PLANT PHYSIOLOGY AND METABOLISM

(DBOT04)

(MSC BOTONY)

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Lesson - 1

PLANT WATER RELATIONS

1.0 Objective

In this lesson, the unique physicochemical properties of water and its transport by diffusion and bulk flow in biological systems, water potential concept and its components are discussed.

1.1 Introduction

Water is the most dominant constituent of living organisms and in a typical non woody plant parts it accounts for more than 70 percent by weight. There are certain desiccation tolerant plants which may have water contents of around 20 percent and dry seeds may contain as little as 5% water, both are considered to be metabolically inactive. The resumption of metabolic activity in them is possible only after the water content has been restored to normal levels.

Water has unique physical and chemical properties that are suited to drive many important processes in the physiology of plants. Over a range of temperatures, water does remain in a liquid state. This thermal property of water is important because most of the biochemical reactions that characterize life can occur in an aqueous medium. The thermal

properties of water also contribute to temperature regulation, helping to ensure that plants do not cool down or heat up too rapidly. Water also has excellent solvent properties which enable the water to act as a suitable medium for the uptake and distribution of mineral nutrients and other solutes required for growth. In addition, water is a transparent medium. This property of water the permits visible light to penetrate the aqueous medium of cells to power photosynthesis or control development.

Water in land plants is part of a very dynamic system. Plants that are actively carrying out photosynthesis are generally subjected to substantial water loss through evapouration from the leaf surfaces. Plants, however, take up equally large quantities of water form the soil to compensate the deficiencies developed in the leaves. This continous and constant flow of water through plants not only provide their growth and survival but also allow all cells to be present in turgid condition, essential to the plant to remain erect in the absence of any skeletal system.

1.2 Physical and Chemical Properties of Water Hydrogen bonding

Water consists of an oxygen atom covalently bonded to two hydrogen atoms. The oxygen is strongly electronegative which means that it has a tendency to attract electrons. So that in the water molecule, the oxygen tends to draw electrons away from the hydrogen. As a result, the two electrons that make up the O-H bond in the water molecule, on the average closer to the oxygen nucleus than to hydrogen. The result is that an oxygen atom carries a partial negative charge and a corresponding positive charge is shared between the two hydrogen atoms. This asymmetric electron distribution makes water a polar molecule. As a whole water is a neutral molecule, but the separation of negative and positive charges generates a strong mutual (electrical) attraction between adjacent water molecules or be-

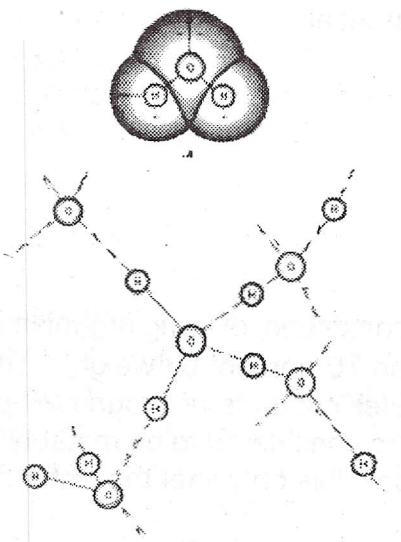

Figure 1.1 (A) Schematic structure of water molecule. (B) The hydrogen bond results from the electrostatic attraction between the partial positive charge on one molecule and the positive negative charge on the next.

tween water and other polar molecules. This is called **hydrogen bonding** (Figure 1.1) and this is largely responsible for many unique properties of water compared with other molecules of similar molecular size. Hydrogen bonding, for example, is the basis for hydration shells that form around biologically important macromolecules such as proteins, nuclei acids and carbohydrates.

Thermal Properties of Water

The thermal properties of water that result from hydrogen bonding are among the most biologically important.

Water remains in a liquid state over the range of temperatures most compatible with life. Boiling and melting points are generally related to molecular size. Smaller molecules undergo state of transitions at lower temperatures than that of larger molecules. Water being a small molecule might be expected to exist primarily in the vapour state at temperatures encountered over most of the earth. However, the melting and boiling points of water are higher than expected when compared with other molecules of similar size such as ammonia and methane. This is because the presence of oxygen in the water induces polarity and opportunity to form hydrogen bonds

Specific Heat and Thermal Conductivity of Water

Specific heat is defined as the amount of energy required to raise the temperature of one gram of substance by 1 ^{o}C. It tells the thermal capacity of a substance. The thermal capacity or specific heat of water is 4.184J g^{-1} $^{o}C^{-1}$. This value is higher than that of any substance except liquid ammonia. In addition liquid water also has a heigh **thermal conductivity** (rapidly conducts heat away from the point of application.) The combination of heigh specific heat and thermal conductivity enables water to absorb and redistribute large amounts of heat energy without correspondingly large increases in temperature. For plant tissues that consist largely of water, this property of water, therefore, enables them to maintain constant internal temperature under conditions of localized overheating due to the heat of biochemical reactions and temperature variations in the surrounding environment.

Melting and Vaporizing Water

Energy is required to cause changes in the state of any substance, such as from solid to liquid or liquid to gas without a change in temperature. The energy required to convert a substance from the solid to the liquid state is known as the **heat of fusion**. The energy required to convert one gram of ice to one gram of liquid water at 0 ^{o}C is 335J g^{-1} (when expressed on a molar basis, the heat of fusion of water is 6.0 kj mol^{-1}, 18 g of water per mole x 335J g^{-1}). This is one of the highest known, second only to ammonia. The high heat of fusion of water is attributed to the large amount of energy necessary to overcome the strong intermolecular forces associated with hydrogen bonding.

The density of ice is another important property. At 0 ^{o}C the density of ice is less than that of liquid water. Unlike other substances, water reaches its maximum density in the liquid state (near 4 ^{o}C) rather than as a solid. This is because the water molecules in the liquid state are able to pack more tightly than in the crystalline state of ice. Consequently ice floats

on the surface of lakes and ponds rather than sinking to the bottom. This is extremely important to the survival of aquatic organisms of all kinds.

Hydrogen bonding also increases the energy required to evaporate water. This is called **heat of vaporization**. The heat of vaporization of water, or the energy required to convert one mole of liquid water to one mole of water vapor is about 44kJ mol^{-1} at 25 °C. Plants absorb this energy from their surroundings to cause evaporation of water from the surfaces of leaves. As a result plants undergo substantial heat loss and maintain pronounced cooling effect on their leaf surfaces. Such heat loss is an important mechanism for temperature regulation in the leaves of terrestrial plants that are often exposed to intense sunlight.

Water as a Solvent

Water is a very good solvent, often called universal solvent because in which most of the substances will be dissolved than in any other common liquid. The excellent solvent properties of water are due to the highly poiar character of the water molecule. So that it has the ability to partially neutralize the electrical attractions between charged solute molecules or ions by surrounding the ion or molecule with one or more layers of tightly oriented water molecules, called a hydration shell. Water which is present in a hydration shell is often referred to as **bound water**. Bound water is important to the stability of biomolecules. It prevents the larger molecules, for example, proteins, from approaching close together to form large aggregates and precipitate.

Cohesion

The strong mutual attraction between water molecules resulting from hydrogen bonding is known as **cohesion**. Because of cohesion, water contains an exceptionally high surface tension, Particularly at interfaces between water and air. Surface tension arises due to the cohesive force, which in between water molecules is much stronger than the interactions between water and air. The result is that the water molecules at the surface are

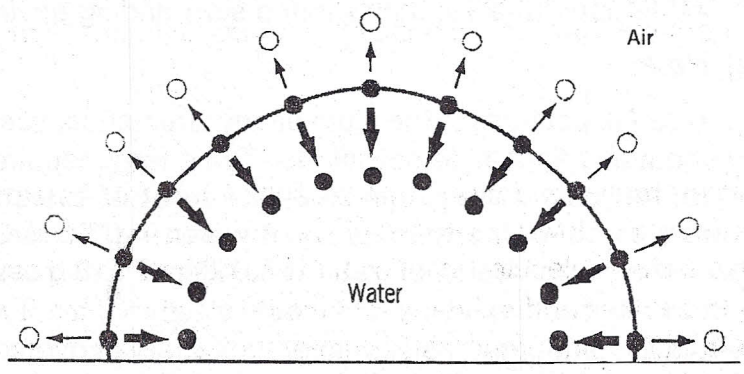

Figure 1.2 Schematic demonstration of surface tension in a water drop. Intermolecular attractions between neighboring water molecules (heavy arrows) are greater than attractions between water and air (light arrows), thus tending to pull water molecules at the surface into the bulk water.

constantly being pulled into the bulk water. The surface thus tends to contract. A high surface tension is the reason that water drops tend to be spherical or its surface will support the weight of small insects. Cohesion is also responsible for the unusually high **tensile strength** of water. It is the maximum tension that an uninterrupted column of any material can withstand without breaking. Although it is a property of metals, under appropriate conditions water columns are also capable of withstanding high tension's on the order of 30 megapascals (MPa). The cohesive forces that attract water molecules to each other will also attract water to solid surfaces known as **adhesion**. Adhesion is an important factor in the capillary raise of water in small diameter conduits.

The combined properties of cohesion, adhesion, and tensile strength help to explain the water rise in capillary tubes and is exceptionally important in maintaining the continuity of water columns in plants.

1.3 Water Transport Processes

When water flows from the soil through plant to the atmosphere it travels through a widely variable medium such as cell wall, membrane, cytoplasm, and air spaces. The mechanisms of water transport also vary with the type of medium. While water flows, substances move from one region to another and this kind of movement of substances along with water in the plant is commonly known as **translocation**. Mechanisms of translocation depending upon the usage of metabolic energy may be classified as either **active** or **passive**. In plants translocation of water is clearly a passive process occurs by one of two physical processes: either **bulk flow** or **diffusion**. In the case of water a special case of diffusion is called as **osmosis**.

1.3.1 Bulk Flow

Bulk flow or mass flow is a pressure driven process by which molecules of the substance move in a mass. For example, water in a river or through a municipal pipes flows in response to the hydrostatic pressure established by gravity. This pressure driven bulk flow of water is the predominant mechanism responsible for long distance transport of water in the plant via xylem.

1.3.2 Diffusion

Diffusion can be interpreted as a direct movement of substances from a region of a high concentration to a region of low concentration by the random thermal motion of individual molecules. That is water molecules in a solution are not static, they are in continuous motion, colliding with one another and exchanging kinetic energy. Such a random thermal motion gives rise to the random but progressive movement of substances from regions of high concentration to regions of low concentration down a concentration gradient. This process was first examined quantitatively by A. Fick. He suggested that the rate of diffusional movement is directly proportional to the concentration gradient. In symbols we write this relation as Fick's first law:

$$J_s = -D_s A\Delta C/l$$

J_s is the flux or amount of material crossing a unit area per unit time (for example, mol $m^{-2}s^{-1}$)

D_s is the diffusion coefficient, a proportionality constant that measures how easily substance **S** moves through a particular medium. The diffusion coefficient is a characteristic of the substance (larger molecules have smaller diffusion coefficients) and depends on the medium (diffusion in air is much faster than diffusion in a liquid). A and l are the cross sectional area and the length of the diffusion path, respectively. ΔC represents the difference in concentration of the subsance S between the two regions. In case of gaseous diffusion, difference in density is expressed in g m^{-3} or vapor pressure (KPa, Kilopascal) in place of concentration. The negative sign in the equation indicates that the flux moves down a concentration or vapor pressure gradient.

Overall Fick's law explains, that the rate of diffusion is directly proportional to the cross sectional area of the diffusion path and to the concentration or vapor pressure gradient and it is inversely proportional to the length of the diffusion path.

Diffusion as is diven by concentration or vapor pressure differences is a significant factor in the uptake and distribution of water, gasses and solutes throughout the plant. Supply of carbon dioxide for photosynthesis and lose of water vapor from the leaves are the two important processes which explains the concept of diffusion.

1.3.3 Osmosis

Movement of a solvent such as water from a region of a high free energy to a region of low free energy through a selectively permeable membrane is known as **osmosis**. Membranes of plant cells are selectively permeable, that is they allow the movement of water and other small uncharged substance across them more readily than the movement of larger solutes and charged substances.

Like molecular diffusion and pressure driven bulk flow, osmosis occurs spontaneously in response to a driving force. In simple diffusion, substances move down a concentration gradient; in bulk flow substances move down a hydrostatic pressure gradient; in osmosis, both types of gradients influence transport. The direction and rate of water flow across a membrane are determined not solely by the concentration gradient of water or by the pressure gradient, but by the sum of these two driving forces. This process can easily be demonstrated in the laboratory using a device known as an **osmometer**.

Osmometer is constructed by closing off the open end of a thistle tube with a selectively permeable membrane. To demonstrate osmosis concentrated solution of sucrose is placed in the hollow core of an inverted thistle tube and a pure water is placed in a beaker. The sucrose solution contained thistle tube is then immersed in the beaker of water. Over a period of time, the volume of solution in the tube will increase. This increase in volume is due to a net diffusion of water across the membrane into the solution. This occurs because the chemical potential of water in pure water in the beaker is higher than the chemical potential of water in the sucrose solution. As the transport of water proceeds, the hight of sucrose solution in the thistle tube increases. Therefore hydrostatic pressure on the membrane

increases. This hydrostatic pressure will tend to press water molecules through the membrane and out of the sucrose solution. When the hydrostatic pressure developed in the tube is sufficient to balance the force driving the water into the solution, further net movement of water through the membrane ceases. Equilibrium with respect to water movement across the membrane has been reached (Figure 1.3). The equilibrium hydrostatic pressure when measured in units of pressure, (force per unit area) is known as **osmotic pressure,** denoted by the Greek symbol π.

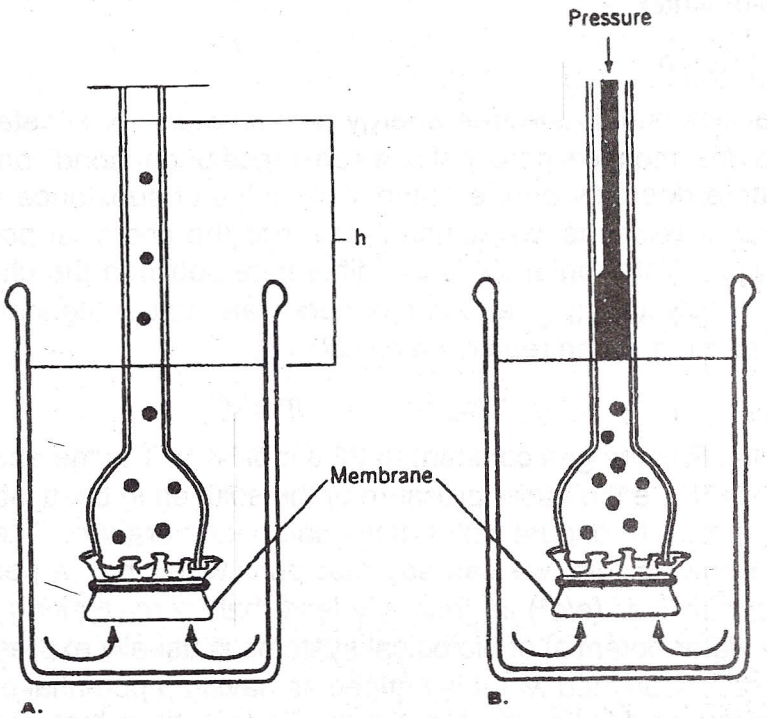

Figure 1.3 A demonstration of osmosis. Initially, water will diffuse across the membrane in response to a water potential gradient. (A) Diffusion will continue until the force of hydrostatic pressure developed in the tube is sufficient to balance the force driving the water into the solution or (B) the pressure applied by the piston.

The magnitude of the osmotic pressure that develops is a function of solute concentration. Isolated solution cannot have an osmotic pressure, but it has only the potential to manifest a pressure when placed in osmometer. For this reason, we say that the solution has an osmotic potential, indicated by the symbol ψ_s. It is convention to define osmotic potential as the negative force of the osmotic pressure ($\psi_s = \pi$).

Most of the water in a mature plant cells is present in the vacuoles. This water moves across the plasma membrane and vacuolar membrane (tonoplast) and the layer of protoplasm between these membranes by osmosis. The most important factor to remember is that osmosis is driven not only by the concentration of dissolved solute but also by pressure differences. Both of these factors influence the overall chemical potential of water, which is the ultimate driving force for water movement in plants.

1.4 Osmosis and Chemical Potential

Osmosis is an energetically spontaneous process. That is water moves down a chemical potential gradient, from a region of high chemical potential to a region of low chemical potential. This means that water moves from out side to inside the cell. Net movement of water stops when there is no longer an energy gradient across the membrane. To understand water transport by osmosis, we need to examine more closely what influences the chemical potential of water.

1.4.1 Chemical Potential

The chemical potential is the free energy per mole of any substance in a chemical system. Therefore, the chemical potential of a substance under conditions of constant pressure and temperature depends on the number of moles of substance that is present. In discussing plant water relations, we generally refer to the chemical potential of water as **water potential** (ψ_w). Water potential is the difference between the chemical potential of water at any point in a system (μ_w) and that of pure water under standard conditions (μ^o_w). With the following formula, it can readily be calculated.

$$\psi_w = \mu_w - \mu^o_w = RT \ln e/e^o$$

In the formula, R is the gas constant (8.32 J $mol^{-1} K^{-1}$), T is the absolute temperature (in degrees Kelvin, or °K), e the vapor pressure of the solution in the system at temperature T, and e^o the vapor pressure of pure water at the same temperature. The expression of RT ln (e/e^o) is zero. Knowing this, we can say that pure water has a petential of zero. In biological systems, however (e/e^o) is generally less than zero, making ln (e/e^o) negative. Consequently, the water potential of biological systems is usually expressed as a negative quantity. Since pure, unconfined water is defined as having a potential of zero, any dilution of water with a solute establishes a potential that is less than that of pure water and is expressed as a negative number. In order to express the water potentials in pressure units, the above water potential equation is divided by the partial molal volume of water (V_w) which is the volume occupied by one mole of water molecules.

$$\psi_w = \mu_w - \mu^o_w / \bar{V}_w = \frac{RT \ln e/e^o}{\bar{V}_w}$$

Where $\mu_w - \mu_w^o$ is the difference between chemical potential of water in solution and that of pure water. The units of above equation are:

$$\frac{erg/mole}{Cm^3/mole} = \frac{erg}{Cm^3}$$ and 1 bar = 0.989 atm = 10^6 dynes/Cm^2

The concept of water potential is actually introduced by R.O. Slatyer and S.A. Taylor in 1960. According to them water potential is proportional to $\mu_w - \mu_w^o$ and the above equation was further simplified as

$$\psi_w = \mu_w - \mu^o_w / V_w = -\pi + P \text{ or simply } \psi_w = -\pi + P$$

Where P is the hydrostatic pressure of the solution, which may be positive, as in turgid cells, or negative, as in xylem water. The symbol π is called osmotic pressure and is the negative of osmotic potential (ψ_s).

The water potential of biological systems is determined by choosing the pure water as a reference state at atmospheric pressure. Under these conditions there is neither hydrostatic pressure nor dissolved solutes; that is both P and π are zero. So that the value of ψ_w for pure water according to the above equation is zero. This is not to say that the chemical potential of pure water is zero. The value of μ^o_w is infact very high but ψ_w for pure water is zero by definition.

1.5 The Components of Water Potential

Water potential of solution may be also defined as the sum of its component potentials, such as solute potential (ψ_s) and pressure potential (ψ_p).

$$\psi_w = \psi_s + \psi_p$$

Solute potential (ψ_S)

The decrease in water potential brought about by dissolved solutes in solution is called **solute potential** or **osmotic potential**. Solutes reduce the free energy of water by diluting the mole fraction of water. Mole fraction of water is the number of water molecules compared with total number of particles.

$$\text{Mole fraction of water} = \frac{\text{moles of water}}{\text{moles of solute + moles of water}}$$

Solute potential in a solution depends on the total number of solute particles in a solution rather than on their kind or their change. For ionic solutes that dissociate into two or more particles, the solute concentration of the solution must be multiplied by the number of dissociated particles. Thus, for example, if we dissolve 0.1 mol of sucrose (non dissociating substance) in 1L of water, we obtain a solution with an osmolarity of 0.1 mol L^{-1} (solute concentration of the solution is expressed as osmolarity: moles of total dissolved solutes per liter of water). If we dissolve ionic substance, for example, sodium chloride in 1L of water, the resulting solution has an osmolarity of 0.2 mol L^{-1} because NaCl dissociates into Na^+ and Cl^- particles. As another example, consider calcium chloride ($CaCl_3$), each mole of which dissociates in solution into 3 moles of ions so that the resulting solution has an osmolarity of 0.33 mol L^{-1}. At 20 °C (293K) the solute potential of 0.2 mol NaCl (-0.244 MPa) and 0.33 mol $CaCl^3$ solutions is equal to solute potential of 0.1 mol sucrose solution (-0.488 MPa). In a solution that consists of several different solutes the solute potential is the sum of the individual solute potentials contributed by each of the solutes.

Pressure (ψ_p)

The term ψ_p is the hydrostatic pressure of the solution. Some times ψ_p is called pressure potential. Positive pressures raise the water potential whereas negative pressures reduce it.

In a laboratory osmometer, this pressure (ψ_p) can be estimated as the difference between atmospheric pressure (0.1 MPa) and the hydrostatic pressure generated by the height of the water column. In cells, this pressure component arises from the force exerted outwardly against the cell walls by the expanding protoplast. This is known as **turgor pressure**. An equal but opposite inward pressure called **wall pressure** is exerted by the cell wall. A cell experiencing turgor pressure is said to be **turgid.** A cell that experiences water loss to the point where turgor pressure is reduced to zero is said to be **flaccid**. The value of ψ_p may also be negative, for example, in the xylem and in the walls between cells, where a tension or negative hydrostatic pressure can develop.

In actuality as water passes through the membrane by osmosis and into a cell, it often encounters resistance from other substances, a factor that contributes to the matric potential (ψ_m). It may be defined as the energy lost (with respect to pure water) as water diffuses and interacts with other substances in the diffusion medium. Since the ψ_m is not applicable and difficult to measure in osmotic systems, we consider it to be negligible when solving problems of osmosis in plant cells.

The osmotic potential of most plant cells is due to the contents of the large central vacuole. Except meristematic cells and highly specialized cells, majority of the cell vacuoles contain 50-80% of the cellular water and dissolved solutes including sugars, organic salts, organic acids and anthocyanin pigments. Most of the remaining cellular water is located in the cell wall spaces, while the cytoplasm accounts around 5-10 percent. The osmotic potential of parenchyma cell is typically in the range of –0.1 to –0.3 MPa.

1.6 Water enters the cell along a water potential gradient

When dealing with water transport at the cell level, the water potential is significantly influenced by two components such as dissolved solutes and hydrostatic pressure.

$$\psi_w = \psi_s + \psi_p$$

The concept of water potential can be fully explained through the following examples. First imagine an open beaker full of pure water at 20 °C (Figure 1.4 A). Since the water is open to the atmosphere, the hydrostatic pressure of the water is the same as atmospheric pressure (ψ_p = OMPa). There are no solutes in the water, so ψ_s = OMPa, therefore, the water potential of water in an open beaker is OMPa ($\psi_w = \psi_s + \psi_p$).

Now imagine dissolving sucrose in the water to a concentration of 0.1 M (Figure 1.4 B). This addition lowers the osmotic potential (ψ_s) to – 0.244 MPa and decrease the water

potential (ψ_w) to – 0.244 MPa.

$\psi_w = \psi_s + \psi_p$; ψ_p = OMPa, ψ_s = o.244 Mpa $\therefore$ $\psi_w = \psi_s$ = -0.244 MPa

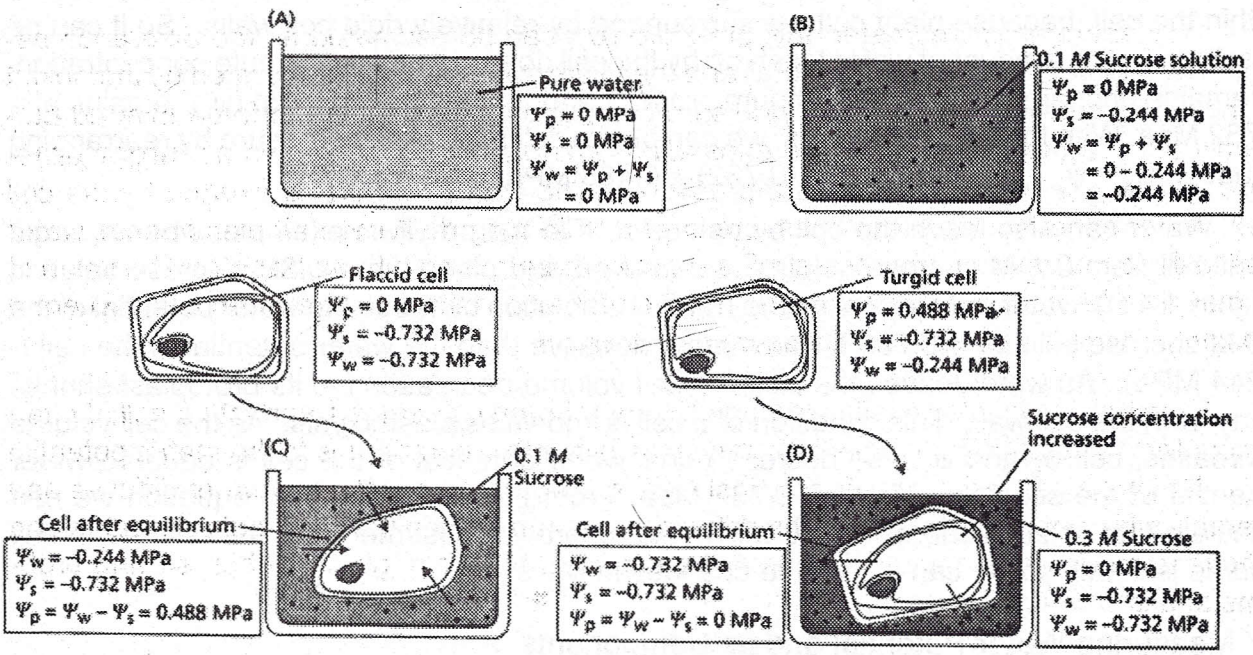

Figure 1.4 Examples illustrating the concept of water potential and its components. (A) Pure water. (B) A solution containing 0.1 M sucrose. (C) After a flaccid cell is dropped in the 0.1 M sucrose solution. (D) After a turgid cell is placed in the 0.3 M sucrose solution.

Next consider a flaccid plant cell (a cell with no turger) that has a total internal solute concentration of 0.3 M (figure 1.4 C). This solute concentration gives an osmotic potential (ψ_s) of –0.732 MPa. Because the cell is flaccid, the internal pressure is zero. So here also the water potential is equal to osmotic potential, which is –0.732 MPa.

What happens if this cell is placed in the beaker containing 0.1M sucrose (see Figure 1.4 C). Water will move from the sucrose solution to the cell because the water potential of the sucrose solution (-0.244 MPa) is greater than the water potential of cell (-0.732 MPa). That is water moves down a water potential gradient. As water moves into the cell, the hydrostatic pressure or turgor pressure (ψ_p) of the Cell increases. Consequently, the cell water potential (ψ_w) increases and the difference between inside and outside water potential ($\Delta\psi_w$) is reduced.

Eventually, cell ψ_p increases enough to raise the cell water potential to the same value as the ψ_w of the sucrose solution. At this point equilibrium is reached ($\Delta\psi_w$ = OMPa), and net water transport ceases. In fact the tiny amount of water taken up by the cell does not significantly affect the solute concentration of the sucrose solution because the volume of sucrose

solution is very much higher than that of the cell. Hence osmotic potential (ψ_s), hydrostatic pressure (ψ_p) and water potential (ψ_w) of the sucrose solution are not altered. Therefore, at equilibrium that water potential of the cell sap is equal to the water potential of sucrose solution in the beaker, which is –0.244 MPa.

A slight increase in cell volume increases a large increase in the hydrostatic pressure within the cell, because plant cell are surrounded by relatively rigid cell walls. So it can be assumed, a tiny amount of water taken up by the cell does not effect its solute concentration. It remains uncharged during equilibrium process and that solute potential (ψ_s) remains at –0.732 Mpa. With these assumptions, we can obtain cell hydrostatic pressure by rearranging equation $\psi_p = \psi_w - \psi_s = (-0.244) – (-0.732) = 0.488$ MPa.

Water can also leave the cell by osmosis. If in the previous example, when a turgid plant cell from 0.1 M sucrose solution is removed and placed in a 0.3M sucrose solution (Figure 1.4 D), water will move from the cell to the solution because the water potential of the 0.3 M sucrose solution (-0.732 MPa) is more negative than the water potential of the cell (-0.244 MPa). As water leaves the cell, the cell volume decreases i.e. its protoplast shrinks away from the cell wall. This condition of a cell is known as plasmolysis. As the cell volume decreases, cell ψ_p and ψ_w also decrease until water potential of the cell is equal to water potential of the solution which is –0.732 Mpa. From the water potential equation we can calculate that at equilibrium $\psi_p = 0$ MPa. As before we assumed that the change in cell volume is small, so we can ignore the changes in ψ_s.

1.7 Measuring Water Potential and its Components

Over the years, very simple and accurate methods have been developed by plant physiologists for assessing water potential and its components in plants because the growth, metabolic activities and the productivity is influenced markedly by water.

Tissue Weight Change Method

Water potential of tissues can be estimated simply by equilibrating preweighed samples of tissue in solution of known osmotic potential. The main objective of this method is to determine the solution with an osmotic potential equivalent to the water potential based on the change in the weight of the tissue.

- If the osmotic potential of the bathing solution is more negative than the water potential of the tissue, water will leave the tissue, as a result the tissue lose weight .
- If the osmotic potential of the bathing solution is less negative than the water potential of the tissue, the tissue will take up water and gain weight.
- The solution at which the tissue neither gains nor loses weight is deemed to have an osmotic potential equivalent to the water potential of the tissue.
- Samples of uniform size are prepared, weighted, and placed in solutions of known molality (0.1, 0.2, 0.3, 0.4) of sorbitol or Mannitol (or) Polyethylene glycol. After allowing 30 minutes for the tissue and bathing solution to come to equilibrium, the tissue is blotted to

remove excess solution and weighed once again, the weight gain or loss is then calculated as a percentage of the original weight and plotted against the concentration of the solution.

- Water potential of the plant tissues is also measured using Thermocouple Psychrometry.
- Osmotic potentials are estimated in leaf epidermal cells and other cells by observing incipient Plasmolysis.
- Negative hydrostatic pressures normally present in xylem vessels can be measured with pressure bomb.

1.8 The importance of water potential

The concept of water potential has two principal uses. First, it is the quantity that governs transport across cell membranes. Second, water potential is used as a diagnostic tool by the plant scientists to assign a precise value to the water status in plant cells and tissues. The lower the water potential in a cell or tissue, the greater is its ability to absorb water. Conversely, the higher the water potential, the greater is the ability of the tissue to supply water to other more desiccated cells and tissues. Thus water potential is used to measure water deficit and water stress in plant cells and tissues.

Figure 1.5 shows some of the physiological changes that plants experience as they become dry under conditions when the transpirational water loss to the atmosphere exceeds the water absorption from the soil. The process that is most affected by water deficit is cell growth. More severe water stress leads to inhibition of cell division, inhibition of wall and protein synthesis, accumulation of solutes, closing of stomata and inhibition of photosynthesis.

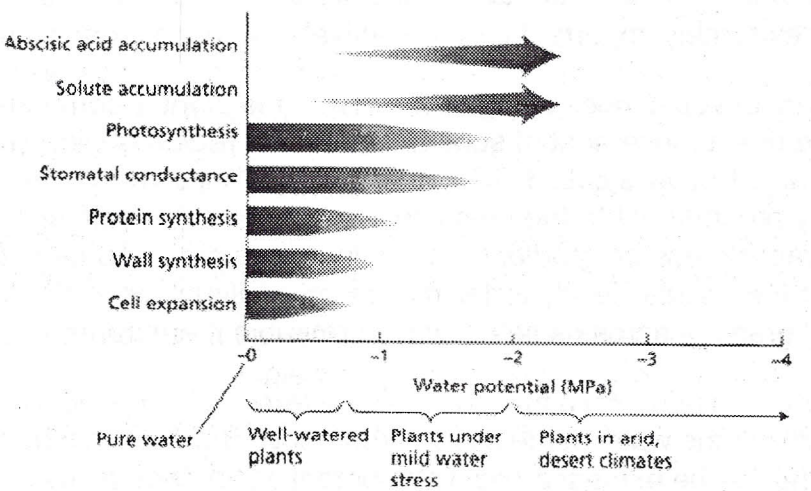

Figure 1.5 Water potential of plants under various growing conditions, and sensitivity of various physiological processes to water potential. The intensity of bar color corresponds to the magnitude of the process. For example cell expansion decreases as water potential falls.

Generally leaves of most plants rooted in wall watered soils are likely to have water potentials between about –0.2 and –0.8 Mpa. With decreasing soil moisture supply, leaf water potential will become more negative than –0.8 MPa and leaf growth rates will decline. Most plant tissues stop growth completely (i.e. will not enlarge) when water potential drops to about – 1.5 MPa.

Leaves of most herbaceous plants usually do not recover if the water potential drops below about –2 to –3 MPa. In contrast, leaves of desert shrubs have a greater ability to survive under conditions of severe drought, perhaps in the range of – 3 MPa to – 6 MPa. Viable, air dried seeds are also likely to have very low water potentials perhaps as low as – 6 MPa to –10 MPa or even much lower depending on the extent of drying and the plant species.

1.9 Summary

Water is the main constituent of living organisms. It possesses certain unique physico-chemical properties that enable water to play many important roles in the physiology of plants. The thermal properties of water contribute to regulation of temperature there by ensuring that plants do not cool down or heat up too rapidly. The solvent properties of water facilitate appropriate medium for the uptake and distribution of mineral nutrients and other solutes required for growth. The transparency of water permits light to penetrate the aqueous medium of cells, and used to perform photosynthesis or control development.

Hydrogen bonding is largely responsible for the main unique properties of water. The most important property of water is that it is a liquid over the range of temperatures most compatible with life. The combination of high specific heat and thermal conductivity enables water to absorb and redistribute large amounts of heat energy without correspondingly large increase in temperature. Unlike other substances, water reaches its maximum density in the liquid state that is extremely important for the survival of aquatic organisms of all kinds.

The movement of substances along with water in the plant is commonly referred to as translocation. The direct movement of substances from a region of high concentration to a region of lower concentration is called diffusion. Over all Fick's law explains that the rate of diffusion is directly proportional to the cross sectional area of the diffusion path and to the concentration or vapor pressure gradient, an it is inversely proportional to the length of the diffusion path. At the cellular level, water moves primarily by osmosis, in response to a chemical potential gradient across a selectively permeable membrane.

Water movement is primarily a function of the difference in chemical potential between the water in the cell and the water in the cell environment. The chemical potential of water in plant water relations can be expressed as water potential, defined as the sum of two easily measured quantities: hydrostatic pressure and osmotic pressure.

In view of the significance of water that influences the growth, metabolic activities and the productivity, many methods and mechanism have been developed by plant physiologists for assessing water potential like tissue weight change method, *Thermo couple* psychrometry, Incipient plasmolysis and pressure bomb.

1.10 Model questions

1. Give an account of thermodynamics of plant water relations. Define osmotic and water potential. Suggest simple method to determine water potential.
2. Write short notes
   a) Significance of water potential
   b) Hydrogen bonding and its contribution to the unique properties of water.

1.11 Reference Books

1. Introductory plant physiology – G.R. Noggle and G.J.Fritz. Prentice Hall of India – New Delhi.
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Dr. G. Rosaiah

Lesson – 2

WATER TRANSPORT THROUGH XYLEM - THE COHESION THEORY

2.0 Objective

In this lesson the upward movement of water from ground level to the top of the tallest trees was described by a variety of mechanisms including root pressure, capillarity and cohesion-tension theory.

2.1 Introduction

Movement of the absorbed water through the vascular system from the xylem terminals in the root to those in the leaf is called ascent of sap. Sometimes, water transport covers a distance of more than 110 meters against a gravitational bull as in the case of a Californian Redwoods (*Sequoia sempervirens*) and an Australian *Eucalyptus regans* (130 meters). The problem that has long held plant physiologists is, the mechanism by which water moves to the top of the tallest tress movement. The forces required to move water to such heights are substantial. One atmospheric pressure supports a column of water 10.3 m or a column of mercury 760 mm in height. To push water from the ground level to the top of the tallest tree requires a top to bottom pressure difference of about 1.0 to 1.5 MPa. So, is evident that water is not pushed to the top of tall trees by atmospheric pressure.

Even a force of 1.0 to 1.5 MPa would not sufficient to move water up the tallest tree. Water moving through the plant will encounter a certain amount of resistance due to irregular wall surfaces and perforation plates of water conducting tracheids and vessels. In addition to this frictional resistance, we must consider gravitation force. The weight of a standing column of water 100 m tall creates a pressure of 1 MPa at the bottom of the water column.

This pressure gradient due to gravity must be added to that required to cause water movement through the xylem. Thus we calculate that a pressure difference of about 2.0 to 3.0 MPa would be required to move water from ground level to the top of the tallest trees.

How can such a pressure be generated? To answer this question, from time to time various theories have been proposed, the prominent being are root pressure, capillarity and cohesion – tension theory. Before we are going to discuss these theories in some detail, we must consider anatomy of water conducting cells.

2.2 Water transport by tracheids and vessels

One of the distinguishing feature of vascular plants is the presence of vascular tissues, the **xylem** and **phloem**, which conduct water and nutrients between the various organs. Xylem tissue is responsible for the transport of water, dissolved minerals, and on occasion, small organic molecules from the root to the aerial organs through the stem. Phloem, on the other hand, is responsible for the translocation of organic substances from sites of synthesis to storage sites or sites of metabolic demand.

Xylem consists of **fibers**, **parenchyma cells**, **tracheary elements**. Fibers are very elongated cells with thickened secondary walls, which provide structural support for the plant Parenchyma cells of the xylem serve as storage as well as the lateral translocation of solutes. Tracheids and vessel elements together known as tracheary elements. They are the most highly specialized of the xylem cells and are the principal water conducting cells. Vessel elements are found only in angiosperms and a small group of gymnosperms called the Gnetales. Tracheids are present in both angiosperms and gymnosperms. When matured, the trachieds and vessels form an interconnected network of nonliving cells devoid of all protoplasm. They have no membranes or organelles. They are like hollow tubes. Tubular and hollow nature of trachieds and vessel elements with their extensive interconnections enables them to transport large volumes of water throughout the plant with great efficiency.

Tracheids are elongated, spindle shaped cells with diameters in the range of 10-50 μm. (Figure 2.1) They generally measure less than 1 cm in length. The cell walls of trachieds are composed of cellulose, hemicellulose and lignin. Because of the high lignin content, secondary walls of trachieds are less permeable to water than are the primary walls of growing cells. On the other hand, the secondary walls provide additional strength, and helps to prevent the cells from collapsing under the extreme negative pressure developed in the actively transpiring plants. Though trachieds conduct water, the thickened secondary walls also provide structural support to the plant.

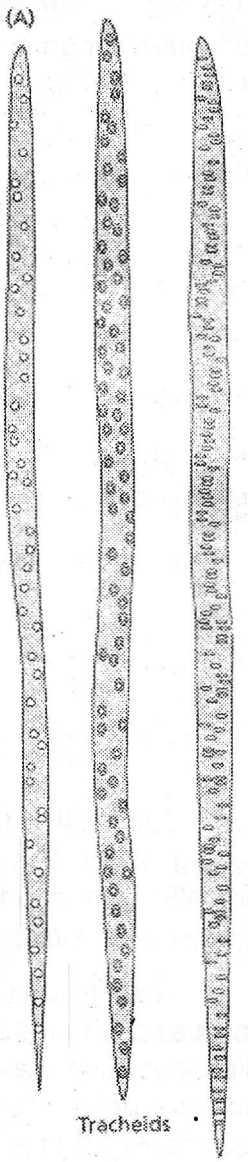

Figure 2.1 The shape of plant tracheids. The walls contain numerous bordered pits.

The movement of water between trachieds is facilitated by small interruptions, known **as bordered pits** *in the secondary walls. Bordered pits do not deposit secondary wall* material during the development of trachieds. Hence, they have only the middle lamella and primary walls to separate the hollow core or lumen of the cell (Figure 2.2). Pits of one tracheid are typically located opposite pits of adjoining tracheid, forming **pit pairs**. The combined middle lamella and primary walls between pit pairs is known as the **pit membrane** and having around 0.3 μm diameter openings that permit the relatively free passage of water and solutes. Bordered pit pairs have secondary wall projections over the pit area. The pit membrane in tracheids of gymnosperms usually have a central thickening called a torus (Figure 2.2). The torus acts like a valve to close the pit by lodging itself in the circular or oval wall thickenings bordering these pits. When pressure is unequal in adjacent vascular elements, the torus drawn toward the element with the lower pressure and seals off the pit.

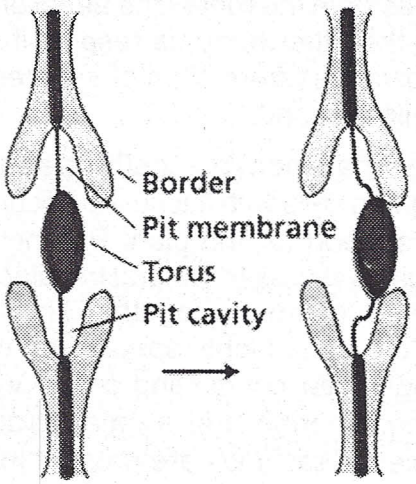

Figure 2.2 Diagram of a bordered pit with a torus eitner contered in the pit cavity or lodged to one side of the cavity, thereby blocking water flow.

Successive trachieds overlap at their tapered ends. As a result they line up in files running longitudinally throughout the plant. Water move between adjacent tranchieds either vertically or longitudinally through the pit pairs by their overlapped regions. The movement of water is facilitated by the openings in the pit membranes.

Vessels are very long tracheary elements made up of individual units, known as **vessel members**. (Figure 2.3) They are arranged end to end in longitudinal series to form a larger unit called a **vessel**. At maturity, the end walls of the vessel members are dissolved away and form perforation plates. If the perforations are elongate and parallel they are called scalariform. If they are net like pattern they are known as reticulate. These perforations allow free flow of water between successive vessel members. However absence of perforation plates at the ends of vessels, that is, the last vessel member in a sequence, facilitates lateral conduction of water from one vessel to the next due to the presence of pit pairs.

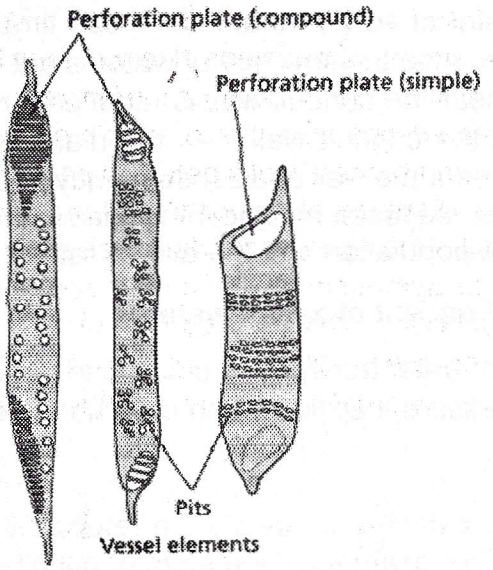

Figure 2.3 Vessel elements with perforation plates and pits.

