as biopolymers, antibiotics, alternative structural materials to plastics
3.	Energy industry	Production of new fuel sources and improved efficiency of energy recovery from existing ones.
4.	Food industry	Production of colourants, sweeteners, preservatives, etc
5.	Fermentation industry	Production of beer, wine, alcohols, amino acids, vitamins, etc.,
6.	Public health	Production of vaccines, drugs, growth hormones, interferons monoclonal antibodies, etc., use of bacteria in waste water treatment and recycling and pollution control

Properties of Water, pH and Buffer

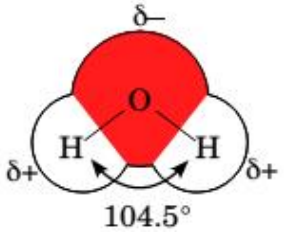
Water is the most abundant substance in living systems, making up 70% or more of the weight of most organisms. The attractive forces between water molecules and the slight tendency of water to ionize are of crucial importance to the structure and function of biomolecules. The water molecule and its ionization products, H^+ and OH^-, profoundly influence the structure, self-assembly and properties of all cellular components, including proteins, nucleic acids and lipids. The noncovalent interactions responsible for the strength and specificity of "recognition" among biomolecules are decisively influenced by the solvent properties of water, including its ability to form hydrogen bonds with itself and with solutes.

Properties of water

- Water is a polar molecule.
- It has hydrogen bonding potential
- It has Specific heat, heat of vaporization
- Nucleophilic
- Ionization
- Water is an ideal biological solvent

Water is a polar molecule

It is a polar molecule: Atoms are held together by polar covalent bonds. This gives the oxygen as light negative charge (δ^-) and each hydrogen as light positive charge (δ^+). Hydrogen atoms are attached to the oxygen at an angle of 104.5°.

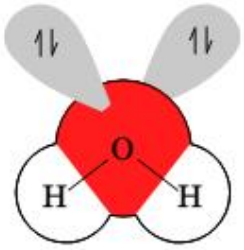


Hydrogen bonding gives water its unusual properties

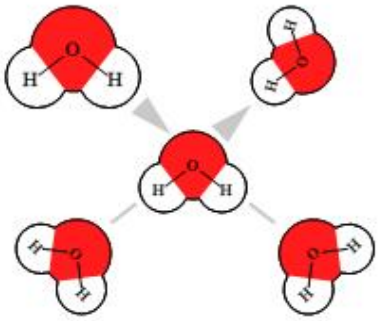
Each water molecule can form up to four hydrogen bonds (with the oxygen accepting two hydrogen bonds, and with each hydrogen acting as an hydrogen bond donor). The arrangement is roughly tetrahedral. The H–O–H bond angle does not allow the formation of a perfect tetrahedron. (Note that in actual water, the average number of hydrogen bonds per water molecule is less than four, probably due to geometry constraints and entropic effects.)

The ability of the water molecule to participate in extensive hydrogen bonding networks is responsible for most of the unusual bulk properties of water, including its high

melting and boiling points, ΔH of vaporization, and surface tension.



A look at the electron structure of the H2O molecule reveals the cause of these intermolecular attractions. Each hydrogen atom of a water molecule shares an electron pair with the central oxygen atom. The geometry of the molecule is dictated by the shapes of the outer electron orbitals of the oxygen atom, which are similar to the sp3 bonding orbitals of carbon. These orbitals describe a rou gh tetrahedron, with a hydrogen atom at each of two corners and unshared electron pairs at the other two corners. The H-OH bond angle is 104.5°, slightly less than the 109.5° of a perfect tetrahedron because of crowding by the nonbonding orbitals of the oxygen atom.



The oxygen nucleus attracts electrons more strongly than does the hydrogen nucleus (a proton); that is, oxygen is more electronegative. The sharing of electrons between H and O is therefore unequal; the electrons are more often in the vicinity of the oxygen atom than of the hydrogen. The result of this unequal electron sharing is two electric dipoles in the water molecule, one along each of the H-O bonds; each hydrogen bears a partial positive charge and the oxygen atom bears a partial negative charge equal to the sum of the two partial positives. As a result, there is an electrostatic attraction between the oxygen atom of one water molecule and the hydrogen of another (Fig. 2–1c), called a hydrogen bond. Hydrogen bonds are relatively weak. Those in liquid water have a bond dissociation energy (the energy required to break a bond) of about 23 kJ/mol, compared with 470 kJ/mol for the covalent O-H bond in water or 348 kJ/mol for a covalent C-C bond. The hydrogen bond is about 10% covalent, due to overlaps in the bonding orbitals, and about 90% electrostatic.

Water as solvent

Hydrogen bonds between water molecules provide the cohesive forces that make water a liquid at room temperature and that favor the extreme ordering of molecules that is typical of crystalline water (ice). Polar biomolecules dissolve readily in water because they can replace water-water interactions with more energetically favorable water-solute interactions. In contrast, nonpolar biomolecules interfere with water-water interactions but

are unable to form water-solute interactions consequently, nonpolar molecules are poorly soluble in water. In aqueous solutions, nonpolar molecules tend to cluster together.

Hydrogen bonds and ionic, hydrophobic (Greek, "water-fearing"), and van der Waals interactions are individually weak, but collectively they have a very significant influence on the three-dimensional structures of proteins, nucleic acids, polysaccharides, and membrane lipids.

Ionization of water

Water spontaneously ionizes to release free protons and free hydroxyl ions. In aqueous media, free H^+ doesn't typically exist. Instead, the dissociated proton is shared between many other water molecules to form transient $H3O^+$ and related species.

$H2O \rightleftharpoons H^+ + OH^-$

For simplicity, in the following discussion the symbol H^+ will be used to reflect the shared ion. Aqueous solutions do contain free hydroxyl ions, although these ions, as with all ions in aqueous solution, have a hydration shell

$$K = \frac{[H^+][OH^-]}{[H2O]}$$

However, in aqueous solution, the concentration of H2O is so high that it is not

significantly altered by the extremely small amount of dissociation that occurs. The effectively constant water concentration is generally included in the equilibrium K term to simplify the calculations.

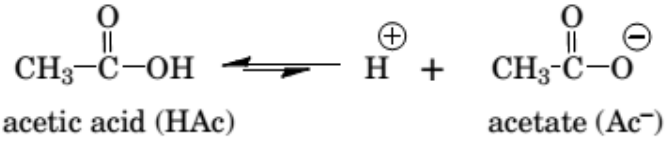
$Kw = [H^+][OH–] = 10^{-14}M^2$

In pure water, therefore, $[H^+] = [OH^-] = 10^{-7}M$

To simplify discussion another term was introduced to describe small concentrations of hydrogen ions: **pH**, which is defined as the negative log of the hydrogen ion concentration. Neutral is defined as pH 7.0, because this is the pH for water with no additional ions present. Because **pH** is a log function, a change of one pH unit (e.g., from pH 7 to pH 6) corresponds to a 10-fold change in hydrogen ion concentration.

Weak acids

Unlike strong acids, some compounds capable of releasing protons do not completely ionize in aqueous solution. For example, acetic acid also dissociates to yield a free proton and acetate ion (the conjugate base of acetic acid):



Again, this process reaches equilibrium, with the dissociation constant given by:

$$K_a = \frac{[H^+][Ac^-]}{[HAc]}$$

For acetic acid, Ka = 1.75 x 10^{-5}M; this indicates that only a small proportion of the acetic acid will dissociate. As with hydrogen ion concentrations, this dissociation constant value is somewhat clumsy to deal with, and therefore, as with pH, it is frequently worth using the negative log of the Ka: pKa = 4.75. By contrast, HCl has a pKa of -7. This illustrates a general principle: a higher pKa corresponds to a higher affinity for protons. To determine the pH, the equilibrium equation can be rearranged to solve for hydrogen ion concentration:

$$[H^+] = K_a \frac{[HAc]}{[Ac^-]}$$

Taking the negative log of both sides gives:

$$\mathrm{pH} = \mathrm{pK}_a - \log \frac{[HAc]}{[Ac^-]}$$

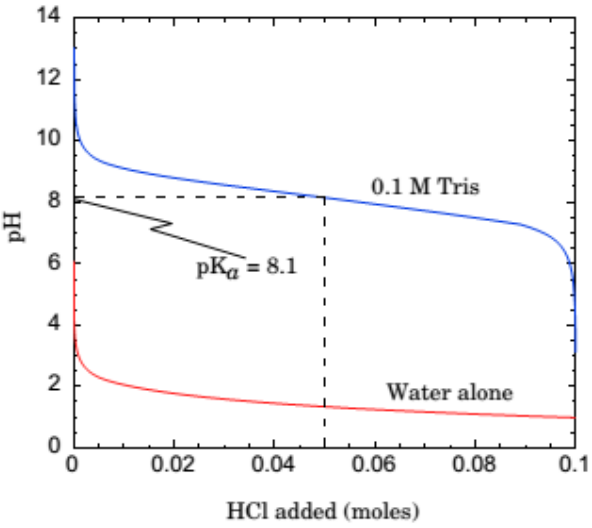
This is the same as:

$$\mathrm{pH} = \mathrm{pK}_a + \log \frac{[Ac^-]}{[HAc]}$$

which is the classic Henderson-Hasselbalch equation. For acetic acid (and for all weak acids), when [Ac^-] = [HAc] the solution pH is equal to the pKa. The Henderson-Hasselbalch equation can be used to solve for the relative amounts of acid and conjugate base present at any pH.

Buffers

Solutions containing mixtures of weak acids and their conjugate bases resist changes in pH due to the addition of either hydrogen ions or hydroxide ions. These solutions are buffers. Buffers are critical for maintenance of pH control both in experiments in vitro and in physiological systems (although physiological systems also use active mechanisms to control pH).



Weak acids have the ability to act as buffers. Because the conjugate base of a weak acid has a relatively high affinity for hydrogen ions, it will absorb any hydrogen ions added to a solution; alternatively, if the pH increases, the weak acid can release hydrogen ions. Adding HCl to water causes a rapid drop in pH to a final pH of 1. On the other hand, in the presence of Tris base (a commonly used buffer compound), the decrease in pH is markedly attenuated, with the final pH being 2 pH units higher. In the titration shown below for 0.1 M Tris, the solution pH changes from a high value to a low value upon addition of the strong acid HCl. The inflection point at 0.5 equivalents of strong acid corresponds to the pKa of the protonated form of Tris. As is apparent in the plot, between about 0.05 and 0.95 equivalents (corresponding to ±1 pH around the pKa), the system resists change in pH. (Note that, to a very good approximation, all of the added protons can be assumed to become bound by the conjugate base; the pH change then results from the altered ratio of conjugate base to weak acid.)

In principle, the Henderson-Hasselbalch equation can be used to calculate the pH of a buffer given the ratio of weak acid and conjugate base, or to calculate the amount of weak acid and conjugate base that must be added to a solution to obtain a desired pH. Because, in most cases, real solutions deviate from ideal behavior, most biochemists obtain the desired pH by titrating a solution while directly measuring pH using a pH meter. The Henderson-Hasselbalch allows an estimate of the amount of strong acid or strong base that must be added to obtain the correct pH, which tends to make buffer preparation less tedious.

Carbohydrates

Based on chemical constitution, the carbohydrates or saccharides are most simply defined as polyhydroxy aldehydes or ketones and their derivatives. But this definition is not entirely satisfactory. Because, presence of free carbonyl group (>C=O) in the simple carbohydrate molecule is not true. Because carbonyl compound reacts with an alcohol to form a hemiacetal. In carbohydrate,we have an aldehyde group which combine with an alcoholic –OH of the same molecule to form an interal hemiacetal and by elimination of H2O between the hemiacetal –OH group of two sugar molecules are formed. So that the definition of carbohydrate may be improved by a polyhydroxy compound that has an aldehyde or a ketone function present, either free or as hemiacetal or acetal. Carbohydrates are composed of carbon, hydrogen and oxygen possessing a common empirical formula $C_n(H2O)_n$.

Occurrence

Carbohydrates are the most abundant of all biochemical compounds and constitute more than 50 percent of the total biochemical matter. They are widely distributed in plants, animals and microbes. They are synthesized in green plants and algae from water and CO_2 using solar energy in a process called photosynthesis.

Physiological role and biological importance

The carbohydrates serve many functions in the living organisms. Some of their vital functions are:

1. Chief source of energy (4 kcal/g).
2. Reserve or storage forms of energy in plants (starch, inulin) and animals (glycogen).
3. Structural elements in plant cell wall (cellulose), exoskeleton of some insects and crustacea (chitin), cell walls of certain microorganisms (peptidoglycans) and skin and connective tissues of animals (mucopolysaccharides).
4. Important components of nucleic acids, co-enzymes and flavoproteins (for example, ribose).
5. They are involved in cell recognition, contact inhibition and also have antigenic

properties of blood group substances.

Classification

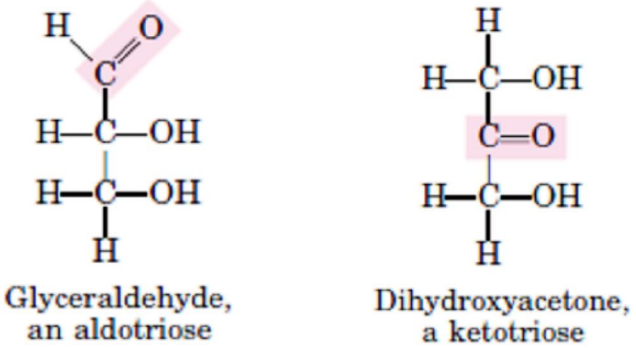
The carbohydrates can be classified into three main groups as: a) monosaccharides, b) oligosaccharides and c) polysaccharides, based on number of monomeric sugar units present. Monosaccharides are the simplest sugars consisting of single polyhydroxy aldehyde or ketone group that cannot be hydrolyzed into smaller units under reasonable mild conditions. They serve as the building-blocks for the more complex sugars.

Oligosaccharides (Greek Oligo 'few') contain from two to ten monosaccharide units joined through glycosidic linkage or bond. They are hydrolysable into constituent monosaccharide units.

Polysaccharides are polymers of monosaccharide units joined in long linear or branched chains through glycosidic bonds. Hydrolysis of polysaccharides yields many units of constituent monosaccharides. Polysaccharides have two major biological functions: a) as a storage form of fuels and b) as structural elements in living organisms.

1. **Monosaccharides (simple sugars)**

Monosaccharides, also called as simple sugars have the empirical formula $(CH_2O)_n$, where n=3 or larger number. They contain a short chain of carbon atoms with one carbonyl group, each of the remaining carbon atoms bearing a hydroxyl group. If the carbonyl group is an aldehyde (-CHO) the sugar is called as an aldose (name ends in 'ose') and if a ketone (C=O) it is a ketose (usually ends in 'ulose'). The simplest monosaccharides are the 3-carbon trioses glyceraldehyde and dihydroxyacetone.



Glyceraldehyde is an aldotriose; dihydroxyacetone is a ketotriose. Other monosaccharides are tetroses (four carbons), pentoses (five carbons), hexoses (six carbons), heptoses (seven carbons) and octoses (eight carbons). Each exists in two series,

ie., aldotetroses and ketotetroses; aldopentoses and ketopentoses; aldohexoses and ketohexoses, etc. Hexoses (both aldoses and ketoses) are the most abundant among monosaccharides. Glucose (aldohexose) is the most abundant monosaccharide; serves as the major fuel for most organisms and the- basic building-block of the many oligo- and polysaccharides. However, aldopentoses are important components of nucleic acids (for example, ribose) and various polysaccharides (for example, xylose and arabinose). Trioses, tetroses and heptoses are important intermediates in carbohydrate metabolism.

Table: Some important monosaccharides.

Name	Major source	Function	Type	No. of carbons
Xylose (wood sugar)	Hydrol,ysis of wood, straw, seed hulls	Constituent of straw, seed hulls wood,	Aldopentose	5
Arabinose	Hydrolysis of gum arabic, cherry tree gum, pectin	Constituent of gum arabic	Aldopentose	5
Ribose	Hydrolysis of nucleic acids	Constituent of nucleic acids	Aldopentose	5
Glucose (dextrose)	Ripe fruits, sweet corn, honey, blood, egg yolk	Energy source	Aldohexose	6
Mannose	Hydrolysis of mannans	Constituent of mannans	Aldohexose	6
Galactose	Hydrolysis of lactose	Constituent of milk sugar	Aldohexose	6
Fructose (levulose)	Honey, sweet fruits	Energy source	Ketohexose	6

General properties

Some of the general properties of monosaccharides are summarized below:

b) Monosaccharides are polyhydroxy aldehydes or ketones and their derivatives having either a potentially free aldehyde or a ketone group.

c) Simplest form of carbohydrates which cannot be hydrolyzed to other sugar units under reasonably mild chemical conditions.

d) Generally monosaccharides are white crystalline solids, insoluble in ether, sparingly soluble in alcohol but readily soluble in water.

e) Most of them have a sweet taste and char when heated.

f) Those with potentially a free aldehydic or a ketonic group are able to reduce metal ions under alkaline conditions. Hence, they are excellent reducing agents.

g) Amphoteric nature i.e., they are capable of reacting as weak acids or weak bases with strong acids or alkalies to form salts.

Stereochemistry

All the monosaccharides except dihydroxyacetone contain one or more asymmetric carbon atom(s) i.e., a single carbon atom having four different substituents and thus are chiral molecules. Subcompounds are capable of existing in two or more isomeric forms that are non-superimposable mirror images of each other. Such com· pounds exist in right-handed forms and are called chiral (hand) compounds. This phenomenon is called chirality (handedness).

Glyceraldehyde contains only one asymmetric carbon atom (carbon atom, 2) and therefore can exist as two different stereoisomers, i.e., as D- and L- glyceraldehyde. The symbols D- and L- designate the absolute configuration of an isomer and not the sign of rotation of plane-polarized light. The structure with -OH group on the right and –CHO group on the top of the asymmetric carbon atom is designated as D-glyceraldehyde. The structure in which the -OH group is to the left and -CHO group on the top is designated as L-glyceraldehyde.

The D- and L-glyceraldehydes are used as reference or parent compounds for designating the absolute configuration of all stereoisomeric compounds. The term configuration refers to the special arrangement of the atoms in a molecule resulting from the double bonds and/or chiral centers. Configurational isomers cannot be interconverted without breaking one or- more covalent bonds.

Enantiomers

Aldoses and ketoses of the L-series are mirror-images of their D-counterparts as shown in Fig. 2.5. These two D- and L- forms of a sugar are known as enantiomers. L-sugars are found in nature, but they are not so abundant as D-sugars.

Diastereoisomers

Two sugars having the same molecular formulae but not the mirror images of each other are known as diastereoisomers. e.g. D-glucose and D-mannose. All these sugars are not mirror images of each other.

Epimers

Two sugars differing only in the configuration around one specific carbon atom are called epimers of each other. Thus, D-glucose and D-mannose are epimers with respect to carbon atom 2, and D-glucose and D-galactose are epimers with respect to carbon atom 4.

Optical activity

All the monosaccharides except dihydroxyacetone contain one are more asymmetric carbon atom(s) and thus are optically active. Optical activity refers to the ability of a compound in solution to rotate the plane of polarization of plane-polarized light when observed in a polarimeter. Optical activity is shown by all compounds capable of existing in two forms that are non-superimposable mirror images of each other.

The optical activity is expressed quantitatively as the specific rotation:

$$[\alpha]_D^{25} = \frac{\text{Observed rotation (in degrees)} \times 100}{\text{Optical path length (dm)} \times \text{Concentration (g/100 mL)}}$$

Where, α = specific rotation in degrees at temperature usually 25°C and the wavelength of the light employed (usually the D line of sodium) is 589.3nm.

If the rotation of the beam of plane-polarized light is clockwise (to the right or rectus as the observer looks towards the light source), the enantiomer is designated as dextrarotatory (dextro, 'd' or '+' symbols) and if it is anticlockwise (to the left or sinister), the enantiomer is designated as levorotatory (levo, 'l' or '-' symbols). For example, the specific rotation of a-D-glucose is + 112.2° (dextrarotatory) and that of D-fructose is -93° (levorotatory). Thus, the symbol '+' and '-' refer to the direction of rotation of the beam of plane-polarized light but not the absolute configuration.

The D-and L-stereoisomers of any given compound have identical physical properties and identical chemical reactivities, with two exceptions: (a) they rotate the plane of plane-polarized light equally but in opposite directions and (b) they react at different rates with reagents that are themselves asymmetric. The equimolar mixture of the D- and L-stereoisomers, known as racemic mixture or racemate (designated as D L-) is optically inactive as the asymmetric carbon atom passes through a symmetrical intermediate during chemical reaction.

Structural aspects

Ring structure and mutarotation

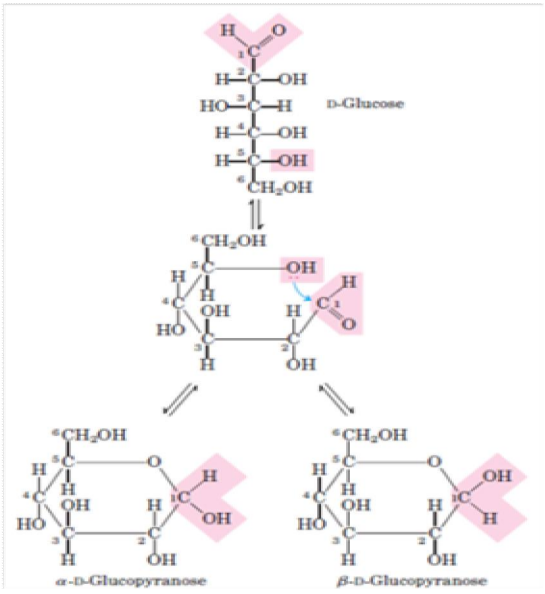
In aqueous solution, many monosaccharides act as if they have one more asymmetric center than is given by the open chain structural formulae. D-glucose may exist in two different isomeric forms differing in specific rotation, α-D-glucose (Crystallizes from a concentrated aqueous solution at 30°C , melting point 146°C), for which $[\alpha]_D^{20}$ = + 112°, and β-D-glucose (Crystallizes from hot glacial acetic acid solution, melting point 148-150°C), for which $[\alpha]_D^{20}$ = +19°. These two sugars do not differ in elementary composition but differ in physical and chemical properties. When the α- and β-isomers of D-glucose are dissolved in water, the optical rotation of each gradually changes with time

and approaches a final equilibrium value of $[\alpha]_D^{20}$ = +53°. This change, called mutarotation is due to the formation of an equilibrium mixture consisting of about one-third α-D-glucose and two thirds β-D-glucose at 20°C.

Although carbohydrates are formally aldehydes or ketones, a sugar like glucose does not readily answer the normal reactions of aldehydes as would be expected; this is because of their existence as cyclic hemiacetals or hemiketals. Aldehydes can react with an alcohol to form a hemiacetal; similarly ketones can react with an alcohol to form a hemiketal.

Anomers

From various chemical considerations it has been deduced that the α- and β-isomers of D-glucose are not open-chain structures in aqueous solution but six-membered ring structures formed by the reaction of the alcoholic hydroxyl group at carbon atom 5 with the aldehydic carbon atom 1 to form a hemiacetal which renders an other chiral center at carbon atom 1, also known as carbonyl carbon atom or anomeric carbon atom. Isomeric forms of monosaccharides that differ from each other only in configuration about the carbonyl carbon atom are known as anomers. Thus, D-glucose will have two anomers designated as α-D-glucose and β-D-glucose.



As hemiacetal or hemiketal formation is reversible, if one of the anomers is dissolved in water, an equilibrium mixture of the two anomers results. This interconversion between the two anomers is due to mutarotation. The cyclic hemiacetal formation in the case of glucose by the reaction of the alcoholic hydroxyl group at carbon 5 with the aldehydic carbon atom 1 results in formation of six-membered ring. The six-membered ring forms of sugars are called pyranoses because they are derivatives of the heterocyclic compound pyran. Thus, the systematic name for the ring form of α-D-glucose is α-D-glucopyranose.

In the case of fructose, the hemiketal is formed by reaction of the hydroxyl group on carbon atom 5 with the carbonyl group at carbon atom 2 to yield a five-membered ring. The five-membered ring forms of sugars are called furanoses as they are derivatives of heterocyclic compound, furan (Fig. 2.8) as suggested by Haworth. The systematic name

for the ring form of α-D-fructose is α-D-fructofuranose.

2. Oligosaccharides

These sugars consist of a short chain of 2 to 10 monosaccharide units linked by the glycosidic bond (s) with the elimination of water molecule (s). The glycosidic bond is formed most frequently between the anomeric carbon of one sugar residue and a hydroxyl group of the other sugar residue. Depending on the number of monosaccharide units that are linked, the oligosaccharides are further classified as disaccharides (two sugar units), trisaccharides (three sugar units), tetrasaccharides (four sugar units), etc. Amongst these, disaccharides are the most important class because of their biological role and relative abundance in natural products.

2.1.Disaccharides ($C_{12}H_{22}O_{11}$) .

These are a group of compound sugars composed of two monosaccharides linked by the glycosidic bond with the elimination of one molecule of water.

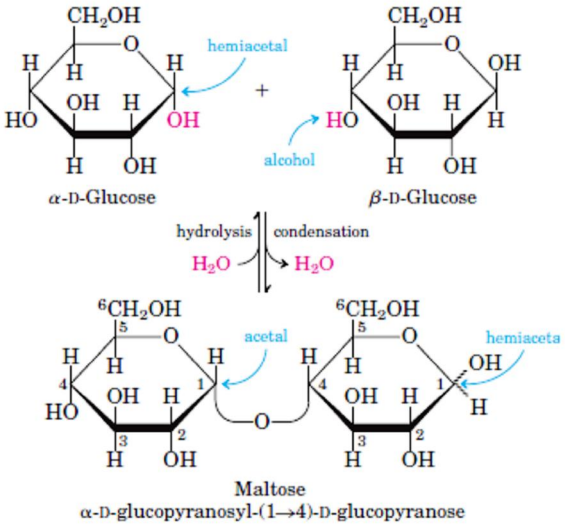
General properties

1. Those with potentially a free aldehyde or a ketone group can reduce Fehling's solution, hence are called reducing disaccharides.
2. The reducing disaccharides have most of the properties of monosaccharides i.e., they can form osazones and show mutarotation, etc.
3. Disaccharides can be hydrolyzed into their constituent monosaccharide units unlike monosaccharides.
4. Some disaccharides may exist in white crystalline solids and are soluble in water and sweet in taste.
5. Disaccharides are not fermented by yeast directly but they are first hydrolyzed to constituent monosaccharides which in turn are fermented.

The most abundant disaccharides in nature are maltose, sucrose and lactose.

2.1.1 Maltose

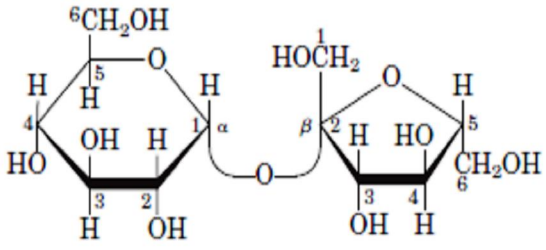
It is a disaccharide formed by linking two units of α-D-glucose through α-*1,4* glycosidic bond with the elimination of one molecule of water (Fig. 2.20). It is a reducing sugar since the -OH group bound to carbon 1 of the glucose residue



is free and can exist in the aldehyde form. It exhibits mutarotation since it exists in both α- and β-forms. Malt prepared from sprouting barely, is an excellent, source of maltose.

2.1.2 Sucrose (α-D-glucopyranosyl-β-D-fructofuranoside)

Sucrose or cane sugar or beet sugar or saccharose or invert sugar is a disaccharide made up of one molecule each of α-D-glucose and β-D-fructose, the linkage involving the potential aldehyde group of carbon atom 1 of glucose and the ketonic group of the carbon atom 2 of fructose (β,2->1) linkage. It is a non-reducing sugar because of the absence of a potentially free aldehyde or ketonic group and forms no osazone. As it does not exist in α- and β-forms, it fails to exhibit mutarotation.

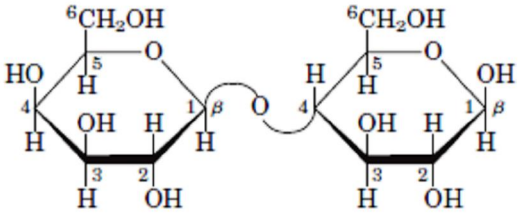


Sucrose
α-D-glucopyranosyl β-D-fructofuranoside
Glc(α1↔2β)Fru

It is hydrolyzed by acid or enzyme sucrase (invertase) into glucose and fructose. The specific rotation of sucrose is +66.5° and after hydrolysis, the specific rotation of the mixture is -19.84°. Such a change in specific rotation from dextro- to levorotatory nature is called 'inversion' and hence the name 'invert sugar'. The reason for the inversion is that fructose is more strongly levorotatory (-93° than glucose which is dextrarotatory (+52.5°). It is the most abundant oligosaccharide and is ubiquitous in plants. It is generally manufactured from sugarcane and sugar beet.

2.1.3 Lactose (4-O-β-D-galactopyranosyl-D-glucopyranose)

Lactose or milk sugar is made up of β-D-galactose and α-(in α-form) or β-(in β-form) D-glucose through β-1, 4 glycosidic bond (Fig. 2.20). It is a reducing sugar, exhibits mutarotation and forms osazone. It reduces Fehling's solution but not Barfoed's reagent and thus can be distinguished from other reducing disaccharides. It is hydrolyzed by the enzyme lactase into its constituent hexoses. It does not ferment as easily as glucose and hence makes an ideal constituent of milk of mammals (about 5 g/100 ml milk). It is not produced in plants.



Lactose (β form)
β-D-galactopyranosyl-(1→4)-β-D-glucopyranose
Gal(β1→4)Glc

2.1.4 Cellobiose (4-O-β-D-glucopyranosyl-D-glucopyranose)

It is a partial hydrolytic product of cellulose, made up of two glucose units joined through β-1, *4* linkage (Fig. 2.20). It is a reducing sugar. It is probably present in only trace

amounts in nature and formed during the digestion of cellulose by the cellulases of microorganisms.

2.1.5 Trehalose (1-O-α-D-glucopyranosyl-1-α-D-glucopyranoside)

It is made up of two glucose units linked through two anomeric carbon atoms. It is a non-reducing sugar. It is the major carbohydrate present in insects and fungi where it serves as a storage carbohydrate from which glucose may be obtained as required.

2.2 Trisaccharides ($C_{18}H_{34}O_{17}$)

A naturally occurring trisaccharide is raffinose [α-D-galactopyranosyl-O-(1,6-α-D-glucopyranosyl-O-(1,2) –β-D-fructofuranoside] found in sugar beet, coffee and other plant materials. It is a non-reducing sugar.

Melezitose[O-α-ODglucopyranosyl(1->3)-O-β-D-fructofuranosyl(2,1)-α-D glucopyranoside] is found in the sap of some coniferous trees.

2.3 Tetrasaccharides

The important one among tetrasaccharides is the stachyose derived from raffinose. Stachyose consists of galactose-galactose-glucose-fructose monosaccharide sugars linked through α-1, 6, α-l, 6 and α-1, 2 glycosidic bonds, respectively. It occurs during germination of seeds.

3. Polysaccharides or glycans $(C_5H_{10}O_5)_n$

These are complex carbohydrates which are polymerized anhydrides of a large but undetermined number of the simple sugars which are joined by glycosidic bonds. Those found in nature contain either five or six carbon monosaccharide units. The bulk of carbon found in nature exists in the form of polysaccharides. These are involved in the majority of biological processes although free monosaccharides and disaccharides occur in many biological fluids and plants.

General properties

Some of the important properties of polysaccharides are as follows:

1. Complex sugars of high molecular weight; polymers of several units of monosaccharides or either derivatives with linear or branched chains.
2. Upon hydrolysis by acids or enzymes, they are broken down into various intermediate products and finally into their consituent monosaccharides or their derivatives.
3. They are tasteless, apparently amorphous, some are crystalline
4. Mostly insoluble in cold water but form a sticky or gelatinous solutions
5. They differ in the nature of their recurring monosaccharides units, in the length of their chains and in the degree of branching

Biological role

Polysaccharides serve two main functions in the living organisms as:

1. Storage form of cellular fuel and
2. Structural elements in animal, plant and microbial systems

Classification

Polysaccharides can be classified in many ways

A. Based on function

1. Structural polysaccharides: These polysaccharides serve as structural components of living organisms. e.g. cellulose (plant cell wall), chitin (exoskeleton of some insects), etc.

2. Storage/reserve/nutrient polysaccharides: These polysaccharides function as reserve or storage form of fuel in living organisms e.g. starch (plants), glycogen (animal cells) etc.

B. Based on composition:

1. Homopolysaccharides: These are made up of single kind of monosaccharide residues or their derivatives. e.g. Starch, glycogen, cellulose, chitin, inulin, etc.
2. Heteropolysaccharides: These are made up of two or more different kinds of monosaccharide units or their derivatives. e.g. Hyaluronic acid, heparin, pectins, gums, mucilages, chondroitins, etc.

Polysaccharides are often called as glycans. Those containing glucose are called as glycans (starch and glycogen); those containing mannose are called mannans and those containing galactose units are called galactans.

Structural polysaccharides

Cellulose

It is the most abundant organic compound of our planet accounting for about 50 per cent of all carbon. It is the principal constituent of cell walls in higher plants forming the main structural element. It is a linear homopolymer of glucose units linked by β-1,4 glycosidic bonds. It is insoluble in water and all organic solvents. It dissolves in conc. H_2SO_4, on diluting the solution and boiling, glucose is formed as final product. Partial hydrolysis of cellulose yields cellobiose, a disaccharide. Cellulase, a β-glucosidase produced by many bacteria and fungi, hydrolyzes cellulose. The large amount of glucose present in cellulose is not available as a source of energy for humans due to the lack of enzymes capable of cleaving the β-1,4 bonds. However, ruminants can effectively use cellulose as they contain a large bacterial population in their rumen capable of hydrolyzing it.

In plant cell walls, cellulose microfibrils are cemented together by other substances important among them being pectin and hemicellulsoe. Pectins contain arabinose, galactose and glacturonic acid while hemicelluloses are homopolymers of D-xylose linked by β- 1,4 bonds. The important sources of cellulose are cotton fibers (98%), jute (50-70%), wood (40-50%), algae and bacteria. Cellulose and its derivatives are widely used in textiles, films and plastics.

Chitin

It is a structural homopolysaccharide made up of N-acetyl glucosamine residues in β-1,4 linkage. It is the principal structural polysaccharide present in the exoskeleton of crustaceous insects, earthworms and mollusks. It is the second most abundant organic substance on earth.

Table: Structure and functions of some polysaccharides

Polymer	Type	Repeating unit	No. of monosaccharide unit	Role
Starch				Energy storage: in plants
Amylose	Homo	A linear polymer of D-glucose residues in (α1→4) linkage.	50-50000	
Amylopectin	Homo	Glucose residues in amylopectin chains are (α1→4); the branch points (Occurring every 24 to 30 residues) are (α1→6) linkages.	Up to 10^6	
Glycogen	Homo	polymer of (α 1→4)-linked subunits of glucose, with (α 1→6)-linked branches,	Up to 50000	Energy storage: in bacteria and animal cells
Cellulose	Homo	D-glucose residues are in (β1→4)linkage	Up to 15000	Structural: in plants, gives rigidity and strength to cell walls
Chitin	Homo	A homopolymer of N-acetyl-D-glucosamine units in (β1→4) linkage.	Very large	Structural: in insects, spiders, crustaceans, gives rigidity and strength to exoskeletons
Dextran	Homo	(α1→6)-linked poly-D-glucose with (α1→3) branches	Wide range	Structural: in bacteria, extracellular adhesive
Peptidoglycane	Hetero-; peptides attached	(β1→4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues	Very large	Structural: in bacteria, gives rigidity and strength to cell envelope
Agarose	Hetero-	D-galactose (β1→4)-linked to 3,6-anhydro-L-galactose that are joined by (α1→3) glycosidic links to form polymer	1000	Structural: in algae, cell wall material
Hyaluronate (a glycosamine -glycan)	Hetero-; acidic	D-glucuronic acid(β1→3) and *N*-acetylglucosamine (β1)	Up to 100000	Structural: in vertebrates, extracellular matrix of skin and connective tissue; viscosity and lubrication in joints

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Peptidoglycan (murein)

It is a structural heteropolysaccharide present in bacterial cell walls. The repeating unit of peptidoglycan is the muropeptide which is a disaccharide composed of N-acetyl-D-glucosamine (NAG) and N-acetyl muramic acid (NAMA) joined by a, β-1,4 glycosidic bond. NAMA consists of a NAG unit which has its C-3 hydroxyl group joined to the hydroxyl group of lactic acid by an ether linkage.. In the peptidoglycan the carboxyl group of each lactic acid moiety is in turn linked to a tetrapeptide consisting of L-alanine, D-isoglutamine, L-lysine and D-alanine (Fig. 2.21). The terminal D-alanine residue of the side chain of one polysaccharide chain is joined covalently with the peptide side chain of an adjacent polysaccharide chain, either directly as in *E.coli* or through a short conneting peptide, e.g. The pentaglycine in *Staphylococcus aureus*.

The peptidoglycan structure of the bacterial cell wall is resistant to the action of peptide-hydrolyzing enzymes, which do not attack peptides containing D-amino acids. However, the enzyme lysozyme, found in tears and in egg white, hydrolyzes the β(1 4) glycosidic bonds of the polysaccharide backbone of the peptidoglycan structure.

Reserve or storage polysaccharides

Starch

It is a principal storage homopolysaccharide of the plant kingdom, made up of D-glucose as repeating units. It is a mixture of two components amylose (about 20%) and amylopectin (about 80%) Amylose consists of long unbranched chains of D-glucose units which are linked by α-1, 4 glycosidic bonds. Its molecular wieght ranges from a few thousands of about 500,000. It gives blue colour with iodine due to the iodine-amylose complex in which iodine molecule is occupying a position in the interior of the helical coil. Amylopectin also has a backbone of α-1, 4 linked glucose units but in addition, branched through α-1, 6 linkages. The average length of branching is from 24 to 30 glucose residues. It gives a purple colour with iodine. Its molecular weight may range from 50,000 to 1,000,000.

Both amylose and amylopectin can be hydrolyzed by the enzymes α- and β-amylases. α-amylases cleave α-1,4 linkages at random to give the mixture of maltose and glucose units while the β-amylases, present in plants remove maltose units succcesively from the non reducing end. The intermediate product left after the cleavage of starch by α and β amylases is

called limit dextrins. Neither of these enzymes can hydrolyze α-1,6 linkages. Microbial glucomylase can act on both α-1, 4 and α-1, 6 likages of starch to yield glucose.

Starch forms the major source of carbohydrates in the human diet and is of great economic importance. The important source of starch are seeds, fruits, tubers, bulbs and cereal grains varying from a few per cent of over 75 per cent. It is also found in some protozoa, bacteria and algae.

Glycogen

It is the storage homopolysaccharide in animals and is often called 'animal starch'. It is present mainly in liver, skeletal muscle and in smaller amounts in all other tissues. It is stored in liver and muscles of animals and split to glucose in the liver to maintain proper concentration of glucose in the blood to furnish energy. The amount of glycogen present in the animal varies widely among the different tissues with diet and physiological state of the body. It is also aabundant in the mollusks while glycogens like polysaccharides are found in some bacteria.

Glycogen is a branched chain of D-glucose units resembling amylopectin of starch. However, the branching through α-1, 6 linkages is more extensive than amylopectin, with 8-10 glucose units between branching points. A glycogen molecule may contain as many as 30,000 glucose units. It is readily dispersed in water to form an opalescent solution which gives a reddish brown colour with iodine. It does not reduce Fehling's solution.

Insulin

It is a storage polysaccharide in the Compositae family (artichokes, dahlias, dandelions, etc). It is a homopolymer made of D-Fructose units linked by β(2→1) bonds.

Dextrans

These are storage polysaccharides of some yeasts and bacteria. They consist of D-glucose units joined by α-1, 6 glycosidic bonds primarily with cross linkages through α-1,2 and and α-1, 3 linkages.

Glycoproteins

Glycoproteins are molecules composed of covalently joined protein and carbohydrates. The carbohydrate is attached to the polypeptide chains of the protein in a series of reactions that are enzymatically catalyzed after the protein component is synthesized.

Glycoproteins in cell membranes apparently have an important role in the group behavior of

cells and other biological functions of the membrane. They form a major part of the mucus that is secreted by epithelial cells, where they perform an important role in lubrication and in the protection of tissues lining the body's ducts. Many other proteins secreted from cells into extracellular fluids are glycoproteins. These proteins include hormone proteins found in blood, such as follicle stimulating hormone (FSH), lutenizing hormone (LH), chorianic gonadotropin; and plasma proteins such as immunoglobulins etc. Glycoproteins are also one of the major components of the cell coats of higher organisms.

The carbohydrate percent within glycoproteins is highly variable. Some glycoproteins such as IgG contain low amounts of carbohydrate (4 %). Human ovarian cyst glycoprotein is composed of 70 per cent carbohydrate and human gastricglycoprotein is 82 per cent carbohydrate. Glycoproteins having a very high content of carbohydrate are called proteoglycans.

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Amino acid and Protein

All proteins are formed from **20** different **amino acids**. All the amino acids have trivial or common names based on the source from which they were first isolated or based on their properties. e.g. **Asparagine** was named so, as it was isolated from asparagus and **glycine** was so named because of its sweet taste (Greek:'glykos' meaning sweet).

All the 20 amino acids, except **proline**, found in proteins have an amino group and a carboxyl group attached to the same carbon atom, namely the **α-carbon**. They differ only in the side chains (R groups). The 20 amino acids found in proteins are referred as the **standard or normal or protein amino acids**.

There are many other amino acids found in nature but do not occur in proteins. They are referred as **non-protein amino acids**.

Classification of protein amino acids

The protein amino acids are classified according to the **chemical nature** of their R groups as **aliphatic, aromatic, heterocyclic and sulphur containing amino acids**. More meaningful classification of amino acids is based on the **polarity of the R groups**. The polarity of the R groups varies widely from totally non-polar to highly polar. The 20 amino acids are classified into four main classes whose structures, three-letter and one-letter symbols are given below

a) Amino acids with non-polar or hydrophobic, aliphatic R groups

This group of amino acids includes **glycine, alanine, valine, leucine, isoleucine and proline.** The hydrocarbon R groups are non-polar and hydrophobic. The side chains of alanine, valine, leucine and isoleucine are important in promoting hydrophobic interactions within protein structures. The minimal steric hindrance of the glycine side chain (hydrogen) allows more flexibility than other amino acids. On the other hand, the imino group of proline is held in a rigid conformation and reduces the structural flexibility of the protein.

b) Amino acids with non-polar aromatic R groups

This group includes **phenylalanine, tyrosine and tryptophan**. All these amino acids participate in **hydrophobic interactions**, which is stronger than aliphatic R groups because of stacking one another. Tyrosine and tryptophan are more polar than phenylalanine due to the presence of hydroxyl group in tyrosine and nitrogen in the indole ring of tryptophan. The absorption of ultraviolet (UV) light at 280 nm by tyrosine, tryptophan and to a lesser extent by

phenylalanine is responsible for the characteristic strong absorbance of light by proteins. This property is exploited in the characterization and quantification of proteins.

b) Amino acids with polar, uncharged R groups

This group of amino acids includes **serine, threonine, cysteine, methionine, asparagine and glutamine.** The hydroxyl group of **serine and threonine**, the sulphur atom of **cysteine and methionine** and the amide group of **asparagine and glutamine**, contribute to the polarity. The R groups of these amino acids are more hydrophilic than the non-polar amino acids.

c) Amino acids with charged R groups

Acidic: The two amino acids with acidic R groups are **aspartic and glutamic acids.** These amino acids have a net negative charge at pH 7.0.

Basic: This group includes **lysine, arginine and histidine** . The R groups have a net positive charge at pH 7.0. The **lysine** has a second -amino group; **arginine** has a positively charged guanidino group; and **histidine** has an imidazole group.

Physical Properties of amino acids

Amino acids are white crystalline substances. Most of them are **soluble in water** and insoluble in non-polar organic solvents (e.g., chloroform and ether). Aliphatic and aromatic amino acids particularly those having several carbon atoms have limited solubility in water but readily soluble in polar organic solvents. They have **high melting points** varying from 200-300^{o}C or even more. They are tasteless, sweet or bitter. Some are having good flavour. **Sodium glutamate** is a valuable flavouring agent and is used in the preparation of certain dishes and sauces.

Amphoteric nature of amino acids

- Amino acids are **amphoteric** compounds, as they contain **both acidic (COOH)** and **basic (NH_2) groups.**
- They can react with both alkalis and acids to form salts.
- In **acid solution** amino acids carry **positive charges** and hence they move towards cathode in an electric field.
- In **alkaline solution**, the amino acids carry negative charges and therefore move towards anode.
- When an amino acid is dissolved in water, it exists as **inner salt** carrying both positive and negative charges.This occurs as a result of dissociation of carboxyl group to release the

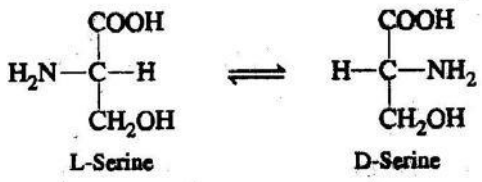
H+ ion, which passes from the carboxyl to the amino group. The amino acids possessing **both positive and negative charges** are called

Zwitterions.

The zwitterion reacts as an **acid** with a base by **liberating a proton (H+)** from the NH3+ group and as a result possesses a **net negative charge**. On the other hand, zwitterions reacts with an acid **as base**, **combining with the proton (H+)** of the acid resulting in the formation of a compound having a **net positive charge**. These reactions are reversible. The **pH** at which the amino acid has no tendency to move either towards positive or negative electrode is called **isoelectric pH or isoelectric point**. At isoelectric pH, the amino acid molecule bears a **net charge of zero.**

Isomerism

- All amino acids except proline, found in protein are **-amino acids** because NH2 group is attached to the -carbon atom, which is next to the COOH group.
- Examination of the structure of amino acids reveals that **except glycine**, all other amino acids possess **asymmetric carbon** atom at the alpha position.
- Because of the presence of asymmetric carbon atom, amino acids exist in **optically active forms**.
- For example, in the steric configuration for serine, the carboxyl group is written on the **top**, while the amino group is written to the **left** in the case of L-serine and to the right in the case of D-serine This distinction will hold good for all the amino acids having asymmetric carbon atoms.



Structure of D- and L-Serine

- 'D' and 'L' do not refer to the optical rotation, but to the steric configuration of amino group to the right and left side of the carboxyl group.

- The **direction of optical rotation** of amino acid is indicated by the symbol **+ or -,** which follows the designation 'D' or 'L'.
- The steric configuration and optical rotation of an amino acid may be simultaneously expressed as D (+) or D (-) and L (+) or L (-).
- L-forms are more common than D-forms and most of the **naturally occurring amino acids** are **L-amino acids.**

Chemical properties

a) Reactions due to amino group

Reaction with formaldehyde (Formal titration)

- Amino acid exists as zwitterion in aqueous medium. If an amino acid solution is treated with excess of **neutralized formaldehyde solution**, the amino group combines with formaldehyde forming **dimethylol amino acid** which is an amino acid formaldehyde complex.
- Hence the **amino group is protected** and the proton released is titrated against alkali.
- This method is used to find out the **amount of total free amino acids** in plant samples.

Reaction with nitrous acid

Nitrous acid reacts with the amino group of amino acids to form the corresponding hydroxyacids and liberate nitrogen gas.

Reaction with ninhydrin

Ninhydrin is a **strong oxidizing agent**. When a solution of amino acid is boiled with ninhydrin, the amino acid is **oxidatively deaminated** to produce ammonia and a ketoacid.

The **keto acid** is decarboxylated to produce an **aldehyde** with one carbon atom less than the parent amino acid. The net reaction is that ninhydrin oxidatively deaminates and decarboxylates - amino acids to CO_2, NH_3 and an aldehyde. The reduced ninhydrin then reacts with the liberated ammonia and another molecule of intact ninhydrin to produce a purple coloured compound known as **Ruhemann's purple**. This ninhydrin reaction is employed in the **quantitative determination of amino acids.** Proteins and peptides that have free amino group(s) (in the side chain) will also react and give colour with ninhydrin.

Reactions due to carboxyl group

Decarboxylation

- The carboxyl group of amino acids is **decarboxylated** to yield the corresponding **amines.** Thus, the vasoconstrictor agent, **histamine** is produced from histidine.
- Histamine stimulates the **flow of gastric juice into the stomach** and the **dilation and constriction of specific blood vessels.**
- Excess reaction to histamine causes the **symptoms of asthma** and various **allergic reactions.**

Essential amino acids

- Most of the prokaryotic and many eukaryotic organisms (plants) are capable of synthesizing all the amino acids present in the protein. But higher animals including man possess this ability only for certain amino acids.
- The amino acids, which are needed for normal functioning of the body but **cannot be synthesized from metabolic intermediates,** are called **essential amino acids**.
- These must be obtained from the **diet** and a deficiency in any one of the amino acids prevents growth and may even cause death.
- **Methionine, Arginine, Threonine, Tryptophan, Valine, Isoleucine, Leucine, Phenylalanine, Histidine, and Lysine are the essential amino acids**
- (Remember **MATTVILPHLy**).

Peptide

- Amino acids are linked together by formation of **covalent bonds.**
- The covalent bond is formed between the **-carboxyl group of one amino acid and the amino group of the next amino acid.**
- The bond so formed between the carboxyl and the amino groups, after elimination of a water molecule is called as a **peptide bond** and the compound formed is a **peptide.**
- The peptide formed between two amino acids is a dipeptide; three amino acids is a tripeptide; few amino acids are an oligopeptide and many amino acids is a polypeptide.
- In writing the peptide structure, the amino terminal (N-terminal) amino acid is written first and carboxyl terminal (C-terminal) amino acid written last.

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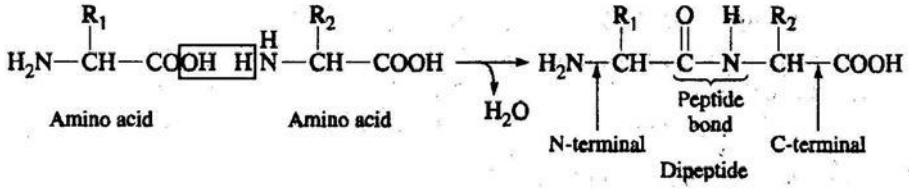
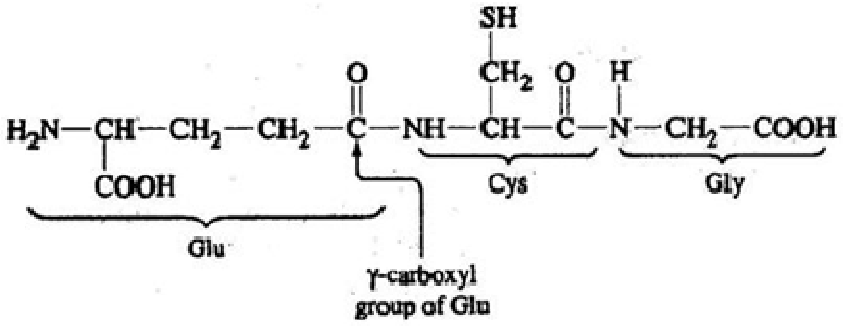


Formation of peptide from amino acids

Peptides of physiological interest

Glutathione is a commonly occurring tripeptide **(glutamyl cysteinyl glycine**) in many living organisms.



Structure of glutathione, a tripeptide

- It has a role in **detoxification of toxic compounds** in physiological system.
- The nonapeptides (nine amino acids), oxytocin and vasopressin are important animal peptide hormones.
- **Oxytocin** induces labor in pregnant women and controls contraction of uterine muscle.
- **Vasopressin** plays a role in control of blood pressure by regulating the contraction of smooth muscles.
- A dipeptide **L-aspartyl-L-phenylalanine**, is of commercial importance. This dipeptide is about 200 times sweeter than cane sugar. The methyl ester of this dipeptide is called as **aspartame** and marketed as an artificial sweetener for **diabetics.**

Protein structure

Protein are polymer of Amino acid , the structure of Protein is rather complex which can be divided into 4 levels of organization

1. **Primary Structure**
2. **Secondary structure**
3. **Tertiary Structure**
4. **Quaternary Structure**

The structure of Protein is comparable with the structure of a building. The amino acids may be considered as the bricks, the wall as the Primary structure, the twists in a wall as the secondary structure, a full fledged self contained room as the tertiary structure. A building with similar and dissimilar rooms will be the quaternary structures.

Primary Structure

The primary structure of a protein refers to the linear sequence of amino acids in the polypeptide chain. The primary structure is held together by covalent bonds such as peptide bonds, which are made during the process of protein biosynthesis or translation. The two ends of the polypeptide chain are referred to as the carboxyl terminus (C-terminus) and the amino terminus (N-terminus) based on the nature of the free group on each extremity. Counting of residues always starts at the N-terminal end (NH_2-group), which is the end where the amino group is not involved in a peptide bond.

Secondary Structure

The conformation of polypeptide chain by twisting or folding is referred to as secondary structure.

The amino acid are located close to each other in their sequence. The most common type of secondary structure are the

a) Alpha helix.

b) Beta pleated sheet.

Both Alpha helix and beta pleated sheet patterns are stabilized by hydrogen bonds between the carbonyl and N-H groups in the polypeptide's backbone.

Alpha Helix

1. The Alpha Helical structure was proposed by Pauling and Corey (1951)

2. The Alpha Helix is the most common spiral, rigid, rod like structure that forms when a polypeptide chain twists into a helical conformation. The screw sense of Alpha Helix can be right handed (right handed) or left handed (anticlockwise), however right handed helices are energetically more favorable. There are 3.6 amino acid residues per turn of the helix and the distance it rises per turn is 0.54 nm each residue is related to the next one by rise of 1.5 A.

3. The Alpha Helix is stabilize by extensive hydrogen bonds in between the N-H group of each amino acid and the carbonyl group of the amino acid four residue away, side chain of amino acids extended outward from the central helix. The hydrogen bond are individually weak but collectively they are strong enough to stabilize the Helix.

4. All the peptide bonds, except the first and the last in polypeptide chain participate in the H-bonding.

Beta Pleated sheets

This is the second type of structure, proposed by Pauling and Corey.

1. Beta pleated sheets form when two or more polypeptide chain segment line up side by side. Each individual segment is referred to as a beta-strand. Rather than being coiled, each Beta strand is fully extended. The distance between adjacent amino acids along a beta strand is approximately 3.5 A.

2. Beta pleated sheets are stabilized by hydrogen bonds that form between the polypeptide backbone N-H and carbonyl groups of adjacent strand, adjacent strand can be either parallel or antiparallel.

3. In parallel Beta pleated sheet structures, the polypeptide chains are arranged in the same direction. However in, antiparallel Beta pleated sheet chain run in opposite directions. Antiparallel beta pleated sheet are more stable than parallel beta pleated sheet because fully collinear hydrogen bonds form.

Tertiary structure

1. The three dimensional arrangement of protein structure referred to a tertiary structure.

2. It is a compact structure with hydrophobic side chain held interior while the hydrophilic groups are on the surface of the protein molecule, this type of arrangement ensures stability of the molecule.

3. The following types of covalent and non-covalent interaction stabilize tertiary structure.

i. Hydrophobic interaction

ii. Electrostatic interaction

iii. Hydrogen bond

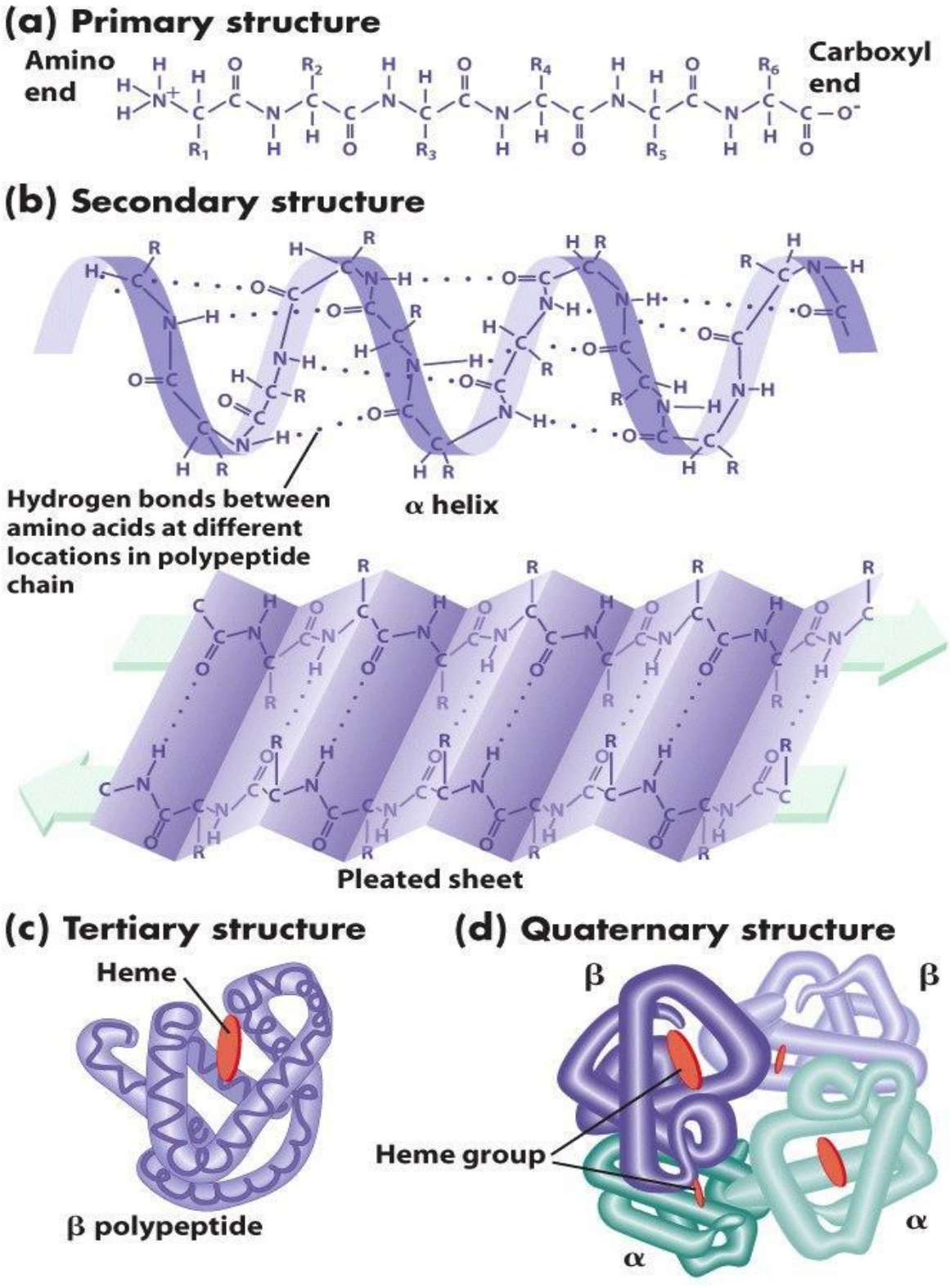
iv. Vander waal forces

v. Covalent bonds.

Quaternary structure

1. Some of the Proteins are composed of two or more polypeptide chains referred t o as subunits. The spatial arrangement of these subunits is known as Quaternary Structure. Subunit in multisubunit protein may be identical or quite different. Multisubunit Proteins in which some or all subunits are identical are referred to as Oligomer and identical units are referred to as protomers.

2. Polypeptide subunits assemble and are held together by the noncovalent interactions such as hydrophobic interactions, ionic bond and hydrogen bonds.


(a) Primary structure
Amino end
Carboxyl end
(b) Secondary structure
Hydrogen bonds between amino acids at different locations in polypeptide chain
α helix
Pleated sheet
(c) Tertiary structure
Heme
β polypeptide
(d) Quaternary structure
β
β
Heme group
α
α

Denaturation and Renaturation of protein

- Renaturation refers to **the attainment of an original, regular three-dimensional functional protein after its denaturation.**
- When active pancreatic ribonuclease A is treated with **8M urea or mercaptoethanol,** it is converted to an inactive, **denatured** molecule.
- When urea or mercaptoethanol is removed, it attains its native (active) conformation.

ENZYMES

Enzyme are biocatalysts – the catalysts of life, a catalysts is defined as a substance that increases the velocity or rate of a chemical reaction without itself undergoing any change in the overall process. It is well known that highly complex synthetic and breakdown reactions take place much more rapidly and easily by the living organism. In the absence of the cell these chemical reactions would proceed too slowly. In the laboratory, hydrolysis of protein by a strong acid at 100 C takes at least a couple of days. The same protein is fully digested by the enzyme in gastrointestinal tract by the enzyme in gastrointestinal tract at body temperature (37 C) with in a couple of hours

An enzyme is a protein that is synthesized in a living cell and catalyzes or speeds up a thermodynamically possible chemical reaction. The enzymes in on way modify the equilibrium constant (K_{eq}) or the free energy change (G) of a reaction.

Terminology

Some of the terms used in enzymology are defined below:

Substrate: The substrate acted upon by the enzyme.

Product: The substrate formed as a result of the enzymatic action

Active site or catalytic site: the site on the enzyme wherein the substrate is bound and is converted into products

Regulatory site: The site other than the active site on the enzyme wherein the effector or modulator is bound and controls the rate of enzyme catalyzed reaction.

Effector or modulator: The substance which binds at an allosteric site (the site other than the catalytic site) of the regulatory enzyme and may stimulate or inhibit the rate of enzyme catalyzed reaction

Holoenzyme: A completely catalytically active enzyme.

Holoenzyme = Apoenzyme + Cofactor

(active) (inactive) (inactive)

Cofactor: The non-protein component of the enzyme molecule required for complete activity. Cofactors can be classified into three groups; coenzymes, prosthetic groups and metal ions.

Enzymes and their Cofactors

Sl.no	Co -factors	Enzymes
1	Fe2+ or Fe3+	Cytochrome oxidase Catalase. Peroxidase. iv. Xanthine Oxidase.
2	Cu2+	Cytochrome oxidase Lysyl oxidase Superoxide Dismutase
3	Zn2+	Carbonic anhydrase Alcohal dehydrogenase Carboxy peptidase
4	Mg2+	Hexokinase Enolase Glucose 6 Phosphatase
5	Mn2+	Arginase Enolase Pyruvate carboxylase
6	K+	Pyruvate Kinase
7	Ni2+	Urease
8	Mo	Dinitrogenase Xanthine oxidase
9	Se	Glutathione peroxidase

Apoenzyme: The protein component of the enzyme.

Coenzyme: The non-protein organic molecule that is not covalently bound and can be readily dissociated from the protein component of the enzyme by dialysis.

Prosthetic group: The non-protein component that is covalently bound and not readily dissociated from the protein component of the enzyme by dialysis.

Zymogen or proenzyme : The precursor form of the enzyme in an inactive form. **Activator:** Any substance that increases the rate of an enzyme-catalyzed reaction.

Inhibitor: Any substance that reduces or inhibits the rate of an enzyme- catalyzed reaction.

Turnover number (molar activity): The number of molecules transformed into product (s) per minute by one molecule of the enzyme.

Specific activity: The amount of enzyme units present per milligram of protein.

Enzyme unit: The amount of enzyme which transforms one micromole of substrate per minute under defined assay conditions.

Michaelis-Menten constants (K_m): The substrate concentration at half the maximal velocity of the enzyme-catalyzed reaction.

General characteristics

The general characteristics of enzymes are as follows:

Specialized proteins

All the enzymes as far as known are specialized proteins that catalyze biochemical reactions. Enzymes show all the properties of protein, i.e., like proteins, enzymes are chemically made up of amino acids as building-blocks linked by peptide bonds; can be hydrolyzed to yield a mixture of constituent amino acids; lose catalytic activity if subjected to extreme pH, temperature, strong acids or bases, organic solvents or other conditions which denature protein and give typical color tests like biuret and FCR (Folin-Ciocalteu reagent) reactions.

Biological organic catalysis

Enzymes are also referred to as biological organic catalysis as:

- Thy enhance the rate of specific chemical reactions.
- They do not shift the eqauilibria of the reactions they catalyze.
- They are regenerated during the course the reaction and
- They are effective in concentrations that are minute as compared with the concentrations of substrates undergoing reaction.

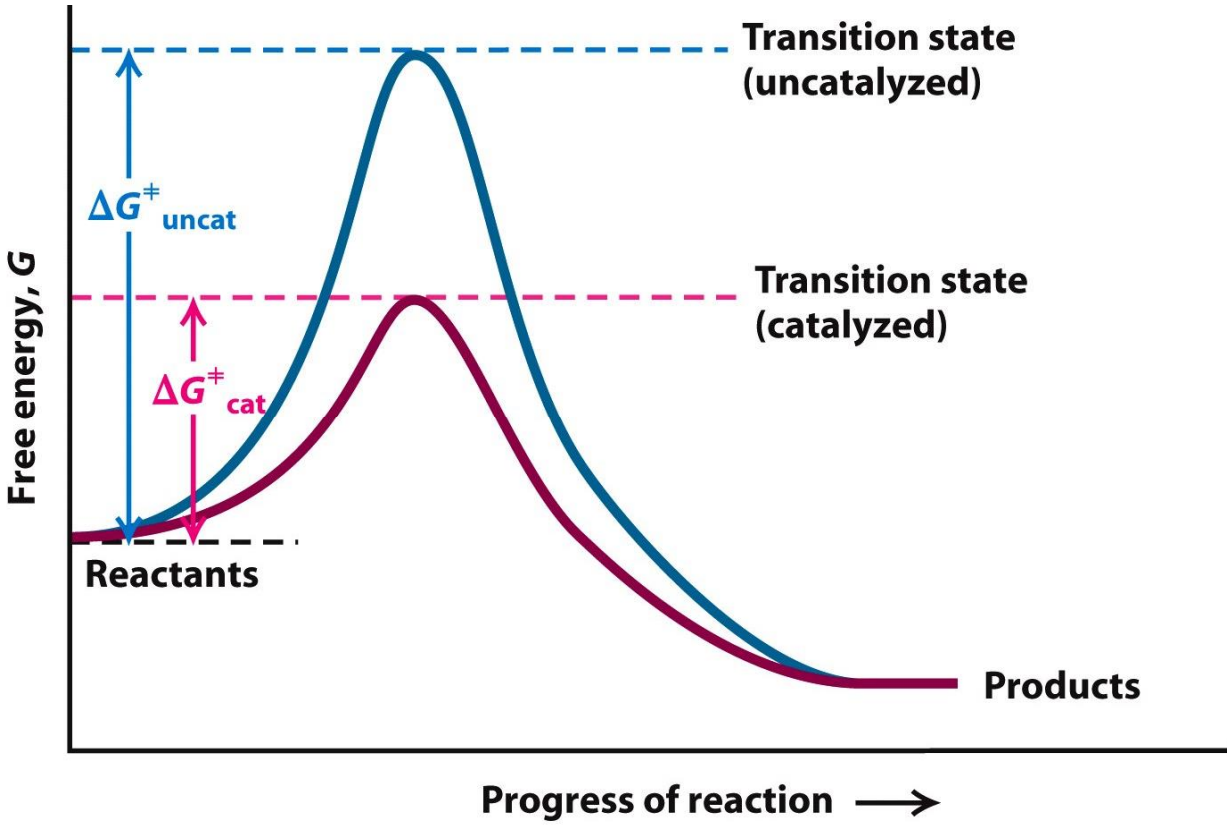


Fig: Activation energy

How an enzyme enhances the rate of chemical reactions?

For a chemical reaction A + B → C + D to occur, three criteria must be met:

1. The reactants, called substrates (A, B) must collide
2. The molecular collision must occur with the correct orientation, and
3. The reactants must have sufficient energy. This energy is called the activation energy which is the amount of energy required to bring all the molecules at a given temperature to the transition sate. More specifically, it is the difference in energy between the ground state of reactants and the transition state.

Enzymes enhance the rate of chemical reaction by decreasing the activation energy of the reaction and with a high probability of correct orientation of reactants. The transition state refers to the state at which all molecules at any given instant possess

enough energy to attain an activated condition before the reactants can be converted to

products (C, D). This state is at the top of the energy barrier separating the reactants and products.

In the normal course, not all molecules acquire sufficient energy to attain the transition state by collision. There are two ways of increasing the reaction rate by:

a) Increasing the temperature which increases thermal motion and energy of molecules capable of entering the transition state, and

b) Lowering the energy of the transition state by the addition of catalysts.

In the enzyme-catalyzed reaction, the lowering of activation energy is achieved by the formation of activated ES (enzyme-substrate) complex by the combination of the enzyme with the substrate. This is clearly represented in Fig(6.1) where, (1) is the energy of activation, the difference in the energy level of reactants, A and B and the transition state for uncatalyzed reaction, (2) it is energy of activation for the formation of ES complex and is much less than (1) and (3), is the difference in energy levels between the reactant and product, i.e., overall free energy change of the reaction which remains the same in both catalyzed and uncatalyzed reactions.

Specificity

Unlike inorganic catalysts enzymes, the biological organic catalysts are more specific toward their substrates and for the type of reactions that catalyze. The term specificity refers to the affinity of the enzyme towards its substrate.

Types of specificity

Enzymes exhibit different types of specificity

- **Absolute specificity:** Some enzymes act on only one substrate. Such enzymes are said to exhibit absolute specificity. For example, succinic dehydrogenase, a key enzyme of TCA cycle catalyzes only the oxidation of succinate to fumarate.

- **Absolute group specificity:** Some other enzymes act on a very small group of substrates having the same functional group but at different rates. Such enzymes are said to exhibit absolute group specificity. For example, alcohol dehydrogenase oxidizes both ethanol and methanol which have common hydroxyl group. Similarly, hexokinase not only phosphorylats glucose but also fructose and mannose.

- **Relative group specificity:** Some other enzymes exhibit relative group specificity. A given enzyme can act upon more than one group of substrates. For example, trypsin catalyzes the hydrolysis of both ester and amide bonds.

- **Stereospecificity:** Many other enzymes show stereospecificity i.e., a given enzyme can act upon only particular stereoisomer. For example, L-amino acid oxidase acts only on L-amino acid but not on its D-form of amino acid. D-amino oxidase acts only on D-amino acid but not on its L-form.

The enzymes are so specific since the active site of each enzyme has the proper shape, size and charge to bind certain substructure only and to catalyze the conversion of these substrates to specific products.

Active site

Enzyme –catalyzed reactions occur at an asymmetric pocket of the enzyme called the active site. The conformation and chemical composition of the active site determines the specificity of enzymatic catalysis. Theoretically, the active site can be subdivided

a) A binding site, which includes the amino acid residues come into contact with the substrate, and (b) a catalytic site, which includes residues directly responsible for catalysis. However, the binding and bond-breaking processes are important in catalysis by enzymes.

In all cases where conformations of enzymes have been determined by X-ray crystallography, the active site has been found to a relatively small area of the enzyme surface. Furthermore, the active site is a specific three – dimensional region having a unique arrangement of amino acid side chains, which are often contributed by amino acids situated quite far apart on the linear sequence of polypeptide chain. For example, the groups on the active site of enzyme lysozyme are contributed by the side chains of glutamate 35, aspartate – 52, tryptophan – 62 and 63 and aspartate – 101 (number after each amino acid refers to its location along the polypeptide chain). The rest of the polypeptide chain may be extremely important in maintaining the correct three – dimensional conformation of the active site.

Theories for enzyme – substrate binding

Two theories have been proposed to explain interaction of substrate and enzyme.

1. Lock and key model

According to the lock and key model proposed by Emil Fisher in 1894, the substrate and enzyme have structural complementarily and fit together like lock and key i.e., the active site of the enzyme has a complementary shape of the substrate to form enzyme- substrate complex. This model has proved to be essentially correct in the case of enzymes known to exhibit absolute specificity.

2. Induced-fit theory

This theory proposed by D.E.Koshland in 1968 suggests that the substrate binds at the active site of the enzyme and then modifies the shape of the active site so that it becomes complementary for the substrate binding. For example, binding of substrate to lysozyme takes place in this way.

Nomenclature

Naming of enzymes

In the past enzymes were named in a haphazard manner as an when they were discovered. The ways in which enzymes are or have been named are listed below:

1. The first enzymes studied have been named for their colour their localization within the body or after the person who discovered them. However, this nomenclature had not been agreeable to many.

2. Later, many enzymes have been named by adding the suffix "ase" to the name of the substrate, for example, urease catalyzes the hydrolysis of arginine to ornithine and urea and so on. However, this nomenclature has not been always practicable.

3. Further, a systematic classification of enzymes has been adopted on the recommendation of an International Enzyme Commission as listed in the 1973 edition of Enzyme Nomenclature with a few exceptions. According to this, enzymes have been classified into six major classes and sets of classes based on the nature and type of reactions catalyzed. According to this, each enzyme is assigned:

A recommended name: It is usually short and appropriate everyday use. **A systematic name:** It identifies the reaction the enzymes catalyzes and

A classification number: It is used where accurate and unambiguous identification of an enzyme is required, as in research journals, abstracts and indexes.

An example is given by the enzyme catalyzing the reaction: ATP + creatine → ADP + phosphocreatine

The recommended name: creatine kinase

The systematic name: ATP: creatine phosphotransferase

Classification number : EC 2.7.3.2, where EC stands for Enzyme Commission, the first digit (2) for the major class name (transferases), the second digit (7) for the subclass

(phosphotransferases), the third digit (3) for the sub-subclass (phosphotransferases with a nitrogenous group as acceptor) and the fourth digit (2) designates creatine kinase.

Enzyme classification

Enzymes can be classified into six major classes based on the nature and type of reactions catalyzed as given below:

1. **Oxidoreductases:** These enzymes catalyze oxidation or reduction reactions by transfer of hydrogen or electrons, e.g., succinic dehydrogenase.

2. **Transferases:** These enzymes are involved in transferring functional groups between donors and acceptors. The amino, acyl, phosphate, one-carbon and glycosyl groups are the major moieties that are transferred e.g., glutamic pyruvic transminase.

3. **Hydrolases:** This group of enzymes can be considered as special class of transferases in which the donor group is transferred to water. The generalized reaction involves the hydrolytic cleavage of C-O, C-N, O-P and C-S bonds. The cleavage of the peptide bond by peptidases is good example of this reaction. The proteolytic enzymes are a special class of hydrolases called peptidases.
4. **Layses:** These enzymes remove the groups of water, ammonia or CO_2 from the substrate to cleave double bond or conversely, add these groups to double bonds e.g., furmarase.
5. **Isomerases:** These are a very heterogeneous group of enzymes that catalyze isomerizations (i.e., structural rearrangements within a molecule) of several types. These include cis-trans, keto-enol, and adose-ketose interconversion. Isomerases that catalyze inversion at asymmetric carbons are either epimerases or racemases. Mutases involve the intramulecular transfer of a group such as phosphoryl group.
6. **Liagases (synthetases):** These enzymes are involved in synthetic reactions where two molecules are joined together at the expense of breakdown of nucleoside-triphosphates. The formation of aminoacyl tRNAs, acetyl coenzyme A, glutamine, and the addition of CO_2 to pyruvate are reactions catalyzed by ligases, e.g. pyruvic carboxylase.

The six major classes of enzymes and the type of reactions catalyzed are summarized in Table below.

Table: Classification of enzymes and types of reactions catalyzed

S.N.	Enzyme class	Reactions catalyzed
1.	Oxidoreductases	Oxidation and reduction of substrates (usually involve hydrogen transfer)
	Dehydrogenases	Transfer of hydrogen atoms from substrate to NAD*
	Oxidases	Transfer of hydrogen atoms from substrate to oxygen
	Oxygenases	Partial incorporation of oxygen to substrate
	Peroxidases	Transfer of electrons from substrate to hydrogen peroxide
2.	Transferases	Transfer of a chemical group (such as a methyl group, amino group, phosphate group from one molecule to another
	Phosphorylases	Addition of orthophosphate to substrate
	Transaminases	Transfer of amino group from one substrate to another
	Kinases	Transfer of phosphate from ATP to substrate
3.	Hydrolases	Cleavage of bonds by the addition of water
	Phosphatases	Removal of phosphate from substrate
	Peptidases	Cleavage of peptide bonds
4.	Lyases	Addition of groups to double bond (-C=C-, C=O, -C=N-)
	Decarboxylases	Removal of carbon dioxide from substrate
5.	Isomerases	Rearrangement of atoms of a molecule
6.	Liagases	Formation of new bonds using energy from (simultaneous) breakdown of ATP
	Synthetases	Joining two molecules together

Factors affecting the rate of enzyme-catalyzed reactions

Several factors are known to influence the rate of enzyme-catalyzed reaction, chief of them being :

1. Enzyme concentration
2. Substrate concentration
3. Temperature
4. pH and
5. Activators and inhibitor

Effect of enzyme concentration

With few exceptions, the initial velocity (V_0) of enzymatic reactions bears a linear relationship with the concentration of the enzyme [E], provided other conditions do not act as limiting factors. If an excess substrate is present, doubling the enzyme concentration usually doubles the rate of formation of end products. This usually applies only at the start of the reaction, for the end-products of the reaction often have an inhibitory effect on the enzyme, and decrease its efficiency. Fig 6.5 is a graph which relates enzyme concentration to enzyme activity, all other factors being held constant. The dotted part is hypothetical, and almost impossible to attain *in vitro;* it may occur to a limited extent in the living cell.

Effect of substrate concentration

The concentration of substrate [S] affects the rate of the enzyme-catalyzed reaction. When the enzyme concentration is kept constant and the substrate concentration varied, the rate of enzyme-catalyzed reaction increases linearly upto a certain concentration of substrate after which a point is finally reached, where the enzyme is saturated with substrate. When this saturation point is reached, further increases in substrate concentration have no influence on rate of the enzyme-catalyzed reaction which is due to saturation of all the active sites of the enzyme by the substrate and partly due to more rapid accumulation of end-products causing inhibition of enzyme. The typical shape of curve obtained in this case is a nonlinear hyperbolic relationship between V_0 and substrate concentration.

Pioneering work on the kinetic studies of enzyme-catalyzed actions was made by A. Brown (1902) and V. Henri (1903). This was further developed by L. Michaelis and M.L. Menten in 1903 and by G.E. Briggs and J.B.S.Haldane in 1926.

To account for hyperbolic relationship. Brown put forward hypothesis that the enzyme (E) reversibly combines with the substrate (S) to form an enzyme-substrate (ES) complex which is decomposed to yield product (P) and the free enzyme in its original from.

$$E + S \underset{}{\overset{K_S}{\leftrightarrows}} ES \xrightarrow{k_{cat}} E + P$$

Based on the work of Briggs and Henri, Michaelis and Menten derived a mathematical equation which is consistent with the empirical data represented They assumed that a rapid equilibrium exists between E, S and ES and that ES-complex breakdown of ES to E and S. This plot is based on the following equation for the behavior of a simple enzyme-catalyzed reaction in which one substrate is converted to one product.

$$E + S \underset{k-1}{\overset{k+1}{\rightleftharpoons}} ES \underset{k-2}{\overset{k+2}{\rightleftharpoons}} E + P$$

Where k_{+1}, k_{-1} , k_{+2} and k_{-2} are the velocity constants for the above four steps. By rate measuring the initial of reaction, the rate constant k_{-2} may be ignored because not be present to make the reverse reaction proceed at significant rate. enough products will

The equation they obtained is called Michaelis-Menten equation which is,

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Where V_o = Initial velocity of the enzymatic reaction

V_{max} = Maximal velocity at infinite substrate concentration, [S] = Substrate concentrtion and

K_m = Michaelis – Menten constant

In Michaelis – Menten equation, when [S] = K_m, then

$$V_0 = \frac{V_{max}[S]}{[S] + [S]}$$

Or $V_o = \frac{V_{max}}{2}$

Hence, K_m can be defined as the concentration of the substrate at which the reaction rate is half the maximal velocity. Usually, K_m has dimension moles per liter and its independent of enzyme concentration.

Determination of K_m and V_{max}

The hyperbolic curve of the V_o verses [S] plot does not permit the determination of exact values of K_m and V_{max}. This limitation can be overcome by plotting the same kinetic data in different ways. The most popular approach is simple by taking reciprocal of both sides of Michaelis-Menten equation to yield a straight-line rate equation, called a Lineweaver-Burk equation which is,

$$\frac{1}{V_o} = \frac{K_M}{V_{max}} \cdot \frac{1}{S} + \frac{1}{V_{max}}$$

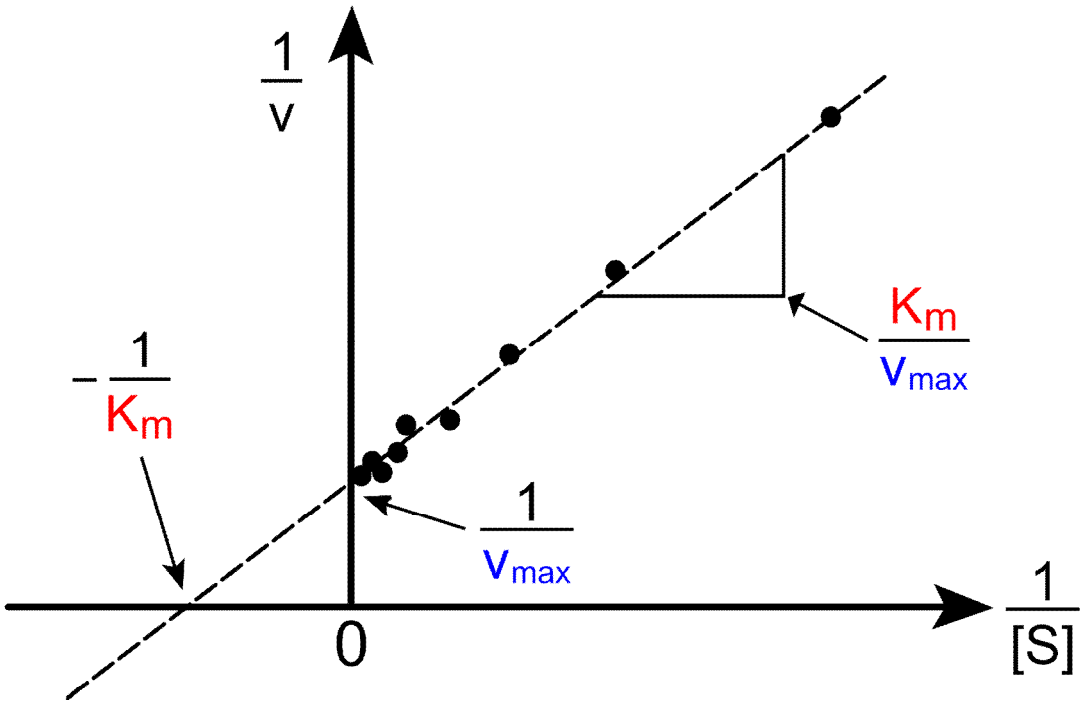


Fig. Lineweaver–Burk plot

By plotting l/V_o versus l/[S], a straight line is obtained having a slope of K_m/V_{max}, an intercept of l/V_{max} on l/v_o axis and an intercept of $-l/K_m$ and l/[S] axis. Such a plot is called a Lineweaver- Burk plot or double reciprocal plot (Fig. 6.7). By determining the value of l/V_{max}, K_m can be determined. K_m can also be directly determined by extrapolation of the line to meet the l/(S) axis where the intercept is $-l/K_m$.

The double-reciprocal plot has the advantage that measurement of the velocity of the reaction can be made at a number of substrate concentrations and then extrapolated back to infinite substrate concentrations at the intercept, which gives a straight-line so that accurate values of V_{max} and K_m can be determined. This also gives variable information on enzyme inhibition.

Significance of K_m and V_{max}

The K_m value which is expressed in molar concentration is characteristic of each individual enzyme. K_m is not a fixed value may vary with structure of substrate, pH and temperature. For enzyme having more than one substrate, each substrate has a characteristic K_m value. K_m helps to evaluate the specificity of action of given enzyme towards similar substrates. The substrate with the lowest K_m value has highest apparent affinity for the enzyme. It also establishes an approximate value for the intercellular level of substrate as the enzymes are not necessarily saturated with their substrates. Knowledge of the K_m of an enzyme can be of consideration value in investigations of metabolic control.

V_{max} represents the efficiency of enzyme action and can be used to compare the catalytic efficiency of different enzymes.

Effect of temperature

As with most chemical reactions, the rate of an enzyme-catalyzed reaction increases with temperature, however, in the case enzymes there is a temperature at which thermal denaturation of protein causes a loss of activity and the rate begins to slow down. For each enzyme there is an optimum temperature at which it is more active and its activity slows down when the temperature is changed in either direction away from this optimum. This is because, temperature changes cause slight changes in the three-dimensional shape of protein, increases in temperature may cause the protein to loosen up and become less compact. Even small changes in the dimensions of its active site can make an enzyme a less efficient catalyst.

The velocity of a chemical reaction is related to temperature according to Vant Hoff's law which states that a rise of $10^{\circ}C$ will double the speed of a chemical reaction.

$$\frac{\text{Velocity at } (T+10^{\circ})}{\text{Velocity at } T^{\circ}}$$

The temperature coefficient is usually expressed by the symbol, Q_{10} which is also applicable to enzyme-catalyzed reactions. For most enzyme-catalyzed reactions. Q_{10} is approximately 2 at lower temperatures, but gradually drops off until the rate is 1 (or lower) at higher temperatures. Fig.6.8 shows the general effect of temperatures (bell shaped curve) on most enzyme-catalyzed reactions. Most enzymes show a temperature optimum between 25 and $37^{\circ}C$ but are inactivated at temperatures above $55^{\circ}C$ although enzymes in thermophilic bacteria are active even at temperature exceeding $55^{\circ}C$.

Effect of pH

The activity of the enzyme is markedly influenced by the pH of the medium in which the reaction occurs. It is usually found that activity is shown over a limited range of pH and within this range, a bell-shaped activity curve (Fig) is often observed when enzyme activity is plotted against pH. Every enzyme has a characteristic pH optimum wherein the activity is at maximum and the activity decreases on either side of this value. Most enzymes have pH

optimum in the region 6-8, which, however, varies with the source of the enzyme, the kind of substrate, the kind of buffer and the temperature. For example, pepsin has a pH optimum around 2, for enzymes of plants and fungi it is 4.0-6.5 and for most enzymes of higher animals it is 6.5-8.0.

At extreme pH values protein denaturation will occur with concomitant loss of enzyme activity. Slight changes in pH which do not denature the protein, may alter the state or degree of ionization on the R groups within the active site, thereby reducing the enzymes ability to bind the substrate and catalyze the reaction. Changes in pH can also alter the charges of the substrate molecules, thus changing the character of the enzyme-substrate complex.

Effect of activators and inhibitors

Activators are specific chemical compounds which accelerate the rate of enzyme-catalyzed reactions. Activators include metal ions, coenzymes and prosthetic groups. Many enzymes are activated by specific metals which are readily removed from the protein. These latter proteins are called metalloenzymes. The ions most commonly found in enzymes and an example of each are : Mg^{2+} in most phosphotransferases; Zn^{2+} in alcohol dehydrogenase; Mn^{2+} in arginase; Fe^{2+} in ferrodoxin; and Cu^{2+} in cytochrome C oxidase.

Substances that reduce the activity of an enzyme are known as inhibitors. The different classes and mode of action inhibitors are discussed below.

Enzyme inhibition

Enzyme inhibition refers to the phenomenon of decrease in the rate of enzyme-catalyzed reactions by specific substances known as inhibitors which include drugs, antibiotics, poisons, antimetabolities as well as products of enzymatic reactions. Inhibition studies are useful in understanding the specificity and the nature of functional groups at the active site of the enzyme and also the enzyme regulation. Two general classes of inhibition are recognized namely, irreversible and reversible inhibitions.

Irreversible inhibition

This type of inhibition is brought about by an irreversible inhibitor which forms a

covalent bond with a specific function, usually an amino acid reside, which may, in some manner, be associated with the catalytic activity of the enzyme. For example, iodoacetate reacts irreversibly with the essential sulfhydryl group of triosephosphate dehydrogenase.

$E\text{-}SH + ICH_2COOH \rightarrow E\text{-}S\text{-}CH_2COOH + HI$

Iodoacetate Enzyme-inhibitor complex

The other example is irreversible inhibition of serine protease such as chymotrypsin by disopropylphosphofluoridate (DIPF) which phosphorylates specific serine residue to yield inactive enzyme.

Reversible inhibition

This kind of inhibition is reversible in nature as the inhibitor which binds does not form a covalent bond with the enzyme molecule. Three distinct types of reversible inhibition are known and they are discussed below:

Competitive inhibition

As the name indicates, the competitive inhibitor (I) which has a structure similar to that of natural substrate (S) of the enzyme (E) reversibly binds at the active site of the enzyme to form an enzyme inhibitor (EI) complex analogous to enzyme-substrate (ES) complex. Thus, the inhibitor competes with the natural substrate for binding at the active site.

Competitive inhibition can be overcome by increasing the substrate concentration which causes the reaction sequence to swing to the right in the above equation.

A classical example of competitive inhibition is that of succinic dehydrogenase (SDH) which readily oxidizes succinic acid to fumaric acid, by malonic acid.

Another example of competitive inhibition is provided by sulpha drugs used to combat microbial infection in humans. These drug are structurally realted to p-aminobenzoic acid (PABA), a vital precursor in the microbial biosynthesis of folic acid and inhibit the synthesis of this vitamin.

Non-competitive inhibition

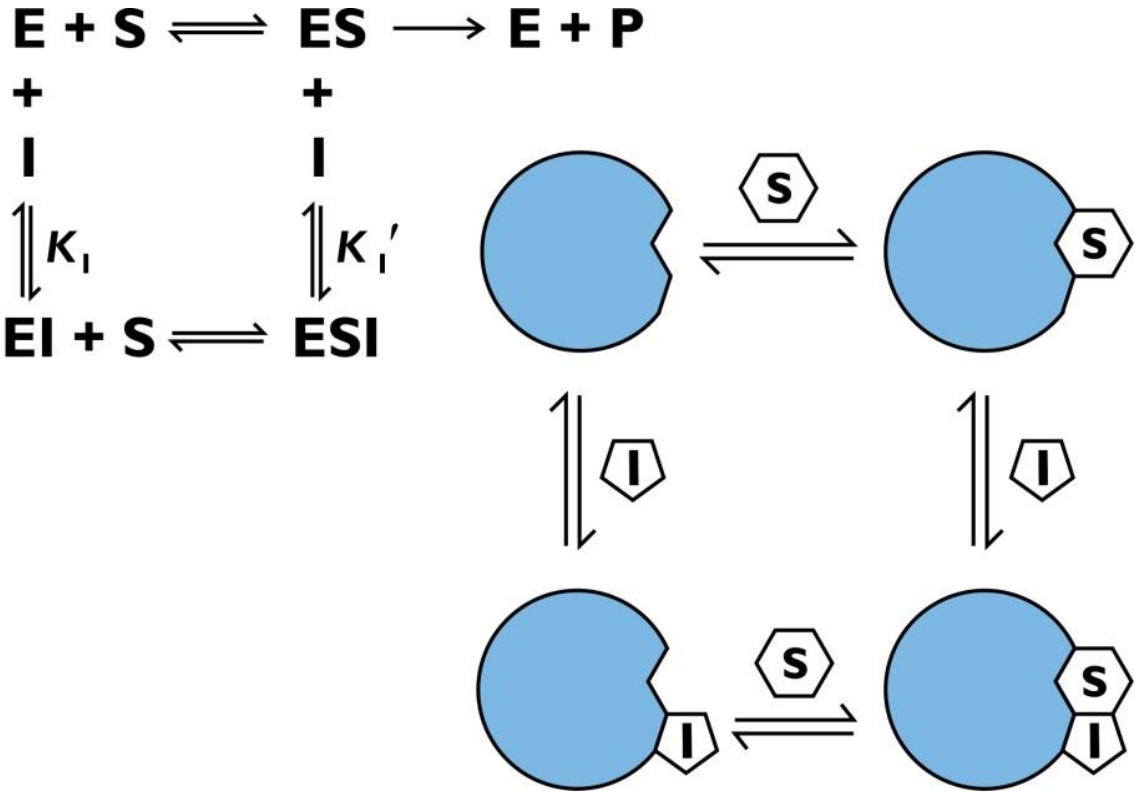
This type of inhibition is brought about by the inhibitors than bind reversibly with either the free enzyme or the enzyme substrate complex as shown below;

Non-competitive inhibition differs from competitive inhibition in that the inhibitor can combine with ES, and S can combine with EI to form both instance EIS. This type of inhibition is not completely reversed by high substrate concentration since the closed sequence of reaction will occur regardless of the substrate concentration.

The examples of non-competitive inhibitors are heavy metal ions and organic molecules which bind reversibly with sulphydryl groups of cysteine residues in the enzymes.

Uncompetitive inhibition

Compounds that combine only with the enzyme-substrate complex but not with the enzyme are called uncompetitive inhibitors. This inhibition is not overcome by high substrate concentrations. The sequence of reaction is



An example of this type of inhibitor is α- ketoglutarate which inhibits the mouse brain γ-aminobutyrate amino transferase.

Use and application of enzymes

Enzymes are widely used in various fields due to their efficiency and specificity. Some of the commercial uses of enzymes are listed in Table 6.5

Table. Some commercial uses of enzymes

Field of application	Purpose	Enzyme used
Fermentation industry	Bread making and brewing	Amylases and proteases
	Clarification of beers and wines	Papain
Food industry	Production of sweetners and sugar syrups	Amylase β galactosidase, glucose isomerase
	Meat tenderizing	Papain, trypsin chymotrypsin and other proteolytic enzymes from microbes
	Cheese making	Rennin / rennet
	Processing and clarification of fruit juices	Pectines
Leather industry	Hydrolysis of proteins of hairs	Proteases
Paper industry	Preparation of partially hydrolyzed starch as surface coating	Amylase
Textile Industry	Removal starch size before dyeing	Amylase
Pharmaceutical industry	As digestive capsules	Pepsin, mixture of amylase, trypsin and lipase
Photography	Recovery of waste gelatin	Ficin
Forensic laboratory	Blood alcohol treatment	Alchol dehydrogenase

Detergents	As biological detergents for partial degradation of polysaccharides and proteins from garments	A mixture of amylase and alkalin and neutral proteases
Medical application	Clearing of blood clots	Streptokinase
	Diagnons of liver disease	Serum aspartate, aminotransferase, alkaline phosphatase, lactate dehydrogenase
	Diagnosis of heart disease	Creatine kinase, aspartate amino-transferase,

IMMOBILZED ENZYMES

Immobilized enzymes are those purified enzymes which are attached to an insoluble support that will stabilize and permit the continuous use of enzymes for various commercial applications. Immobilization of enzymes will also increase the efficiency and also reduce the cost of production of industrially important products that are beneficiary to the mankind.

METHODS OF IMMOBILIZATION

There are a number of methods of enzyme immobilization. Some of the methods are as follows:

1. Adsorption

The purifired enzymes are adsorbed to insoluble support materials using alumina, bentonite, cellulose anion and cation exchangers, resins, glass, hydroxyapatite, kaolinite, etc.,

Covalent linkage

Insoluble support materials polyacrylamide, nylon, sephadex, silica glass beads and bifunctional reagents like 1, 5- diflouro-2,4-dinitro-benzene, dimethyladipalmidate, glutaraldehyde, etc., are employed for enzyme immobilization by this method the support materials require activation before enzyme attachment.

3. Matrix entrapment

This method involves the polyacrylamide gels polymerized in a solution containing the enzyme.

4. Encapsulation

Here, the enzymes are enclosed in a capsule usually generated from cellulose nitrate or nylon which is permeable to small ions and molecules.

Use and applications of immobilized enzymes

The use of immobilized enzymes may be broadly classified into preparative and analytical.

The preperative type includes production of sugar syrups from corn starh, preparation of fructose- glucose mixture, large scale preparation of L-amino acids from racimic D,L- amino acids, antibiotics, hormones, interferons,etc.,

Analysis of blood sugar using immobilized glucose dehydrogenase is an example of analytical type of application. This enzyme oxidizes D-glucose to D-glucono-δ-lactone in the presence of NAD^+ which is reduced to NADH and H^+ . The NADH formed in measured spectrophotometrically.

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Lipid and Fats

Lipids are a heterogeneous group of fats and fat-like substances which are insoluble in water but get dissolved in nonpolar organic solvents like chloroform, benzene, ether, etc.They are of three kinds.

1. Simple Lipids. They are esters of fatty acids and alcohols. Additional groups areabsent. Simple lipids include true fats and waxes. True fats are esters of fatty acids and trihydric alcohol glycerol. They are often distinguished into.fats and oils. At ordinary temperature fats are solid while oils are liquid. In waxes glycerol is replaced by a long chainalcohol. For the sake of coherence **cutin** and **suberin** may also be included in this group.

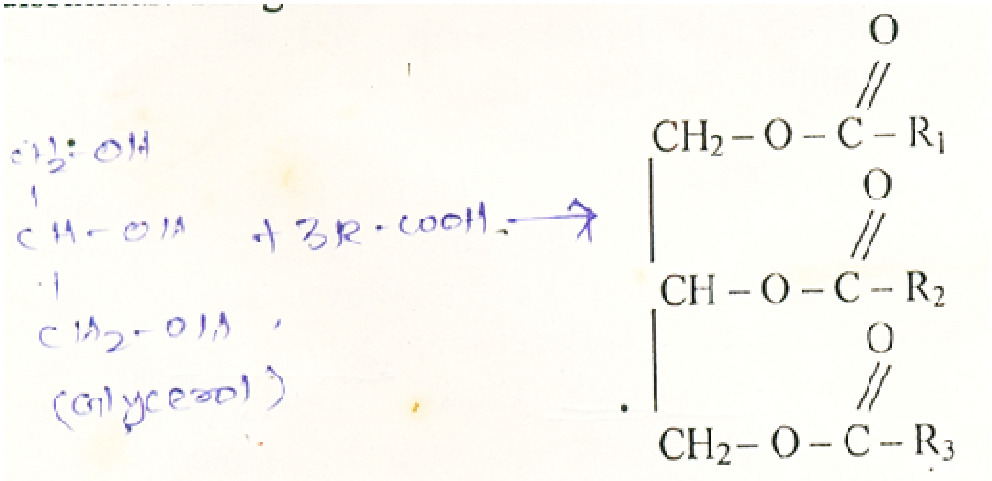
2. Compound Lipids. Lipids having additional groups, besides fatty acids andalcohol are described as compound Iipids. The common compound lipids are phospholipids,s hinoli ids glycolipids, suifolipids, etc. Phospholipids contain phosphate and anothercompound like come, ethanolamine, serine, etc. They are constituents of cellular membranes. Glycolipids possess a sugar (i.e., glucose, galactose) at the terminal group of a diglyceride. Sulfolipids similarly have a sulphur group.

3. Derived Lipids. They include hydrolysis-products of lipids which are soluble in fat solvents, i.e., fatty acids, Sterols.

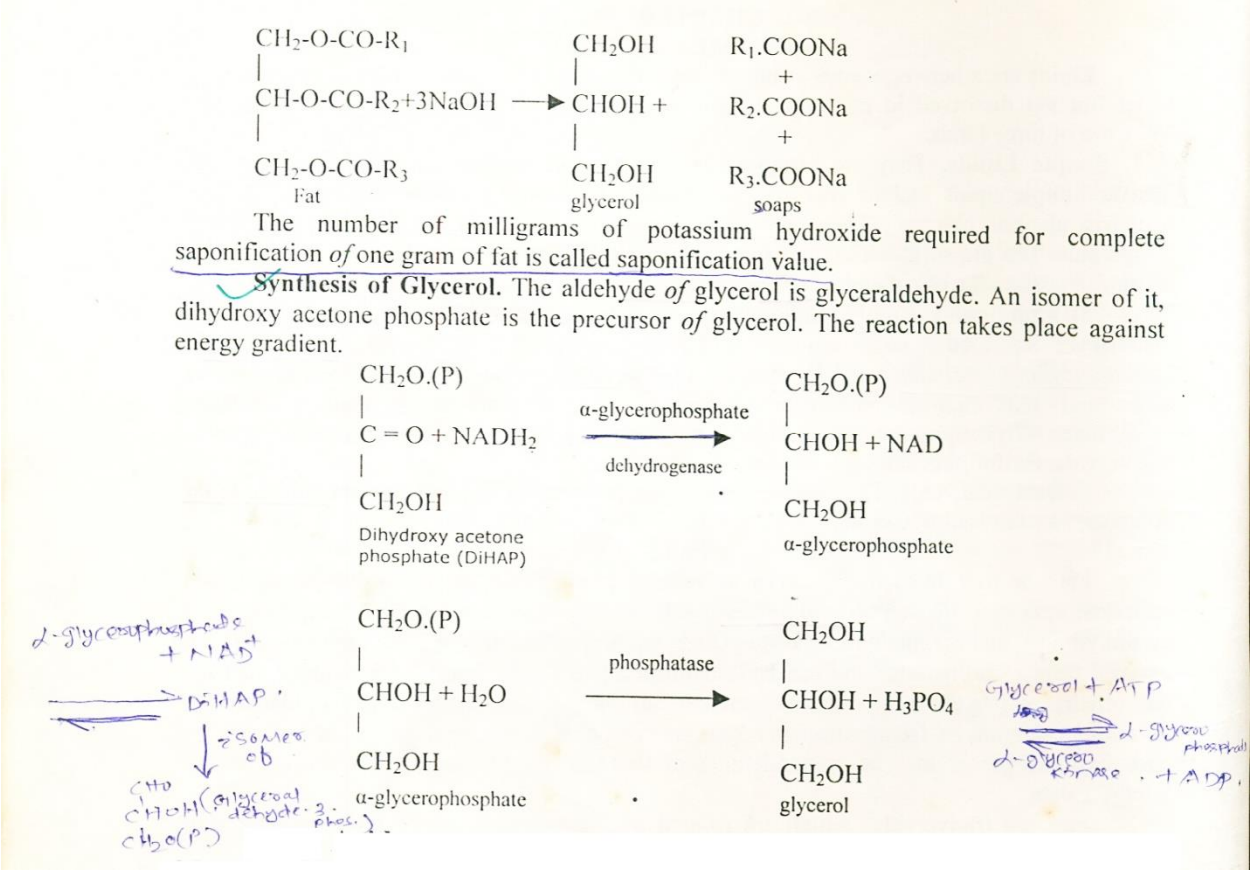
Fats:

Fats or true fats chiefly serve as reserve food. They are generally stored inside spherical vesicles called oil or ligid bodies. Each oil body is surrounded by a membrane. Like carbohydrates, fats are made of C, H and 0 but the proportion of oxygen is very low. Fats are derived from carbohydrates and can be transformed into them. Being more reduced than the carbohydrates, fats consume more oxygen and can release more energy during oxidation. On an average a gram of fat produces 9.3 kcal, as compared to 4.1 kcal given out by complex carbohydrates per gram. The preponderance of fats and oils in seeds is due to their higher energy value.

Fats are triglycerides which are formed by the esterification of three molecules of fatty acids with one molecule of alcohol glycerol. The three fatty acids may be similar or dissimilar. The general formula of fats can be written as follows:



Here R – C is derived from the fatty acid. At each esterification a molecule of water is given out. Hydrolysis breaks ester linkages so that the two components of fats can be regenerated. Hydrolysis can' occur in the presence of alkalies, this yields salts of fatty acids or soaps; the process of hydrolysis of fats by alkali solutions is termed as saponification.



The number of milligrams of potassium hydroxide required for complete saponification *of* one gram of fat is called saponification value.

Synthesis of Glycerol. The aldehyde *of* glycerol is glyceraldehyde. An isomer of it, dihydroxy acetone phosphate is the precursor *of* glycerol. The reaction takes place against energy gradient.

Fatty Acids

They are organic acids having carboxylic group (COOH) and a short or long carbon chain. The carboxyl group is hydrophilic while the hydrocarbon chain is lipophilic. A number of fatty acids have been reported in plants. Almost all of them possess even number of carbon atoms. The following are the categories of fatty acids found in plants.