The exact length of vessels is difficult to ascertain. It is highly variable. In maples, vessels range from 40-60 μm in diameter. Whereas in some **Quercus** species vessels range from 300-500 μm in diameter. Similarly length of the vessels in maple are account for 4 cm or less, but in some cases they may reach 30 cm to 30 m length (*Quercus*). Because of extensive branching of the vasucular system and the large number of lateral connections between the overlapping tracheary elements, the xylem constitutes a single continous, interconnected system of water conducting conduits between the extremes of the plant from the tip of the longest root to the outermost margins of the highest leaf.

Vessels are more advanced than trachieds. Gymnosperms entirely consist of trachieds in their xylem. Compared to gymnosperms angiosperms do possess trachieds but the bulk of the water is conducted through vessels efficiently because vessels are larger in size.

Jean L.M.Poiseuille, a French scientist, developed an empirical equation to show the relationship between flow rate and size of conduits.

$$J_v = \Delta P \ \pi r4/8\eta$$

When a fluid is pressure driven, the volume flow rate (Jv) is a function of the viscosity of the liquid (η) the difference in pressure or pressure drop (ΔP) and the radius of the conduit (r).

The above equation can be applied to movement of water in the xylem, because water movement in the xylem is driven by a difference in pressure between the soil and the leaves. The key point to remember is that the volume flow rate is directly proportional to the fourth power of the radius.

The volume flow rate through a vessel with a dimeter of 200 μm is 5 times that of the tracheid having a diameter of 40 μm (that is 5^4 = 625 times). The high rate of flow in the larger vessels occurs because the flow rate of water is not uniform across the conduit. The flow rate of molecules near the conduit wall is reduced by friction, due to adhesive forces between the water and the conduit wall. As the diameter of the conduit increases, the proportion of molecules near the wall and consequently subject to these frictional forces will decrease. In other words, the faster moving molecules in the center of the conduit constitute a larger proportion of the population and the overall rate of flow increases accordingly.

2.3 The mechanism of ascent of xylem water :

The mechanism of water transport in plants can be explained by the three prominent theories such as root pressure, capillarity and cohesion – tension theory.

2.3.1 Root Pressure

Plants sometimes exhibit a phenomenon referred to as **root pressure** or **positive hydrostatic pressure**. For example, if the stem of a well watered herbaceous plant is cut off above the soil line, the stump will often exude sap from the cut xylem for many hours. If a monometer is sealed over the cut stump, positive pressures can be recorded. This pressure is known as root pressure because the forces which give rise to the exudation, originate in the root. These pressures can be as high as 0.05 to 0.5 MPa.

Root pressure can be understood as a positive hydrostatic pressure in the xylem. It has its basis in the structure of roots and the active uptake of mineral salts from the soil. The xylem elements are located in the central core of a root, called the stele, which in turn is surrounded by a layer of endodermis. Water can move into or out of the stele only by first passing through the membranes of the endodermis and then through the plasmo-desmatal connections. This is because of the Casparian band, which is present in the radial and transverse walls of the endodermal cells, prevents the movement of water through the apoplastic space of the endodermis.

Further, the roots absorb ions from the dilute soil solution and transport them into the xylem. The active build up of solutes in the xylem sap leads to a decrease in the xylem osmotic potential (ψ_s) and thus a decrease in the xylem water potential (ψ_w). In response to the lowered water potential, water moves from the surrounding cortical cells into the xylem through the membranes of the endodermal cells. Since the Casparian band prevents the free return of water to the cortex, a positive hydrostatic pressure is developed in the xylem. In effect, the whole root acts like an osmotic cell in which the endodermis behaves as a differentially permeable membrane, the ions accumulated in the xylem represent the dissolved solute, and the xylem elements are the vertical tube. The question to be answered at this point is whether root pressure can account for the rise of sap in a tree. For several reasons, the answer to this question is no. The measured root pressure values are only in the range of 0.05 to 0.5 MPa, which are no more than 16 percent of that required to move water to the top of the tallest trees. In addition, root pressure has not been detected in all species and is not always detectable even those species which do exhibit. Root pressure is most prominent

in well-hydrated plants under high humidity where there is little transpiration. Under drier conditions, when transpiration rates are high, water is taken up so rapidly into the leaves and lost to the atmosphere, the xylem is present under tension i.e negative pressure. So root pressure clearly cannot serve as the mechanism for the ascent sap in all cases.

Plants that develop root pressure frequently exhibit exudation of liquid from the leaves, a phenomenon known as **guttation**. Positive xylem pressure causes exudation of xylem sap through structures called the hydathodes. They are located near terminal tracheids of the bundle ends around the margins of leaves. The 'dewdrops' that can be seen on the tips of grass leaves in the morning are actually guttation droplets exuded from such specialized pores.

2.3.2 Water rise by capillarity

The movement of water small distance up a glass capillary tube is called **capillarity**. When a glass capillary tube is inserted into a volume of water, water will rise in the tube to some level above the surface of the surrounding water. This upward movement of water in such a small diameter tube is due to the interaction of several forces. These include adhesion between water and polar groups along the capillary wall, surface tension which tends to minimize surface area, and the force of gravity acting on the water column. Adhesive forces attract water molecules to polar groups along the surface of the tube. When the water to wall forces are strong, water flows upward along the wall. As water flows upward along the wall, strong cohesive forces between the water molecules act to pull the bulk water up the lumen of the tube. This rise will continue until these lifting forces are balanced by the downward force of gravity acting on the water column.

The rise of water in a capillary tube is inversely proportional to the radius of the tube. The smaller the tube, the higher the capillary rise which may be calculated by the following formula :

$$\text{Capillary rise} = \frac{14.9 \times 10^{-6}\ m^2}{\text{Radius}}$$

Where both capillary rise and radius are expressed in meters. For a xylem vessel with 25 mm radius, the capillary rise is about 0.6 m. This rise is only 0.08 mm for a vessel having longer radius of 200 um. On the basis of these numbers, capillarity in tracheids and small vessels might account for the rise of xylem sap in small plants to a height of less than 0.75m. The capillarity mechanism there fore, is inadequate to explain the water movement in the tallest tress.

2.3.3 Cohesion tension theory

Henry H. Dixon, an Irish botanist, and John Joley, a physicist in 1894 developed the idea of cohesion-tension mechanism of ascent of sap. The work was published in book form by Dixon with a great wealth of experimental details in 1914. The theory states that

transpiration pull or tension, cohesion property of water and adhesion between water and cell walls are collectively responsible for the upward movement of water in the tallest trees. Water movement through the xylem is a bulk flow caused by pressure difference ($\Delta\psi_p$) between the roots and the tree tops. A negative pressure or tension created due to transpiration is transmitted through the continuous water column within the xylem to the root. The breaking of the water column is prevented by cohesive forces between adjacent molecules of water and adhesion between water molecules and cell walls. Therefore, the cohesion theory for the ascent of sap has three basic components, the driving force, the cohesion of water and the hydration of cell wall (adhesion).

The driving force

The driving force is the gradient in decreasing water potentials from the soil through the plant to the liquid – air interface at the evaporating surfaces within the leaf. Water covers the surfaces of the leaf mesophyll cells as a thin film. As water evaporates from this thin film, the air – liquid interface retreats into the small spaces between cellulose microfibrils and the angular junctions between adjacent cells. This creates very small curved surfaces (Figure 2.4). As the radii of these curved surfaces progressively decrease, surface tention at the air-water interface generates an increasingly negative pressure, which in turn tends to draw more liquid water toward the surface. That is, as water evaporates from the leaf mesophyll cells, it causes a decrease in water in water potentials (ψ_w) of those cells in direct contact with the air spaces of the leaf. Due to the negative water potentials of the surface cells, water moves into them from the deeper cells of the leaf. In an attempt to equate water potential value, the leaf cells ultimately tend to draw water from the veins of the leaf, thus subjecting the water in the xylem to a state of tension or negative pressure. Because the water column is continuous, this negative pressure or tension, is transmitted through the column all the way to the soil. This top to bottom pressure difference ($\Delta\psi_p$) developed between the roots and the tree tops via the stem is called the driving force. This driving force drives the upward movement of water in plants from the roots to the surface of mesophyll cells in the leaf.

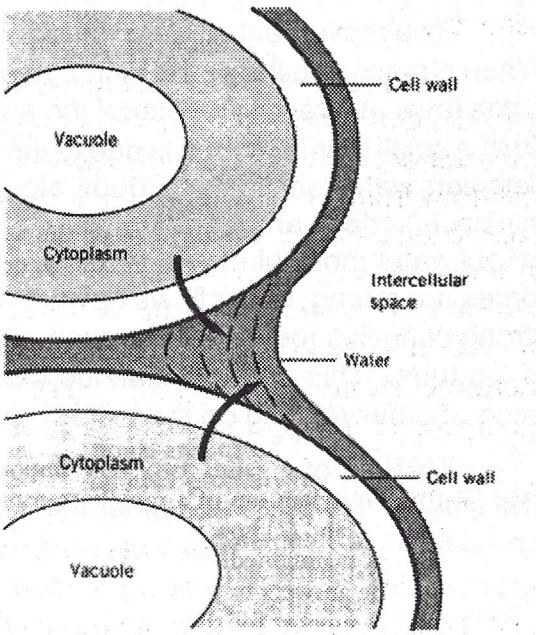

FIGURE 3.13 Tension (negative pressure) in the wa column. Evaporation into the leaf spaces causes the water–air interface (dashed lines) to retreat into the spaces between and at the junctions of leaf mesophy cells. As the water retreats, the resulting surface ter pulls water from the adjacent cells. Because the wat column is continuous, this tension is transmitted through the column, ultimately to the roots and soi water.

The cohesion theory raises two very important questions.

1. Is the xylem sap of a rapidly transpiring plant under tension ?
2. How is the integrity of very tall water columns maintained ?

Several lines of evidence made it clear that xylem water is under significant tension. When the xylem of a rapidly transpiring plant is served, it is sometimes possible to hear the sound of air being drawn rapidly into the wound. If served beneath the surface of a dye solution, the dye will be rapidly taken up into tracheary elements in the immediate vicinity of the wound. Kramer and Kozlowski (1960) by using Dendrographs observed a decrease in diameter of woody stems during excessive transpiration period. This will happen because the stem is slightly elastic and the tension in the water column pulls the tracheary walls inward. In the evening, when transpiration declines, the tension is released and stem diameter recovers.

In a pressure bomb technique adopted by Scholander and co-workers (1965), a shoot is excised and sealed in a pressure chamber with only the cut surface of the stem exposed. If the water column in the xylem is under tension, as it normally is in a transpiring shoot, it will withdraw from the surface when the stem is cut. Pressuring the chamber will force the water back to the surface. The pressure required to bring the water back to the surface is considered of equal magnitude but of opposite sign to the tension that existed in the xylem prior to excision. With such a device, scholander and others have measured tensions on the order of –0.5 to –2.5 MPa in rapidly transpiring temperate zone trees. The weight of evidence, therefore, clearly supports the hypothesis that the xylem water column is literally pulled up the tree in response to transpiration.

The cohesion of water

The integrity of tall water columns in the xylem depends on tensile strength of the water. Tensile strength is a measure of the maximum tension a material can withstand before breaking. The water molecules have strong mutual attraction (cohesion) due to which they cannot be easily separated from one another (high tensile strength). A variety of factors including the diameter of conduit, the properties of conduit walls, and presence of any dissolved gasses or solute will influe the tensile strength of water. It has been estimated that a pure water, free of dissolved gasses, is able to withstand tensions as low as –25 to –30 MPa at 20°C. This is approximately 10 times greater than the –2.5 to –3.0 MPa required to pull an uninterrupted water column to the top of the tallest trees.

Adhesion

Cell wall polysaccharides have a great affinity for water molecules. Wall surfaces usually have a net negative charge that attracts the slightly positive sides of the polar water molecules. This called hydration. The wall surface which can bind with water is called matrix. Water can be held by hydrophilic wall surfaces with tension in the order of –100 MPa to –300 MPa. Gravity, that is the weight of water column in the xylem vessel, cannot remove water against such powerfull forces.

There is only one relevant but refutable objection to cohesion tension theory. Xylem water contains several dissolved gasses such as CO_2, O_2, and nitrogen. When the water column is under tension, there is a tendency of these gasses to come out of solution. As a result, submicroscopic bubbles first form at the interface between the water and the walls of xylem elements, probably in small, hydrophobic crevices or pores in the walls. This phenomenon is sometimes called 'air seeding'. These small bubbles may redissolve or they may coalesce and expand within the water column of a conduit. This phenomenon of bubble formation is known as **cavitation**. The resulting gas bubble form an obstruction, called **embolism**, (Gr. embolus = Stopper), in the conduit. Cavitation or embolism of the xylem breaks the continuity of the water column and stops water transport.

However, the impact of xylem cavitation on the plant is minimized by several means. Tracheids and vessels constitute multiple, parallel, interconnected pathways for water movement. When cavitation blocks water movement within the cavitated vessel, it does not completely stop water movement in the cell file. Because tracheary elements are interconnected through wall pits, water can detour around the blocked vessel by moving through adjacent tracheary elements (Figure 2.5).The spread of the vapour bubble throughout the xylem is eventually stopped by an end wall that lacks a perforation plate. Gas bubbles may also be eliminated from the xylem. At night, when transpiration is low, xylem ψ_p increases and the water vapor and gases may simply dissolve back into the solution of the xylem.

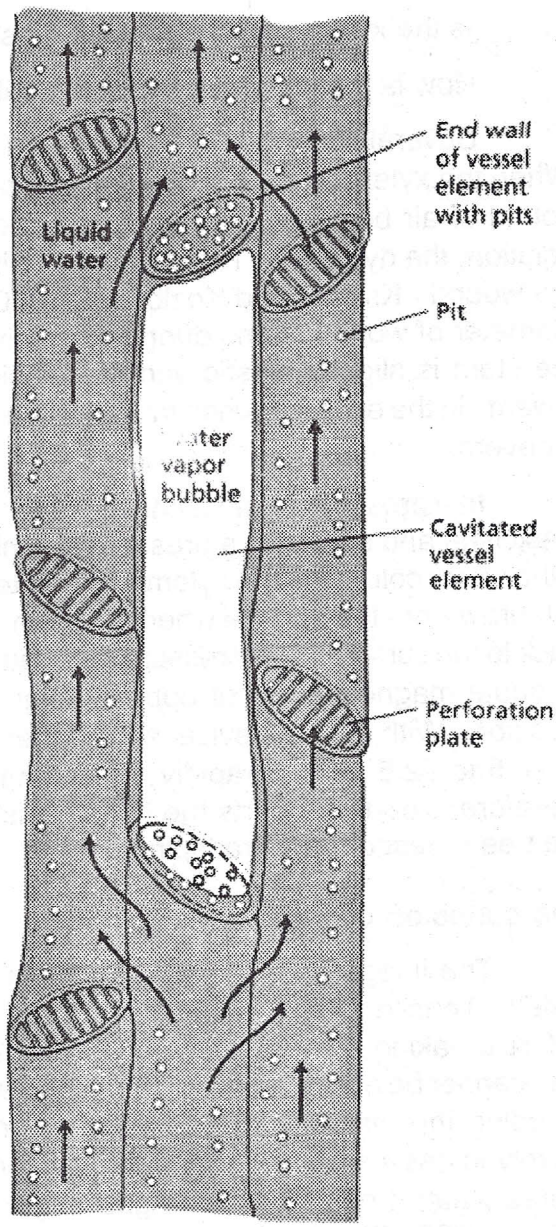

Figure 2.5 Water detours around a cavitated vessel element. Water can detour around the blocked vessel by moving through adjacent tracheary elements.

So, from the above discussion it is clear that the cohesion tension theory is now the available mechanism to explain the mechanism of ascent of sap in the tallest trees and it has got the following essential features.

1. Water inside the xylem forms a continuous column from top to bottom.
2. Water evaporates from the mesophyll cell surfaces (transpiration), due to which driving force or pulling force develops putting the water column inside the xylem under tension.
3. The tension may cause a break in the water column but due to the cohesive and adhesive property of water the continuous column does not break.

2.4 Summary

In plants, water is mainly transported through an interconnected system of open conduits formed as series of tracheary elements. Xylem and phloem transports water and mineral nutrients between various organs. Translocation of water in the xylem is facilitated by components like fibres, parenchyma cells, tracheary elements. The phenomenon of water absorption and transport to all the aerial parts is very common process. But the question, how the integrity of the xylem water column is maintained and how it moves to the top of the tallest trees, has attracted the attention of many plant physiologists. In this connection several mechanisms have been proposed, but the only one to have stood the test of time combines transpiration with the strong cohesive forces of water.

H.H. Dixon proposed the cohesion theory in 1914 that gave the detailed account of movement of water through the plant. Although aspects of the cohesion tension theory of sap ascent are intermittently debated, an overwhelming body of evidence supports the idea that water transport in the xylem is driven by pressure gradient. High transpiration generates negative pressure in the xylem water may cause cavitation (embolisms) in the xylem. But the structure of trachied or vessel members play a major role for minimizing the effect of embolism.

2.5 Model questions

1. Describe the path of water from the time it enters the root until it escapes as water vapour from the leaf surface.
2. Write short notes on

a. Cavitation and embolism

b. Root pressure

c. Cohesion theory of ascent of sap

d. Anatomy of water conduction

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Dr. G. Rosaiah

Lesson – 3

TRANSPIRATION – LOSS OF WATER

3.0 Objective

In this lesson the process of transpiration, the structure of stomata and mechanisms for stomatal opening and closure, and the role of vapor pressure differences in directing the exchange of water between leaves and atmosphere have been discussed.

3.1 Introduction

Plants absorb large quantities of water from the soil and translocate to the variou parts of the plant. Of all the water absorbed by the plant, it retains less than five percent o water to maintain growth and even less is used biochemically. The balance passes through the plant to be lost as water vapor, a phenomenon known as transpiration. The quantitative importance of transpiration has been indicated by a variety of studies over the years. In his classic 1938 physiology book E.C. Miller revealed that a single maize plant might transpire as much as 200 litres of water over its life time and this transpirational water loss is approximately 100 times of its own body weight. Similarly a single, 14.5 meter open grown *Silver mapple* tree may lose as much as 225 litres of water per hour. Whether there is any positive advantage to be gained by transpiration is a point of discussion, but the potential for such massive amounts of water loss clearly has profound implications for growth, development, productivity and even survival of plants.

3.2 The process of transpiration

Transpiration is an inevitable phenomenon in which large amounts of water from the plant are lost in the form of water vapor to the atmosphere. From the vascular termini water moves to the leaf parenchyma, from which evaporation and loss of water takes place. There are two pathways for this movement of water to the atmosphere. They are **cuticular** and **stomatal**, and are strongly tied to leaf anatomy. The outer surfaces of a typical vascular plant leaf are covered with multilayered waxy deposit called the cuticle. The principal component of the cuticle is cutin, which is a heterogenous polymer of long chain 16 or 18 carbons containing hydroxylated fatty acids. Cutin forms an extensive polymeric network that in association with cuticular waxes, which are mixtures of long chain saturated hydrocarbons, alcohols, aldehydes and ketones, forms multilayered thickening on the leaf surfaces. This layer offers extremely high resistance to diffusion of both liquid water and water vapor from the underlying cells. Though it is meant to check transpiration, it is rarely completely impermeable due to some cracks in it, the more so when the layer is thin. So a small amount of water absorbed may be lost from the underlying mesophyll cells to the bulk air through the cuticle. This is known as **cuticular transpiration**. The major pathway involves movement of liquid water from the leaf parenchyma in the form of vapor, to the airfilled intercellular spaces and from there to the atmosphere through the stomatal pores in the epidermis. This is known as **stomatal transpiration** and this process accounts for 90 to 95 percent of the water loss from the leaves. Sometimes, loss of water also takes place through the lenticels of stems and fruits and this is called **lenticular transpiration**. Both cuticular and lenticular transpirational processes account for 5 to 10 percent of the water loss and is insignificant when compared to the amount of water lost by stomatal transpiration.

3.3 Stomatal transpiration

Diffusion of water vapor through occasional interruptions of epidermis and the overlying cuticle, called the stomata (sing- stoma), is known as stomatal transpiration.

3.3.1 Structure of stomata

Stomata are minute pores in the epidermis of leaves and are evolved for the exchange of gasses between the internal air spaces and the ambient atmosphere. They are found in the leaves of virtually all higher plants (angiosperms and gymnosperms) and most lower plants (mosses and ferns) with the exception of submerged aquatic plants and liverworts. In higher plants they are found on most aerial parts including nonleafy structures such as floral parts and stems, although they may be nonfunctional in some cases. The frequency and distribution of stomata is quite variable and depends on a number of factors including species, leaf position, ploidy level and growth conditions. A frequency (number of stomata per unit leaf area) in the range of 20 to 400 stomata mm^{-2} of leaf surface is quite common. In some cases frequencies of 1000 mm^{-2} or more have been reported. The leaves of herbaceous monocots such as grasses usually contain stomata on both the upper (adaxial) and lower (abaxial) surfaces with roughly equal frequencies. The herbaceous dicots also contain stomata on both sides of their leaves, but the frequency is usually lower on the upper surface. Most woody dicots and tree species have stomata only on the lower leaf

surface while floating leaves of aquatic plants (i.e water lily) have stomata only on the upper surface.

The opening or pore of a stoma is surrounded by a pair of unique cells called **guard cells**. In most cases the guard cells are in turn surrounded by specialized, differentiated epidermal cells called **subsidiary cells**. The opening together with its bordering guard cells and subsidiary cells is called the **stomatal complex**. The distinguishing feature of the stomatal complex is the pair of guard cells that functions as a hydraulically operated value. Guard cells take up water and swell to open the pore when CO_2 is required for photosynthesis and lose water to close the pore when CO_2 is not required or when water stress overrides the photosynthetic needs of the plant. That is they have the capacity to undergo reversible turgor changes that in turn regulate size of the pore between them.

Anatomically there are two basic types of guard cells: the **graminaceous type** and **elliptic type** (Figure 3.1).

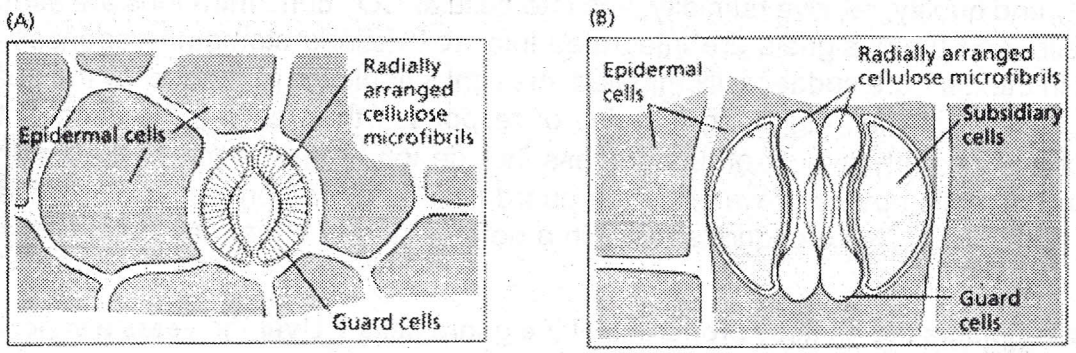

Figure 3.1 The radial alignment of the cellulose microfibrils in guard cells of (A) a kidney shaped stoma and (B) a graminaceous type stoma.

The graminaceous type of guard cells are largely restricted to members of the Gramineae and a few other monocots such as palms. These guard cells have a characteristic dumbbell shape, with bulbous ends. The pore proper is a long slit located between the two 'handles' of the dumbbells. These guard cells have a thickened cell walls toward the lumen and are always flanked by a pair of differentiated epidermal cells called subsidiary cells. In grasses, the dumbbell shaped guard cells function like beams with inflatable ends. As the bulbous ends of the cells increase in volume and swell, the beams (handles) are separated from each other and the slit between them widens (Figure 3.1B).

Elliptic or kidney shaped guard cells are so called because of the elliptic shape of the opening. In surface view, these guard cells resemble a pair of kidney beans with their concave sides opposed. In cross section the cells are roughly circular in shape, with a ventral wall bordering the pit and a dorsal wall adjacent to the surrounding epidermal cells. The mature guard cells have characteristic wall thickenings that cause guard cells to become concave during stomatal opening. This thickening pattern is associated with the alignment of their cellulose microfibrils. In ordinary cells with a cylindrical shape, cellulose microfibrils

are oriented transverse to the long axis of the cell. As a result, the cell expands in the direction of its long axis, since the cellulose reinforcement offers the least resistance at right angles to its orientation (Figure 3.1A). In guard cells the microfibril organization is different where the cellulose microfibrils fanning out radially from the pore. Thus the cell girth is reinforced like a steel-belted radial tire, and the guard cells curve outward during stomatal opening.

3.3.2 Mechanism of stomatal movements

More than 90 percent of the CO_2 and water vapor exchanged between a plant and its environment passes through the stomata. Stomata are therefore involved in controlling two very important but competing processes such as uptake of CO_2 for photosynthesis from the atmosphere and transpirational water loss to the atmosphere. So it is important discuss the stomatal functioning in view of the photosynthetic productivity and crop yields of higher plants.

Guard cells function as multisensory hydraulic valves. Environmental factors such as light intensity and quality, relative humidity, and intercellular CO_2 concentrations are sensed by guard cells, and these signals are integrated into well defined stomatal responses. If leaves kept in the dark are suddenly illuminated, the light stimulus is perceived by the guard cells as an opening signal, triggering a series of responses that result in opening of the stomatal pore. The early events of this process include ion uptake, decrease in osmotic potential, and osmotic uptake of water by the guard cells and the consequent increase in hydrostatic pressure. These changes result in a deformation of guard cells with widened pore.

What regulates the osmotic properties of the guard cells ? Over the years a variety of mechanisms have been proposed to explain osmotic concentrations of guard cells. The botanist H. Von Mohl had proposed in 1856 that turgor changes in guard cells provide the driving force for stomatal movements, and the plant physiologist F.E. Leoyd hypothesized in 1908 that these turgor changes depend on starch-sugar interconversions, a concept that led to a starch-sugar hypothesis of stomatal movements. Guard cell chloroplasts contain large, prominent starch grains and their starch content decreases during stomatal opening and increases during closing. Starch, an insoluble polymer of glucose, does not contribute to the cell's osmotic potential, but the hydrolysis of starch into soluble sugars causes an increase in the osmotic potential of guard cells. In the reverse process, starch synthesis decreases the sugar concentration, resulting in a lowering of the cell's osmotic potential with associated stomatal closing.

This hypothesis was widely accepted until the discovery of potassium fluxes in guard cells by S. Imamura in 1943, which were later confirmed by M.Fujino and R.A. Fischer. In the late 1960s it became evident that potassium levels are very high in open guard cells and very low in closed guard cells. Potassium concentrations can increase severalfold in open stomata, from 100 mM in the closed state to 400 to 800 mM in the open state, depending on the plant species and the experimental conditions. An accumulation of K^+ in guard cells is now accepted as a universal mechanism in stomatal opening (Figure 3.2).

The accumulation of ions by most plant cells is driven by the proton-pumping H^+-ATPase located on the plasma membrane. Proton pumping by an ATP driven H^+-ATPase which is one of the initial events in stomatal opening, is evidenced from several lines o research. First, the fungal toxin fusicoccin, which is known to stimulate active proton pump ing from the inside to out side of the cell by the H^+-ATPase, stimulates stomatal opening Second, vandate (VO_3^-) which inactivates the proton pump, and carboxyl cyanide m chlorophenylhydrazone (CCCP) which abolishes H^+-ATPase generated proton gradient, inhibits stomatal opening. Thus proton extrusion is the initial event that causes a voltage or potential difference across the membrane because of unequal distribution of anionic and cationic charges. In addition, proton pumping also generates a pH gradient of about 0.5 to 1 pH unit. Energy stored in the resulting electrochemical proton gradient, also known as the proton motive force, provides a driving force for the passive uptake of potassium ions via voltage-regulated potassium channels (Figure 3.2).

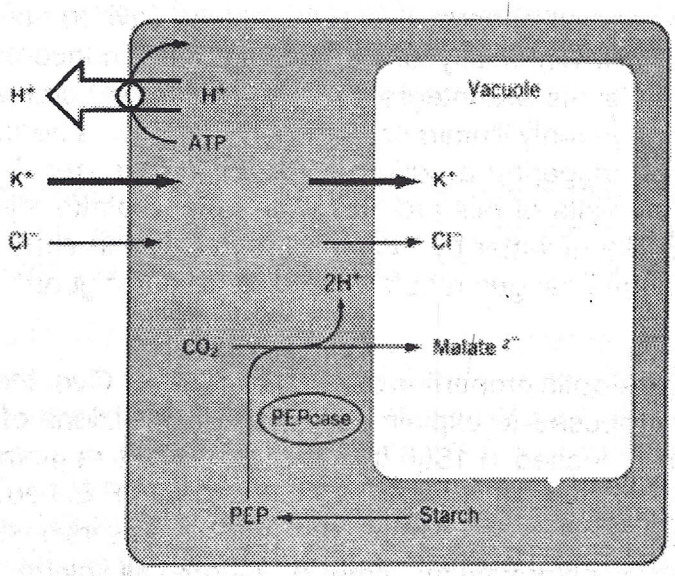

Figure 3.2 A proposed model for stomatal opening. Potassium uptake is driven by an ATPase – proton pump located in the plasma membrane. The accumulation of ions in the vacuole lowers the water potential of the guard cell, thereby stimulating the osmotic uptake of water and increased turgor.

In order to maintain electrical neutrality, excess K^+ ion accumulated in the cells must be balanced by a counter ion carrying a negative charge. According to the model shown in Figure 3.2, charge balance is achieved partly by an influx of chloride ion (Cl^-), and partly by organic anions such as malate^{2-}. In species of the genus *Allium*, such as onion (*Allium cepa*), K^+ ions are balanced by Cl^-. In most species, however, potassium fluxes are balanced by varying amounts of Cl^- and the organic anion malate^{2-}.

Like potassium, chloride is taken up into the guard cells during stomatal opening and extruded during stomatal closing. It is thought to be taken up through a Cl^- - H^+ symporter. Malate, on the other hand, is synthesized in the guard cell cytosol, in a metabolic pathway

that uses carbon skeletons generated by starch hydrolysis. The malate content of guard cells decreases during stomatal closing.

The accumulation of K^+ Cl^- and malate in the vacuoles of the guard cells would lower the osmotic potential. As osmotic potential decreases, the water potential also decreases and water consequently moves into the guard cells. As water enters the cell, turgor pressure increases, which in turn causes reversible deformation of the guard cells to open the stomata. At present this remains a working model for stomata opening since many of the details have yet to be experimentally verified.

Stomatal closure has not received the much attention as that of opening. Its closure is effected simply by a reversal of events leading to stomatal opening. According to the presently available information, signals for stomatal closure stimulate the uptake of Ca^{2+} into the cytosol. Ca^{2+} uptake, thus intiating a chain of signal transduction events that includes opening anion channels to allow release of Cl^- and $malate^{2-}$. A loss of these anions then depolarize the membrane. Membrane depolarization is accompanied by the passive diffusion of potassium ions into the adjacent subsidiary and epidermal cells through opened K^+ channels.

When coming to the source of ATP to drive an ATPase proton pump in guard cells, it seems that ATP is generated either by photosynthesis in those guard cells that contain chloroplasts or from oxidation of carbon through normal respiratory pathways.

Stomatal closure also occurs in response to water stress. This kind of stomatal movement is called **hydroactive closure**, which takes place when the plant senses a water deficit and initiates a specific mechanism to induce closure. The mechanism for hydroactive closure involves the same ion fluxes normally associated with closure, but is triggered by water deficit in the leaf and is mediated by the hormone abscisic acid (ABA). ABA is a normal constituent of leaves, where it is synthesized at low rates in an unstressed mesophyll cells and accumulates in the chloroplasts. In an actively photosynthesizing leaf, the pH of the chloroplast stroma is normally higher (pH 7.5 to 8.0) than that of the cytosol (pH 6.0 to 6.5). This pH difference leads to a large accumulation of ABA in the chloroplast. Moderate water stress causes a decrease in the pH of the chloroplast stroma and an increase in the cytosolic and apoplastic pH. Such changes in pH then causes the release of ABA from the chloroplast stroma into the apoplastic space. ABA from apoplastic space is carried to the guard cells through the transpiration stream and initiates stomatal closure. Because of its ability to stimulate stomatal closure and thus reduce transpirational water loss, ABA has been referred to as an "anti-transpirant or stress hormone".

We will now examine the driving force for leaf transpiration.

3.4 The driving force for transpiration

The driving force for water loss is difference in water vapor concentration. The difference in water vapor concentration expressed as $C_{wv\,(leaf)} - C_{wv}\,(air)$ According to Fick's law of

diffusion, molecules will diffuse from a region of high concentration to a region of low concentration, or, down a concentration gradient. Vapor pressure is proportional to vapor concentration so that water vapor will also diffuse down a vapor pressure gradient; that is, from a region of high vapor pressure to a region of lower vapor pressure. Stomata are located such that, when open, they provide a path for the movement of water vapor between the internal air space and the bulk atmosphere surrounding the leaf. Because of this relationship, this space is referred to as substomatal space. This substomatal air space of a leaf is normally saturated or very nearly saturated with water vapor. This is because the mesophyll cells which border the air space present a large, exposed surface area for evaporation of water. On the other hand, the atmosphere which surrounds the leaf is usually unsaturated and may often have a very low water content. These circumstances create a gradient between the high water vapor pressure in the substomatal air space of the leaf and lower water vapor pressure of the external atmosphere. This difference in water vapor pressure drives the movement of water vapor molecules from the internal air spaces of the leaf to the surrounding bulk air.

3.5 Transpiration ratio

The effectiveness of plants in regulating water loss while allowing sufficient CO_2 uptake for photosynthesis can be assessed by a parameter called the **transpiration ratio.** This value is defined as the amount of water transpired by the plant divided by the amount of CO_2 assimilated by photosynthesis.

$$\text{Transpiration ratio} = \frac{\text{Moles of } H_2O \text{ transpired}}{\text{Moles of } CO_2 \text{ fixed}}$$

For a typical plant in which the first stable product of carbon fixation is a three-carbon compound, about 500 molecules of water are lost for every molecule of CO_2 fixed by photosynthesis, giving a transpiration ratio of 500. Some times the reciprocal of the transpiration ratio, called the **water use efficiency**, is cited. Plants with a transpiration ratio of 500 have a water use efficiency of 1/500, or 0.002.

The large ratio of H_2O efflux to CO_2 influx depends on two factors. First, the concentration gradient driving water loss is 50 times larger than that driving CO_2 influx. In large part, this difference is due to the low concentration of CO_2 in air (about 0.03%) and the relatively high concentration of water vapor within the leaf air spaces. Second, CO_2 diffuses about 1.6 times more slowly through air than water does (the CO_2 is larger than H_2O and hence has a smaller diffusion coefficient).

3.6 Significance of transpiration

Transpiration has got immense significance in plant life as it is of great benefit to the plant. Though a large amount of absorbed water is lost during transpiration, which is no doubt a harmful effect.

The theory of evolution states that any harmful feature should be eliminated by natural selection. But transpiration is obvious in any land plant. So it is definitely advantageous inspite of its harmful features or in other words, the benefit is much greater than the harm. For that reason transpiration is often said to be 'a necessary evil'. The advantage of transpiration is a sort of 'victory by default'.

Transpiration is not only beneficial but also essential in the life of land plants for the absorption of CO_2 required for photosynthesis. Besides the gaseous exchange, it gives cooling effect by regulating the temperature in the leaves of terrestrial plants that are often exposed to intense sun light and provides a driving force for the ascent of sap from ground level to the top of the tallest trees.

3.7 Summary

Transpiration is an inevitable phenomenon in which large amounts of water are continuously lost in the form of vapor to the surrounding atmosphere from the surfaces of aerial parts as a consequence of their structural organization. There are three types of transpiration – stomatal, cuticular, and lenticular. The major portion of water (90 to 95%) is transpired through stomata. The mechanism of transpiration is mainly dependent on the mechanism of stomatal opening and closing. The stomatal movement depends on the increase or decrease in the osmotic potential of the guard cells. These osmotic changes result in changes in water potentials, causing movement of water in and out of the guard cells. According to the current hypothesis, the osmotic potential of guard cell and consequently, the size of the stomatal opening, is determined by the extent of K^+ accumulation in the guard cells. This theory also known as proton transport concept can explain the obvious facts occurring during stomatal opening namely (a) excretion of H^+ from guard cells, (b) uptake of K^+ into the guard cell vacuole, (c) uptake of Cl^- into the vacuole and (d) production of organic acid, particularly $malate^{2-}$. Stomatal closure involves a several of events leading to opening. Under conditions of water deficit, the ABA acts as an antitranspirant and closes the stomata. The gradient in the water vapor concentration between the internal air spaces of the leaf and the surrounding atmosphere, is the driving force of transpiration.

3.8 Model questions

1. Explain how guard cells regulate the size of the stomatal aperture
2. Write short notes on :
   a) Transpiration ratio
   b) Structure of stomata
   c) Role of ABA in the mechanism of stomatal closure

3.9 Reference books

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3. Plant Physiology – F.B. Salisbury and C.W.Ross – CBS Publishers – New Delhi
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Dr. G. Rosaiah

LESSON: 4

INORGANIC NUTRITION OF PLANTS

4.0 Objective

In this lesson, the criteria for essentiality of mineral nutrients, classification of nutrients on the basis of their relative requirement and biological functions, mineral nutritional studies through various techniques and nutrient roles and their deficiency symptoms are discussed.

4.1 Introduction

Plants must have a supply of raw materials from the environment to obtain energy and construct new cellular components. The supply and absorption of chemical compounds needed to maintain their metabolism, growth and development may be defined as **nutrition.** The chemical compounds required by an organism are termed as **nutrients.** Plants absorb these chemical elements through their roots principally as inorganic ions from the soil. The study of how plants absorb and assimilate these inorganic ions is called **mineral nutrition.** This area of plant research has now been playing an important position in designing modern agriculture and environmental protection.

Much of the ground work for modern nutritional studies was aimed with an interest to get high agricultural yields. In this context, we must give first credit to French plant physiologist **"de Saussure".** In 1804 de Saussure clearly demonstrated that the inorganic mineral elements contained in the ash of plants are obtained from the soil via the root system. He suggested that some of the chemical elements found might be indispensable (i.e., essential) to plant growth. Serious study of the identify of mineral nutrients required for plant growth was then began by others who followed de Saussure. C.S.Sprengel (1787-1859) working in Germany had of the opinion that soil might be unproductive if it is defficient even in single element necessary for growth. Further, the French agronomist Boussingault stressed about the quantitative relationships between the effects of fertilizer and nutrient upstake on crop yield. By the middle of the 19th century, the technique of growing plants in defined nutrient solutions in the complete absence of soil has been established particularly through the efforts of the

two German plant physiologists, Sachs and Knop (1860). Finally J.B. Lowers and J.H. Gilbert working in the England had successfully converted insoluble rock phosphate to soluble phosphate called super phosphate. By the end of the century the use of nitrogen, phosphorous and potassium (N-P-K) fertilizers in agriculture was well established. According to one estimate, the use of these fertilizers in agriculture rose steadily from 112 million metric tons in 1980 to 143 million metric tons in 1990.

On the basis of nutritional requirements for carbon containing compounds organisms have been classified as **autotrophs** and **heterotrophs.** Plants, most algae and a few bacteria are **autotrophic** (self nourishing) organisms. They live in an entirely inorganic environment, taking in CO_2 from the atmosphere and water and mineral nutrients from the soil. In contrast to autotrophs, most bacteria, fungi and all members of the animal kingdom depend for their existence on energy-rich organic molecules previously synthesized by autotrophs. Hence, they are called **heterotrophs.** The autotrophic ability of the green plants has a key role in the nature because that provides continuous cycling of inorganic ions between organisms and their environment. Plants having this ability of absorbing inorganic nutrients from the soil solution through their large surface area of roots made Epstein (1972, 1974) to call them as "**miners**" of earth's crust.

4.2 Nutrient sources

The nutrients indispensable for growth and development of green plants are derived from three environmental sources, the atmosphere, water and the soil. The atmosphere furnishes CO_2 and O_2. Water is a second source of nutrition that gives hydrogen atoms and O_2. Carbon and most of the O_2 derived from CO_2 (also from the H_2O) together with hydrogen make up 90% of the dry weight of the plant. Soil is a third environmental source of mineral nutrients. Mineral elements of soil are mainly derived from the parent rock and from decaying plant and animal debris. Majority of the plants absorb mineral ions mostly by roots from the soil. However, certain species of higher plants that grow as epiphytes absorb minerals from air borne dust particles, which come in contact with their surfaces and then dissolve in dew or rain water.

4.3 Criteria for essentiality

The inorganic chemical elements that have a clear physiological role in the growth and development of a plant and whose absence prevents a plant from completing its life cycle are defined as **essential elements.** Analysis of plant reveals the presence of a large number of mineral elements. In fact, all elements found in a plant are not essential for its growth and development. A large number of them are non-essential. In order to find out the essentiality of an element for a plant, three criteria have been proposed by Arnon and Stout (1939) and Epstein (1972).

1. A deficiency of the element makes it impossible for the plant to complete its life cycle.
2. The deficiency is specific for the element in question. It can not be replaced by any other element.
3. The element is directly involved in the nutrition of the plant and not causing some other element to be more readily available or antagonizing the effect of another element.

These three requirements form the criteria for essentiality. According to the first criterion an element is said to be absolutely essential, if a plant is unable to produce viable seed in the absence of that element. By the second criterion the essential role of elements, for example **Mg** in chlorophyll and **N** in proteins can not be compensated by any other element. The third criterion has got less importance in deciding essentiality, but there are few cases in which it has been applicable. The growth promoting effects of selenium, for example, resulted from the ability of the selenate ion to inhibit the absorption of the phosphate, which was other wise absorbed by the plants in toxic amounts. Some recent studies have shown that some of the essential elements can be partially replaced by others, for example, magnesium by manganese, potassium by rubidium and chlorine by bromine.

Based on these criteria, at present 17 chemical elements are recognized to be essential for the growth of higher plants (Table 4.1)

TABLE 4.1 The essential nutrient elements of higher plants and their concentrations considered adequate for normal growth.

Element	Chemical Symbol	Available Form	Concentration in Dry Matter (mmol/kg)
Macronutrients			
Hydrogen	H	H_2O	60,000
Carbon	C	CO_2	40,000
Oxygen	O	O_2, CO_2	30,000
Nitrogen	N	NO_3^-, NH_4^+	1,000
Potassium	K	K^+	250
Calcium	Ca	Ca^{2+}	125
Magnesium	Mg	Mg^{2+}	80
Phosphorous	P	HPO_4^-, $H_2PO_4^-$	60
Sulfur	S	SO_4^{2-}	30
Micronutrients			
Chlorine	Cl	Cl^-	3.0
Boron	B	BO_3^{3-}	2.0
Iron	Fe	Fe^{2+}, Fe^{3+}	2.0
Manganese	Mn	Mn^{2+}	1.0
Zinc	Zn	Zn^{2+}	0.3
Copper	Cu	Cu^{2+}	0.1
Nickel	Ni	Ni^{2+}	0.05
Molybdenum	Mo	MoO_4^{2-}	0.001

4.4 Macro and Micronutrients

The seventeen mineral essential elements as shown in table 4.1 are divided into **macronutrients** and **micronutrients**. Such a distinction was proposed according to their relative concentrations found in plant tissue or required in nutrient solutions.