1. Saturated Fatty Acids [C_nH_{2n} O_2 or CH_3. $(CH_2)_{n-2}$ COOH]. They do not have double bonds. The series starts with formic acid which has the formula of CH_2O_2. The first three of this series (upto $C_3H_6O_2$, propionic acid) are not found in fats. Butyric acid ($C_4H_8O_2$) is present in traces in sesame oil. The common saturated fatty acids are *caproic acid* ($C_6H_{12}O_2$, coconut), *caprylic acid* ($C_8H_{I6}O_2$, palm nut), *capric acid*($C_{10}H_2O$,palm nut), *lauric acid* (C_{12} $H_{24}O_2$, coconut and some others), *myristic acid*($C_{14}H_{28}O_2$, nutmeg butter), *palmitic acid* (C_{16} $H_{32}O_2$, most vegetable fats), stearic acid ($C_{18}H_{36}O_2$, in many vegetable fats), *arachidic acid* ($C_{20}H_{40}O_2$, ground nut and others).

2. Unsaturated Fatty Acids. These fatty acids have one or more double bonds in between pairs of carbon atoms. On account of the presence of double bonds fatty acids contain less number of hydrogen atoms than required for saturation.

Fats formed of unsaturated fatty acids have low melting point. They are, therefore, *oils* at ordinary temperatures. They can combine with oxygen and others in order to satisfy the unsaturated linkages. Consequently these fats solidify. They are often named as drying oils. This property of these fats to solidify on exposure to air is made use of in paint industry. The edible oils are changed into fats by the addition of hydrogen to their double bonds. The process is described as hydrogenation and is used in preparation of vegetable ghee.

The common unsaturated fatty acids are oleic acid ($C_{18}H_{34}O_2$ or CH_3-$(CH_2)_7$-CH = CH-$(CH_2)_7$-COOH) in several vegetable oils, *Linoleic acid* ($C_{18}H_{32}O_2$ or CH_3-$(CH_2)_4$ - CH = CH - CH_2 - CH = CH - $(CH_2)_7$-COOH, in several vegetable oils), *Linolenic acid* ($C_{18}H_{30}O_2$ or CH_3-CH_2-CH = CH- CH_2-CH = CH - CH_2- CH = CH-$(CH_2)_7$-COOH, linseed oil.)

3. Other Fatty Acids. They include (i) *Saturated Hydroxy Acids* withgeneral formula C_n, $H_{2n}O_3$, e.g., juniperic acid (C_{12} $H_{24}O_3$), sabinic acid ($C_{16}H_{32}O_3$); (ii) *Unsaturated Hydroxy Acids, e.g.,*ricinoleic acid ($C_{18}H_{34}O_3$) or CH_3 - $(CH_2)_5$ - CHOH - CH_2 - CH = CH - $(CH_2)_7$ - COOH); *(iii) Saturated Dihydroxy Acids, e.g.,* ($C_{18}H_{36}O_4$), (Castor *oil*); *(iv) Saturated Dibasic acids, e.g.* ($C_{22}H_{42}O_4$), (Japan Wax); (v) *Cyclic Acids*, e.g., chaul-moogric acid.

$[C_{18}H_{32}O_2$ or $C_{17}H_{31}(CH_2)_{12}COOH]$ (CH = CH, CH = CH)

Synthesis of Fatty Acids

Acetyl-CoA and malonyl-CoA are important in the synthesis of fatty acids. Acetyl-CoA can be obtained from pyruvic acid or acetic acid.

$CH_3.CO.COOH + HS.CoA + NAD \xrightarrow{CH_3.CO\ Dehydrogenase} S.CoA + NADH_2 + CO_2$
Pyruvic acid Acetyl-CoA

$CH_3.COOH + ATP + HS.CoA \xrightarrow{CH_3.CO\ Synthetase} S.CoA + AMP + iPP$
Acetic acid Acetyl-CoA

Malonyl-CoA is formedfrom acetyl-CoA in aerial parts by the addition ofa molecule bf carbon dioxide ATP is required. The enzyme which catalyses the reaction is named as acetyl-CoA carboxylase. Mn^{2+} and biotin are essential for the reaction.

$CH_3.CO \sim S.CoA + CO_2 + ATP \xrightarrow{COOH.CH_2.CO\ Carboxylase} S.CoA + ADP + iP$
Acetyl-CoA Malonyl-CoA

It is also formed *from* oxaloacetate in roots through decarboxylation to malonate and then reacting with coenzyme A.

$HOOC\text{-}CO\text{-}CH_2\text{-}COOH \longrightarrow HOOC\text{-}CH_2\text{-}COOH + CO_2$
Oxaloacetic acid Malonic Acid

$HOOC\text{-}CH_2\text{-}COOH + HS.CoA + ATP \xrightarrow{Synthetase} HOOC\text{-}CH2\text{-}CO\text{-}S.CoA + AMP + iPP$
Malonic Acid

Synthesis of fatty acids occurs in chloroplasts, leucoplasts, mitochondria or inside special vesicles. There are two pathways for thesynthesis of fatty acids-de novo pathway and elongationpathway.

1. De Novo Pathway. The pathway is catalysed by an enzyme complex (made up of up 7 enzymes), known as fatty acid synthetises It requires Acetyl CoA and malonyl CoA. Both acetyl CoAand malonyl CoA become reactive after combining with acyl carrier proteins (HS.ACP).The twocondense and give rise to aceto-acetyl S.ACP liberation of a molecule of CO_2. The product undergoes reduction twice through the agency of $NAD(P)H_2$. The double reduction is intervened by dehydration. It produces butyryl~S.ACP. The latter combines further with malonyl~S.ACP to form propionylderivative. The process is repeated till a.16-C

long fatty acid (palmitic acid) is formed. It is freed from the acyl carrier protein. De novo pathway occurs on the inner envelope of the plastid towards the stromal face.

Acetyl ~S.CoA + HS.ACP Acetyl ⟶ S.ACP + HS. CoA
2C 2C

Malonyl Transacylase

Malonyl~S.CoA + HS.ACP ⟶ Malonyl ~ S.ACP + HS. CoA
3C 3C

Acetyl Malonyl ACP-Condensing Enzyme

Acetyl~S.ACP + Malonyl~S.ACP ⟶ Acetoacetyl~S.ACP+CO_2+HS.ACP
2C 3C 4C

dehydrogenase

Acetoacetyl~S.ACP+$NADPH_2$/$NADH_2$ ⟶ α-hydroxybutryl~S.ACP+$NADP^+$/NAD^+
4C 4C

dehydrogenase

α-Hydroxybutyryl~S.ACP ⟶ Crotonyl~S.ACP+H_2O
4C 4C

dehydrogenase

Crotonyl~S.ACP+$NADPH_2$/$NADH_2$ ⟶ Butyryl~S.ACP+$NADP^+$/NAD^+
4C 4C

* The six enzymatic reactions alongwith ACP mentioned above are detailed below:

I. Priming of the system by acetyl-CoA

A. ACP-Acytyl transferase reaction

H_3C-C(=O)-S-CoA + ACP-SH ⟶ H_3C-C(=O)-S-ACP + CoA-SH
Acetyl CoA Acetyl-ACP

B. Transfer to β–ketoacyl-ACP synthase

H_3C-C(=O)-S-ACP + synthase-SH ⟶ H_3C-C(=O)-S- synthase + ACP-SH
Acetyl ACP Acetyl-synthase

II. ACP-malonyl transferase reaction

(malonyl transfer to system)

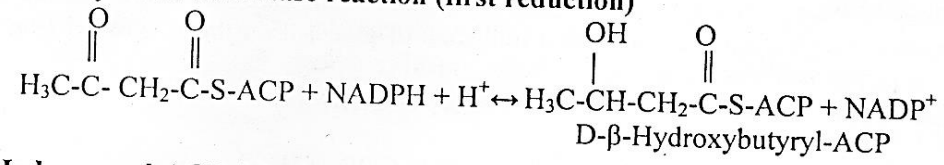
OOC-CH_2-C(=O)-S-CoA + ACP-SH ⟶ OOC-CH_2-C(=O)-S-ACP + CoA-SH
Malonyl-CoA Malonyl-ACP

III. β-Ketoacyl-ACP synthase reaction (condensation)

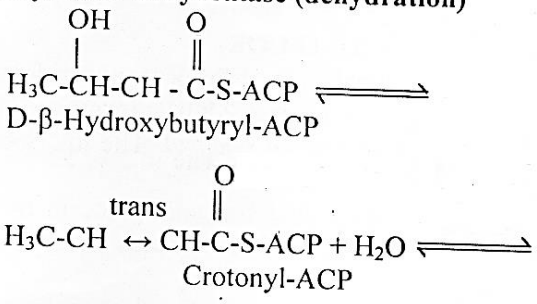
H_3C-C(=O)-S-synthase + OOC-CH_2-C(=O)-S-ACP ⇌
Acetyl-synthase Malonyl-ACP

H_3C-C(=O)-CH_2-C(=O)-S-ACP + CO_2 + synthase-SH
Acetoacetyl-ACP

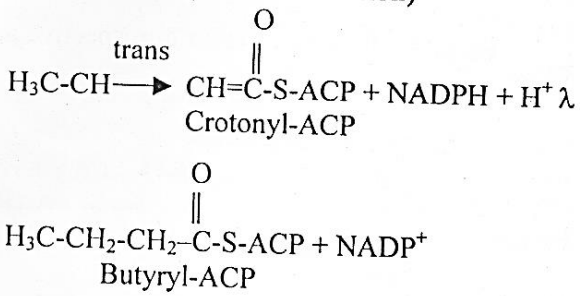
IV. β-Ketoacyl-ACP reductase reaction (first reduction)



V. β-Hydroxyacyl-ACP dehydratase (dehydration)



VI. β-Enoyl-ACP reductase (second reduction)



This reaction sequence is repeated, the malonyl-ACP now condensing with the butyryl ACP to give ACP ester of a 6C acid and so on until the full even 'number chain length of 16 C atoms is obtained,

2. Elongation Pathway. In this case a preformed fatty acid chain (16 C) is lengthened by the addition of two carbon groups from acetyl or malonyl compounds. Both of them react in the form of their CoA or ACP derivative. A separate elongase enzyme is required for each addition of 2 carbon groups.

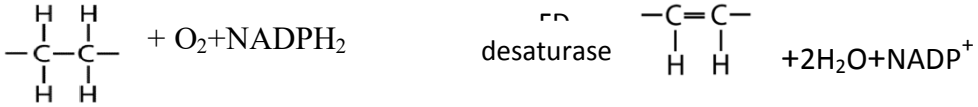
Acetyl ~ S.ACP + Palmityl ~ S.ACP + $2NADPH_2$/ $NADH_2$ ⟶
Stearyl~ S.ACP + $2NAD^+$ $INADP^+$ + HS.ACP

Malonyl ~ S.ACP + Palmityl ~ S.ACP + $2NADH_2$/$NADHP_2$ ⟶
Stearyl~ S.ACP + CO_2 +$2NAD^+$/$NADP^+$ + HS.ACP

Synthesis of unsaturated fatty acids

The double bonds of a unsaturated fatty acids can be produced by two methods.

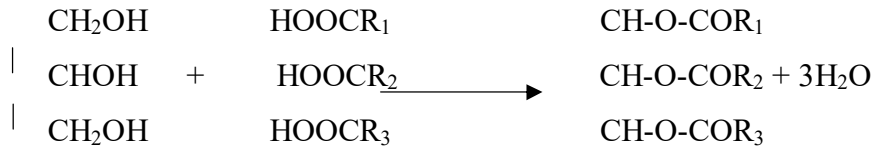
(a) In the presence of a desaturase, oxidised ferredoxin, molecular oxygen and a reductant ($NADPH_2$), two hydrogen atoms are taken up from the already saturated fatty acids to produce a double bond. Each such double bond is incorporated by a separate desaturase enzyme.



(b) Double bond is placed in β-y position during chain formation due to the failure of second reduction after condensation.

Condensation of Fatty Acids and Glycerol

Both glycerol and fatty acids combine together as soon as they are formed. This is the final step in fat synthesis. Three molecules of fatty acids undergo esterification with a molecule of glycerol on the surface of Endoplasmic reticulum as follows:

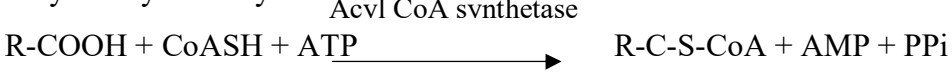


The three fatty acids combining in a molecule of fat or triglyceride are similar only in rare cases (e.g., in fat palmatin - palmitic acid, $C_{15}H_{31}COOH$). In most of the fats, all three fatty acids are different (partial Random Distribution Theory),The intermediate type having two similar fatty acids is also widespread.

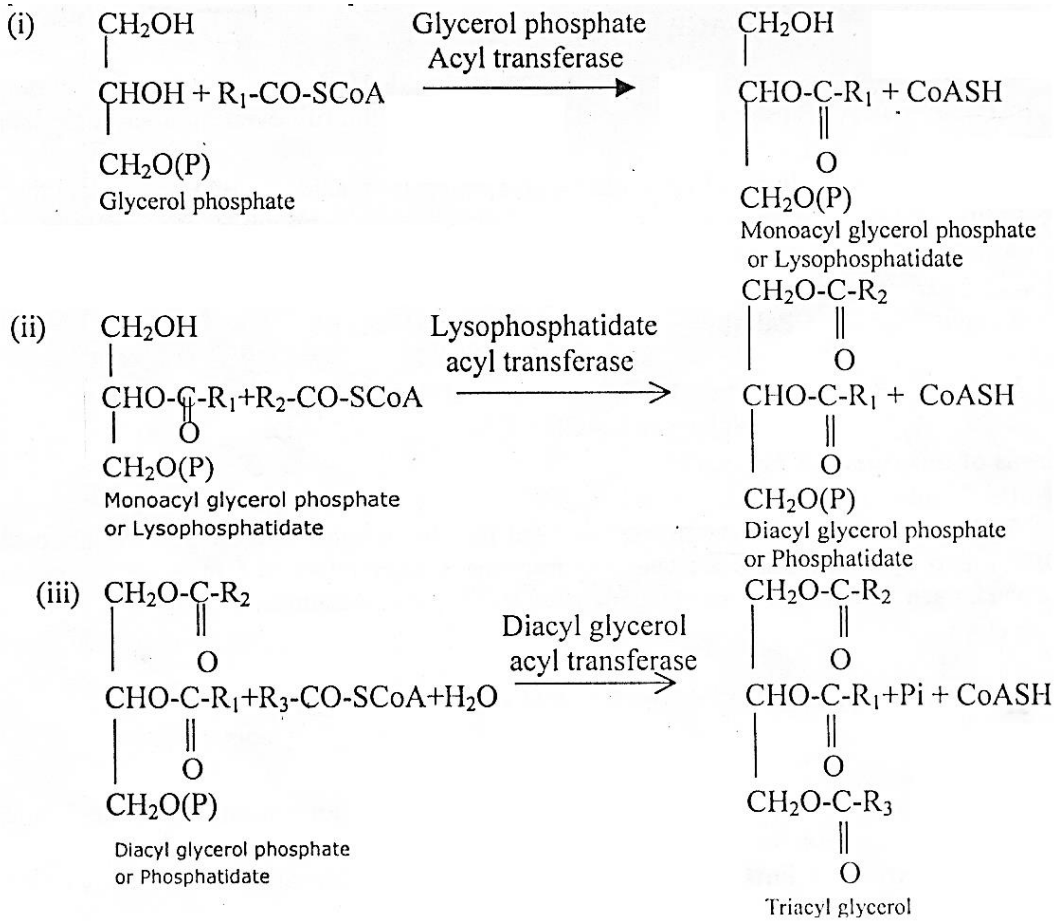
Condensation of glycerol and fatty acids does not take place in the form mentioned above. Glycerol is reactive as glycerol phosphate while fatty acids take part in the reaction as fatty acyl-CoA or fatty acyl-SACP.

Three fatty acid molecules join sequentially to a glycerol phosphate molecule to form a triacylglycerol.

The fatty acids are first activated by their conversion to respective acyl derivatives by the enzyme acyl CoA synth



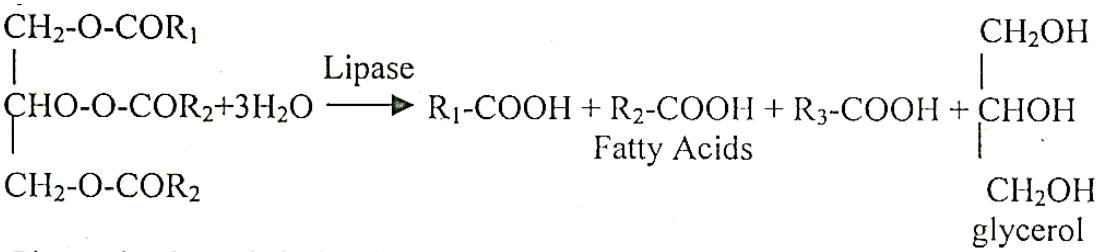
* The esterification of glycerol phosphate with this fatty acyl-CoA (RCO-SCoA) takes place in three successive steps;



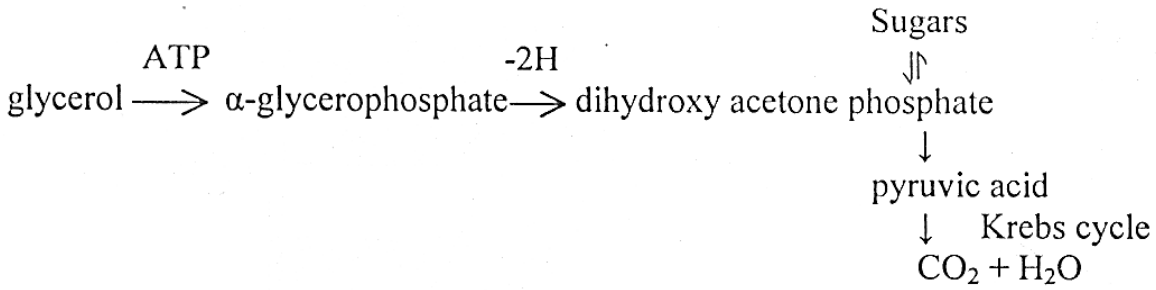
The enzymes carrying out acylations of glycerol phosphate are part of a membrane' bound t~cyl glycerol synthetase complex.

Digestion of Fats and Oxidation of Fatty Acids

Fats are hydrolysed into its constituents, glycerol and fatty acids, in the presence of enzyme lipase. It may occur in one or three steps.



Glycerol released during hydrolysis of fats can take part in both synthesis of sugars and oxidative metabolism in Krebs or TCA cycle.

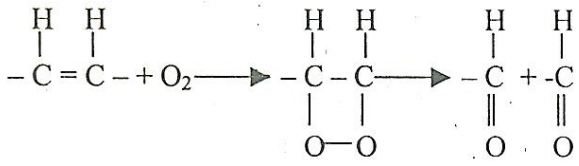


Oxidation of Fatty Acids

Fatty acids are further oxidised by three methods - α-oxidation, peroxidation and β-oxidation

1. α-OXIDATION. It was first described by rytartin and Stumpf in 1959 in the cotyledons of groundnut. In the presence of a complex peroxidase, terminal acidic group is lost and aldehyde group is generated at the a-carbon. The aldehyde is further oxidised to the acidic radical in the presence of water and NAO+ by fatty Acyl Dehydrogenase (Fig.2, P-84). α-oxidation occurs in fatty acids having more than 12-carbon atoms.

2. PEROXIDATION PATHWAY. 'This is employed in the catabolism of unsaturated fatty acids. Here molecular oxygen directly takes part in oxidation through the agency of enzyme Iipoxidase or lipoxygenase. It gives rise to hydroperoxy compounds which break further into aldehydes.



The β-oxidation Pathway

Itis a complicated but by far the most important process revealing the oxidation of fatty acids. For simplicity, it can be studied with the help of following headings.

(A) Mechanism of β-oxidatlon. It is a major process of fatty acid catabolism and is most commonly found. It consists of sequence of degradative steps, e.g. dehydration, hydration, dehydration and release of acetyl CoA. Stumpf and Barber (1965) demonstrated these steps in germinatingpeanut cotyledons. The process occurs in the following sequence:

(i) Fatty acids are first esterified by the coenzyme A in the presence of enzyme thiokinase which is found associated with microsomes. This reaction is referred to as thiokinase type of reaction. The other steps are as in figure.

(ii) *Acyl* dehydrogenase type of reaction occurs in which two hydrogen atoms are given off to reduce FAD into $FADH_2$.

(iii)Enoylhydrase type of reaction occurs and in presence of an enzyme there is addition of one molecule of water.

(iv) β-hydroxyacyl dehydrogenase type of reaction occurs and there is a further loss of hydrogen which converts NAD+ to $NADH_2$.

(v) β-oxoacyl-thiolase type of reaction occurs. This enzyme catalyses the reaction involving the productio,n of acetyl-coenzyme A and thioester of the remaining fatty acid.

This chain now re-enters combining with CoA. This time it is a short of 2 carbon atoms. Similarly this cycle removes every time two carbon units from the fatty acid and the process continues or repeated till there is complete oxidation of fatty acids. It is commonly called β-oxidation helix.

The enzymes required for fJ-oxidation are found located in the microsomes or glyoxysomes.

Acetyl-CoA produced during β-oxidation of fatty acids can be utilized in either liberation of energy in the Krebs cycle or building up of sugars and other metabolites through the glyoxylate cycle **.** All the enzymes required for the operation of glyoxylate cycle like malate synthetase, malic dehydrogenase, oxaloacetate decarboxylase, etc. have been found to be present in germinating fatty seeds. Krebs cycle does not operate during the germination of fatty seeds. ' Furthermore, Rebeiz et al (1965) could not find recognisable mitochondria in the cotyledonary tissue. Glyoxylates cycle is, therefore, a method of conversion of fats into carbohydrates during the germination of fatty seeds**.**Beevers and his eo-workers (1967-69) have found out that both β-oxidation of fattyacids and glyoxylate cycle operate in glyoxysomes.

Acetyl CoA reacts with oxaloacetic acid to form citric acid, just as in the Krebs cycle (reaction 1). After isocitric acid (6 carbons) is formed (reaction 2), it undergoes cleavage by an enzyme unique to this cycle, called isocijratelyase. Succinate (4 carbons) and glyoxylate (2 carbons) are produced (reaction 3). The glyoxylate reacts with another acetyl CoA to form malate and free coenzyme A (CoASH, reaction .4). This reaction is catalyzed by another enzyme restricted to the glyoxylate pathway called malate synthetase. This malate is transported to the cytosol where it is converted to sugars.

Succinate produced in reaction 3 moves to the mitochondria for further processing. Here it is oxidized by Krebs cycle reactions 5, 6, and 7 to oxaloacetate (OAA), releasing $NADH_2$ and $FADH_2$. Both $NADH_2$ and $FADH_2$ are oxidized by the mitochondrial electron-transport system with O2 to form H20 and ATP. Reactions 8 and 9 are transaminations between alpha-keto acids and amino acids that require exchange transport of such molecules between mitochondria and glyoxysomes. Their main function seems to be regeneration of the OAA needed to maintain reaction I of the glyoxylate pathway. Without these reactions, OAA is converted mainly to malate (reaction 10), because of the large amounts of $NADH_2$ produced in β-oxidation.

The malate produced by malate synthetase (reaction 4) is enough to account for all of the fatty-acid carbons converted to sucrose carbons (in fact, even more). This malate is first oxidized to OAA by a cytoplasmic NAD^+-malate dehydrogenase (reaction 11), then the OAA is decarboxylated and phosphorylated with ATP to yield CO_2 and phosphoenolpyruvate, PEP (reaction 12). This reaction is catalyzed by an enzyme called PEP carboxykinase. It is likely that ATP produced in the mitochondria during oxidation of $NADH_2$ and F ADH_2 somehow becomes available to drive reaction 12. Once PEP is formed it can readily undergo reverse glycolysis to form hexose phosphates. Sucrose derived from these hexose phosphates is then transported via the phloem to the growing roots and shoot, where it provides much of the carbon needed for growth of those organs.

An overall summary of the conversion of a fattyacid (palmitic) to a sugar (sucrose) is as follows:

$$C_{16}H_{32}O_2 + 11O_2 \longrightarrow C_{12}H2_2O_{11} + 4\ CO_2 + 5\ H_2O$$

Palmitic acid Sucrose

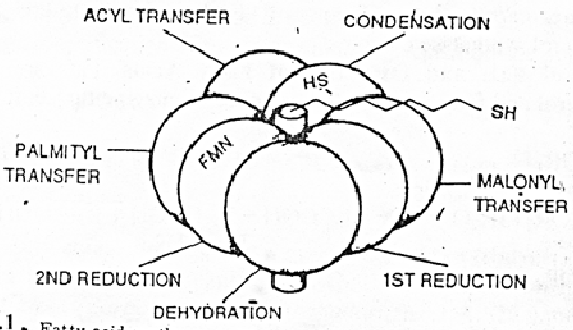


Fig. 1. Fatty acid synthase multienzyme complex. The complex is a dimer of 2 identical polypeptide monomers, 1 and 2 each consisting of 6 separate enzyme activities and the acyl carrier protein (ACP). (*Courtesy Harper, 1988*).

(both receives 2 electron)

neutral

FAD → Flavine Adenine dinucleotide

NAD^+ → Nicotinamide dinucleotide

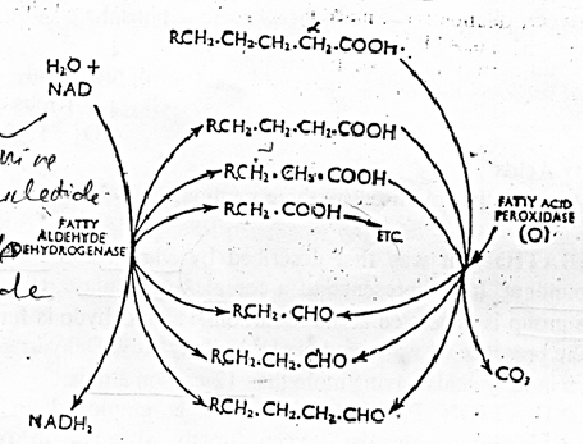
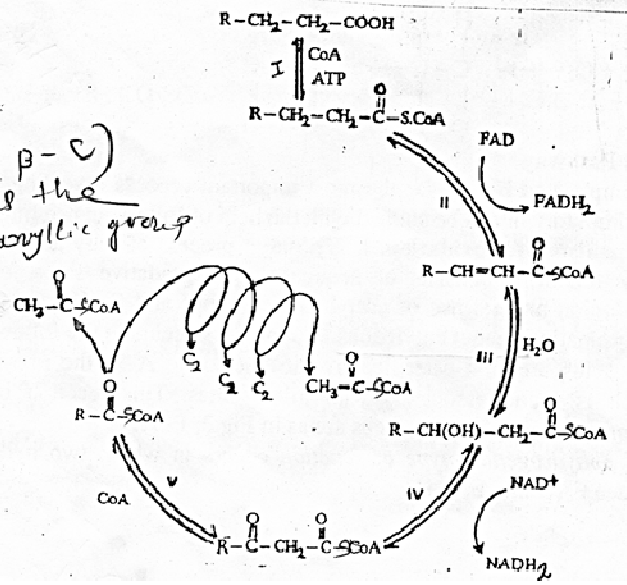


Fig. 2. α-Oxidation of fatty acids.

Why β ? (β - C)

bcz it b cleaves the C next to carboxylic group



IMPORTANCE OF LIPIDS

1. True fats and oils form an important food reserve in plants. They have the maximum caloric value per gram of the organic matter.
2. The reserve fat can transform itself into carbohydrates, thus in many seeds carbohydrates are converted into fats during maturation but the process is reversed during seed germination.
3. Seed and spore lipids are helpful in providing thermal insulation. They also prevent the loss of moisture.
4. Except for B and C all other vitamins are insoluble in water but soluble in fats. Fats act as their carrier and protect them from oxidation.
5. Several of the plant fats are edible. The edible oils or fats are not only required for the sypply of energy, they also enhance palatability of food.
6. Oils which contain unsaturated fatty acids (e.g. linseed oil having linolenic acid) are used in paint industry. On exposure to air they combine with oxygen to produce peroxides. The latter polymerise and become though and solid to form a protective layer.
7. As suberin the lipids make the cork cells impermeable.
8. Cutin and cuticle help the plant in reducing cuticular or epidermal transpiration.
9. Waxes are strongly hydrophobic. They function as protective surface coating against transpiration in land plants of arid areas.
10. Waxes protect the floating leaves of the aquatic plants against wetting.
11. Phospholipids form an important component of all cell membranes.

Some naturally occurring fatty acids

Saturated Fatty Acid:

Common name	Symbol	Systematic name	Formula	Source
Lauric	12:0	n-dodecanoic	$CH_3(CH_2)_{10}COOH$	Coconut oil, palm oil
Myristic	14:0	n-tetradecanoic	$CH_3(CH_2)_{12}COOH$	Butter, wool fat
Palmitic	16:0	n-hexadecanoic	$CH_3(CH_2)_{14}COOH$	Plant, animal fats
Stearic	18:0	n-octadecanoic	$CH_3(CH_2)_{16}COOH$	Plant, animal fasts
Arachidic	20:0	n-cicosanoic	$CH_3(CH_2)_{18}COOH$	Peanut oil
Behenic	22:0	n-docosanoic	$CH_3(CH_2)_{20}CO$	

			OH	
Lignoceric	24:0	n-tetracosanoic	$CH_3(CH_2)_{22}CO$ OH	
Cerotic acid	26:0	n-hexacosanoic	$CH_3(CH_2)_{24}CO$ OH	Bee wax, wool fat etc.

Unsaturated Fatty Acids

Common name	Un-saturation	Systematic name	Formula	Source
Monoenoic				
Palmitoleic (16:1)	9	Cis-9-hexadecenoic	$CH_3(CH_2)_5CH = CH(CH_2)_7COOH$	Sardine oil
Olelc (18:1)	9	Cis-9-octadecenoic	$CH_3(CH_2)_5CH =CH(CH_2)_7COOH$	Olilve oil
Gadoleic (20:1)	9	Cis-9-eicosenoic	$CH_3(CH_2)_9CH =CH(CH_2)_7COOH$	
Erucic (22:1)	13	Cis-13-docosenoic	$CH_3(CH_2)_7CH =CH(CH_2)_{11}COOH$	Rapeseed oil
Dicnoic				
Linoleic (18:2)	9,12	Cis-cis-9, 12-octadecadienoic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7$ COOH	Cotton seed, soyabean oil
Trienoic				
Linolenic (18:3)	9,12,15	All cis-9, 12, 15 octadecatrienoic	$CH_3CH_2CH=$ $CHCH_2$ $CH=CHCH_2$ $CH=CH$ $(CH_2)_7$ COOH	Linseed oil
Linolenic (18:3)	6,9,12	All cis-6, 9, 12 octadecatrienoic	$CH_3(CH_2)_4CH=$ $CHCH_2$ $CH=CHCH_2$ $CH=CH$ $(CH_2)_4$ COOH	
Eleostearic (18:3)	9,11,13	All cis-9, 11, 13 octadecatrienoic	$CH_3(CH_2)_3CH=$ $CHCH$ $=CHCH$ $CH(CH_2)_7$ COOH	Tung oil
Tetraenoic				
Arachidonic (20:4)	5,8,11,14	All eis-5, 8, 11, 14,Eicosttetraenoic	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2$ $CH=CHCH_2CH=CH(CH_2)_3COOH$	Animal fats adrenal phosphatides
Hydroxy Fatty Acids				
Ricinoleic (18: 1)	9 mono-OH	12-hydroxy, cis-o-octadecanoic	$CH_3(CH_2)_5CH(OH)CH_2$ $CH=CH$ $(CH_2)_7COOH$	Castor oil

Dihyydroxy Stearic (18:0)	-OH	9, 10 dihydroxy-octadecanoic	$CH_3(CH_2)_7CH(OH)$ $CH(OH)$ $(CH_2)_7COOH$	Castor oil
Cyclic Fatty Acids				
Malvalic (17:1)	CH_2	eis-8, 9-methy Leneheptadecen oic	$CH_3(CH_2)_7C=C(CH_2)_6$ COOH CH_2	

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Carbohydrate Metabolism

One of the main attributes of living matter is its ability to extract, transform and use energy from the environment for maintenance, growth and development. This is achieved by a constant breakdown, turnover and resynthesis of the macromolecular components of living organisms. During digestion, the complex mixture of carbohydrates, fats and proteins are broken down into a simpler form glucose, amino acids, fatty acids and glycerol which can be absorbed by the organism to maintain its orderly structure at the expense of energy derived from these nutrients. This principle is manifested in the process of metabolism.

The sum total of all the enzymatic reactions occurring in the cell is collectively called metabolism (Greek –'metabolos' means changeable). The reaction sequences occurring within organisms in an orderly and regulated way are known as metabolic pathways and the compounds formed as a result of metabolism are called metabolites. Because metabolism proceeds in a stepwise manner through many intermediary metabolites, the term intermediary metabolism is used to denote the chemical pathways of metabolism.

Functions of metabolism

Cellular metabolism has four specific functions

To obtain chemical energy, generally as ATP through the degradation of energy-rich nutrient biomolecules from the environment or through capture of solar energy.

To transform nutrient biomolecules into the building-blocks or precursors needed for the synthesis of macromolecular cell components.

To assemble such building-blocks into proteins, nucleic acids, lipids and other cell components and

To form and degrade biomolecules required in specialized functions of cells.

Metabolism is broadly divided into two categories.

Catabolism

It refers to all the degradative reactions involving degrading or breakdown of various complex molecules to smaller and simple molecules. The chemical energy of metabolites is conserved in the form of ATP.

Anabolism

It referes to all the synthetic reactions involving synthesis of various molecules from smaller and simpler precursor molecules. This process requires the input of chemical energy in the form of ATP.

In living systems both catabolism and anabolism occur concurrently and simultaneously. The energy released during catabolism is required for anabolic reactions and for many other cellular activities like muscular contraction, active transport, osmotic works, catabolic reactions are oxidative whereas anabolic reactions are reductive in nature.

The pathway of reactions that is accessible to both catabolism and anabolism is called amphibolic pathway (Greek- 'amphi' means 'both') which can be used catabolically to bring about completion of the degradation of small molecules derived from catabolism or can be used anabolically to furnish small molecules as precursors in biosynthetic reactions.

Stages of metabolism

Catabolic and anabolic reactions take place in three major stages. In stage I of catabolism, large nutrient molecules are degraded to their major building-blocks. Thus, polysaccharide are degraded to yield hexoses or pentoses; lipids to yield fatty acids, glycerol and other components; and proteins to yield their component amino acids. In stage II of catabolism, the many different products of stage I are collected and converted into smaller number of still simpler intermediates. Thus, the hexoses, pentoses and glycerol aredegraded via the three-carbon intermediate pyruvic acid to yield a single two-carbon species, the acetyl group of acetyl-CoA. Similarly, the various fatty acids and amino acids are broken down to form acetyl-CoA and few other products. Finally, the acetyl groups of acetyl-CoA as well as other products of stage II, are channeled into stage III, the final common catabolic pathway, in which they are ultimately oxidized into carbon dioxide and water.

During anabolism, the intermediates formed in stage III are converted into building-block molecules of stage II which are finally assembled into macromolecules of stage I.

The pathways of catabolism differ from the pathways of anabolism. Whereas, catabolic pathways are convergent with many nutritional macromolecules being converted to common end products biosynthetic pathways are divergent, with a few precursors being converted to many different products.

Glycolysis

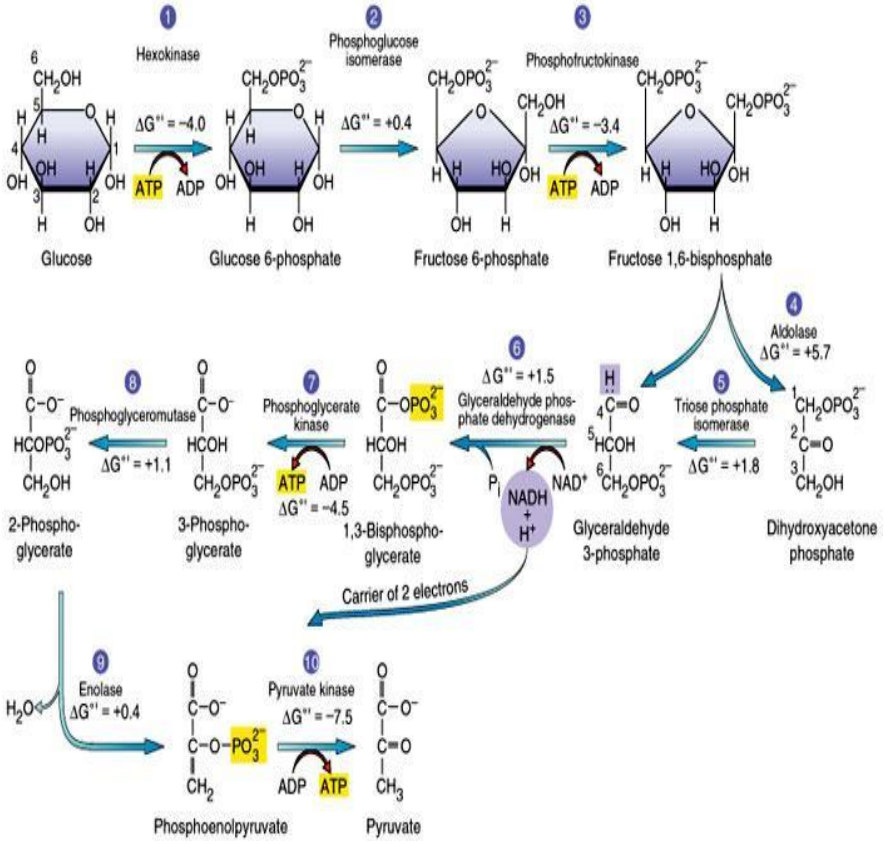
The term 'glycolysis'is derived from the Greek words 'glycos', sugar (sweet) and 'lysis', dissolution. The sequence of reactions by which glucose is degraded anaerobically to pyruvate with the concomitant production of ATP is called glycolysis.

Glycolysis is one of the several catabolic pathways known generally as anaerobic fermentations, by which many organisms extract chemical energy from various organic fuels in the absence of molecular oxygen. It is the most ancient type of biological mechanism for obtaining energy from nutrient molecules.

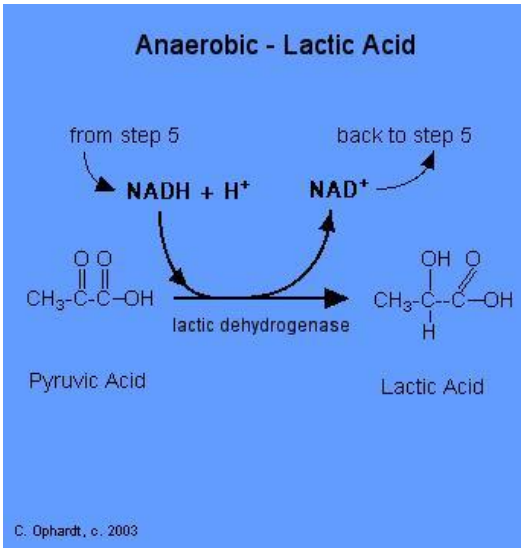
Moreover, in most animals glycolysis serves as an important emergency mechanism capable of yielding energy for short periods when oxygen is not available. Most higher animals have retained the capacity for anaerobic degradation of glucose to pyruvate and then to lactate as a preparatory pathway for aerobic metabolism of glucose, which harvests most of the energy contained in glucose.

Overall view of glycolysis

The sequence of reactions from glucose to pyruvate is often called as Embden-Meyerhof-Parnas (EMP) pathway.



Under anaerobic glycolysis, pyruvic acid is converted to lactic acid as follows:



In a cell, the molecule which accepts energy from the breakdown of fuel molecules and donates this energy to cell functions is ATP. ATP is synthesized from ADP and inorganic phosphate and 7 kcal/mole must be available to achieve this reaction. This energy is obtained from the catabolism of carbohydrates, fats and proteins.

There are two mechanisms by which energy is coupled to the formation of ATP.

1) Substrate level phosphorylation

2) Oxidative phosphorylation.

Substrate level phosphorylation occurs when one of the phosphate groups in a metabolic intermediate is transferred to ADP to form ATP. In contrast oxidative phosphorylation couples inorganic phosphate to ADP directly. In the breakdown of glucose, many of the chemical reactions involve the removal of hydrogen atoms from various intermediates. These reactions require a coenzyme, to which the hydrogen atoms are transferred. As a result of such hydrogen transfers, some of the chemical energy in glucose molecule is transferred to the coenzyme molecule. The process of oxidative phosphorylation then uses this energy to synthesize ATP.

The energy release in glycolysis is as follows:	
Aerobic glycolysis :	
Through substrate level phosphorylation	4 ATP
Through oxidative phosphorylation	6 ATP
Total	10 ATP
Input	2 ATP
Net energy yield	**8 ATP**
Anaerobic glycolysis : =	4 ATP
Through substrate level phosphorylation	
Input	2 ATP
Net energy yield	**2 ATP**

The end product of glycolysis, pyruvic acid is converted to acetyl CoA.

Importance and regulation of glycolysis

Glycolysis is the central pathway of glucose metabolism in most organisms, including microorganisms, plants and humans. It is only the first stage in the overall degradation of glucose to CO_2 and H_2O. Under anacerobic conditions, one glucose molecule is degraded into two pyruvate molecules releasing two ATP molecules. Under aerobic conditions, pyruvate can be oxidized to CO_2 and H_2O with a release of large quantity of free energy that is also captured in ATP molecules. Hexoses, other than glucose, such as fructose and galactose, are enzymatically converted into intermediates of the glycolytic pathway as are certain pentoses. An ancillary function of glycolysis is the production of 2,3 – diphospho-D-glycertate, an allosteric inhibitor of hemoglobin.

Regulation of glycolysis occurs primarily by inhibition of the allosteric enzymes hexokinase, phosphofructokinase and pyruvate kinase, each of which catalyzes a thermodynamically inrreversiable reaction of glycolysis.

Alcoholic fermentation

In organisms like brewer's yeasts, which ferment glucose to ethanol and CO_2 rather than to lactic acid, the fermentation pathway is identical to that described for glycolysis except for the terminal step catalyzed by lactate dehydrogenase, which is replaced by two other enzymatic steps.

In the first step, pyruvate is decarboxylated to acetaldehyde and CO_2 by the enzyme pyruvate decarboxylase which is absent in animal tissues:

In the final step of alcoholic fermentation, acetaldehyde is reduced to ethanol regenerating NAD^+ from NADH formed during anaerobic glycolysis in the presence of alcohol dehydrogenase.

TCA cycle

The TCA cycle also known as citric acid cycle is a cyclic sequence of reactions of almost universal occurrence in aerobic organisms. It is the process in which acetate (in the form of acetyl -CoA) is oxidized completely to CO_2 and H_2O. The electrons removed from the substrates as they are oxidized are transferred through a series of electron carrying proteins to molecular oxygen via electron transport chain to form water and large quantity of chemical energy in the form of ATP is produced in the process known as oxidative phosphorylation. All these processes take place within the same organelle, the mitochondrion in eukaryotic organisms unlike the glycolytic reactions which occur in the cytosol of the cell.

H.A. Krebs, in 1937 postulated the citric acid cycle involving cycle of reactions which accounted for the oxidation of pyruvic acid to CO_2 and H_2O. Hence, the TCA cycle is also known as Krebs cycle in his honour.

Reactions of the TCA cycle

The enzymatic reactions of the TCA cycle take place within the inner mitochondrial compartment, whereas others are attached to the inner mitochondrial membrane. Some of the enzymes also occur in the cytosol of some tissues.

The oxidation of pyruvate to acetyl-CoA

Pyruvate which is formed by anaerobic breakdown of glucose via glycolytic sequence undergoes oxidative decarboxylation to yield a acetyl-CoA catalyzed by pyruvate dehydrogenase complex (PDC) which is localized in the mitochondrial membrane of higher organisms.

This reaction itself is not part of the TCA cycle but is obligatory for the entry of all carbohydrates (via pyruvate) to the TCA cycle.

The pyruvate dehydrogenase complex is a multienzyme complex composed of three enyzmes. a) Pyruvate dehydrogenase b) dihydrolipoyl transacetylase and c) dihydrolipoyl dehydrogenase. In the initial reaction, pyruvic acid is decarboxylated to form CO_2 and a acetyl complex of TPP that is bound to pyruvate dehydrogenase. The two carbon acetyl group is next transferred to an oxidized lipoic acid moity that is covalently bound to the enzyme, dihydrolipoyl transacetylase. In the third reaction, the acetyl group is transferred to coenzyme A to form acetyl –CoA, which dissociates from the enzyme in a free form. The reduced lipoic acid moiety of the dihydrolipoyl dehydrogenase is then reoxidiaed to the cyclic lipoyl form by

the third enzyme of the complex, dihydrolipoyl de hydrogenase, a flavoprotein that contains FAD. Finally, the reduced flavin co-enzyme is reoxidized by NAD^+ and NADH is produced. The reactions involved in TCA cycle

Energetics of the TCA cycle

The overall reaction of the TCA cycle may be written as Acetyl CoA + $3NAD^+$ + FAD + GDP + P_i + $2H_2O$ - $2CO_2$ + NADH +$FADH_2$ + GTP + $2H^+$ + CoAS

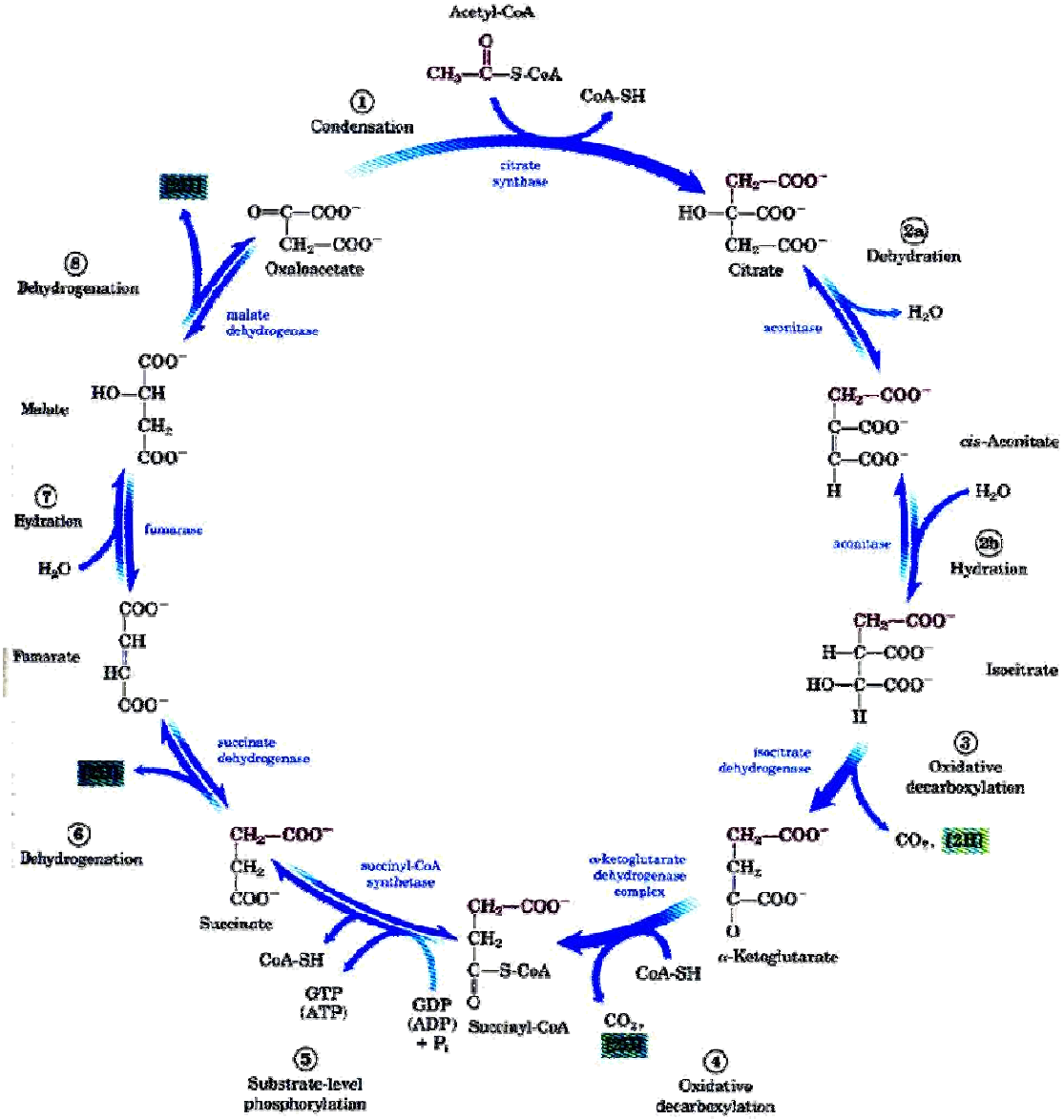


Fig: TCA cycle

Regualtion of the TCA cycle.

The flow of pyruvate in to the TCA cycle is regulated through the activities of the enzymes as summarized below.

Production of energy during the cellular oxidation of glucose .

Reaction step	ATP used (-) or formed (+) per glucose molecule		Comments
	Anaerobic	Aerobic	
Glycolysis			
Glucose glucose-6-phosphate	-1	-1	
Fructose-6-phosphate	-1	-1	
fructose-1, 6-diphosphate			
(2) Glyceraldehyde-3-phosphate		+6	2NADH = 6ATP (Oxidative phosphorylation)
(2)I, 3-diphosphoglycerate			
(2)I, 3-Diphosphoglycerate	+2	+2	
(2) 3-Phosphoglycerate			
(2) Phosphoenolpyruvate			
(2) Pyruvate	+2	+2	
Pyruvateacetly-CoA		+6	2NADH=6ATP
TCA cycle			
Isocitrate α-ketoglutarate		+6	2NADH =6ATP
α-ketoglutarate succinyl-CoA		+6	2NADH =6ATP
Succinyl-CoASuccinate		+2	2GTP=2ATP
Succinatefumarate		+4	$2FADH_2$ = 4ATP
MalateOxaloacetate		+6	2NADH = 6ATP
Total Net →	+2	+38	(all by oxidative phosphorylation) except succinyl CoA to succinate step

1. The activity of the pyruvate dehydrogenase complex which furnished a major portion of the acetyl-CoA input into the cycle is diminished by the ATP dependent phosphorylation of the dehydrogenase component and is activated by dephosphorylation of the phosphoenzyme.
2. The concentration of oxalocetate and its condensation with acetyl-CoA to yield citrate catalyzed by citrate synthetase which is regulated by the negative modulators succinyl-CoA, NADH and fatty acyl-CoA.
3. The activity of NAD^+ - linked isocitrate dehydrogenase is regulated by ADP, the positive modulator.
4. The activity of œ-ketoglutarate dehydrogenase is regulated by succinyl-CoA (product inhibition) and
5. The activity of succinate dehydrogenase is promoted by high concentrations of succinate, phosphate and ATP and is inhibited by oxaloacetate.

The TCA cycle is also regulated by the concentrations of its various intermediates because some of the reactions also function in bio-synthesis of other compounds as summarized below:

b) α-ketoglutarate and oxaloacetate are enzymatically transminated into glutamate and aspartate amino acids, respectively.

α-Ketoglutarate + alanine → glutamate + pyruvate Oxalocetate + alanine → aspartate + pyruvate

c) Citrate serves as a precursor of extra mitochondrial acetyl-CoA for fatty acid biosynthesis through the ATP-citrate lyase reaction.

Citrate + ATP + CoA → acetyl-CoA + oxaloacetate + ADP + $þ_i$

d) Sccinyl-CoA can be removed from the cycle for heme biosynthesis.

e) In plants and some microorganisms, a modification of the TCA cycle (the glyoxalate cycle) allows the sysnthesis of citric acid cycle intermediates from acetyl-CoA.

In order to maintain the flow of acetyl-CoA into the cycle, intermediates must be replenished by special enzymatic reactions called anaplerotic (‘filling-up’) reactions. This occurs through two specialized enzymatic reactions catalyzed by pyruvate carboxylase and phosphoenolpyruvate carboxykinase. The former transforms pyruvate to oxalocetate and the latter transforms phosphoenolpyruvate to oxaloacetate.

The TCA cycle is the final common pathway for the oxidation of the major fuel molecules of the cell. The oxidation products of carbohydrates, fatty acids and amino acids converge in this pathway. Thus, it plays a central role in metabolism

Biological oxidation electron transport chain and oxidative phosphorylation

Biological oxidation

Oxidation may be defined as the addition of oxygen ($2\ Mg+O_2 – 2\ MgO$), the loss of hydrogen ($CH_3CHO + +H_2O – CH_3COOH + 2H^+$) or by the most general definition: the loss of electrons. Thus, in the first two examples, a magnesium atom also loses two electrons to oxygen, and a carbon atom also loses two electrons to oxygen in the formation of magnesium oxide and acetic acid, respectively.

Electron Transport chain (ETC)

In electron transport chain or respectively chain, the oxygen-dependent oxidation reduction reactions involve intermediate electron carriers intervening in the flow of electrons between reduced metabolite and the electron acceptor, the oxygen. Each intermediate electron carrier would first participate in its oxidized state as an acceptor of electrons and then be converted to its reduced state. In the reduced state the carrier, as a donor, would then transfer electrons to the next carrier, as a donor, would then transfer electrons to the next carrier in its oxidized state and in doing so, it would be reconverted back to original oxidized state. The final carrier would transfer electrons to molecular oxygen, the terminal electron acceptor in respiration which would be reduced to water.

Components of the electron transport chain

Oxidation-reduction enzymes generally are more complex in structure and mechanism. Most of the members of the electron-transport chain are embedded in the mitochondrial inner membrane and exceedingly difficult to extract in soluble form and purify.

There are five different kinds of electron carriers that participate in the transport of electrons from reduced substrates as they are oxidized in mitochondria. These are (a) pyridine nucleotide, $NAD^{+;}$ (b) flavin nucleotides, FMN or FAD, (c) coenzyme Q or ubiquinone, (d) a family of cytochrome (cyt. b, c, c_1, a, a_3) and (e) non-heme iron-sulfur proteins. These components are discussed below.

Nicotinamide adenine dinucleotide (NAD^+)

The NAD^+ is the initial electron acceptor in the oxidation of several substrates like isocitrate, malate, α–ketoglutarate, pyruvate and glutamate among others.

In these reactions, only the nicotinamiide moiety of NAD^+ is reversibly oxidized and reduced. Out of two electrons and two protons removed from substrates two electrons and one proton combine with NAD^+ and the second proton is released in the medium. The enzymatic reduction of pyridine is ring of NAD^+ or $NADP^+$ is accompanied by a spectral change and is stereospecific.

Flavoproteins

The prosthetic groups FMN and FAD (Fig.) are tightly bound to their dehydrogenases (flavoproteins). The active protion of FMN or FAD that participates in the oxidoreduction is the isolloxazine ring of the riboflavin moiety, which is reversibly reduced.

These flavoproteins accept two electrons and a proton from NADH or two electrons and two protons from succinate. The transfer of electrons from NADH of flavin coenzyme.

Ubiquinone (coenzyme Q)

It is a lipid-soluble electron-carrying coenzyme also participates in the transport of electrons from organic substrates to oxygen in the respiratory chain of mitochondria. This coenzyme is a reversibly reducible quinone with a long isoprenoid side chain. It occurs in animals, plants and microbes. Several ubiquinons are known differing only in the length of isoprenoid side chain which has 6 isoprene units in some microbes and 10 in the mitochondria of animal tissues. In plant tissues the closely related plastoquinones perform similar functions in photosynthetic electron transport. The oxidized and reduced forms of unbiquinone are shown in

Cytochromes

The cytochormes are electron-transferring heme-proteins containing iron-porphyrin as prosphetic groups and are found only in aerobic cells. All cytochromes undergo reversible Fe (II) – Fe (III) valence changes during their catalytic cycles. Their reduced forms cannot be oxidized by molecular oxygen, with the exception of the terminal cytochrome of mitochondrial respiration, namely, cytochrome a, or cytochrome c oxidase which also contains tightly bound copper. There are at least five different types of

cytochromes (b, c, c_1, a and a_3) in the mitochondria of mammals.

The porphyrins are considered as derivatives of parent tetrapyrrole compound, porphyrin. The porphyrins are named and classified on the basis of their side-chain substituents. Of these protoporphyrin IX, generally known as heme (Fig 8.4) is the prosphetic group of cytochromes b, c and c_1. The protoporphyrin IX forms quadridenate chelate complex with Fe (II).

Cytochromes a and a_3, together called cytochrome c oxidase, the respiratory enzyme contain different iron-porphyrin prosthetic group called heme A (Fig 8.4). The cytochromes are distinguished from one another by their characteristics absorption spectra and variation in redox potentials. The function of cytochromes is to participate in electron transport reactions with the iron atom of the heme group undergoing a reversible oxidation-reduction.

Non-heme iron-sulfur proteins

This type of protein was encountered as ferredoxin in plants, in nitrogen fixation and photosynthesis before it was recognized to function in mitochondrial electron transport. Most of the iron present in many mitochondria is complexed to protein other than cytochromes and these are called non-heme iron proteins. The iron-sulfur proteins contain iron and acid-labile sulfur in equimolar amount (Fig 8.5). The iron atoms, usually two or more, are arranged in an iron-sulfide bridge that in turn is bonded to cysteine residues in protein. All the Fe S-proteins are characterized by low E_o'-values indicating a role as electron carries. In the oxidized state, both iron atoms in the model are in Fe^{3+} state. When reduced, one iron becomes Fe^{2+}.

The pathway of electron transport

The sequence of electron-transfer reactions in the respiratory chain from NADH to oxygen is now fairly well established NADH is the form in which electrons are collected from any different substrates through the action of NAD^+-linked dehydrogenases. These electrons funnel into the chain via the flavoprotein in NADH dehydrogenase. On the other hand, other respiratory substrates are dehydrogenated by flavin-linked dehydrogenases, such as succinate dehydrogenase and acyl-CoA dehydrogenase which funnel electrons into the chain via ubiquinone. NAD+ and ubiquinone thus serve to collect reducing equivalents from respiratory substrates oxidized by pyridine-linked and flavin-linked dehydrogenases. From ubiquinone, electrons are transferred through a specific order of the cytochrome carriers b, c_1, c and aa_3 and ultimately delivered to O_2, the terminal electron acceptor.

The points of entry of electrons from various substrates and the sites of inhibition of electron transport by rotenone, amytal and cyanide . The symbols FP designats flavoprotein; FP_1 – NADH dehydrogenase; Fe-S – iron sulfur centers (their positions in chain are still uncertain); Q-ubiquinone (coenzyme Q).

The sequence of electron carriers is supported by many types of evidences. However, only two important evidences will be mentioned. First, the sequence is consistent with the standard redox potentials of the different electron carriers, which become more positive as electrons move from substrate to O_2. Second, the intact mitochondria could be very carefully disrupted to sub-mitochondrial fragments which are still capable of complete electron transport. Further, disruption of these fragments yields four separate respiratory complexes (I, II, III IV), each of which is capable of catalyzing a portion of the complexes *in vitro* indicate that complexes, I, III and IV will transfer electrons from NAD^+ to O_2 while complexes II, III and IV are required for the oxidation of succinate.

Inhibitors of electron transport

Inhibitors that block specific carriers in the electron-transport chain have yielded valuable information on the sequence of electron carriers in the respiratory chain. Three inhibitors have been found to block electron transport – rotenone and amytal act between NADH and ubiquinone of the chain and are believed to act on NADH dehydrogenase; and antibiotic antimycin A blocks the chain between cytochromes b to c and the third inhibitor cyanide blocks the chain from cytochrome aa_3 to oxygen, inhibiting cytochrome c oxidase enzyme

Oxidative phosphorylation

As mentioned earlier, electron transport from NADH to oxygen proceeds with a very large decrease in free energy, which is the part conserved by the coupled phosphorylation of ADP to ATP in a process known as oxidative phosphorylation. While, the oxidative phosphorylation is associated with electron transport, the substrate level phosphorylation involves synthesis of ATP from ADP and energy rich compounds such as 1,3-diphosphoglycerate and phosphoenolpyruvate in glycolysis.

Oxidative phosphorylation is fundamental to all aspects of cellular life in aerobic organisms since it is the principal route of ATP synthesis Mitochondria, present in all aerobic eukaryotic cells are the sites of oxidative phosphorylation. The enzymes of electron transport and oxidative phosphorylation are located in inner membrane of

mitochondria.

Two striking characteristics of the electron transport process relevant to the mechanism of energy conservation during electron transport are a large number of sequential electron-transferring steps resulting in stepwise release of energy and the H^+ ions are absorbed and released at some of these steps suggesting that proton exchanges are involved in energy conservation.

Coupling of oxidative phosphorylation to electron transport

The ATP formation from ADP is coupled to the electron transport chain. Three molecules of ATP are synthesized at three separate points in the chain when electrons flow from NADH to oxygen in the chain. These sites have been established by measurement of þ/O ratios with different substrates in the presence and absence of respiratory inhibitors. The þ/O ration corresponds to the number of molecules of inorganic phosphate ($þ_i$) used up per gram atom of oxygen consumed. The þ/O ration is 3.0 for NAD^+ linked electron transport and 2.0 for flavin-linked electron transport.

The three sites of ATP synthesis in the respiratory chain are a site I, between NAD^+ and ubiquinone, (b) site II, between cytochromes b and c, and (c) site III, between cytochrome a and oxygen.

Uncoupling and inhibition of oxidative phosphorylation

Oxidative phosphorylation is influenced by a number of chemical agents, which can be grouped into three major classes; (a) the uncoupling agents which allow electron transport to continue but prevent the phosphorylation of ADP to ATP, e.g. 2-4-dinitrophenol (b) agents which inhibit both electron transport and phsohprylation e.g., oligomycin, and (c) inophores which inhibit oxidative phosphorylation by utilizing the energy of electron transport for the cation transport as they complex with K^+, Na^+ or other cations e.g., gramicidin, valinomycin, etc. (antibiotics).

Mechanism of oxidative phosphorylation

Three major hypotheses have been proposed to explain the coupling of phosphorylation to electron transport : (a) the chemical-coupling hypothesis which postulates formation of a high-energy covalently bonded intermediate, (b) the conformational-coupling hypothesis which postulates an intermediate high-energy conformational state, and (c) the chemiosmotic-coupling hypothesis which is most consistent, postulates that an electrochemical gradient of H^+ ion across the inner membrance of mitochondria is the coupling vehicle.

Agents that interfere with Oxidative phosphorylation

Types of interference	Compound	Target
Inhibition of electron transfer	Cyanide, Carbon monoxide	Inhibit Cytochrome oxidase
	Antimycin	Block electron transfer from cytochrome b to cytochrome C1
	Myxothiazol, Rotenone, Amytal, Peiricidin A	Prevent electron transfer from Fe-S centre to ubiquinone
Inhibition of ATP synthase	Aurovertin	Inhibit F1
	Oligomycin, Ventrucidin	Inhibit F_0 and CF_0
	DCMU - 3- (3,4-dichlorophenyl) -1,1 dimethylurea	Block proton flow through Fo and CFo
Uncoupling of photophosphorylation from electron transfer	FCCP- Cyanide-p-trifluromethoxyphenylhydrazone, DNP- 2,4 dinitrophenol	Hydrophobic proton carriers
	Valinomycin	K+ ionophore
	Thermogenin	In brown fat, form proton conducting pores in inner mitochondrial membrane
Inhibition of ATP – ADP exchange	Atractyloside	Inhibit adenine nucleotide translocase.

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Amino acid degradation

Amino acids are important as they are the precursors of proteins. In plants these are synthesized from organic acids (produced during glycolysis & Kreb's cycle) & inorganic nitrogen (NH_3). The inorganic N enters into plants either in the form of nitrate or ammonium. The nitrate is first reduced to ammonium & then incorporated into aminoacids. Leguminous plants possess Symbiotic N fining bacteria in there root nodules and they use atmospheric N to synthesize aminoacids. The micro-organism differs in their capacity to synthesize amino acids.

All amino acids contain at least one nitrogen atom, which forms their α-amino group. Some amino acids contain additional nitrogen atoms in their side chains. Nitrogen has no use in energy metabolism and has to be eliminated. There are three key processes in metabolic nitrogen elimination:

1. Transamination: Transamination removes the α-amino group from one amino acid and transfers it to α-ketoglutarate. This leads to the accumulation of glutamate.
2. Deamination: Deamination is of two types namely Oxidative (using, NAD + & FAD^+) and Non-oxidative
3. Ammonia Assimilation: Enzymes catalysing the process are Glutamate Dehydrogenase, Glutamine synthatase and Carbomoy - Phosphate Synthetas-I.

Transamination

The transfer of an amino ($-CH_2$) group from on aminoacids to a keto acid is known as transamination. About 17 AAs are synthesized from glutamic acid through transmination.

The C-skeleton of the aminoacids is first converted to keto acids (by transamination) which meet one or more of the following fates.

(i) Utilized to generate energy

(ii) Used for the synthesis of glucose

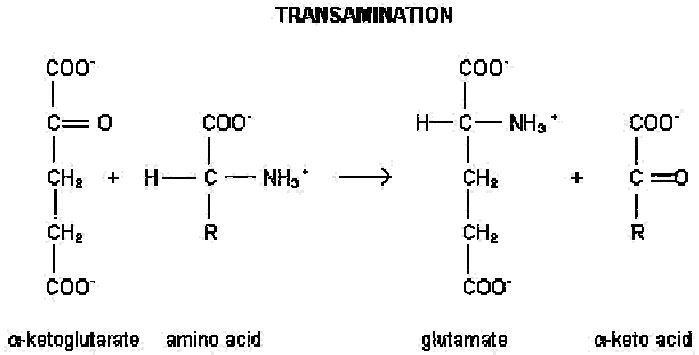
(iii) Diverted for the formations of fat or ketone bodies.

(iv) Involved in the production of non-essential amino acids.

This process involves the inter-conversion of a pair of aminoacids and a pair of keto acids, catalyzed by a group of enzymes called transaminases. During the synthesis of various AAs, first glutamic acid is produced by reductive amination & the other aminoacids are synthesized by the transfer of its amino group to various keto acids.

- All transaminases require pyridoual phosphate (PLP), a coenzyme derived from vitamin B_6.

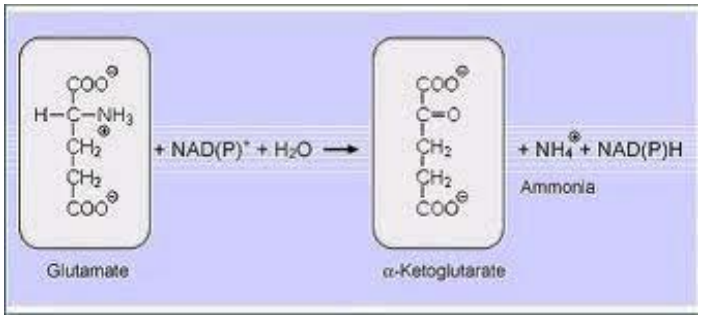
- It picks up amino group from donor AAs & converted into pyridoxamine phosphate.
- There is no free NH_3 liberated; only the transfer of amino group occurs.
- Transamination is reversible.
- It involves both catabolism (degradation) & anabolism (synthesis) of aminoacids.
- Transamination diverts the excess aminoacids towards energy generation.
- The AAs undergo transamination to finally concentrate nitrogen in glutamate. Glutamate is the only AA that undergoes oxidative deamination to a significant extent to fibrated $NH_3$for urea synthesis.
- All AAs except lysine, threonine, proline & hydroxyproline participate in transamination.



Deamination

- The removal of amino group from the aminoacids as NH_3 is deamination.
- It results on the liberation of ammonia for urea synthesis.
- Deamination may be either oxidative or non-oxidative.

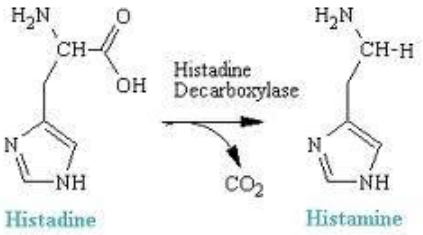
Oxidative Deamination: Amino group from amino acid is oxidatively removed in this process. Glutamate dehyrogenase enzyme catalyses the following reaction.



Non oxidative deamination: - Non oxidative deamination takes place in absence of water and the corresponding unsaturated dicarboxylic acid is formed

Aspartic acid + H_2 _____ Fumaric acid + NH_3

Decarboxylation:- In decarboxylation reactions, the CO_2 from the corresponding amino acids are removed and biologically active amines are formed.



Ammonia assimilation:

The ammonia released during metabolism of amino acids is toxic to the cells if it is not incorporated into synthetic processes. There are three reactions that can catalyze the incorporation of the nitrogen atom as NH3 into organic compounds.

These reactions are those catalysed by glutamic dehydrogenase, glutamine synthetase and carbamyl phosphate synthetase.

Carbomyl phosphate Synthetase

CO_2 + 2ATP ---------- Carbomyl phosphate + 2ADP + H_3PO_4

$$NH_3 + \text{glutamate} + ATP \xrightarrow[Mg^{2+}]{GS} \text{glutamine} + ADP + P_i \quad (1)$$

$$\text{glutamine} + \alpha\text{-ketoglutarate} + NADPH \xrightarrow{GOGAT} 2\,\text{glutamate} + NADP^+ \quad (2)$$

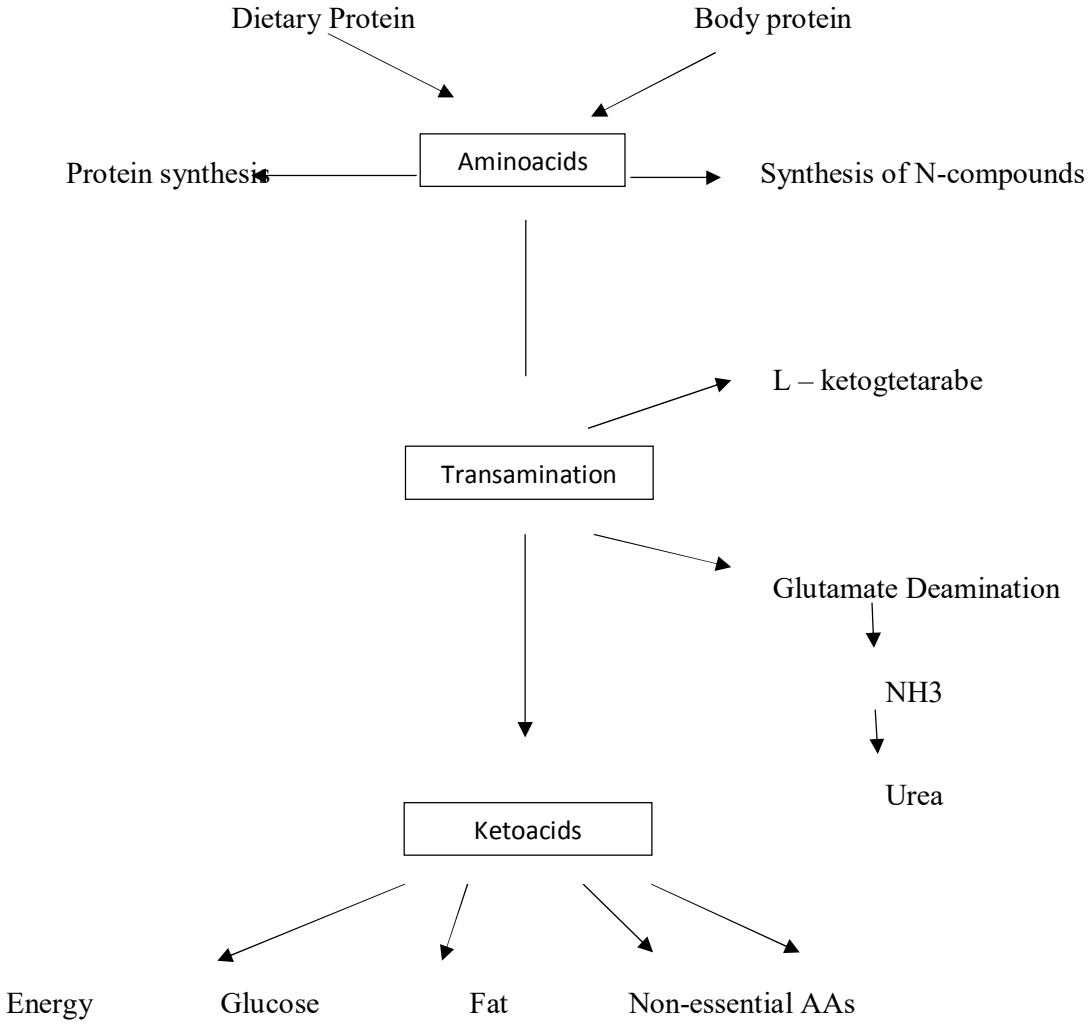
$$NH_3 + \alpha\text{-ketoglutarate} + NADPH \xrightarrow{GDH} \text{glutamate} + NADP^+ \quad (3)$$

After removal of amino groups, the carbon skeleton of amino acids is converted to intermediates of TCA cycle or their precursors.

- The C skeleton finally has one or more of the following fates :
  - Synthesis of glucose
  - Formation of lipids-fatty acids & ketone bodies.
  - Synthesis of non-essential aminoacids.

The C skeletons of the 20 standard amino acids (or the amino acids of proteins) are degraded to α-ketoglutarate, Sucoinyl COA, fumarate oxaloacetate, acetyl CoA & acetoacetate.

An overview of amino acid degradation



Classification of AAs based on fate of C-skeleton

Glycogenic (glycogenic)	Glycogenic & Ketogenic	Ketogenic
Alanine	Phenylalanine*	Leucine*
Arginine*	Isoleucine*	Lysine*
Aspartate	Tyrosine	
Cysteine	Tryptophan*	
Glutamine		
Glutamate		
Glycine		
Histidine		
Hydroxyproline		
Methionine*		
Proline		
Serone		
Threonine*		
Valine*		

***Essential AAs**

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Biosynthesis of Nucleic Acid

Transcription

Transcription is the process by which DNA is copied (*transcribed*) to mRNA, which carries the information needed for protein synthesis. Transcription takes place in two broad steps. First, pre-messenger RNA is formed, with the involvement of RNA polymerase enzymes. The process relies on Watson-Crick base pairing, and the resultant single strand of RNA is the reverse-complement of the original DNA sequence. The pre-messenger RNA is then "edited" to produce the desired mRNA molecule in a process called *RNA splicing*.

Formation of pre-messenger RNA

The mechanism of transcription has parallels in that of DNA replication. As with DNA replication, partial unwinding of the double helix must occur before transcription can take place, and it is the RNA polymerase enzymes that catalyze this process.

Unlike DNA replication, in which both strands are copied, only one strand is transcribed. The strand that contains the gene is called the *sense* strand, while the complementary strand is the *antisense* strand. The mRNA produced in transcription is a copy of the sense strand, but it is the antisense strand that is transcribed.

Ribonucleotide triphosphates (NTPs) align along the antisense DNA strand, with Watson-Crick base pairing (A pairs with U). RNA polymerase joins the ribonucleotides together to form a pre-messenger RNA molecule that is complementary to a region of the antisense DNA strand. Transcription ends when the RNA polymerase enzyme reaches a triplet of bases that is read as a "stop" signal. The DNA molecule re-winds to re-form the double helix.

Transcription is a three step process namely initiation, elongation and termination.

Initiation

Just before initiation, RNA polymerase and accessory proteins bind to a DNA molecule upstream of the initiation point. The DNA is unwound to separate and expose the strand to be transcribed. Then, the RNA polymerase complex binds to a promoter sequence, which establishes initiation of transcription. Polymerase begins to synthesize a strand of RNA complementary to one side of the DNA strand, moving into the coding sequence portion of the gene being transcribed.

Elongation

During elongation, a lengthening RNA molecule is produced by DNA polymerase as it reads the DNA triplet code on the template strand. The polymerase will continue reading the template until it reaches a sequence that provides a signal indicating that transcribed region is at an end. Another RNA polymerase can attach to the promoter to begin synthesizing another RNA before the first one is finished.

Termination

Termination of transcription is triggered when the RNA polymerase encounters a particular DNA sequence, causing the polymerase to lose affinity for the DNA template. At this point, RNA polymerase disengages from the DNA and the RNA molecule is released for translation or post-transcriptional processing.

Transcription Factors

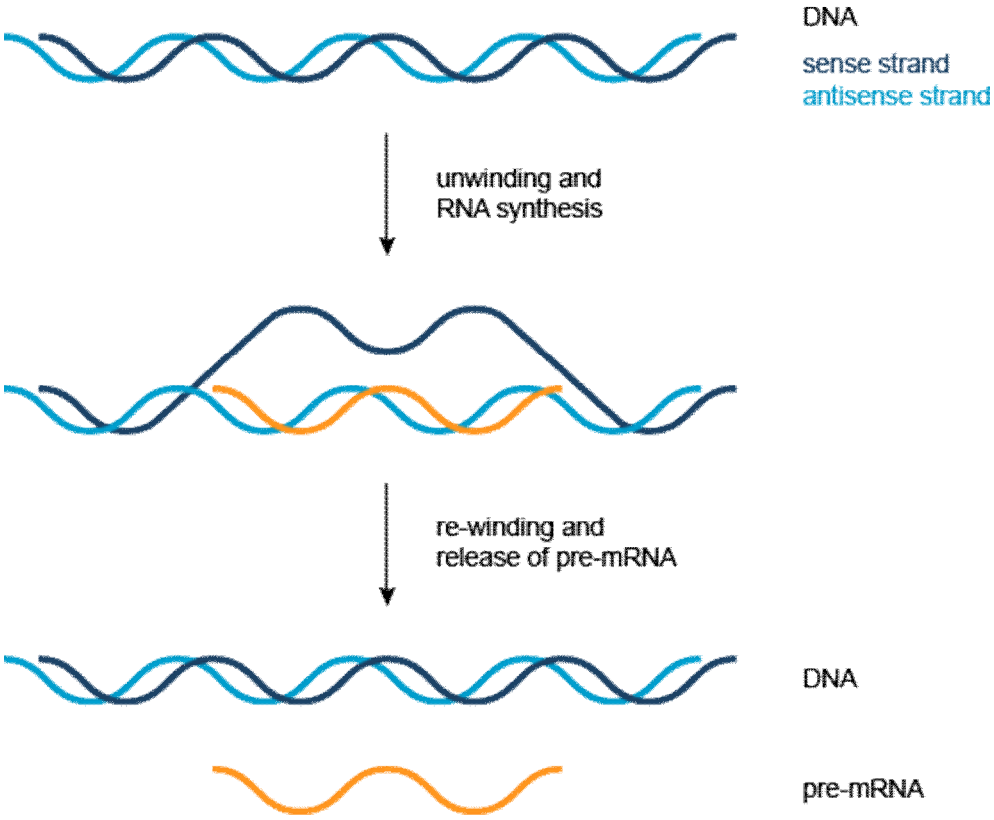
Other proteins besides RNA polymerase are required for transcription. These proteins are called transcription factors. They may bind to RNA polymerase, interact with other transcription factors, or bind to DNA directly to affect transcription. Transcription factors are required for proper assembly of the initiation complex, and have important functions in elongation and termination.

Regulation of Transcription

The efficiency and degree to which transcription occurs is regulated by the aforementioned transcription factors as well as DNA binding proteins. Suppressor proteins attach to DNA to block initiation, preventing certain genes from being transcribed. Other molecules can interact with suppressors, causing them to leave their DNA binding sites, allowing transcription to proceed.

Eukaryotic and Prokaryotic Transcription

The different cell organization and complexities of eukaryotes and prokaryotes make for some significant differences in transcription. Transcription occurs in the nucleus in eukaryotes and in the cytoplasm in prokaryotes (since they have no nucleus). Eukaryotic mRNA is post-transcriptionally modified with a 3-foot poly-A tail and 5-foot cap. Eukaryotic RNA often contains non-protein coding sections called introns, which are removed after transcription. No such modifications are made in prokaryotes. Prokaryotic transcription requires fewer proteins than eukaryotic transcription.


DNA
sense strand
antisense strand
unwinding and
RNA synthesis
re-winding and
release of pre-mRNA
DNA
pre-mRNA

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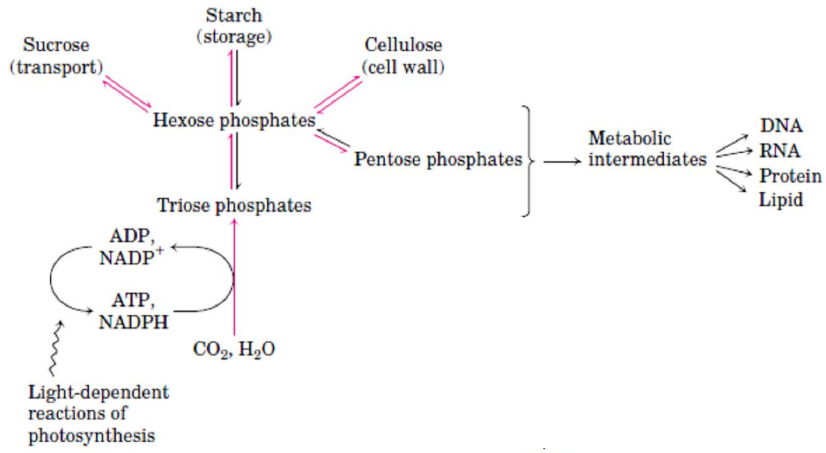
Biosynthesis of Carbohydrate

Photosynthetic Carbohydrate Synthesis:

Plants and photosynthetic microorganisms, by contrast, can synthesize carbohydrates from CO_2 and water, reducing CO_2 at the expense of the energy and reducing power furnished by the ATP and NADPH that are generated by the light-dependent reactions of photosynthesis. Plants (and other autotrophs) can use CO_2 as the sole source of the carbon atoms required for the biosynthesis of cellulose and starch, lipids and proteins, and the many other organic components of plant cells.



Glycogen biosynthesis:

Glycogen synthesis takes place in virtually all animal tissues but is especially prominent in the liver and skeletal muscles. In liver, glycogen serve as a reservoir of glucose, readily converted in blood glucose for distribution to other tissues. In muscle, glucose produced from the breakdown of glycogen is metabolised via glycolysis to provide ATP energy from muscle contraction.

The starting point for synthesis of glycogen is glucose-6-phosphate derived from free glucose in a reaction catalyzed by the isozymes hexokinase (in muscle) and glucokinase (in liver):

D-Glucose + ATP → D-glucose 6-phosphate + ADP

However, some ingested glucose takes a more roundabout path to glycogen. It is first taken up by erythrocytes and converted to lactate glycolytically; the lactate is then taken

up by the liver and converted to glucose 6-phosphate by gluconeogenesis.

To initiate glycogen synthesis, the glucose 6-phosphate is converted to glucose 1-phosphate in the phosphoglucomutase reaction:

Glucose 6-phosphate ⇌ Glucose 1-phosphate

The product of this reaction is converted to UDP-glucose by the action of UDP-glucose pyrophosphorylase, is a key step of glycogen biosynthesis:

Glucose 1-phosphate + UTP → UDP-glucose + PP_i

UDP-glucose is the immediate donor of glucose residues in the reaction catalyzed by glycogen synthase, which promotes the transfer of the glucose residue from UDP-glucose to a nonreducing end of a branched glycogen molecule.

Gluconeogenesis:

Carbohydrate biosynthetic processes are the central pathway that leads to the formation of carbohydrates from non-carbohydrate precursors.

- ✓ Biosynthesis of glucose is an absolute necessity in all mammals, because brain and nervous system as well as the erythrocytes, testes, renal medulla, embryonic tissues require glucose from the blood as their sole fuel source. The human brain alone requires oven 120 g of glucose each day.
- ✓ The formation of glucose

Pentose Phosphate Pathway:

The *oxidative* pentose phosphate pathway (phosphogluconate pathway or hexose monophosphate pathway) brings about oxidation and decarboxylation at C-1 of glucose 6-phosphate, reducing $NADP^+$ to NADPH and producing pentose phosphates.

■ NADPH provides reducing power for biosynthetic reactions, and ribose 5-phosphate is a precursor for nucleotide and nucleic acid synthesis. Rapidly growing tissues and tissues carrying out active biosynthesis of fatty acids, cholesterol, or steroid hormones send more glucose 6-phosphate through the pentose phosphate pathway than do tissues with less demand for pentose phosphates and reducing power.

■ The first phase of the pentose phosphate pathway consists of two oxidations that convert glucose 6-phosphate to ribulose 5-phosphateand reduce $NADP^+$ to NADPH. The second phase comprises nonoxidative steps that convert pentose phosphates to glucose 6-phosphate, which begins the cycle again.

■ In the second phase, transaldolase (with TPP as cofactor) and transketolase catalyze the

interconversion of three-, four-, five-, six-, and seven-carbon sugars, with the reversible conversion of six pentose phosphates to five hexose phosphates. In the carbon-assimilating reactions of photosynthesis, the same enzymes catalyze the reverse process, called the *reductive* pentose phosphate pathway: conversion of five hexose phosphates to six pentose phosphates.

■ A genetic defect in transketolase that lowers its affinity for TPP exacerbates the Wernicke- Korsakoff syndrome.

■ Entry of glucose 6-phosphate either into glycolysis or into the pentose phosphate pathway is largely determined by the relative concentrations of $NADP^+$ and NADPH.

Pentose Phosphate Pathway of Glucose Oxidation:

In most animal tissues, the major catabolic fate of glucose 6-phosphate is glycolytic breakdown to pyruvate, much of which is then oxidized via the citric acid cycle, ultimately leading to the formation of ATP. Glucose 6-phosphate does have other catabolic fates, however, which lead to specialized products needed by the cell. Of particular importance in some tissues is the oxidation of glucose 6-phosphate to pentose phosphates by the pentose phosphate pathway (also called the phosphogluconate pathway or the hexose monophosphate pathway. In this oxidative pathway, $NADP^+$ is the electron acceptor, yielding NADPH. Rapidly dividing cells, such as those of bone marrow, skin, and intestinal mucosa, use the pentoses to make RNA, DNA, and such coenzymes as ATP, NADH, $FADH_2$, and coenzyme A.

In other tissues, the essential product of the pentose phosphate pathway is not the pentoses but the electron donor NADPH, needed for reductive biosynthesis or to counter the damaging effects of oxygen radicals. By maintaining a reducing atmosphere (a high ratio of NADPH to $NADP^+$ and a high ratio of reduced to oxidized glutathione), they can prevent or undo oxidative damage to proteins, lipids, and other sensitive molecules. In erythrocytes, the NADPH produced by the pentose phosphate pathway is so important in preventing oxidative damage that a genetic defect in glucose 6-phosphate dehydrogenase, the first enzyme of the pathway, can have serious medical consequences.

Oxidative reaction of Pentose Phosphates and NADPH

The first reaction of the pentose phosphate pathway is the oxidation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase (G6PD) to form 6-phosphoglucono-δ-lactone, an intramolecular ester. $NADP^+$ is the electron acceptor, and the overall equilibrium lies far in the direction of NADPH

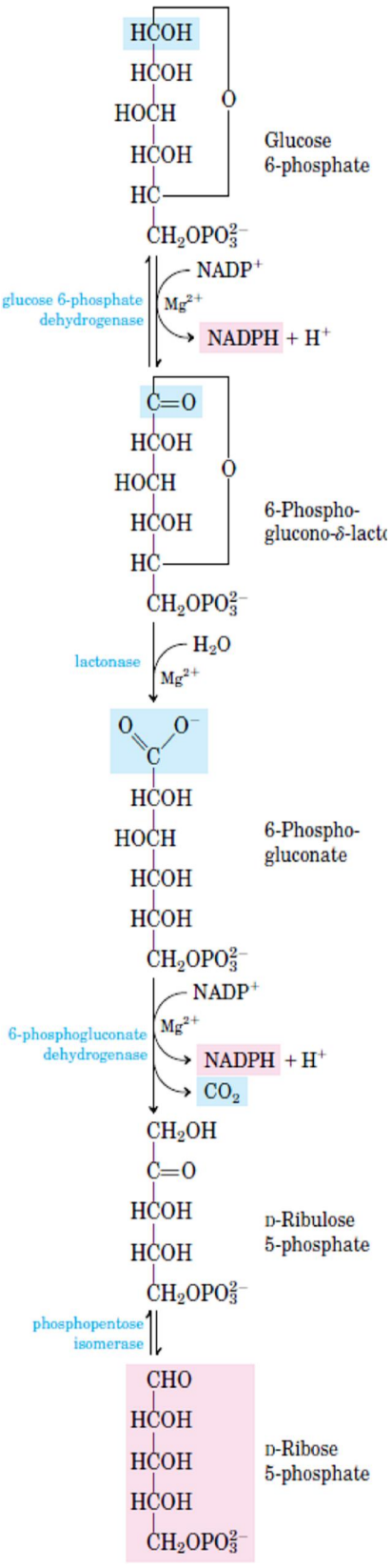


Fig.: Oxidative reaction of Pentose Phosphate Pathway

formation. The lactone is hydrolyzed to the free acid 6-phosphogluconate by a specific lactonase, then 6-phosphogluconate undergoes oxidation and decarboxylation by 6-phosphogluconate dehydrogenase to form the ketopentose ribulose 5-phosphate. This reaction generates a second molecule of NADPH. Phosphopentose isomerise converts ribulose 5-phosphate to its aldose isomer, ribose 5-phosphate. In some tissues, the pentose phosphate pathway ends at this point, and its overall equation is
precursor for nucleotide synthesis.

Glucose 6-phosphate + $2NADP^+$ + H_2O → Ribose 5-phosphate + CO_2 + 2NADPH + $2H^+$

The net result is the production of NADPH, a reductant for biosynthetic reactions, and ribose 5-phosphate, a precursor for nucleotide synthesis.

Nonoxidative Phase of Pentose Phosphates Pathway

In this nonoxidative phase, ribulose 5-phosphate is first epimerized to xylulose 5-phosphate. Then, in a series of rearrangements of the carbon skeletons six five-carbon sugar phosphates are converted to five six-carbon sugar phosphates, completing the cycle and allowing continued oxidation of glucose 6-phosphate with production of NADPH. Continued recycling leads ultimately to the conversion of glucose 6-phosphate to six CO_2. Two enzymes unique to the pentose phosphate pathway act in these interconversions of sugars: transketolase and transaldolase. Transketolase catalyzes the transfer of a two-carbon fragment from a ketose donor to an aldose acceptor. In nonoxidative part of the pentose phosphate pathway, transketolase, a thiamine pyrophosphate (TPP) dependent enzyme transfers C-1 and C-2 of xylulose 5-phosphate to ribose 5-phosphate, forming the seven-carbon product sedoheptulose 7-phosphate. The remaining three-carbon fragment from xylulose is glyceraldehyde 3-phosphate.

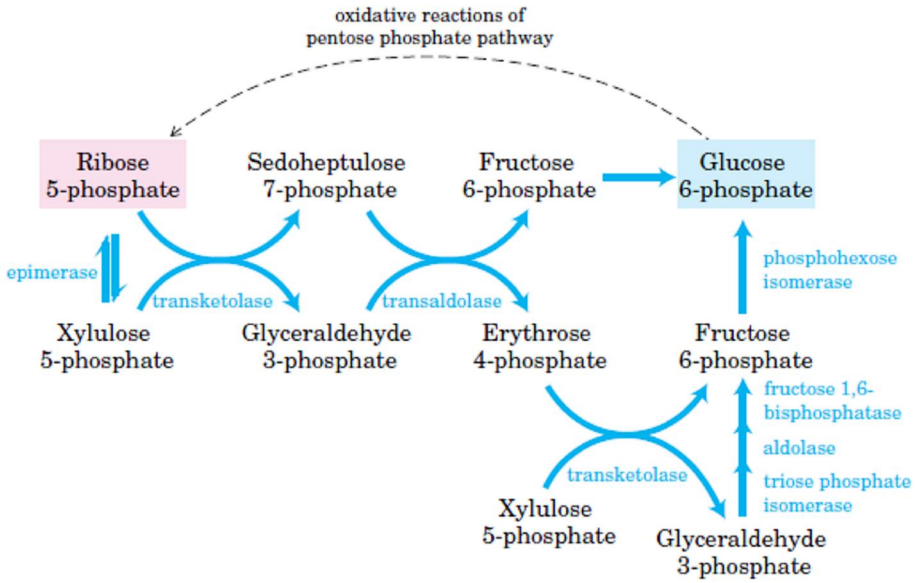


Fig.: Nonoxidative Phase of Pentose Phosphates Pathway

Transaldolase catalyzes a reaction similar to the aldolase reaction of glycolysis: a three-carbon fragment is removed from sedoheptulose 7-phosphate and condensed with glyceraldehyde 3-phosphate, forming fructose 6-phosphate and the tetrose erythrose 4-phosphate. Now transketolase acts again, forming fructose 6-phosphate and glyceraldehyde 3-phosphate from erythrose 4-phosphate and xylulose 5-phosphate. Two molecules of glyceraldehyde 3-phosphate formed by two iterations of these reactions can be converted to a molecule of fructose 1,6-bisphosphate as in gluconeogenesis, and finally fructose bis phosphatase-1 (FBPase-1) and phosphohexose isomerase convert fructose 1,6-bisphosphate to glucose 6-phosphate. The cycle is complete: six pentose phosphates have been converted to five hexose phosphates.

All reactions of the nonoxidative part of the pentose phosphate pathway are readily reversible and thus also provide a means of converting hexose phosphate to pentose phosphate. This is essential in the photosynthetic fixation of CO_2 by plant.

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Lipid Biosynthesis

Lipids play a variety of cellular roles, some only recently recognized. They are the principal form of stored energy in most organisms and major constituents of cellular membranes. Specialized lipids serve as pigments (retinal, carotene), cofactors (vitamin K), detergents (bile salts), transporters (dolichols), hormones (vitamin D derivatives, sex hormones), extracellular and intracellular messengers (eicosanoids, phosphatidylinositol derivatives), and anchors for membrane proteins (covalently attached fatty acids, prenyl groups, and phosphatidylinositol). The ability to synthesize a variety of lipids is essential to all organisms. The biosynthetic pathways for some of the most common cellular lipids by assembling these water-insoluble products from water-soluble precursors such as acetate. Like other biosynthetic pathways, these reaction sequences are endergonic and reductive. They use ATP as a source of metabolic energy and a reduced electron carrier (usually NADPH) as reductant. The biosynthesis of fatty acids, the primary components of both triacylglycerols and phospholipids. The synthesis of cholesterol, a component of some membranes and the precursor of steroids such as the bile acids, sex hormones, and adrenocortical hormones.

Fatty Acid Synthase Receives the Acetyl and Malonyl Groups

Before the condensation reactions that build up the fatty acid chain can begin, the two thiol groups on the enzyme complex must be charged with the correct acyl groups. First, the acetyl group of acetyl- CoA is transferred to the Cys OSH group of the β-ketoacyl-ACP synthase. This reaction is catalyzed by acetyl-CoA–ACP transacetylase. The second reaction, transfer of the malonyl group from malonyl-CoA to the OSH group of ACP, is catalyzed by malonyl-CoA–ACP transferase (MT), also part of the complex. In the charged synthase complex, the acetyl

Step 1 Condensation The first reaction in the formation of a fatty acid chain is condensation of the activated acetyl and malonyl groups to form acetoacetyl-ACP, an acetoacetyl group bound to ACP through the phosphopantetheine OSH group; simultaneously, a molecule of CO_2 is produced. In this reaction, catalyzed by *β*-ketoacyl-ACP synthase (KS), the acetyl group is transferred from the Cys-SH group of the enzyme to the malonyl group on the -SH of ACP, becoming the methyl-terminal two-carbon unit of the new acetoacetyl group. The carbon atom of the CO_2 formed in this reaction is the same carbon originally introduced into malonyl- CoA from HCO_3^- by the acetyl-CoA

carboxylase reaction. Thus CO_2 is only transiently in covalent linkage during fatty acid biosynthesis; it is removed as each two-carbon unit is added.

By using activated malonyl groups in the synthesis of fatty acids and activated acetate in their degradation, the cell makes both processes energetically favorable, although one is effectively the reversal of the other. The extra energy required to make fatty acid synthesis favorable is provided by the ATP used to synthesize malonyl-CoA from acetyl-CoA and HCO_3.

Step 2 Reduction of the Carbonyl Group The acetoacetyl-ACP formed in the condensation step now undergoes reduction of the carbonyl group at C-3 to form D-*β*-hydroxybutyryl-ACP. This reaction is catalyzed by *β*-ketoacyl-ACP reductase (KR) and the electron donor is NADPH. Notice that the D-*β*-hydroxybutyryl group does not have the same stereoisomeric form as the L-*β*-hydroxyacyl intermediate in fatty acid oxidation

Step 3 Dehydration The elements of water are now removed from C-2 and C-3 of D-_-hydroxybutyryl-ACP to yield a double bond in the product, *trans*-Δ^2- butenoyl-ACP. The enzyme that catalyzes this dehydration is *β*- hydroxyacyl-ACP dehydratase (HD).

Step 4 Reduction of the Double Bond Finally, the double bond of *trans*-_2-butenoyl-ACP is reduced (saturated) to form butyryl-ACP by the action of enoyl-ACP reductase (ER); again, NADPH is the electron donor.

The Fatty Acid Synthase Reactions are repeated to Form Palmitate

The Production of the four-carbon, saturated fatty acyl–ACP completes one pass through the fatty acid synthase complex. The butyryl group is now transferred from the phosphopantetheine -SH group of ACP to the Cys-SH group of *β*-ketoacyl-ACP synthase, which initially bore the acetyl group. To start the next cycle of four reactions that lengthens the chain by two more carbons, another malonyl group is linked to the now unoccupied phosphopantetheine -SH group of ACP. Condensation occurs as the butyryl group, acting like the acetyl group in the first cycle, is linked to two carbons of the malonyl-ACP group with concurrent loss of CO_2. The product of this condensation is a sixcarbon acyl group, covalently bound to the phosphopantetheine -SH group. Its *β*-keto group is reduced in the next three steps of the synthase cycle to yield the saturated acyl group, exactly as in the first round of reactions in this case forming the six-carbon product. Seven cycles of condensation and reduction produce the 16-carbon saturated palmitoyl group, still bound to ACP. For reasons not well understood, chain elongation by the synthase complex generally stops at this point and free palmitate is released from the ACP

by a hydrolytic activity in the complex. Small amounts of longer fatty acids such as stearate (18:0) are also formed. In certain plants (coconut and palm, for example) chain termination occurs earlier; up to 90% of the fatty acids in the oils of these plants are between 8 and 14 carbons long. We can consider the overall reaction for the synthesis of palmitate from acetyl-CoA in two parts. First, the formation of seven malonyl-CoA molecules:

7 Acetyl-CoA + $7CO_2$ + 7ATP →7 malonyl-CoA + 7ADP + 7Pi

Then seven cycles of condensation and reduction:

Acetyl-CoA + 7 malonyl-CoA + 14NADPH + $14H^+$ → Palmitate + $7CO_2$ + 8 CoA +$14NADP^+$ +$6H_2O$

The overall process is

8 Acetyl-CoA + 7ATP + 14NADPH +$14H^+$ →Palmitate + 8 CoA + $6H_2O$ + 7ADP + 7Pi + $14NADP^+$

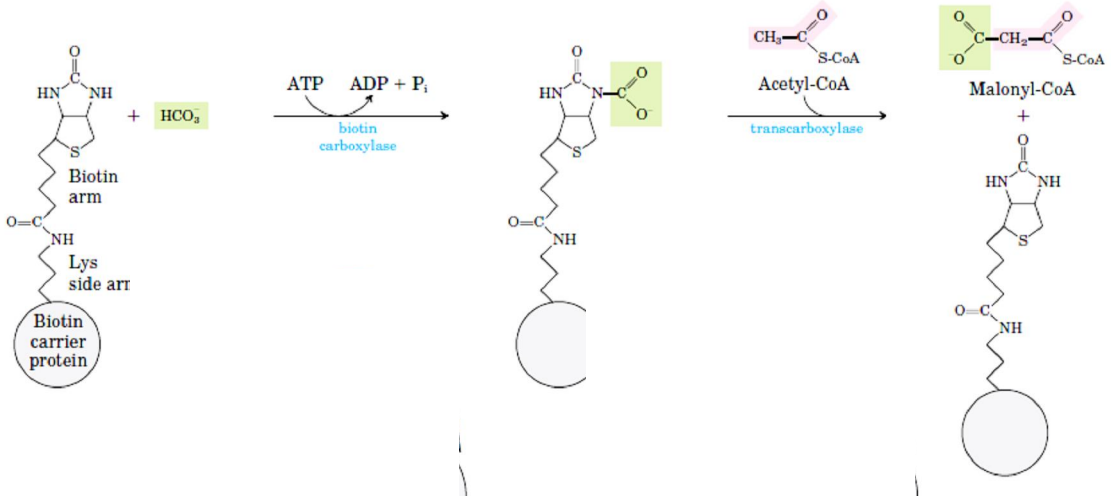


Fig.1: Formation of Acetyl-CoA from Malonyl-CoA

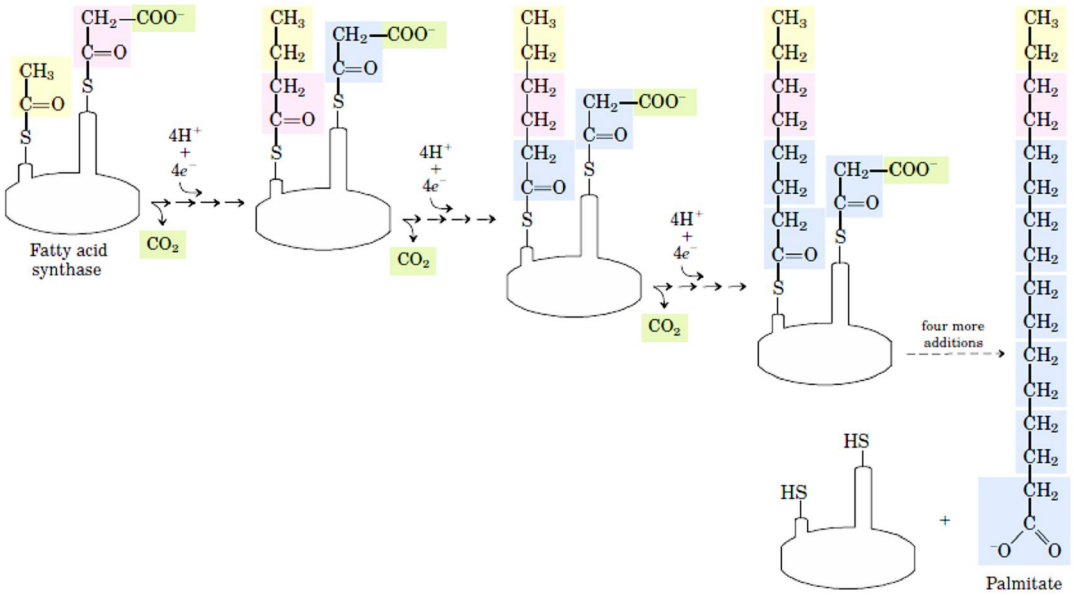
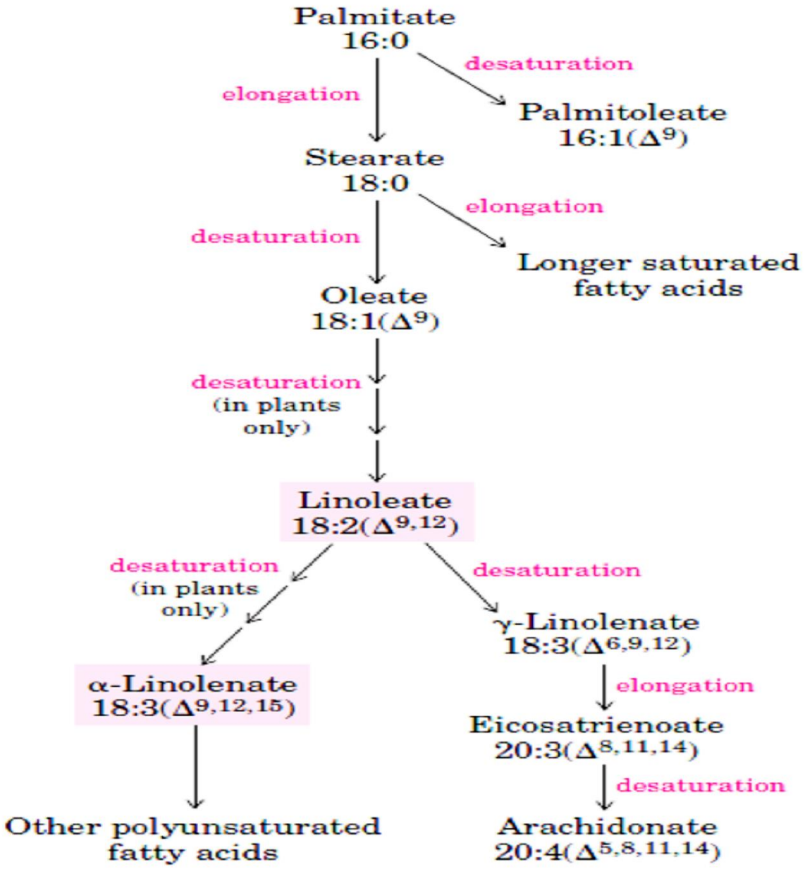


Fig.3: Process of Fatty acid Synthesis

Long-Chain Saturated Fatty Acids Are Synthesized from Palmitate

Palmitate, the principal product of the fatty acid synthase system in animal cells, is the precursor of other long-chain fatty acids. It may be lengthened to form stearate (18:0) or even longer saturated fatty acids by further additions of acetyl groups, through the action of fatty acid elongation systems present in the smooth endoplasmic reticulum and in mitochondria. The more active elongation system of the ER extends the 16-carbon chain of palmitoyl-CoA by two carbons, forming stearoyl-CoA. Although different enzyme systems are involved, and coenzyme A rather than ACP is the acyl carrier in the reaction, the mechanism of elongation in the ER is otherwise identical to that in palmitate synthesis: donation of two carbons by malonyl-CoA, followed by reduction, dehydration, and reduction to the saturated 18-carbon product, stearoyl-CoA.