According to this classification, nine elements viz., hydrogen (H), carbon (C), oxygen (O), nitrogen (N), potassium (K), calcium(Ca), magnesium (Mg), phosphorous (P), and sulfur (S) are called macronutrients. They are required in large amounts i.e., in excess of 10 m mol Kg^{-1} of dry weight. The macronutrients are largely involved in the structure of bimolecules. Some macronutrients like Ca and Mg serve as regulators in addition to their structural role. The remaining eight elements such as iron (Fe), boron (B), manganese (Ma), zinc (Zn), copper (Cu), nickel (Ni), molybdenum (Mo) and chlorine (Cl) are the micronutrients. Some times the micronutrients are called **minor** or **trace** elements because they are required in relatively small quanties i.e., less than 30 m mol Kg^{-1} of dry weight. Actually the designations minor or trace are quite unsatisfactory, because there is nothing minor or trace about the essentiality of the micronutrients. Micronutrients are mainly involved in catalytic and regulatory functions particularly as an enzyme activators.

The division of plant nutrients as macronutrients and micronutrients is some what arbitrary. In many cases, the differences between macronutrient and micronutrient content of plant tissues are not so large as those indicated in Table 4.1. The Fe or Mn content of plant tissues, for example, the leaf mesophyll is sometimes nearly as high as the content of **S** or **Mg.** Further, many elements may be present in concentrations greater than the plants minimum requirements. So in physiological point of

Table 4.2
Classification of plant mineral nutrients according to biochemical function

Nutrient element	Functions
Group 1	**Nutrients that form the organic compounds of plants**
N	Constituent of amino acids, amides, proteins, nucleic acids, nucleotides, coenzymes, hexoamines, etc.
S	Component of cysteine, cystine, and methionine, and proteins. Constituent of lipoic acid, coenzyme A, thiamine pyrophosphate, glutathione, biotin, adenosine-5'-phosphosulfate, and 3-phosphoadenosine.
Group 2	**Nutrients that are important in energy storage or structural integrity**
P	Component of sugar phosphates, nucleic acids, nucleotides, coenzymes, phospholipids, phytic acid, etc. Has a key role in reactions in which ATP is involved.
B	Complexes with mannitol, mannan, polymannuronic acid, and other constituents of cell walls. Involved in cell elongation and nucleic acid metabolism.
Si	Deposited as amorphous silica in cell walls. Contributes to cell wall mechanical properties, including rigidity and elasticity.
Group 3	**Nutrients that remain in ionic form**
K	Required as a cofactor for more than 40 enzymes. Principal cation in establishing cell turgor and maintaining cell electroneutrality.
Na	Involved with the regeneration of phosphoenolpyruvate in C_4 and CAM plants. Substitutes for potassium in some functions.
Mg	Required by many enzymes involved in phosphate transfer. Constituent of the chlorophyll molecule.
Ca	Constituent of the middle lamella of cell walls. Required as a cofactor by some enzymes involved in the hydrolysis of ATP and phospholipids. Acts as a second messenger in metabolic regulation.
Mn	Required for activity of some dehydrogenases, decarboxylases, kinases, oxidases, peroxidases. Involved with other cation-activated enzymes and photosynthetic O_2 evolution.
Cl	Required for the photosynthetic reactions involved in O_2 evolution.
Group 4	**Nutrients that are involved in electron transfers**
Fe	Constituent of cytochromes and nonhame iron proteins involved in photosynthesis, N_2 fixation, and respiration.
Cu	Component of ascorbic acid oxidase, tyrosinase, monoamine oxidase, uricase, cytochrome oxidase, phenolase, laccase, and plastocyanin.
Zn	Constituent of alcohol dehydrogenase, glutamic dehydrogenase, carbonic anhydrase, etc.
Mo	Constituent of nitrogenase, nitrate reductase, and xanthine dehydrogenase.
Ni	Constituent of urease. In N_2-fixing bacteria, constituent of hydrogenases.

Source: After Evans and Sorgel 1966 and Mengel and Kirkby 1987.

view it is difficult to justify the classification of plant nutrients into macronutrients and micronutrients depending on element concentration in plant tissues. Mengel and Kirkby (1987) therefore proposed an alternative and more meaningful system for nutrient division. They divided essential plant nutrients into four basic groups on the basis of their biochemical behaviour and physiological function (Table 4.2).

The first group includes the elements such as C,H,O,N and S that form the organic compounds of the plant. Plants assimilate these elements through biochemical reactions involving oxidation and reduction.

Phosphorous, boron and silicon constitute second group of elements. They are important in energy transfer reactions or in maintaining structural integrity. They are often present in plant tissues as phosphate, borate, and silicate esters in which the elemental group is bound to hydroxyl groups of sugars.

The third group of plant nutrients is made up of K, Na, Mg, Ca, Mn and Cl. In the plant cell they are present in the free ionic state or are adsorbed to indiffusible organic anions, for example, carboxylic groups of the pectins. They are important as cofactors of enzymes and in the regulation of osmotic potentials.

Members of the fourth group includes Fe, Cu, Zn, Mo and Ni. These elements have important roles in reactions involving electron transfer.

Elements other than those given in Table 4.1 can also accumulate in plant tissues. For some plants, sodium (Na), silicon (Si), cobalt (Co), selenium (Se), and alluminum (Al) have now been established as an essential elements. These elements are not required to all higher plants. Hence they are referred to as **beneficial elements** rather than essential elements. All Chemical elements except C, H and O are mineral elements.

4.5 Techniques used in nutritional studies

Several methods have been proposed for the study of plant nutrition, but following methods are very common in use.

4.5.1 **Ash analysis:** In this method of elemental study, the plants are subject to high temperatures of about 600^{0}C in a muffle furnace. At this temperature the organic materials will be oxidized and driven off as water and carbon dioxide. The small quantity of white or grey matter left as residue is called **ash.** It contains all the mineral elements that were absorbed from the soil. The mineral elements of this ash are determined even in micro and semi micro quantities by spectrophotometric, colorimetric, turbidimetric, flame photometric and titrametric methods. This method is a crude one because in the ash mineral elements are not found in pure form but occur in the state of oxides.

4.5.2 Solution culture : This technique is used to understand the kinds and amounts of elements essential to plants. In solution culture technique, the plants are grown with their roots immersed in a nutrient solution containing only inorganic salts. This technique of growing plants in a defined nutri-

ent solution without soil is called **hydroponics** (Greek hudor = water; ponos = working). Woodward (1699) for the first time used water culture technique. This method in real sense was worked out further by Sacks (1860), Knop (1865), and Arnon and Hoagland (1940). Nutrient solutions formulated by Sachs (1860) and Knop (1865) are given in table 4.3 and 4.4. respectively.

Table 4.3 The composition of Sachs' nutrient solution

SALT	FORMULA	APPROXIMATE CONCENTRATION(mM)
Potassium nitrate	KNO_3	9.9
Calcium phosphate	$Ca_3(PO_4)_2$	1.6
Magnesium sulfate	$MgSO_4.7H_2O$	2.0
Calcium sulfate	$CaSO_4$	3.7
Sodium chloride	NaCl	4.3
Iron sulfate	$FeSO_4$	trace

Table 4.4 The composition of Knop's nutrient medium

SALT	FORMULA	APPROXIMATE CONCENTRATION(mM)
Calcium nitrate	$Ca(NO_3)_2$	0.8
Potassium nitrate	KNO_3	0.2
Potassium dihydrogen phosphate	KH_2PO_4	0.2
Magnesium sulfate	$MgSO_4 7 H_2O$	0.2
Iron sulfate	$FeSO_4$	trace

In the begining it was thought that these nitrient solutions may contain all the minerals required by the plant. But these nutrient solutions used in their experiments have chemicals that were contaminated with other elements such as boron or molybdenum that are now known to be essential. Table 4.5 shows a more modern formulation for a nutrient solution. This nutrient medium is called modified Hoagland solution, named after D.R.Hoagland. A modified Hoagland solution contains all the mineral elements needed for rapid plant growth. This nutrient medium is specialized in having high concentrations of mineral elements than those found in the soil in addition to nitrogen source both as ammonium (NH_4^+) and nitrate (NO_3) and iron as ferric sodium ethylene diamine tetraacetic acid (NaFeEDTA) or diethylene triaminepenta acetic acid (NaFeDTPA). The high initial level of elements allows the plants to be grown in a medium for extended periods without replenishment of the nutrients. Supplying nitrogen in a balanced mixture of NH_4^+ (cation) and NO_3^- (anion) tends to reduce the rapid rise in medium pH. Generally iron can precipitate out of solution as iron hydroxide when it is supplied in the form of Fe SO_4 or $Fe(NO_3)_2$. This problem can be eliminated by providing iron in the form of iron chealator (NaFeEDTA or NaFeDPTA) complex which is readily available to the plants when compared to iron supplied in the form of Fe SO_4 (or) Fe $(NO_3)_2$ to the nutrient solution (Table 4.5).

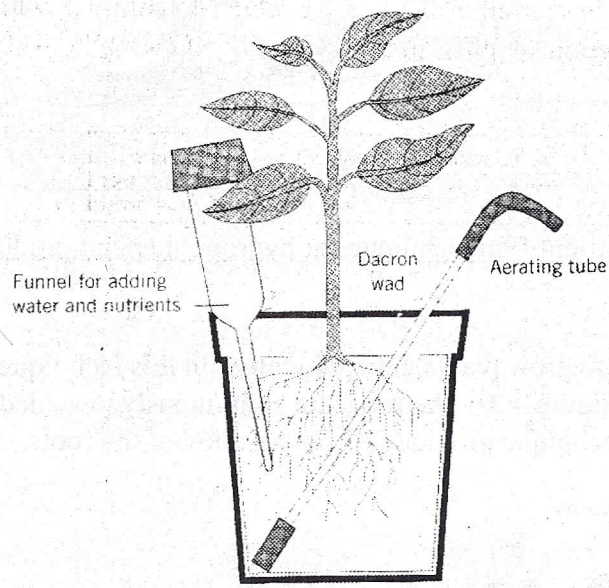
Compound	Molecular weight	Concentration of stock solution	Concentration of stock solution	Volume of stock solution per liter of final solution	Element	Final concentration of element	
	g mol^{-1}	m*M*	g L^{-1}	mL		*μM*	ppm
Macronutrients							
KNO_3	101.10	1,000	101.10	6.0	N	16,000	224
$Ca(NO_3)_2 \cdot 4H_2O$	236.16	1,000	236.16	4.0	K	6,000	235
$NH_4H_2PO_4$	115.08	1,000	115.08	2.0	Ca	4,000	160
$MgSO_4 \cdot 7H_2O$	246.48	1,000	246.49	1.0	P	2,000	62
					S	1,000	32
					Mg	1,000	24
Micronutrients							
KCl	74.55	25	1.864	2.0	Cl	50	1.77
H_3BO_3	61.83	12.5	0.773		B	25	0.27
$MnSO_4 \cdot H_2O$	169.01	1.0	0.169		Mn	2.0	0.11
$ZnSO_4 \cdot 7H_2O$	287.54	1.0	0.288		Zn	2.0	0.13
$CuSO_4 \cdot 5H_2O$	249.68	0.25	0.062		Cu	0.5	0.03
H_2MoO_4 (85% MoO_3)	161.97	0.25	0.040		Mo	0.5	0.05
NaFeDTPA (10% Fe)	558.50	53.7	30.0	0.3–1.0	Fe	16.1–53.7	1.00–3.00
Optional[a]							
$NiSO_4 \cdot 6H_2O$	262.86	0.25	0.066	2.0	Ni	0.5	0.03
$Na_2SiO_3 \cdot 9H_2O$	284.20	1,000	284.20	1.0	Si	1,000	28

Source: After Epstein 1972.

Note: The macronutrients are added separately from stock solutions to prevent precipitation during the preparation of the nutrient solution. A combined stock solution is made up containing all micronutrients except iron. Iron is added as sodium ferric diethylenetriaminepentaacetate (NaFeDTPA, trade name Ciba-Geigy Sequestrene 330 Fe; see Figure 5.2); some plants, such as maize, require the higher level of iron shown in the table.

[a] Nickel is usually present as a contaminant of the other chemicals, so it may not need to be added explicitly. Silicon, if included, should be added first and the pH adjusted with HCl to prevent precipitation of the other nutrients.

In the simplest form of solution culture a seedling is supported in the lid of a clean glass container containing dissolved mineral elements in double glass distilled water (Figure 4.1). The container in which the plants are grown is usually painted black or covered with an opaque material to protect the roots from direct light and to check algal growth in the medium. Aeration to the roots growing in the medium of a container is provided by aerating tube. This is to prevent the development of anoxic conditions in the nutrient solution. Anoxia inhibits the respiration of root cells and reduces nutrient uptake. In this technique in order to ascertain the essentiality of a particular mineral element to the growth and development of plants, every time only one element is left out from the solution and the plant is grown on it. In the absence of that particular element if plant shows some deficiency symptoms, and if these symptoms were disappear on supplying the missing element, then that elements is decided as an essential element.



Successful solution or hydroponic culture requires frequent replenishment of nutrient solutions to prevent particular ion depletion and associated changes in the P^H due to root absorption. In order to over come this problem, some investigators grow the plants in a non-nutritive medium, such as acid-washed quartz sand, perlite or vermiculite. Seedlings are raised on either of these solid media filled in a containers. Plants grown in this way can then be watered by daily application of fresh nutrient solution. In this technique nutrient solution to the plants is provided in three ways: (1) by pouring over solid medium (called slope culture), (2) by dripping on to the solid medium at suitable intervals from a reservoir (drip culture) and (3) by pumping solution up from bottom of the container (sub-irrigated culture). This fill and empty process is repeated on a regular basis which serves both to replenish the nutrient solution and to aerate the roots. By eliminating one element at a time and by comparing the growth of the plant in its presence and absence, the essentiality of that element to the plant can be decided.

4.5.3 Nutrient film growth technique

An alternative hydroponic system that is often used at commercial level is called the **nutrient film growth system**. In this technique plants are grown in a tube or trough placed on a slight incline (Figure 4.2). The nutrient solution is pumped as a thin film from a reservoir to the elevated end of the tube by a pump. The solution then flows down the shallow trough surrounding the plant roots. This technique therefore allows the plant roots to be bathed continuously in a thin film of aerated nutrient solution. In this system, the composition and P^H of the nutrient solution can be controlled automatically.

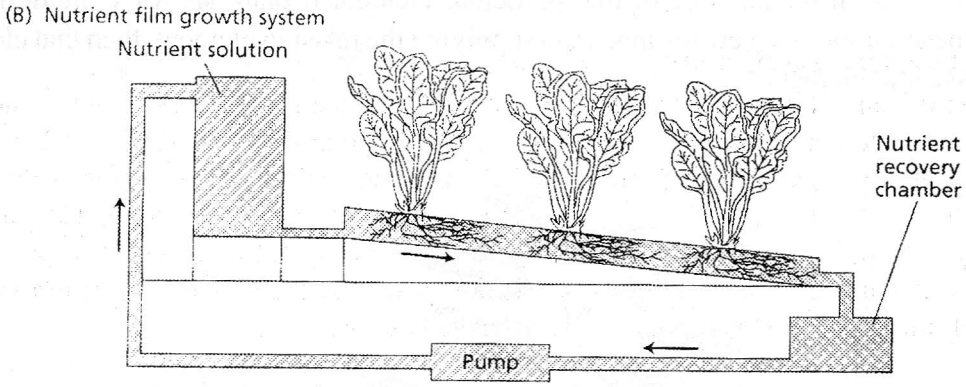


Figure 4.2 The nutrient film technique for hydroponic plant production.

4.5.4 Aeroponics

Another alternative is to grow plants **aeroponically**. In this technique, plants are grown with their roots suspended in air (Figure 4.3). The roots are continuously provided with a nutrient mist of known concentration. This technique provides proper aeration to the roots. But at the same time it

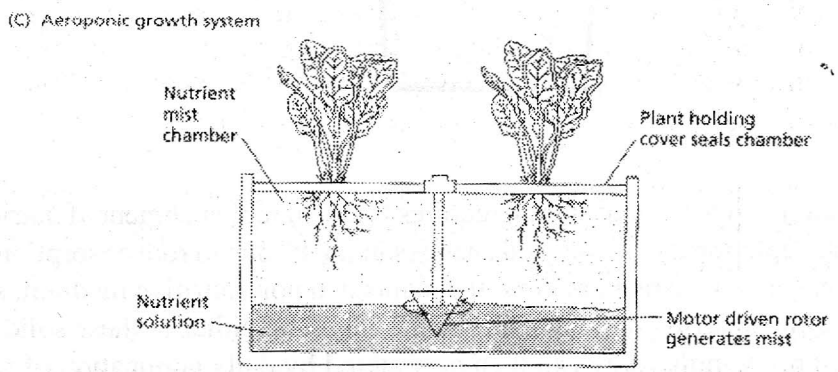


Figure 4.3 Diagram of a typical aeroponic system.

requires high quantity of nutrient solution than it is in hydroponic culture. For this reason and other technical difficulties, the use of aeroponics is not wide spread.

Recently, the techniques to measure concentrations of elements as low as 10^{-8} g ml^{-1} in plants, soils and nutrient solutions have been improved tremendously. These include the use of atomic

absorption spectrophotometers or atomic emission spectrometers. They are now used to measure mineral elements. These techniques require vaporization of the elements at temperatures above 5000K. In the vaporous state the element, depending on the temperature will either absorb (atomic absorption) or emit (atomic emission) light at very narrow wavelength bands. The wavelength of light absorbed or emitted is characteristic to each element. The wavelength and energy of the light absorbed or emitted is quantified by the spectrometer. The quantity of absorbed or emitted energy is proportional to the concentration of the element in the sample. A single sample solution containing concentrations of more than 20 elements can be measured with great sensitivity in less than 1 minute by these techniques.

4.6 Nutrients roles and deficiency symtoms

Each mineral element has one or more structural or functional roles in the plant. Inadequate supply of an essential element results in nutritional disorder, which is manifested by characteristic deficiency. Such visually observed symptoms include stunted growth of roots, stems or leaves and chlorosis or necrosis of various organs. Characteristic symptoms often help in determining the necessary functions of the element in the plant. Knowledge of nutrient deficient symptoms also helps agriculturists and foresters to determine how and when to fertilize the crops. The deficiency symptoms and the functional roles of mineral elements in plants are described below.

Carbon, hydrogen and oxygen: Although these are not minerals in true sense but as they are very much important and necessary for life, therefore, they are included in the list of mineral elements. Air and soil are the sources from which they are supplied in the form of CO_2 and H_2O. They are required in the structural backbone of all organic molecules.

A deficiency of carbon produces rapid starvation of the plant, while a deficiency of water leads to desiccation.

Nitrogen: Soil is the chief source of nitrogen. Plants absorb this element as inorganic nitrate ion (NO_3^-) or ammonium ion (NH_4^+) from the soil solution. Actually nitrogen is not a mineral element but it has been included in the list because it is normally obtained by the plant from soil. Soil gets this element by the activity of atmospheric N_2 fixing bacteria and cyanobacteria.

Nitrogen is a constituent of amino acids, proteins, nucleic acids, chlorophyll and certain hormones and lipids. Thus the processes like protein and chlorophyll synthesis and role of other biomolecules are related to nitrogen nutrition.

Nitrogen deficiency causes a slow and stunted growth and chlorosis (yellowing) of leaves. Chlorosis generally confined to older leaves near the base of the plant. Under severe nitrogen deficiency, these leaves become completely yellow or tan and fall off the plant. Younger leaves do not show these symptoms initially, because nitrogen can be mobilized from older leaves. Thus, a nitrogen deficient plant may have light green upper leaves and yellow or tan lower leaves (i.e., older). Nitrogen deficiency is also manifested in the form of slender and often woody stems. This woodiness may be due to the accumulation of excess carbohydrates, that in the absence of nitrogen, can not be used in the synthesis of amino acids and other nitrogen containing compounds. Also the carbohydrates that are not used in nitrogen metabolism may be used in synthesizing anthocyanins. This condition is exhibited as a

purple coloration in stems, petioles and underside of leaves. Further excess nitrogen supply gives a high shoot/root ratio and delay in the onset of flowering.

Potassium : Potassium is an essential element for all living organisms. Plants taken up this element as monovalent cation K^+. It is produced in the soil from parent rocks such as felspar, mica and glauconite by weathering processes.

Potassium does not serve as the component part of any organic compound. Within plants it occurs as the cation K^+. It serves as an activater of a number of enzymes including enzymes involved in photosynthesis and respiration. Potassium also functions in starch and protein synthesis. Further, potassium as an osmoregulator can control the plant movements such as opening and closure of stomatal guard cells and daily changes in the orientation of leaves (sleep movements).

Like nitrogen, potassium is highly mobile element in the plants. Its deficiency symptoms therefore, first appear in older leaves. The first observable symptom of potassium deficiency is mottled or marginal chlorosis followed by necrotic lesions (spots of dead tissue) at the leaf tips, margins, and between veins. In many monocots, these necrotic lesions may initially form at the leaf tips and margins and then extend towards the leaf base. The potassium deficient plants may have weak, slender, and shortened stems with increased susceptibility to root-rotting fungi. These effects causes the plant to be easily bent to the ground (lodging).

Phosphorous : Phosphorous in soils occurs mainly in the form of phosphoric acid (H_3PO_4). Plants absorb this element either as monovalent ($H_2PO_4^-$) or divalent (HPO_4^{2-}) or triphosphate anions. The availability of these phosphorous forms to the plant depends on soil P^H. This is because that phosphoric acid contains more than one proton, each with a different dissociation constant. At a soil P^H less than 6.8, it exists as $H_2PO_4^-$ and between p^H 6.8 and 7.2, it is predominantly $HPO^2{}_4^{-}$ Soils with p^H greater than 7.2 i.e., alkaline soils have phosphorous mainly in the form of trivalent HPO_4^{3-}. This form of phosphorous is not available for uptake by plants. Further, the tendency of phosphorous to form insoluble complexes with aluminum and iron at neutral P^H and with calcium and magnesium in alkaline soils, made it a limiting element in soils. Plant roots infected with micorrhizal fungus have enhanced uptake of phosphorous.

Phosphorous is a constituent of many vitally important compounds like sugar-phosphate intermediates of photosynthesis and respiration, phospholipids of cellular membranes, nucleotides of energy metabolism and DNA and RNA make up. Another organic P containing compound called phytin is present mainly in seeds. Phosphorous in the phytin form of seeds is regarded as a P reserve.

Plants suffering from phosphorous deficiency are retarded in growth. The shoot/root dry matter ratio is usually low. Generally the symptoms of phosphorous deficiency appear on the older leaves, which are often a darkish green colour. In the extreme situation, the leaves may be malformed and contain small spots of dead tissue called necrotic spots. The stems of many annual plant species contain a reddish coloration due to anthocyanin formation in phosphorous deficient conditions.

Sulfur: Plants mainly absorb sulfur in the form of divalent sulfate anion (SO_4^{2-}). Sulfur oxidizing soil microorganisms release it to the soil solution from iron sulfides and elemental sulfur.

Sulfur is a constituent of amino acids such as cysteine and methionine which contribute disulfide bridges in the tertiary structure of proteins. It is also a constituent of vitamins like thiamine, biotin and coenzyme A. Iron-sulfur proteins that contain Fe-S, 2Fe-2S and 4Fe-4S clusters to catalyze electron transfer reactions of photosynthesis, respiration and nitrogen fixation involve sulfur as an integral part. The characteristic adour of brassicaceae (crucifers) members such as cabbage, onion, garlic and turnips is due to the presence of sulfur as constituent of volatile mustard oils such as thiocyanates and isothiocyanates.

Sulfur deficiency is responsible for chlorisis, stunting of growth and anthocyanin accumulation. Many of these symptoms are similar to those of nitrogen, because both of them are constituents of proteins. However, the chlorosis caused by sulfur deficiency appear initially in young leaves rather than on the old leaves as in nitrogen deficiency. This is due to its inability to mobilize in most plant species.

Calcium : Calcium in the soil occurs as Ca bearingAl-silicates, Ca phosphates and Ca carbonates such as calcite ($CaCO_3$) or dolomite ($CaCO_3MgCO_3$). The weathering of these Ca bearing primary minerals release Ca^{2+} cations to be taken up by the plants.

Calcium is a constituent of middle lamellae of cell walls. It is also used in the mitotic spindle formation during cell division. In various signal transduction pathways, calcium acts as a second messenger. As a second messenger calcium by binding with a protein calmodulin, forms Ca^{2+} - calmodulin complex in plant cells. This complex regulates the activities of number of enzymes necessary to produce response.

The deficiency of calcium mainly appears in the meristematic regions, where cell division and wall formation are more rapid. Calcium is relatively immobile and the symptoms typically appear first in young tissues such as the tips of roots or young leaves. The young leaves are deformed and necrotic with downward hooking. The root system of a calcium deficient plant may appear brownish, short and give 'slippery' to the touch due to the deterioration of the middle lamella.

Magnesium : Plants taken up magnesium as a divalent Mg^{2+} cation from the soil solution where it is held as an exchangeable base.

Magnesium is a constituent of chlorophyll porphyrin ring structure. It is also required to stabilize ribosome structure and to activate enzymes involved in respiration, photosynthesis and the synthesis of DNA and RNA.

A characteristic symptom of magnesium deficiency is a chlorosis due to the break down of chlorophyll at interveinal regions. The chlorosis appears first in the older leaves because of the mobility of magnesium. This interveinal pattern of chlorosis due to Mg deficiency results because chlorophyll in the vascular bundles remains unaffected for longer periods than the chloroplasts in the cells between the leaf veins.

Iron : Plant species absorb iron in the form of ferric (Fe^{3+}) or ferrous (Fe^{2+}) ions. The availability of iron to plants increases with increasing acidity of the soil. Iron deficiencies are therefore common in

neutral or alkaline soils where the more available form of Fe^{3+} is converted into insoluable hydrous oxides ($Fe_2O_3.3H_2O$). In such cases plants exhibit some specialized iron uptake mechanisms. These include the synthesis and release of iron chelating substances like caffeic acid and phytosiderophores..

Under conditions of iron stress plant roots also exhibit enhanced proton secretion into the soil surrounding the roots. This causes acidification of the rhizosphere soil. Acidification of the rhizosphere then encourages the chelation of the Fe^{3+} with caffeic acid. This complex then moves to the root surface where Fe^{3+} is reduced to Fe^{2+} by the root plasma membrane bound reducing enzyme. This reaction causes iron to enter the root cells and releases chelator to the rhizosphere soil for the next round of iron absorption.

The role of plant phytosiderophores in iron uptake processes are a recent discovery. Phytosiderophores are highly specific iron binding ligands found in the members of the family Graminae. These ligands convert insolule iron into soluble form by forming a complex called **iron phytosiderophore complex** or **ferrisiderophore complex**. The entire ferrisiderophore complex is taken into the root cell, where the iron is subsequently reduced to Fe^{2+} and released for use by the cell.

Iron is a component of heme containing cytochromes and non-heme iron sulfur proteins. Both these proteins are important in the oxidation-reduction reactions of photosynthesis and respiration. Iron of these proteins as an electron carrier reversibly oxidized from fe^{2+} to fe^{3+} stae during electron transfer. Iron is also a constituent of several oxidase enzymes such as catalase and peroxidase.

Like magnesium iron deficiency results in a interveinous chlorosis. In contrast to magnesium deficiency the symptoms of iron appear initially on the younger leaves, because the mobility of iron in the plant is very low and cannot be withdrawn from the older leaves. Although iron is not a constituent of chlorophyll, the leaves become chlorotic because it is required for the synthesis of some thylakoid electron transport proteins. Under conditions of extreme or prolonged deficiency, the whole leaf become white due to impaired protein synthesis.

Boron : Boron is probably taken up by plants as the undissociated boric acid (H_3BO_3). The role of boron in plant nutrition is least understood of all the plant nutrients. The available evidence, however suggests that it is required for cell elongation, membrane function, nucleic acid synthesis and hormone responses. In addition, boron is known to stimulate pollen tube germination and elongation.

Boron deficiency gives the roots stubby and bushy appearance. This is due to inhibition of both cell division and elongation in primary and secondary roots. Inhibition of cell division and elongation in boron deficient plants is accompanied by an increased activity of enzymes that oxidize the hormone indole-3-acetic acid (IAA) and a decrease in RNA content possibly through impaired synthesis of RNA precursor uracil. Other symptoms of born deficiency include shortened internodes resulting a bushy or rosette appearance. The most well known born deficiency symptoms are stem crack in celery and heart rot in sugar beet.

Copper : Copper is taken up by the plants as a divalent cupric ion, Cu^{2+}. Its availability to plants is influenced by several factors such as soil organic matter, P^H and microorganisms.

Copper is a constituent of many oxidative enzymes such as plastocyanin, cytochrome oxidase, amide oxidases, super oxide dismutase and polyphenol oxidase. Most of these copper bound enzymes are involved in oxidation and reduction reactions while some react with O_2 and reduce it to H_2O_2 or H_2O.

The initial symptom of copper deficiency is the production of dark green leaves, which may contain necrotic spots. Necrotic spots appear first at the tips of the young leaves and then extend toward the leaf base along the margins. Under extreme copper deficiency, loss of young leaves occur particularly in fruit trees. This condition is known as summer dieback in citrus trees.

Zinc : Plants use zinc in the form of divalent cation (Zn^{2+}).

As a plant nutrient zinc functions as an activator of many enzymes such as alcohol dehydrogenase, carbonic anhydrase, lactate dehydrogenase, glutamic dehydrogenase, alkaline phosphatase and carboxy peptidase. It is also required for the synthesis of tryptophan, a precursor of indote-3-acetic acid (IAA).

Zinc deficiency is characterized by shortened internodes and smaller leaves, a condition usually refered to as "*little leaf*". These symptoms may result from loss of the capacity to produce the sufficient amounts of IAA. This is because that zinc is required for the synthesis of hormone precursor tryptophan.

Manganese : Manganese exists in the soil as a divalent, trivalent and tetravalent forms, but it is absorbed largely as the divalent manganous cation Mn^{2+}.

Manganese acts as an activator of several enzymes in plant cells. Enzymes like decarboxylases and dehydrogenases involved in the tricarboxylic acid cycle are specifically activated by manganese. It is an important component of the oxygen evolving complex (OEC) of photosynthesis, where it is in the form of manganoprotein causes the photolysis of water and consequent evolution of oxygen on the lumen side of the thylakoid membranes.

The major symptom of manganese deficiency is interveinous chlorosis with a small necrotic spots on younger or older leaves. Manganese deficiency is also responsible for grey speck of cereals, a disorder characterized by the appearance of greenish-grey oval-shaped spots on the basal regions of young leaves.

Molybdenum : Plants absorb molybdenum in the form of molybdate (MoO_4^{2-}) ions.

Molybdenum is an essential component of nitrate reductase and nitrogenase. Nitrate reductase catalyses the reduction of nitrate to nitrite for its assimilation into amino acids and nitrogenase, an enzyme of nitrogen fixing organisms converts atmospheric nitrogen to ammonia.

Molybdenum deficiency is indicated by chlorosis and necrosis between veins of the older leaves. Plants that can depend on nitrate or symbiotic nitrogen as source of nitrogen are usually subjected to molybdenum deficiency. In such plants *whiptai* is a common disorder in which young leaves are twisted and deformed. Premature flower fall or prevention of flower formation are other molybdenum deficiency symptoms.

Nickel : Nickel has only recently been added to the list of essential elements. Plants absorb it in the form of monavalent Ni^+ cation.

Nickel is an integral part of two enzymes namely urease and hydrogenase. Urease catalyses the hydrolysis of urea into NH_3 and CO_2 , whereas hydrogenase in nitrogen fixing bacteria catalysis the recycling of hydrogen gas generated during N_2 fixation.

Nickel deficiency in plants is very rare, because they require this element at very low quantities. A nickel deficient plant accumulate urea in its leaves. Such leaves show leaf tip necrosis.

Chlorine : It occurs commonly in soils as chloride anions (Cl^-) and moves freely in soil solution from which it is available to plants. Most of the plants absorb chlorine at levels much higher than is needed for normal functioning.

Along with manganese, chloride is required for water splitting reactions of the photosynthesis. It is a counter ion in the maintenance of electrical neutrality across the energy tranducing membranes. Like potassium, it is one of the osmotically active solute in the vacuole. Chloride is also required for cell division in both leaves and roots.

Plants deprived of chloride tend to exhibit reduced growth, wilting of the leaf tips and a general chlorosis. Roots of chlorine deficient plants may appear stunted and thickened near the root tips.

Other nutrients : In addition to the 17 essential elements described, for some plants there is a requirement of some additional elements such as sodium, silicon, cobalt and selenium. Plants require these elements at quantities that cannot be detected reliably through the presently available analytical techniques. Hence, they are called as beneficial elements.

Sodium is required as a micronutrient for most of the plant species that have C_4 and CAM pathways of carbon fixation. In these plants, sodium ions (Na^+) are required for regenerating phosphoenol pyruvate from pyruvic acid. Phosphoenolpyruvic acid is the first CO_2 accepter in C_4 and CAM plants. Sodium deficiency in these plants causes chlorosis and necrosis.

Silicon is a beneficial element particularly to grasses. In these plants it is present as a constituent of cell walls of epidermal cells. Its deficiency leads to lodging of these plants.

Cobalt is necessary for the growth of both symbiotic and asymbiotic nitrogen fixing microorganisms. In the absence of this beneficial element, nitrogen nutrition of legumes may be effected.

Generally **selenium** is toxic to most plant species. However, certain plant species called seleniferous indicators such as *Astragalus bisulcatus* and *A. pectinatus* accumulate high selenium that would be toxic to most other plants. The exact role of selenium in these plants is not yet known. Plants containing high selenium content exhibit sickness known as alkali poisoning.

Plants require every essential mineral element at a particular concentration. The concentration of the nutrient at which plants show maximum growth or yield is defined as **critical concentration**

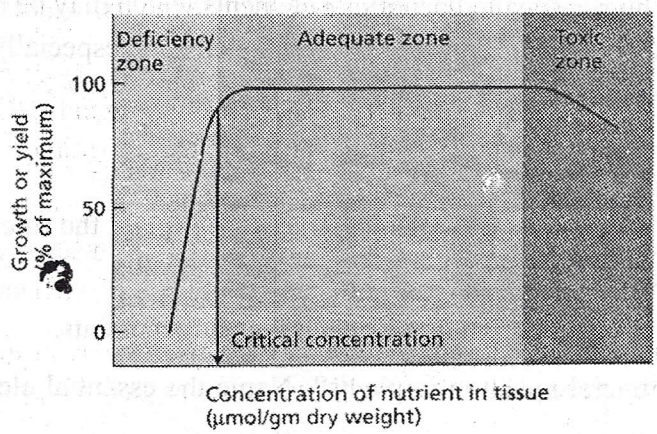


Figure 4.4 Relationship between growth and the nutrient content of the plant tissue.

(Figure 4.4). As the nutrient level in the tissue sample increases above the critical concentration, a point is reached at which additional increases of mineral nutrient has no effect on growth or yield. Such a nutrient concentration is called **adequate concentration**. When the nutrient content in a tissue sample is low, growth is reduced. At this point the nutrient level is said to be deficient. As the nutrient content of the tissue increases above the adequate level, growth or yield declines because of toxicity. For example, the critical concentration of copper in plants ranges from 4 to 15 $\mu g\ g^{-1}$ of tissue dry weight. The growth is reduced, when it is present below 4 µg and become toxic in excess of 20 µg.

4.7 Summary

Plants are autotrophic organisms. They require mineral elements to be used in biosynthesis and energy production. Studies of plant nutrition have shown that plants require seventeen elements obtained from the soil. These 17 elements are considered essential because it has been demonstrated that in their absence all plants are unable to complete a normal life cycle. The seventeen essential elements are classified as macronutrients and micronutrients, depending on the relative amounts required.

Macronutrients (C, O, H, N, P, K, S, Ca and Mg) are needed in large quantities and micronutrients (Fe, B, Cu, Zn, Mn, Mo, Ni and Cl) are used in very small amounts. Techniques such as ash analysis, solution culture and atomic absorption or atomic emission spectophotometers are used in the study of mineral elements essential for plant life.

Each essential mineral element has a role to play in the biochemistry and physiology of the plant. A plant that is deficient either in macronutrient or micronutrient exhibits a nutritional disorder with characteristic symptoms. Nutritional disorders occur because nutrients serve as components of organic compounds, in energy storage, to maintain plant structures, as enzyme cofactors, and in electron transfer reactions.

There are some additional nutrients called beneficial elements which may be required by some plants in order to satisfy some special requirements. Essential elements, especially micronutrients, may be toxic when present above the critical concentration.

4.8 Model questions

1. What are the criteria for essentiality of mineral elements? Explain the role of N P and K in plant growth and development.
2. Discuss the symptoms and effects of micronutrient deficiency in plants.
3. What do you mean by mineral nutrition in plants? Name the essential elements with their roles.
4. Write short notes on :
   (a) Iron as plant nutrient
   (b) Sulfur and calcium deficiency
   (c) Solution culture

4.1 Reference Books

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Dr.G. ROSAIAH

LESSON: 5

MINERAL SALT UPTAKE AND MEMBRANE TRANSPORT PROTEINS

5.0 Objective

In this lesson mineral salt absorption by diffusion, facilitated diffusion and active transport, membrane transport proteins such as channels, carriers and pumps, symplast and apoplastic transport of ions from the roots to the shoots are discussed.

5.1 Introduction

As described in the previous lesson that plants require 17 elements for their growth and development. Except C, H, O all the remaining elements are present in the soil solution in dissociated condition so that plants adsorb them in the form of ions. Mineral ions are found either as soluble fractions of soil solution or as adsorbed ions on the surfaces of negatively charged inorganic and organic soil particles. Mineral cations such as ammonium (NH_4^+), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), manganese (Mn^{2+}) and others adsorb to the negative surface charges of soil particles. These ions are not easily lost when the soil is leached by water, and they provide a nutrient reserve available to plant roots. Mineral nutrients adsorbed in this way can be replaced by other cations in a process known as cation exchange capacity. For example, H^+ replaces K^+. A soil with higher cation exchange capacity generally has a larger reserve of mineral nutrients.

Mineral anions such as NO_3^-, Cl^-, SO_4^{2-}, and others occur as soluble fractions of soil solution in low concentrations and are potentially available for absorption by plant roots. Because of their negative charges, however, these ions will be subjected to repulsion with negatively charged soil particles so that they may be leached by water moving through the soil if not absorbed by the plant roots.

As we know that plant cells are separated from their environment by a plasma membrane. Plasma membrane is made up of proteins and non polar lipids. It acts as a biological barrier for the

free passage of charged solutes and ions in and out of the cell. The transport of charged nutrients across this biologically impermeable membrane takes place through the transport proteins. Molecular and ionic movement between the soil and the plant and also from cell to cell across the membrane is called transport or absorption. From time to time various theories have been proposed by different workers to explain the mechanism of ion absorption. These different theories are however categorized into three fundamental concepts such as diffusion, facilitated diffusion and active transport. These three processes now make up the basic language of transport across the membrane of all organisms. These three basic models of transport are schematically shown in the Figure 5.1 and are described in detail in the following sections.

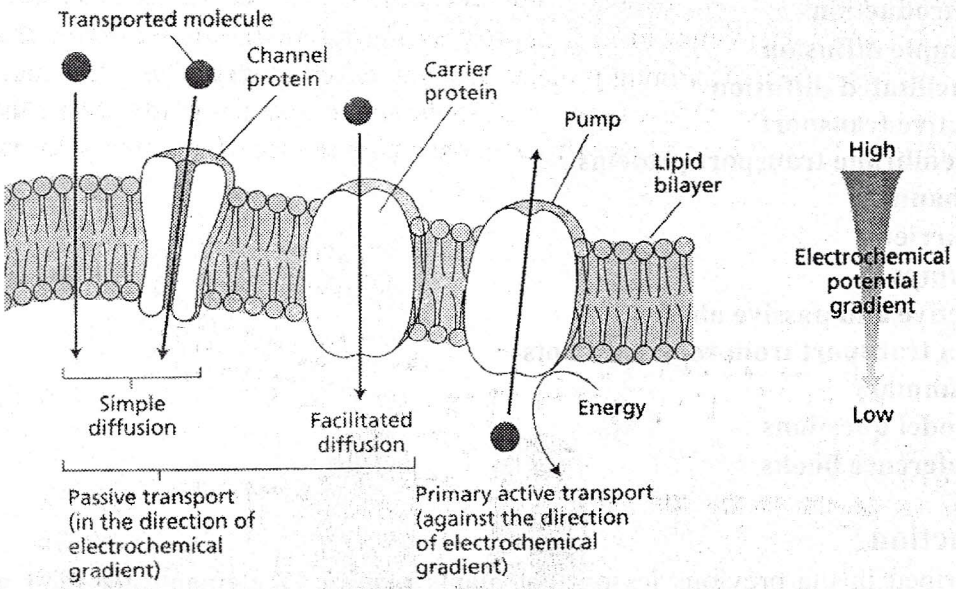

Figure 5.1 The transport of ions and solutes across membranes by simple diffusion, facilitated diffusion and active transport

5.2 Simple diffusion

Diffusion is the process by which molecules intermingle as a result of their random thermal agitation. Such agitation gives rise to the random but progressive movement of substances from regions of high concentration (or high free energy) to regions of low concentration (or low free energy) down a concentration gradient. Fick discovered that the rate of diffusional movement of molecules is directly proportional to the concentration gradient. This law in symbols for a membrane bound cell can be written as :

$$Js = - PA (C_s^o - C_s^i)$$

Where Js is the flux density or rate of transport which specifies the amount of substance s crossing a unit membrane per unit time. **P** is the permeability coefficient that measures the velocity

(in cm s^{-1}) with which the substance **s** moves through the membrane. $C_s^o - C_s^i$ is the concentration *difference of substance* ***s*** *between outside and inside of the cell. The negative sign in the equation* indicates that the flux moves down a concentration gradient. This law says that a substance will diffuse faster when the concentration gradient becomes steeper or when the permeability coefficient is increased. *Because the membrane is lipid in character and those substances that have high* permeability coefficient such as nonpolar susstances move rapidly across the biological membrane. Nonpolar substances such as O_2, CO_2 and NH_3 enter the cell through simple diffusion process. Water is a highly polar molecule. Its permeability coefficient in lipid layer is very low. Although it is a polar molecule it diffuses rapidly and freely across the membranes from its high concentration to low concentration. Previously it was suggested that the free movement of water across the membranes takes place by simple diffusion. However, the recent molecular genetic studies indicated that the water transport occurs by membrane integrated proteins which form a water-selective channels across the membrane. These channel forming proteins are now called **aquaporins**. Aquaporins facilitate water movement across the membrane in response to the water potential gradient on either side of the membrane (Figure 5.2). Just how aquaporins achieve a high degree of selectivity for water remains unclear.