Palmitate
16:0
desaturation
elongation
Palmitoleate
16:1(Δ9)
Stearate
18:0
elongation
desaturation
Longer saturated
fatty acids
Oleate
18:1(Δ9)
desaturation
(in plants
only)
Linoleate
18:2(Δ9,12)
desaturation
(in plants
only)
desaturation
γ-Linolenate
18:3(Δ6,9,12)
α-Linolenate
18:3(Δ9,12,15)
elongation
Eicosatrienoate
20:3(Δ8,11,14)
desaturation
Other polyunsaturated
fatty acids
Arachidonate
20:4(Δ5,8,11,14)

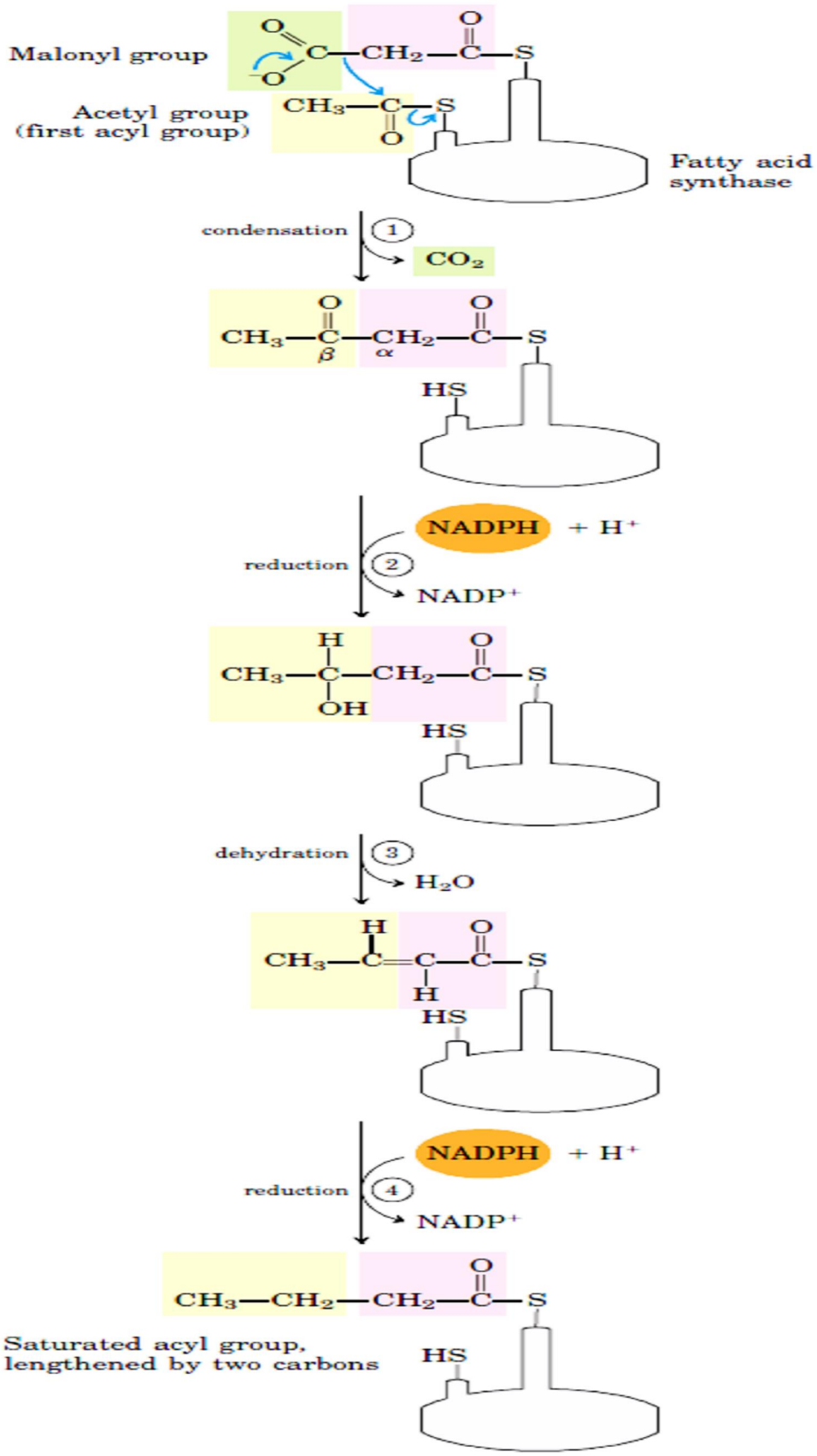


Fig. 2: A four step sequence lengthens a growing fatty acyl chain by two carbons

CONCEPT OF PLANT BIOTECHNOLOGY

What is Biotechnology?

The origin of Biotechnology can be traced back to prehistoric times, when microorganisms were already used for processes like fermentation. In 1920's *Clostridium acetobutylicum* was used by Chaim Weizman for converting starch into butanol and acetone, latter was an essential component of explosive during World War- II. This raised hopes for commercial production of useful chemicals through biological processes, and may be considered as the first rediscovery of biotechnology in the present century. Similarly, during World War-II (in 1940's) , the production of penicillin (as an antibiotic discovered by Alexaner Flemming in 1929) on a large scale from cultures of *Penicillium notatum* marked the second rediscovery of biotechnology. The third rediscovery of biotechnology is its recent reincarnation in the form of recombinant –DNA technology, which led to the development of a variety of gene technologies and is thus considered to be greatest scientific revolution of this century.

Biotechnologies, as world indicate, is the product of interaction between the science and technology.

Definition of Biotechnology:

1. Biotechnology is the application of biological organisms, system or processes to manufacturing and service industries.
2. Biotechnology is the integrated use of biochemistry , microbiology and engineering science in order to achieve technological application of the capabilities of micro-organism, cultured tissue cells and part thereof.
3. Biotechnology is "a technology using biological phenomenon for copying and manufacturing various kinds of useful substances."
4. Biotechnology is "the controlled use of biological agents such as micro-organisms or cellular components for beneficial use. (U.S National Science Foundation)

Application of Biotechnology

Following are some of the field s where biotechnology innovations are playing important roles:

1. Tissue Culture Techniques in Biotechnology:

An important aspect of all biotechnology processes is the culture of either the microorganism or plant or animal cells or tissues and organs in artificial media. While members in culture are used in recombinant DNA technology and in variety of industrial processes, plant cells and

tissues are used for a variety of genetic manupulation. For example, another culture is used for haploid breeding , gametic and somatic cell or tissue culture are used for tapping gametoclonal and Somaclonal variation or for production of artificial seeds. Transformation of protoplast in culture leads to production of useful transgenic plants.

2. Gene Technology as a Tool for Biotechnology:

Most biotechnology companies make use of gene technology or genetic engineering which involves recombinant DNA and gene cloning. Most recently, extensive use of newly discovered polymerase chain reaction (PCR) has also been made for gene technology.

3. Hybridization and Monoclonal Antibodies in Biotechnology:

Rapid progress has been made in hybridoma technique and monoclonal antibodies which is extremely used in human health care. Enzyme conjugated antibodies are being used for detection of viruses both in plants and animals using ELISA test. Immunotixins are being produced from gene fusion so that the toxic drugs meant for killing tumour cells may be carried to the target sites with the help of specific antibodies.

4. Biotechnology in Medicine:

In the field of medicine, insulin and interferon synthesized by bacteria have already been released for use. A large number of vaccines for immunization against deadly diseases, DNA probes and monoclonal antibodies for diagnosis of various diseases, and human growth hormone and other pharmaceutical drugs for treatment of disease are being released.

5. Biotechnology and Protein Engineering:

Protein engineering will lead to production of superior enzymes and storage proteins. Biochemistry has also provided us with remarkable in the form of immobilized enzymes system, which allowed the production of variety of substances. E.g. High- fructose corn syrup using an immobilized enzyme, glucose isomerase.

6. Biotechnology in Agriculture:

Biotechnology has also revolutionized research activities in the area of agriculture which include following:

i) Plant cell, tissue and organ culture.

ii) Genetic engineering leading to transformation followed by regeneration of plants to give transgenic plants carrying desirable traits like disease resistance, insect resistance and herbicide resistance.

iii) Somatic hybrids between sexually incompatible species permitting transfer of desirable traits from wild or unrelated species to our crop plants.

iv) Transgenic animals produced in mice, pigs, goats, chicken, cows, etc. It is suggested that some of these will eventually be used as bioreactor to produce drugs through their milk, blood or urine, this area has sometimes been described as molecular farming.

7. Biotechnology and Environment:

Biotechnology methods have been devised for some environmental problems like i) Pollution control ii) depletion of natural resources for non-renewable energy. iii) restoration of degraded lands and iv) biodiversity conservation. For instance, microbes are being developed to be used as bio pesticides, bio fertilizers, biosensors etc. and for recovery of metals, cleaning of spilled oils, etc.

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APPLICATION OF PLANT BIOTECHNOLOGY IN CROP IMPROVEMENT

Plant tissue culture comprises a set of in vitro techniques, methods and strategies that are part of the group of technologies called plant biotechnology. Tissue culture has been exploited to create genetic variability from which crop plants can be improved, to improve the state of health of the planted material and to increase the number of desirable germplasms available to the plant breeder. With selected laboratory material typically taking one or two decades to reach the commercial market through plant breeding, this technology can be expected to have an ever increasing impact on crop improvement as we approach the new millenium. The applications of various tissue culture approaches to crop improvement are as follows:

1. Micro propagation helps in mass multiplication of plants which are difficult to propagate through conventional methods.
2. Some perennial crop plants like ornamental and fruit crops can not be propagated through seeds. The vegetative propagation like grafting, budding are tedious and time consuming. In such crops micro propagation helps in rapid multiplication.
3. Rapid multiplication of rare and elite genotypes such as Aromatic and Medicinal plants.
4. Isolation of *in vitro* mutants for a large number of desirable character Eg:- Isolation of biochemical mutants and mutants resistant to biotic (pest and disease) abiotic (salt and drought, cold, herbicide etc) stresses through the use of somaclonal variation
5. Screening of large number of cells in small space.
6. Cross pollinated crops like cardamom, Eucalyptus, coconut, oil palm do not give true to type plants, when multiplied through seed. Development of genetically uniform plants in cross pollinated crops is possible through tissue culture
7. In case of certain horticultural crops orchids etc seed will not germinate under natural conditions, such seed can be made to germinate *in vitro* by providing suitable environment.
8. Induction of flowering in some trees that do not flower or delay in flowering. E. g:- Bamboo flowers only once in its life time of 50 years
9. Virus free plants can be produced through meristem culture

10. Large amount of germplasm can be stored within a small space and lesser cost for prolonged periods under *in vitro* condition at low temperature. The preservation of cells tissues, organs in liquid Nitrogen at – 196^0C is called cryopreservation
11. Production of secondary metabolites. E. g:- Caffeine from *coffea arabica,* Nicotine from *Nicotiana rustica.*
12. Plant tissue culture can also be used for studying the biochemical pathways and gene regulation.
13. Anther and pollen culture can be used for production of haploids and by doubling the chromosome number of haploids using cholchicine, homogygous diploids can be produced. They are called dihaploids.
14. In case of certain fruit crops and vegetative propagated plants where seed is not of much economic impor tant, triploids can be produced through endosperm culture.
15. Inter specific and inter generic hybrids can be produced through embryo rescue technique which is not possible through conventional method. In such crosses *in vitro* fertilization helps to overcome pre-fertilization barrier while the embryo rescue technique helps to over come post fertilization barrier.
16. Somatic hybrids and cybrids can be produced through protoplast fusion (or) somatic hybridization
17. Ovary culture is helpful to know the physiology of fruit development.
18. Development of transgenic plants.

Advantages of tissue culture

- Rapid multiplication within a limited space
- It is not time bound and not season bound
- Free from pests and diseases

Limitations (or) Disadvantages

- Laborious, costly, special risk is required.

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NUTRITIONAL REQUIREMENTS OF TISSUE CULTURE

Preparation of composition of Murashige and Skoog medium

The isolated plant tissues are grown on a suitable artificially prepared nutrient medium called culture medium. The medium is substrate for plant growth and it refers to the mixture of certain chemical compounds of form a nutrient rich gel (or) liquid for growing cultures, whether cells, organs (or) plantlets. The culture media has to supply all the essential mineral ions required for *in vitro* growth and morphogenesis of plant tissue.

The major constituents of most plant tissue culture methods are:

1) Inorganic nutrients: micronutrients/microelements and macronutrients/ microelements
2) Carbon source
3) Organic supplements
4) Growth regulators
5) Solidifying agents

1) Inorganic Nutrients: A variety of mineral elements (salts) supply the macro & micro nutrients required in the life of plant.

Elements required in concentration 0.5 mM are referred to as macronutrients and those required in less than 0.5 mM concentration are considered as micro nutrients

Macronutrients

They include six major elements N, P, K, Ca, Mg and S present as salts in the media which are essential for plant cell and tissue growth

Nitrogen is the element which is required in greatest amount. It is most commonly supplied as a mixture of Nitrate ions (KNO_3) and Ammonium ions (NH_4 NO_3).

Phosphorous is usually supplied as phosphate ion of Ammonium, sodium and Potassium salts. Other major elements Ca, Mg, S, are also required to be incorporated in the medium

Micro nutrients

These are Mn, Zn, B, Cu, Mo, Fe, Co, I etc.

Iron is generally added as a chelate with EDTA. (Ethylene Diamino Tetra Acetic acid) In this form iron is gradually released and utilized by living cells and remains available up to a PH 8.

Some of the elements important for plant nutrition and their physiological function.

Element	Function
Nitrogen (N2)	Component of proteins nucleic acids some co-enzymes
Phosphrous (P)	Component of nucleic acids energy transfer component Of intermediate in respiration and photosynthesis
Potassium (K)	Regulates osmotic potential principal in organic cation
Calcium (Ca)	Cell wall synthesis, Membrane function cell signalling.
Magnesium (Mg)	Enzyme co-factor component of chlorophyll
Sulphur (S)	Component of some amino acids (Methionine, cysteine) some co-factors
Chlorin (Cl)	required for photosynthesis
Iron (Fe)	Electron transfer as a component of cytochromes
Managanese (Mn)	Enzyme co-factor
Cobalt (Co)	Component of some vitamins
Copper (Cu)	Enzyme co-factor electron transfer reaction
Zinc (Zn)	Enzyme co-factor chlorophyll biosynthesis
Molybdenum (Mo)	Enzyme co-factor component of nitrate reductase.

Preparation of Nutrient Medium:

The nutrients required for optimal growth of plant organ tissue and protoplast *in vitro* generally vary from species to species even tissues from different parts of a plant may have different requirements for satisfactory growth.

Carbon source

Plants cells and tissues in culture medium lack autotrophic ability and therefore need external carbon for energy. The most preferred carbon energy source in plant tissue culture is sucrose. It is generally used at a concentration of 2-5%. While autoclaving the medium sucrose is converted to Glucose and Fructose. In the process first Glucose is used and then Fructose.

Glucose supports good growth while fructose less efficient. Maltose, Galactose, lactose, and mannose are the other sources of carbon. Most media contain myoinositol at a concentration of approximately 100 mg l-1 which improves cell growth.

Organic supplements

Vitamins

Plants synthesize vitamins endogenously and these are used as catalysts in various metabolic processes. When plant cells and tissues are grown in *in vitro* some essential vitamins are synthesized but only in suboptimal quantities. Hence it is necessary to supplement the medium with required vitamins and amino acids to get best growth of tissue. The most commonly used vitamins is thiamine (vitamin B) the other vitamin which improve growth of cultured plants are Nicotinic acid, Pantothenic acid, Pyridoxine (B6), Folic acid, Aminobenzoic acid. (ABA)

Amino acids

Cultured tissues are normally capable of synthesing Amino acids necessary for various metabolic processes. In spite of this the addition of Amino acid to the media is important for stimulating cell growth in protoplast cultures and for establishing cell culture. Among the amino acids glycine is most commonly used. Amino acids Glutamine Aspargine, Arginine, Cystine are the other common sources of organic Nitrogen used in culture media

Other organic supplements

These include organic extracts E. g:- Protein (casein) hydrolysate, coconut milk, yeast & malt extract, ground banana, orange juice, Tomato juice, Activated charcoal. The addition of activated charcoal to culture media stimulates growth and differentiation in orchids, Carrot and Tomato Activated charcoal adsorbs inhibitory compounds & darkening of medium occurs. It also helps in to reduce toxicity by removing toxic compounds

E.g:- Phenols produced during the culture permits un hindered cell growth

Antibiotics

Some plant cells have systematic infection of micro organisms. To prevent the growth of these microbes it is essential to enrich the media with antibiotics.

E.g:- Streptomycin or Kanamycin at low concentration effectively controls systemic infection and do inhibit the growth of cell cultures

Growth regulators

These include auxins, cytokinins, gibberillins, ABA. The growth differentiation and organogenesis of tissue occurs only on the addition of one (or) more of these hormones to the medium.

Auxins

Auxins have the property of cell division, cell elongation, elongation of stem, internodes, tropism, Apical dominance abscission and rooting commonly used auxins are

IAA (Indole 3-Acetic Acid), IBA (Indole 3-Butyric Acid), 2,4-D (Dichloro Phenoxy Acetic Acid), NAA (Naphthylene Acitic Acid), NOA (Naphthoxy Acitic Acid)

The 2,4 -D is used for callus induction where as the other auxins are used for root induction.

Cytokinins

Cytokinins are adenine derivatives which are mainly concerned with cell division, modification of apical dominance, and shoot differentiation in tissue culture. Cytokinins have been shown to activate RNA synthesis and to stimulate protein and the enzymatic activity in certain tissues. Commonly used Cytokinins are -

BAP (6-Benzylamino purine) or BA (Benzy adenine), 2ip (Isopentyl adenine), Kinetin (6 – furfur aminopurine), Zeatin (4 – hydroxy 3 methyl trans 2 butinyl aminopurine)

Gibberillins and Abscisic acid

GA_3 is most common gibberillin used in tissue culture. It promotes the growth of the cell culture at low density. Enhances callus growth and simulates the elongation of dwarf or stunted plantlets formation from adventive embryos formed in culture.

ABA in culture medium either stimulates or inhibits culture growth depending on specie s. It is most commonly used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis.

Solidifying agent

Gelling and solidifying agents are commonly used for preparing semisolid or solid tissue culture media. Agar (polysaccharide obtained from marine sea weeds) is used to solidify the medium. Normally 0.5-1% Agar is used in the medium to form a firm gel at the pH typical of plant tissue culture media. Use of high concentration of agar makes the medium hard and prevents the diffusion of nutrients into tissues.

pH

Plant cells and tissues require optimum pH for growth and development in cultures. The pH affects the uptake of ions, hence it must be adjusted below 5-6.0 by adding 0.1N NaOH (or) HCl. Usually the pH higher than six results in a fairly hard medium where as pH below five does not allow satisfactory solidification of medium.

Preparation of Nutrient Media

The nutritional requirement for optimum growth of plant organ, tissue and protoplast *in vitro* generally vary from species to species even tissues from different parts of plant may have different requirement for satisfactory growth. Therefore no single media as such can be suggested as being entirely satisfactory for all types of *in vitro* culture. In order to formulate a suitable medium for a new system a well known basal medium such as Ms (Murashige and Skoog), B5 (Gamborg et al) etc.

The composition of MS media is given below

Macro salts Concentration

NH_4NO_3 ……	1.65 g
KNO_3 ……	1.90 g
$CaCl_2$. 2 H_2O ……	0.44 g
$MgSO_4.7H_2O$ …….	0.37 g
KH_2PO_4 ……	0.17 g

Micro salts

$FeSO_4.7H_2O$ ……	27.80 mg
Na_2EDTA .2H $_2O$ ……	. 33.60 mg
Kl …….	0.83 mg
H_3BO_3 ……	6.20 mg
$MnSO_4.4H_2O$ …….	22.30 mg
$ZnSO_4.7H_2O$ ……	8.60 mg
Na_2MoO_4 .2H_2O ……	0.25 mg
$CuSO_4$ .5 H_2O ……	0.025 mg
$CoCl_2$ .6H_2O ……	0.025 mg

Organic supplements

Myo-inositol ……	100.00 mg
Nicotinic acid ……	0.05 mg
Pyridoxine HCl ……	. 0.05 mg
Thiamine HCl …….	0.05 mg
Glycine ……	0.20 mg
Sucrose …….	20.00 gm

Growth regulators as per need

Gelling agent (only for solid medium)

Agar …… (0.5-1%) 6-8 g/lit.

pH …… 5.8

By making minor quantitative and qualitative changes a new media can develop to accommodate the specific requirements of the desired plant material.

Preparation of the medium

The most suitable method for preparing media now -a-days is to use commercially available dry powdered media. These media contains all the required nutrients. The powder is dissolved in distilled water generally 10% less than final volume of medium and after adding sugar, agar and other desired supplements. The final volume is made up with distilled H_2O. The pH is adjusted and medium is autoclaved, Another method of preparing media is to prepare concentrated stock solutions by dissolving required quantities of chemicals of high purity in distilled water. Separate stock solution are prepared for different media components

1. Major salts
2. Minor salts
3. Iron
4. Organic nutrients except sucrose

For each growth regulator a separate stock solution is prepared. All the stock solutions are stored in proper glass or plastic containers at low temperature in refrigerators.

Stock solution of Iron is stored in amber coloured bottles. Substances which are unstable in frozen state must be freshly added to the final mixture of stock solution at the time of medium preparation, Contaminated (or) precipitated stock solution should not be used.

The following sequential steps are followed for preparation of media

1. Appropriate quantity of Agar and sucrose is dissolved in distilled water.
2. Required quantity of stock solution, heat stable growth hormones (or) other substances are added by continuous stirring
3. Additional quantity of distilled water is added to make final volume of the medium.
4. While stirring the pH of the medium is adjusted by using 0.1 N NaOH (or) HCl
5. If a gelling agent is used heat the solution until it is clear.
6. Medium is dispensed into the culture tubes, flasks, (or) any other containers.
7. The culture vessels are either plugged with non-absorbent cotton wool rapped in cheese cloth or closed with plastic caps.

8. Culture vessels are sterilized in autoclave at 121oC 15Psi (1.06kg / cm2) for about 15- 20 min
9. Heat labile constituents are added to the autoclaved medium after cooling to 30-40^{0}C under a Laminar airflow cabinet.
10. Culture medium is allowed to cool at room temperature and used or stored at 4^{0}C (1or 2 days).

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TECHNIQUES OF *IN VITRO* CULTURE

In *in vitro* culture, plants are multiplicated by cloning the plants themselves. In such manner, we can obtain a large number of specimens identical to each other and to the specimen introduced *in vitro* at the beginning of the process.

Types of Cultures

1) Callus culture:-

Callus culture may be derived from a wide variety of plant organs roots, shoots, leaves (or) specific cell types. Eg:- Endosperm, pollen. Thus when any tissue (or) cell cultured on an agar gel medium forms an unorganized growing and dividing mass of cells called callus culture.

In culture, this proliferation can be maintained more (or) less indefinitely by subculturing at every 4-6 weeks, in view of cell growth, nutrient depletion and medium drying.

Callus cultures are easy to maintain and most widely used in Biotechnology. Manipulation of auxin to cytokinin ratio in medium can lead to development of shoots or somatic embryos from which whole plants can be produced subsequently.

Callus culture can be used to initiate cell suspensions which are used in a variety of ways in plant transformation studies.

Callus cultures broadly speaking fall into one of the two categories.

1) Compact 2) friable callus

In compact callus the cells are densely aggregated. Where as in friable callus the cells are only loosely associated with each other and callus becomes soft and break a part easily. It provides inoculum to form cell suspension culture.

2) Organ Culture

The organ culture refers to the *in vitro* culture and maintenance of an excised organ primordia or whole or part of an organ in a way that may allow differentiation and preservation of the architecture and/or function.

Importance of Organ Culture:

(i) Organ culture provides an excellent experimental system to define the nutrients and growth factors normally received by the organ from other parts of the plant body and from its external environment.

(ii) Organ culture is particularly valuable in studies of the interdependence of organs for growth hormones and other growth factors.

(iii) Cultured organs may be ideally suited for studying specific problems in morphogenesis and for investigating the sites of biosynthesis of specific metabolites and growth compounds.

(iv) Organ culture also opens up a new avenue for the developments in agriculture and horticulture.

3) Cell Suspension culture

When friable callus is placed into a liquid medium (usually the same composition as the solid medium used for callus culture) and then agitated single cells and / or small clumps of few to many cells are produced in the medium is called suspension culture.

Liquid cultures may be constantly agitated generally by a gyratory shaker of 100-250 rpm to facilitate aeration and dissociation of cell clumps into small pieces.

Suspension cultures grow much faster than callus cultures, need to be sub-cultured at every week, allow a more accurate determination of the nutritional requirement of cells and even somatic embryos.

The suspension culture broadly grouped as A) Batch culture B) Continuous culture

A) Batch culture

A batch culture is a cell suspension culture grown in a fixed volume of nutrient culture medium. Cell suspension increases in biomass by cell division and cell growth until a factor in the culture environment (nutrient or oxygen availability) becomes limiting and the growth ceases. The cells in culture exhibit the following five phases of a growth cycle.

i. Lag phase, where cells prepare to divide

ii. Exponential phase, where the rate of cell division is highest.

iii. Linear phase, where cell division shows but the rate of cells expansion increases.

iv. Deceleration phase, where the rates of cell division and elongation decreases.

v. Stationary phase, where the number and size of cells remain constant.

When cells are subcultured into fresh medium there is a lag phase. It is the initial period of a batch culture when no cell division is apparent. It may also be used with reference to the synthesis of a specific metabolite or the rate of a physiological activity. Then follows a period of cell division (exponential phase). It is a finite period of time early in a batch culture during which the rate of increase of biomass per unit of biomass concentration (specific growth rate) is constant and measurable. Biomass is usually referred to in terms of the number of cells per ml of culture. After 3 to 4 cell generations the growth declines. Finally, the cell population reaches a stationary phase during which cell dry weight declines. It is the terminal phase of batch culture growth cycle where no net synthesis of biomass or increase in cell number is apparent.

In batch culture, the same medium and all the cells produced are retained in the culture vessel (e. g. culture flask 100-250 ml). The cell number or biomass of a batch culture exhibits a typical sigmoid curve. Batch cultures are maintained by sub-culturing and are used for initiation of cull suspensions.

B) Continuous culture:-

These cultures are maintained in a steady state for a long period by draining out the used (or) spent medium and adding the fresh medium. such subculture systems are either closed (or) open type.

i) Closed:-

The cells separated from used medium taken out for replacement and added back to the suspension culture. So that the cell biomass keeps on increasing

ii) Open:-

Both cells and the used medium are takenout from open continuously cultures and replaced by equal volume of fresh medium. The replacement volume is so adjusted that cultures remain at sub-maximal growth indefinitely.

4) Embryo Culture

Embryo culture is a technique in which isolated embryos from immature ovules or seeds are cultured in vitro with an ultimate objective of obtaining a viable plant. Young embryos are removed from developing seeds and are placed on a suitable nutrient medium to obtain seedlings. The cultured embryos generally do not complete development, but germinate prematurely to give rise to seedling. Sometimes, embryos from mature seeds may also be used for embryo culture, e.g., in Iris, Orchids etc. So far, it has not been possible to culture

embryos before a certain stage of development, for example, before the globular stage in the case of barley. But in case of some species, e.g., Brassica, very young embryos of few cells has been successfully cultured. Young embryos need to be isolated with their suspensors intact as it provides gibberellins to the developing embryo. This technique has been employed as a useful tool for direct regeneration in species where seeds are dormant, recalcitrant, or abort at early stages of development. Embryo culture also finds use in the production of inter specific hybrids between in viable crosses, whose seeds are traditionally condemned and discarded because of their inability to germinate. In plant breeding programs, embryo culture goes hand in hand with in vitro control of pollination and fertilization to ensure hybrid production. Besides this, immature embryos can be used to produce embryogenic callus and somatic embryos and or direct somatic embryos.

Types of Embryo Culture:

A. Mature Embryo Culture:

Mature embryos are isolated from ripe seeds and cultured in vitro. Mature embryo cultures are carried out in the following conditions:

i. When the embryos remain dormant for long periods.
ii. Low survival of embryos in vivo.
iii. To avoid inhibition in the seed for germination.
iv. For converting sterile seeds to viable seedlings.

Seed dormancy in plant species is a common occurrence. This may be due to chemical inhibitors or mechanical resistance exerted by the structures covering the embryo. Seed dormancy can be successfully bypassed by culturing the embryos in vitro. Embryo culture is relatively easy as they can be grown on a simple inorganic medium supplemented with energy source (usually sucrose). This is possible since the mature embryos excised from the developing seeds are autotrophic in nature.

B. Embryo Rescue: Embryo rescue involves the culture of immature embryos to rescue them from unripe or hybrid seeds which fail to germinate. This approach is very useful to avoid embryo abortion and produce a viable plant. Wild hybridization involving crossing of two different species of plants from the same genus or different genera often results in failure. This is mainly because the normal development of zygote and seed is hindered due to genetic barriers.Consequently, hybrid endosperm fails to develop leading the abortion of hybrid embryo. The endosperm may also produce toxins that ultimately kill the embryo. In the normal circumstances, endosperm first develops and supports embryo development

nutritionally. Thus, majority of embryo abortions are due to failure in endosperm development. Embryo abortion can be avoided by isolating and culturing the hybrid embryos prior to abortion. The most important application of embryo rescue is the production of interspecific and inter-generic hybrids from wild plant species.

5) Anther/Pollen Culture:

The culture of anthers or isolated micropsores (pollen) to produce haploid plants is known as anther culture or pollen culture. The underlying principle is to stop the development of pollen cell whose fate is normally to become a gamete, i.e. a sexual cell, and to force its development directly into a plant. First time, haploid plants were discovered in *Datura stramonium* by A.D. Bergner in 1921. Guha and Maheshwari (1964) pioneered the formation of embryos from anthers of *Datura innoxia* grown in vitro. After this, haploid plants have been produced via anther culture in more than 170 species. Embryos can be produced via a callus phase (indirect androgenesis) or be a direct recapitulation of the developmental stages characteristic of zygotic embryos (direct androgenesis). Direct androgenesis mimics zygotic embryogenesis; however, neither a suspensor nor an endosperm is present. At the globular stage of development, most of the embryos are released from the pollen cell wall (exine). They continue to develop, and after 4 to 8 weeks, the cotyledons unfold and plantlets emerge from the anthers. Direct androgenesis is primarily found among members of the tobacco (Solanaceae) and mustard (Cruciferae) families. During indirect androgenesis, the early cell division pattern is similar to that found in the zygotic embryogenesis and direct androgenic pathways. After the globular stage, irregular and a synchronous division occur and callus is formed. This callus must then undergo organogenesis for haploid plants to be recovered. The cereals are among the species that undergo indirect androgenesis.

The early cell divisions that occur in cultured anthers have been studied and it has been known that late uninucleate to early binucleate microspores are the best explants for embryogenesis. In this case, the somatic embryos develop into haploid plants. Doubled haploids can then be produced by chromosome-doubling techniques. Thus microspore culture enables the production of homozygous (at every locus) plants in a relatively short period as compared to conventional breeding techniques. These homozygous plants are useful tools in plant breeding and genetic studies. In addition, haploid embryos are used in mutant isolation,

gene transfer, studies of storage product biochemistry, and physiological aspects of embryo maturation.

6) Ovule Culture:

Ovule culture is an elegant experimental system by which ovules are aseptically isolated from the ovary and are grown aseptically on chemically defined nutrient medium under controlled conditions.

An ovule is a mega sporangium covered by integument. Ovules are attached with placenta inside the ovary by means of its funiculus. An ovule contains a megaspore or an egg cell. After fertilization, a single cell zygote is formed which ultimately leads to form a mature embryo possessing shoot and root primordia.

Ovules can be isolated and cultured in nutrient medium. In vitro ovule culture helps to understand the factors that regulate the development of a zygote through organised stages to a mature embryo. Alternatively, it may be possible to germinate pollen in the same culture as the excised ovule and to induce *in vitro* fertilisation and subsequently embryo production.

Application of *in vitro* culture

In vitro tissue culture can be applied in the following are of plant tissue cultures

i. Micropropagation
ii. Embryo rescue/culture
iii. Anther/pollen/ovary/ovule culture
iv. Production of secondary metabolites
v. Genetic Transformations
vi. Somatic hybridization etc.

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MICROPROPAGATION

Introduction

Micropropagation is the clonal propagation of a selected genotype using in vitro culture techniques. It is generally done by culturing apical shoots, auxillary buds and meristematic tissues. This is in fact replacement of vegetative propagation ensuring faster rate of multiplication. George Morel in 1960 produced orchid plants on a commercial scale for the first time. Now this method is used for production of commercially important plants such as banana, apple, strawberry, orchids etc. Murashige in 1974 recognized four stages of micropropagation:

1. Stage I: Establishment of culture
2. Stage II: Multiplication of shoots in culture
3. Stage III: Rooting
4. Stage IV: Hardening of tissue culture raised plants

Stage I - Establishment of culture:

The purpose of stage-I is to initiate axenic culture. This stage begins with the excision of meristem tissue from an identified stock plant. This explant is treated with anti microbial chemicals to remove contaminating organisms. Using aseptic techniques the explants are cultured into appropriate nutrient medium. A rapidly proliferating culture is established under optimum levels of light and temperature. The major constraints in establishing sterile cultures of woody tissues are microbial contamination and interference of phenolic exudates. Use of fungicides and antibiotics limits the microbial infection to some extent. Use of antioxidants like ascorbic acid, PVP and charcoal helps in eliminating the interfering phenolic exudates. Contamination in tissue culture can originate from two sources through carryover of microorganisms on the surface and/or in the tissues of explants or through faulty procedures in the laboratory. For most micropropagation work the explant of choice is an apical or axillary bud. The developmental stage of the explant is an important factor. The explant must be physiologically competent to survive the initial culture and elicit the appropriate response. In general, younger tissues such as terminal or axillary shoot buds regenerate better than older and mature tissues of the same stem. The age of the stock plant, physiological age of the explant and its developmental stage as well as its size can determine the success of a

procedure (Franclet, 1987). In general the ingredients of culture medium in this stage are determined by the kind of response desired e.g. axillary shoot formation or adventitious shoot formation or callusing etc., Supplementation of cytokinins like BA, TDZ in media is desired for axillary shoot formation whereas auxins like NAA, 2,4-D is preferred for callusing. Carbohydrate as carbon source is one of the important component of the plant tissue culture medium. Sucrose is the most commonly used carbon source in the media. Most of the earlier studies strongly suggested the use of sucrose as carbon source. Depending on the mineral requirements of different plant species, several media compositions have been formulated. The most commonly used basal medium is Murashige and Skoog's medium.

Stage II - Proliferation of shoots in culture:

In this stage, shoots are proliferated in culture, which then serves as the source of shoots for subsequent propagation as well as the material that is required to maintain the stock. Multiplication of shoots is achieved by sub culturing the shoot cultures at regular intervals in appropriate medium. Variant plants may arise from the cultures maintained in vitro for a long period. For the purpose of micropropagation, usage of shoots from the cultures maintained for several passages and having higher subculture numbers is avoided to ensure genetic fidelity.

Stage III – Rooting:

In this stage the shoots obtained from multiplication media are separated and shifted to conditions that favor root initiation and shoot elongation. Rooting in shoots can be induced either in vitro or extra vitrum. For rooting in vitro, shoots are cultured either in growth regulator free medium or subjected to pulse treatment of auxin prior to transferring to an auxin free medium for root induction. For ex vitro rooting, shoots are excised as small cuttings (micro cuttings), treated with commercial rooting mixture and planted in soil. These are then placed in a high humidity chamber for rooting. Rooting ex vitro has several advantages:

• It is easier to stick a cutting in soil than to plant a rooted plantlet.

• Labor-intensive in vitro operations of single shoots are avoided.

• The root system produced ex vitro simultaneously establishes in soil.

• Possibility of damaging the roots while transferring the plant to soil is avoided. These damages cause root or stem diseases.

• For difficult-to-root plants it is easier and cheaper to create appropriate conditions for Ex vitro rooting.

Stage IV – Hardening of tissue culture raised plants:

This stage involves transfer of plantlets from aseptic condition to green house and ultimately to the final location (environment). Plantlets develop within culture vessel under aseptic condition, on a medium containing sugar and nutrients to allow heterotrophic growth and in an atmosphere with high relative humidity and low levels of light, these all contribute to a phenotype that cannot survive the environmental conditions when directly placed in green house or field. Thus it is necessary to acclimatize plantlets gradually to ensure survival until they develop new leaves that are more adapted to ambient conditions under which plants are normally grown. High relative humidity has to be maintained during hardening process to protect the plants from desiccation and enable them to initiate new roots and shoots.

Advantages of Micro propagation:

Micro propagation technique is preferred over conventional practice as it has many advantages:

1. Millions of clonal plants can be regenerated by micro propagation in a year which would take years to propagate by conventional methods. The initial plant tissue needed as explants for micropropagation is also very small.
2. Many plant species are highly resistant to conventional bulk propagation practices. Micropropagation helps in bulking up rapidly new cultivars of important traits that would otherwise take many years to bulk up by conventional methods.
3. The in vitro techniques provide a method for speedy international exchange of plant materials. If handled properly, the sterile status of the culture eliminates the danger of disease introduction. Thus the period of quarantine is reduced or unnecessary.
4. The in vitro stock can be quickly proliferated at any time of the year. Also, it provides year round nursery for ornamental fruit and tree species.
5. Production of disease free plants: Meristem tip culture is generally employed in cases where the aim is to produce disease-free plants. It has been demonstrated that shoot apices of virus-infected plants are frequently devoid of viral particles or contain very low viral concentration. Though chemotherapeutic and physical agents have been used for production of virus-free plants but with limited success. In vitro culture has

become the only effective technique to obtain virus free plants in potato, Dianthus, Chrysanthemum, gladiolus etc. from stocks systematically infected not only with virus but with various other pathogens.

6. Seed production: For seed production in some of the crops, a major limiting factor is the high degree of genetic conservation required. In such cases micropropogation (axillary bud proliferation method) can be used. For example, production of F1 hybrid seed lines in crops like onion to provide an alternative to difficult backcrossing methods, and the production of asparagus for producing high quality supermale and female homozygous lines from which desirable all male hybrids can be produced.
7. Germplasm storage: Plant breeding programs rely heavily on the germplasm. Preservation of germplasm is a means to assure the availability of genetic materials as the need arises. Most seeds and vegetative organs have a limited storage life, research on germplasm preservation has concentrated on the development of procedures to extend usable life spans. Since meristem cells are highly cytoplasmic and non-vacuolated, a high percentage of cells can be expected to survive cryopreservation procedures. Meristems are genetically stable and can be regenerated into pathogen free plants. Meristems have been identified as excellent material for germplasm preservation of crop species with seed borne viruses also.
8. Artificial seeds: The concept o artificial or synthetic seeds (i.e. encapsulated embryos) produced by somatic embryogenesis has become popular. The aim of somatic embryo (SE) encapsulation is to produce an analog to true seeds which is based on similarity of SE with zygotic embryo with respect to gross morphology, physiology and biochemistry. Two types of synthetic seeds have been developed, namely hydrated and desiccated.

Disadvantages of micro propagation

Many problems are associated with micro propagation. The major problems are:

1. Extensive requirements and sophisticated facilities with trained manpower are needed.
2. Though precautions of high order are normally taken during culture, but there are chances of contamination by various pathogens. Contamination could cause very high losses in a short time.
3. The problems of genetic variability may be pronounced in some forms of culture. Shoot tip culture tends to remain stable while those system utilize adventitious shoots or multiplication of callus, genetic variability is pronounced. Source of variability

may be due to chimera breakdown, aberrant cell division in callus or cell suspension, epigenetic effects and pre-existing genetic variability.

4. It has been experienced that during repeated cycles of in vitro shoot multiplication, cultures show water soaked almost transluscent leaves. Such shoots exhibit a decline in the rate of growth multiplication, become necrotic and may eventually die. This phenomenon is referred as vitrification or hyperhydration. This is due to morphological, physiological and metabolic dearrangements occurring during intensive shoot multiplication in culture.

   Some preventive measures to reduce hyper hydration includes (i). Increasing concentration of agar. (ii). Overlaying medium with paraffin; (iii). Using dessicant such as $CuSO_4$ (iv) Bottom cooling of the culture vial to improve aeration (v) Lowering cytokinin by another and (vi) Manipulating NH_{4+} or salt concentrations in the culture medium.

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ORGANOGENESIS AND SOMATIC EMBRYOGENESIS AND SYNTHETIC SEED PRODUCTION TECHNOLOGY

Organogenesis

In culture, the explant develops into callus tissue in a medium containing either a particular concentration of auxin or a definite auxincytokinin ratio.

Such medium is known as callus inducing or initiation medium. Proliferation of callus mass in a relatively unorganised way will continue for a prolonged period, if the callus tissue is maintained in the same medium through a number of subcultures. But the main objective in plant tissue culture is to regenerate a plant or plant organ from the callus culture.

The regeneration of plant or plant organ only takes place by the expression of cellular totipotency of the callus tissue. The callus tissue during its growth in callus inducing medium shows an extremely limited expression of totipotency, but in a certain number of plant species, this potentiality can be enhanced and extended by the adjustment of nutritional and hormonal conditions in culture.

Scattered areas of actively dividing cells, known as meristematic centres, develop as a result of differentiation and their further activity results in the production of root and shoot primordia. Skoog and his co-workers at Wisconsin, in their studies with tobacco stem pith culture, demonstrated that the initiation and the type of organ primordia formed from the resulting callus culture could be controlled by appropriate adjustment of the relative levels of the auxins and cytokinins.

With high auxin—low cytokinin roots develop, with low auxin—high cytokinin shoot buds develop; at intermediate levels undifferentiated callus tissue develops (Skoog and Miller, 1957). The expanded expression of totipotency of the callus tissue offers considerable potential for tissue culture technique as it is possible to grow the root or shoot or both. The production of adventitious roots and shoots from cells of tissue culture is called organogenesis.

Somatic embryogenesis

Development of embryo- like structures from somatic tissues, which can develop into whole plants is called somatic embryogenesis. The initiation and development of embryos from somatic tissues in plant culture was first recognized by Steward *et. al.,* (1958) and Reinert (1958,1959) in cultures of *Daucus carota*. Somatic embryos may develop from sequential sages of somatic embryo development. Somatic embryos may develop from a single cell or

from group of cells. Repeated cell divisions may lead to the production of a group of cells that develop into an organized structure known as a "globular –stage embryo". Further development results in heart and torpedo-stages. Polarity is established early in embryo development. Signs of tissue differentiation become apparent in the globular stage and apical meristems are apparent in heart-stage embryos. There are two routes of somatic embryogenesis:

1. Direct embryogenesis: The embryo is formed directly from the explants tissue without the production of intervening callus. This occurs through pre-embryogenic determined cells (PEDC), where the cells are committed to embryonic development and need only to be released. Such cells are found in embryonic tissues (e.g scutellum of cereals), certain tissues of young in vitro grown plantlets (e.g. hypocotyls in *Daucus carota*, *Ranunculus scleratus*, *Linum usitatissimum*, *Brassica napus*), nucellus and embryo-sac (within ovules of mature plants).

2. Indirect embryogenesis: In indirect somatic embryogenesis, callus is first produced from the explants. Embryos can then be produced from the callus tissue or from a cell suspension produced from the callus. A callus can be established from explants from a wide range of carrot tissues by placing the explants on solid medium (e.g Murashige and Skoog) containing 2,4-D ($1mgl^{-1}$). This callus can be used to produce a cell suspension by placing it in agitated liquid MS medim containing 2,4-D ($1mgl^{-1}$). This cell suspension can be maintained by repeated subculturing into 2,4-D containing medium. Removal of the old 2,4-D containing medium and replacement with fresh medium containing abscisic acid($0.025\ mgl^{-1}$) results in the production of embryos.

SYNTHETIC SEED AND THEIR SIGNIFICANCE

Artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under in vitro or ex vitro conditions, and that retain this potential also after storage is known as synthetic seeds Earlier, synthetic seeds were referred only to the somatic embryos that were of economic use in crop production and plant delivery to the field or greenhouse. In the recent past, however, other micropropagules like shoot buds, shoot tips, organogenic or embryogenic calli, etc. have also been employed in the production of synthetic seeds. Thus, the concept of synthetic seeds has been set free from its bonds to somatic embryogenesis, and links the term not only to its use (storage and sowing) and product (plantlet) but also to other techniques of micropropagation like organogenesis and enhanced axillary bud proliferation system. Implementation of synthetic seed technology requires manipulation of in vitro culture systems for large-scale production of viable materials that are able to convert into plants, for encapsulation. Somatic embryogenesis organogenesis and enhanced axillary bud proliferation systems are the efficient techniques for rapid and large scale in vitro multiplication of elite and desirable plant species. Through these systems a large number of somatic embryos or shoot buds are produced which are used as efficient planting material as they are potent structures for plant regeneration either after having minor treatment or without any treatment with growth regulator(s). Because the naked micropropagules are sensitive to desiccation and/or pathogens when exposed to natural environment, it is envisaged that for large scale mechanical planting and to improve the success of plant (in vitro derived) delivery to the field or greenhouse, the somatic embryos or even the other micropropagules useful in synthetic seed production would necessarily require some protective coatings. Encapsulation is expected to be the best method to provide protection and to convert the in vitro derived propagules into ‘synthetic seeds’ or ‘synseeds’ or ‘artificial seeds’. The encapsulation technology has been applied to produce synthetic seeds of a number of plant species belonging to angiosperms and gymnosperms. Nevertheless, their number is quite small in comparison to the total number of plant species in which in vitro regeneration system has been established. Production of artificial seeds has unravelled new vistas in plant biotechnology. The synthetic seed technology is designed to combine the advantages of clonal propagation with those of seed propagation and storage. To mimic the natural seeds, embryos from cultures are encapsulated in a nutrient gel containing essential organic/inorganic salts, carbon source, plant hormones and antimicrobial agents and coated

completely to protect the embryos from mechanical damages during handling and to allow the development and germination to occur without any undesirable variations. Several agents have been attempted for encapsulation and sodium alginate complexing with calcium chloride is found to be the most suitable. By this method, two types of synthetic seeds are prepared: hydrated and desiccated.. The desiccated synthetic seeds are produced from somatic embryos either naked or encapsulated in polyoxyethylene glycol (Polyoxr) followed by their desiccation. Desiccation can be achieved either slowly over a period of one or two weeks sequentially using chambers of decreasing relative humidity, or rapidly by unsealing the petri dishes and leaving them on the bench overnight to dry. Such types of synseeds are produced only in plant species whose somatic embryos are desiccation tolerant. On the contrary, hydrated synthetic seeds are produced in those plant species where the somatic embryos are recalcitrant and sensitive to desiccation. Hydrated synthetic seeds are produced by encapsulating the somatic embryos in hydrogel capsules.

The production of synthetic seeds for the first time by Kitto and Janick involved encapsulation of carrot somatic embryos followed by their desiccation. Of the various compounds tested for encapsulation of celery embryos, Kitto and Janick selected polyoxyethylene which is readily soluble in water and dries to form a thin film, does not support the growth of micro-organisms and is non-toxic to the embryo. Janick et al. have reported that desiccated artificial seeds were produced by coating a mixture of carrot somatic embryos and callus in polyoxyethylene glycol. The coating mixture was allowed to dry for several hours on a Teflon surface in a sterile hood. The dried mixture was then placed on a culture medium, allowed to rehydrate, and then scored for embryo survival. The Plant Cell Culture Technology Group of Nuclear Agriculture and Biotechnology Division had initiated research on synthetic seeds in the late 1980s working with sandalwood and mulberry. Eventually other crop systems such as banana, cardamom and rice have also been taken up for the production of synthetic seeds.

In general, the method used in synthetic seed production is as follows: The propagules (embryos/axillary buds/shoot tips) are carefully isolated from aseptic cultures and blots dried on filter paper, and are then mixed in sodium alginate prepared in nutrient medium. The propagules are then picked up manually by forceps and dropped into a solution of calcium chloride for 40 minutes. After the incubation period, the beads (synthetic seeds) are recovered by decanting the calcium chloride solution and washing them in sterile water 3 to 4 times

before culturing on nutrient medium or on different substrates such as filter paper, cotton or soil for their growth and conversion to plants.

In 1984 Redenbaugh et al. developed a technique for hydrogel encapsulation of individual somatic embryos of alfalfa. Since then encapsulation in hydrogel remains to be the most studied method of artificial seed production. A number of substances like potassium alginate, sodium alginate, carrageenan, agar, gelrite, sodium pectate, etc. have been tested as hydrogels but sodium alginate gel is the most popular. Hydrated artificial seeds consist of somatic embryos individually encapsulated in a hydrogel. To produce hydrated synthetic seeds, the somatic embryos are mixed with sodium alginate gel (0.5–5.0% w/v) and dropped into a calcium salt solution [$CaCl_2$ (30–100 mM), Ca $(NO_3)_2$ (30–100 mM)] where ion-exchange reaction occurs and sodium ions are replaced by calcium ions forming calcium alginate beads or capsules surrounding the somatic embryos. The size of the capsule is controlled by varying the inner diameter of the pipette nozzle. Hardening of the calcium alginate is modulated with the concentrations of sodium alginate and calcium chloride as well as the duration of complexing. Usually 2% sodium alginate gel with a complexing solution containing 100 mM Ca^{2+} is used and is found to be satisfactory. However it is found that for the production of synthetic seeds of carrot, 1% sodium alginate solution, 50 mM Ca^{2+} and 20–30 min time period were satisfactory for proper hardening of calcium alginate capsules. Use of a dual nozzle pipette in which the embryos flow through the inner pipette and the alginate solution through the outer pipette results in positioning of the embryo in the centre of the beads for better protection.

Despite the fact that the technology is an exciting and rapidly growing area of research in plant cell and tissue culture, practical implementation of the technology is constrained due to the following main reasons:

- Limited production of viable micropropagules useful in synthetic seed production.
- Anomalous and asynchronous development of somatic embryos.
- Improper maturation of the somatic embryos that makes them inefficient for germination and conversion into normal plants.
- Lack of dormancy and stress tolerance in somatic embryos that limit the storage of synthetic seeds.

- Poor conversion of even apparently normally matured somatic embryos and other micro propagules into plantlets that limit the value of the synthetic seeds and ultimately the technology itself.
- Facilities required are costly and economic considerations may not justify their use in commercial propagation of many kinds of plants.
- Errors in maintenance of identity, introduction of an unknown pathogen, or appearance of an unobserved mutant may be multiplied to very high levels in a short time.

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SOMATIC HYBRIDIZATION AND CYBRIDS

Introduction

Protoplast fusion and somatic hybridization techniques provide the opportunity for bypassing reproductive isolation barriers, thus facilitating gene flow between species. Fusion of protoplasts is accomplished by the use of PEG [poly (ethylene glycol)]. Protoplast fusion has helped in the development of somatic hybrids or cybrids (cytoplasmic hybrids). Protoplasts offer the possibility of efficient and direct gene transfer to plant cells. DNA uptake has been found to be easier in protoplasts than into intact plant cells.

Purified protoplasts once obtained from any two different sources (can be different tissues, different plants or species or different genera), they can be fused together to form somatic hybrids. This non-conventional method of genetic recombination involving protoplast fusion under in vitro conditions and subsequent development of their product to a hybrid plant is known as somatic hybridization. First, somatic hybrid plant of *Nicotiana glauca*(+) *N. langsdorfii* was reported by Carlson in 1972. Protoplasts can be induced to fuse by variety of fusogens or electrical manipulations which induce membrane instability. Most commonly reported fusion inducing agents are sodium nitrate (used by Carlson), high pH/ Ca^{2+} concentration and Polyethylene glycol (PEG) treatment. Sodium nitrate treatment results in low frequency of heterokaryon formation, high pH and high Ca^{2+} concentration suits few plant species whereas PEG is the most favored fusogen for its reproducible high frequency of heterokaryon formation and low toxicity. However, treatment with PEG in presence of high pH/ Ca^{2+} is reported to be most effective in enhancing heterokaryon formation and their survivability.

A more selective, simpler, quick and non toxic approach is electrofusion which utilizes electric shock or short pulse of high voltage to promote membrane fusion between two cells. Many useful somatic hybrid plants produced by electrofusion have been reported like Nicotiana plumbaginifolia(+) *N tobacum*, *Solanum tuberosum*(+) *S. charcoense* (resistant to Colorado potato beetle).

Somatic Hybrids:

Protoplast population following fusion treatment is heterogeneous mixture of unfused parents, homokaryons, heterokaryons, fused protoplasts with independent two nuclei etc thus necessitating selection of stable hybrids. Therefore, some identification and selection system should be incorporated into each parental cell line before fusion. Usually, two parental cell

lines with differing requirements as selective screens make selection of hybrid convenient as only those fused cells that possess complementary traits of both parents will thrive. Resistance to antibiotics, herbicides and ability to grow on specific amino acid analogs are few of the selection methods used. Besides these, two different vital stains which do not affect viability of cells like fluorescein isothiocynate (FITC) and rhodomine isothiocyanate (RITC) have been successfully used. Under fluorescent microscope, FITC stained cells appear green and RITC stained protoplasts appear red whereas fused cells having both RITC and FITC fluoresce yellow.

Cybrids:

Cybrids or cytoplasmic hybrids are cells or plants containing nucleus of one species but cytoplasm from both the parents. Cybrids are produced in relatively high frequency by either irradiating (with X-rays or gamma rays) the protoplasts of one species prior to fusion in order to inactivate its nuclei or by preparing enucleate protoplasts of one species and fusing them with normal protoplasts of the other species.

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APPLICATION OF SOMATIC HYBRIDS AND CYBRIDS IN CROP IMPROVEMENT

Genetic recombination in asexual or sterile plants: Protoplast fusion has overcome the impediment of reproduction in haploid, triploid and aneuploid plants. Also, genomes of asexually reproducing plants can be recombined using this approach viz. protoplasts isolated from dihaploid potato clones have been fused with protoplasts of *S. brevidens* to produce hybrids of practical breeding value.

Genetic recombination between sexually incompatible species: The incompatibility barriers in sexual recombination at interspecific or intergeneric levels are also overcome by somatic hybridization. Generally, somatic hybrids are used for transfer of useful genes such as disease resistance, abiotic stress resistance or genes of industrial use for e.g. Datura hybrids (*D. innoxia*+ *D discolor*, *D. innoxia*+ *D stramonium*) show heterosis for scopolamine (alkaloid) content which is 20-25% higher than in parent species and therefore has industrial application.

Cytoplasm transfer: Somatic hybridization minimizes the time taken for cytoplasm transfer to one year from 6-7 years required in back cross method. Also, this method allows cytoplasm transfer between sexually incompatible species. Cybrids have cytoplasm from both parents but nucleus of only one. Nucleus of other parent is irradiated. This approach has been potentially used to transfer two desirable traits – cytoplasmic male sterility (CMS) and resistance to atrazine herbicide, both coded by cytoplasmic genes in Brassica to different crops like tobacco, rice etc. Cybrids also provide opportunity for combining mitochondria of one species with chloroplasts of another species, and of generating recombinant organelles, especially mitochondria. This may be an important objective in case of several alloplasmic lines in which one organelle confers desirable characters, while the other has deleterious effects. An alloplasmic line has nucleus of one species and cytoplasm from a different species. For example *Brassica napus* alloplasmic lines (produced sexually by repeated back crossing) carrying radish (*Raphanus sativus*) cytoplasm (*Oagura cytoplasm*) are male sterile (due to mitochondrial genome) but show chlorophyll deficiency under low temperature (due to chloroplast genoe). Cybrids were produced between CMS (*Oagura cytoplasm*) *B. napus* and normal male fertile *B. napus*. Some of the cybrid regenerants were male sterile but did

not show chlorophyll deficiency uder low temperature, these cybrids contained radish mitochondria and *B. napus* chloroplasts.

Genetic Transformation: Protoplasts can take up micro-organisms, cytoplasmic organelles, isolated chromosomes and naked or plasmid DNA etc. They can be used for genetic transformation by PEG or liposome mediated deliveries or by electroporation.

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SOMACLONAL VARIATION AND CRYOPRESERVATION

Plants derived from tissue culture has been variously referred to as somaclones or calliclones or protoclones and the variations displayed by such plants are simply called 'somaclonal variation'. Variants obtained using callus cultures are referred as "**Calliclones**" (Skirvin, 1978) while variants obtained using protoplast cultures are known as "**Protoclones**" (Shepard et al. 1980). According to Larkin and Scowcroft (1986), 'somaclonal variation is the genetic variability which is regenerated during tissue culture.'

Accordingly, the plants derived from cell and tissue cultures are termed as '**somaclones**', and the plants displaying variation as 'somaclonal variants'. Another term suggested by Evans et al. (1984) as '**gametoclonal variation**' for those variations arising in cell cultures of gametic origin like, in pollen and microspores cultures, to distinguish them from somatic cell derived regenerants. However, generally the term somaclonal variation is used for genetic variability present among all kinds of cell/plants obtained from cell cultures *in vitro*. Plants regenerated from tissue and cell cultures show heritable variation for both qualitative and quantitative traits. Several useful somaclonal variants have been obtained in large number of plant species such as, potato, sugarcane, banana, tomato etc. Chaleff (1981) labeled plants regenerated from tissue cultures as R_0 generation and their successive sexual generations as R_1, R_2 and so on.

Variations for karyotype, isoenzyme characteristics and morphological variations in somaclones have been commonly observed. Such variations manifest themselves as heritable mutation and persist in the plant population even after transplantation to the field.

Sometimes, phenotypic variations may arise in the progeny of plants regenerated from culture, but after the transplantation to the field, the plants exhibit the parental characteristic during their further growth and development. Such variations are not considered as somaclonal variations.

Steps Involved in Somaclonal Variation

I. Growth of callus or cell suspension cultures for several cycles.
II. Regeneration of a large number of plants from such long term cultures.
III. Screening for desirable traits in the regenerated plants and their progenies. For example, *invitro* selection to select agronomically desirable somaclones for tolerance

to various biotic and abiotic stresses, herbicides, high salt concentration and extremes of temperature.

IV. Testing of selected variants in subsequent generations for desirable traits.

V. Multiplication of stable variants to develop new breeding lines.

Characteristics of Somaclonal variants

I. It must involve useful characters.

II. It should be superior to the parents in the character(s) in which improvement is sought.

III. The improved character(s) must be combined with all other desirable characters of the parent, and

IV. The variations must be inherited stably through successive generations by chosen means of propagation.

Mechanisms of Somaclonal Variation:

The variations could also arise in tissue culture due to physiological changes induced by the culture conditions. Such variations are temporary and are caused by **epigenetic changes**. These are non-heritable variations and disappear when the culture conditions are removed. The somaclonal variation may be attributed to either:

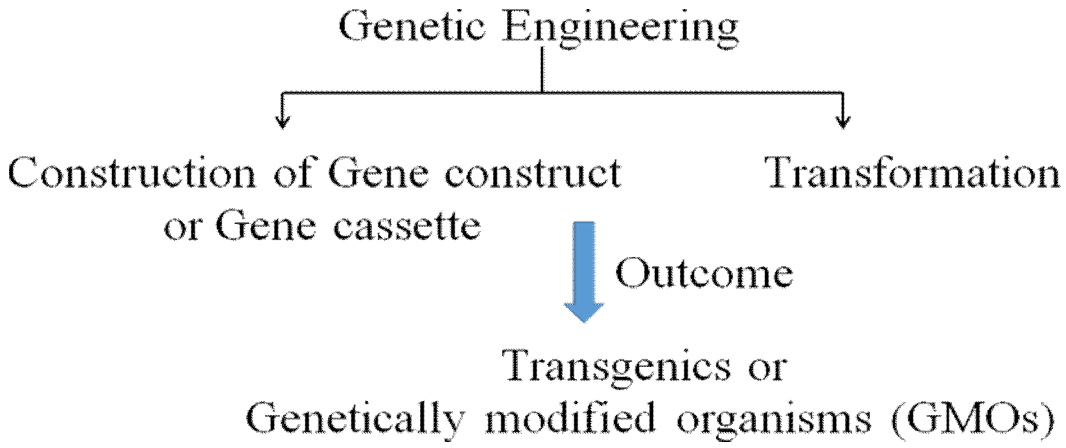
(i) Pre-existing variation in the somatic cells of the explant (genetic)

(ii) Variation generated during tissue culture (epigenetic). Often both factors may contribute.

iii) Several molecular basis for somaclonal variation have been proposed, which include changes in chromosome number, point mutations, somatic crossing over and sister chromatid exchange, chromosome breakage and rearrangement, somatic gene rearrangement, DNA amplification, changes in organelle DNA etc.

GENETIC ENGINEERING

- Genetic engineering is the process by which the genome of an organism can be modified using recombinant DNA (rDNA) technologies.
- The transfer of specifically constructed gene assemblies using transformation techniques is called Genetic Engineering.



- **Gene :** A gene can be defined as the fragment of DNA (or RNA in case of virus) that contains coding and noncoding sequences joined together with regulatory sequences.

Or

A gene can be defined as the fragment of DNA (or RNA in case of virus) that controls a discrete hereditary characteristic, usually corresponding to a single protein or RNA. This includes the entire functional unit, encompassing coding (exons) and noncoding sequences (introns and regulatory sequences).

- Coding sequences (CDS): CDS codes for amino acid or functional RNA molecules like rRNA & tRNA.
- Regulatory sequences: A DNA sequence responsible for regulating gene expression. Regulatory sequences contains:

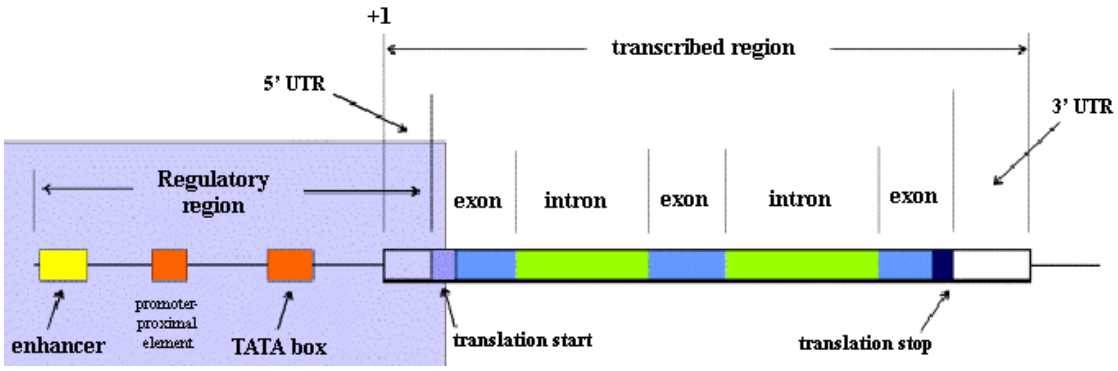


Figure 1. Schematic diagram of a typical gene.

Basic Definitions:

Transcription start:	Base 1 of the primary transcript
Transcription stop:	Where the RNA polymerase falls off the DNA
Translation start:	The AUG (Methionine) codon
Translation stop:	The termination codon (TAA, TGA, TAG)
Polyadenylation site:	3' end of the mRNA
5' untranslated region:	Between base 1 and the AUG, often called the leader
3' untranslated region:	Between the termination codon and polyadenylation site
5' flanking region:	From -1 continuing upstream in a 5' direction
3' flanking region: in 3' direction	Usually from polyadenylation site continuing downstream
Regulatory region:	Refers to enhancer and promoter

- **Promoter -** DNA sequence which determines the site of transcription initiation for a RNA polymerase and is required for basal transcription. Most promoters contain several sequences or motifs that contribute to promoter function. For example, the CCAAT box and the TATA box.

- **Enhancer** - A type of regulatory sequence in eukaryotic DNA (rarely in prokaryotes) that may be located at a great distance upstream or downstream from the promoter which it

influences. Binding of specific proteins to an enhancer stimulates or decreases (silencer) the rate of transcription of the gene. Features thought to be unique to enhancers are that they function in either orientation and can function 5', 3' or within the gene. Enhancers work by binding transcription factors, of which we will see much more in future lectures. Transcription factors either have activating or repressive domains, or recruit activator or repressor proteins, which in turn influences gene expression.

- Exons and introns which represent the coding and noncoding regions are present in a eukaryotic gene. Introns are absent in prokaryotes. The introns are removed by splicing and the exons are translated in tandem to yield the functional polypeptide that further undergoes post translational modification to become functional. These functional polypeptides (proteins) are targeted to various organelles in the cell or exported out of the cell for carrying out various intracellular and extracellular processes respectively.

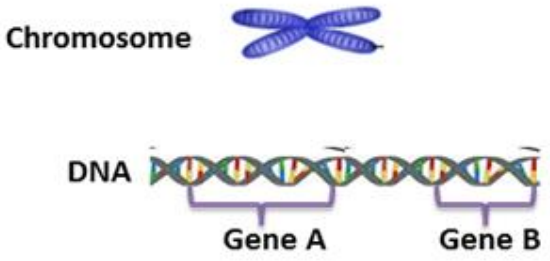


Figure 2. Organization of genes in DNA of a chromosome

Genome: Genome is the complete set of genetic information of a cell or an organism; in particular, the complete sequence of DNA/RNA that carries this information. In diploid organisms, it refers to the haploid set of chromosomes present in a cell. Depending on its localization, genome may be nuclear or organellar.

Organellar genomes are of two types: mitochondrial and chloroplast genome. Genome size of organisms differs significantly between different species. The size of the genome governs the size and complexity of an organism. However, many small sized organisms, in fact have bigger genomes than their larger counterparts.

Various organisms have different sized genome as can be seen in the table below.

Species	Organism	Genome Size (Mb)
Rice	Plant	430
Triticum aestivum	Plant	16000
Homo sapiens	Mammal	3200
Arabidopsis thaliana	Plant	125
Drosophila melanogaster	Insect	180
Caenorhabditis elegans	Nematode worm	97
Saccharomyces cerevisiae	Yeast	12.1
Escherichia coli	Bacterium	4.64
Haemophilus influenzae	Bacterium	1.83

Table 1. Genome size of various organism.

The genome contains all the genes present in the nucleus of a cell. Gene varies in size from a few hundred DNA/RNA bases to more than few thousand bases. The haploid set of chromosome contains the total genome of the organism.

The bacterium *Mycoplasma genitalium* has a small genome size of 0.58Mb and the plant *Triticum aestivim* has a large genome size of 16000Mb. The genome size in human is 3200Mb.

Role of genes within cells:

a) Genes contain the instructions for each cell to make proteins and RNAs.
b) Within the cell the DNA performs act as information repository including instructions in making the component molecules of the cells.
c) Pass on the information to the next generation.
d) The mere presence of DNA does not implicate a cell to be alive and functional. Mammalian red blood cells (RBCs) discard nucleus during developmental process and thus lacks DNA in mature state.
e) Genes are transcribed to RNA which are processed to various forms like mRNA, tRNA, rRNA etc. mRNA are translated to proteins depending on the regulatory signals. tRNA and rRNA serve as the components of translational machinery.

f) New functions of RNA are also being discovered like regulatory (miRNA, siRNA etc) and catalytic (ribozymes) functions.

g) Proteins are structural components of cells, enzymes, hormones, various signalling molecules, receptors and other factors which are involved in performing the chemistry of life and are essential for the normal body function, for example, sugar conversion to energy and metabolite (small molecules) production in cell.

Genetic code:

a) The genetic code (Table 1) is the set of instructions that translates the information encoded in genetic material (mRNA or DNA sequences) into proteins (amino acid sequences) by living cells.

b) The genetic code is a triplet code (i.e. a group of three adjacent nucleotides) called codon. This three nucleotide codon in nucleic acid sequence specifies a single amino acid.

c) Epigenetic effects, however is not stored using the genetic code.

d) These codons are always written with the 5′-terminal nucleotide to the left.

e) The code is unambiguous i.e. each triplet specifies only a single amino acid.

f) The genetic code is degenerate i.e. more than one triplet codon can code for a single amino acid. (61 codons code for 20 amino acids)

g) Three codons do not specify any amino acid but acts as terminal sites (stop codons), signalling the end of protein coding sequence. They are namely UAA (Ochre), UAG (Amber) and UGA (Opal).

h) AUG is an initiation codon that signals the initiation of translation and also codes for methionine. In some mRNA, GUG and UUG also act as initiation codon.

The genetic codes for each 20 amino acids were defined by pioneering works of Marshall W. Nirenberg and Har Gobind Khorana in 1964.

First letter →	Second letter → U	Second letter → C	Second letter → A	Second letter → G	Third letter →
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

Table 2. Genetic code.

Gene expression: The genes contain the information needed to make functional molecules called proteins. However, some genes produce other molecule (tRNA, rRNA, microRNA etc) that assist or regulates the protein expression and assembly. These complex events within each cell consist of two main steps: transcription and translation. In prokaryotes where there is no nuclear membrane, both transcription and translation occur in the cytoplasm whereas in eukaryotes, transcription occurs inside the nucleus and the translation occurs in the cytoplasm.

Central Dogma: The 'Central Dogma' is the process by which the instructions in DNA are converted into a functional product. It was first proposed in 1958 by Francis Crick, discoverer of the structure of DNA.

The central dogma (Figure) of molecular biology explains the flow of genetic information, from DNA to RNA, to make a functional product, a protein.

The central dogma states that the pattern of information that occurs most frequently in our cells is:

a) From existing DNA to make new DNA (DNA replication)
b) From DNA to make new RNA (transcription)
c) From RNA to make new proteins (translation)

- Reverse transcription is the transfer of information from RNA to make new DNA, this occurs in the case of retroviruses, such as HIV. It is the process by which the genetic information from RNA is assembled into new DNA.

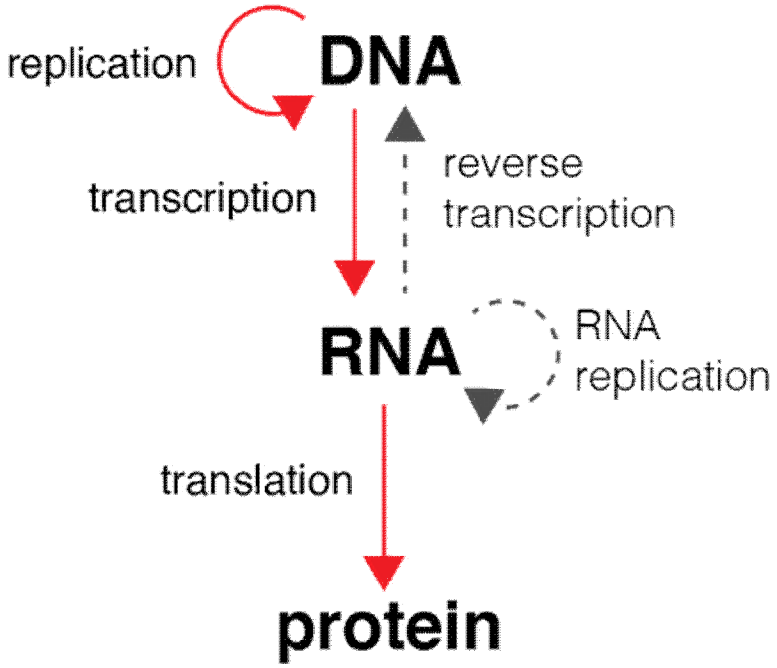


Figure 3. Central dogma of life

Types of gene expression:

Constitutive expression: Housekeeping genes are essential and necessary for sustaining life, and are therefore continuously expressed. E.g. *Actin, Ubiquitin* e.t..c. are plant housekeeping genes which are expressed throughout the development.

Induction and repression: The expression levels of some genes fluctuate in response to external signals. Also, under a certain situation, some genes show higher expression level, while others show lower expression levels. The former is called induced expression and the latter is called repressed expression.

Regulatory elements:

Gene expression is a complex multi-step process. Amongst various steps involved, transcription initiation is a vital key point in controlling gene expression.

Fundamental elements that regulate the process of transcription are:

a) cis-acting elements comprising of special DNA sequences.
b) trans-acting elements comprising of regulatory proteins.
c) DNA –protein interaction.
d) Protein-protein interaction
e) RNA polymerase

cis-acting elements: In Latin “cis”, means "same side as". Cis acting elements are thus a region of DNA or RNA that regulates the expression of genes located on the same molecule. The cis-regulatory elements are often binding sites for one or more trans-acting factors. Cis-element may be located upstream to the coding sequence of the gene it controls (in the promoter region or further upstream 5'), in an intron, or 3' to the gene's coding sequence, either in the untranslated or untranscribed region.

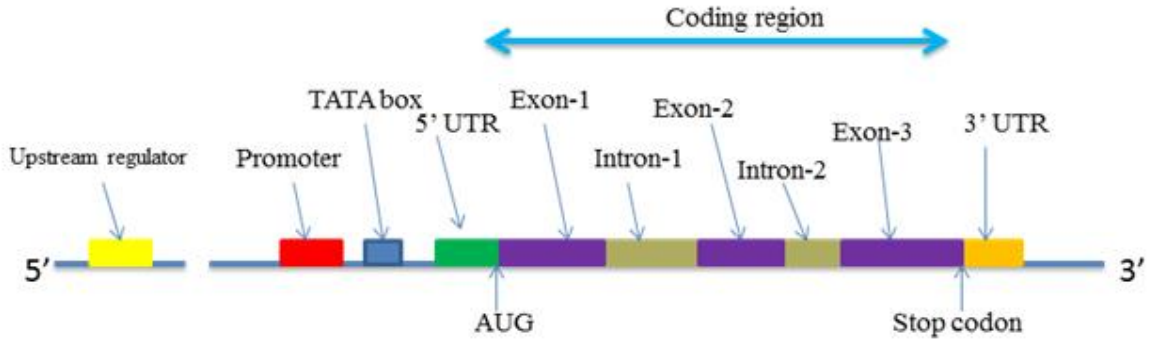


Figure 4. Structure of eukaryotic gene with upstream regulatory elements

Examples of cis-acting elements:

Prokaryotic systems:

Promoter is a DNA sequence where RNA polymerase binds to initiate transcription. There are two promoter sequences in prokaryotic systems known as -10 (Pribnow box or Pribnow-Schaller box) and -35 sequences.

Operator is regulatory sequence of DNA located immediately upstream of the structural gene that controls transcription of an operon.

Inducers are located upstream of promoter region.

Downstream regulatory sequences comprise of GC-rich inverted repeats followed by four adenine (A) residues signal the termination of transcription.

Trans-acting elements:

The protein factors which regulate the expression of gene by binding to cis acting DNA sequence are termed as trans-acting elements. Trans-acting molecules generally have two domains: DNA binding domain (which binds to cis elements) and protein binding domain (required for activation or suppression of transcription). Transcription initiation is a tightly regulated process controlled by trans-acting elements both in prokaryotes as well as eukaryotes.

DNA – Protein interactions

Gene regulatory proteins and the transcription factors are capable of binding to the DNA based on the interaction of amino acids of the protein with the nucleotides of the DNA. The regulation is implemented through various interactions between cis-acting elements and trans-acting factors. Examples of some DNA binding proteins are:

- Eukaryotic TATA-binding protein
- σ subunit of bacterial RNA polymerase etc.

There are four types of structures of DNA binding proteins:

- Zinc finger proteins
- Helix loop Helix protein
- Leucine zipper proteins
- Homeodomain proteins

Recombinant DNA (rDNA):

Single chimeric DNA formed by combining two or more different fragments of DNA from diverse organisms is generally called as recombinant DNA (rDNA) and the method applied to create recombinant DNA is called recombinant DNA technology.

The organism, from which the candidate DNA is isolated, is called **Donor organism**. The organism which will accept the foreign gene is called **Host organism**. Genetic material from one organism is selected and then artificially introduced to a host organism. if the foreign recombinant DNA integrates into the host genome, it gets replicated along with the genome and then express the foreign protein. Paul Berg, Herbert W. Boyer and Stanley N. Cohen are the pioneers of recombinant DNA technology (early 1970). A hybrid of the *SV40* mammalian DNA virus genome and phage λ was one of the recombinant DNA molecules to be first engineered.

Methods involved in Recombinant DNA Technology:

Molecular cloning is a process for creating recombinant DNA and generally involves the following steps:

(1) Selection of a cloning vector
(2) Selection of a host organism
(3) Preparation of a vector DNA
(4) Preparation of DNA to be cloned (insert)
(5) Creation of recombinant DNA vector (having foreign DNA)
(6) Introduction of recombinant vector into host organism
(7) Selection of recombinant clones (clone having desired insert)
(8) Screening and multiplication of recombinant clones

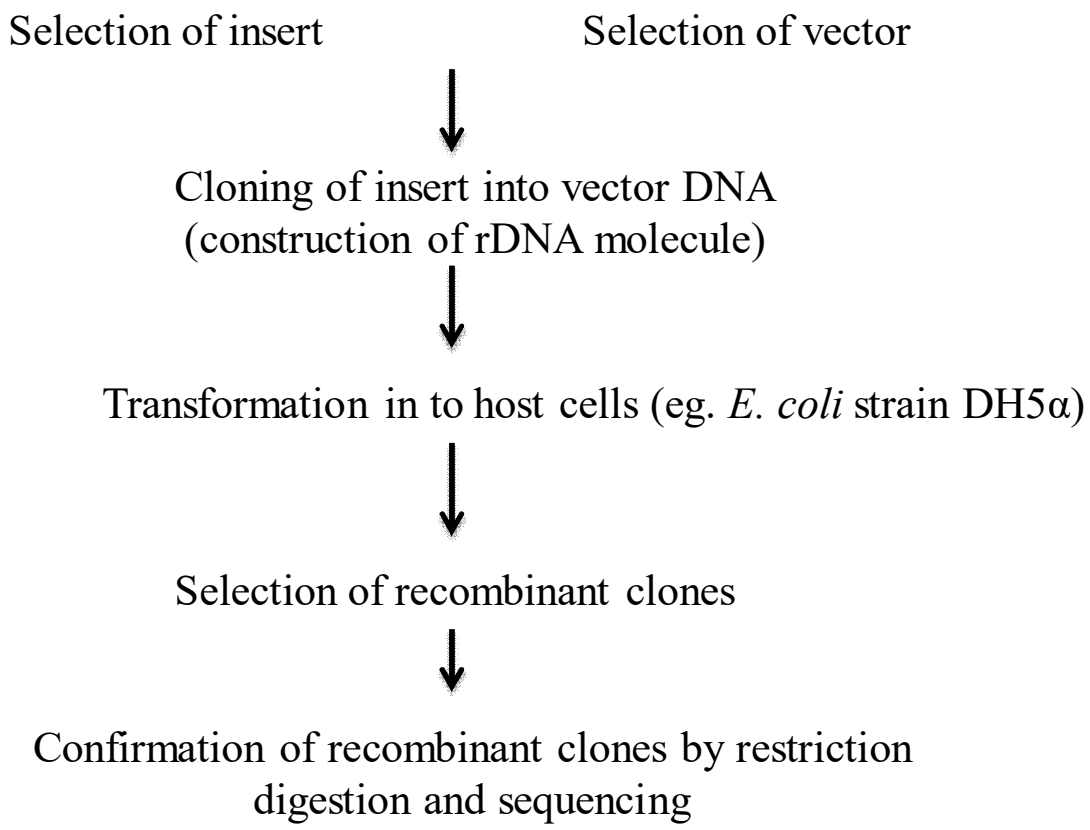


Figure 5. Steps in Gene Cloning.

Choice of host organism:

a) A good host should have the following properties:

b) Easy to grow and transform.

c) Do not hinder replication of recombinant vector.

d) Do not have restriction and methylase activities.

e) Deficient in recombination functions so that the introduced recombinant vector is not altered.

f) Easily retrievable from the transformed host.

g) Various hosts are used in rDNA technology depending on the goal: For example bacteria, yeast, plant cells, animal cells, whole plants and animals.

Prokaryotic systems such as *E. coli* are commonly used due to various advantages like, they have-

a) Easy to culture/multiply
b) Easy to transform,
c) Easy to isolate
d) Well studied expression system,
e) Compact genome,
f) Versatile,
g) Widely available, and
h) Rapid growth of recombinant organisms with minimal equipment.

Only **disadvantage** is that they lack post-translational modification (PTMs) machinery required for eukaryotic proteins.

Eukaryotic systems are difficult to handle in contrast to bacterial hosts. They are favoured for expression of recombinant proteins which require post translational modification and only if they can grow easily in continuous culture.

----------**X**----------

DIRECT AND INDIRECT METHODS OF GENE TRANSFER IN PLANT

Introduction

Plants are important sources of many important products, such as food, fibers, medicines, energy which benefits the need of human beings. Human has been cultivating these plants to meet their desired products. Theses selective plants were improving for better quality and quantity of product by breeding to meet the need of growing human population on Earth. The plant breeders are depended on the existing gene pool and sexual compatibility of the plant species, which is a limitation.

The genetic material of species were altered by the incorporation of the foreign DNA following the molecular biology methods are called transformation and if these techniques performed directly or indirectly in the plant cells are called plant genetic transformation. In the year 1981, the first successful gene transformation was demonstrated in the tobacco plant using the soil bacterium *Agrobacterium tumefaciens*. Until now more than 100 different plant species has been transformed with the desired foreign genes using *A. tumefaciens* or other available methods.

The plant genetic transformation has become a versatile method for the production of agricultural and medicinal value product for benefits of human society. The transfers of gene in to plants cells are difficult because plant cells are impermeable which acts as barrier to diffuse through cell membrane. Due to diversity of plant species and their diverse genotypes variety of gene transformation methods has been developed to overcome the barrier in plants.

PLANT GENETIC TRANSFORMATION METHODS

The various available plant transformation methods has been discussed in this chapter such as biological methods; *Agrobacterium tumefaciens* and protoplast mediated transformation, Chemical methods; calcium phosphate, co-precipitation and lipofection, physical methods, electroporation, biolistics, agitation with glass beads, vacuum infiltration, silicon carbide whisker, laser microbeams, ultrasound, and shock-wave mediated.

The plant genetic transformations are classified into direct and indirect gene transfer methods.

Indirect gene transfer methods

For indirect gene transformation methods, two bacterial strain, *Agrobacterium tumefaciens*, or *Agrobacterium rhizogenes*, has been discover to transfer the desired gene into plant cells however *Agrobacterium tumefaciens* is widely used.

Agrobacterium tumefaciens

Among the various available vectors for plant transformation the Ti plasmid of *Agrobacterium tumefaciens* has been widely accepted. Agrobacterium was first discovered in grape plant in the year 1897 by Fridiano Cavara. In 1907, crown gall disease was reported in plant caused by T-DNA of Ti-plasmid of *Agrobacterium*. *Agrobacterium* has been characterized as gram-negative bacteria or soil phytopathogen that belongs to the rhizobiaceae family. Agrobacterium prefer to infect mostly the dicotyledonous plants and at infection or wound site produces an unorganized growth of cells that known as crown gall tumors. This bacterium harbors the tumor inducing plasmid known as Ti plasmids which are exploited to transfer desired gene into target plant tissue. Agrobacterium got the natural ability to transfer the T-DNA, part of Ti plasmid, into the plants genome and due to this unique ability, *Agrobacterium* known as natural genetic engineer of plants. The unwanted sequence of T-DNA region of Ti-plasmid is the crucial region to replace with foreign desire gene.

Molecular basis of Agrobacterium-mediated transformation:

Vectors for gene transfer:

Vectors usually contain the selectable markers to recognize the transformed cells from the untransformed cells, multiple rare restriction sites and bacterial origins of replication (e.g. ColE1). However, these features in vectors do not help in transfer of gene and integration into plant nuclear genome. Ti-plasmid of *Agrobacterium* has wide host range and capable to transfer gene that make them preferred vector over other available vectors.

The T-DNA nucleotide sequence end are flanked by 25 bp direct repeat sequences known as left border (LB) and right border (RB) and both border sequences collectively known as T-DNA border. Plasmid DNA comprising the T-DNA with border sequences called as mini or micro Ti-plasmid. Nucleotide sequences of T-DNA borders are essential and play an important role to transfer the T-DNA into the plant cell upon infection if present in cis orientation but border sequence itself does not get transfer. Any DNA sequence flanked by repeat of 25 bp in the correct orientation can be transfer to plant cells and similar attribute

were exploited with *Agrobacterium* mediated gene transfer to produce transgenics of higher plants. It was shown in experiment only right border sequence have been used and observed an enhance sequence or sometimes refer overdrive sequence located upstream to right border sequence also necessary for high-efficiency transfer of T-DNA. However, the left-border sequence has little activity alone.

b. Structure and functions of Ti Plasmids

Agrobacterium harbor a large Ti-plasmid of 200-800 kbp which contains four main regions; T-DNA, vir region, origin of replication, region enabling conjugative transfer and o-cat region. The molecular understanding of crown gall disease caused created an opportunity to develop the gene transfer system in plant.

T-DNA:

It has been demonstrated that T-DNA of Ti-plasmid of *Agrobacterium* transfer to plant nuclear genome that cause the crown gall disease. If the Agrobacterium killed with antibiotics then also undifferentiated callus can be cultivated in *in vitro* retaining the tumorous properties. This property represents the oncogenic transformation of crown gall tissues and has the ability to form tumor if grafted onto a healthy plant. T-DNA, a small and specific element of Ti plasmid are of ~ 24kbp size which comprises the following important regions, (i) two tms genes responsible for indole acetic acid (an auxin) biosynthesis and tmr gene responsible for isopentyladenosine 5'-monophosphate (a cytokinin) synthesis. This is the reason when T-DNA sequence transferred to the plant nuclear genome leads to form crown gall due to the synthesis of two phytohormones, auxin and cytokinin (ii) os region responsible for synthesis of unusual amino acid or sugar derivatives, known as opines. Opines metabolism is the chief feature of crown gall disease formation. Two common opines, octopine and nopaline, synthesized in the plant cells from octopine and nopaline synthase, respectively. *Agrobacterium* strain determine the type of opine to be produce and not by host plant. Based on type of opine produced, Ti-plasmid further described as octopine type Ti - plasmid or nopaline type Ti - plasmid

Virulence gene

Vir gene is also essential for T-DNA transfer and unlike border sequence of T-DNA vir gene can function even in trans orientation. T-DNA and *vir* gene present on two different plasmids does not affect the T-DNA transfer provided both are present in same *Agrobacterium* cells. The virulence region of Ti-plasmid are of approximately 35 kbp in size

and organized in eight operons known as Vir A, Vir B, Vir C, Vir D, Vir E, Vir F, Vir G and Vir H. All operon except Vir A and Vir H are polycistronic in nature.

Origin of replication:

In general, the *Agrobacterium* has broad host range of origin of replication for multiplication in different type of host plant.

Mechanism of T-DNA transfer to the plant genome:

The foreign gene transfer using the *Agrobacterium* has been considered a highly improved form of bacterial conjugation. The complete Ti-plasmid does not transfer to plant instead only a small segment or T-DNA get transferred and integrated into the plant genome. Virulence (vir) genes responsible for T-DNA transfer are located in a separate part of Ti-plasmid. T-DNA carries the genes for unregulated growth and also to synthesize opines in the transformed plant tissues. These genes are not necessary to transfer the T-DNA and therefore this region generates an opportunity to be replaced with the desired foreign genes.

a. Recognition and induction of *vir* genes: Plants secrets number of chemotactic signal or phenolic compounds but acetosyringone and β-hydroxyacetosyringone particularly and certain monosaccharides are recognized to induce the *vir* genes expression, processing, transfer and may also for integration of T-DNA into the plant genome. The bacteria respond well to the simple molecules like sugars and amino acids but not to the acetosyringone compounds to the injured plant cells. The *vir* genes induce only after attachment of agrobacterium to the plant cells. The chemotactic signal turns on Vir A by autophosphorylation and it will phosphorylates Vir G genes. Vir A and G gene expressed constitutively at low level. Vir A and G product encodes a membrane-bound sensor kinase and cytoplasmic regulator protein, respectively. It is well established that the signal transduction process involves Vir A autophosphorylation and then subsequently transfer phosphate to Vir G. Mainly *vir* G gene along with additional gene on the *Agrobacterium* chromosome encode transcriptional activator they play an important role to regulate the other *vir* genes. The list of *vir* genes of Ti-plasmid of *Agrobacterium* and their function are summarized in Table 1.

Table 1. List of Vir locus of Ti-plasmid of Agrobacterium and their function.

S.No	Virulence Gene	Functions
1	*Vir* A	Encodes acetosyringone receptor protein which activates Vir G by phosphorylation leading to expression of other vir genes
2	*Vir* B	Encodes membrane protein which involved in conjugal tube formation for T-DNA transfer Vir B11 has the ATPase activity
3	*Vir* C	Encodes helicase enzyme for unwinding of T-DNA
4	*Vir* D	Vir D1 Topoisomerase activity, Vir D2 is an endonuclease
5	*Vir* E	Vir E2 Single strand binding protein(SSBP) binds to T-DNA during transfer
6	*Vir* F	Activity not known
7	*Vir* G	Master controller DNA binding protein, Vir A activates the Vir G by phosphorylation, Vir G dimerise and activates expression other vir genes
8	*Vir* H	Activity not known

b. Transfer of T-DNA to plant cells: Transfer of T-DNA initiated by Vir D1 and D2 product an endonuclease enzyme which specifically recognizes T-DNA border sequences. These enzymes create nick either on single strand or double strand at T-DNA border sequences which leads to release of ss-T-DNA from the Ti-plasmid. This process is enhanced by the Vir C2 and 12 proteins by recognizing and binding to the enhancer elements. Vir D2 binds covalently to 5'end of processed ss T-DNA forming an immature T-DNA complex. Single stranded T-DNA intermediate favored by the octopine type Ti-plasmid whereas double stranded favored by nopaline type Ti–plasmid. Vir D2 protein protects the T-DNA intermediate complex from nuclease degradation to target the DNA to cytoplasm and nucleus then integrate into plant genome. T-DNA intermediate are coated with Vir E2 which encodes a single stranded DNA binding protein (SSBP). The induction of *vir* gene expression forms the conjugative pilus to transfer the T-DNA to plant cells. Vir B gene operon product also involved to make part of conjugative pilus. Vir B and Vir D product then transport the T-DNA complex through membrane channel a type IV secretion system to cytoplasm of plant

cell. Vir D4 protein acts as a linker facilitating the interaction of processed T-DNA complex with membrane channel. Vir B2 to Vir B11 and Vir D product are important for forming a membrane-associated export apparatus includes hydrophobicity, membrane-spanning domains, and/or N-terminal signal sequences in cytoplasm. The interaction between Vir B7 and Vir B9 help to form heterodimer that stabilizes the other Vir B proteins. Membrane channel is composed of Vir D4 and Vir B11 protein which is necessary for transport of T-DNA complex. Vir B protein also serves as ATPases to provide energy for channel assembly or export process. Vir B1 has transglycosidase activity which utilizes to assemble other Vir proteins. With the T-DNA complex the other Vir protein H and F also transported into plant cells which are necessary for efficient transport of T-DNA complex and nuclear transport. Next, Vir D2 and Vir E2 protein play an important role for nuclear transport of T-DNA complex because it contains the nuclear localization signal. The nucleus of injured plant cells often associated with the cytosolic membrane facilitating the rapid transfer of T-DNA into nucleus without much exposure to the cytosolic environment. After reaching to the nucleus, T-DNA probably integrate to plant genome by illegitimate recombination process exploiting naturally occurring chromosome breaks.

Ti-plasmid derivatives for gene transfer

T-DNA of Ti-plasmid transferred and express in to plant cells because T-DNA carry the promoter element and polyadenylation site similar to eukaryotic one. This sequence acquired by agrobacterium may be during the evolution of Ti-plasmid. Ti-plasmid of *Agrobacterium* can transfer its T-DNA to the plant genome due to this specific reason Ti-plasmid qualified as natural vector to engineer the plant cells. The wild type Ti-plasmid is suitable due to presence of oncogenes on the T-DNA which causes the uncontrolled growth of plant cells. So the oncogenes region of T-DNA must disarmed to be qualified as successful natural vector for revival of plant well. T-DNA should be left with the left- and right border sequence and the *nos* gene in modified vector. The plant cells when transform with *Agrobacterium* containing modified vector, no tumor should produce and nopaline production will be evident for positive transformation. To make the screening easier to identify the transformed plants cells, selectable marker like drug or herbicide resistance can be inserted on T-DNA because the enzymatic assay for nopaline at every step of transformation is a cumbersome process. Nopaline positive cells could be culture to callus tissues provided the required phytohormones.

The modified vector of Ti-plasmid is also not convenient for plant transformation due to the large size that makes them difficult for manipulation. The absence of unique restriction enzymes sites in T-DNA sequence is another problem to manipulate this vector.

This problem was resolved by constructing an intermediate vector, in which T-DNA was subcloned into *E. coli* plasmid vector for easy manipulation. However, intermediate vector does not replicate in *Agrobacterium* and also lacked conjugation functions. To succeed the gene transfer process the triparental mating was introduced mixing the three bacterial strains like: a) *E. coli* strain carrying the recombinant intermediate vector; b) *E. coli* strain containing the helper plasmid to mobilize intermediate vector in trans; c) *Agrobacterium* carrying the Ti-plasmid. Conjugation between *E. coli* strains- a and b, transferred them to the c, recipient *Agrobacterium*. Homologous recombination occurs between the T-DNA and intermediate vector and forms a large cointegrate plasmid, from where recombinant T-DNA transferred to plant genome. Intermediate vector has been widely used but large cointegrate vector still not required for gene transformation in plant.

T-DNA sequence is not essential and the necessary *vir* gene region can functions in trans during the transfer the gene by Ti-plasmid to the plant. Therefore, *vir* gene and disarmed T-DNA sequence part of Ti-plasmids can supply on separate plasmids in *Agrobacterium* and this principle were termed T-DNA **binary vector** system. In binary vector system, maintaining the T-DNA on a shuttle vector is beneficial because the copy number is not determined by Ti-plasmid and not dependent on recombination. This event makes the identification of transformats much easier. The gene of interest to transfer including origin of replication and antibiotic resistance genes will be maintained on T-DNA region in binary vector system, whereas *vir* gene maintained on separate replicon known as Vir helper plasmid. The *vir* gene products will help processing the T-DNA and export further to plant cells (Figure 1 & 2). Ti-plasmid with *vir* gene region but without the T-DNA sequence will be transformed into the *Agrobacterium*. T-DNA sequence will introduced in to *Agrobacterium* by triparental mating or methods like electroporation. To achieve the efficient transformation the binary vector should have some properties: a) right and left border sequence of T-DNA; b) Selectable marker gene compatible to plant usually antibiotic or herbicide resistance; c) Multiple rare-cutting restriction endonuclease site on T-DNA and the *lacZ* gene for blue-white screening and cos site for preparing cosmid libraries; d) Origin(s) of replication for *E. coli* and *Agrobacterium* facilitate the replication in broad host range; e) Antibiotic-resistance genes in binary vector for selection in both *E. coli* and *Agrobacterium*.

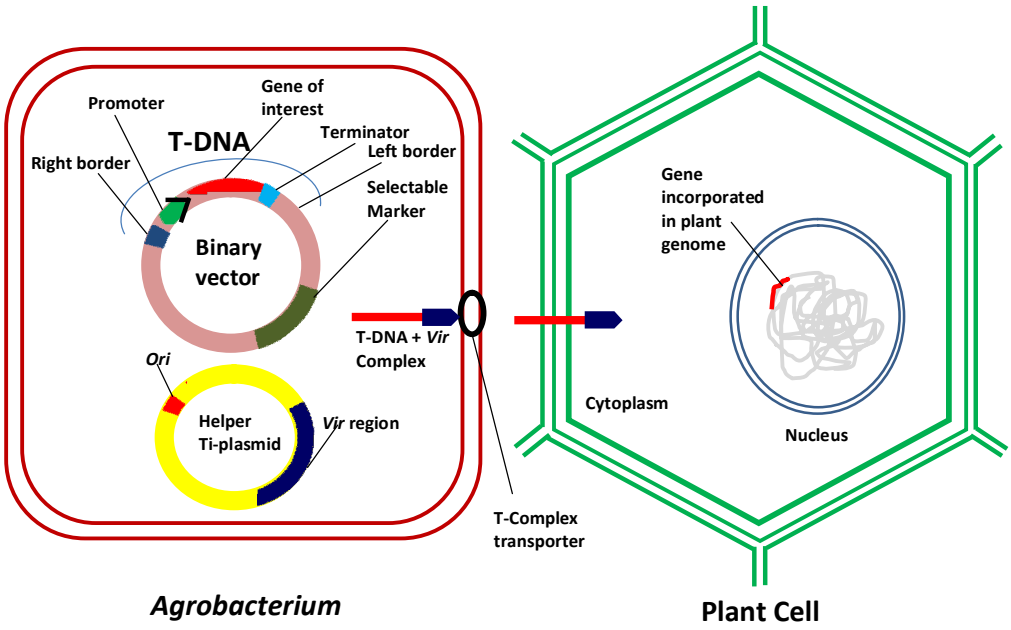


Figure 1. ***Agrobacterium*** **–mediated gene transformation process in host plant cells.**

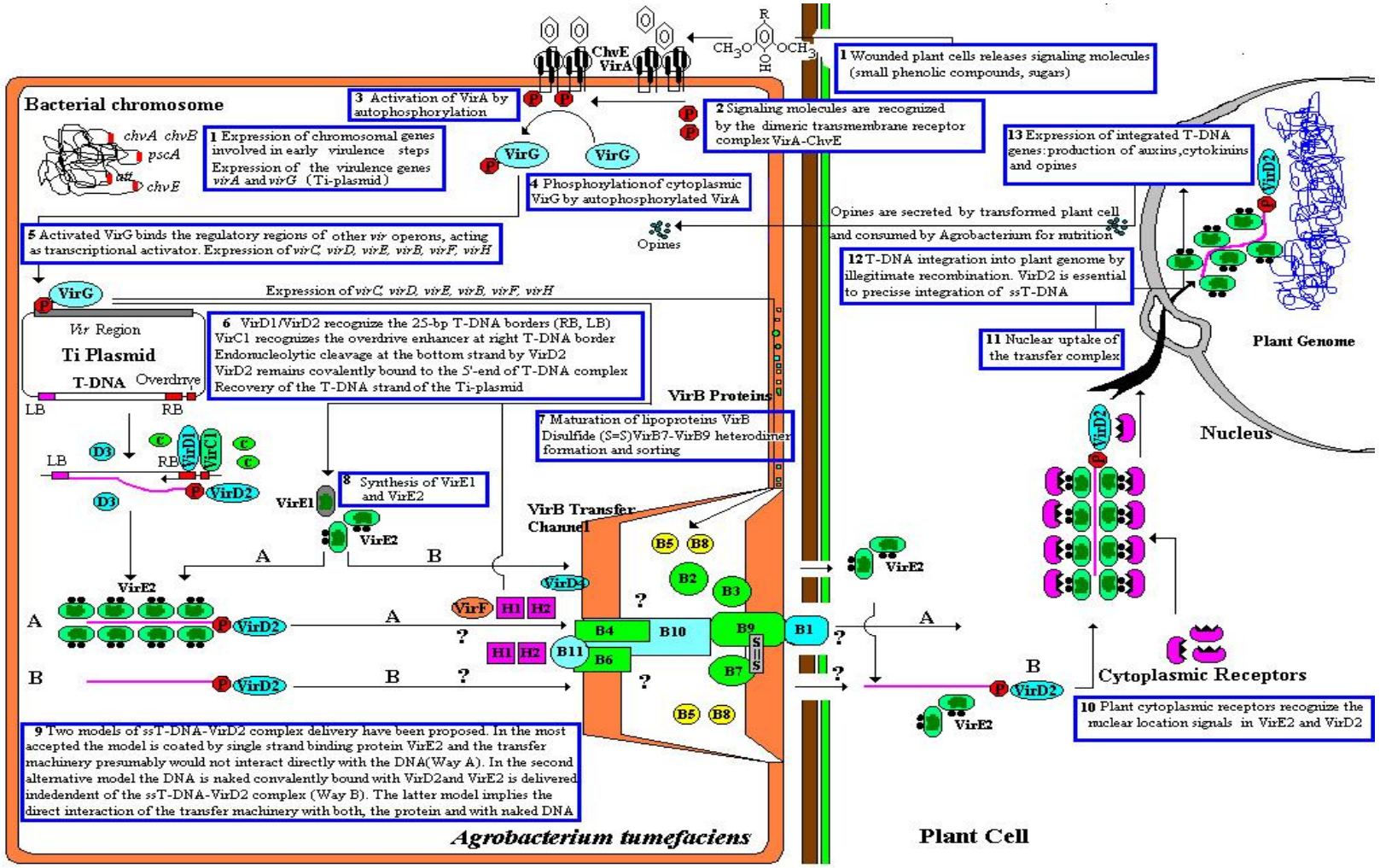


Figure 2. Mechanism of T-DNA transfer from Agrobacterium to the host cell.

***Agrobacterium*-mediated gene transformation in plants**

For the transfer of gene in dicot plants, a few millimeters diameter leaves were surface sterilized and inoculated in medium containing *Agrobacterium* cells transformed with recombinant disarmed binary or cointegrate vector. The leaf disk was first grown for two days and then transferred to the medium containing the kanamycin and carbenicillin. In the medium, kanamycin added because the foreign chimeric gene has kanamycin resistance gene for selection and carbenicillin to kill *Agrobacterium* cells. The shoots were usually developed in 2-4 week time from the leaf disk. The grown shoots were removed from callus and transfer to the medium containing auxin for root development. The roots were developed in 2-3 week time and then plantlets were transplanted to the soil. This is a superior, simple and rapid methods compared to methods where transformed plant were recovered from the protoplast-derived callus which transformed with agrobacterium by co-cultivation.