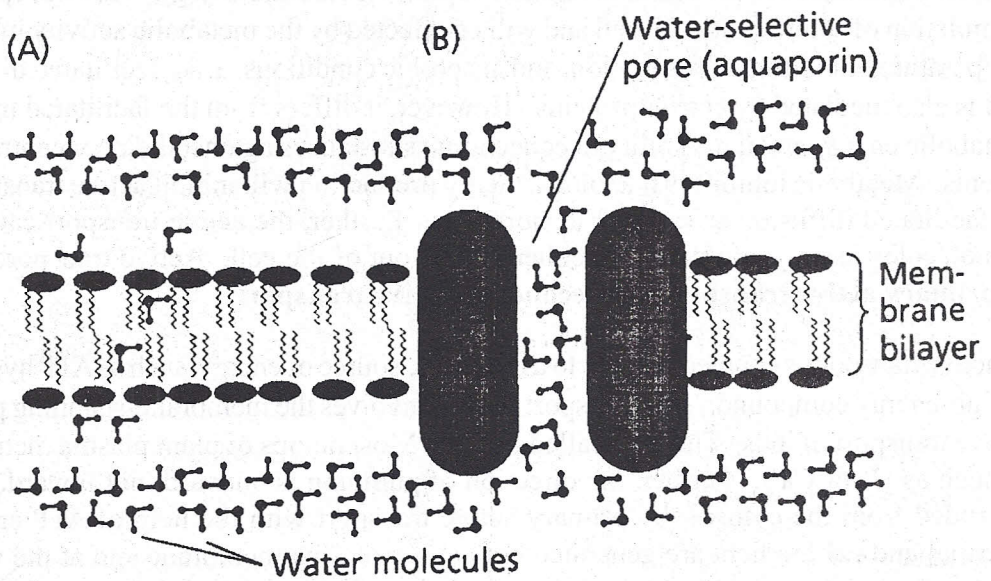

Figure 5.2 Water movement through a water selective pore formed by integral membrane protins aquaporins

5.3 Facilitated diffusion

The plasma membrane will not permit the free passage of most substances particularly charged solutes or ions. However, it was found that certain charged ions entered cells far more quickly than would be expected on the basis of their diffusion through a lipid bilayer. The reason is that biological membranes contain transport proteins that facilitate the passage of ions and other polar molecules. The rate of diffusion of certain solutes across selectively permeable membranes is therefore greatly

increased by using transport proteins, sometimes called carriers, permeases and transporters. It is truly a diffusion process by which charged nutrients can cross the membrane with the help of membrane integrated carrier proteins. This carrier aided diffusion process is called **facilitated diffusion**. Each carrier is selective and will transport only closely related solutes. A concentration gradient spanning the membrane drives the movement of molecules. If the concentration gradient disappears, net inward movement ceases. Remember that this transport is reversible, if the solute concentration is greater inside the cell that is the solute will move outward.

The movement of molecules by either simple or facilitated diffusion across the plasma membrane is called **passive transport**. In passive transport molecules will always proceed spontaneously down a concentration or chemical gradient until equilibrium is reached wit ut a direct input of metabolic energy. These processes does not cause an accumulation of solu against its concentration or electrochemical gradient.

5.4 Active Transport

The movement of solute molecules against a concentration or chemical gradient is termed **active transport**. It is not a spontaneous process and requires input of metabolic energy. This transport will lead to an accumulation of solute inside the cell and will be affected by the metabolic activity inhibitors such as low temperature, inhibitors of respiration, and anaerobic conditions. Like facilitated diffusion, active transport is also mediated by carrier proteins. However, it differs from the facilitated transport in its use of metabolic energy and in its ability to concentrate substances against their concentration or chemical gradients. Metabolic inhibitors that block energy production will inhibit active transport but will not affect facilitated diffusion at least for a short time. Further, the active transport causes the movement of molecules in only one direction either into or out of the cell. Active transport can be dissected into **primary active transport** and **secondary active transport.**

Primary active transport is coupled directly to a metabolic source of energy, such as ATP hydrolysis or some other high-energy compound. This transport system involves the membrane-spanning proteins to carry out active transport of ions. They are called pumps. Most pumps of plant plasma membranes transport ions such as H^+ or Ca^{2+}. Further, the direction of pumping is outward, not inward. When protons are extruded from the cytosol by primary active transport with the help of ATP energy, a membrane potential and pH gradient are generated both at the plasma membrane and at the vacuole membrane. That is the inside of the cytoplasm becomes electrically negative and alkaline and the out side of the membrane becomes electrically positive and acidic. This gradient of electrochemical potential for H^+ across the membrane is called proton motive force (PMF or Δp). It represents stored free energy in the form of the H^+ gradient.

The proton motive force generated by primary active transport is used to drive the transport of many other organic and essential mineral nutrients against their concentrations by another transport mechanism called the secondary active transport. It is a carrier mediated co-transport system. In this mechanism the solutes are actively transported across a membrane against their electrochemical potential gradients by coupling of the uphill transport of one solute to the downhill transport of another. The secondary active transport is of two types : the **symport system** and the **antiport system .**

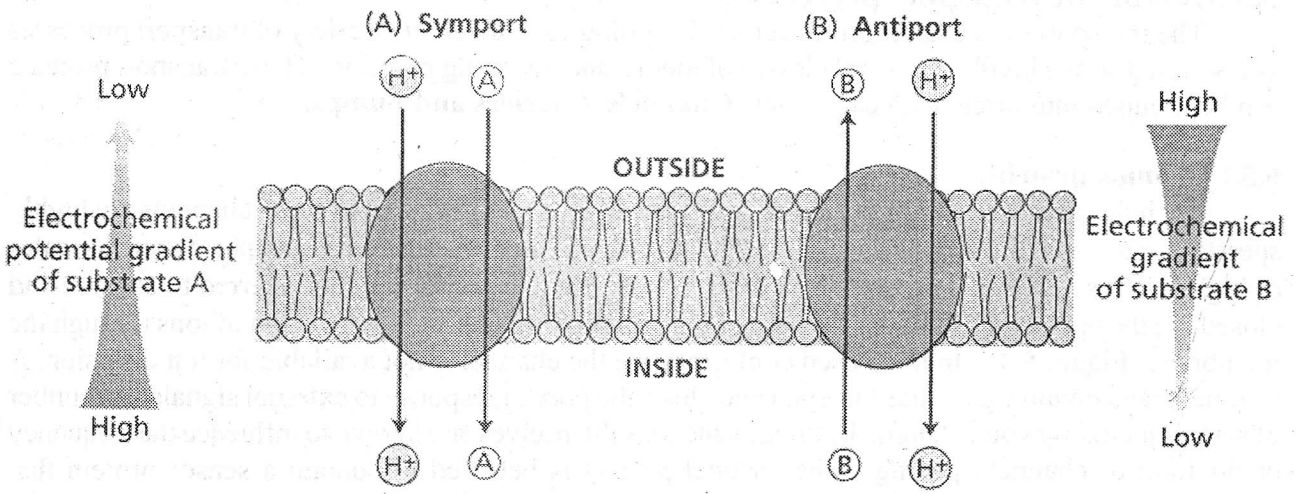

Figure 5.3 Secondary active transport typs. (A) Symport system. The energy dissipated by a proton moving back into the cell is coupled to the uptake of one substrate molecule into the cell. (B) Antiport system. The energy dissipated by a proton moving back into the cell is co,[;ed tp tje active transport of the solute out of the cell. In both cases, the substrate under sonsideration is moving against its electrochemical potential gradient.

A symport simultaneously transports two different substances across the membrane in the same direction. In plants and fungi, the sugars and amino acids are taken up by symport with protons. For example, when glucose is supplied to a plant cell bathed in a simple solution of mineral salts, a reduction in the membrane potential, an increase in external pH, and uptake of glucose occur simultaneously. The decrease in membrane potential is due to the positive charges (H^+) that move into the cell along with glucose. However, this membrane depolarization is transitory, because the reduced membrane voltage allows the primary active transport H^+ pump to work faster and thereby restore the membrane voltage and pH gradient in the presence of continuing glucose uptake. Similarly, transport of Cl^-, NO_3^-, $H_2PO_4^-$, K^+, sucrose, amino acids, and other substances enter the cell via specific secondary active proton symport systems.

An antiport simultaneously transports two different molecules across the membrane in opposite directions. For example, sodium transport across the membranes takes place by secondary active antiporter system in which sodium is transported out of the cell in response to the inward movement of protons.

In both types of secondary transport, the ion or solute being transported along with the protons is moving against its gradient of electrochemical potential, so that its transport is active. However, the energy driving this transport is provided by the proton motive force rather than by ATP hydrolysis. Typically, a cell depends on ATP driven primary active transport system in order to set up the proton motive force. Many other ions and organic substrates can then be transported by a secondary active transport system with the help of energy of proton motive force i.e by simultaneously carrying one or two H^+ down their energy gradient. Thus H^+ ions circulate across the membrane, outward through the primary active transport, and back into the cell through the secondary transport system.

5.5 Membrane transport proteins

The transport of ions and solutes across the biological barrier by a variety of transport processes as discussed above involves several classes of membrane-spanning proteins. These transport proteins can be grouped into three main categories: **Channels, Carriers and Pumps.**

5.5.1 Channel proteins

Channels are transmembrane ion or solute transport proteins. Most of these channels are highly specific for one or a limited number of ion species, which can diffuse through an open channel at rates as high as 10^8 s^{-1}. Channel proteins may exist in two different conformations referred to as open and closed. In the open conformation, the core of the protein forms a pore for diffusion of ions through the membrane (Figure 5.4). In the closed conformation, the channel is not available for ion diffusion. A channel may contain a gate that can open and close the pore in response to external signals. A number of signals including voltage, light, hormones and ions themselves, are known to influence the frequency or duration of channel opening. The channel protein is believed to contain a sensor protein that responds to the appropriate stimulus by changing the conformation of the protein and opening the gate.

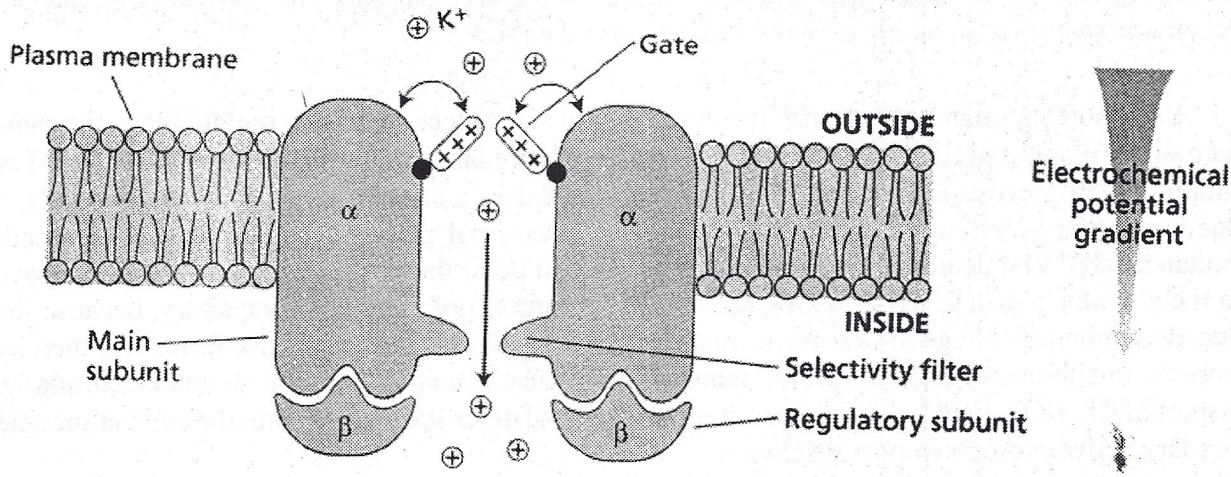

Figure 5.4 A model of a voltage gated K^+ channel in a plant plasma membrane. The gate opens or. closes the pore in response to the membrane potential. Selectivity filter and regulatory subunit may also be present.

Transport through a channel is always passive. Its specificity of ion transport depends on pore size and electric charge rather than on selective binding. The region of the channel that determines its specificity is called the **selectivity filter**.

In guard cell plasma membranes, two types of gated anion channels have been distinguished on the basis of how long the gates remain open in response to a prolonged stimulus. They are designated as **R-type channels** (rapidly activated) and **S-type channels** (slowly activated). R-type channels open and close very rapidly in response to a voltage stimulus while S-type channels remain open for the duration of the stimulus. Rapid and slow type channels have also been identified in vacuolar membranes, and they are referred to as fast vacuolar (FV) or slow vacuolar (SV) channels.

5.5.2 Carrier proteins

Carrier proteins are also known as transporters or simply porters. In transport mediated by a carrier, the particular solute or ion being transported is initially bound to a specific site on the carrier protein like an enzyme-substrate binding. Binding of the solute causes a conformational change in the carrier protein, which exposes the solute to the solution on the other side of the membrane. Release of the solute at the other surface of the membrane completes the transport and the protein then reverts to its original conformation, ready to pick up another solute. Typically, carriers may transport 100 to 1000 ions or molecules per second, which is about 10^6 times slower than transport through a channel.

Carrier-mediated transport can be either passive or active. Facilitated diffusion, a passive process that takes place through these carriers, transport the substances down their concentration gradients without an additional input of metabolic energy. Carrier mediated secondary active transport involves two types of transmembrane carriers called **symporters** and **antiporters**. These carriers contain two sites on the out side of the membrane to bind a proton and a solute. Proton binding at the first site causes the second site to be exposed. This site binds the ion or solute that is being actively transported. With both molecules bound, the transporter undergoes a conformational change that exposes the binding sites to the opposite side of the membrane. The cycle is completed by diffusion of the proton and substrate molecule away from their binding sites, causing the transporter to regain its original, or "relaxed" confirmation (Figure 5.5).

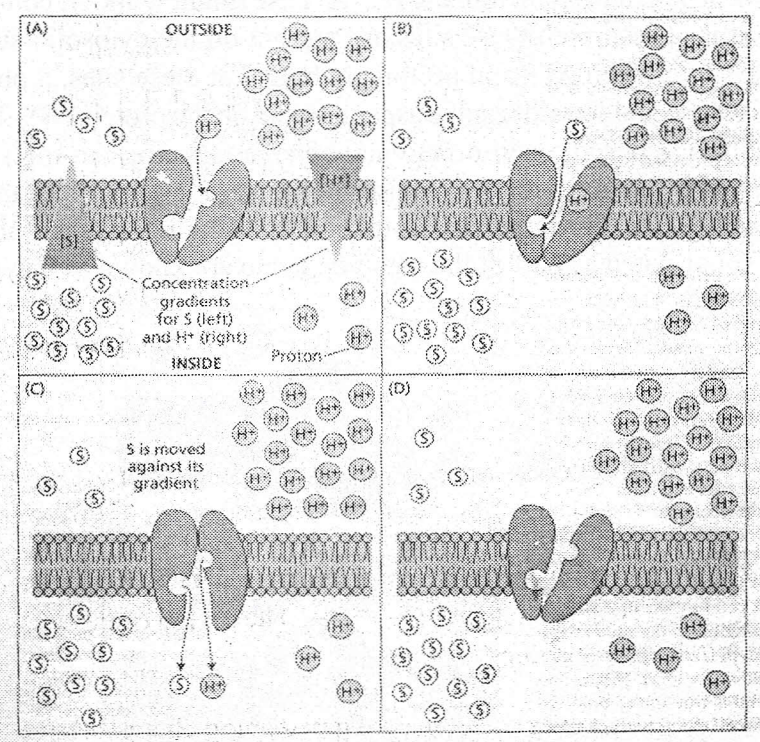

Figure 5.5 A model showing the mechanism of secondary active transport. (A) Initial conformation of the carrier with binding sites on the out side of the membrane. (B) H^+ binding exposes the substrate binding site of the carrier. (C) The binding of H^+ and S causes the carrier to be present in another conformation that exposes both the sites to the inside of the cell. (D) Release of a proton and substrate into the cytosol restores the original conformation of the carrier.

5.5.3 Pumps

The membrane proteins that carry out primary active transport are called pumps. The pumps can use the energy of ATP hydrolysis to establish a proton gradient across the membrane. Hence, they are also known as **ATPase-proton pumps**. These pumps are large multiprotein complexes found in the plant plasma membranes and tonoplasts. ATPase-proton pumps may be either **electrogenic** or **electroneutral**. Electrogenic pumps cause the net movement of charge across the membrane, while electroneutral pumps, as the name implies, involve no net movement of charge. For example, Na^+/K^+ - ATPase of animal cells pumps three Na^+ ions out for every two K^+ ions in, resulting in net outward movement of one positive charge. The Na^+/K^+ - ATPase is therefore an electrogenic ion pump. In contrast, the H^+/K^+ - ATPase pumps one H^+ out of the cell for every one K^+ in, so there is no net movement of charge across the membrane. Therefore, the H^+/K^+ - ATPase is an electroneutral pump.

H^+ - ATPase is the electrogenic proton pump of plant plasma membranes. This pump creates the proton gradient across the membrane by translocating protons from the cytosol to the surrounding apoplastic cell wall space with the use of ATP energy. This proton gradient, together with the normal membrane potential, establishes a proton motive force that tends to move protons back across the membrane. This proton motive force is the primary source of energy to drive the secondary active transport, necessary for the active transport of many other substances.

Figure 5.6 shows how a plasma membrane H^+ - ATPase might work. It contains ATP and proton binding sites. When it is at rest, both these sites are present on the cytosolic side with an occluded pore in the membrane (A). When ATP and proton are bound at these sites, a phosphate group from ATP is transferred to specific amino acid residue asparatic acid on the protein (B). This phosphorylation then causes the protein to undergo a conformational change, opening the transport pathway to the outside and simultaneously closing it on the cytosolic side (C). The proton then leaves its binding site, and the protein comes to its original conformation with the removal of phosphate group (D). ATPases that are phosphorylated as part of the catalytic cycle are known as P-type ATPases.

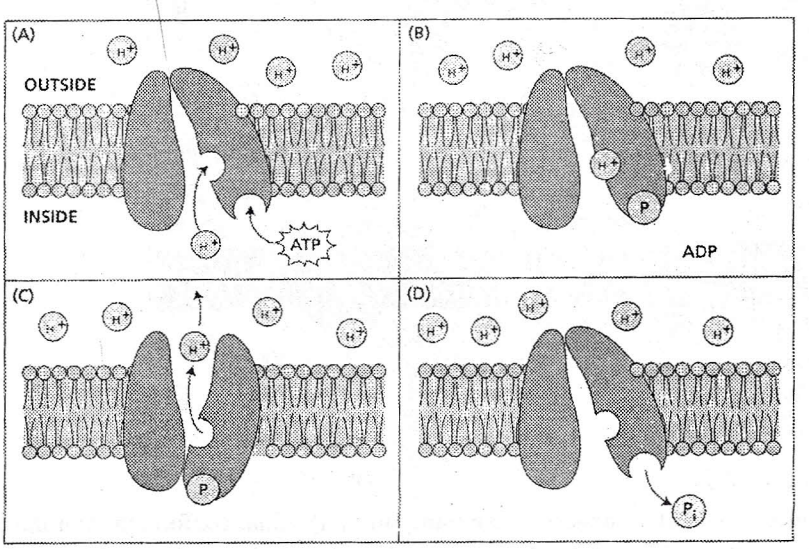

Figure 5.6 A model showing the transport of H^+ against its chemical gradient by an electrogenic H^+-pump (see the text for details).

According to molecular studies, the plasma membrane H^+-ATPase consists of a single polypeptide chain with a molecular mass of 100 kDa. Different tissues of the plant have various H^+-ATPase isoforms to regulate transport in different ways for each tissue. Like other enzymes, the plasma membrane H^+-ATPase is regulated by the concentration of substrate (ATP), pH and temperature. Further, H^+- ATPase causes the translocation of a single ion (H^+) in one direction. This kind of transport is called **uriport system** and H^+-ATPase as **uniporter protein.**

Tonoplast contained H^+-ATPase is known as **vacuolar H^+-ATPase** or **V–ATPase.** This electrogenic pump translocates protons from the cytoplasm into the lumen of vacuole.

V–ATPase is a large enzyme complex made up of at least ten different subunits with a molecular mass of about 750 kDa. It differs both structurally and functionally from the plasma membrane H^+-ATPase because it does not involve the formation a phosphorylated intermediate. Like F–ATPases of chloroplasts and mitochondria, the catalytic subunits of V-ATPases are organized into a peripheral catalytic complex, V_1, and an integral membrane channel complex, V_o. Figure 5.7 shows how V-ATPases might work. Hydrolysis of ATP by the V_1 catalytic complex drives the rotation of the V_1 stalk. The rotation of the V_1 stalk, in turn, drives the rotation of the V_o complex. When the V_o complex turns, protons are transported from one side of the membrane to the other.

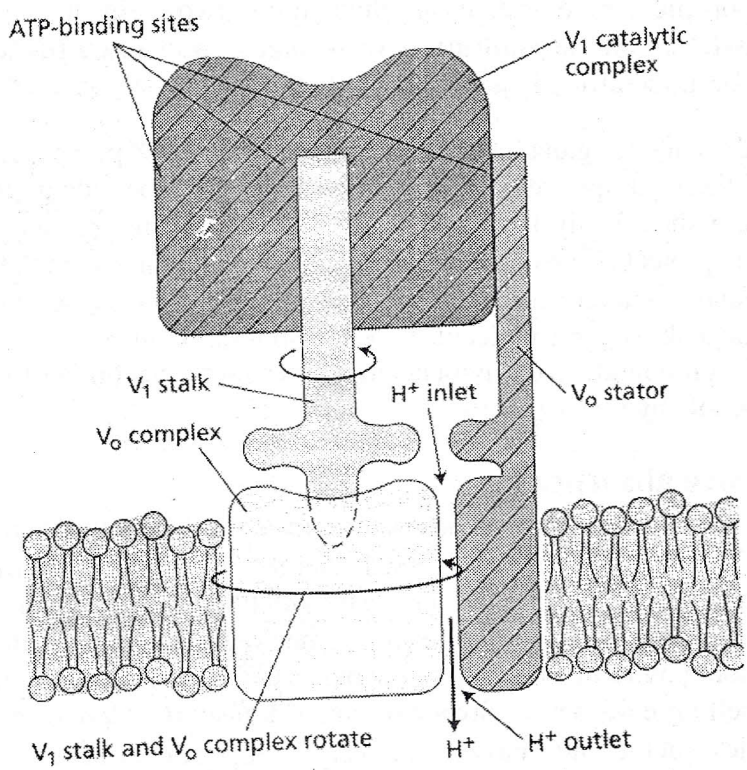

Figure 5.7 Vacuolar H^+-ATPase and its organization in the tonoplast.

tissue (or be carried passively by water flow) exclusively through the cell wall without crossing any membranes. The apoplast is the continuous system of cell walls and intercellular air spaces in plant tissues. Ions may also move via the symplast pathway, which consists of the entire network of cell cytoplasm interconnected by cytoplasmic bridges called **plasmodesmata**. These two pathways enable the ions to reach the endodermis, a boundary layer between the stele and the cortex. At the endodermis, water and ion movement into the stele through the apoplast pathway may be obstructed by the Casparian strip. The Casparian strip is a band of radial cell walls in the endodermis that is impregnated with the waxlike, hydrophobic substance suberin. Suberin acts as a barrier to water and solute movement. Hence the only possible route for ions to pass through the endodermis is to enter the symplast by some carrier or channel mediated transport at the cell membrane. In all cases, ions must enter the symplast before they can enter the stele because of the presence of Casparian strip. Symplastic connections facilitate ions passive movement from cell to cell until they arrive at a xylem parenchyma cell in the stele. At this point the ions are unloaded into the xylem vessels (Figure 5.8).

Figure 5.8 The radial path of ion movement through a root. Arrows indicate the alternate paths that may be taken by nutrient ions as they move from the soil solution into the vascular elements in the stele. Arrows with circles indicate active transport of ions across plasma membranes.

Once ions have been taken up into the symplast of the root at the epidermis or cortex, they must be loaded into the tracheids or vessel elements of the stele to be translocated to the shoot. Since the xylem tracheary elements are devoid of cytoplasm and consist only of nonliving, water filled tubes, the ions must exit the symplast by crossing a plasmamembrane a second time. Release of ions into the xylem thus requires a transfer from the symplast into the apoplast. The process whereby ions exist the symplast and enter the conducting cells of the xylem is called **xylem loading**. At one time, it was thought that this transfer was simply a passive leakage, but the evidence now suggests that ions are actively secreted from the xylem parenchyma. The plasma membranes of xylem parenehyma cells contain proton pumps, water channels, and a variety of ion channels specialized for influx or efflux. The flux of ions from the xylem parenchyma cells into the xylem tracheary elements, rather than being due to simple leakage, is under tight metabolic control through regulation of the plasma membrane H^+ - ATPase and ion efflux channels.

5.8 Summary

Molecular movement between different compartments of biological systems is known as transport. Plants exchange solutes with their environment and among their tissues and organs. Transport between cells is specifically controlled by their plasma membranes. Solutes may cross a membrane by simple diffusion, facilitated diffusion or active transport. In simple diffusion and facilitated diffusion transport of solutes occur down a chemical gradients called passive transport. Movement of solutes against a chemical potential gradient is known as active transport.

Facilitated diffusion involves channels as solute transporters while the active transport is mediated by membrane spaning pumps and carrier proteins. Only active transport achieves accumulation of ions against an electrochemical gradient. It requires a source of metabolic energy, normally in the form of ATP.

transport. Because ions carry an electrical charge so that they will diffuse in response to a gradient in electrical potential as well as chemical potential. That is for ions acted upon by an electrical gradient, cations are attracted to a negative electropotential whereas anions are attracted to a positive electropotential. Ion movement is thus dependent on an electrochemical potential gradient and the electrical properties of the cell or its transmembrane potential.

Generally living cells are negatively charged as compared with the outer medium. This is because the cytosol contains a large number of fixed or nondiffusible charges such as the carboxyl ($RCOO^-$) and amino ($R.NH_4^+$) groups of proteins. At the same time, cells use metabolic energy to actively pump cations like H^+, Ca^{2+} and Na^+ into the exterior space. As a result a voltage or potential difference will develop across a membrane due to this unequal distribution of cations and anions. For this reason the passage of ions through the plama membrane or tonoplast must be considered in relation to the prevailing electrical potential gradient as well as the concentration gradient between the outer solution (medium) and inner solution (cytoplasm). Positively charged potassium ions, for example, will naturally be attracted to a region with a preponderance of negative charges.

The relationship between transmembrane potential gradient and ion distribution across the membrane can be expressed quantitatively by the Nernst equation :

$$\Delta Enj = \frac{2.3\ RT}{zF} \times \log \frac{C_j^i}{C_j^o}$$

Where ΔEn = the electrical potential difference for the ion j

C_j^i / C_j^o = the ratio of the molar concentrations inside and outside the cell

R = gas constant

F = Faraday constant (96,500 J V^{-1} $mole^{-1}$)

z = Valency or charge for ion j. The value of z for a univalent cation would be 1 while for calcium or magnesium it would be 2. For chloride or nitrate it would be –1 and for sulphate it would be –2.

This equation allows us to say whether the ion is transported passively or actively across the membrane. In order to apply the equation, it is necessary to first measure the transmembrane potential and the concentrations of ions both inside and outside the cell. Deviations from the concentrations predicted by the Nernst equation are considered evidence that either active uptake or expulsion of the ions is involved. If the measured internal concentrations are approximately equal to the calculated Nernst value, it can be assumed that the ion has been distributed passively. If the measured concentration is greater than predicted, active uptake is probably involved, and, if lower, the ion is actively expelled from the cell (Table 5.1).

Table 5.1 The uptake of selected ions by maize roots

Accumulation ratio			
Ion	**C^o(m)**	**C^i(m)**	**$[C^i/C^o]$**
K^+	0.14	160	1142
Na^+	0.51	0.6	1.18
NO_3^-	0.13	38	292
SO_4^{2-}	0.61	14	23

C^o and C^i are the ion concentrations of the medium and root tissue respectively.

5.7 Ion transport from roots to shoots

So far, we have seen the ion transport at cellular level. In this section we will discuss the pathways by which ions can move from root to shoot via the xylem.

Mineral nutrients absorbed by the root are carried to the shoot by the transpiration stream moving through the xylem. When an ion enters the root, it may be transported across the root into the xylem by apoplast pathway or symplast pathway. In the apoplast pathway, ions can diffuse across a

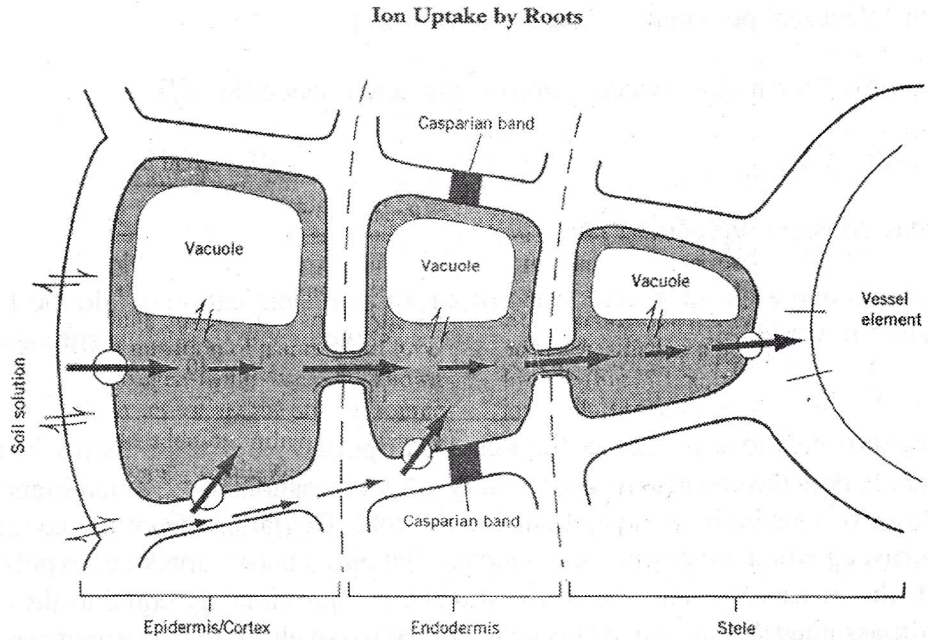

FIGURE 5.11 The radial path of ion movement through a root. Arrows indicate the alternate paths that may be taken by nutrient ions as they move from the soil solution into the vascular elements in the stele. Arrows with circles indicate active transport of ions across plasma membranes.

As we know that plant cells enlarge primarily by the uptake of water into the large central vacuoles so that the osmotic pressure of the vacuole must be maintained sufficiently high for water to enter from the cytoplasm. The electrogenic proton pumping V–ATPases of tonoplasts do this job. They are capable of generating a proton motive force across the tonoplast by pumping protons from the cytoplasm into the vacuole. The accumulation of H^+ inside the vacuole due to V–ATPase activity accounts for the fact that the pH of the vacuolar sap becomes low i.e about 5.5 compared to the cytoplasmic pH of 7.0 to 7.5. The electrical component of the resulting proton motive force drives the uptake of anions such as Cl^- and $malate^{2-}$ into the vacuole and the pH gradient is used to drive the uptake of cations and sugars into the vacuole via secondary transport (antiporter) systems.

Vacuolar membranes in addition to V-ATPases contain another type of proton pump called **H^+ - Pyrophosphatase** (H^+-PPase). This pump consists of a single polypeptide with a molecular mass of 80 kDa. It is driven by the energy obtained from the hydrolysis of inorganic pyrophosphate (PPi). It is a inducible pump and is induced by low O_2 levels (hypoxia) or by chilling. Under these conditions ATP levels are depleted resulting in an inactivation of V–ATPase. In order to maintain essential cell activities under these stress conditions cells operate H^+-PPase to regulate the ionic traffic and metabolites between the cytosol and the vacuolar sap across the tonoplast membrane.

Tonoplasts also contain still another large group of active transport proteins known as the **ATP – binding cassette (ABC) transporters** for the transport of large organic molecules into the vacuole. They are energized directly by ATP hydrolysis to pump organic molecules across a membrane. This family of active transport membrane proteins are divided into two main subclasses: **the multidrug resistance proteins (MDRs)** and the **multidrug resistance – associated proteins** (MRPs). Both types of proteins have been identified in plants, but only the MRPs were studied in detail.

Plant MRPs are also called as glutathione conjugate pumps or GS-X pumps, because the molecules that are transported by these pumps are covalently attached to the tripeptide glutathione. The GS-X pumps of plant cells are specifically localized on the vacuolar membrane where they function in herbicide detoxification, protection against oxidative damage, pigment accumulation, and the storage of antimicrobial compounds. A family of enzymes called glutathione transferases (GSTs) are responsible for attaching glutathione to the organic molecule to be transported. Compounds such as anthocyanins, IAA, various phenolic compounds and phytochelatins after their attachment to the glutathione are transported into the vacuole by GS-X pumps.

5.6 Active and passive absorption

The concentrations of some ions inside the cell may reach levels much higher than in the surrounding medium. This phenomenon is expressed quantitatively by the **accumulation ratio** which can be defined as the ratio of the concentration inside the cell (C^i) to the concentration outside the cell (C^o). For example, the internal concentration of K^+ in maize roots is more than 1000 times greater than it is in the surrounding nutrient solution. In the past, an accumulation ratio greater than one has been considered compelling evidence in favour of active transport. Conversely, an accumulation ratio less than one implies that the solute has been actively excluded or extruded from the cell. Actually this is true for uncharged solutes like sugars. But for charged solutes or ions, the accumulation ratio is not always a valid indication to say whether the ion is accumulated either by passive or active

Channels are transport proteins that span the membrane, forming pores through which solutes diffuse down their gradient of elechemical potentials. Carriers bind a solute on one side of the membrane and release it on the other side.

In plants, a family of H^+-pumping ATPases provide the primary driving force for transport across the plasma membrane. Further, V-ATPases and H^+-Pyrophosphatases serve this function at the tonoplast. In addition, vacuolar membranes also contain ATP-binding cassette transporters that use the energy of ATP directly to transport large organic molecules into the vacuole. The gradient of electrochemical potential generated by H^+ pumping is used to drive the transport of other substances in a process called secondary transport. Secondary active transport is mediated by symporters and antiporters.

The relationship between the voltage difference across the membrane and the distribution of ion at equilibrium is described by the Nernst equation. Deviations from the concentrations prediated by the Nernst equation are considered evidence that either active uptake or expulsion of the ions is involved.

Solutes move between cells either through the apoplast or from cytoplasm to cytoplasm via the symplast. Cytoplasm of neighboring cells are connected by plasmodesmata, which facilitate symplast transport. When an ion enters the root, it may be taken up into the cytoplasm of an epidermal cell or it may diffuse through the apoplast into the root cortex and enter the symplast through a cortical cell. From the symplast, the ion is loaded into the xylem and transported to the shoot.

5.9 Model Questions

1. Describe the mechanism of nutrient uptake by active transport process.
2. What are membrane transport proteins and what role do they play in nutrient uptake?
3. Write short notes on :
   (a) Accumulation ratio
   (b) Apoplast and symplast
   (c) ABC transporter proteins

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Dr. G. ROSAIAH

LESSON: 6
ENZYMES

6.0 Objective

In this lesson chemical nature and properties of enzymes, enzyme specificity, IUB system of enzyme classification, activation energy, mechanism of enzyme action and significance of Km value are discussed.

6.1 Introduction

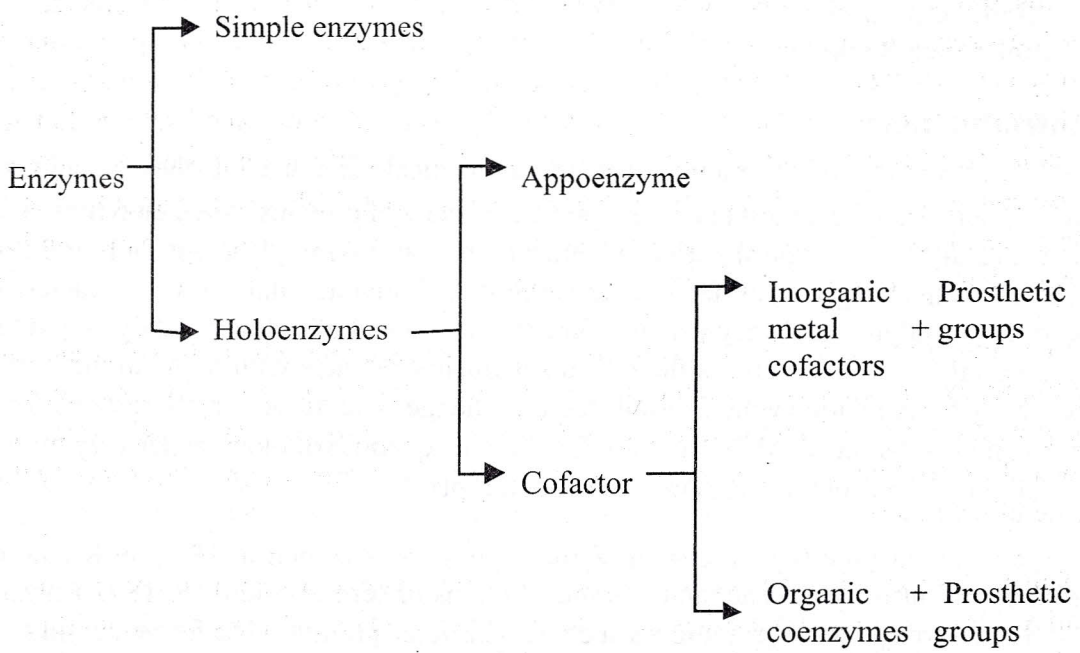
Life is composed of enormous variety of biochemical reactions. All these reactions in a living cell are supported by the largest and most specialized class of proteins called **enzymes**. Enzymes are biological catalysts that control various chemical reactions taking place within living cells without themselves being changed or utilized. These exceptional organic catalysts are characterized by their extraordinary catalytic efficiency which is far greater than that of ordinary catalysts and their greater reaction specificity which permits them to distinguish substrates with quite similar structures and their capacity for regulation which permits them to change catalytic activity in response to substances other than their substrates. Above all they function in aqueous solutions under very mild conditions of temparature, atmospheric pressure and at neutral pH.

The name enzyme (Greek en= in; zyme = yeast) was coined in 1878 by Kuhne to describe something present in yeast that catalyses the reactions of fermentation. In 1897 Edward Buchner prepared a cell free juice by grinding yeast cells. This juice promoted the fermentation of sugars like that by original intact yeast cells. His experiment is considered as starting of modern enzyme chemistry. In 1926 James Sumner isolated a urease enzyme in crystalline form from Jackbean which he found proteinaceous in nature. Protein nature of enzymes was confirmed further by Northrop and Kunitz (1930) through their studies on proteolytic enzymes such as pepsin, trypsin and chymotrypsin. Later on with the help of modern techniques, the first amino acid sequence of bovine pancreatic ribonuclease A in 1963 and the first x-ray structure of lysozyme from hen eggwhite in 1965 were determined. So far, nearly 2000 enzymes have been obtained in pure form and are characterized at least to some extent.

6.2 Chemical nature of enzymes

Except catalytic RNA enzymes called ribozymes all other enzymes are proteins. Some enzymes are made up of only protein molecules. For example, trypsin, pepsin, urease, protease and amylase involve only aminoacid residues in the catalysis of chemical reactions and such ones are called **simple enzymes**. Others called **conjugated enzymes** require a non-protein component in addition to the enzyme protein for catalytic activity. In such an enzyme, the protein portion is called **apoenzyme** and the non-protein moiety as **co-factor**. Individually these parts does not have any catalytic activity. A complete catalytically active apoenzyme co-factor complex is known as the **holoenzyme**.

The cofactors can be considered as the chemical teeth of conjugated enzymes. They may be either inorganic metal ions, such as Fe^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, K^{+} and Mo or complex organic molecules such as TPP, FAD, NAD, CoA, lipoate, tetrahydrofolate and pyridoxal phosphate. Organic cofactors, also known as **coenzymes** are either vitamins or derivatives of vitamins. A metal ion cofactor or coenzyme molecule that functions by having firm or covalent association with the enzyme protein is known as **prosthetic** group.



Further, depending on the number of polypeptide chains (subunits), enzymes may be described as **monomeric** (one subunit) or **oligomeric** (several subunits) enzymes. Some enzymes catalyse the same reaction but are present in several forms. They are called **isoenzymes**, which are frequently coded by different genes and consequently have different protein structures. They act on the same substrate by residing in different tissues or organs (e.g. PEP carboxylase, lactate dehydrogenase).

6.3 Properties of enzymes

Enzymes, like other proteins have molecular weights that range from about 12,000 to over 1 million. They are colloidal in nature and provide large surface area for reactions to take place. Being

proteins they may be lost their native conformation when exposed to higher temperatures. Enzymes have been evolved in living cells to lower the activation energies selectively for reactions that are needed for cell survival. An enzyme catalysed reaction reaches its equilibrium much faster than that of uncatalysed reaction. In a reaction process enzyme is not used up and the equilibrium will not be affected. In addition, enzymes exhibit high turnover number, defined as the number of substrate molecules converted to products by each enzyme molecule in one minute.

Another characteristic feature that distinguishes the enzymes from inorganic catalysts is their greater substrate specificity. For that they contain a specific region or pocket called the **active site**. The molecule that is bound by the active site and acted upon by the enzyme is called the **substrate**.

6.4 Enzyme specificity

Specificity refers to the ability of an enzyme to descriminate between two competing substrates. A particular enzyme attacks only a particular substrate. Enzymatic specificity can be distinguished into steriochemical specificity, reaction specificity, group specificity and substrate specificity.

Most of the biological reactions are extremely specific in forming stereoisomers. Isomers that have same structural formula but differ in the arrangement of atoms or groups around carbon are known as sterioisomers. Most enzymes exhibit a high degree of specificity towards one stereoisomeric form of the substrate. For example, fumarase catalyses an addition of water to fumarate but not to its cis-isomer malate. Similarly, trypsin hydrolyses polypeptides composed of L-amino acids but not those consisting of D-amino acids. The enzymes involved in glucose metabolism are specific for D-glucose residues. A substrate of the wrong chirality will not fit into an enzymatic binding site like your right hand can not fit into your left hand glove.

Enzymes are specific in catalyzing only one of the reactions which the substrate can undergo. For example, oxaloacetate can undergo several reactions viz., reduction to give malate, decarboxylation to give pyruvic acid, amination to give asparitate and with acetate gives citrate. Each of the reactions of oxaloacetate is catalysed by its own separate enzyme which catalyses only that reaction and none of the others.

Substrate specificity of an enzyme varies from one enzyme species to other. It may be absolute or relative. For example, urease has absolute specificity where it catalyses the hydrolysis of urea only. Relative specificity may be group dependent or bond dependent. For example, trypsin hydrolyses residues of only lysine and arginine while chymotrypsin hydrolyses only aromatic aminoacids. Enzymes like proteases, glycosidases and lipases are bond specific enzymes which can hydrolyse peptide, gycosidic and ester bonds, respectively.

6.5 Nomenclature and classification of enzymes

Enzymes are commonly named by adding the suffix "**ase**" to the name of the enzyme's substrate or the type of reaction they catalyse. The only major exceptions to this are the proteolytic enzymes whose names usually end with-`in', e.g. **trypsin, pepsin, tyalin**.

Enzymes that are named in this fashion gives only little information about their substrates or reactions which they catalyse. For example, enzyme **lactase** catalyses the hydrolysis of lactose into glucose and galactose, **urease** catalyses the hydrolysis of urea and **sucrase** which acts on sucrose. The name of these enzymes indicate the substrate of the enzyme it acts upon. The names of other enzymes e.g. **transcarboxylase** indicate the nature of the reaction without specifying the substrates. Some enzyme names such as **catalase**, **tyalin** and **trypsin** even do not indicate on what substrates they can act. Sometimes, the enzymes have the names that are greater confusion among the scientists. **Fumarase**, for example, by analogy with other hydrolases might be supported to catalyse a hydrolytic reaction, but in fact, it hydrates **fumarate** to form **malate**. Due to lack of the systematic approach in naming and classifying enzymes in the beginning, this traditional practice further allowed the same enzyme to have two or more names or different enzymes to have the one name. In order to overcome this inconsistency and also in view of the rapidly increasing new enzymes, a system for naming and classifying enzymes has been formulated by the International Union of Biochemistry (IUB, 1964). A commission on enzymes (EC) appointed by IUB was suggested certain rules and regulations for the systematic arrangement of all enzymes. According to this system, enzymes are divided into six major classes of reactions that they catalyse.

Table 1 Enzyme classification according to IUB.

S.No.	Class	Type of reaction catalysed
1	Oxidoreductases	Transfer of electrons (H^-)
2	Transferases	Transfer of functional groups
3	Hydrolases	Hydrolysis reactions
4	Lyases	Addition of groups to double bonds, or formation of double bonds by removal of groups (not by hydrolysis)
5	Isomerases	Isomerisation by transfer of groups within molecules
6	Ligases	Bond formation coupled with ATP Hydrolysis

Each class is further divided into several sub-classes which in turn into sub-subclasses with enzymes in a serial number. Each enzyme is assigned two names, a trivial (or recommended) and systematic name and a four digit code number. Its recommended name is used in routine laboratory work and the systematic name is referred in case of any ambuiguity. The systematic name includes the name of the substrate (s) followed by a word ending in "-ase". This word is either one of the six major classes of enzymes or their sub-divisions. For example, an enzyme catalyzing the conversion of glucose into glucose-6-phosphate has the trivial name **hexokinase** and the systematic name **ATP: glucose phosphotransferase**.

$$ATP + D\text{-glucose} \rightarrow ADP + D\text{-glucose - 6-phosphate}$$

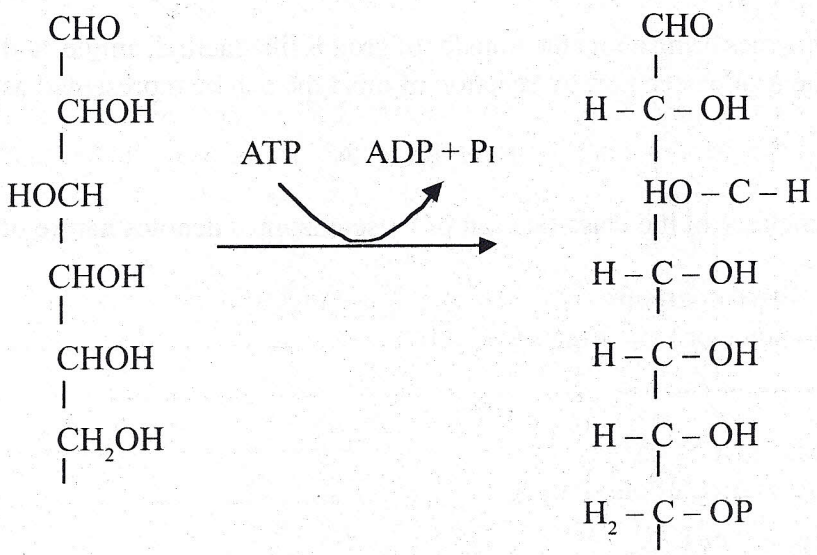
Further, when a reaction involves two types of overall change, e.g. oxidation and decarboxylation, the second function is indicated in brackets. For example, isocitrate undergoes oxidative decarboxylation in the presence of **isocitrate dehydrogenase**. Its systematic name therefore contains

its second function, the decarboxylation in brackets as isocitrate : NAD^+ oxidoreductase (decarboxy-*lating).*

$$\text{Isocitrate} + NAD^+ \rightarrow \alpha\text{ - ketoglutarate} + CO_2 + NADH$$

Enzyme code number consists of four digits separated by dots. The first number indicates the enzyme's major class, the second number denotes its sub-class, the third number designates its sub-subclass and fourth number is the enzyme's serial number in its sub-sub class.

For example ATP: glucose phosphotransferase (hexokinase) has the classification number E.C. 2.7.1.1.



D-Glucose D-Glucose -6-phosphate

Here "EC" stands for Enzyme Commission, the first number (2) denotes the class name transferase (see table 6.1), the second digit (7) describes the sub class phosphotransferase; the third digit (1) tells sub-sub class phosphotransferases with a hydroxyl group as acceptor (in this case the OH group on 6-carbon in glucose); and the fourth digit (1) is the enzyme's serial number in its sub – subclass.

The six main classes of enzymes are now described in some detail.

1. Oxidoreductases : These enzymes catalyse oxidation – reduction reactions by transferring hydrogen or hydride (H^-) ions from donars to acceptors. The second digit in the enzyme classification number of oxidoreductases reveals about the hydrogen or electron donar participated in that reaction.

For example :

E.C.1.1 enzymes are oxidoreductases acting on (>CH.OH) alcohol group of electron donars.

E.C.1.2 enzymes are oxidoreductases acting on aldehyde or ketone (>C=O) group of H or e^- donors.

The third digit in the code number of oxidoreductases indicates the hydrogen or electron acceptors (NAD and NADP, cytochromes, O_2 etc.) involved in a reaction.

For example, E.C. 1.1.1 enzymes are oxidoreductases acting on CHOH group of hydrogen donars with NAD^+ or $NADP^+$ as hydride ion acceptor.

E.C. 1.4.3 are oxidoreductases with primary amines as an electron donors and O_2 as an electron acceptor.

Thus an enzyme acting on ethyl alcohol with NAD^+ as hydride ion acceptor has the systematic name **alcohol: NAD^+ oxidoreductase** and the classification number **E.C. 1.1.1.1**. Its trivial name is **alcohol dehydrogenase**.

Similarly, an enzyme that catalyses the D-amino acid into an oxoacid by using O_2 as an electron acceptor is known as **D-amino acid: oxygen oxido-reductase** (deaminating) with E.C. 1.4.3.3. Its trivial name is **D-amino acid oxidase**.

2. Transferases : These enzymes bring about the transfer of groups like methyl, amine, and phosphate from the group donar to the group acceptor. A reaction of this type can be represented as

$$Ax + B \rightarrow Bx + A$$

The second digit (sub class) in the classification of these enzymes denotes nature of the group transferred. For example :

2.1 Transferring 1 – carbon groups,
2.2 Transferring aldehyde or keto groups (>C=O),

$$-\overset{\overset{O}{\|}}{C}-R$$

2.3 Acyl transferases (- C – R),
2.4 Glycosyl transferases (Carbohydrates),
2.6 Amino transferases ($-NH_3^+$),
2.7 Phosphotransferases.

Third digit (sub-sub class) of this class indicates about the type of group being transferred.

For example:
2.1.1 enzymes are methyl transferases,
2.1.2 enzymes are hydroxy methyl transferases,
2.4.1 enzymes are hexosyl transferases,
2.4.2 enzymes are pentosyl transferases,
2.7.1 enzymes are phosphotransferases with an alcohol group as acceptor,
2.7.2 enzymes are phosphotransferases with a carboxyl group as acceptor.

In case of phosphotransferases, the third digit describes the type of group acceptor. The trivial names of this sub-class usually have the suffix "- kinase" (e.g hexokinase, phosphofructokinase).

3. Hydrolases : These enzymes catalyse the hydrolytic cleavage of bonds such as ester, ether, peptide, glycosidic and acid anhydride by direct addition of water. A reaction catalyzed by hydrolases can be represented as $A - X + H_2O \rightarrow X - OH + HA$.

The second and third digits in the classification number of hydrolases describes about the nature and type of bonds that are cleaved. For example :

3.1 hydrolases acting on ester bonds,

3.1.1 hydrolases which catalyse carboxy ester bonds (- C(=O) – O-),
3.1.2 hydrolases which catalyse thiol ester bonds,
3.2 hydrolases cleaving glycosidic bonds,
3.2.1 hydrolases hydrolyzing N-glycosidic bonds.

For example, **glucose-6-phosphate phosphohydrolase** with enzyme code 3.1.3.9 catalyzes Glucose-6-P + H_2O → Glucose + Pi. In short it is called **glucose-6-phosphatase.**

4. Lyases : This group of enzymes involve in addition or removal of groups by attacking the bonds like C-C, C-O, C-N, C-S, without involving water. Enzymes such as aldolases, carboxylases, synthases, and hydratases are come under this category.

The second and third digit in the enzyme code of this class indicates the type of bond broken and the type of group removed, respectively.

For example :

4.1 lyases catalyzing C-C bonds,

4.2 lyases catalyzing C-O bonds,

4.3 lyases catalyzing C-N bonds,

4.4 lyases catalyzing C-S bonds,

4.1.1 lyases involving in removal of carboxyl group (i.e. CO_2),

4.1.2 lyases involving in removal of aldehyde group (- CHO),

4.2.1 lyases involving in addition of water across the double bonds.

For example, **pyruvate carboxylyase** having the enzyme code E.C. 4.1.1.1. and the trivial name **pyruvate decarboxylase**, catalyses

$$CH_3 - C(=O) - COO^- \rightleftharpoons CH_3 - CHO + CO_2$$

Malate hydro-lyase (E.C. 4.2.1.2) called by trivial name **fumarate hydratase,** catalyses

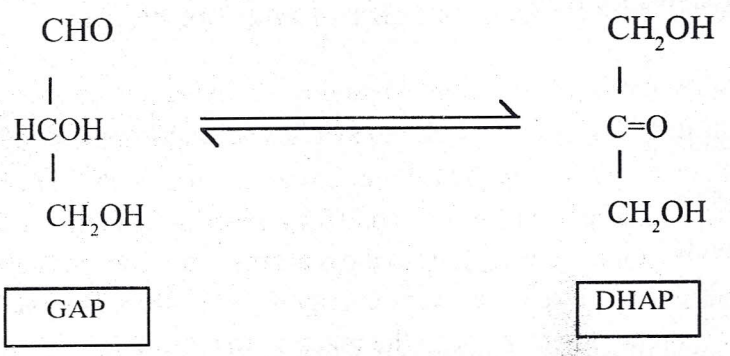
$$^-OOC - CH = CH\text{-}COO^- + H_2O \rightarrow {}^-OOC - CHOH.\ CH_2.\ COO^-.$$

5. Isomerases : This class contains enzymes that catalyse isomerisation reactions i.e intramolecular arrangements like optical (dextro to levo), geometrical (cis to trans) or position (aldose to ketose) isomers.

The second digit in the classification number of this enzyme class indicates type of isomerisation and the third digit denotes type of the compound undergoing isomerisation. For example :

5.1 Recemases and epimerases,
5.1.1 Acting on aminoacids,
5.1.3 Acting on carbohydrates,
5.2 Cis – trans isomerases,
5.3 Intramolecular oxidoreductases,
5.3.3 Inter conversion of aldoses and ketoses,
5.4 Intramolecular transfer reactions.

For example, **triose phosphate isomerase** catalyses the interconversion reaction between the glyceraldehydes-3-phosphate (aldotriose) and the dihydroxy acetone phosphate (ketotriose)



This enzyme has given the systematic name **glyceraldehyde-3-phosphate ketol isomerase** and a classification number E.C. 5.3.3.1.

Similarly, in the presence of **alanine racemase** (E.C. 5.1.1.1) L-alanine is transformed into D-alanine.

L-alanine ⇌ D-alanine

6. Ligases : These enzymes with the help of ATP energy, catalyse the systhesis of new bonds like C-C, C-S, C-O and C-N in the linking of two molecules.

The first digit in the classification describes the type of bond synthesized and the third number indicates the bond being formed.

6.1 Ligases forming C-O bonds,
6.1.1 Ligases are acid-RNA ligases (linkage of amino acids to tRNA),
6.2 Ligases forming C-S bonds,
6.2.1 Ligases are acid – CoA ligases (linkage of acetate to coenzyme A),
6.3 Ligases forming C-N bonds,
6.3.1 Ligases are acid – ammonia ligases (e.g. linkage of glutamate to ammonia),
6.3.2 Ligases are acid – aminoacid ligases (e.g. peptide bond synthesis),
6.4 Ligases forming C-C bonds,
6.4.1 Ligases are carboxylases (catalyses the addition carbon dioxide).

For example, **acetyl – CoA synthetase** (E.C. 6.2.1.1) catalyses the condensation of acetate and CoA to yield acetyl-CoA. The energy required to drive this reaction is supplied by ATP.

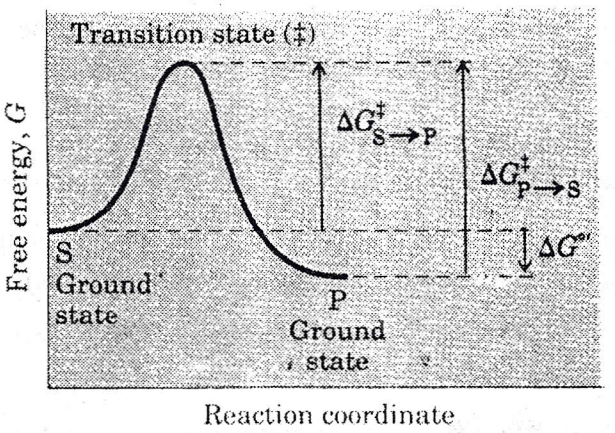
$$CH_3 - COOH + HS\text{-}CoA + ATP \rightarrow CH_3\text{-}\overset{\overset{O}{\|}}{C}\text{-}S\text{-}CoA$$

6.6 The mode of enzyme action or the mechanism of enzyme action

In the absence of enzymes, most of the biochemical reactions in living systems do not occur at a reasonable rate to meet the cells demand for energy and to synthesise cellular constituents. This is because the greater stability of all biomolecules in the cellular environment which is characterized to be aqueous, mild temparature and neutral pH. Further, the path of a reaction, whether it is exergonic (releasing energy) or endergonic (consuming energy), from the reactant to product contains an energy barrier called activation barrier. This barrier represents the energy required for alignment of reacting groups, bending or stretching of existing bonds prior to the reaction to takes place. In other words, the reacting molecules must have to reach the unstable condition before they are converted into products. This unstable state that the reacting molecules attained with the input of energy is called **transition state**.

6.6.1 Activation energy

The amount of energy needed to bring the colliding molecules to the transition state (reaction state) is called **activation energy**. It is the difference between the energy levels of the ground state and the transition state molecules in a chemical reaction. For example, formation of water from oxygen and H_2 has a very large and negative (-237 kJ) free energy i.e. exergonic. Although it is energetically favourable, if we were to simply mix O_2 and H_2 together in a container, no measurable formation of water would occur for many years. This is because the rearrangement of O_2 and H_2 molecules to form water require that the existing chemical bonds of the reactants be broken, for which activation energy is required. The situation for an exergonic reaction that proceeds with a net release of free energy is shown in Figure 6.1.



A

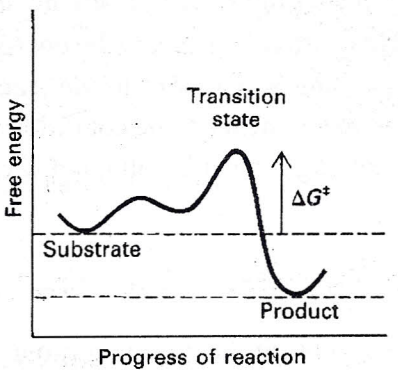
S = Substrate } in ground state
P = Product }

++ = S or P at transition state

$\Delta G^{++}{}_{S \rightarrow P}$ = Activation energy required for the S to form P

$\Delta G^{++}{}_{p \rightarrow S}$ = Activation energy required for the P to form S.

$\Delta G^{\circ\prime}$ = Overall free energy change



B

Figure 6.1 Enzymes accelarate reactions by decreasing ΔG^{++}, the free energy of activation. The free-energy profiles of uncatalyzed (A) and catalyzed (B) reactions are compared.

The rate of a given chemical reaction therefore depends on the height of the activation energy. A higher activation energy corresponds to a slower reaction rate. Thus, factors such as raising the temparature or increasing the reactant concentrations should increase the reaction rates by elevating

their kinetic energy and collision frequency. Addition of catalysts also increase reaction rates by lowering activation energies. In biological systems enzymes do this job.

6.2.2 Enzymatic catalysis

Enzymes are extraordinary catalysts that cause the rate enhancements which are often in the range of 10^7 to 10^{14}. They do this function by providing an energetically favourable specific environment within themselves to a given reaction. In an enzyme catalysed reaction the enzyme's active site temporarily binds with the substrate (s) to form enzyme substrate complex. Then, as the reaction proceeds, the product (P) is released and the enzyme (E) is returned to its original state (Figure 6.2).

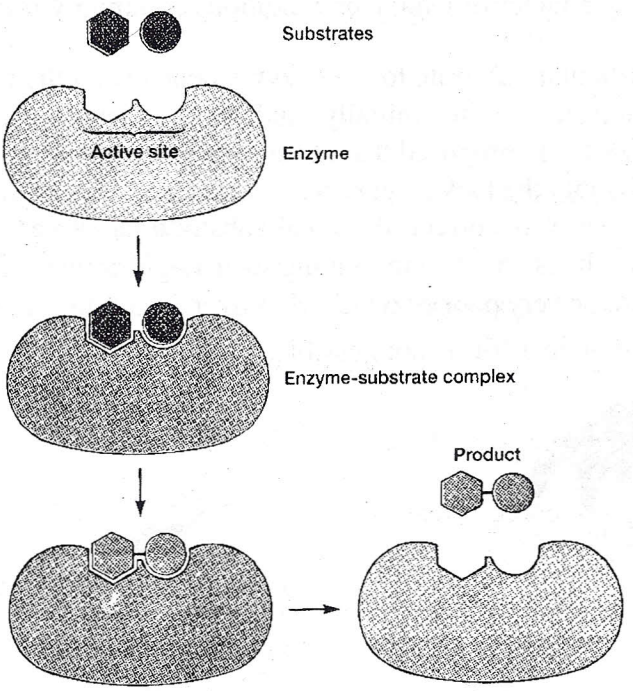


Figure 6.2 Enzyme function. The formation of the enzyme-substrate complex and its conversion to products is shown

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$$

ES and EP are complexes of the enzyme with the substrate and the product, respectively. The bidirectional arrows in the above equation indicates that an enzyme which catalyses the reaction S→P also catalyses the reaction from P→S. It is important to remember that enzymes do not affect the energetics or the equilibrium of a reaction. Their only role is to speed up the reaction rates by lowering the activation energy. As a result the reaction reaches equilibrium very quickly.

How do enzymes lower the activation energies of chemical reactions ? The enzyme is generally much larger than the substrate (S). The active site or catalytic site present on the surface of an enzyme contains catalytic functional groups such as specific amino acid chains, metal ions and co-enzymes. These catalytic functional groups on enzymes can interact transiently with the correct substrate, form-

ing an enzyme substrate complex. The interaction between the substrate and the enzyme in this complex is further guided and stabilized by weak bonds such as electrostatic bonds, hydrogen bonds, van der Waals forces and hydrophobic interactions. These weak interactions that takes place between substrate and enzyme in the ES complex are accompanied by a small release of free energy called **binding energy**. Enzymes use this binding energy to lower the activation energies of reactions.

It has been estimated that formation of a single weak interaction in the ES complex between the enzyme and the substrate releases free energy that ranges from about 4 to 30 kJ/mole. The total amount of free energy available from formation of so many such weak interactions are estimated to be 60 to 80 kJ/mole. Enzymes use this energy in the breaking and making of bonds during catalysis and cause rate enhancements by a factor of ten under conditions commonly found in cells.

The binding of a particular substrate to the enzyme depends on the precisely defined arrangement of catalytic groups in its active site. Initially, studies on enzyme specificity were carried out by Emil Fischer in 1890 to 1894. He proposed that the substrate must have a matching shape to fit into the active site like a key fits into the lock (Figure 6.3). This theory is then became known as the **lock and key theory**. If this theory is correct, the boud substrate has a very little chance to undergo changes in order to form products. An enzyme having such a rigid active site completely complementary to its substrate is said to be very poor enzyme. Moreover, in certain cases a catalytic activity of an enzyme is observed even though a 'fit' is not possible.

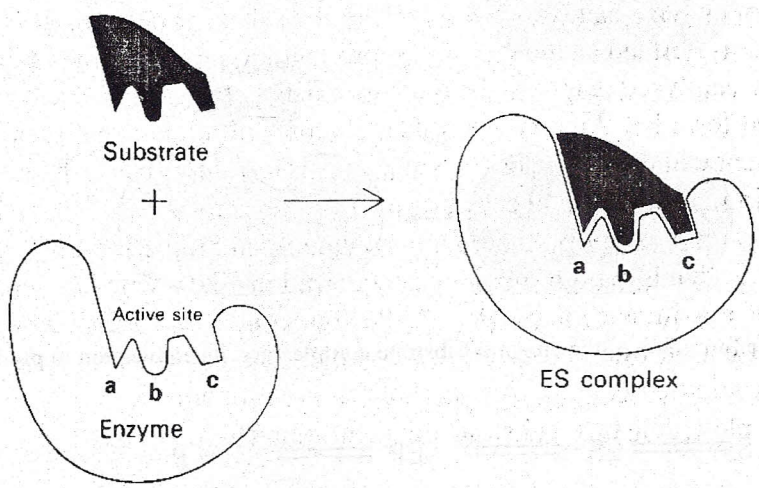


Figure 6.3 Lock-and-key model of the interaction of substrates and enzymes. The active site of the unbound enzyme is complementary in shape to that of the substrate.

The modern concept of enzymatic catalysis was proposed by Daniel E. Koshland (1958). He suggested that the shapes of active sites of many enzymes are modified by binding of the substrate. According to this model, the active sites of enzymes assume shapes that are complementary to that of the substrate (s) transition state only after substrate is bound. This substrate induced fit is called **induced fit theory**. The induced fit theory says that the enzyme active sites are complementary not to the substrates *per se* but to the transition states of reactions they catalyse (Figure 6.4).

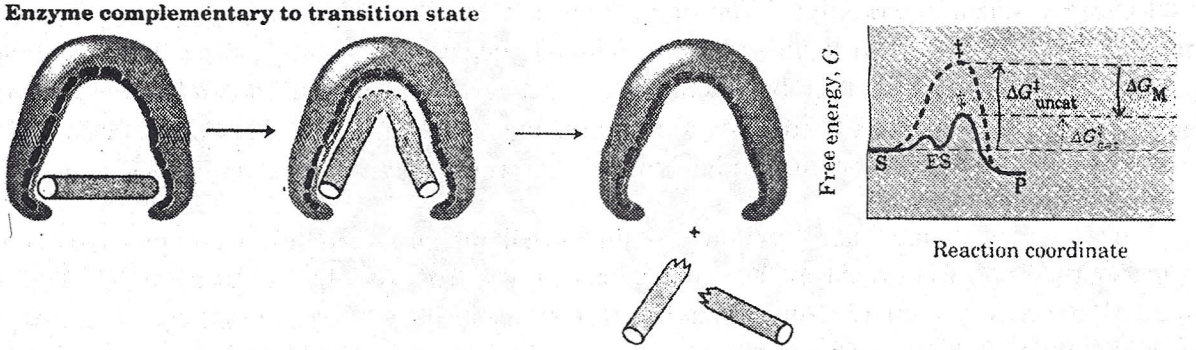


Figure 6.4 An imaginary enzyme showing induced-fit model of the interaction of substrates and enzymes with corresponding reaction coordinate. Here the active site of an enzyme is complementary to the reaction transition state.

This hypothesis says that some weak interactions are involved in the formation of enzyme substrate complex. After that the boud substrate is elevated to higher energy level (transition-state) with the help of maximum possible number of weak interaction takes place between the substrate and the enzyme. The energy released by the formation of these interactions decreases activation energy with following rate enhancements.

6.7 Enzyme kinetics : Michaelis constant (K_m)

Enzyme activity can be influenced by a variety of factors, the major one being the substrate concentration. The rate of enzyme catalysis V varies with the substrate concentration [S] in a manner as shown in Figure 6.5 V is defined as the number of product molecules formed per second. At any given point in an enzyme catalysed reaction, the enzyme exists in two forms, the free or uncombined form E and the combined form ES. At very low substrate concentrations, an enzyme makes product slowly because the frequency of collision between reactants is too slow. Here, the rate is proportional to substrate concentration (Point A in the figure 6.5). The velocity increases as the substrate concentration is increased upto to a point where the enzyme is said to be saturated with its substrate (Point C in the figure 6.5). That is at high substrate concentration all enzyme molecules are present in ES form and working at maximal velocity (V_{max}). At this condition further increase in substrate concentration have no effect on rate. After the ES complex breaks down to yield the product, the enzyme is free to catalyse another reaction. A hyperbolic curve is obtained when the initial velocity of an enzymatic reaction is plotted against substrate concentration (Figure 6.5).

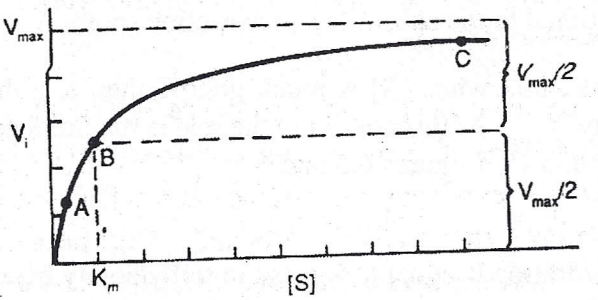


Figure 6.5 Effect of substrate concentration on the velocity of an enzyme-catalyzed reaction.

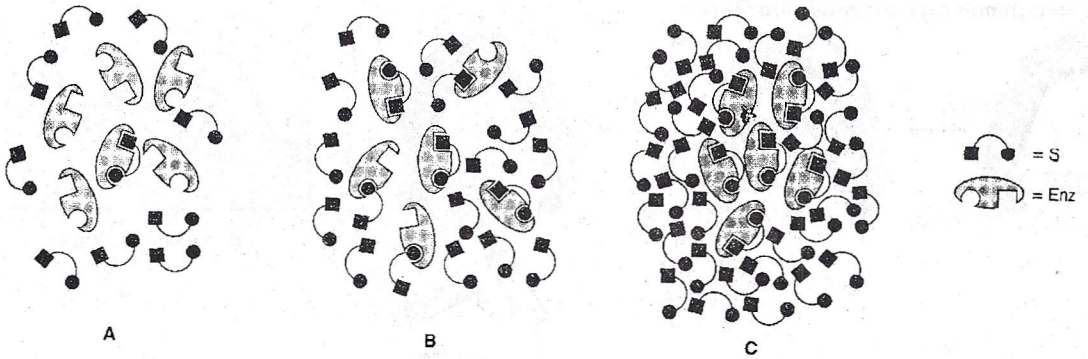


Figure 6.6 Representation of an enzyme at low (A), at high (C), and at the K_m concentration of substrate (B). Points A,B and C correspond to those of figure 6.5.

In order to explain the hyperbolic shpae of the enzymatic reaction curve, Leonor Michaelis annd Maud Menten in 1913 derived a mathematical equation called **Michaelis – Menten equation** on the basis of two basic observations found in the formation and break down of ES. According to their first observation, the enzyme first combines with its substrate to form an enzyme substrate complex in a relatively fast reversible step.

$$E + S \rightarrow ES$$

The breakdown of ES complex to form the product and the free enzyme takes place in a slower second step.

$$ES \rightarrow E + P$$

In this model the rate of the overall enzyme reaction depends on the rate at which ES breaks down to form the product. On the basis of this basic hypothesis, Michaelis and Menten proposed an equation

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

which accounts for the kinetic data given in Figure 6.6 and can be interpreted as follows :

1. At very low substrate concentration, when (s) is much less than K_m, the initial velocity becomes,

$$V = V_{max} [S] / K_m$$

That is the rate is directly proportional to the substrate concentration (point A in figures 6.5 and 6.6).

2. At high substrate concentration, when [S] is much greater than K_m, the initial velocity, V, becomes maximal velocity, V_{max}. At this condition the rate is maximal and is independent of substrate concentration (point C in figures 6.5 and 6.6).

$$V = V_{max}$$

3. When the substrate concentration is equal to K_m, the initial velocity becomes half the maximal velocity (point B in figures 6.5. and 6.6).

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

The substrate concentration that produces half maximal velocity is called the K_m value or **Michaelis constant**. It has the dimensions of molar concentration. This value is used as a measure of apparent affinity of an enzyme for its substrate. The lower the K_m value, the lower the substrate concentration at which an enzyme catalyses its reaction. Typically the values of K_m lies in the range of 10^{-2} to 10^{-5}. moles/litre for various enzymes. The K_m value also gives an idea regarding the type of inhibition of an enzyme caused by an inhibitor.

6.8 Summary

Enzymes are biocatalysts that catalyse specific reactions. They made up of a protein component, the apoenzyme, and often a nonprotein cofactor that may be a prosthetic group, a coenzyme, or a metal activator. Enzymes are known to be absolutely stereospecific, both in binding substrates and catalyzing reactions.

IUB system classifies enzymes systematically. In this system each enzyme contains a recommended name, systematic name, and classification number, which is indicative of the type of reaction catalyzed by the enzyme.

Enzymes speed chemical reactions by binding substrates at their acive sites and lower the activation energies.. They use binding energy derived from weak interactions in lowering activation energies. The active site of an enzyme is complementary to reaction transition state.

The rate of an enzyme – catalyzed reaction increases with substrate concentration at low substrate levels and reaches a plateau, the maximum velocity (V_{max}) at saturating substrate concentrations. The Michaelis constant (K_m) is the substrate concentration that the enzyme requires to achieve half maximal velocity.

6.1. Model Questions

1. Write a critical account on the nature and classification of enzymes.
2. Describe the mode of enzyme action.
3. Write short notes on :
   (a) K_m value
   (b) Activation energy
   (c) Chemical nature of enzymes
   (d) Enzyme specificity

6.2. Reference Books

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Dr. G.ROSAIAH

Lesson – 7

PHOTOSYNTHESIS – LIGHT REACTIONS

7.0 Objective

In this lesson, the principal pigments involved in photosynthesis, the structure and biochemical compartmentation of the chloroplast, red drop and Emerson effect, the photosynthetic electron transport chain, its organization in thylakoid membrane, and its role in generating NADPH and ATP are described.

7.1 Introduction

Life on earth depends on energy derived from the Sun. Photosynthesis is the only known process of biological importance that can harvest this energy. Literally, the term photosynthesis means "synthesis using light". Green plants, cyanobacteria and pigment containing prokaryotes carry out this process. They are called **photoautotrophs**. Photoautotrophs can use light as energy source and CO_2 as carbon source for the synthesis of carbohydrates. Photoautotrophs contain a photosynthetic apparatus that enables them to absorb light energy and package it as a bond energy in the form of ATP and NADPH. The energy of these compounds is used to drive the reactions that are involved in the fixation of carbon dioxide into carbohydrates. The carbohydrates, in turn provide cellular source of

energy and of starting materials for the synthesis of proteins, lipids, nucleic acids and other cellular constituents not only in the photoautotrophs that produce them but also in nonphotosynthetic organisms that directly or indirectly consume photosynthetic organisms. In addition, a large fraction of the earth's energy resource such as coal, oil and gas (fossil fuels) is due to the activity of photosynthesis in ancient times. It has been estimated that photosynthesis annually fixes more than 10^{11} tons of carbon which represents the storage of over 10^{18} kJ of energy. Over half of this photosynthetic CO_2 fixation on earth is carried out by microorganisms.

Photosynthesis as a whole is divided into **light reactions** and **dark reactions**. In the light reactions, light energy is trapped and converted to chemical energy. This energy is then used to fix CO_2 in the dark reactions (Figure 7.1).

$$6CO_2 + 12\,H_2O \xrightarrow[\text{Chlorophyll}]{\text{Light}} C_6H_{12}O_6 + 6O_2 + 6H_2O$$

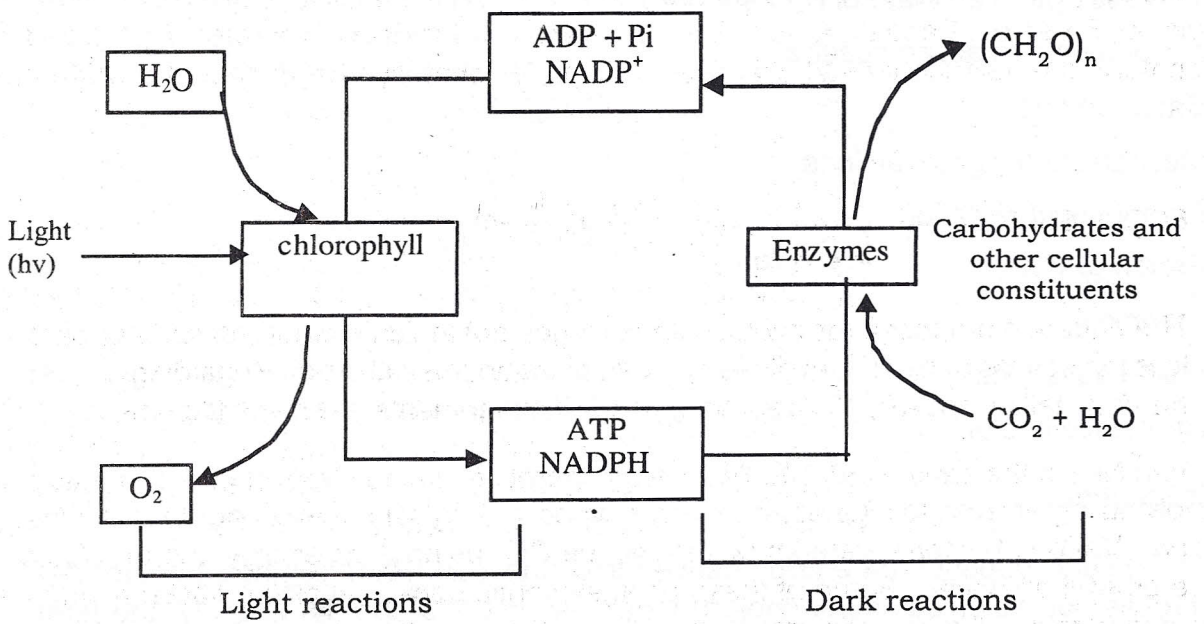


Figure 7.1 Plant photosynthesis. The light and dark reactions together make up photosynthesis. The energy flows from light to high energy intermediate compounds (ATP and NADPH) and then to long term energy bonds connecting carbon atoms of organic molecules.

7.2 Historical Background

Establishing the overall chemical equation of photosynthesis required several hundred years and contributions by many scientists. The beginning of understanding of photosynthesis goes back to the 1970's when composition of air by Lavoisier (1774) in France and discovery of O_2 by Joseph Priestly (1771) were made. In 1771, Joseph Priestly observed that a spring of mint growing in air in which a candle had burned out improved the air so that another candle could burn. He had discovered oxygen evolution by plants. A Dutchman, Jan Ingenhousz, documented the essential role of light in photosynthesis in 1779. Later, after three years a Swiss clergyman, Jean Senebier (1782) reported that on exposure of green plants to sunlight, CO_2 is absorbed by leaves and O_2 is liberated. What was the fate of the carbon in the CO_2 ? Ingenhousz (1796) replied this question and reported that it was derived from CO_2 during photosynthesis. In 1804, Nicholas de Saussure, reported that water plays an important role and thus light acts on both CO_2 and H_2O. In 1818, Pelletier and Caventou identified chlorophyll as the green pigment of plant tissues and by 1837 chlorophyll was shown to the chloroplast pigment. Later on, a German Physician, Robert Mayer (1845) opined that light when absorbed by plants is changed to chemical energy in the process of photosynthesis. Sachs (1862) observed starch grains inside chloroplasts during photosynthesis, since then they are regarded to be the sites of photosynthesis. Starch is used up when the leaves are kept in darkness. The chemical nature of photosynthesis was provided by the 19th century workers including Blackman, Hill, Warburg, Calvin, Robinowitch, Govindjee, Emerson, Bendall, Arnon, Amesz, Wolken and their co- workers. By the end of 19th century, the balance overall chemical reaction for photosynthesis could be written as follows:

$$6CO_2 + 6H_2O \xrightarrow[\text{Chloroplast}]{\text{Light}} C_6H_{12}O_6 + 6O_2$$

The equation proposed for photosynthesis does not indicate whether water or carbon dioxide is the source of oxygen evolved in green plant photosynthesis. Regarding this problem, the Van Neils discovery, Hill reaction, and ^{18}O experiments are more important.

Van Neil in the early 1930s studied the comparative biochemistry of photosynthesis in O_2 evolving organisms (oxygenic) and non oxygen (anoxygenic) evolving photosynthetic bacteria. Various bacteria were known to reduce CO_2 using light energy and an electron source other than water. Some of these photosynthetic non-O_2 evolving bacteria such as green and purple sulfur bacteria can utilize H_2S as a source of electron in the assimilation of CO_2 and produce sulfur as a byproduct, whereas some other bacteria, namely purple non-sulfur bacteria use organic acids like succinate as an electron source and produce fumarate as a byproduct. By comparison, algal and higher plant photosynthesis requires the presence of H_2O as an electron source. Molecular oxygen is one of the products of this reaction. The following equations illustrate the two types of photosynthesis.

$$2H_2S + CO_2 \xrightarrow[\text{light}]{\text{Green and purple sulfur bacteria}} (CH_2O) + 2S + H_2O$$

$$2H_2O + CO_2 \xrightarrow[\text{light}]{\text{Algae and higher plants}} (CH_2O) + O_2 + H_2O$$

The apparent similarity between bacterial and higher plant photosynthesis prompted Van Neil to propose a general equation for photosynthesis.

$$2H_2A + CO_2 \xrightarrow[\text{pigments}]{\text{light}} (CH_2O) + 2A + H_2O$$

Where H_2A is a oxidizable substrate, which in the case of algae and higher plants is water, and in bacteria it may be H_2S or other inorganic or organic substance and A is a byproduct (O_2 in higher plants and sulfur in purple and green sulfur bacteria). According to Van Neils hypothesis, the O_2 evolved in higher plant photosynthesis comes from H_2O and not from CO_2, and the actual assimilation of CO_2 is not light dependent. The photochemical act in this case provides the energy necessary to transfer the hydrogen needed for the reductive steps in CO_2 assimilation.

In 1939 Robert Hill conducted experiments with isolated chloroplasts and found that isolated chloroplasts can evolve oxygen in the presence of light, water and suitable hydrogen acceptor. He noticed salt of ferric iron (ferrioxalate, ferricyanide) to be satisfactory hydrogen acceptors. With ferricyanide, the reaction may be written as

$$2H_2O + 4\,[Fe(CN)_6]^{3-} \xrightarrow[\text{chloroplast}]{\text{light}} 4\,[Fe(CN)_6]^{4-} + 4H^+ + O_2$$

The evolution of O_2 in this experiment occurs in the absence of CO_2, indicating that H_2O being the sole source of O_2 evolved in photosynthesis. This light driven split up of water in the absence of CO_2 fixation became known as **Hill reaction**.

A much more direct line of evidence regarding the evolution of O_2 in the photosynthesis of higher plants is given by Samuel Ruben and his associates in 1941 through their ^{18}O studies on *Chlorella.* If photosynthesis proceeds in the presence of $H_2{}^{18}O$ and normal CO_2, molecular oxygen containing the heavy isotope is evolved.

$$2H_2{}^{18}O + CO_2 \xrightarrow[\text{Chloroplasts}]{\text{light}} {}^{18}O_2 + CH_2O + H_2O$$

In contrast, if photosynthesis takes place in the presence of normal H_2O and $C^{18}O_2$, normal molecular oxygen is evolved.

$$2H_2O + C^{18}O_2 \xrightarrow[\text{Chloroplasts}]{\text{light}} O_2 + (CH_2{}^{18}O_2) + H_2{}^{18}O$$

From this discussion we can assume with reasonable certainity that water provides the hydrogen necessary for the reductive steps leading to the assimilation of CO_2.

7.3 Structure of the Photosynthetic Apparatus

In various groups of photoautotrophs, photosynthetic apparatuses are compartmentalised in discrete highly specialized organelles called **plastids**. The photosynthetic plastids of prokaryotic cells found in bacteria and blugreen algae are represented by isolated and freely lying lamellae in the protoplasm. Schachman, Pradec and Stamier (1952) designated these membranous structures as **chromatophores**. Cyanobacterial photosynthetic apparatus is made up of concentrically arranged circular layers of thylakoid membranes lying freely in the protoplasm without any distinction of grana and stroma regions (Figure 7.2).

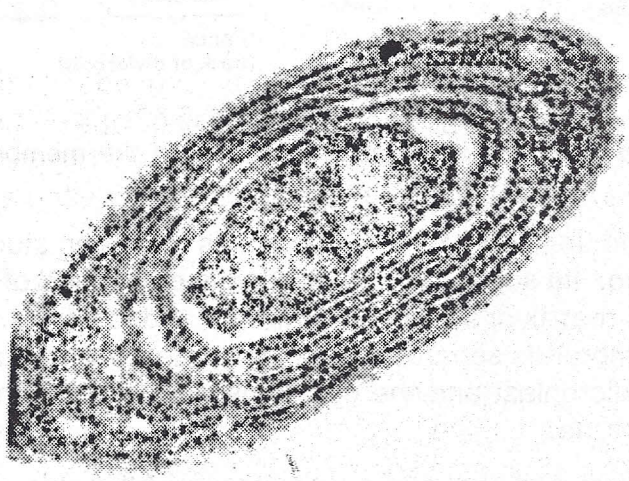


Figure 7.2 Cyanobacterial thylakoids and phycobilisomes. *Synechococcus lividus* with an extensive thylakoid system. The phycobilisomes lining these thylakoids are clearly visible as granules.

In other algae, the photosynthetic lamellae are found closely arranged running parallal to each other but still there is no distinction of grana and stroma regions. These plastids are given the general name **chromoplasts**. In higher plants and bryophytes chlorophyll containing photosynthetic plastids are called **chloroplasts**. A chloroplast is an advanced, well organized and complicated photosynthetic apparatus.

7.4 The Chloroplast

In photosynthetic eukaryotes, photosynthesis takes place in the subcellular organelle known as the chloroplast. A typical higher plant chloroplast is discoid in shape with a diameter of 3 to 10 μm (Figure 7.3). They are located in the cytosol of the cell. The number of chloroplasts in a mesophyll cell is typically in the range of 20 to 60, although values of several hundred have been reported for some species. Furthe, the chloroplasts in palisade mesophyll cells are generally longer and more numerous than in the spongy mesophyll cells.

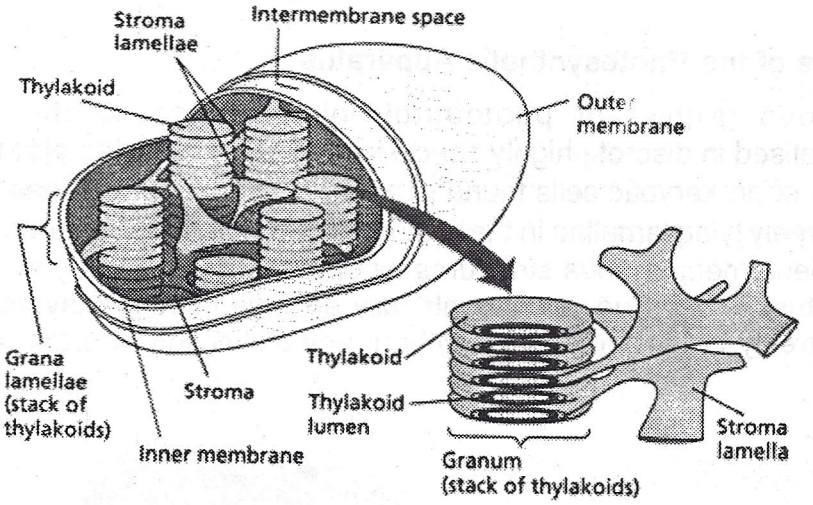


Figure 7.3 Schematic picture of the overall organization of the membranes in the chloroplast of higher plants.