In case of monocot plants, only few of the monocots plant such as rice, maize, wheat, barley, and sugarcane were reported susceptible to *Agrobacterium* infection with the modified culture condition and transformation procedures. The use of explant, embryo and apical meristem and supervirulent strain of *Agrobacterium* like AGL-1 was the key factor for successful transformation. Transformation efficiency in rice was achieved by adding acetosyringone in co-cultivating medium of *Agrobacterium* and rice embryos.

Agrobacterium rhizogenes:

A. rhizogenes is another bacterial strain use to transfer the gene of interest to the plant cells. The molecular understanding of hairy root diseases helps to utilize this *Agrobacterium* for gene transfer system, analogous to *A. tumefaciens*. This *Agrobacterium* harbor the Ri-plasmid which are responsible to produce the characteristic hairy root disease symptoms upon infection to dicotyledonous plant. Ri-plasmid also has T-DNA region which transfer into the plant nuclear genome. T-DNA integrates into the plant genome and in turn *iaaM* and *iaaH* gene induced to produces excess phytohormones tryptophan 2-monooxygenase and indoleacetamide hydrolase, respectively. There are no major differences observed between the Ri plasmid and Ti-plasmid. However, it is not accepted commercially because of problem involved in scale-up of transformed roots.

Direct DNA transfer to plants

Direct gene transfer methods can also called as vector free methods because no living vector used to transfer gene to plant cells. Many physical and mechanical methods were developed that facilitate the entry of this foreign DNA into plant cells.

Protoplast Transformation

The protoplast cells are capable to take up the gene of interest from their surrounding liquid environment. After entry of gene of interest into protoplast, it gets integrated into the genome of transfected cells. The selectable marker can be also added with the gene of interest that required for the selection of desired gene in the protoplast. The gene transfer process can be induce and accelerated under influence of some chemicals like polyethylene glycol (PEG). Alternatively the electroporation methods can also be used for gene transfer to the protoplast. The putative protoplasts containing gene of interest were grown on selective medium where protoplast regenerate their cell wall, cell division begin and eventually produces the callus. Callus then produces the roots and shoots by inducing with phytohormones. The major problems often observe the regeneration of host plant from the protoplast however dicots found more responsive than the monocots.

Electroporation

Electroporation method was first demonstrated studying gene transfer in mouse cells and can be applied also with bacterial, fungal and plant cells. It is a simple and efficient method for integration of gene of interest into protoplast or intact plant cells. For electroporation, with high voltage (1.5 kV) short duration and with low voltage (350V) long duration of pulse was used for gene transfer. Electroporation pulse increases the permeability of membrane by disrupting the phospholipid bilayer of protoplast. This is in turn facilitating the entry of gene of interest into cells if present on protoplast membrane. The target cells can be pre-treated with enzymes or wounded for ease of gene transfer process. Without any form of pre-treatment also gene transfer has been successfully achieved in immature rice, maize and wheat embryos by electroporation method.

The efficient and successful gene transfer by electroporation methods depends on the following factor like applied electric field strength, electric pulse length, temperature, DNA conformation, DNA concentration, and ionic composition of transfection medium, etc. PEG can also be used to stimulate and enhance the uptake of liposome and also improve the transformation efficiency. Successful transfer of gene of interest using electroporation methods has been already achieved in maize, petunia, rice, sorghum, tobacco crops. Some new measure has been suggested to increase the transformation efficiency such as i) uses of

1.25kV/cm, ii) add first DNA followed PEG, iii) heat shock at 45°C for 5 minutes and iv) use linear DNA in place of circular. The modified condition are suitable to transfer gene of interest in both monocot and dicot protoplast.

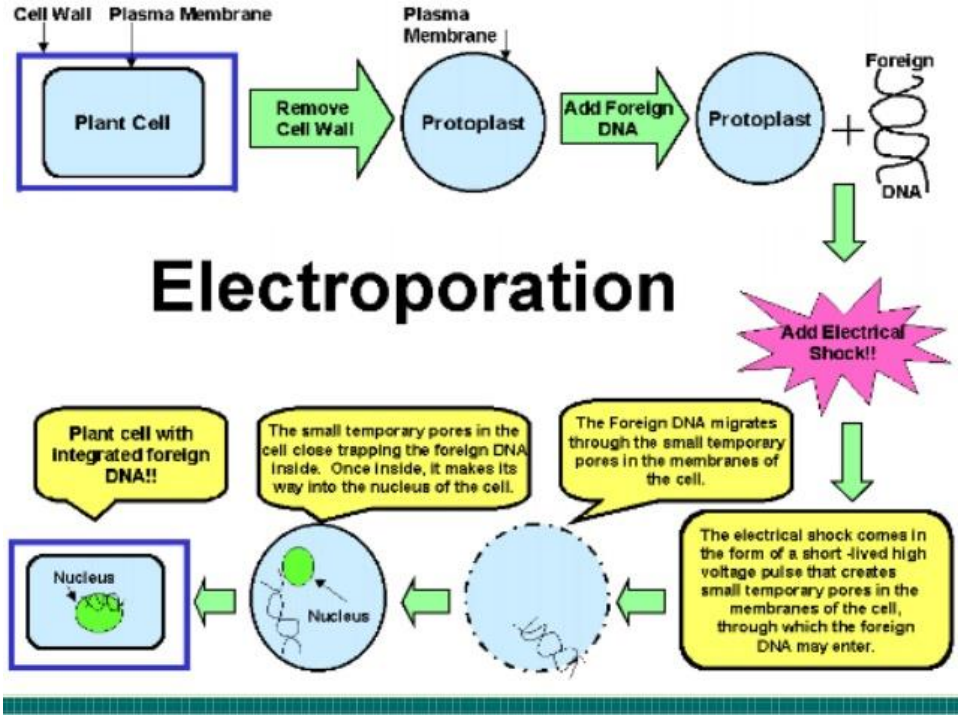


Figure. Overview of electroporation.

Particle bombardment

Particle bombardment methods of gene transfer also known as biolistics, or particle gun, or gene gun, or short gun, or microparticle gun, or projectile bombardment (Figure 3) method. This method is especially useful when some of the live plant tissue like intercellular organelles, leaves, meristem, immature embryos, callus or suspension cultured cells, live pollen, which are impermeable to foreign DNA. The type of plant material used for DNA delivery is not a limitation in this method because the DNA delivery is governed by physical parameters. Plant cell wall is hard and not easy to delivers anything from outside so the powerful particle bombardment method is very useful for efficient gene transfer in plant. Particle bombardment method was developed at Cornell University. The gene of interest, DNA or RNA coated to tiny biologically inert high density particles like gold or tungsten of 1-3 pico to micrometer size are place on the target tissue in vacuum condition. Then gene coated high density particles are accelerated for high velocity (1400 ft/sec) by powerful shot using gene gun to enter inside the tissue membrane. The explosive charge like cordite

explosion or shock waves initiated by high voltage can be used to get the high velocity acceleration in the gene gun. The success of particle bombardment method are governed by some factors such as particle size, acceleration (for penetration and determine the tissue damage), amount and conformation of DNA. These four factors must optimize for each species and type of target tissue using for gene transfer for success of gene transfer. Using this method the first successful transgenic were produced in soybean transferring gene to meristem tissues which were isolated from immature seeds. Gene gun method has been successfully used to transfer gene of interest in crops like barley, cotton, maize, oat, papaya, rice, soybean, sugarcane, tobacco, wheat. This method has also been used to transfer gene of interest for transient expression in onion, maize, rice and wheat.

Over the years the particle bombardment methods has been modified for better control over particle delivery, efficient transformation of gene and enhanced reproducibility of transformation states. For example, particle bombardment based on electric discharge has been designed for gene transfer in recalcitrant cereals and legume crops. Other gene gun modification includes like pneumatic apparatus, particle inflow gun using flowing helium and device utilizing compressed helium.

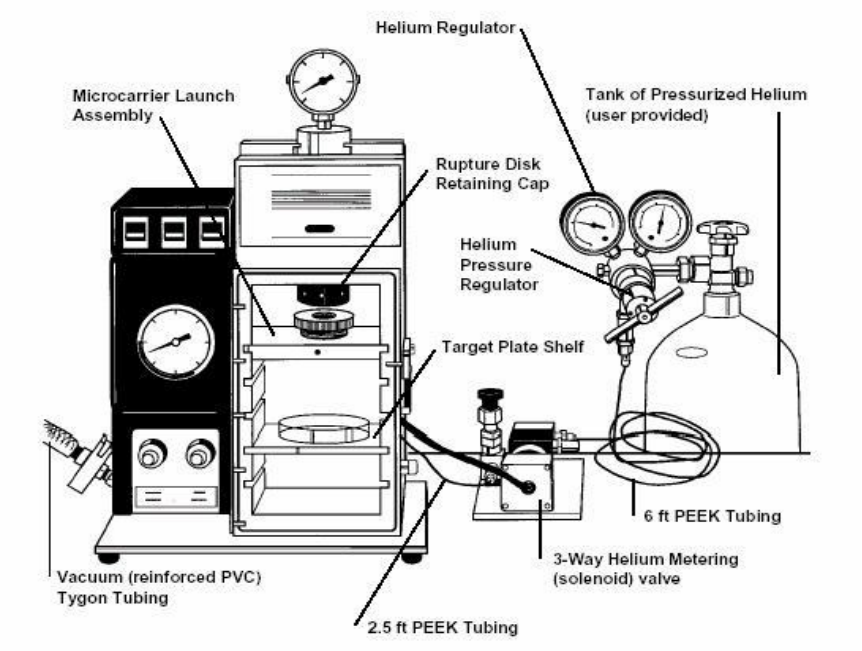


Figure 3. Particle bombardement.

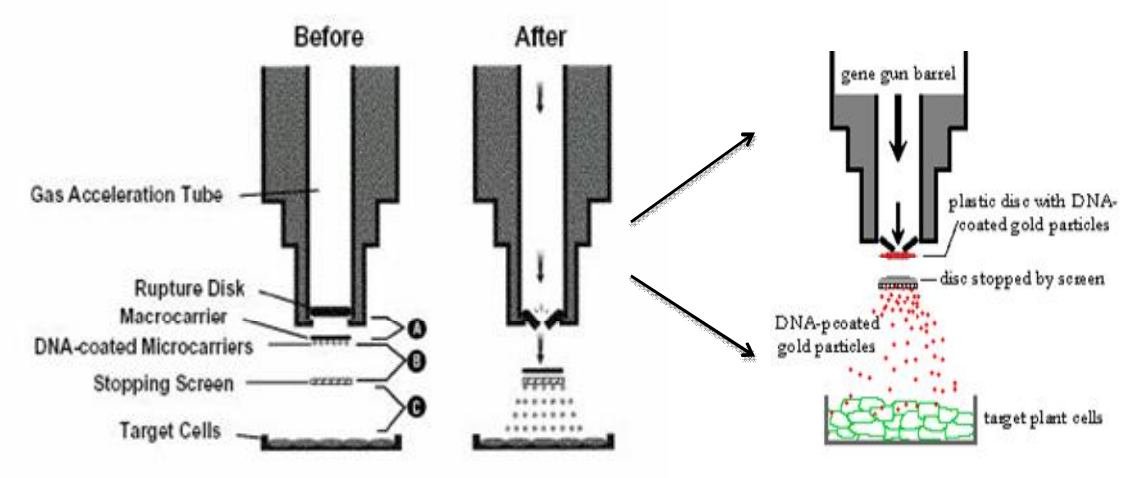


Figure 4. The Particle bombardement process.

Microinjection

Microinjection is widely used and efficient technique for transfer of desire gene into animal cells, tissues or embryo cells nuclear genome. This technique is not efficient to direct transfer gene to the plant cells. In this method gene transfer to the cytoplasm or nucleus of recipient protoplast or plant cells were performed with the glass micropipette of 0.5-10µM diameter needle tip. The target recipient cells for gene transfer are many such as immature embryos, meristems, immature pollen, germinating pollen, isolated ovules, embryogenic suspension cultured cells, etc. The recipient cells were immobilized on a solid support like depression slide under suction and then the cell membrane and nuclear envelope of plant cells were penetrated with the glass micropipette tip under specialized micromanipulator microscope set up. The modified and improved method of microinjection termed as holding pipette method, in which the protoplasts positioned on a depression slide close to already placed micro-drop desired DNA solution. Now with the holding pipette hold the protoplast and DNA were injected into protoplast nucleus by injection pipette. Many genetic manipulation experiments were widely performed using this technique for cell modification, silencing of gene etc. This microinjection technique where also performed and demonstrated successful gene transformation and transient expression in green algae, *Acetabularia*. The drawback of this microinjection technique is that process is very slow, expensive, required highly trained technician and only a part of plant transformed with the desired gene. However, the success rate of transfer of gene is very high. This technique has been employed successfully in oilseed rape (*Brassica napus*) and obtained the transgenic chimera.

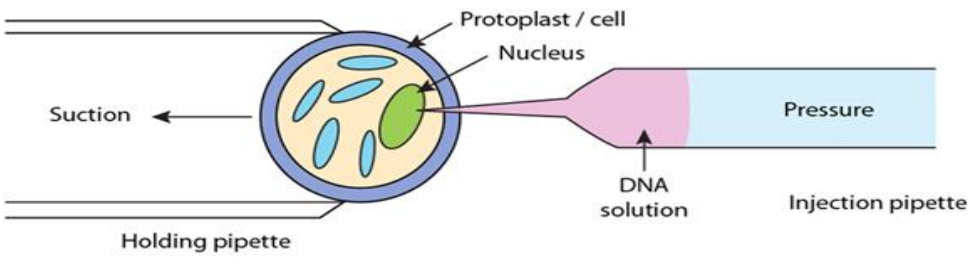


Figure 5. Microinjection process.

Sonoporation:

In this methods the explant like leaves are chopped into pieces and using the ultrasound waves creates the permeability function in cell wall. Through this permeable cell wall, the gene of interest can be uptake from surround environment by cell wall. Sonoporation process uses the sound waves which help to form tiny bubbles that enhance the DNA entry into cell walls. The explants were further transferred to the culture nutrient medium for the growth of shoots and roots.

Calcium phosphate mediated:

Calcium phosphate mediated gene transformation method was also considered promising for plant cells. In this method the desired gene with Ca^{2+} ions precipitated and forms calcium phosphate which coats the cells and released inside the plant cells. Using this method the desired gene can be transferred to study the molecular, biochemical, cellular, genomic and proteomic aspects in *in vitro* and *in vivo* of plant cells.

Lipofection:

Lipofection is a liposome-mediated gene transfer method. This method employs a liposome containing desired gene which induced by PEG to transfer of gene and then fuse into protoplasts. Liposome is cationic in nature and made up of phospholipid layer similar as cell membrane. Liposome and target cells adhere and form aggregates easily because of similar phospholipid bilayer (Felgner et al., 1987). The aggregate of liposome and cell wall are positively charge which enhances the efficiency of negatively charged DNA uptake. The desired gene enter into protoplast by endocytosis process of liposome that includes adhesion of the liposomes to the protoplast surface, liposomes fusion at the site of adhesion and then finally release of DNA inside the protoplast cell. There are many advantages with this method over other gene transfer method like desired gene not exposed to nuclease, stability due to encapsulation, low cell toxicity, high degree of reproducibility and suitable for wide

range of cell types. Lipofection method of gene transfer has been successfully used in number of plant species like tobacco, petunia, carrot.

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TRANSGENIC PLANT AND THEIR APPLICATION

Introduction

Plants containing transgenes i.e gene or genes usually from an unrelated organism, as a result of modification done by genetic engineering are called transgenic plants. Transgenic technology has enabled transfer of genes from many organism or even synthetic DNA sequences into the genome of any plant species. This technology can be and has been used to tailor plant genomes to meet various human needs. Some of the successful examples of transgenic crops of economic values are concerned with: (1) Herbicide resistance (2) Insect resistance (3) Pathogen resistance. (4) Improvement of crop traits for food quality and products. In doing so, transgenic plants are addressing some of the oldest problems in crop production.

Herbicide Resistance

The first transgenic application to be widely adopted in agriculture was resistance to herbicides. Weeds are generally killed by use of chemical herbicides by many farmers because they are cost-effective and efficient at killing weeds but these herbicides are not selective, meaning that they kill the target weeds as well as the crop plant. Using single-gene traits in transgenic plants can provide a very specific way to protect the crop plant from the effects of a given herbicide. Herbicides generally work by targeting metabolic steps that are vital for plant survival. For example, glyphosate kills plants by inhibiting the production of certain amino acids that the plant requires for survival. Glyphosate is the active ingredient in the herbicide RoundUpTM . Thus, crops such as soybean and corn that have been engineered to be resistant to glyphosate were given the name “RoundUp Ready.” Glyphosate works by binding to and inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is active in the shikimate pathway leading to the synthesis of chorismate-derived metabolites, including the aromatic amino acids (tyrosine, phenylalanine, and tryptophan) (Fig.1). To make plants resistant to glyphosate, a form of the EPSPS enzyme that is functional in plants but is not affected by the herbicide was used. In addition to being present in plants, the EPSPS protein can also be found in bacteria. So scientists at Monsanto, the inventors of RoundUp, looked for and identified a form of EPSPS from a soil bacterium that was not sensitive to treatment with glyphosate. The EPSPS gene from the bacterium was then isolated and transferred into plants through particle bombardment method where its

expression was regulated by putting it downstream of a strong promoter, the cauliflower mosaic virus 35Spromoter, which drives gene expression throughout the plant. The normal plant version of EPSPS is encoded by DNA in the nuclear genome. Following translation of the mRNA sequence to amino acid sequence in the cytoplasm, EPSPS is transported into the chloroplast, where the shikimate pathway is active. To ensure that the bacterial form of EPSPS would make its way into the chloroplast after the protein was synthesized, a short DNA sequence encoding a chloroplast transit peptide was fused to the 5` end of the bacterial EPSPS open reading frame. This transit peptide sequence fused at the amino terminus of the bacterial EPSPS serves as an intracellular signal for proper protein localization. The transit peptide sequence originated from a gene encoding a protein normally found in the chloroplasts that carries out carbon fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Once the bacterial EPSPS gets into the chloroplast, it can function in place of the plant enzyme during the biosynthesis of aromatic amino acids. RoundUp Ready soybeans were one of the first transgenic crops to be approved and used on a large scale.

Once they were commercialized, they gained rapid acceptance by farmers and are now the most popular transgenic plant in the world.. Because it is so effective at selectively killing weeds and not the herbicide-resistant crop plant, more farmers using glyphosate have adopted “no-till(age)” or “low-till(age)” methods, resulting in less soil erosion and lower fuel costs because they take fewer trips through a field. Furthermore, because animals do not make aromatic amino acids, they do not possess the shikimate pathway that is the target of glyphosate and so the herbicide has low toxicity in animals. In 1996, the first year they were commercially available, RoundUp Ready soybeans made up about 2% of the total soybeans grown in the United States. By 2000, that amount had risen to 54%, and in 2005 it was up to 87%. Now, glyphosate resistance has been engineered into a large number of crops that are grown globally, including Latin America and Asia. Predictably, adoption of glyphosate-resistant crops has resulted in a vast increase in the amount of this herbicide applied worldwide; however, there has been a decrease in the use of other herbicides especially on soybean.

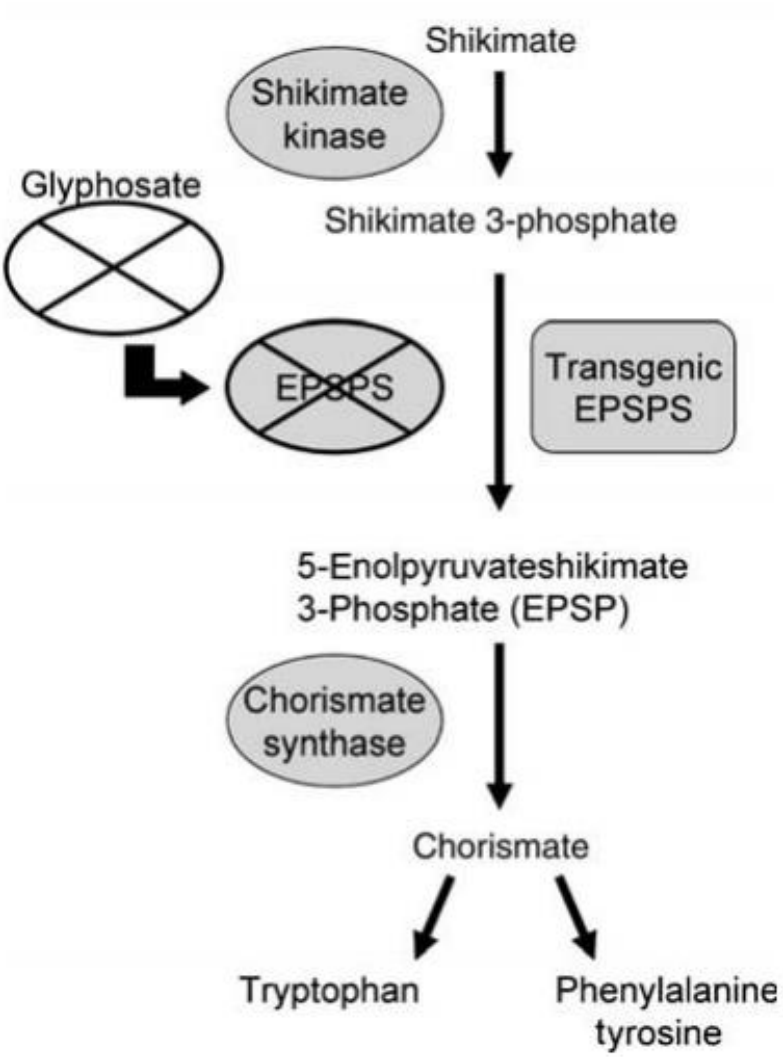

Figure.1.*Resistance to glyphosate in RoundUp Ready TM plants is engineered by expressing a form of the 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EPSPS) enzyme that is resistant to the herbicide. In the absence of this transgenic enzyme, glyphosate inhibits the plant EPSPS and ultimately blocks the synthesis of chorismate, the branchpoint precursor to the essential aromatic amino acids: tryptophan, phenylalanine, and tyrosine. The transgenic EPSPS is unaffected by glyphosate, and can carry out the synthesis of EPSP leading to chorismate production.*

An alternative strategy to engineer herbicide resistance is to express a protein that will inactivate the herbicide if it is sprayed onto plants. This is the approach used in resistance against the herbicide glufosinate, the active ingredient in the product LibertyTM, generating a trait in crop plants often called “LibertyLink.” Glufosinate kills plants by inhibiting the plant enzyme glutamine synthetase (GS), which is responsible for synthesis of the amino acid

glutamine. As part of the chemical reaction that produces glutamine, GS utilizes excess plant nitrogen in the form of ammonium that is incorporated into the amino acid. When GS is inhibited in glufosinate-treated plants, ammonium concentrations inside the plant rise to toxic levels (Fig. 2). The glufosinate compound is naturally produced in some Streptomyces bacteria. In addition to having phytotoxic activity, glufosinate also serves as an antibiotic because it is toxic to some other bacteria. Bacterial strains that are resistant to glufosinate produce an enzyme, encoded by the bargene, called phosphoinothricine acetyl transferase(PAT) (Thompson et al. 1987). The bargene was isolated from a strain of Streptomyces hygroscopicus, which degrades glufosinate, and has been transferred into several crop plants. The LibertyLink trait is currently widely used in transgenic corn, canola, and cotton varieties. Similar to the strategy in making LibertyLink crops, resistance to the herbicide bromoxynil (BuctrilTM) was engineered by expressing the protein of a bacterial gene that will inactivate the herbicide. Bromoxynil kills plants by inhibiting function of photosystem II, a crucial component of photosynthesis. Buctril-resistant cotton is already widely grown in the United States, and other resistant to this herbicide, such as tobacco and potato, are nearing final stages of commercialization.

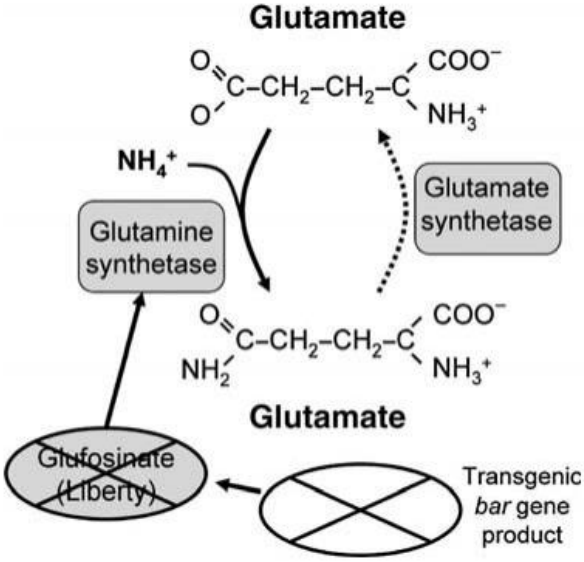

Figure 2. *Resistance to glufosinate in LibertyLinkTM plants is engineered by expressing an enzyme that directly targets and inactivates the herbicide. Glufosinate kills plants by inhibiting glutamine synthetase. This enzyme is responsible for production of the amino acid glutamine in a reaction that can sequester excess nitrogen by incorporating ammonia (NH4+). If this enzyme is inactivated by glufosinate, excess ammonia accumulates and the*

plant is killed. An enzyme encoded by the bacterial bargene in transgenic plants inactivates glufosinate.

2. Insect Resistance

Insect damage to crops poses a problem for farmers worldwide. In spite of the great amounts of money and effort spent on attempts to control insect pests, staggering losses to insects are still incurred before and after harvest. A number of proteins with negative effects on insects have been tested as potential weapons for use in engineering insect-resistant transgenic crops. Genes for several proteins have been expressed in transgenic plants and were shown to inhibit insect growth or cause higher insect death rates. These include genes for protease inhibitors, which interfere with insect digestion; lectins, which kill insects by binding to specific glycosylated proteins; and chitinases, enzymes that degrade chitin found in the cuticle of some insects. Although each of these genes has been shown to have some negative impact when consumed by insects and may have some utility in insect control, none have been as effective or widely adopted as genes encoding endotoxins from the bacterium Bacillus thuringiensis(Bt). The natural insecticidal activity of Bt endotoxin proteins represents an attractive alternative to synthetic chemical pesticides, which often have nonselective toxic effects on beneficial insects, birds, fish, and mammals. The transgenic plant produces its own insecticidal protein that is delivered only to insects that dare eat the plant.

A bacterial species Bacillus thuringiensis producing Bt toxins was first isolated and described in 1915. Bacillus thuringiensis causes bacterial m disease affecting silkworms (Bombyx mori) and was detrimental for silkworm production. However, it was later noted that Bt had toxic effects on caterpillar larvae of most Lepidoptera species (moths and butterflies), which gives the Bt species great potential as a tool for protecting crop plants. In later years, additional strains of Bt were identified that are toxic to Coleoptera (beetles), Diptera (flies and mosquitoes), and even nematodes. The specificity of insecticidal activity of Bt on a particular insect species is determined by the form(s) of the cry gene(s) carried by the bacterium. Only certain species of insects are controlled by particular endotoxins. The cry genes encoding the toxic proteins in Bt take their name from the crystal inclusions formed inside the bacterium when it enters into its spore-forming stage. These crystals often contain more than one specific type of cry gene product. Before they become toxic, the cry-encoded Bt proteins exist as protoxins and must be activated inside the insect digestive tract. Once they are ingested by a susceptible insect, the crystals break down in the alkaline environment of the insect midgut, generally dissolving at pH 8.0. At that point, the termini of the Bt

protoxin proteins are cleaved by specific proteases inside the gut, yielding the toxic protein. The active protein will then bind to specific protein receptors on the insect microvillar membrane of the midgut (Fig. 3). In most cases, when Bt proteins are expressed in transgenic plants the entire coding region of the protoxin is not transferred to the plant. Rather, a shortened version of the gene will typically be expressed because levels of Bt protein accumulation are higher using this strategy. After binding to a receptor, the active Bt toxin will enter the insect cell membrane, where multiple copies of the protein will oligomerize and form pores. This results in ion leakage through the membrane, which causes membrane collapse from osmotic lysis. Once the membranes on the epithelia of the gut cells are disrupted, the insects effectively starve and die. In the case of a true B. thuringiensis infection, bacterial cells would form spores during the latter stages of infection and insect collapse, thereby readying themselves for subsequent infections of other insects. In transgenic plants, susceptible insects usually stop feeding within a few hours after feeding on the plants, and die a short time later.

It is generally the presence or absence of specific forms of midgut receptors that determines whether a particular insect species is susceptible to a given Bt protein (Hofmann et al. 1988). For example, the most widely deployed cry genes in transgenic plants are members of the cry1A gene family, which are toxic to a broad range of Lepidoptera pests. However, this form of Bt has relatively little effect on Coleoptera species because the insects lack the specific receptors that recognize Cry1A proteins. Likewise, some beetle species, such as the Colorado potato beetle (Leptinotarsa decemlineata), are targeted by the Cry3A Bt toxin, whereas most lepidopterans are unaffected. Therefore, specific cry genes have been expressed in transgenic crops to tailor varieties to control specific pests and not affect non target species. For example, several variations ofcry1A genes have been transferred to corn to control European corn borer (Ostrinia nubilali), a lepidopteran pest that feeds on the insides of corn stems; whereas cry3Bb1 expression has been used in corn varieties to control western corn rootworm (Diabrotica virgifera) larvae, a coleopteran species that feeds primarily on roots. By using this strategy, varieties resistant to a particular insect pest can be effective in growing regions where particular pests are problematic. Because of the steps necessary to activate them and their target sites in the digestive tract, the Cry toxins are not effective as contact insecticides. Rather, insects are killed only when the toxins are ingested. This means that most non target and beneficial insects are not affected in fields of Bt crops. Furthermore, most insect and non insect species lack the specific membrane receptors for Bt and often have

digestive conditions that degrade the Bt toxin if it is consumed; therefore Bt is essentially nontoxic for most arthropods, animals, and birds (and humans). In fact, Bt sprays (the intact microbes) are considered to be so safe that certified organic food production in the United States allows for the direct application of Bt crystalline spores on plants immediately prior to harvest as a control for insects. Organic growers use Bt in this form as a valuable tool for insect control. One disadvantage of this approach in comparison to transgenic Bt production in plants is that Bt applied externally to plant surfaces does not penetrate the plant tissue and is not very stable, since it breaks down with time and exposure to ultraviolet light. Even so, because organic producers sometimes depend on application of Bt as a management tool, they are especially concerned about the possibility of Bt-resistant insect populations developing because of the growingand widespread application of engineered Bt crops.

As with herbicide-resistant crops, adoption of Bt transgenic crops has also been extensive. Damage by insects can be a severe problem in cotton, and this crop is heavily treated with synthetic chemical pesticides in many production schemes. In 2005 transgenic cotton represented almost 80% of the total of that crop grown in the United States, and it is widely grown in other parts of the world, including China. Transgenic corn is now grown on well over 50% of all the acreage in the United States. In the case of both cotton and corn, traits of herbicide and insect resistance are often combined in the same plant lines as "stacked" traits.

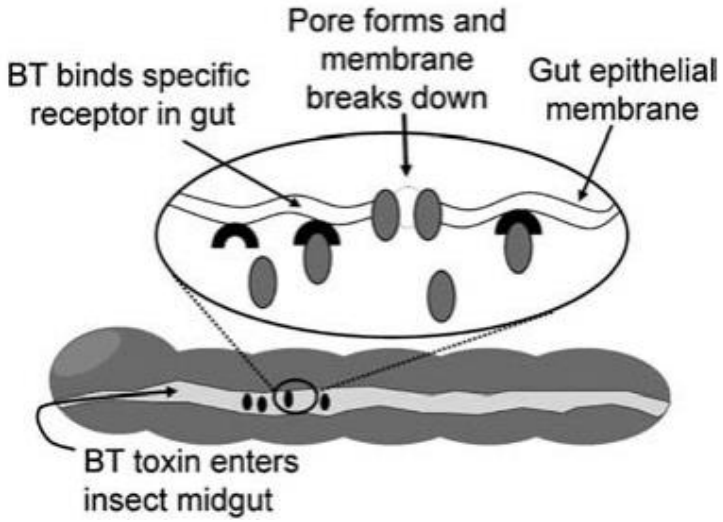

Figure 3.*The Bt toxin binds to very specific receptors on the epithelial membrane of the insect gut. The toxin then forms channels in the membrane that leads to ion leakage and*

ultimately, death of theinsect. This mode of action explains the specificity of Bt (from the presence of the necessary receptors) and also shows why the toxin needs to be eaten by the insect to function

Pathogen Resistance

Plant pathogens such as viruses, fungi, and bacteria are a severe and constant threat to agricultural crop production. Multiple transgenic approaches have been used to attempt plant disease control, although relatively few of these have yet made their way into the field of production. The most effective way to control pathogens in a field setting is to use plants that are resistant to the problem pathogen. Resistance to a particular pathogen can often be conferred by a single plant gene (an Rgene), the product of which is active in recognition of the presence or activity of a single virulence factor from the pathogen (encoded by an Avr gene). In plant pathogen systems, this relationship is known as a gene-for-gene interaction (Fig. 4). Plant breeders have historically taken advantage of this system, although it can sometimes take many years to identify a plant line with the desired resistance and to breed that trait into useful cultivars. Another disadvantage to the breeding approach is that unwanted or undesirable genes may sometimes be linked to the Rgene, and it can be difficult to separate them from the Rgene using traditional breeding methods. Finally, useful Rgenes are sometimes not easy to transfer because of barriers in crossing different species. Therefore, the ability to clone and transfer a single Rgene from one plant variety or species to another represents an encouraging option to adapt and speed up the process. A promising approach at engineering resistance is seen in the application of a specific resistance gene to ward off a bacterial disease in rice (Ronald 1997). Bacterial blight is a destructive disease of domesticated rice (Oryza sativa) in Africa and Asia, caused by the pathogen Xanthomonas oryzae pathovaroryzae. Scientists looking for alternative sources of resistance to bacterial blight identified a wild relative of rice, O. longistaminata, native to Mali, which is resistant to the pathogen but has very low quality and yield in terms of grain production. Through careful genetic and molecular studies, an Rgene called Xa21was isolated from the wild species. This gene has been introduced into domesticated rice using particle bombardment and it confers strong resistance against strains of X. oryzae carrying the Avrgene recognized by Xa21. Through efforts of scientists scattered across the globe, the Xa21gene has been incorporated into several rice varieties of agricultural importance. The use of transgenic rice as a food crop is still controversial, and its adoption has been slow compared to crops like soybean, corn, and cotton. So although transgenic lines of blight-resistant rice are poised for application,

they are currently not widely grown for food production. At least one-third of the world's population, including many developing countries, depends on rice as the major source of calories they consume

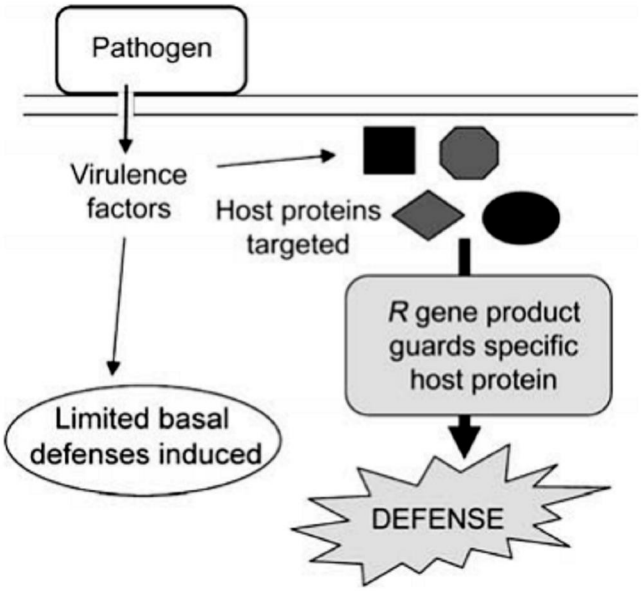

Figure 4. *Resistance to specific strains of plant pathogens can be conferred by the protein product of a single resistance (R) gene. Most plantRgenes function by recognizing the activity or presence of a specific virulence factor from the pathogen. In addition to the ability to induce basal defenses, these pathogen "effectors" are also active in attacking various host proteins. The protein products of Rgenes guard against pathogens via surveillance of specific targeted host proteins. When these R-gene mediated defenses are triggered, the plant responds with a hypersensitive response and rapid activation of defense gene expression.*

Therefore, development of disease-resistant rice could potentially make a major impact on alleviating hunger. It has been known for decades that a previous inoculation with a virus can often protect a plant from subsequent infections by closely related viruses. This form of immunization of the plant has been known ascross-protection and has been employed with active viruses in limited cases. Crop plants can be intentionally inoculated with mild strains of a virus in the hope that this will protect the plant against future viral outbreaks. Much like vaccination with live viruses in humans, this strategy does have certain risks. In the case of inoculating with mild strains of a plant virus, there is a chance that the mild strain will present a drag on yield or that a virulent strain will emerge from the population and cause severe disease. With the advent of genetic engineering in plants, it became possible to express just a portion of plant viruses within the host. It turns out that this approach can likewise lead to resistance to closely related viruses.

Most plant viruses are relatively simple in terms of their genetic makeup, consisting of just a few genes carried by either an RNA or DNA genome encased in a protein coat. By expressing a portion of the viral genome constitutively in plants, a system of specific targeting of incoming, similar RNA sequences can be activated in a potential host plant. This RNA silencing system is active in many organisms, including humans, and might have evolved partially as a surveillance– protection system against invading viruses.

A great success story using RNA-mediated virus resistance has developed in the production of papaya in Hawaii (Gonsalves 1998). Virtually the entire production of this crop in Hawaii was threatened in the mid-1990s by the spread of the papaya ring spot virus (PRSV). Infection with the virus was so common, and the effects on yield were so severe by the late-1990s that many fields had already been abandoned. By expressing the coat protein gene of a mild strain of PRSV in papaya (Fig. 5), transgenic plants were made resistant to incoming pathogenic viruses (Fitch et al. 1992). Varieties of transgenic papaya were first introduced commercially in Hawaii in 1998, and so far, the transgenic lines have remained virus-resistant over the years. Just as in other transgenic crops, after the initial transgenic transformation in a single variety, the gene of interest was transferred to other desirable commercial varieties using standard breeding techniques. A similar approach has been used successfully to control cucumber mosaic virus (CMV) in transgenic squash production. A particularly exciting application of RNA-mediated virus resistance might be viable in the control of the feathery mottle virus in sweet potato in Africa. Sweet potato serves as a staple crop in some countries, such as Kenya, and viral diseases can be especially severe there and in developing countries. Transgenic varieties resistant to this virus have been developed and might be an effective tool in managing production and increasing yields.

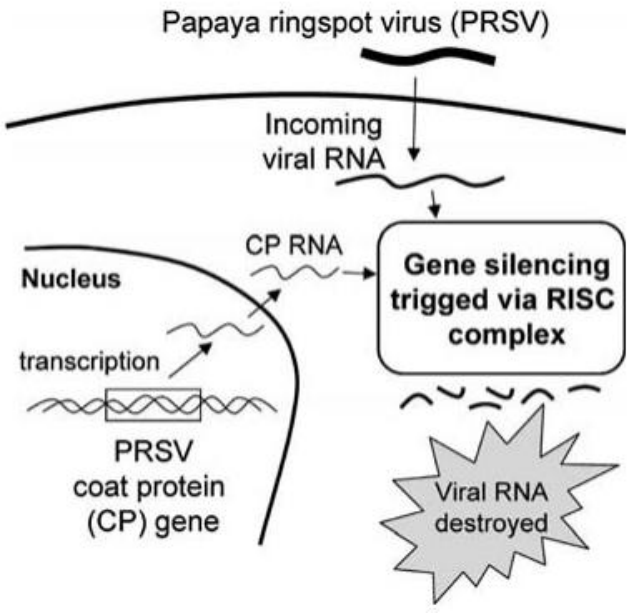

Figure 5. *Transgenic resistance to papaya ringspot virus (PRSV) is possible because of the process of RNA-mediated gene silencing. To make virus-resistant plants, a portion of the coat protein (CP) gene of PRSV was transferred to and expressed in transgenic papaya plants. Following transcription, the RNA triggers targeted, sequence-specific degradation of similar RNA sequences, such as that found on incoming PRSV viral RNA. The initial degradation of RNA is carried out by an enzyme called DICER, and the process is mediated by an enzymatic structure called the RNA-induced silencing complex(RISC). Ultimately, this can lead to RNA cleavage, as well as blockage of transcription or translation of the target gene.*

In the early years of commercialization of plant biotechnology, efforts and products focused on traits that aid in the growing of crop plants, such as resistance to herbicides or insects—these are called input traits. It is likely that many future applications of plant biotechnology will also target output traits, centered on improved plant-based products that will find their way to consumers.

Improvement of crop traits for food quality and products.

Nutritional Improvements

Humans depend on plants as food for survival. In addition to the calories that they provide, plants produce nutrients, vitamins, and essential amino acids that we require. Through methods in biotechnology, efforts are being made to take advantage of the capacity of plants for chemical synthesis to improve or alter the nutritional values of plants. One of the best known examples of nutritional improvement of a food crop has been the development of Golden Rice, a transgenic plant that produces high levels of β-carotene or provitamin A in the grain (Ye et al. 2000). Over one-third of the world's population depends on rice as a major comIS ponent of their diet. Dietary vitamin deficiencies still a serious problem in developing countries in parts of southern Asia and sub-Saharan Africa, where rice is a staple and there is a lack of a diverse diet including meat, fruits, and vegetables. Providing vitamin A supplements as capsules to children and new mothers is one approach to solving this problem, but to be effective, supplements need to be administered several times per year, which can present logistical challenges in many areas. An alternative strategy is to provide provitamin A in the form of β-carotene in rice.

Carotenoids are a subset of compounds based on a five-carbon building block, which can be assembled into multimers to form complex molecules. Carotenoids are 40-carbon compounds produced from the precursor molecule via a biochemical pathway localized in plastids. The 40-carbon backbone of β-carotene is phytoene, which is assembled by combination of two 20-carbon geranylgeranyl diphosphate (GGPP) molecules by the enzyme phytoene synthase Double bonds are then added to phytoene through a series of desaturation steps to produce lycopene, an antioxidant compound found in most plants and that contributes to the red color of tomatoes. Finally, lycopene can be converted to β-carotene by the enzyme lycopene cyclase. Rice grains naturally produce GGPP, and so the addition of an active phytoene synthase gene expressed in rice grains under the control of a seed endosperm-specific promoter led to the production of phytoene. Transgenic plants were produced via particle bombardment in which genes for phytoene synthase, phytoene desaturase, and a lycopene cyclase were co-transformed. These transgenic rice plants had grains with a bright yellow coloring, which was confirmed to come from the presence of β-carotene and led to the name "Golden Rice" (Ye et al. 2000). Later on it was found that plants expressing just the phytoene synthase and the desaturase produced β-carotene, indicating that rice grains already contained the metabolic activity to convert lycopene to β-carotene.

The gene for the desaturase originated from a bacterium, Erwinia spp., whereas the other genes originated from daffodil. The bacterial desaturase enzyme actually performs metabolic steps normally carried out by two separate plant enzymes. Because the daffodil gene products are normally found in plastids, they already contained sequences for a plastid transit peptide to direct newly synthesized proteins to the proper cellular location. The bacterial gene-encoding desaturase was modified by addition of a transit peptide to direct it to plastids following translation.

Golden Rice produces carotene levels sufficient to impart a visible yellow color (Fig.6). One concern with these plants, however, has been that the accumulation levels of β-carotene might not be sufficient to provide enough of the compound to be of nutritional benefit. An improved version of transgenic rice referred to as Golden Rice 2, using a phytoene synthase gene from corn rather than daffodil, was subsequently produced that accumulated levels of carotenoids over 20 times higher than in the original Golden Rice. It is estimated that by eating modest amounts of Golden Rice 2, enough β-carotene can be provided to overcome vitamin A deficiency.

Figure 6. Golden rice having yellow color (a) and normal rice with white color (b).

The large-scale dissemination of Golden Rice has been controversial. Advocates maintain that this rice can provide provitamin A to millions of undernourished children who need it. Rice is already widely grown and consumed in the target regions, and so packaging the technology in this form takes advantage of an existing means to distribute and administer the nutrient. Opponents of the technology counter that development of this product is a tactic used by the biotechnology industry to drive acceptance of transgenic foods worldwide. Many opponents also contend that vitamin supplements and food fortification are superior methods for fighting the problem of vitamin A deficiency. Clearly, this rice has the potential to help malnourished children, but contentious issues must be resolved before it is accepted worldwide. At the very least, development of Golden Rice demonstrates that it is possible to alter the natural abilities of plants to synthesize complex chemicals, and to enhance their nutritional value.

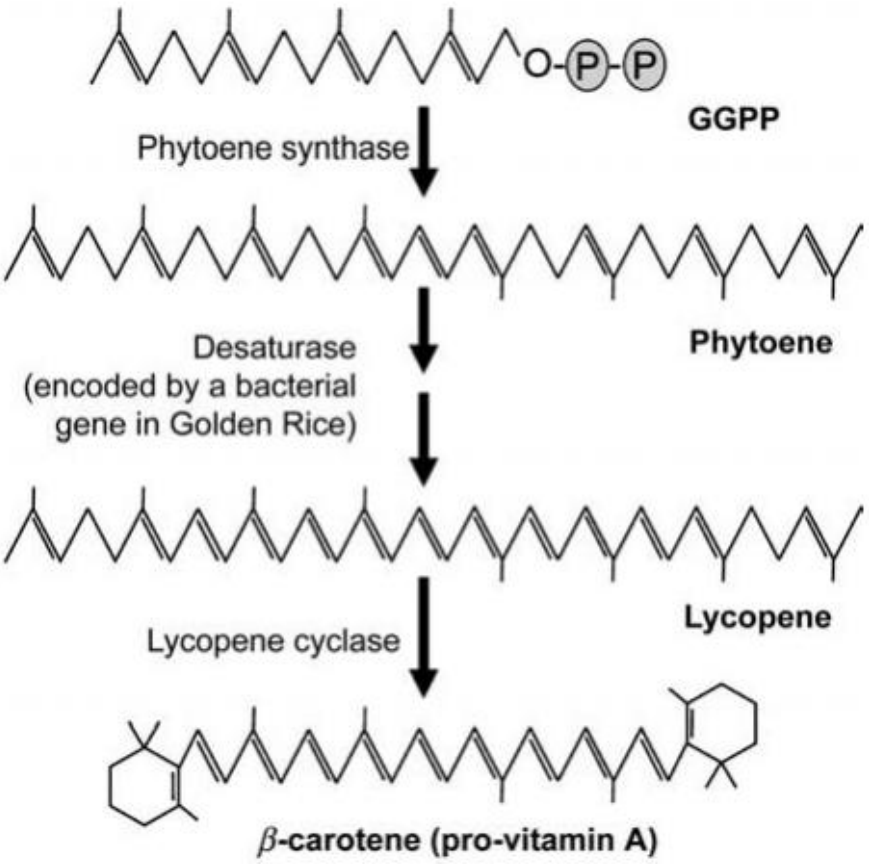

Figure 7. *The production of β-carotene in Golden Rice was made possible by high-level, tissues specific expression of the necessary enzymes in rice. Rice grains normally produce geranylgeranyl–diphosphate (GGPP). A gene-encoding phytoene synthase was transferred to rice from daffodil (for the original Golden Rice) or maize (in Golden Rice 2), and this led to production of phytoene in rice grains. A desaturase enzyme necessary to add double bonds to the structure was provided by transfer of a bacterial gene to rice (the two arrows at this step represent the multiple reactions that are necessary to add all double bonds). Finally, lycopene was converted in rice grains by an endogenous lycopene cyclase activity to the yellow-orange end product, β-carotene.*

Slow Ripening Tomato

In Tomato, enzyme polygalacturonase(PG) degrades pectin which is the major component of fruit cell wall. This leads to the softening of fruits and deterioration in fruit quality. Transgenic tomatoes have been produced which contain antisense construct of the gene encoding PG. These transgenics show a drastically reduced expression of PG and markedly slower ripening and fruit softening (Fig.8). This has greatly improved the self life and general quality of tomato fruits. Such tomatoes are marketed in U.S.A under the name flavr Savr.

Figure 8. Flavr savr Tomato has better self life and so the fruit softening is delayed (a) in comparison to others (b).

Modified Plant Oils

The fatty acids produced by plants are the source of oils used in foods, and also have applications in cosmetics, detergents, and plastics. Oilseed rape (Brassica napus) has been used as a plant oil source for many years. Canola is the common name for the cultivated form of this plant, and has been bred through traditional means to contain low levels of harmful glucosinolates and erucic acid. By engineering canola with a thioesterase gene that originated in the California bay tree (Umbellularia californica), the oils that accumulate contain much higher levels of beneficial fatty acids. The "bay leaf" thioesterase enzyme expressed in canola causes premature chain termination of growing fatty acids, and results in accumulation of 12-carbon lauric acid and 14-carbon myristic acid. The overall level of lipids is not increased in these plants, as the increase in the short-chain molecules is matched by a decrease in the amount of long-chain fatty acids such as the 18-carbon oleic and linoleic acids. These short-chain fatty acids make the canola oil much more suitable as replacement for palm and coconut oils in products such as margarine, shortenings, and confectionaries.

Soybean oil is also used in a variety of food and industrial applications. By decreasing the levels of the enzyme called D12-desaturase in transgenic soybeans, the amount of oleic acid can be increased. To decrease levels of enzyme expression, the normal soybean fad2 gene encoding D12-desaturase was repressed using a technique called gene silencing, whereby a second copy of the gene is introduced into the plant. By over expressing a second copy of the target gene, a response in the plant is triggered to shut down expression of both the endogenous gene and the transgene. In this case, silencing the fad2 gene results in higher levels of oleic acid and corresponding lower levels of two other 18-carbon fatty acids and linoleic and linolenic acids. The only differences in the structures of these three fatty acids are the number of double bonds in the chain. As a result, high oleic acid soybeans have low levels of saturated fats and trans fats. This can alleviate the need for the hydrogenation

process that is often used to make soybean oil suitable for foods like margarine, resulting in a healthier product. It also keeps the oil in a liquid form and makes it more heat-stable for cooking applications.

Pharmaceutical Products

Plant-manufactured pharmaceuticals (PMPs) are one of the most widely discussed applications of transgenic plants. The tremendous variety and potency of chemicals produced in plants has been long recognized, as many have powerful effects on human health and physiology (salicylic acid, cocaine, morphine, taxol, etc.). In addition to being able to produce complex metabolites, plants can also produce high levels of specific proteins when a novel transgene is introduced. Production of human and animal oral vaccines in plants has been proposed as an attractive approach, especially in areas of the world where infrastructure and costs might limit storage, transfer, and administration of traditional vaccines. By including an immunogenic protein in a food, vaccination could be effected using a product that is easily grown and stored and that could be administered via consumption of the food source. For example, production of the surface antigen of the hepatitis B virus in transgenic potato has been demonstrated in clinical trials to lead to an immune response in humans consuming the potatoes. Production of proteins in transgenic bananas is also often cited as a potential source for these oral vaccines. There are several potential problems with this approach, such as the timing of administering the vaccine, dosage, and the ability of the protein to induce immunity on oral administration. Nonetheless, this strategy might have application in some specific instances for humans or in vaccination of farm animals.

Antibodies are large, complex proteins with the powerful ability to recognize and bind to specific molecular targets. Plants do not normally produce antibodies, but it has been repeatedly demonstrated that they can form functional antibodies when the encoding genes are expressed transgenically. One of the more promising approaches is the production of a specific monoclonal antibody that recognizes a cell surface protein of Streptococcus mutans, a bacterium that is one of the major causes of tooth decay. By binding to its surface, the antibody interferes with the bacteria's binding to tooth enamel. The planned applications for this product, produced in tobacco and called CaroRX, would be primarily in toothpastes and mouthwashes.