The structure and development of chloroplasts has been studied largely with the help of electron microscope. Its structural organization is composed of four regions or compartments: **the envelope**, **matrix** or **stroma**, **thylakoids** and the **lumen**. The envelope consists of outer and innermembranes separated by an **intermembrane space** of 10 nm. The ionic traffic between the chloroplast and the cytosol is regulated by the selectively permeable inner envelope membrane.

The inside unstructured background substance of the chloroplast is called **matrix** or **stroma**. The stroma contains all of the enzymes responsible for photosynthetic carbon reduction, including **ribulose-1, 5-bisphosphate carboxylase/oxygenase** or **Rubisco**. This protein accounts for half of the total chloroplast protein. Stroma also contains enzymes for a variety of other metabolic pathways as well as DNA, RNA, and ribosomes necessary to produce some of the chloroplast's own proteins.

Stroma is embedded by a complex system of internal membranes known as **thylakoids**. Most of the thylakoids appears to be very closely associated with each other. These stacked thylakoid membranes are known as **grana lamellae** (each stack is called granum), while the exposed membranes in which stacking is absent are known as **stroma lamellae**.

The thylakoid membranes contain the chlorophyll and carotenoid pigments and are the site of the light dependent energy conserving reaction of photosynthesis. These thylakoids are lipoproteinaceous bilayer membranes. They contain lipids and proteins. Phospholipids, galactolipids and sulfolipids are major lipids in thylakoid membranes. These thylakoid membrane lipids contain a high proportion of highly unsaturated fatty acids, the major one being — linolenic acid which can comprise as much as 90 percent of the total fatty acid content. Because of this, the thylakoid membranes maintain a high degree of fluidity, which is essential for efficient photochemical functioning of the membranes.

The proteins of thylakoid membranes in association with pigments are organized into five major intrinsic and extrinsic pigment protein complexes. They are designated as (1) PS II- Pigment protein complex, (2) Cytochrome b_6f complex, (3) PS I-pigment protein complex, (4) light harvesting chlorophyll a/b complex, and (5) ATP synthase complex. The first three complexes are involved in light driven electron and proton transport while fourth complex acts solely as a light harvesting antenna and has no photochemical activity. ATP synthase of thylakoids catalyzes the synthesis of ATP from ADP and Pi during photosynthetic electron transport.

The PS II-Pigment protein complex, along with its antenna chlorophylls and associated electron transport proteins, is located predominantly in the grana lamellae (stacked regions, Figure 7.4). The PS I -Pigment protein complex and its associated antenna pigments, electron transfer proteins, and ATP synthase complex, are found almost exclusively in the stroma lamellae (unstacked grana regions) and at the edges of the grana lamellae. The cytochrome b_6f complex that connects the photosystems is evenly distributed. This spatial separation between photosystems I and II indicates that a strict one-to-one stoichiometry between the two photosystems is not required.

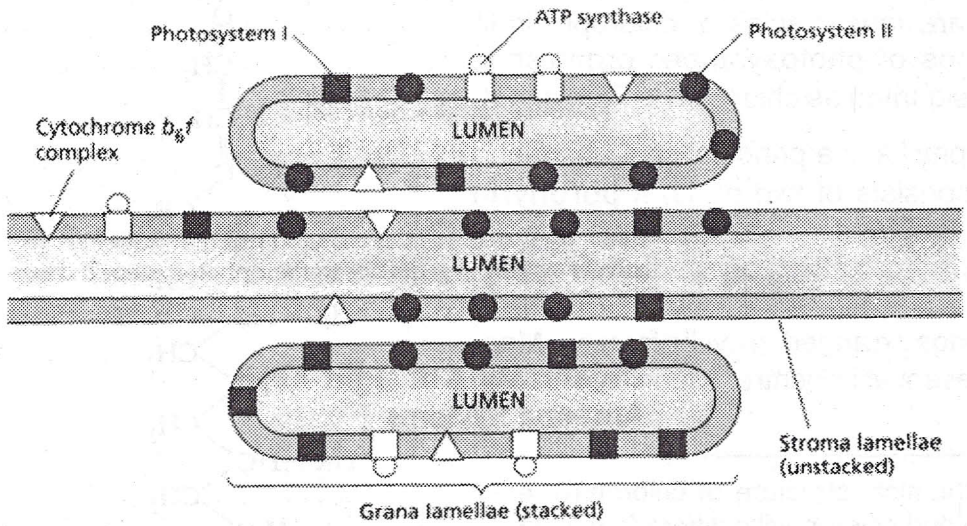


Figure 7.4 Organization of the pigment – protein complexes of the thylakoid membrane. Photosystem II is located predominantly in the stacked regions of the thylakoid membrane; PS I and ATP synthase are found in the unstacked regions protruding into the stroma. Cytochrome b_6f complexes are evenly distributed.

The interior space of the thylakoid is known as the **lumen**. The lumen is the site of water oxidation and, consequently, the source of oxygen evolved in photosynthesis. It is also a place of protons storage during photosynthetic electron transport and these protons are used to drive ATP synthesis.

Chloroplasts also contain starch grains, which represent stored photosynthate, and lipid droplets, called **plastoglobuli**. Plastoglobuli appear to function primarily as lipid storage bodies and may contain particularly large amounts of the electron carrier plastoquinone. Chloroplasts of a aging and senescing leaves may contain an iron binding protein called **phytoferritin**.

7.5 Pigments

Photosynthesis is a photobiological phenomenon that requires the participation of a molecule to absorb light energy. A molecule capable of absorbing light is known as **pigment**. Pigment molecules process the energy and information content of light into a form that can be used by the organism. The principal pigments found in plants are chlorophylls carotenoids and phycobilins.

Chlorophylls

There are four species of chlorophylls in different types of photosynthetic organisms. They are designated as chlorophyll **a**,**b**,**c** and **d**.

Chlorophyll a is a principal light absorbing pigment. It consists of two parts, a **porphyrin head** and a long hydrophobic hydrocarbon tail also called **phytol tail**. A porphyrin is a cyclic tetrapyrrole, made up of four nitrogen containing pyrrole rings arranged in cyclic fashion. Magnesium is present at its centre. A long lipid soluble

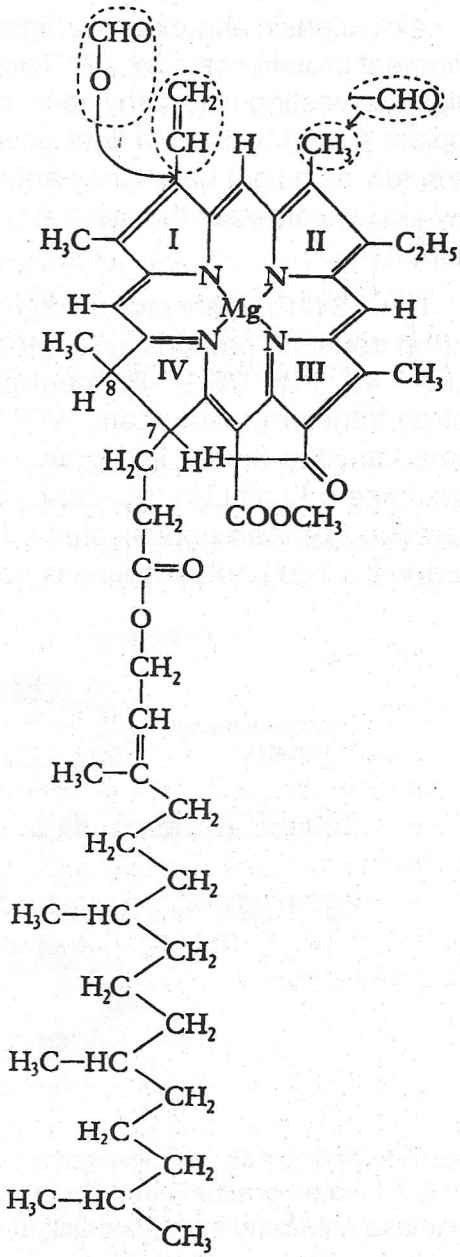


Figure 7.5 Chemical structure of chlorophyll a. Chlorophyll b and chlorophyll c differs from chlorophyll a in having formyl group in place of methyl group, ad-O-CHO group in place of $-CH = CH_2$ respectively shown by dashed lines.

hydrocarbon phytol tail can extend from ring IV of porphyrin head. The chemical structure of *chlorophyll a is shown in Figure 7.5.*

Chlorophyll b differes from chlorophyll a, only in having formyl group (CHO) on ring II in place of methyl group. Chlorophyll b is found in virtually all higher plants and green algae. It is absent in cyanobacteria. The principal difference between chlorophyll a and chlorophyll c which is found in the diatoms, dinoflagellates and brown algae, is that chlorophyll c lacks the phytol tail. Chlorophyll d, found only in the red algae, is similar to chlorophyll a except that (-O-CHO) group replaces the (-$CH=CH_2$) group on ring II.

Chlorophyll a has absorption maxima in the blue (435 nm) and red (663 nm) region of the visible spectrum (Figure 7.6). Chlorophyll does not absorb strongly in the green (490-550nm). The strong absorbance in the blue and red, and transmittance in the green is responsible for the chlorophyll to have characteristic green colour. In thylakoid membranes, chlorophylls are present as water insoluble pigment protein complexes.

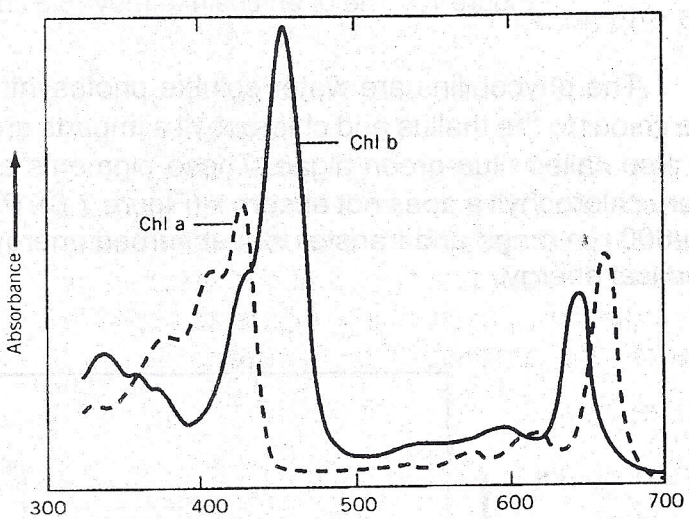


Figure 7.6 Absorption spectra of chlorophyll a (broken line) in comparision with chlorophyll b (solid line) in acetone.

Phycobilins

There are three photosynthetic phycobilins found only in cyanobacteria and red algae. They are **phycoerythrin** (also known as phycoerythrobilin), **phycocyanin** (phycocyanobilin) and **allophycocyanin** (allophycocyanobilin). These pigments differ from chlorophyll a in containing open chain tetrapyrrole with covalently linked protein molecule (Figure 7.7). A pigment that contains protein as an integral part of the molecule is known as a **chromoprotein**. Hence phycobilins are chromoproteins. On the surface of thylakoid membranes, the phycobiliproteins are organized into large macromolecular antenna complexes called **phycobilisomes**.

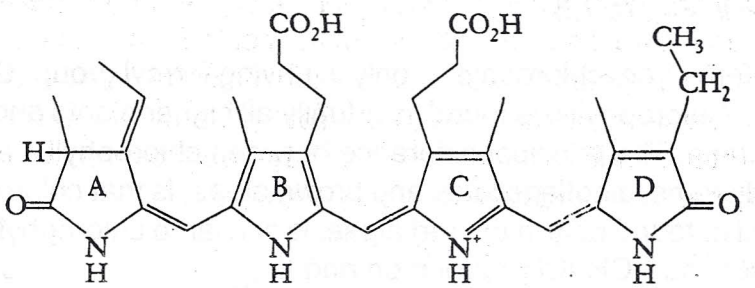


Figure 7.7 The open chain tetrapyrrole chromophore of pycocyanin.

The phycobilins are water soluble photosynthetic accessory pigments. They impart blue colour to the thallus and chlorophyll a imparts green. For this reason, the cyanobacteria are also called blue-green algae. These pigments absorb light energy in the green region where chlorophyll a does not absorb it (Figure 7.8). Phycobilins absorb light energy between 500-600 nm range and transfer that absorbed energy to chlorophyll a for its conversion into chemical energy.

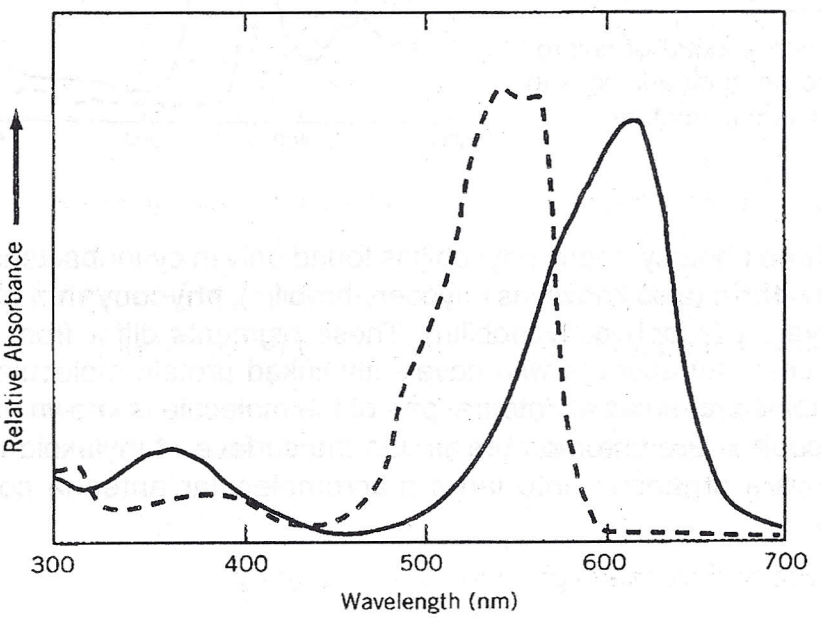


Figure 7.8 Absorption spectra of phycocyanin (solid line) and phycoerythrin (broken line) in dilute buffer. Note that the phycobilins absorb strongly in the 500-600 nm range where chlorophyll absorption is minimal.

Carotenoids

Carotenoids are C_{40} terpenoids. This group of pigments includes **carotenes** and **xanthophylls**. Carotenes are carbon and hydrogen containing orange or red – orange coloured long hydrocarbon pigments. β–Carotene is the major carotenoid in algae and higher plants. α-Carotene is present in minor amounts. Both β-Carotene and α-carotene have a cyclized ends. γ-Carotene found in the bacteria, have only one end cyclized, whereas, lycopene of tomatoes, has both ends open (Figure 7.9).

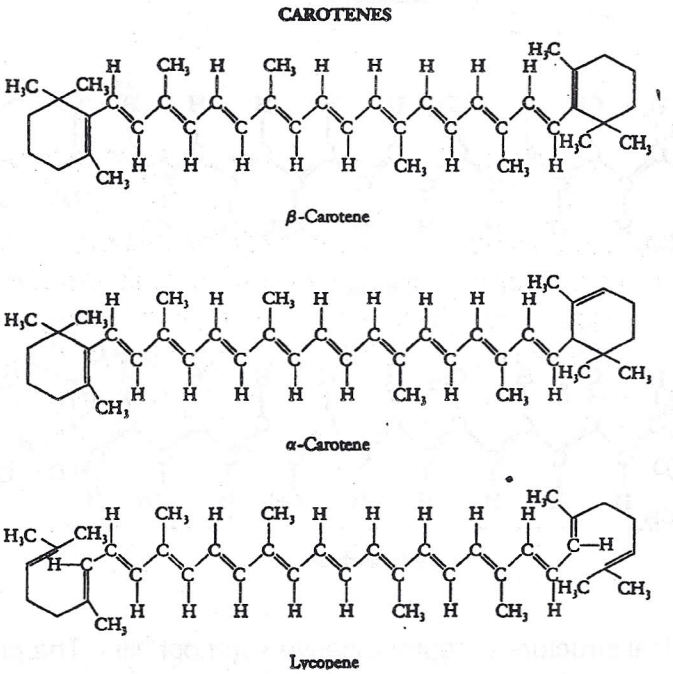


Figure 7.9 The chemical structures of β-carotene, α-carotene and lycopene.

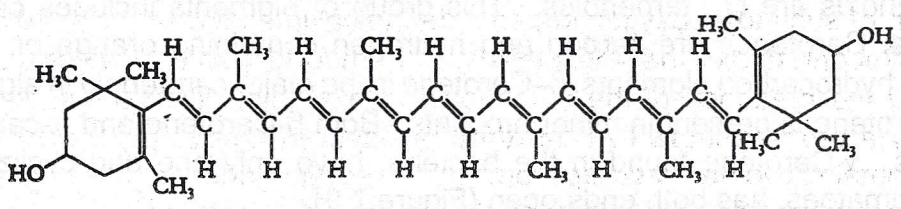
The oxygenated, yellow carotenoids are called xanthophylls. Lutein, zeaxanthin and violaxanthin are principal xanthophylls found in higher plants (Figure 7.10).

Like chlorophylls, carotenoids in the thylakoid membranes are present as water insoluble pigment protein complexes. Carotenoids may serve two principal functions during the course of photosynthesis.

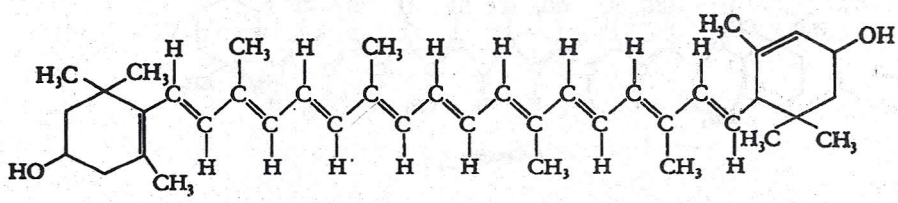
1) They absorb and transfer light energy to chlorophyll a.
2) They protect the photosynthetic system from photooxidative damage.

As an accessory light harvesters, these pigments absorb light strongly between 460 nm and 490 nm (Figure 7.11) and transfer that absorbed energy to chlorophyll a. β–Carotene is the main pigment involved in this function. Xanthophylls on the other hand, are mainly involved in protecting the chlorophyll a against its photooxidative damage.

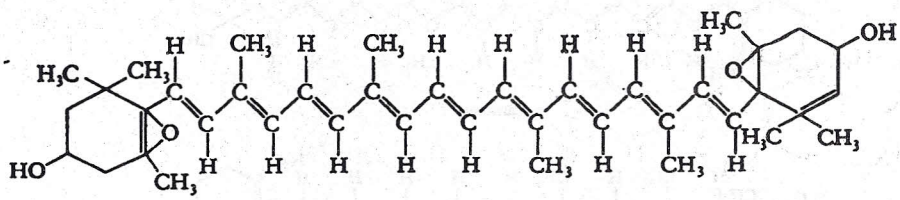
XANTHOPHYLLS



Zeaxanthin

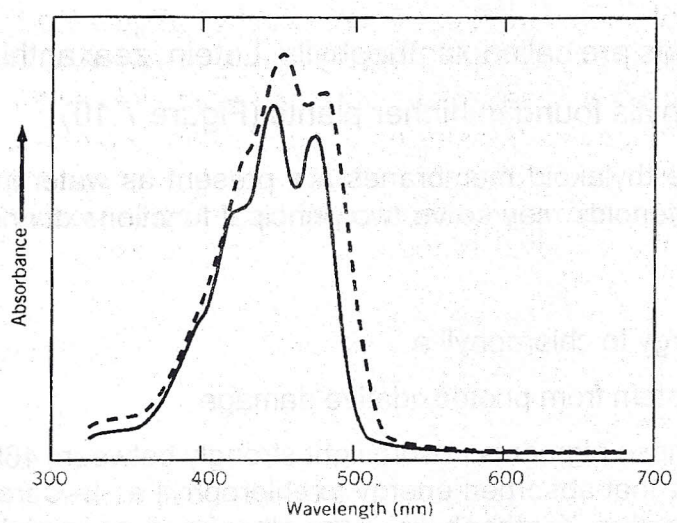


Lutein



Violaxanthin

Figure 7.10 The chemical structure of representative xanthophylls. The principal distinction between the carotenes and the xanthophylls is that xanthophylls contain oxygen and carotenes do not (Figure 7.9).



Photooxidation is a potential problem in all plants. During periods of peak irradiance, plants absorb more energy than they can utilize in the reduction of CO_2. For example, rapidly growing plants may utilize only less than 50 percent of absorbed light, while other species, such as evergreen may utilize as little as 10 percent. Any excess absorbed energy must be dissipated. If not, the reduced products of PS I, particularily ferredoxin, may react with oxygen instead of $NADP^+$, and produce

Figure 7.11 Absorption spectra of α-carotene (solid line) and β-carotene (broken line).

a toxic form of oxygen known as **a superoxide radicle** (O_2^-). It is highly reactive, and with that potential it will oxidize and destroy not only chlorophyll but also organic molecules in the cell. Formation of such toxic oxygen superoxide radical in chloroplasts is prevented by the carotenoids. Recent studies have established an important link between excess energy dissipation and the presence of the xanthophyll zeaxanthin. Zeaxanthin is formed from violaxanthin by a process known as **xanthophyll cycle** (Figure 7.12). Violaxanthin is a diepoxide. It contains two epoxy groups, one on each ring. Under conditions of excess light, violaxanthin is converted to zeaxanthin through the removal of those two oxygens by a

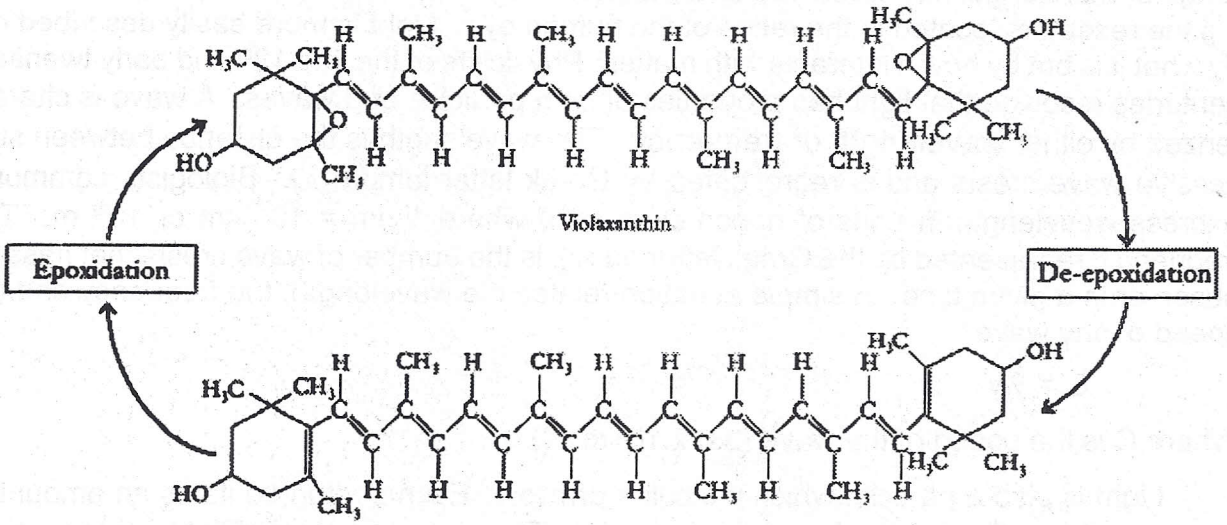


Figure 7.12 The Xanthophyll cycle.

process known as **de-epoxidation**. De-epoxidation is a two step process: removal of the first oxygen generates an intermediate antheraxanthin containing only one epoxy group and removal the second oxygen yields zeaxanthin. De-epoxidation is induced by light, low pH and reduced ascorbate. Zeaxanthin contains increased number of carbon-carbon double bonds and can accept a downhill transfer of energy from excited chlorophyll (Figure 7.13). Under low light, the energy of excited chlorophyll (Chl*) is preferentially transferred to be used in photosynthesis. As irradiance increases, zeaxanthin is formed from violaxanthin and an increasing proportion of excitation energy is transferred to zeaxanthin to be dissipated as heat. The xanthophyll cycle is thus operates as an effective switch, generating zeaxanthin whenever dissipation of excess energy is required and its formation is stopped under conditions of low light when more of the energy is required for photosynthesis.

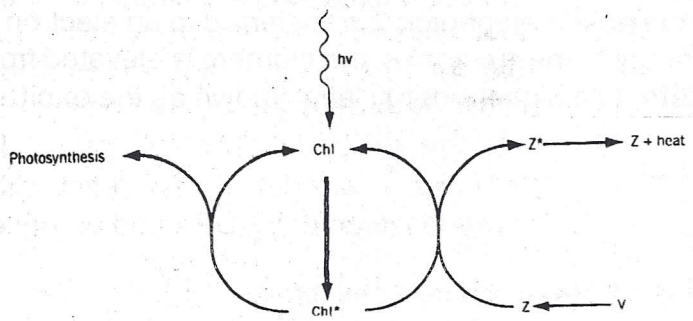


Figure 7.13 Energy dissipation by zeaxanthin.

7.6 The Absorption and Fate of Light Energy

What actually happens when a pigment molecule absorbs light ? This question may be answered by first considering the nature of light.

Nature of light

Light is a form of radiant energy. It can be defined as that portion of the electromagnetic spectrum that causes the physiological sensation of vision in humans or defined by the range of wavelengths between 400 and approximately 700 nanometers capable of stimulating the receptors located in the retina of the human eye. Light is more easily described not by what it is but by how it interacts with matter. Physicists of the late 19th and early twentieth centuries resolved that light has properties of both particles and waves. A wave is characterized by either **wavelength** or **frequency**. The wavelength is the distance between successive wave crests and is represented by Greek letter lamda (λ). Biologists commonly express wavelength in units of nanometers (nm) where 1 nm = 10^{-7} cm or 10^{-9} m. The frequency, represented by the Greek letter nu (ν), is the number of wave crests that pass an observer in a given time. A simple equation relates the wavelength, the frequency and the speed of any wave.

$$C = \lambda\nu$$

Where C is the speed of the wave (3.0×10^8 m s^{-1}).

Light is also a particle, which we call a **photon**. Each photon contains an amount of energy that is called a **quantum** (plural quanta). The energy content of light is not continuous but rather is delivered in these discrete packets, the quanta. The energy (E) of a photon depends on the frequency and wave length of the light according to a relation known as Planck's law:

$$E_q = hc/\lambda = h\nu$$

Where h is Planck's constant (6.626×10^{-34} Js photon $^{-1}$). Accordingly, the quantum energy of radiation is inversely proportional to its wavelength or directly proportional to its frequency. Since both h and c are constants, the energy of a photon is easily calculated for any wavelength of interest. For example, the energy carried by a mole of photons of red light is 181 kJ mol^{-1} and a mole of photons of blue light is 274 kJ mol^{-1}.

Absorption of light

Absorption of light by pigment molecule is a rapid event, occurring within a femtosecond ($=10^{-15}$s). During that time the energy of the absorbed photon is transferred to an electron in the pigment molecule. As a result, the energy of the electron in the pigment is elevated from a low energy level, called the **ground state**, to a higher energy level known as the **excited**, or **singlet state**.

$$Chl + h\nu \longrightarrow Chl^*$$

According to the Einstein. Stark law of photochemical equilance, a single photon can excite only one electron. The number of excited electrons thus equal to the number of photons absorbed. Furthermore, the total energy of a photon is used in exciting the electron; a one-half or one-third photon is not used. This is because that the quanta cannot be subdivided and electron cannot be partially excited. This is the reason why photons of red and blue light are more effective in exciting chlorophylls. These photons contain energy that matches the energy required to raise the energy of the electron to one of the higher allowable energy states. Absorption of blue light excites the chlorophyll to a higher energy state (second excited level) than absorption of red light because the energy of photons is higher when their wave length is shorter.

An excited molecule has a very short life time and in the absence of any chemical reaction with other molecules in its environment, the excess energy of the excited electron may be dissipated in several different ways and return to the ground state (Figure 7.14). The energy of the excited electron (either the first or second excited level) may be emitted as heat and the pigment molecule thereby return to ground state. Frequently, however, a portion of the energy is emitted as thermal energy (heat) and the balance as radiant energy (photon). If this situation occurs, the emitted photon is less energetic than the absorbed photon and of longer wavelength. If this photon reemission occurs within 10^{-9}s, the process is called **fluorescence**. Chlorophyll has a unique fluorescence that is the same regardless of the wavelength (400 to 700 nm) of radiant energy used to excite the molecule. If the energy of the excited molecule is emitted as heat or fluorescence, this energy is unavailable for transformation into chemical work.

Excitation energy may be transferred between pigment molecules by a process known as **inductive resonance** or **radiation less transfer**. This process requires the pigment molecules to be very close together and in this the fluorescent emission band of the donor molecule becomes the absorption band of the receptor molecule. Inductive resonance accounts for much of the transfer of energy between accessory pigments and chlorophylls in the chloroplast.

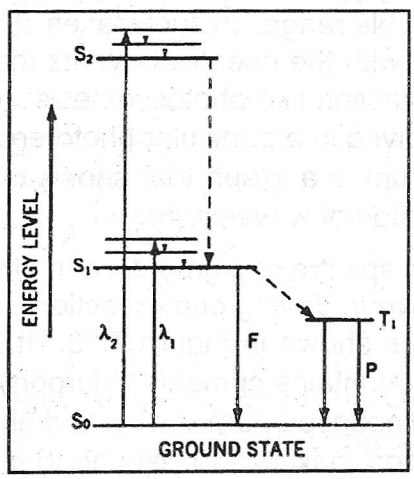


Figure 7.14 The absorption of light by a pigment. A nonexcited pigment is said to be in the ground state S_0). S_1 and S_2 are excited singlet states achieved by absorption (solid arrows) of λ_1 (red) and λ_2 (blue), respectively. Dashed arrow represent radiationless decay through which energy is given up primarily as heat. Flourescence (F) is the emission of light from the lowest excited singlet state. T_1 represents excited triplet state. Energy from the triplet state may be lost by radiationless decay or by delayed emission of light known as phosphorescence (P).

The excited pigment molecule may also revert to another type of excited state, called the **triplet state**. The triplet state differs from the singlet state in the spins of valence electrons. The electron in the triplet state do not emit energy so quickly because, the electron must first reverse spin before dropping to the ground state. In some cases, the triplet state electron persists for as long as 10^{-3}s before energy emission occurs. This long-lived energy emission process is called **phosphorescence**. The longer life time of the triplet state, however is sufficient to allow for photochemical reactions to occur. This could take the form of an oxidation reaction in which the energetic electron is actually given up to an acceptor molecule. When this occurs, the pigment is said to be **photooxidized** and the acceptor molecule becomes reduced. Photooxidation of chlorophyll is the primary photochemical event in the process of photosynthesis.

7.7 Absorption and Action Spectra

An absorption spectrum is a measure of the extent to which a given substance absorbs the light of different wavelengths. It is a function of the relationship between absorption which may be expressed as absorbance (A) or optical density (OD) and wavelength expressed in nanometers. The absorption spectra of chlorophyll a and b as determined with a spectrophotometer was shown in Figure 7.6. The absorption spectra of chlorophylls and accessory pigments provide indirect evidence of the wavelengths of light that are absorbed for the process of photosynthesis. The absorption maxima of these most important photosynthetic pigments should give a hint as to the quality of light most effective in the process of photosynthesis.

In plant or any living organism, a great number of pigments have the ability to absorb radiant energy. In fact, all large organic molecules including proteins and nucleic acids absorb radiations in wavelengths not far removed from the visible range. In such cases, it is difficult to decide which specific pigment is actually coupled with the chemical events that follow light absorption and constitute a photophysiological reaction like photosynthesis. A common procedure in attempting to identify the pigment involved in a particular photoreaction is to determine an **action spectrum**. An action spectrum is a graph that shows the effectiveness of light in a particular process plotted as a function of wavelength.

A comparison of an action spectrum with the absorption spectra of pigments, can provide useful clues to the identity of the pigment that is effective in driving photoreaction. A typical action spectrum for photosynthesis in a green plant is shown in Figure 7.15. It is compared with the absorption spectrum of a leaf extract that contains primarily chlorophyll and some carotenoid. Note that the action spectrum has pronounced peaks in the red and blue regions of the electromagnetic spectrum and these action maxima correspond to the absorption maxima for chlorophyll. The close correspondence between the absorption spectrum of chlorophyll and the action spectrum of photosynthesis strongly supports the notion that chlorophyll is responsible for absorbing the radiant energy used to drive the photosynthesis.

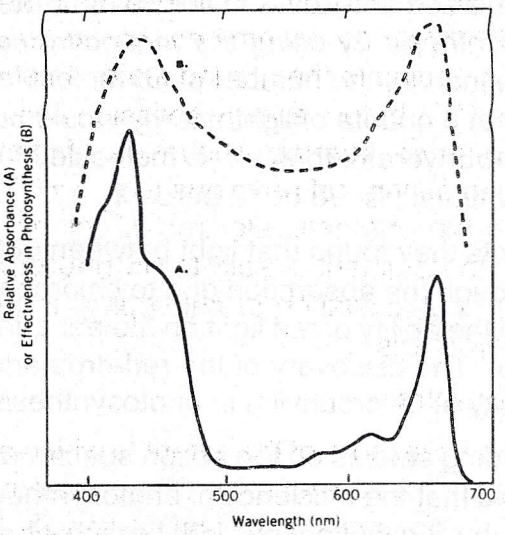


Figure 7.15 A typical action spectrum for photosynthesis (B) compared with the absorption of leaf extract (A). The action spectrum has peaks in the blue and red regions of the spectrum, which correspond with the principal absorption peaks for the pigment in solution.

7.8 Emerson Effect

While studying the photosynthetic role of accessory pigments in algae, several investigators, working independently, observed a curious phenomenon. They found that light absorbed directly by chlorophyll a was less efficient in photosynthesis than light absorbed by the accessory pigments such as phycocyanin in cyanobacteria and both pycocyanin and phycoerythrin in red algae. The absorption and action spectrum of the red alga, *Porphyra nereocystis*, shown in Figure 7.16, clearly illustrate this phenomenon. There is a conspicuous lack of activity in 675 and 680 nm regions, although thallus spectrum shows definite

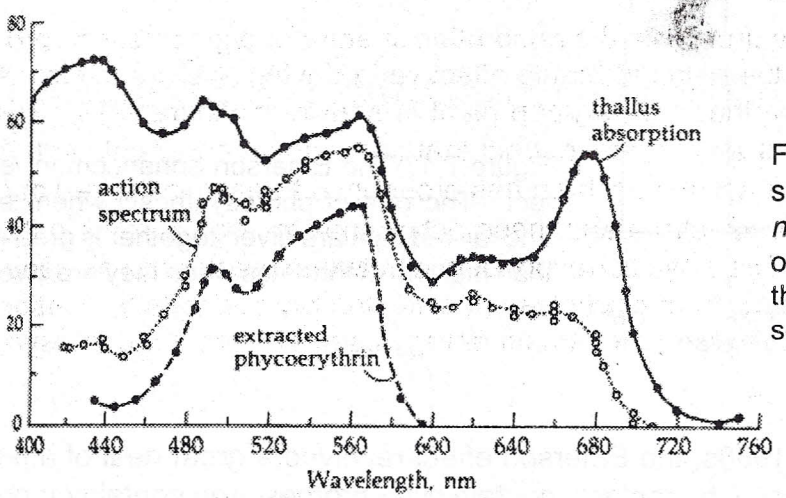


Figure 7.16 The absorption and action spectra of red alga *Porphyra nereocystis*. Note a conspicuous lack of activity at 675 and 680 nm, although thallus spectrum shows definite absorption over that range.

absorption peak over that range. This superiority of the accessory pigments to chlorophyll a was further worked out by Emerson and Lewis in the 1943. Emerson and Lewis worked on the quantum yield of photosynthesis by using the monochromatic light of different wavelengths. Quantum yield is defined as the number of O_2 molecules released for light quanta absorbed. Emerson found that 8 quanta of light energy would be required for the reduction of one molecule of CO_2 to carbohydrate, that is one molecule of O_2 is produced. Thus, the per quantum yield of photosynthesis is 1/8 or 12 percent.

In a series of experiments they found that light between 680 to 720 nm is inefficient in exciting photosynthesis, although the absorption due to chlorophyll in this spectral region is still strong. This deficiency in the ability of red light on the per quantum yield of photosynthesis was termed the **red drop**. The discovery of the red-drop added another chapter to the mystery concerning the activity of chlorophyll a in photosynthesis.

In continuing and extending studies on the action spectra of photosynthesis, Emerson and his co-workers discovered that the efficiency of photosynthesis at wavelengths exceeding 680 nm can be restored by a simultaneous application of a shorter wavelength. The effect of the two superimposed beams of light on the rate of photosynthesis exceeds the sum effect of both beams of light used separately. In other words, illumination a sample with quanta of orange/red and far-red light wavelengths simultaneously give a higher rate of O_2 evolution than the sum of the two effects measured separately (Figure 7.17). This photosynthetic enhancement is referred to as the Emerson enhancement effect, which can be represented as follows

$$E = \frac{[\text{Rate of } O_2 \text{ evolution in combined beams}] - [\text{Rate of } O_2 \text{ evolution at short wavelength alone}]}{\text{Rate of } O_2 \text{ evolution in far-red beam}}$$

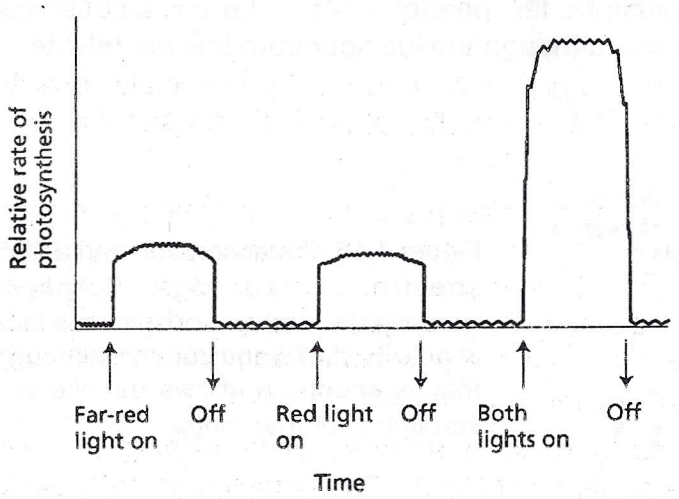


Figure 7.17 The Emerson enhancement effect. The rate of photosynthesis when red and far-red light are given together is greater than the sum of the rates when they are given apart.

In the late 1950s and early 1960s, the Emerson effect received a great deal of attention, particularily by Louis Duysens. Chloroplasts contain cytochromes, iron containing pro-

teins that function as intermediate electron carriers in photosynthesis. Duysens found that when a sample of red alga was illuminated with long wavelength light, the cytochrome became mostly oxidized. If light of a shorter wavelength was also present, the effect was partly reversed. These antagonistic effects can be explained by a mechanism involving two photochemical events: one that tend to oxidize the cytochrome (far-red light) and one that reduces it (green light) Figure 7.18).

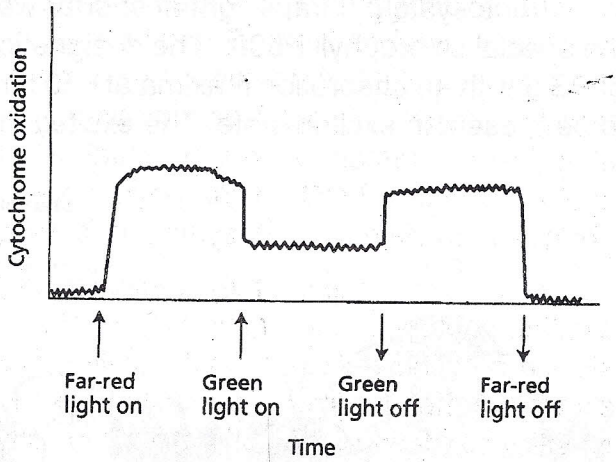


Figure 7.18 Antagonistic effects of light on cytochrome oxidation. Far-red light is very effective in oxidizing the cytochrome f in the chloroplast. If green light is also present, some of the cytochrome becomes reduced. The two wagelengths have opposite effects – hence the term "antagonistic". This experiment was carried out by Duysens on red alga.

We know now that in the red region of the spectrum, one of the photoreactions, known as photosystem I (PS I), absorbs preferentially far-red light of wavelengths greater than 680 nm, while the second, known as photosystem II (PS II), absorbs light of 680nm well and is driven very poorly by far-red light. The wave length dependence explains the enhancement effect and the red drop effect. Another difference between the photosystems is that photosystem I produces a strong reductant, capable of reducing $NADP^+$, and a weak oxidant. PS II produces a very strong oxidant, capable of oxidizing water, and a weaker reductant than the one produced by PS I. This reductant reduces the oxidant produced by PS I, which explains the antagonisitic effect. These properties are shown schematically in the so called z-scheme (Figure 7.21).

7.9 Mechanism of Light Reactions

Photosynthetic light reactions in higher plants are carried out by four major protein complexes: The photosystem II, cytochrome b_6f complex, the photosystem-I and the light harvesting complexes (Figure 7.19).

The PS II reaction center core in higher plant chloroplasts is a heterodimeric protein with D_1 and D_2 as polypeptides. This heterodimer binds all the redox components necessary for primary photochemical reaction. These include reaction center light processing special chlorophyll a called P680, primary electron acceptor pheophytin, quinones and electron donor Z. This core in thylakoids in turn occurs along with the associated antenna components CP43 and CP47, cytochrome-b*559*, and the oxygen evolving complex (OEC).

In addition, each photosystem II is surrounded by a chloropyll containing complex known as light – harvesting complex II, designated as LHC II. This complex contains 50 to 60 percent of the total chlorophyll and with a chlorophyll a/b ratio of about 1.2. It also contains most of the xanthophylls. LHC II functions as a extended antenna system for harvesting additional light energy.

Photosystem II traps light at shorter wavelength (≤ 680 nm) and transfer its energy to the special chlorophyll P680. The designation P680 reveals that it is a special chlorophyll-a of PS II with an absorption maxima at 680 nm. Light absorption causes this special pigment to be present in excited state. The excited form of P680 is designed as P680*. The excited

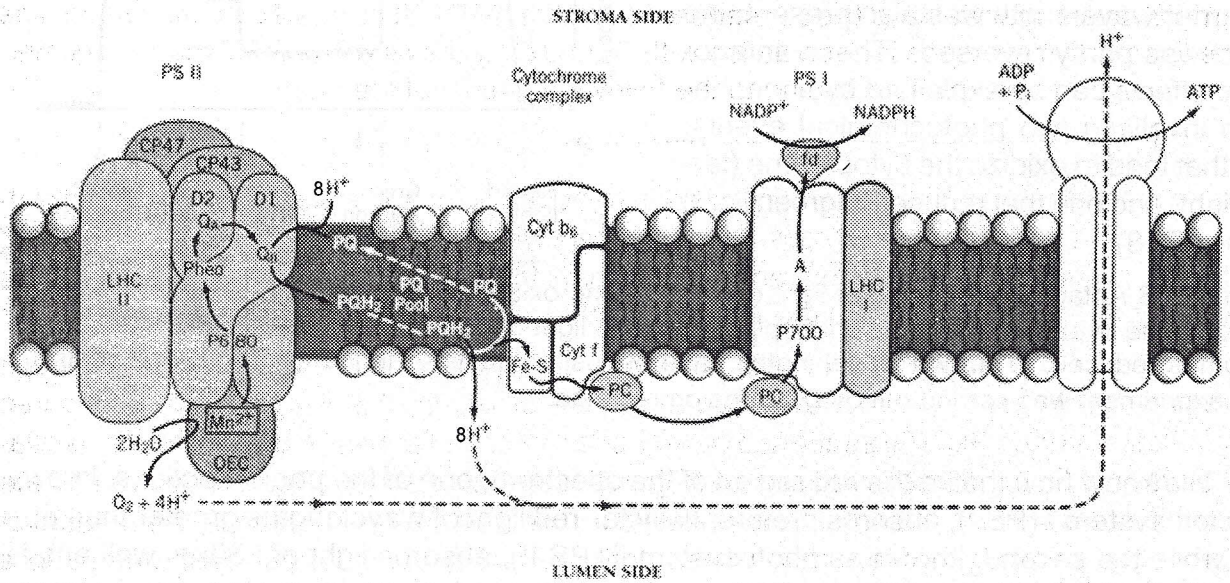


Figure 7.19 The transfer of electrons and protons in the thylakoid membrane is carried out vertically by four protein complexes. Water is oxidized and protons are released in the lumen by PS II. PS I reduces $NADP^+$ to NADPH in the stroma, via the action of ferredoxin (Fd) and the flavoprotein ferredoxin – NADP oxidoreductase (Fp). Protons are also translocated into the lumen by the action of the cytochrome b_6f complex and contribute electrochemical proton gradient. These protons must then diffuse to the ATP synthase enzyme, where their diffusion down the electrochemical gradient is used to synthesize ATP in the cytosol.

*P680** is rapidly photooxidised (within picoseconds, 10^{-12} sec) by donating its excited or high energy electron to pheophytin (Pheo). Pheophytin is a primary electron acceptor in PS II. It is a form of chlorophyll a in which the magnesium ion has been replaced by two hydrogens. This initial photochemical act results in the formation of $P680^+$ and $Pheo^-$. This reaction represents the conversion of light energy into chemical energy. Reduced pheophytin quickly passes its electron to an other electron acceptor called Q_A and then down the electron transport chain to P700 via plastoquinone, cytochrome b_6f complex and plastocyanin.

Plastoquinone is a lipid soluble mobile electron carrier. It shuttles electrons in between PS II and cytochrome b_6f complex. Cytochrome b_6f complex is a multiprotein membrane spanning complex. It is composed of cytochrome b_6 (Cyt b_6), cytochrome f and rieske iron-sulfur protein [Fe – S]. Plastocyanin (PC) is a copper-binding small protein present on the luminal surface of thylakoid membrane. It is an electron carrier between cyt b_6f complex and P700.

Oxidised P680 is a very strong oxidant and is able to extract electrons from the oxidation of water through oxygen evolving complex (OEC). As a result P680 is reduced and is again available for next round of excitation.

Oxygen evolving complex is a manganese containing multiprotein. It is responsible for the oxidation of water and the consequent evolution of O_2. It is located on the lumen side of the thylakoid membrane in association with D_1 and D_2 proteins of the PS II reaction center. Water is oxidized by PS II according to the following chemical reaction :

$$2H_2O \longrightarrow O_2 + 4e^- + 4\ H^+$$

This equation indicates that four electrons are removed from two water molecules, generating an O_2 molecule and four H^+ ions. Water is very stable molecule. Oxidation of water to form molecular O_2 is very difficult, and the photosynthetic oxygen evolving complex is the only known biochemical system that carries out this reaction.

The chemical mechanism of photosynthetic water oxidation is not yet known, although there is a great deal of indirect evidence about the process. If a sample of dark adapted chloroplasts is exposed to a sequence of very brief, intense flashes, a characteristic oscillatory pattern of O_2 production is observed (Figure 7.20). Little or no oxygen is evolved on the first two flashes and the maximal O_2 is released on the third flash. Thereafter, the amount of O_2 produced peaks with every fourth flash until eventually the yield per flash damps to a constant value. This remarkable result was first observed by Pierre Joliot in the 1960 and later on by Kok and coworkers in 1970. The periodicity of O_2 evolution observed by these workers indicates that each O_2 evolving complex cycles through five different states called S state mechanism, consists of S_0 to S_4. Each of the transitions between S_0 and S_4 is a photon driven redox reaction; that from S_4 and S_0 results in the release of O_2. The observation that O_2 evolution peaks at the third rather than the fourth flash in the beginning indicates that the OEC's resting state is predominantly S_1 rather than S_0. The oscillations gradually damp out because a small fraction of the reaction centers fail to be excited or become doubly excited by a given flash of light so that the reaction centers eventually lose synchrony.

The S states represent successively oxidized states of an Mn-containing oxygen – evolving complex, which binds two H_2O molecules so as to facilitate O_2 formation.

Next a light driven charge formation similar to that involving P680, has also occurred in the reaction center PS I. PS I contains P700 chlorophyll a as a special pigment molecule on its polypeptides. The P700 absorbs light at a wavelength of 700 nm. In addition, the complex also contains chlorophyll a, primary electron acceptor (A) and several iron-sulfur (Fe-S) proteins. This core in turn is surrounded by light harvesting complex I(LHC I). It contains 20 percent of the total thylakoid chlorophyll with chlorophyll a/b ratio of about 4/1. When light above 680nm is harvested and transferred to P700, the P700 is excited. Excited P700 then donates its high energy electron to primary acceptor (A) and then to iron-sulfur proteins. Finally, the electron is used to reduce ferredoxin on the cytoplasmic side of the membrane. In the presence of an enzyme ferredoxin – $NADP^+$ oxidoreductase, the electron of ferredoxin is transferred to $NADP^+$ to produce NADPH. The electron deficiency in $P700^+$ is finally

fulfilled by withdrawing an electron from reduced plastocyanin. In this electron transport system the light driven electrons move from water to $NADP^+$ in a non-cyclic route through the two separate photosystems and the intervening cytochrome complex. This kind of electron transport is termed as **non-cyclic electron transport**. This is represented in the form of Z scheme (Figure 7.21).

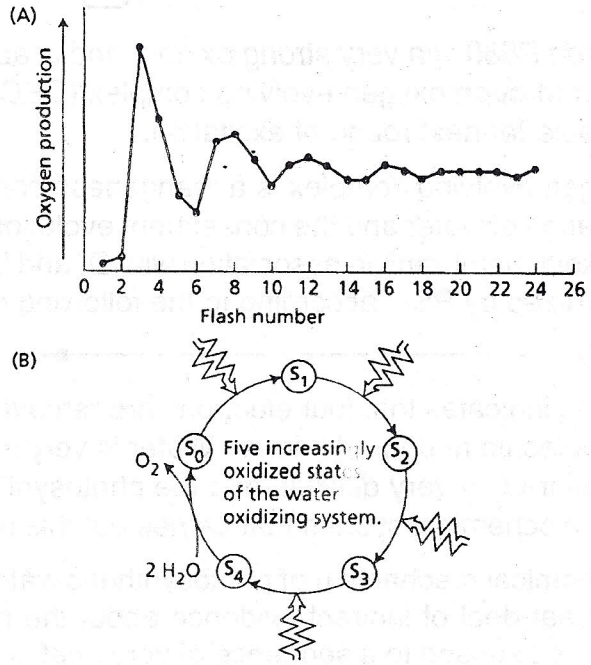


Figure 7.20 The pattern of O_2 evolution in flashing light (A) and the S state mechanism (B) for O_2 evolution in a sample of dark – adapted chloroplasts.

In addition to non-cyclic electron transport, the like higher plants, under conditions of high ATP demand or low light intensities also employ another mode of electron transport called **cyclic electron transport**. In the cyclic pathway (Figure 7.22) the electron moves in a cyclic route through a series of electron carriers and back to the oxidized P700. In this case ferredoxin transfers the electron back to PQ rather than to $NADP^+$. The electron then returns to P700 passing through the cytochrome complex and plastocyanin.

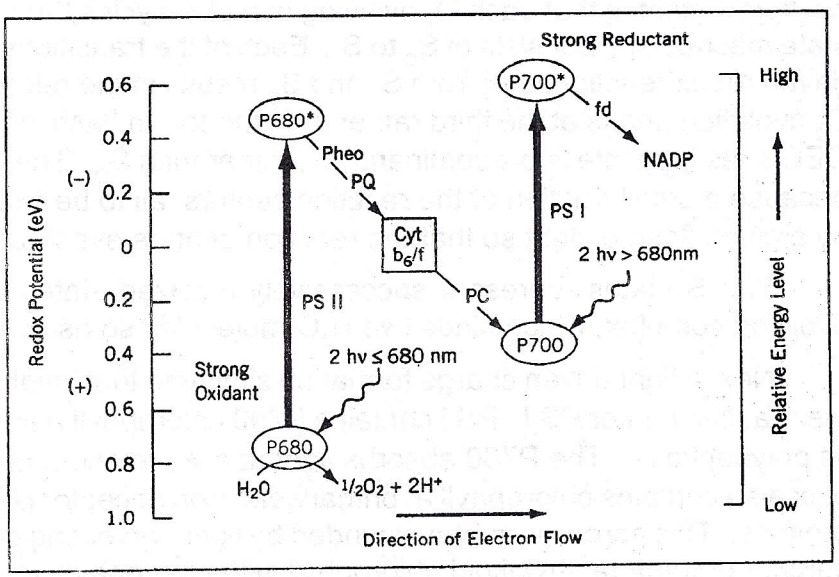


Figure 7.21 The Z scheme for photosynthetic electron transport. The redox components are placed at their approximate midpoint redox potentials. The vertical direction indicates a change in energy level. The horizontal direction indicates electron flow.

In photosynthetic non-cyclic electron transport light energy is absorbed at two points, the PS II and PS I. A portion of this absorbed energy is conserved as NADPH (218 kj mol^{-1}) while the remaining balance can be conserved as ATP by photophosphorylation. In cyclic electron transport light is absorbed only by P700 and is converted into ATP.

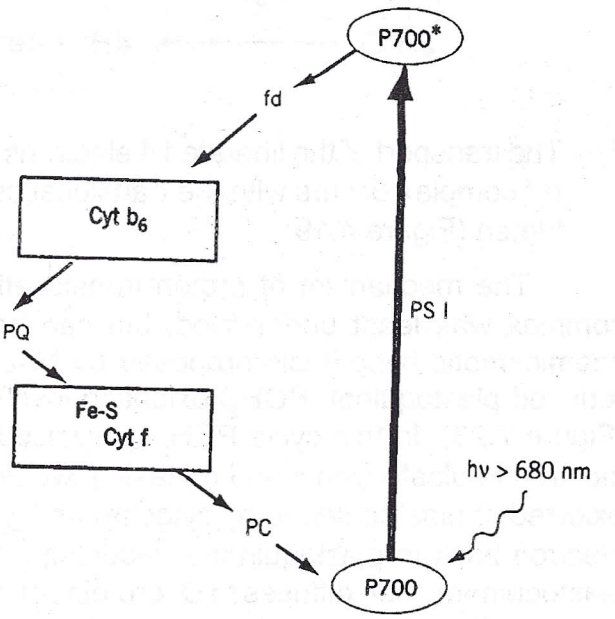


Figure 7.22 Cyclic electron transport. PS I units operating independently of PS II may return electrons from P700* to P700 through ferredoxin (fd), plastoquinone (PQ), and the cytochrome complex. No NADPH is produced by cyclic electron transport, but the energy can be used to produce ATP.

7.10 Photophosphorylation

Light driven synthesis of ATP by thylakoids is known as photophosphorylation. Two types of photophosphorylations are now known: the **non-cyclic photosphorylation** and **cyclic photophosphorylation.**

The formation of ATP in association with non-cyclic electron transport is known as non-cyclic photophosphorylation, while its synthesis by cyclic electron transport is referred to as cyclic photophosphoryaltion. ATP synthase complex of thylakoid membranes catalyses the synthesis of ATP from ADP and Pi in both cyclic and non-cyclic photophosphorylations.

ATP synthase is made up of two parts called F_0 and F_1. F_0 is a hydrophobic transmembrane protein that contains channel for proton translocation from the lumen to the cytoplasm. F_1 is a hydrophilic peripheral membrane protein present on the cytoplasmic side of the membrane in access with ADP and Pi. ATP synthesis is an energy requiring reaction (endergonic). ATP synthase complex then uses energy of the proton gradient which is developed across the membrane i.e. in between the thylakoid lumen and the cytoplasm during photosynthetic electron transport.

7.10.1 Proton gradient

Proton gradient formation is a light driven process by which accumulation of protons (H^+) takes place in the lumen. In other words a part of light energy is primarily conserved in the form of proton gradient. It is developed in two ways :

1) The evolution of one molecule of O_2 from $2H_2O$ molecules releases four protons into the thylakoid lumen.

$$2H_2O \xrightarrow{\text{Light}} 4H^+ + 4e^- + O_2$$

2) The transport of the liberated 4 electrons (to produce 2 NADPH) through the cytochrome b_6f complex occurs with the translocation of nearly 8 protons from the cytoplasm to the lumen (Figure 4.19).

The mechanism of proton translocation across the membrane by the cytochrome complex was least understood, but can be explained on the basis of widely accepted chemiosmotic hypothesis proposed by Mitchell (1961). According to this hypothesis, the reduced plastoquinol (PQH_2) oxidation involves a cyclic series of reactions called **Q cycle** (Figure 7.23). In this cycle PQH_2 is oxidized to semiquinone anion (PQ^-) by passing one electron to plastocyanin and releasing two protons into the lumen. The semiquinone is fully oxidized to plastoquinone by cytochrome b_6. Reduced cytochrome b_6 ultimately passes the electron back to plastoquinone, reducing it to the semiquinone. The semiquinone form of plastoquinone then diffuses to D_1 protein of PS II and reduced to PQH_2 by taking one electron from the D_1 and two protons from the cytoplasm. Thus for each electron passing from plastoquinone to plastocyanin, two protons are translocated from the cytoplasm into the lumen of the thylakoid. According to this scheme, each molecule of evolved O_2 by non-cyclic electron transport contributes 12 protons to the gradient -8 from the Q-cycle and 4 from water oxidation. For cyclic electron transport, the number of protons would be 2 for one turn of electron.

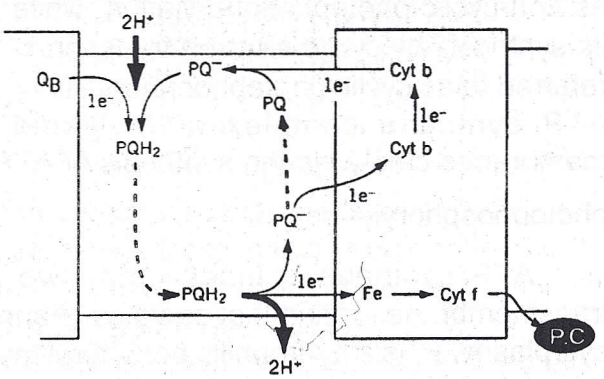


Figure 7.23 The Q-cycle, a model for coupling electron transport from platoquinol to cytochrome with the translocation of protons across the thylakoid membrane. Two protons are translocated for each electron that passes through the electron transport chain.

It is generally agreed that ATP synthase produces one ATP for every three protons it transports out of the thylakoid lumen. Non-cyclic electron transport in thylakoids, therefore results in the production of 12/3 = 4 molecules of ATP per molecule of O_2 evolved (although this quantity is subject to revision) or around one half of an ATP per photon absorbed (4/8 = 1/2 ATP per light quanta absorbed)

The overall light reaction can be represented as :

$$2H_2O + 2NADP^+ + 4\ ADP + 4\ P_i \xrightarrow[\text{Pigments}]{8h\nu} 2NADPH + 4\ ATP + 2H^+$$

Summary

Photosynthesis is the storage of solar energy carried out by photoautotrophs. Light is absorbed mainly by the antenna complexes, which contain chlorophylls, accessory pigments and proteins. They are located in the thylakoid membranes of the chloroplast. Absorbed photons excite chlorophyll molecules, and these excited chlorophylls can dispose of this energy as heat, fluorescence, phosphorescence or chemical work.

Photosynthetic accessory pigments transfer the energy to a specialized chlorophyll-protein complex known as a reaction center. The reaction center contains multiple subunit protein complexes and hundres of chlorophylls. The antenna complexes and the reaction centers are integral components of the thylakoid membrane.

Photosynthetic accessory pigments such as carotenoids may function in a light-harvesting mode, but their best understood function is photoprotection. The capacity of zeaxanthin to absorb excess light energy and dissipate it as heat helps to protect chlorophyll against photooxidative damage.

Plants and some photosynthetic prokaryotes have two reaction centers, photosystem I and photosystem II, that function in series. The photosystems are spatially separated: photosystem I is found exclusively in the nonstacked stroma membranes, photosystem II largely in the stacked grana membranes. The reaction center chlorophylls of photosystem I absorb macimally at 700 nm, those from photo system II at 680nm. The electron transport between these two complexes are mediated by cytochrome b_6f complex. Plastocyania and plastoquinone are mobile electron carriers in the photosynthetic electron transport chain. PS II and PS I together carry out non-cyclic electron transport, oxidize water to molecular O_2, and reduce $NADP^+$ to NADPH. Photosystem I alone is involvedin cyclic electron transport.

During electron transport, a portion of energy of absorbed photons is initially stored in the form of a pH gradient (potential energy) across the thylakoid membrane. This energy is quickly converted into chemical energy during ATP formation by action of an enzyme complex known as the ATP synthase. This energy is quickly converted into chemical energy during ATP formation by action of an enzyme complex known as the ATP synthase. The phosphorylation of ADP by the ATP synthase is driven by a chemiosmotic mechanism. ATP synthesis occurs either by non-cyclic photophosphorylation on cyclic photophosphorylation. NADPH and ATP generated in the light driven electron transport serves as energy source for carbon reduction.

7.13 Model Questions

1. Describe the structure, nature and functions of various photosynthetic pigments.
2. Explain the generation of ATP and NADPH by the photosynthetic electron transport chain
3. Write short notes on
   a. Xanthophyll cycle
   b. Cyclic photophosphoxylation

c. Structure of chloroplast

d. Oxygen evolving complex (OEC).

7.13 Reference Books

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2. Plant Physiology – R.M. Devlin and F.H.Witham – CBS Publishers and distributors – New Delhi
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Dr. G. Rosaiah

Lesson – 8

PHOTOSYNTHESIS
CARBON FIXATION MECHANISMS

8.0 Objective

In this lesson the path of carbon in C_3, C_4 and CAM plants and mechanism of photo-respiration are explained.

8.1 Introduction

In the previous lesson we saw how light energy is harnessed to generate ATP and NADPH. In this lesson we discuss how these products are used to synthesize carbohydrates and other substances from CO_2.

The feat of reducing atmospheric CO_2 to sugar phosphates at the cost of reducing power and ATP generated during light induced electron transport takes place in the chloroplast. The elucidation of the pathway by which carbon reduction takes place posed a formidable problem until the 1940s. The introduction of a stable radio-isotope of carbon (^{14}C) and the availability of sensitive but reliable analytical techniques such as **paper chromatography** and **autoradiography** permitted Melvin Calvin, James Bassham and Andrew Benson at the University of California, Berkeley, to inverstigate this pathway. They did so by tracing the metabolic fate of the radioactive label from $^{14}CO_2$ as it passed through a series of photosynthetic intermediates. The basic experimental strategy they used was to expose growing cultures of *Chlorella* to $^{14}CO_2$ for varying times and under differing illumination conditions.

and then drop the cells into boiling ethanol so as to disrupt them while preserving their labeling pattern. The radioactive products were subsequently separated and identified by the paper chromatography coupled with autoradiography. The results obtained by them led to the conclution that carbon reduction is achieved by a cyclic process. This process is then named as **Calvin cycle**. It is also called by several other names: **Calvin Benson cycle, Reductive pentose phosphate cycle** (RPP) and more recently as **Photosynthetic carbon reduction cycle** (PCR).

Some of Calvin's earliest experiments indicated that algae exposed to $^{14}CO_2$ for a minute or more had synthesized a complex mixture of labeled metabolic products including sugars and amino acids. By inactivating the algae within 5 seconds of their exposure to $^{14}CO_2$, it was found that the first stable radioactively labeled compound formed was 3-phosphoglyceric acid. This result immediately suggested that the 3PGA was formed by the carboxylation of a C_2 compound. Subsequent work failed to find any such compound. The actual carboxylation reaction however, was discovered through an experiment in which illuminated algae had been exposed to $^{14}CO_2$ for 10 minutes so that the levels of their photosynthetic products had reached a steady state. The CO_2 was then withdrawn. In doing so, the carboxylation product, 3-phosphoglyceric acid, decreased in concentration (figure 8.1) because it is taken up by reactions further along the pathway. The concentration of ribulose-1, 5-bisphate (RuBP) however, simultaneously increased. The conclusion drawn was that RuBP is the Calvin cycles carboxylation substrate. While the search for the carboxylation substrate was going on, several other photosynthetic intermediates had been identified through chemical degradation and labeling pattern studies.

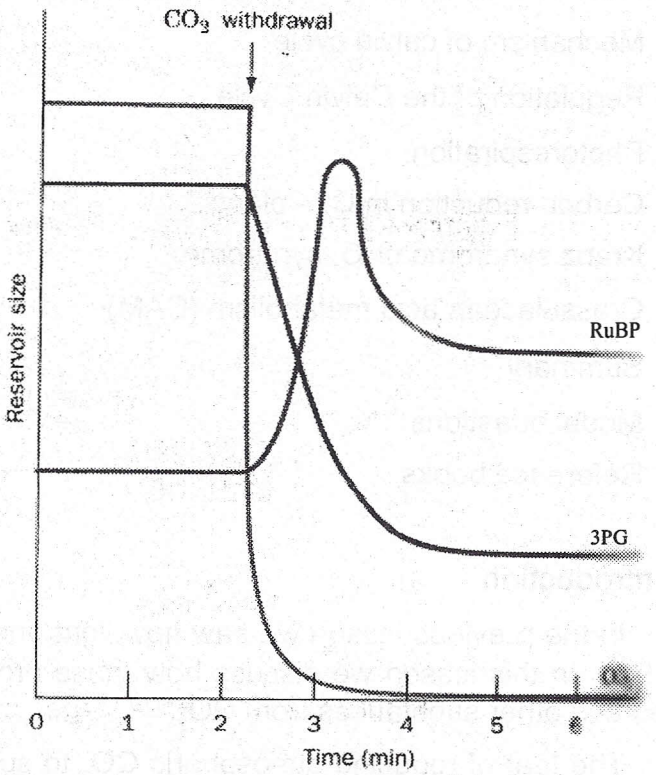


Figure 8.1 An experiment showing the levels of 3PG and RuBP in illuminated algae exposed to $^{14}CO_2$ at steady state and during a period in which the CO_2 is abruptly withdrawn. In the absence of CO_2, the 3PG concentration rapidly decreases because it is taken up by the reactions of the Calvin cycle but cannot be replenished by them. Conversely, the RuBP concentration transiently increases as it is synthesized from the residual pool of Calvin cycle intermediates but, in the absence of CO_2, cannot be used for their regeneration.

8.2 Overview of Calvin cycle

In the Calvin cycle, CO_2 and H_2O from the environment are enzymatically combined with a five-carbon acceptor molecule to generate two molecules of a three carbon 3-phosphoglyceric acid, the first stable intermediate in the Calvin cycle. This part of the cycle is called the **carboxylation** stage. The 3-phosphoglycerate is reduced to Glyceraldehyde-3-phosphate, (GAP) a three carbon carbohydrate by use of ATP and NADPH generated photochemically by a process what is known as **reduction stage**. At this point one GAP can be blend off from the cycle for use in biosynthesis. The cycle is completed by regeneration of the five carbon acceptor ribulose-1,5-bisphosphate (Figure 8.2).

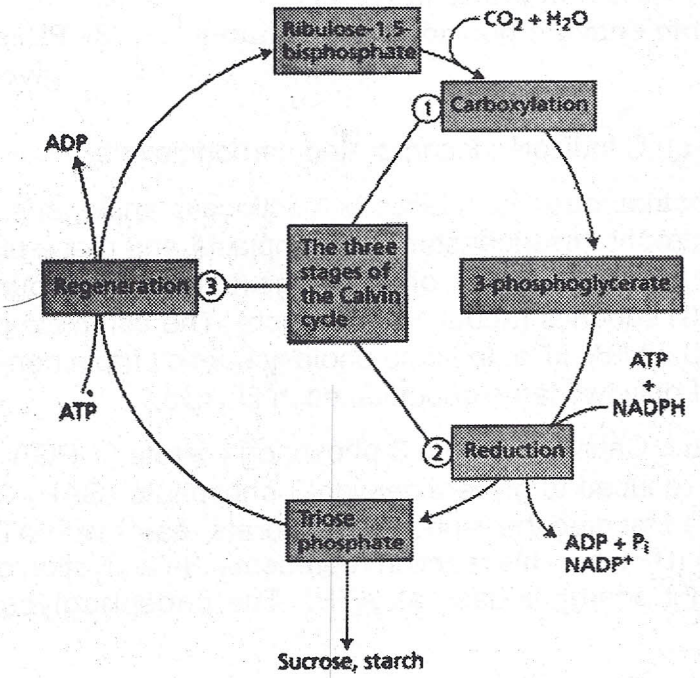
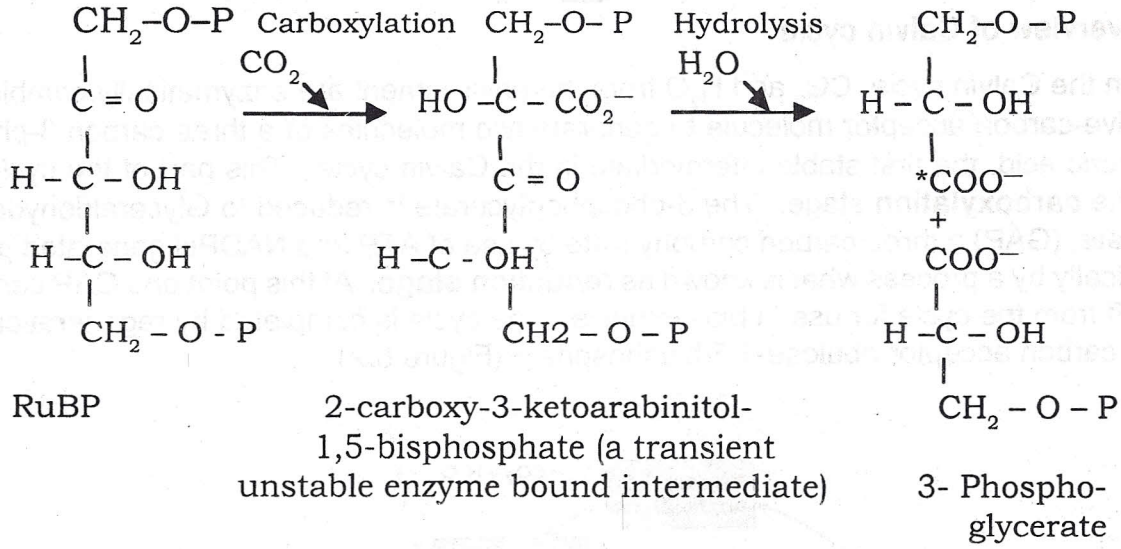


Figure 8.2 The three stages of Calvin cycle.

8.3 Mechanism of Calvin cycle

Calvin cycle as a whole consists of 13 enzymatic reactions which are described below.

1) Carbon dioxide is first accepted by ribulose-1, 5-bisphophate and forms an unstable enzyme bound intermediate 2-carboxy-3-ketoarabinitol-1,5-bispshosphate. This is further hydrolyzed to yield two molecules of the stable product 3-phophoglycerate. This carboxylation reaction is catalyzed by the enzyme **ribulose-1,5-bisphosphate carboxylase/oxygenase** generally called **rubisco**.



RuBP

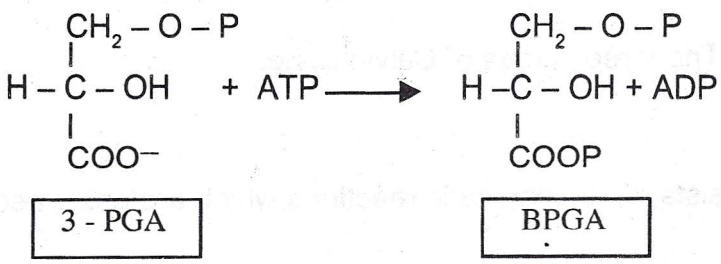
2-carboxy-3-ketoarabinitol-1,5-bisphosphate (a transient unstable enzyme bound intermediate)

3- Phospho-glycerate

(*Symbol on C indicates incorporated carbondioxide)

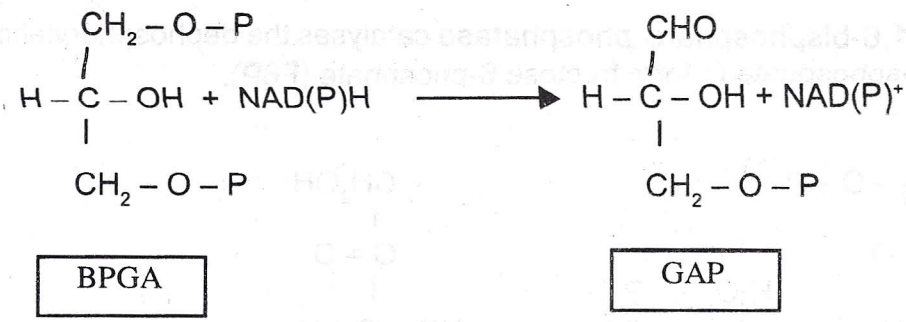
In various groups of photoautotrophs, rubisco is functionally analogous and structurally diverse. Oxygenic phototrophs (cyanobacteria) chloroplasts and many photosynthetic bacteria contain a form of the enzyme made up of eight large (L) catalytic subunits (about 55 kDa each) and eight small (S) subunits (about 14 kDa each). The complete protein (L_8S_8) has the molecular mass of about 560 kDa. In some photosynthetic purple non-sulphur bacteria, rubisco is composed of only two large subunits, each 50 kDa.

2) In the reduction stage of the Calvin cycle, the 3-phosphoglycerate (3-PGA) formed as a result of carboxylation is reduced to glyceraldehyde-3-phosphate (GAP). This reaction occurs in two steps. In the first step the 3-phosphoglycerate reacts with ATP and forms 1-3- bisphosphoglycerate (BPGA). This reaction represents the activation of PGA and it is the first step where light energy is used as ATP. The **phosphoglycerate kinase** catalyses this reaction.



3 - PGA BPGA

3) In the second step,-1,3-bisphosphoglycerate is reduced to glyceraldehyde-3- phosphate by using the NAD(P) H generated by the light reactions. This reaction is regulated by the enzyme **glyceraldehyde-3-phosphate dehydrogenase**.



4) The phosphoglyceraldehyde molecule then converted into dihydroxy acetone phosphate (DHAP) by the enzyme **triose phosphate isomerase**. This triose is available for the biosynthesis of sugars and other cellular components. If this triose is completely used for biosynthesis, CO_2 fixation would be stopped due to the shortage of acceptor RuBP. Thus a third process must follow i.e. the regeneration of the CO_2 acceptor. This is accomplished by a series of reactions involving 4-,5-,6-, and 7- carbon sugars (Figure 8.3).

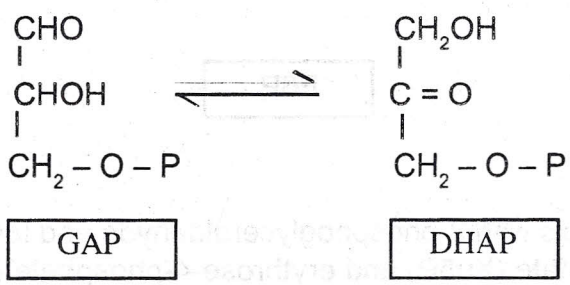
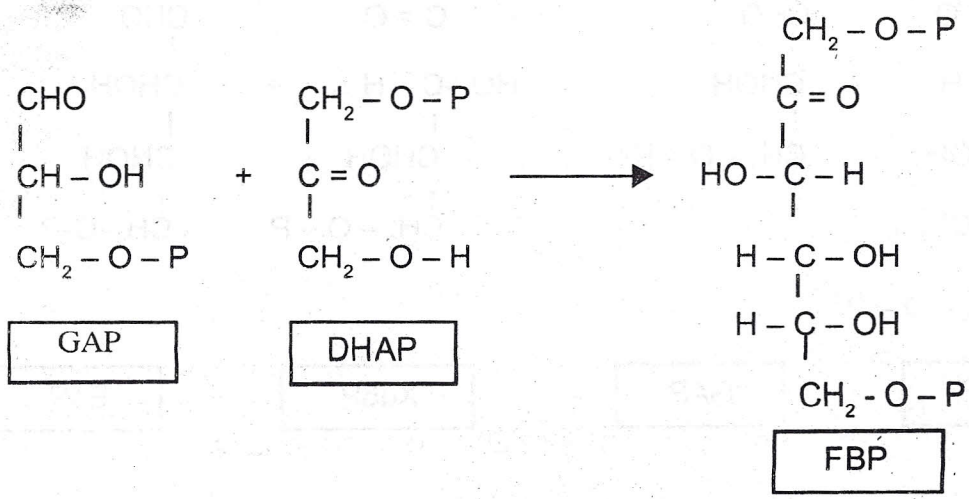
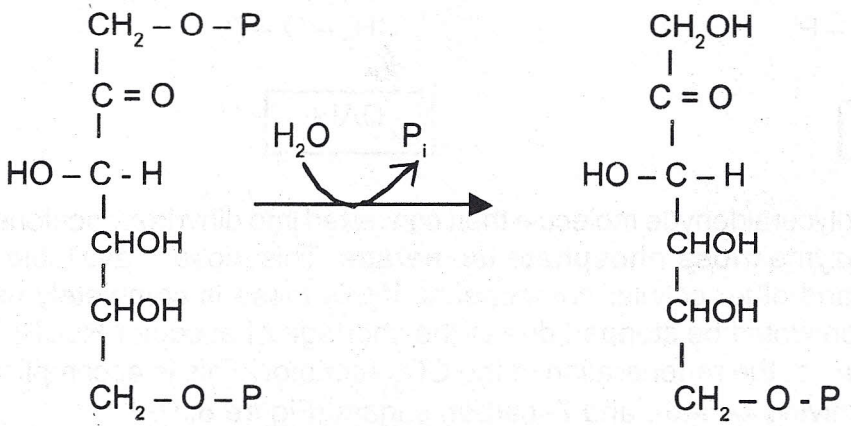


Fig.8.3 shows that for every three turns of the cycle i.e. the uptake of three CO_2, there is sufficient carbon to regenerate the required number of acceptor molecules as well as one additional triose phosphate available for sugar formation.

5) In the presence of enzyme **aldolase**, the 3-phosphoglyceraldehyde and dihydroxy-acetone phosphate undergo condensation to yield fructose-1,6-bisphosphate (FBP).

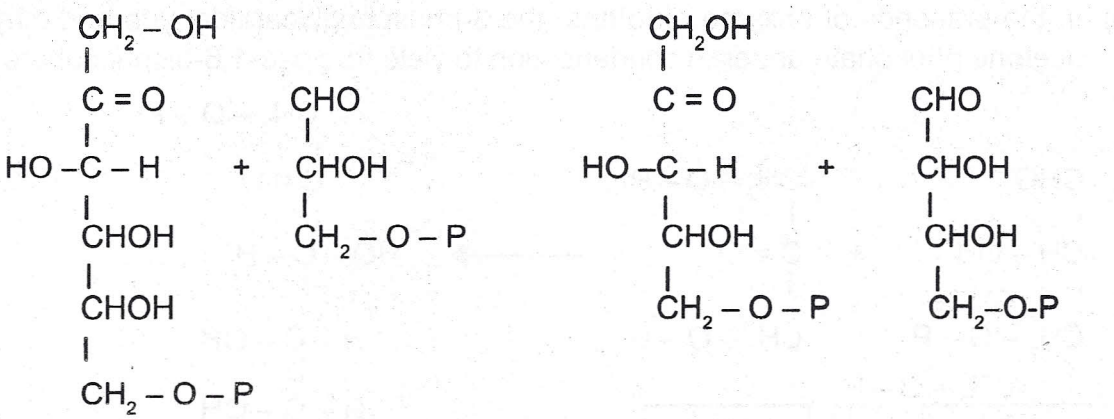


6) Fructose-1,6-bisphosphate phosphatase catalyses the dephosphorylation of fructose-1,6-bisphosphate to form fructose 6-phosphate (F6P).



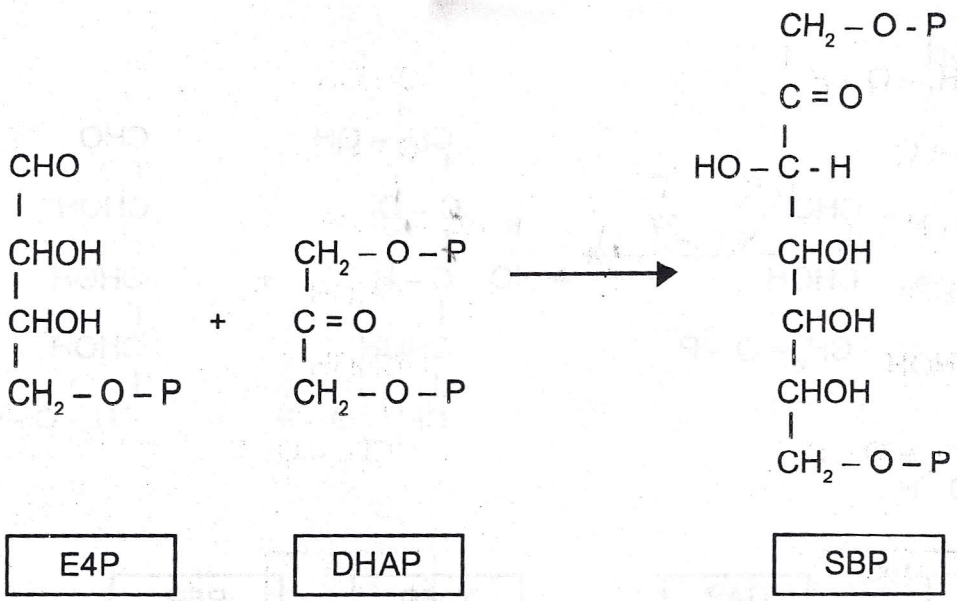
FBP F6P

7) Fructose-6-phosphate then reacts with 3-phosphoglyceraldehyde and form one molecule each of xylulose-5-phosphate (Xu5P) and erythrose-4-phosphate (E4P) in the presence of an enzyme **transketolase**.

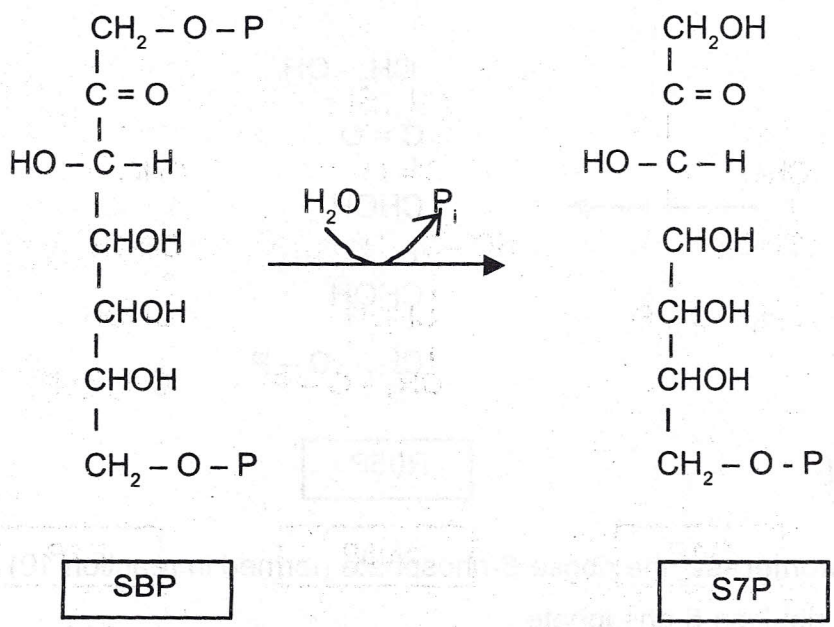


F6P GAP Xu5P E4P

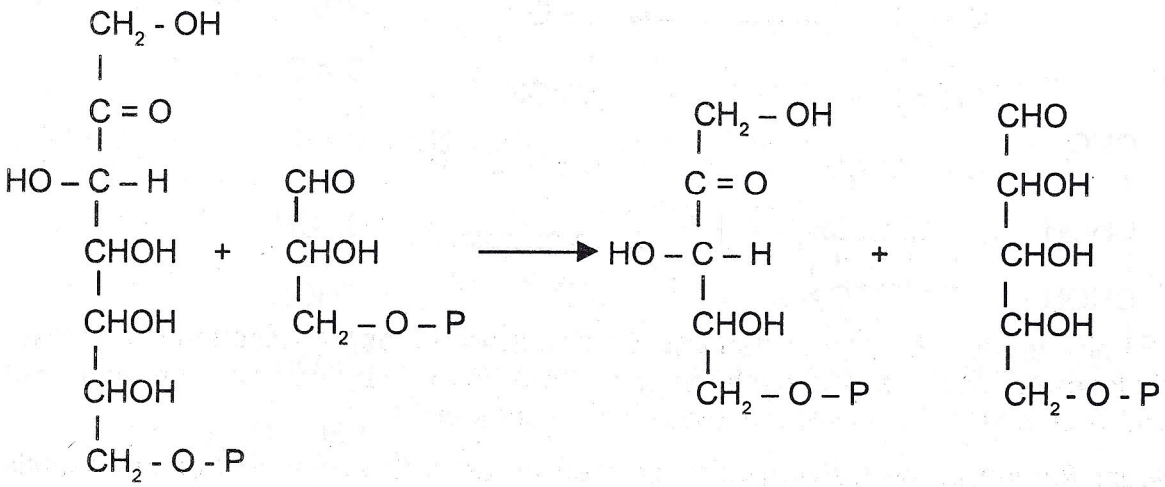
8) Erythrose-4-phosphate combines with a molecule of dihydroxyacetone phosphate to form sedoheptulose-1,7-bisphosphate (SBP) in the presence of **aldolase**.



9) Sedoheptulose-1,7-bisphosphate is further hydrolysed by way of a specific **phosphatase** to give sedoheptulose-7-phosphate (S7P).