To date, the vast majority of transgenic biopharmaceuticals are produced using E. coli, yeast, or mammalian cell cultures. The strategy of producing pharmaceutical proteins in plants could have several advantages. Transgenic plants offer the economies of scale to grow

and harvest large amounts of biomass expressing the target product on relatively little land. Some applications for therapeutic proteins such as serum factors, hormones, or antibodies have traditionally relied on human or animal sources. By using plants, the risk of transferring unknown infectious agents from the donor source can be greatly reduced because plants typically do not carry animal pathogens. The idea of producing therapeutic proteins in crop plants is not accepted by everyone. Opponents worry that food products could be contaminated with tissue of plants intended for drug production. Companies that rely on commodities for products to which certain consumers may be sensitive have also opposed transgenic crops expressing pharmaceuticals.

A prominent example was when a large beverage company opposed a pharmaceutical company who wished to grow transgenic rice near rice fields that would be used in their beverage product. Another potential hurdle is the differences in glycosylation of proteins that occur in plants and animals. The sugar moieties added to proteins can vastly affect their function and immunogenicity, and some patterns of plant glycosylation can cause unwanted allergic reactions in humans. To be used in humans, these proteins would need to be produced so that they do not elicit an immune response in the patient.

Biofuels

With demands for energy increasing worldwide and supplies of fossil fuels being depleted, finding alternative and renewable energy sources has become an important goal for plant scientists. Both ethanol (ethyl alcohol) and biodiesel produced using plant materials can be adapted relatively easily to existing fuel storage, movement, and uses with existing infrastructure and machinery. Applications using transgenic plants have the potential to increase the efficiency of biofuel production on several fronts. Ethanol offers several attractive features as an energy source; it is biodegradable and renewable, and burns cleaner than do most fossil fuels. Ethanol is produced by yeast driven fermentation of carbohydrates (sugars). In the United States corn is currently the dominant source for fermentable sugars. In this case, the complex carbohydrates of starch in corn grains are first converted to simple sugars, which the yeast can then use to produce ethanol. One suggested approach to improve ethanol production is to transgenically engineer plants to produce higher levels of the enzymes responsible for the initial steps of starch breakdown. The genes encoding enzymes such as amylase, which degrades starch into simpler sugars, could possibly be expressed at high levels in corn grains or in other plants, resulting in higher percentages of readily fermentable sugars. The considerable inputs necessary for growing corn, in terms of nitrogen

fertilizer, fuel, and pesticides, mean that it is likely not going to be an efficient long-term solution as a source for ethanol production. In Brazil, sugarcane is the plant source of choice for making ethanol, as the high levels of simple sugars make it superior for fermentation. In addition, sugarcane is a perennial crop that can be more easily grown with fewer inputs. The success of the Brazilian adoption of ethanol as a fuel source is widely touted as an example of how existing infrastructure and practices can be adapted for conversion to reliance on biofuels.

The use of plant material high in celluloseas a source for ethanol production is also being widely studied. The conversion of high-cellulose materials into fermentable sugars is an inefficient process, and so it is not currently viable as a method for biofuel production. However, plant materials such as corn stover (stalks and leaves), wood chips, or biomass crops such as perennial grasses contain energy that could potentially be converted to ethanol. Biomass crops, such as switchgrass or fast-growing trees such as willow or poplar, have advantages in that large amounts of biomass can be harvested multiple times from the same plants, and that they will grow efficiently with less need for watering and fertilizers. Although they are currently not efficient, improved methods for this cellulytic conversion of plant material to ethanol may hold some of the best promise for sustainable fuel production from plants. Transgenic approaches are being explored to produce cellulose that would be more easily converted to simple sugars by microbes for alcohol production, or in grasses and woody plants with decreased levels of lignin that can interfere with cellulose degradation. In addition, identification and engineering of microbes that can degrade lignin or more readily convert cellulose and sugars to ethanol are also being explored. There are a number of investigators searching for ways to modify plant feed stocks for eventual more facile cellulosic ethanol production. One idea is to encode cellulases and other cell-wall-degrading enzymes by the transgenic biomass crops directly.

Diesel fuel made from plant material, biodiesel, can also represent an alternative to fossil fuels. Diesel currently accounts for approximately 20% of the fuel consumed for transportation in the United States; therefore, finding a renewable replacement could have a considerable impact on the need for oil throughout the world. Biodiesel is produced from oilseed crops such as soybean and canola, through a process called transesterification.

The properties of biodiesel are slightly different from those of petroleum-based diesel, but biodiesel can be used alone as a fuel or in a blend of the two types of fuel. Although there are currently no transgenic applications to improve biodiesel production in oilseed crops, the

two major sources for biodiesel (soybean and canola) are most often grown as transgenic plants. Because of the economic, environmental, and political concerns associated with fossil fuel consumption, the use of plants for biofuel production will almost certainly continue to increase and develop with new strategies. Genetically engineered biofuel crops will likely not be food or feed crop plants for several reasons as noted above, food companies could be opposed to altering food crops for fuel purposes if there is a viable chance of accidental mixing of fuel and feed.

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PCR AND ITS APPLICATIONS

Introduction:

Polymerase chain reaction (PCR) is a widely employed technique in molecular biology to amplify single or a few copies of DNA, generating millions of copies of a particular DNA sequence. The polymerase chain reaction results in the selective amplification of a target region of a DNA or RNA molecule. PCR has been extensively exploited in cloning, target detection, sequencing etc. The method consists of thermal cycles of repeated heating followed by cooling of the reaction mixture to achieve melting and primer hybridization to enable enzymatic replication of the DNA.

History:

By 1971, a “repair synthesis" process was reported which was an artificial system containing primers and templates that can allow DNA polymerase to copy target gene. The DNA polymerases initially employed for *in vitro* experiments were unable to withstand these high temperatures. In 1976, Chien*et al* discovered a novel DNA polymerase from the extreme thermophile *Thermus aquaticus* which naturally dwell in hot water spring (122 to 176 $^{\circ}$F). The enzyme was named as *Taq* DNA polymerase which is stable upto 95°C. In 1985, Kary Mullis invented a process Polymerase Chain Reaction (PCR) using the thermo-stable *Taq* polymerase for which he was awarded Nobel Prize in 1993.

Fig 1: PCR Thermo cycler

Basic Protocol for Polymerase Chain Reaction:

Components and reagents:

A basic PCR set up requires the following essential components and reagents :

1. Template DNA containing the DNA region (target) to be amplified.

2. Primers that are complementary to the 5' ends of each of the sense (Forward primer) and anti-sense strand of the DNA target (Reverse primer).

3. *Taq* polymerase or other thermostable, high fidelity DNA polymerase (Pfu polymerase isolated from *Pyrococcus furiosus*).

4. Deoxyribonucleotide triphosphates (dNTPs), which are the building-blocks for a newly synthesized DNA strand.

5. Buffer solutions to provide a suitable chemical condition for optimum activity and stability of the DNA polymerases.

6. Divalent cations (eg. magnesium or manganese ions). They act as a co-factor for Taq polymerase which increases its polymerase activity. Generally Mg^{2+} is used, but Mn^{2+} can be applied to achieve PCR-mediated DNA mutagenesis. This is because higher Mn^{2+} concentration leads to higher error rate during DNA synthesis.

Procedure:

Typically, PCR is designed of 20-40 repeated thermal cycles, with each cycle consisting of 3 discrete temperature steps: denaturation, annealing and extension. The thermal cycles are often proceeded by a temperature at a high range (>90°C), and followed by final product extension or brief storage at 4 degree celsius. In PCR cycles, the temperatures and the duration of each cycle is determined based on various parameters like the type of DNA polymerase used, the melting temperature (Tm) of the primers, concentration of divalent ions and dNTPs in the reaction etc. The various steps involved are:-

a) Initial Denaturation

b) Denaturation

c) Annealing

d) Extension

e) Final extension

Denaturation at 94^{o}C: During the denaturation, the double stranded DNA melts open to single stranded DNA.

Annealing at 54^{0}C: The primers are jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

Extension at 72^{0}C: This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template

than the forces breaking these attractions. Primers that are on positions with no exact match get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template.

APPLICATIONS OF PCR

• The polymerase chain response is utilized by a wide range of researchers in a continually expanding scope of experimental orders. In microbiology and atomic science, for instance, PCR is utilized as a part of exploration labs in DNA cloning techniques, Southern smudging, DNA sequencing, recombinant DNA innovation, to give some examples. In clinical microbiology labs PCR is priceless for the analysis of microbial diseases and epidemiological studies. In nourishment science PCR has turn out to be progressively essential to the horticultural and sustenance businesses as an important distinct option for customary identification strategies [47]. PCR is additionally utilized as a part of crime scene investigation labs and is particularly valuable on the grounds that just a little measure of unique DNA is needed, for instance, adequate DNA can be gotten from a bead of blood or a solitary hair .

• Constant PCR (or qPCR) is right now utilized as a part of all applications set up of customary, legacy PCR. Constant PCR has applications in all branches of organic science. Applications incorporate agrarian and nourishment commercial ventures, quality expression examination, the conclusion of irresistible ailment and human hereditary testing. Because of their capacity in fluorimetry the continuous machines are additionally good with option enhancement routines.

• PCR can be utilized for hereditary testing, where an example of DNA is broke down for the vicinity of hereditary illness transformations.

• PCR can be utilized as a major aspect of a touchy test for tissue writing, key to organ transplantation .

• PCR can be utilized for HIV test (vicinity of the HIV infection that causes AIDS can be resolved utilizing PCR on platelets. PCR tests have been created with the goal that it can recognize one viral genome among the DNA of more than 50,000 host cells.

• PCR can be utilized for Genetic fingerprinting (scientific science) can remarkably separate one individual from the whole populace of the world [54]. Moment tests of DNA can be detached from a wrongdoing scene and contrasted with that from suspects or from a DNA database of prior confirmation or convicts.

• PCR can be utilized for DNA fingerprinting can help in Parental testing (DNA sequencing).

• PCR can be utilized for DNA cloning - It can concentrate fragments for insertion into a vector from a bigger genome, which may be accessible in little amounts\.

• PCR can be utilized for the investigation of examples of quality expression. Tissues or individual cells can be examined at diverse stages to see which qualities have get to be dynamic or which have been exchanged off .

• PCR can at the same time increase a few loci from individual sperm has significantly upgraded hereditary mapping by considering chromosomal hybrids after meiosis .

• PCR can be utilized to build up connections among species in human studies and developmental science.

• PCR can be utilized to help recognize antiquated human stays in paleohistory.

• PCR can be utilized PCR to intensify DNA from terminated bugs saved in golden for 20 million years.

• PCR can be utilized to identify the vicinity of a quality moved into a living being (transgene).

MOLECULAR MARKERS AND ITS APPLICATIONS

Introduction

Markers are any trait of any organism that can be identified with confidence and relative ease which can be also followed in mapping population. In general, markers are heritable entities associated with economically important trait under the control of polygenes. In particular, molecular markers were used in genome scans to select those individuals that contain the greatest proportion of favorable alleles from the recurrent parent genome. Molecular breeding is currently standard practice in many crops. Molecular breeding may be defined as genetic manipulation performed at DNA molecular levels to develop characters of interest in plants, including genetic engineering or gene manipulation, molecular marker-assisted selection, genomic selection, etc. However, molecular breeding also implies molecular marker-assisted breeding (MAB) which may be defined as the novel application of molecular markers, in permutation with linkage maps and genomics, to alter and improve plant traits on the basis of genotypic assays.

In the current scenario, the DNA markers become the marker of choice for improving genetic diversity of crops and had revolutionised the plant biotechnology. Gradually, methods are developing more specifically, rapidly and cheaply to assess genetic variation. Results obtained after successful usage of molecular markers indicated that when inbreed lines were unrelated, a measurement of relative relationship based on proportion of homomorphic marker loci, was significantly correlated with a measure of relationship based on yield. Conversely, when lines were related the correlations were low. With the rise of DNA marker technology, several types of DNA markers and molecular breeding strategies are now available to plant breeders and geneticists, helping them to over- come many of the problems faced during conventional breeding. In this chapter, basic qualities of molecular markers, their characteristics, usage and their applications will be discussed.

GENETIC MARKERS: CONCEPT AND TYPES

Genetic markers are the biological features that are determined by allelic forms of genes or genetic loci and can be transmitted from one generation to another, and thus they can be used as experimental probes or tags to keep track of an individual, a tissue, a cell, a nucleus, a chromosome or a gene. Genetic markers that are positioned in near vicinity to genes may be stated as gene ‘tags’. Such markers do not intrude the phenotype of the trait of

interest as they are located only adjacent or 'linked' to genes regulating the trait. All genetic markers occupy definite genomic positions within chromosomes (like genes) called 'loci' (singular 'locus'). The quality of a genetic marker is typically measured by its heterozygosity in population of interest.

Genetic markers may differ in key features like genomic abundance, level of polymorphism, locus specificity and reproducibility. No marker is superior to all others for a wide range of applications. The selection of appropriate marker depends on specific application, presumed polymorphism level, economical variability. Genetic markers were originally used in genetic mapping to determine the order if genes along the chromosomes. In 1913, Alfred Sturtevant generated the first genetic map using sex morphological traits (termed 'factors') in fruit fly *Dorsophila melanogaster* and soon after, Karl sax produced evidence for genetic linkage in common bean . Since these pioneer studies, genetic markers have evolved from morphological markers through isozymes markers to DNA markers. Today, genetic markers are used in both basic plant research, for gene isolation, plant breeding for characterizations of germplasm, for marker assisted introgression of favourable alleles and for variety protection.

Genetic markers used in genetics and plant breeding can be classified into two categories: classical markers and DNA markers.

Classical markers are further classified as morphological markers, cytological markers and biochemical markers.

DNA markers developed into many systems based on different polymorphism detecting techniques or methods (southern blotting, PCR , and DNA sequencing), such as RFLP, AFLP, RAPD, SSR, SNP, etc.

Classical Markers

Morphological Markers

Use of markers as a supportive tool for selection of plants with preferred traits had started in plant breeding long time ago. In early history of plant breeding, markers were used usually for visual phenotype character identification such as leaf shape, flower color, pubescence color, pod color, seed shape, hilum color, type and length, fruit shape, etc. These morphological markers mostly signify genetic polymorphisms which can be simple to identify and manipulate. Therefore, they are usually used in construction of linkage maps by

classical two- and/or three- point tests. Some of these markers which are linked with other agronomic traits and thus can be used as indirect selection criteria in practical breeding. In scientific terms, **morphological markers are those traits that are scored visually, or morphological markers are those genetic markers whose inheritance can be followed with the naked eye**.

The main **advantages** of using morphological markers are easily monitoring and economically viable. However, molecular markers are severely affected by the external environment. Such markers regularly cause major alternations in the phenotype which leads to failure of breeding programs. Morphological markers are also limited in number and appears late in plant development which makes scoring almost impossible. Also, Morphological markers are dominant in nature.

The best example for successful usage of morphological marker that could be considered is selection of semi dwarfism in rice and wheat leading to high yield cultivation of crop. This could be regarded as a suitable illustration for effective use of morphological markers to modern breeding.

Cytological markers: Cytological markers are used for the identification of structural features of chromosomes. Cytological markers can be revealed by chromosome karyotypes and bands. The distributional differences of euchromatin and hetrochromatin in chromosomes including its color, width, order and position in banding patterns can be studied using cytological markers. For example, Q bands are formed by quinacrine hydrochloride, G bands are visualized by Giemsa stain, and R bands are the reversed G bands. The applications of identified chromosome landmarks are not only for classification of normal chromosomes and uncovering of chromosome mutation, but also broadly used in physical mapping and linkage group identification. The physical maps created using combination of morphological and cytological markers lays a perfect basis for genetic linkage mapping with the assistance of modern molecular techniques. However, direct usage of cytological markers has been very limited in genetic mapping and plant breeding.

Molecular markers: Molecular markers have become the marker of choice for the study of plant genetic diversity. Molecular markers are heritable differences in nucleotide sequences of DNA at the corresponding position on homologous chromosome of two dfferent individuals, which followa simple Mendelian pattern of inheritance. Over the last two decades, the advent of molecular markers has revolutionized the entire scenario of biological

sciences. DNA-based molecular markers are a versatile tool in the fields of taxonomy, physiology,

embryology, genetic engineering, etc. They are no longer looked upon in simple DNA fingerprinting markers in variability studies or in mere forensic tools. Ever since the development of molecular markers, these are constantly being modified to enhance the utility and to bring about automation in the process of genome analysis. The discovery of PCR (polymerase chain reaction) was a landmark in this effort and proved to be a unique process that brought about a new class of DNA profiling markers. This facilitated the development of marker- based gene tags, genetic mapping, map-based cloning of agronomically important genes, genetic diversity studies, phylogenetic analysis, and marker-assisted selection of desirable genotypes etc. Thus, giving new dimensions to breeding and marker-aided selection, that can reduce the time span of developing new and improved varieties and the dream of super varieties come true. These DNA markers offer several advantages over traditional phenotypic markers, as molecular markers are not environmentally influenced and provides data that can be analyzed objectively. The existence of various molecular techniques and differences in their principles and methodologies require careful consideration in choosing one or more of such marker.

Types of Molecular Markers

Due to the rapid developments in the field of molecular genetics, varieties of different techniques have emerged to analyze genetic variation during the last few decayed. These genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications. The most appropriate genetic marker has depend on the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and knowhow, time constraints and financial limitations.

A. Biochemical Marker - Allozymes (Isozyme)

Isozymes analysis has been used for over 60 years for various research purposes in biology, viz. to delineate phylogenetic relationships, to estimate genetic variability and taxonomy, to study population genetics and developmental biology, to characterization in plant genetic resources management and plant breeding. Isozymes were defined as structurally different molecular forms of an enzyme with, qualitatively, the same catalytic function. Isozymes originate through amino acid alterations, which cause changes in net charge, or the patial

structure (conformation) of the enzyme molecules and also, therefore, their electrophoretic mobility. After specific staining the isozyme profile of individual samples can be observed (Hadacová & Ondrej 1972, Vallejos 1983, Soltis & Soltis 1989).

Allozymes are allelic variants of enzymes encoded by structural genes. Enzymes are proteins consisting of amino acids, some of which are electrically charged. As a result, enzymes have a net electric charge, depending on the stretch of amino acids comprising the protein. When a mutation in the DNA results in an amino acid being replaced, the net electric charge of the protein may be modified, and the overall shape (conformation) of the molecule can change. Because of changes in electric charge and conformation can affect the migration rate of proteins in an electric field, allelic variation can be detected by gel electrophoresis and subsequent enzyme-specific stains that contain substrate for the enzyme, cofactors and an oxidized salt (e.g. nitro-blue tetrazolium). Usually two, or sometimes even more loci can be distinguished for an enzyme and these are termed isoloci. Therefore, allozyme variation is often also referred to as isozyme variation isozymes have been proven to be reliable genetic markers in breeding and genetic studies of plant species, due to their consistency in their expression, irrespective of environmental factors.

Advantages:

Isoenzymes/ allozymes markers are the oldest among the molecular markers and has following advantages-

i. Doesn't require DNA extraction or the availability of sequence information, primers or probes, they are quick and easy to use.

ii. Simple analytical procedures, allow some allozymes to be applied at relatively low costs, depending on the enzyme staining reagents used.

iii. Allozymes are codominant markers that have high reproducibility.

Disadvantages: The main weakness of allozymes is

i. relatively low abundance and low level of polymorphism.

ii. Like phenotypic markers they may be affected by environmental conditions.

iii. Stage and tissue specific.

B. DNA based Molecular Markers: A molecular markers a DNA sequence that is readily detected and whose inheritance can be easily be monitored. The uses of molecular markers are based on the naturally occurring DNA polymorphism, which forms basis for designing strategies to exploit for applied purposes. A marker must to be polymorphic i.e. it must exit in different forms so that chromosome carrying the mutant genes can be distinguished from the chromosomes with the normal gene by a marker it also carries. Unlike protein markers, DNA

markers segregate by following simple Mendelian Genetics and they are not affected by the environment. DNA is easily extracted from plant materials and its analysis can be cost and labour effective.

Characteristics of ideal DNA marker

1) Highly polymorphic in nature: It must be polymorphic as it measures for various genetic diversity studies.

2) Codominant inheritance: determination of homozygous and heterozygous states of diploid organisms.

3) Frequent occurrence in genome: A marker should be evenly and frequently distributed throughout the genome.

4) Selective neutral behaviours: The DNA sequences of any organism are neutral to environmental conditions or management practices.

5) Easy access (availability): It should be easy, fast and cheap to detect.

6) Easy and fast assay

7) High reproducibility

8) Easy exchange of data between laboratories.

Types of DNA markers:

1. Hybridization based DNA marker
2. PCR based DNA marker
3. Sequencing based DNA marker

1. Hybridization based DNA marker:

a) Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of particular Restriction Endonucleases, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another. This technique is mainly based on the special class of enzyme i.e. Restriction Endonucleases. In RFLP analysis, restriction enzyme-digested genomic DNA is resolved by gel electrophoresis and then blotted (Southern 1975) on to a nitrocellulose membrane. Specific banding patterns are then visualized by hybridization with labeled probe.

Advantages:

i) RFLPs, being co-dominant markers, can able to detect homozygous *vs* heterozygous.

ii) RFLPs are generally found to be moderately polymorphic.

iii) High reproducibility

Disadvantages:

i) Requirement of large quantities (1–10 µg) of purified, high molecular weight DNA

ii) The requirement of radioactive isotope makes the analysis relatively expensive and hazardous.

iii) The assay is time-consuming and labourintensive.

iv) Their inability to detect single base changes restricts their use in detecting point mutations occurring within the regions at which they are detecting polymorphism.

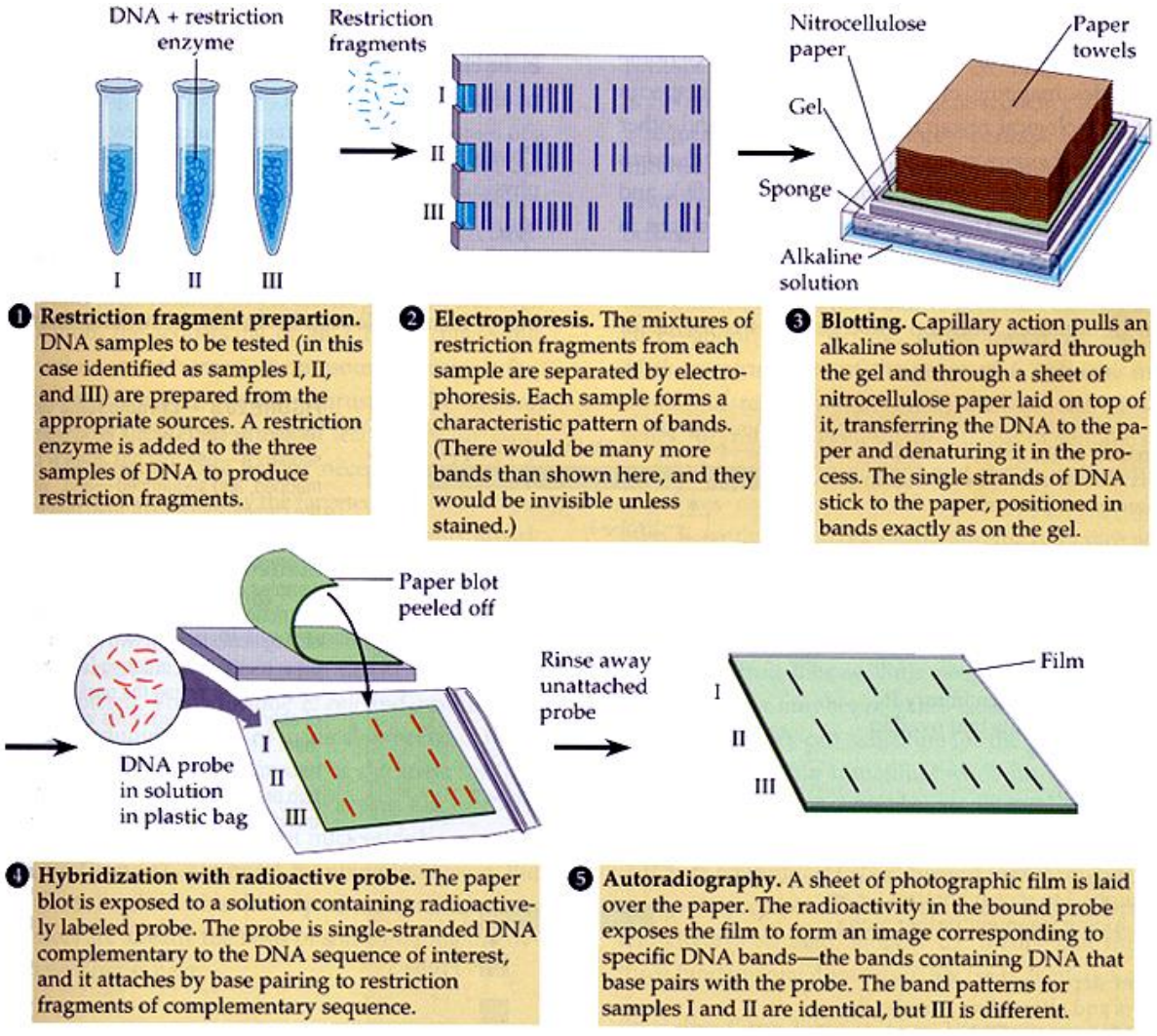

Figure 1. Steps involved in a Southern blot analysis.

PCR based DNA marker

a) Random Amplified Polymorphic DNA (RAPD)

RAPD is a PCR-based technology. The method is based on enzymatic amplification of target or random DNA segments with arbitrary primers. In 1991 Welsh and McClelland developed a new PCR-based genetic assay namely randomly amplified polymorphic DNA (RAPD). This procedure detects nucleotide sequence polymorphisms in DNA by using a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is formed through thermo cyclic amplification. On an average, each primer directs amplification of several discrete loci in the genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals (William et al.1993). RAPDs are DNA fragments amplified by the PCR using short synthetic primers (generally 10 bp) of random sequence. These oligonucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1–10 genomic sites simultaneously. Amplified products (usually within the 0.5–5 kb size range) are separated on agarose gels in the presence of ethidium bromide and view under ultraviolet light and presence and absence of band will be observed. These polymorphisms are considered to be primarily due to variation in the primer annealing sites, but they can also be generated by length differences in the amplified sequence between primer annealing sites.

Advantages:

i) The main advantage of RAPDs is that they are quick and easy to assay.

ii) Low quantities of template DNA are required, usually 5–50 ng per reaction.

iii) Since random primers are commercially available, no sequence data for primer construction are needed.

Disadvantages:

i) The main drawback of RAPDs is their low reproducibility

ii) The inherent problems of reproducibility make RAPDs unsuitable markers for transference or comparison of results among research teams working in a similar species and subject.

iii) RAPD, being dominant markers, can't able to detect homozygous *vs* heterozygous.

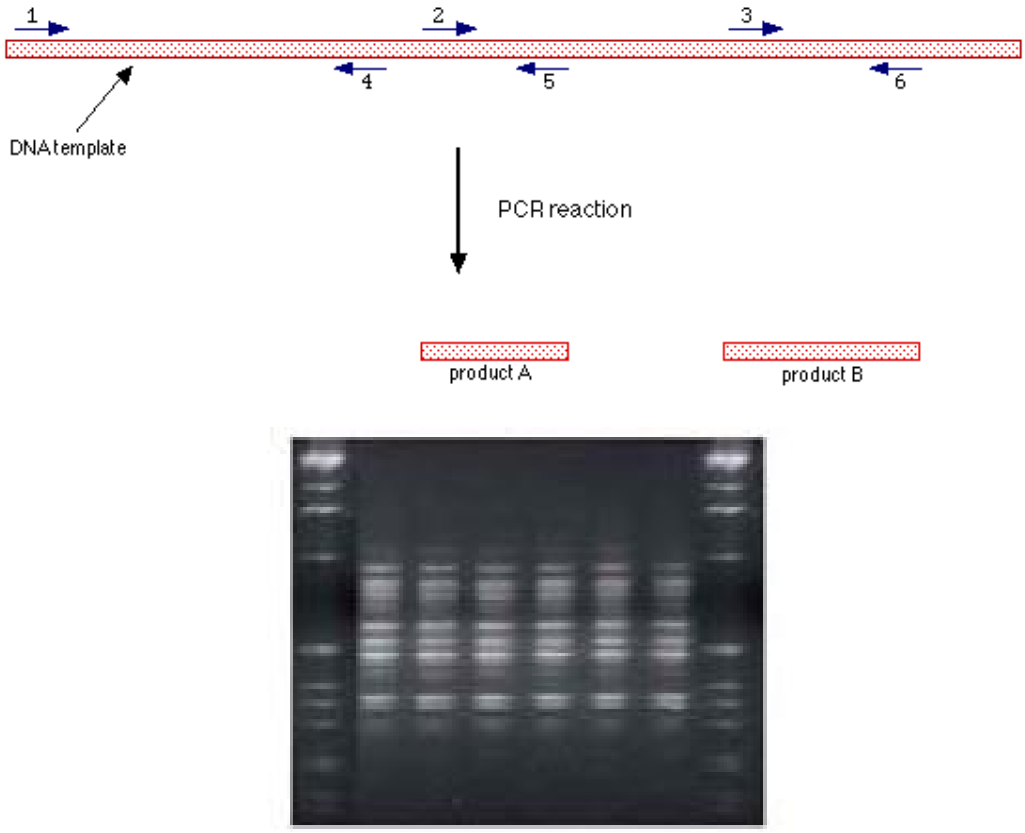

Figure 2. RAPD analysis. In the upper panel binding of random primes to the double stranded DNA has been shown. Gel picture showing the RAPD pattern.

b) AFLP (Amplified Fragment Length Polymorphism)

Amplified fragment length polymorphism (AFLP), which is essentially intermediate between RFLPs and PCR. AFLP is based on a selectively amplifying a subset of restriction fragments from a complex mixture of DNA fragments obtained after digestion of genomic DNA with restriction endonucleases. Polymorphisms are detected from differences in the length of the amplified fragments by polyacrylamide gel electrophoresis (PAGE) (Matthes et al. 1998)or by capillary electrophoresis. The technique involves four steps: (1) restriction of DNA and ligation of oligonucletide adapters; (2) preselective amplification; (3) selective amplification; (4) gel analysis of amplified fragments. AFLP is a DNA fingerprinting technique, which detects DNA restriction fragments by means of PCR amplification. AFLP involves the restriction of genomic DNA, followed by ligation of adaptors complementary to the restriction sites and selective PCR amplification of a subset of the adapted restriction fragments. These fragments are viewed on denaturing polyacrylamide gels either through

autoradiographic or fluorescence methodologies. AFLPs are DNA fragments (80–500 bp) obtained from digestion with restriction enzymes, followed by ligation of oligonucleotide adapters to the digestion products and selective amplification by the PCR.

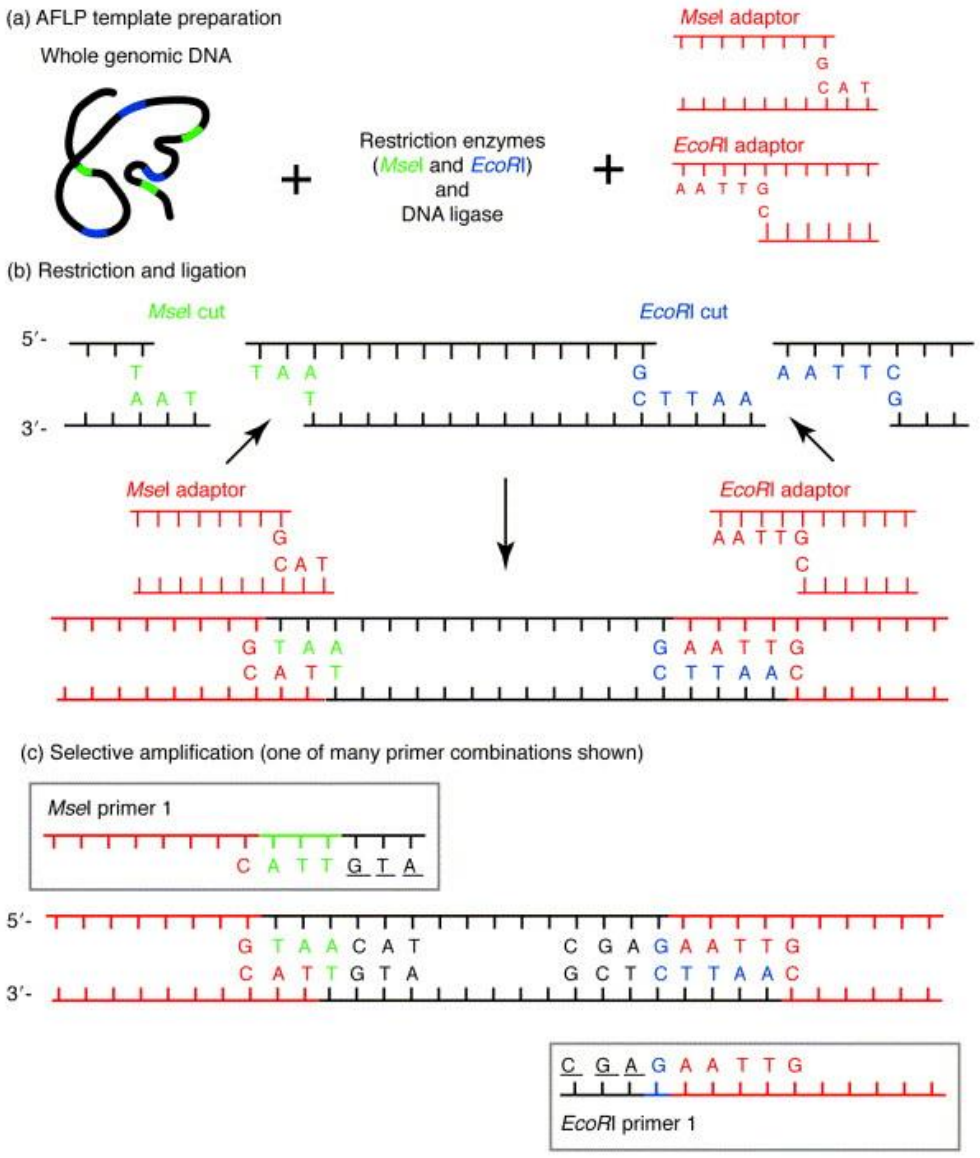

Figure 3: Schematic representation of the AFLP analysis principle.

Advantages:

i) high genomic abundance and considerable reproducibility in nature

ii) no sequence data for primer construction are required

iii) high number of polymorphic bands will be formed by single reaction

Disadvantages:

i) Dominant in nature
ii) Technically demanding
iii) Need to use kits

Minisatellites, Variable Number of Tandem Repeats (VNTR)

These loci contain tandem repeats that vary in the number of repeat units between genotypes and are referred to as variable number of tandem repeats (VNTRs) (i.e. a single locus that contains variable number of tandem repeats between individuals) or hypervariable regions (HVRs) (i.e. numerous loci containing tandem repeats within a genome generating high levels of polymorphism between individuals). Minisatellites are a conceptually very different class of marker. They consist of chromosomal regions containing tandem repeat units of a 10–50 base motif, flanked by conserved DNA restriction sites. A minisatellite profile consisting of many bands, usually within a 4–20 kb size range, is generated by using common multilocus probes that are able to hybridize to minisatellite sequences in different species.

Advantages:

i) The main advantages of minisatellites are their high level of polymorphism and high reproducibility.
ii) No sequence information is required for designing primers.
iii) Co-dominant in nature
iv) Occurs throughout the genome

Disadvantages:

i) Require synthesis of large number of primers

Sequence based molecular marker

Simple sequence repeats (SSRs), also known as microsatellites were recognized and presented a preference for many genetic researcher for its low to high-throughput approaches. They are random tandem repeats of short nucleotide motifs (2 - 6 bp). SSRs are high frequency polymorphic sequences present in animals and plants, and utilized to study relationship between inherited traits within a species. Microsatellite markers are the sequences from noncoding regions of genome.

Polymorphism is based on the variation in the number of repeats in different genotypes. In recent years, SSR markers can easily be developed *in silico* due to the availability of large-scale gene (expressed sequence tag) EST sequence information for many plant species. The high degree of polymorphism as compared to RFLPs and RAPDs, their co-

dominant nature and locus specific make them the markers of choice for a diversity of purposes including practical plant breeding. Therefore, (SSRs) have become a marker of choice for an array of applications in plants due to extensive genome coverage and hyper variable nature.

Inter Simple Sequence Repeats (ISSRs)

It includes the enhancement of DNA fragments present at an amplifiable separation in the middle of two indistinguishable microsatellite rehash locales situated in inverse bearings. The procedure utilizes microsatellites as basis as a part of a solitary preliminary PCR response focusing on numerous genomic loci to increase basically ISSR of diverse sizes. The microsatellite repeats utilized for ISSRs can be dinucleotide, trinucleotide, tetranucleotide, or pentanucleotide. This primer utilized can be either unanchored or all the more for the most part secured at 3` or 5` end with one to four ruffian bases reached out into the flanking groupings. ISSRs utilize longer primer (15-30 mers) when contrasted with RAPD (10 mers), which allow the resulting utilization of a high annealing temperature prompting higher stringency. In addition, ISSRs, as RAPDs, may have reproducibility issues. ISSR examination can be connected in studies including hereditary character, parentage, clone and strain recognizable proof, and taxonomic investigations of firmly related species and in addition in quality mapping studies.

3. Sequencing based DNA marker

Single Nucleotide Polymorphisms (SNPs)

SNP is a single nucleotide polymorphism in which single base difference between two DNA sequences of samples, SNPs are typically biallelic and generated due to substitutions/point mutations (transversion and transition) or as a result of deletion/insertion of nucleotides.

SNPs provide the simplest and ultimate form of molecular markers as a single nucleotide base is the smallest unit of inheritance, and therefore they can provide a great marker density. High density of SNPs over other markers makes it advantageous to find more probability of polymorphisms in a target gene at best closely linked to a locus of interest. Typically, SNP frequencies are in a range of one SNP 100 - 300bp in plants. SNPs are present within coding, non-coding regions or in the intergenic regions between genes at different frequencies in different chromosomal segments.

SNP discovery methods are broadly categorized into four segments: hybridization with allele-specific oligonucleotide probes, oligonucleotide ligation, enzymatic cleavage, and

single nucleotide primer extension. In principle, the SNP methods show differences between a probe of known sequence and a target DNA containing the SNP site. The target DNA sections are typically PCR products and mismatches with the probe reveal SNPs within the amplified target DNA segment. The mismatched DNA can be sequenced to identify SNP polymorphisms.

Now a day, it is very gainful and easier to quickly identify a large number of SNPs within limited time frame in any plant species. This is due to the emergence of the third generation DNA sequencing technologies. The advantage of this new sequence technology are expected to further reduction in sequencing costs to $1 per mega base compared to $60, $2, and $1 expected costs for sequences generated by next generation sequencing (Thudi et al., 2012).

Table. Comparison of Molecular Markers

Feature	RFLP	RAPD	AFLP	SSRs	SNPs
DNA Require (μ g)	10	.02	.5-1.0	.05	.05
DNA quality	High	High	Moderate	Moderate	High
PCR based	No	Yes	YES	YES	YES
No. of Polymorph loci analyzed	1-3	1.5-50	20-100	1-3	1
Ease of use	Not Easy	Easy	Easy	Easy	Easy
Amenable to automation	Low	Moderate	Moderate	High	High
Reproducibility	High	Unreliable	High	High	High
Development Cost	Low	Low	Moderate	High	High
Type of probes/primers	Low copy DNA or cDNA clones	10 bp random nucleotides	Specific sequence	Specific sequence	Allelespecific PCR primers
Effective multiplex ratio	Low	Moderate	High	High	Moderate to high
Marker index	Low	Moderate	Moderate to high	High	Moderate
Genotyping throughput	Low	Low	High	High	High
Primary application	Genetics	Diversity	Diversity and genetics	All purposes	All purposes

MARKER-ASSISTED SELECTION (MAS)

MAS procedure and theoretical and practical considerations

Marker-assisted selection (MAS) refers to such a breeding procedure in which DNA marker detection and selection are integrated into a traditional breeding program. Taking a single cross as an example, the general procedure can be described as follow:

Select parents and make the cross, at least one (or both) possesses the DNA marker allele(s) for the desired trait of interest.

a) Plant F_1 population and detect the presence of the marker alleles to eliminate false hybrids.
b) Plant segregating F_2 population, screen individuals for the marker(s), and harvest the individuals carrying the desired marker allele(s).
c) Plant F2:3 plant rows, and screen individual plants with the marker(s). A bulk of F3 indi-
   viduals within a plant row may be used for the marker screening for further confirmation in case needed if the preceding F2 plant is homozygous for the markers. Select and harvest the individuals with required marker alleles and other desirable traits.
d) In the subsequent generations ($F_4$and F5), conduct marker screening and make selection similarly as for F2:3s, but more attention is given to superior individuals within homozygous lines/rows of markers.

f. In F5:6 or F4:5 generations, bulk the best lines according to the phenotypic evaluation of target trait and the performance of other traits, in addition to marker data.

g. Plant yield trials and comprehensively evaluate the selected lines for yield, quality, resistance and other characters of interest.

In MAS, phenotypic evaluation and selection is still very helpful if conditions permit to do so, and even necessary in cases when the QTLs selected for MAS are not so stable across en- vironments and the association between the selected markers and QTLs is not so close. Moreover, one should also take the impact of genetic background into consideration. The presence of a QTL or marker does not necessarily guarantee the expression of the desired trait. QTL data derived from multiple environments and different populations help a better understanding of the interactions of QTL x environment and QTL x QTL or QTL x genetic background, and thus help a better use of MAS. In addition to genotypic (markers) and phe-

notypic data for the trait of interest, a breeder often pays considerable attention to other important traits, unless the trait of interest is the only objective of breeding.

There are several indications for adoption of molecular markers in the selection for the traits of interest in practical breeding. The situations favorable for MAS include:

The selected character is expressed late in plant development, like fruit and flower fea- tures or adult characters with a juvenile period (so that it is not necessary to wait for the plant to become fully developed before propagation occurs or can be arranged)

• The target gene is recessive (so that individuals which are heterozygous positive for the recessive allele can be selected and/or crossed to produce some homozygous offspring with the desired trait)

• Special conditions are required in order to invoke expression of the target gene(s), as in the case of breeding for disease and pest resistance (where inoculation with the disease or subjection to pests would otherwise be required), or the expression of target genes is highly variable with the environments.

• The phenotype of a trait is conditioned by two or more unlinked genes. For example, selection for multiple genes or gene pyramiding may be required to develop enhanced or durable resistance against diseases or insect pests.

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BIOTECHNOLOGY REGULATIONS

Biosafety is all about ensuring safety and security of both, ecology and human health so that biological integrity is maintained. It is about minimizing the perceived risks to environment and human health from the handling of Genetically Modified Organisms (GMOs) developed through modern biotechnology. It is remarkable that Convention on Biological Diversity (CBD) addresses the conservation and sustainable use of biodiversity. Governments from 130 countries agreed the Cartagena Protocol on Biosafety in Montreal in January 2000. It sets out rules for risk assessment, risk management, Advance Informed Agreement (AIA), technology transfer, and capacity building. AIA procedures will take care of the transgenic plants introduced into the environment intentionally, which may threaten biodiversity.

22.2. Biosafety concerns

Transgenesis of has been in use for over 20 years for genetic improvement of crop plants. Transgenic crops generally carry foreign genes inserted randomly in the genome, and their commercialization is frequently prevented by public concern over ***health and environmental safety issues***. Transgenic crop products are the most highly regulated items in the world. In recent years, there have been calls in the United States to relax some of the rules for their oversight. But, controversies over the safety of transgenic food products continue to resonate, particularly in Europe, Africa and recently in the Far East. Numerous national and international scientific panels have concluded that food derived through transgenic approaches is as safe as food produced otherwise. In fact, the food-borne pathogens pose a much greater threat to human health. However, scary stories continue to appear in the media and questions continue to be asked about the adequacy of current regulatory systems to determine the safety of our food, transgenic or otherwise (DeFrancesco, 2013). It is thought people would show more preference for GM foods if they were eco-friendly.

The great success of GM crops has had an enormous impact on world crop production and cultivation pattern of agricultural species (James 2006). The extensive environmental release and cultivation of GM crop varieties have aroused tremendous biosafety concerns and debates worldwide (Stewart et al. 2000). Biosafety issue has already become a crucial factor in constraining the further development of transgenic biotechnology and wider application of GM products in agriculture. There are a number of ***biosafety related concerns*** in general, but the most important ones envisaged as ***ecological risks*** can be summarized as follows.

(1) Direct and indirect effects of toxic transgenes (e.g. the *Bt* insect-resistance gene) to non-target organisms (O'Callaghan et al. 2005; Oliveira et al. 2007); Insect pests may develop resistance to crops with Bt (*Bacillus thuringiensis*) toxin.

(2) Influences of transgenes and GM plants on biodiversity, ecosystem functions and soil microbes (Giovannetti et al. 2005; Oliveira et al. 2007); It may lead to monoculture and threaten crop genetic diversity with a possible genetic erosion over a period of time.

(3) Transgene escape to crop landraces and wild relatives through gene flow and its potential ecological consequences (Wilkinson et al. 2000; Snow et al. 2003; Lu and Snow 2005; Mercer et al. 2007); Potential transfer of genes from herbicide-resistant crops to wild or weedy relatives thus creating "superweeds".

(4) Potential risks associated with the development of resistance to biotic-resistance transgenes in the target organisms (Dalecky et al. 2007; Li et al. 2007a, b; Wu 2007).

Among the above environmental biosafety issues, transgene escape from a GM crop variety to its non-GM crop counterparts or wild relatives has aroused tremendous debates worldwide (Ellstrand et al. 1999; Ellstrand 2001, 2003; Lu and Snow 2005). This is because transgene escape can easily occur via gene flow that may result in potential ecological consequences if significant amounts of transgenes constantly move to non-GM crops and wild relative species. Despite the potential benefits of transgenic crops, there are also concerns regarding the possible environmental and agronomic impacts if the transgenes escape and get established in natural or agricultural ecosystems. From an agronomic point of view, the transfer of novel genes from one crop to another may have many implications, including depletion in the quality of seeds leading to a change in their performance and marketability. Concerns over the ecological impacts of transgenic crops largely depend upon whether or not a crop has wild relatives and the ability to cross pollinate them. If crops hybridize with wild relatives and gene introgression occurs, wild populations could incorporate transgenes that change their behavior and they could present a serious threat as weeds or competitors in natural communities. This is particularly true when these transgenes can bring evolutionary selective advantages or disadvantages to crop varieties or wild populations. It is therefore essential to properly address the most relevant questions relating to the transgene outflow and its potential environmental consequences on a science-based altitude.Biosafety, as currently discussed in the International “Convention on Biological Diversity” (CBD) and designed to create internationally binding protocols on biosafety.

The application of biotechnology to food and agriculture can bring not only potential risks and benefits as any technology can, but also concerns about the human dimensions coupled with biotechnology. These include both positive and negative impacts on stake holders, social institutions, economy and communities.

Different areas associated with biosafety include:

(*i*) Agriculture and food system issues

(*ii*) Market and consumer issues

(*iii*) Institutional issues, business issues and

(*iv*) Social issues

Agriculture and food system issues. These include the impact of biotechnology on the organisation, structure and behaviour of agricultural industry; further, the coexistence of conventional organic and biotechnology oriented agriculture; the capacity of the food system to

segregate genetically-modified commodity and product of specific markets; impacts on competitions involved, trade in agricultural commodities and the economical impacts of establishing

oversight, standard regulations and public policies concerning biotechnology. **Market and consumer issues.** These include various limitations which come to the rescue of consumer demand for overall against the products of agricultural biotechnology, the needs, desires and concerns of consumers in domestic and international markets; the influence

of culture, advertising, product labelling, scientific information and recent new events on consumer decision making about the use of biotechnology products; different methods for most

effectively increasing the understanding on which publication and primary decision making concerning biotechnology is based. **Institutional issues and business issues.** These include the impacts of biotechnology on individual forms or group of forms about buying or selling biotechnology products and processes; changes in business practices, alliances and domestic and international markets including markets in Third World countries.

Social issues. These include the needs of various public to secure meaningful information for involvement in decision making on development and by use of agricultural biotechno-

logy; the role of civic engagement at the local, state and national levels; perceived and actual risksbenefits to consumers and the general environmental protection, agro-terrorism; research vandalism, and their impacts on Third World nations.

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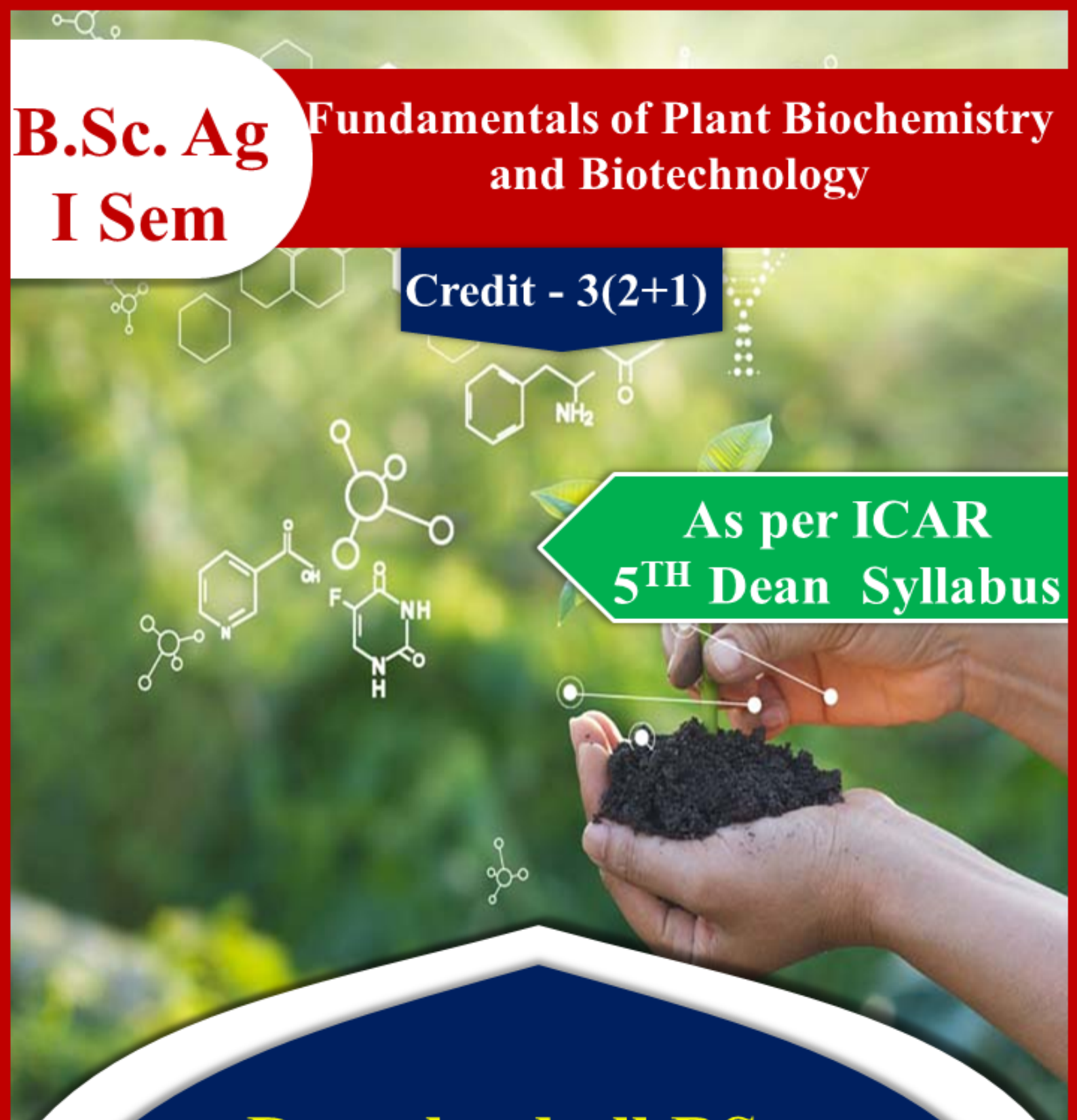

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