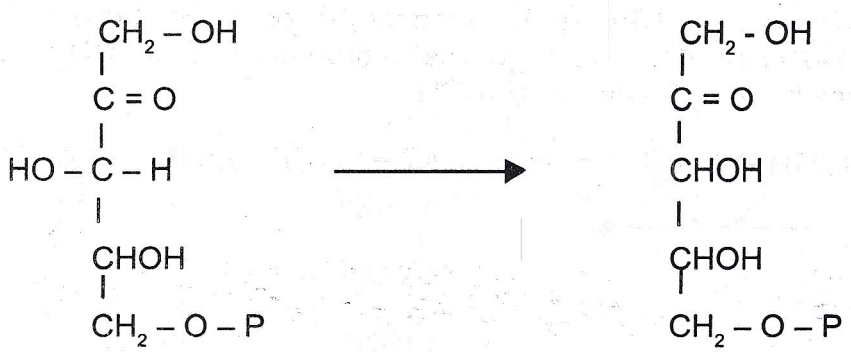


10) Sedoheptulose-7-phosphate donates a two carbon unit to the molecule of glyceraldehyde-3-phosphate via **transketolase** and produces ribose-5-phosphate (R5P) and xylulose-5- phosphate.



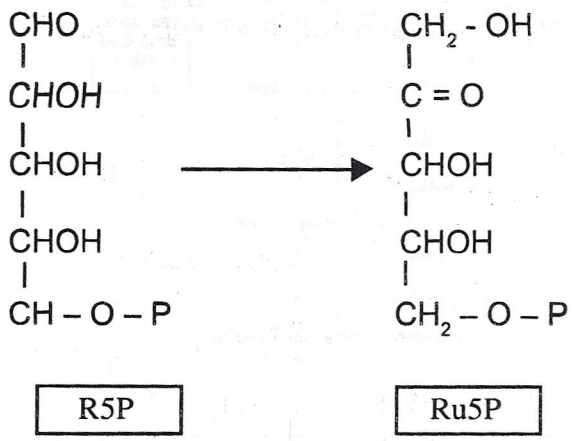
S7P GAP Xu5P R5P

11) The two molecules of xylulose-5-phosphate formed in reactions 7 and 10 are converted to two ribulose-5-phosphate (Ru5P) sugars by an **epimerase**.



Xu5P Ru5P

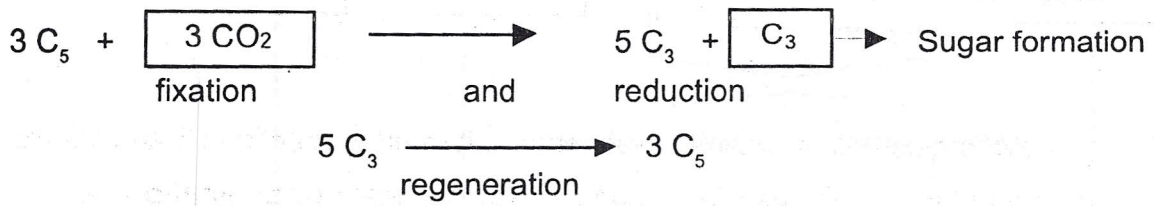
12) By the action of **isomerase**, the ribose-5-phosphate (formed in reaction 10) is also converted to ribulose-5-phsophate.



13) Finally, in the presence of **ribulose-5-phosphate kinase** (phosphoribulo kinase), ribulose-5-phosphate is phosphorylated with ATP and regenerating the three molecules of initial CO_2 acceptor ribulose-1,5-bisphosphate.

Sugar formed in the Calvin cycle can then be used to synthesize other essential molecules.

The carbon balance of the whole cycle can be described schematically as follows :



A total of 9 ATP and 6 NADPH are required for the formation of one glyceraldehyde-3-phosphate (GAP) from CO_2. In otherwords incorporation of one CO_2 into an organic material requires three ATP and two NADPH.

$$3\ CO_2 + 9\ ATP + 6\ NADPH.H^+ \longrightarrow GAP + 9\ ADP + 8\ P_i + 6NADP^+$$

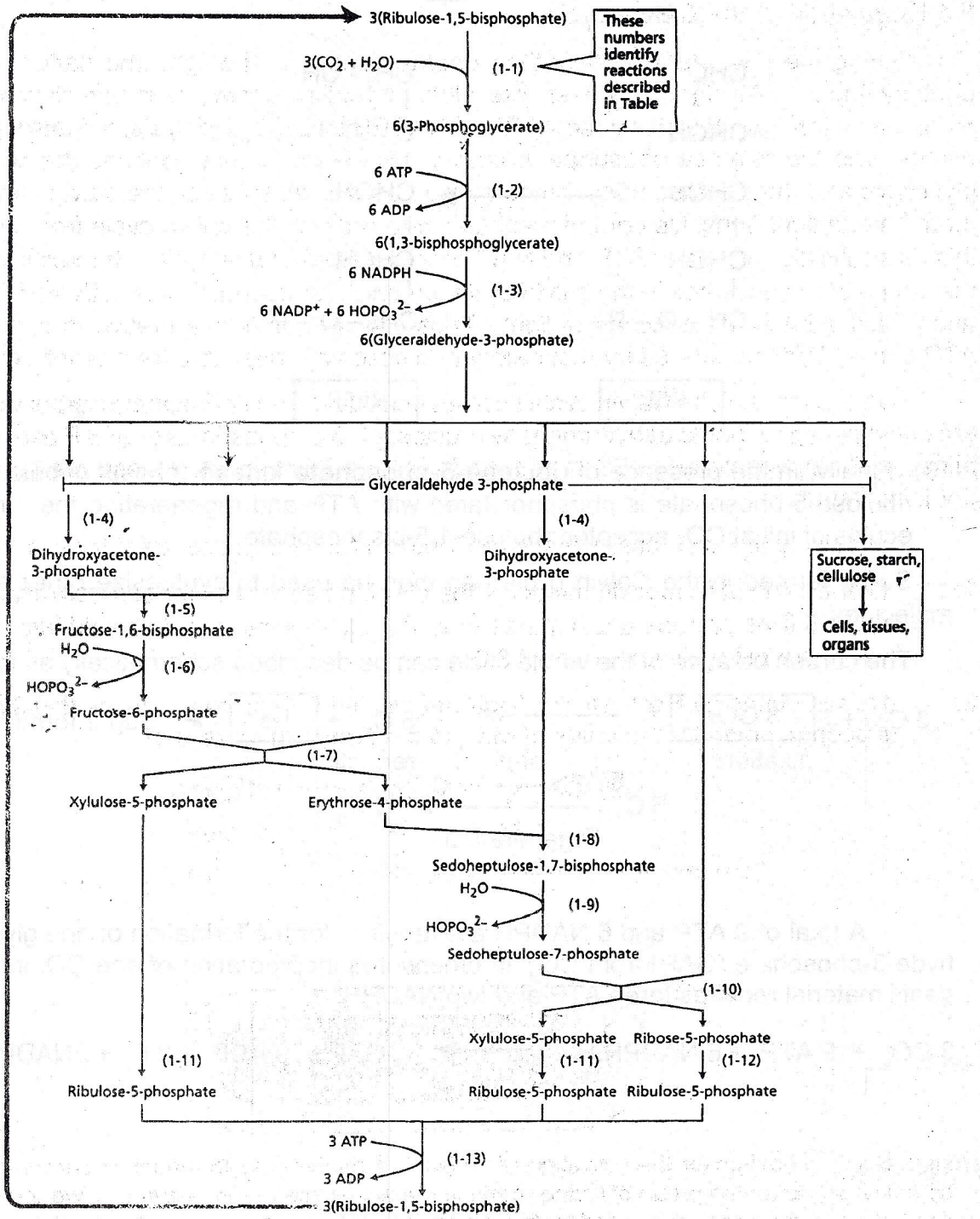


Figure 8.3 Reactions of Calvin cycle 1, RuBP carboxylase/oxygenase; 2, Phsophoglycerate kinase; 3, Glyceraldehyde-3-phosphate dehydrogenase; 4, Triosephosphate isomerase; 5, Aldolase; 6, Fructose-1, 6-bisphosphate phosphatase; 7, Transketolase; 8, Aldolase; 9, Sedoheptulose-1,7-bisphosphate phosphatase; 10, Transketolase; 11, Phosphoribulo epimerase; 12, Ribose-5-phosphate isomerase; and 13, Phosphoribulo kinase.

8.4 Regulation of the Calvin cycle

During the day, plants satisfy their energy needs via the light and dark reactions of photosynthesis. At night, however, like other organisms, they must use their nutritional reserves to generate their required ATP and NADPH through glycolysis, oxidative phosphorylation and the pentose phosphate pathway. Since the stoma contains the enzymes of glycolysis and the pentose phosphate pathway as well as those of the calvin cycle, plants must have a light-sensitive control mechanism to prevent the calvin cycle from consuming this catabolically produced ATP and NADPH in a wasteful futile cycle. Studies have shown that some of the enzymes in the calvin cycle are photoregulated, that is activated in the light and deactivated in the absence of light. Thus calvin cycle is inoperative in darkness and ATP and NADPH generated by respiratory processes are available for anabolic processes.

Five enzymes of the Calvin cycle such as ribulose-1, 5-bisphosphate carboxylase, glyceraldehydes-3 phosphate dehydrogenase, fructose-1, 6-bisphosphatase (FB P ase) sedoheptulose-1, 7-bisphosphatase (SB P ase) and ribulose-5 phosphate kinase are known to be photoactivated.

The activity of RuBP carboxylase responds to four light dependent factors:

1. It varies with pH. Upon illumination, the pH of the stroma increases from around 7.0 to about 8.0 as protons are pumped from the stroma into the thylakoid lumen. RuBP has sharp pH optimum near pH 8.0.
2. It is stimulated by Mg^{2+}. During light-induced influx of protons into the thylakoid lumen is accompanied by the efflux of Mg^{2+} to the stroma (Figure 8.4).

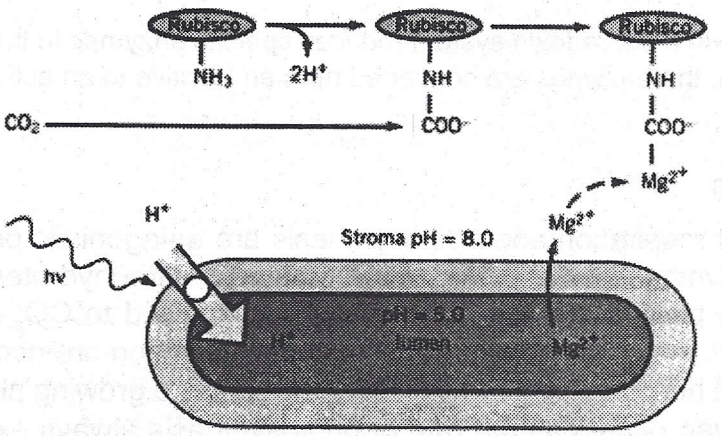


Figure 8.4 Mechanism for the activation of rubisco. It involves the formation of carbomate –Mg^{2+} complex on the amino group of lysine within in the active site of the enzyme. Two protons are released, so activation is enhanced by the increase in pH and Mg^{2+} concentration that result from illumination.

3. It is allosterically activated by NADPH, which is produced by illuminated PSI.
4. Full activity of RuBP carboxylase also requires the presence of its activating enzyme rubisco activase.

FBPase and SBPase are also activated by increased pH, Mg^{2+} and NADPH. The action of these factors is complemented by a second regulatory system that responds to the redox potential of the stroma. In this mechanism, a photoactivated PS I reduces soluble stroma ferredoxin (Fd). The reduced ferredoxin reduces ferredoxin thioredoxin reductase, which, in turn, reduces the disulfide linkage of thioredoxin. Thioredoxin, a 12 kDa protein that occurs in many types of cells, contains a reversibly reducable cystine disulfide group. Reduced thioredoxin activates both FBPase and SBPase by a disulfide exhange reaction (Figure 8.5). Subsequent deactivation of the enzymes in the dark is not well understood, but clearly the sulfhydryl groups are in some way reoxidized and the enzymes rendered inactive.

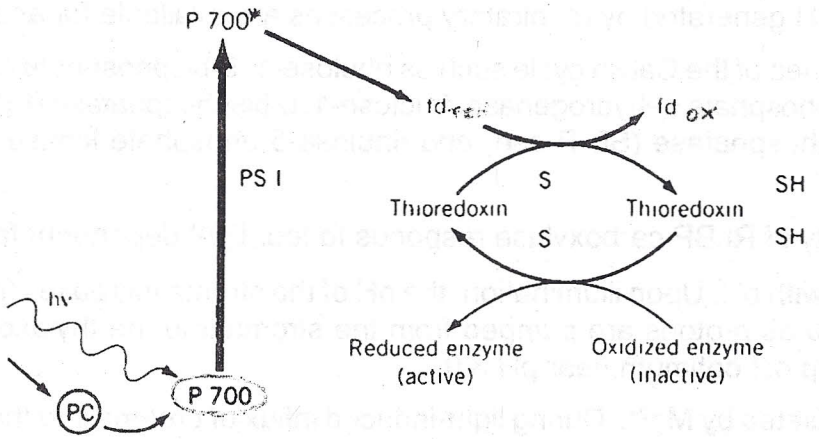


Figure 8.5 The ferredoxin – thioredoxin system reduces specific enzymes in the presence of light. On reduction, the enzymes are converted from an inactive to an active state.

8.5 Photorespiration

As we know that respiration and photosynthesis are antagonistic processes. During the process of photosynthesis CO_2 is incorporated into the carbohydrates while in respiration carbohydrates so produced in photosynthesis are oxidized to CO_2 and H_2O with the release of energy as ATP. When photosynthesis and respiration are occur at equal rates, the net photosynthesis becomes zero. A gain in dry weight of a growing plant, called the net photosynthesis however, occurs as the rate of photosynthesis always exceeds the rate of respiration. The total or gross photosynthesis of a growing plant is obtained by adding the rate of respiratory CO_2 release from the leaf to the value of net photosynthesis as shown in the following relation.

Rate of total photosynthesis = rate of net photosynthesis + rate of respiration in the light.

Net photosynthesis is usually expressed in terms of CO_2 uptake on a leaf area basis because the leaf is the main photosynthetic organ. The range of net photosynthesis is

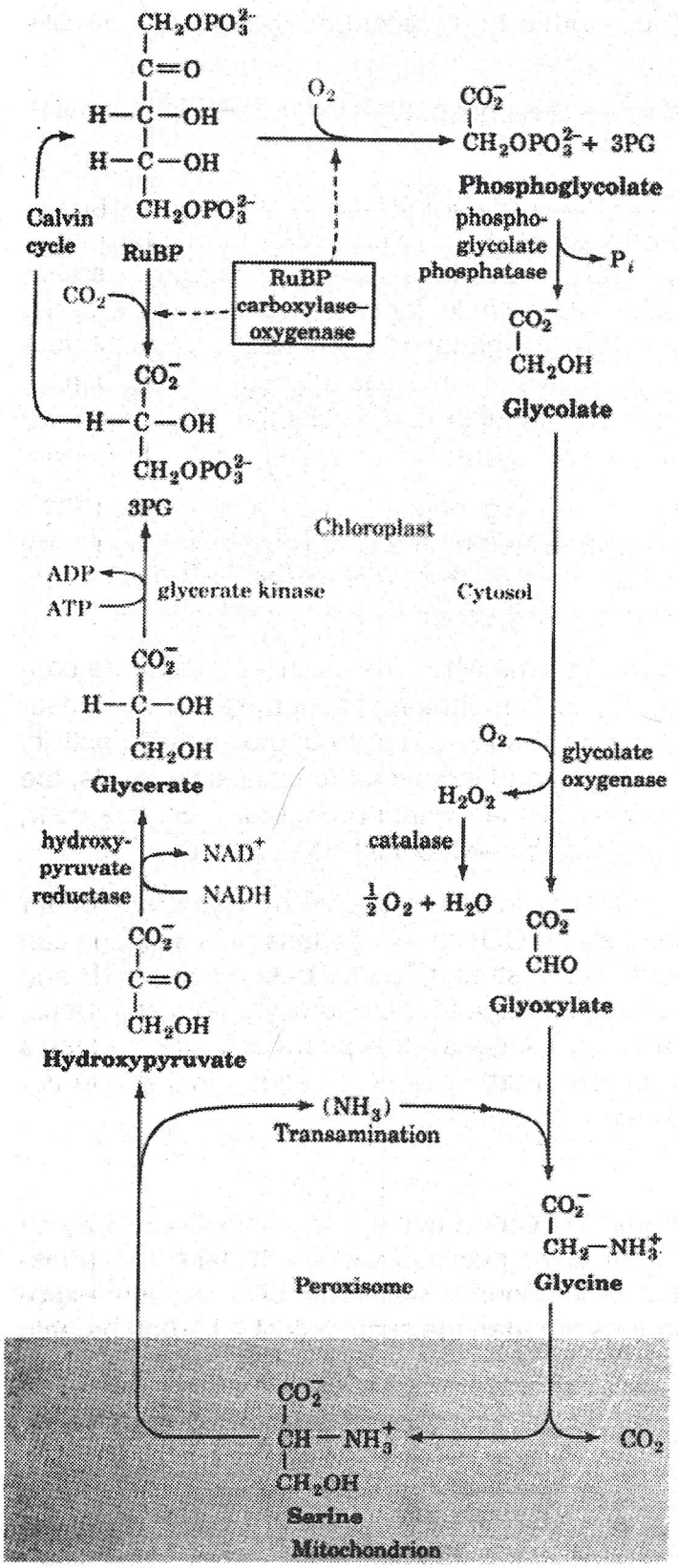
generally from 15 to 35 mg CO_2 dm^{-2} leaf surface h^{-1} for C_3 plants and 40 to 80 mg CO_2 dm^{-2} leaf surface h^{-1} for C_4 plants, in full sunlight and at the natural concentrations of O_2 (21 percent) and CO_2 (0.33 per cent) in the external atmosphere. This difference in the rates of net productivity between C_3 and C_4 plants led to the conclusion that there is a difference in the rates of CO_2 release from the leaves of C_3 plants in both light and dark from the leaves of C_3 plants. In case of C_4 plants, the rates of respiration in the light and dark are almost same, whereas in C_3 plants respiratory CO_2 release is much higher in the light than the dark. This difference in the rates of CO_2 release by a C_3 leaf between the light and the dark periods led to the discovery of **photorespiration** by John-P.Decker in 1955. This process involves the reoxidation of products just previously assimilated in photosynthesis. The photorespiratory pathway involves activities of at least three different cellular organelles namely the chloroplast, the peroxisome and the mitochondrion.

Mechanism of Photorespiration

Photorespiration is the uptake of O_2 and the release of CO_2 by the illuminated chlorophyllous tissues. This process results from the biosynthesis of a two carbon compound glycolate in chloroplasts and its subsequent metabolism in the same leaf cell. Glycolic acid is synthesized in green leaves in the light but not in the dark. Its synthesis in illuminated leaves has been shown to depend on the concentrations of CO_2 and O_2 in the external atmosphere. An increase in O_2 concentration in the external atmosphere will increase the amount glycolic acid synthesized in an illuminated leaf. Reverse effect will be observed with increasing CO_2 concentration. Thus only a small amounts of glycolic acid are produced when a C_3 plant is held in a chamber at about 2 percent oxygen. But glycolic acid is formed rapidly and accumulated in relatively large amounts in C_3 plants held in air with the natural concentration of O_2 (= 21 percent).

This competition between O_2 and CO_2, with O_2 promoting glycolic acid synthesis and inhibiting photosynthesis and CO_2 inhibiting glycolic acid synthesis and promoting photosynthesis, has led to the conclusion that glycolic acid is synthesized in vivo as a result of the oxygenation of a member of the PCR cycle-ribulose-1, 5-bisphosphate. In fact, the enzyme RuBPcase not only catalyzes the addition of CO_2 to RuBP but also catalyzes the addition of O_2 to RuBP. Because of this dual function, this enzyme is also called RuBP carboxylase / Oxygenase or Rubisco or RuBPCO.

In 1971 W.L. Ogren and George Bowes have showed that carbons 1 and 2 of RuBP were the precursors for the synthesis of glycolic acid. When O_2 is added to the RuBP during oxygenation, the bond between carbon atoms 2 and 3 is broken to yield one molecule each of 2-phosphoglycolate and 3-phosphosglycerate (Figure 8.6). This was confirmed by non-radioactive stable oxygen isotype (^{18}O) studies. The 3-phosphoglycerate is available for further metabolism by the PCR cycle, but the 2-phyphoglycolate is rapidly dephosphorylated to glycolate in the chloroplast. This reaction is catalyzed by the chloroplast containing **phophoglycolate** phosphatase and the resulting glycolate is transported to peroxisomes.



Peroxisomes are organelles that have a close spatial relationship with chloroplasts and mitochondria. They are especially numerous in mesophyll cells of C_3 plants. They are unique in having two oxidative enzymes such as **glycolic acid oxidase** and **catalase**. The glycolic acid oxidase is a flavin containing oxidase, i.e flavin adenine mononucleotide (FMN) is its coenzyme. It catalyzes the oxidation of glycolate to glyoxylate and hydrogen peroxide. The H_2O_2 produced in this reaction is a powerful and potentially harmful oxidizing agent and it is rapidly decomposed to H_2O and O_2 by the catalase. Glyoxylate is converted to glycine by a **transaminase**.

Glycine is then transported to a mitochondrion where two molecules of glycine are converted to one molecule of serine plus one molecule each of CO_2, ammonia, and NADH. This oxidative decarboxylation is complex and involves more than one enzyme such as **glycine decarboxylase** and serine **hydroxymethyl transferase**. This reaction is the source of CO_2 in photorespiration.

The serine then leaves the mitochondrion, returning to a peroxi some where the amino group is given up in a transamination reaction to form

Figure 8.6 The photorespiration pathway for the metabolism of the phosphoglycolate produced by the RuBP carboxylase catalyzed oxidation of RuBP. Note that two glycines required to form serine +CO_2.

hydroxypyruvate. The hydroxypyruvate is converted to glycerate by the enzyme **NADH-*hydroxypuruvate reductase*.**

Glycerate is transported to chloroplast where it is phosphorylated to 3-PGA using ATP. The PGA enters Calvin cycle.

Since photorespiration involves metabolism of a 2-C glycolate, it is known as **C_2 glycolate cylce** or **photosynthetic carbon oxidation cycle** (PCO) which involves complex interactions between photosynthesis, photorespiration, and various aspects of nitrogen metabolism in at least three different cellular organelles. As a whole, for each two turns of the cycle, two molecules of phosphoglycolate are formed by oxygenation of Rubisco. Of these four carbon atoms, one is lost as CO_2 and three are converted into one molecule of 3-phosphoglycerate which is returned to the chloroplast. In other words, 75 percent of the carbon lost by the oxygenation of RuBP is recovered by the PCO cycle and returned to the Calvin cycle.

Although 75 percent of the carbon lost by the oxygenation is recovered through PCO cycle, still that some of the ATP and NADPH generated by the light reactions is uselessly dissipated in addition to a net loss of one carbon. When this process has no known metabolic function and considered it as a wasteful one, why do plants contain it ?

It is thought that photosynthesis evolved at a time when the earth's atmosphere contained large quantities of CO_2 and very little O_2 so that photorespiration was of no consequence. When O_2 began to accumulate in the atmosphere due to photosynthetic activity and by the time that the atmospheric content of O_2 had increased to significant levels, the bifunctional nature of the enzyme had been established without recourse. By this view, then, the oxygenase function is an evolutionary hangover that has no useful role.

Another possible explanation is that photorespiration is important, especially under conditions of high light intensity and low inter cellular CO_2 concentrations (this situation can arise when stomata are closed in response to water stress), to dissipate excess ATP and NADPH from the light reactions, thus preventing damage to the photosynthetic apparatus. Furthermore, recent evidence with transgenic plants suggests that photorespiration protects C_3 plants from photooxidation and photoinhibition. Further work is needed to improve our understanding of the function of photorespiration.

8.6 Carbon reduction in C_4 plants

Until the late sixties it was presumed that the Calvin cycle is the unique pathway by which all the photoautotrophs assimilate CO_2. In some plants, however, ^{14}C-labeled 3-phosphoglyceric acid is not one of the early labeled compounds observed following brief exposure to $^{14}CO_2$. In 1957 Kortschak and colleagues reported the synthesis of 4-carbon organic acids (malate and asparate) as the first stable products of photosynthesis in sugarcane leaves. Later Karpilov in 1960 reported a similar observation in the case of maize leaves. These observations which suggested a variation of carbon assimilation from the traditional Calvin cycle pathway stimulated a thorough investigation of this observation by Hatch and Slack. They proposed a cyclic mechanism in which carbon was first incorporated into a C4 acid, the oxaloacetic acid. Since in some plants the first stable product of CO_2 reduction is a

4-C acid and hence these have been designated as the C_4 plants and the cycle as C_4 – cycle and carboxylation cycle.

Of the approximately 300,000 species of flowering plants, about 1500 species spread over in 18 different angiosperm families (3 monocots, 15 dicots) are reported to be C_4 plants (Raghavendra and Das 1978). Most of them are grasses. C_4 plants occur largely in tropical regions because they grow faster under hot and sunny conditions than the so called C_3 plants. Well known C_4 plants are maize, sorghum, sugarcane, italian millet, crabgrass and Bermuda grass. C_4 group also included certain dicots such as **Amaranthus** and a few species of **Atriplex, Tribulus**, etc. The basic C_4 cycle consists of four steps. In the first step, fixation of CO_2 by the carboxylation of phosphoenol pyruvate in the mesophylls takes place to form a moderately unstable C_4 acid, the oxaloacetate (OAA). This is a key reaction in the C_4 cycle. In this reaction, the enzyme **phosphoenolpyruvate carboxylase** (PEPcase) catalyses the β-carboxylation of phosphoenol pyruvate (PEP) using the bicarbonate ion HCO_3^- as the substrate. Oxaloacetate produced in this reaction is quickly reduced either to malate or tansaminated to asparate. Both are stable and C_4 acids. In the second step, these acids are transported from the mesophyll cells to the bundle sheath cells via the cytoplasmic connections known as **plasmodesmata**. In the third step, the C_4 acid in the bundle sheath cell undergoes a oxidative decarboxylation and the resulting CO_2 is then reduced to carbohydrate via the Calvin cycle. In the fourth step, the C_3 acid either pyruvate or alanine that remains after decarboxylation is then transported back into the mesophyll cell. Here (if alanine, it is converted to pyruvate), the pyruvate is phosphorylated to regenerate the original acceptor molecule, PEP (Figure 8.7). One interesting feature of the cycle is that the enzyme that catalyzes

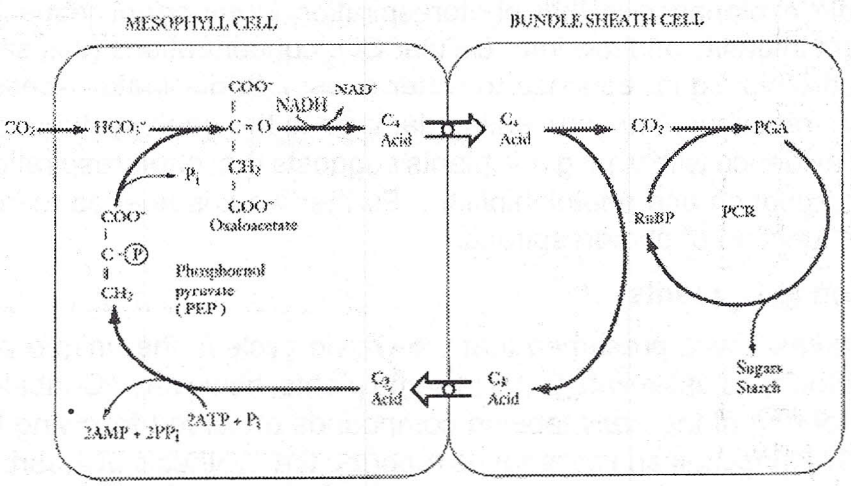


Figure 8.7 The basic C_4 cycle involves two cell types and proceeds through four stages: (1) Fixation of CO_2 into a 4C-acid in the mesophyll cells; (2) transport of the 4C-acid from the mesophyll cells to the bundle sheath cells; (3) decarboxylation of the four-carbon acid to yied CO_2; and (4) transport of the residual three-carbon acid back to the mesophyll cells, where the original CO_2 acceptor, PEP, is regenerated.

this regeneration, **pyruvate orthophosphate dikinase**, consumes two "high energy" phosphate bonds through the conversion of ATP to AMP. The regeneration of ATP from AMP requires the incorporation of two phosphate bonds, one to form ADP and second to form ATP. CO_2 is thereby concentrated in the bundle sheath cells at the expense of two ATP molecules per CO_2. *Photosynthesis in C_4 plants therefore* consumes a total of five ATP molecules per CO_2 fixed versus the three ATP molecules by the Calvin cycle.

There are three variations of the basic C_4 pathway (Figure 8.8). The variations differ principally in the C_4 acid transported into the bundle cells and in the manner of decarboxylation. They are named after the enzymes that catalyze their decarboxylation reactions. Accordingly they are designated as **NADP-malic enzyme type (NADP-ME), NAD-malic enzyme type** (NAD-ME) and the **PEP-carboxy Kinase type** (PCK).

The primary carboxylation reaction catalyzed by PEP carboxylase, which is common to all three variants, occurs in the cytosol of the mesophyll cells and uses HCO_3^- rather than CO_2 as a substrate. In the NADP-ME tyne, the oxaloacetate is rapidly

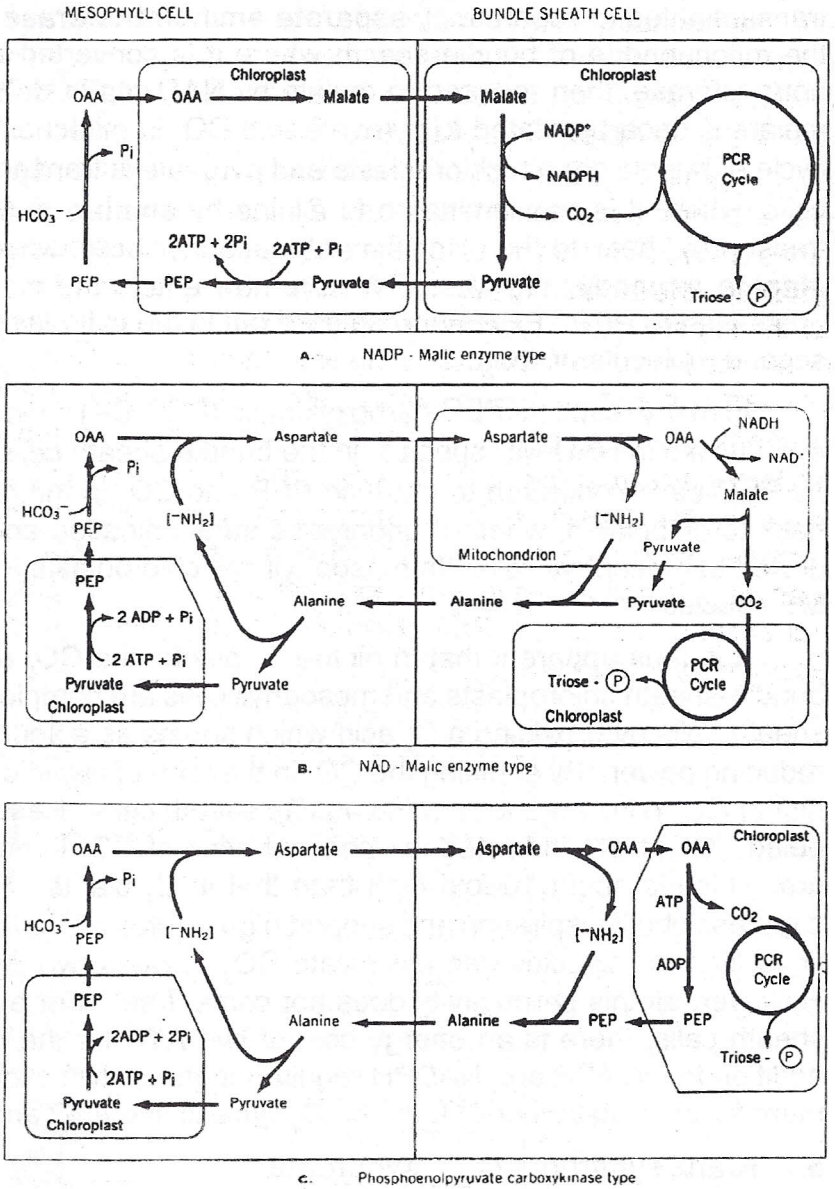


Figure 8.8 The three variants of the C_4 photosynthetic carbon cycle.

reduced to malate in the mesophyll chloroplasts by NADPH using **NADP: malate dehydrogenase**. The NADPH necessary for the reduction of oxaloacetate to malate is produced by the light driven photosynthetic electron transport of mesophyll cell chloroplasts. The malate is then transported to the chlorplast of bundle-sheath cell where it undergoes oxidative de-

carboxylation to yield pyruvate, CO_2 and NADPH. The CO_2 released within the bundle-sheath cell is converted to carbohydrate by the Calvin cycle. The pyruvate is transported back to the mesophyll cell where it is phosphorylated to from PEP. The phosphorylation reaction, catalyzed by the enzyme, pyruvate-orthophosphate dikinase requires two ATP. This reaction takes place in the mesophyll chloroplast of all three variants.

In NAD-Malic enzyme type, oxaloacetate produced in the cytosol of mesophyll cells is transaminated to asparate by **asparate aminotransferase**. The asparate is transported to the mitochondria of bundle sheath where it is converted back to OAA by as parate aminotransferase, then reduced to malate by **NAD-malic dehydrogenase** (NAD.MDH). The malate is decarboxylated to pyruvate and CO_2 in mitochondria. CO_2 is used by the Calvin cycle of bundle sheath chloroplasts and pyruvate is transported to cytosol of bundle-sheath cells, where it is transaminated to alinine by **alanine aminotransferase**. The alanine is transported back to the cytoplasm of mesophyll cells, where it is converted to pyruvate by **alanine aminotransferase**. Pyruvate now enters the mesophyll chloroplasts, where it is processed to PEP. PEP is transported out in the cytoplasm, where it is used for accepting second molecule of CO_2.

In many respects PEP-carboxykinase (PEP-CK) species are similar to NAD.ME. However, unlike in NAD-ME species, in the bundle-sheath cells oxaloacetate is decarboxylated by **PEP-carboxykinase** to produce PEP and CO_2 in the chloroplasts. PEP is transported back to cytoplasm, where it undergoes transamination and the resulting alamine is transported to mesophyll cells. In mesophyll cell chloroplasts it is converted to PEP as in NAD-ME species.

It is thus apparent that in all the C_4 plants, the CO_2 reduction is primarily resticted to bundle-sheath chloroplasts and mesophyll cells are complementing the functions of bundle-sheath cells by providing a C_4 acid which serves as a source of CO_2 and in some cases of reducing power. By shuttling the CO_2 in the form of organic acids it is possible to build much higher CO_2 concentrations in the bundle sheath cells. Results of studies employing radiolabeled $^{14}CO_2$ have indicated the concentration of CO_2 in bundle-sheath cells may reach 60 μm, which is about 10-fold high than that in C_3 plants. Higher CO_2 concentration would suppress photorespiration and support higher rates of photosynthesis. Under optimal conditions, C_4 crop species can assimilate CO_2 at rates two to three times that of C_3 species. However, all this productivity does not come 'free'. For every CO_2 build up in the bundle-sheath cells, there is an energy cost of two ATP for the regeneration of PEP. This is in addition to the ATP and NADPH required in the Calvin cycle. Thus the net energy requirement for assimilation of CO_2 by the C_4 cycle is five ATP and two NADPH.

8.7 Kranz syndrome or C_4 syndrome

Plants displaying C_4 metabolism possess certain unique characteristic features. These include the specialized leaf anatomy (Kranz anatomy), formation of C_4 acids as first photosynthetic products, low CO_2 compensation point, low degree of discrimination against ^{13}C, insensitivity of net photosynthesis to O_2 at and below natural levels, lack of photorespiration, higher temperature optimum and low transpiration ratio. These anatomical, physiological

and biochemical characteristics that are used to distinguish C_4 plants from C_3 plants, are collectively referred to as the **C_4 syndrome** or **Knanz syndrome**.

Kranz leaf anatomy

In C_4 plants such as maize and sorghum, the vascular bundles are surrounded by a tightly fitted layer of parenchyma cells known as bundlesheath. This tightly packed bundle sheath cells in turn are surrounded by more loosely arranged chloroplast containing mesophyll cells (Figure 8.9). Bundlesheath lacks intercellular spaces between the individual cells of the sheath and between the sheath cells and the enclosed vascular tissue. It also extends over the ends of the vascular bundles within the leaf tissue. Thus all substances moving between leaf mesophyll cells and leaf vascular tissue must necessarily pass through the bundle-sheath cells. Because of the wreath like configuration of these bundle sheath cells, originally observed by the German anatomist G.Haberlandt, this arrangement is known as **kranz anatomy** (German Kranz=Wreath). The bundle-sheath cells also contain other organelles such as mitochondria and peroxisomes in addition to large number of chloroplasts.

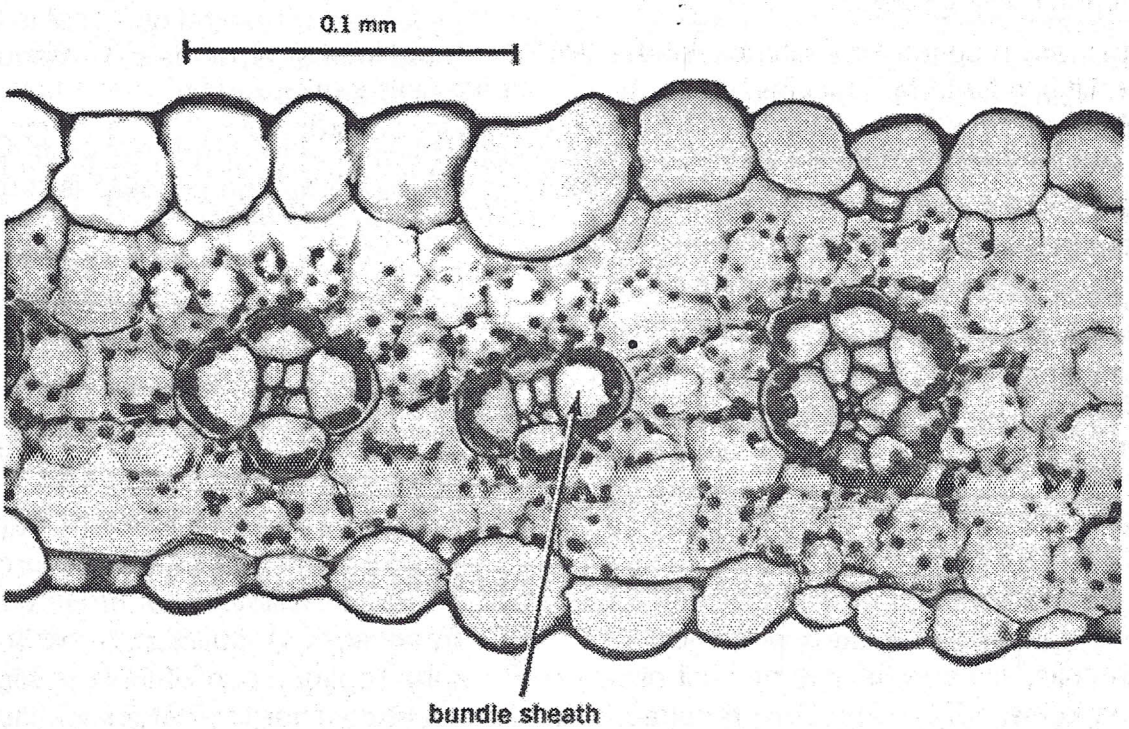


Figure 8.9 Cross section of a C_4 maize showing the arrangement of bundle sheath and mesophyll cells. Note high concentration of chloroplasts in bundle sheath cells.

The mesophyll and bundlesheath cells are in close contact with one another and are connected by plasmodesmata. Biochemical studies have revealed that leaf mesophyll chlo-

roplasts and bundlesheath chloroplasts contain specific unique arrays of enzymes. The C_4 pathway characterized by the presence of PEP carboxylase is located in the mesophyll cells and enzymes responsible for converting CO_2 to sugars, starch and other assimilation products by the Calvin cycle are located in the bundle-sheath cells.

The bundlesheath cells in leaves of C_3 plants are either completely absent or when present have fewer organelles (chloroplasts and mitochondria) than the surrounding cells or none at all.

Low CO_2 compensation point

C_4 plants have low CO_2 compensation concentration. This is measured by placing a shoot or a leaf in a small air tight glass chamber. Upon illumination with bright light, the CO_2 concentration in the chamber (monitored by infrared analyzer) will decrease because the initial rate of CO_2 fixation in photosynthesis is greater than the rate of CO_2 release through respiration. Starting with the natural concentration of CO_2 in air, (0.33 percent or 330 ppm) over a period of time, the concentration in the chamber will be reduced to a range between about 0 to 5 and 20 to 100 ppm CO_2 depending on the species. At this point, the rate of CO_2 fixation in photosynthesis just balances the rate of CO_2 released in respiration. That is CO_2 released in respiration is exactly compensated by CO_2 fixed in photosynthesis. This equilibrium CO_2 concentration is called the carbon dioxide **compensation point.**

The net photosynthesis is zero at the CO_2 compensation point. Net fixation of CO_2, therefore, occurs only when the CO_2 concentration in the external atmosphere is greater than the CO_2 compensation concentration. When measured at about 25°C and 21 percent O_2, C_3 plants have CO_2 compensation points in the range of 20 to 100 µl CO_2 while C_4 plants have values in the range of 0 to 5 µl CO_2 l^{-1}. So it can be said that there is a very little leakage of CO_2 into the external atmosphere from the C_4 plants.

Low degree of discrimination against ^{13}C

The C_4 plants have low degree of discrimination against ^{13}C than C_3 plants. ^{13}C and ^{12}C are the two stable nonradioactic isotopes of carbon and in combination with O_2 which are present as $^{13}CO_2$ and $^{12}CO_2$ in the atmosphere. If there is no pollution input into the atmosphere both $^{12}CO_2$ and $^{13}CO_2$ are present in the ratio of 98.8 percent and 1.1 percent, respectively. Both $^{12}CO_2$ and $^{13}CO_2$ are absorbed in photosynthesis so that it can be expected that photosynthates may contain ^{13}C and ^{12}C in the same ratio as they are in the atmosphere. However, this ratio of ^{13}C to ^{12}C in carbon containing constituents is slightly lower in tissues of terrestrial plants than in the external atmosphere. This discrimination against ^{13}C is that $^{13}CO_2$ has a higher mass (45) than $^{12}CO_2$ (44) and its rate of movement in the gas phase through stomatal pores and intercellular spaces in leaves as compared to that of $^{12}CO_2$ is relatively slow which accounts for the discrimination against $^{13}CO_2$ in photosynthesis. There is a difference between C_4 and C_3 plants with respect to this discrimination. The carbon containing compounds in C_4 plants are more enriched with ^{13}C than C_3 plants, that is $^{13}C/^{12}C$ ratios in C_4 plants are higher than those in C_3 plants. Isotopic compositions are expressed in $\delta^{13}C$ units.

An interesting consequence of this carbon isotope fractionation effect is that carbon materials in sugarcane (a C_4 plant) have higher $\delta^{13}C$ values (on an average = -14 parts per thousand) than those in a C_3 sugar beet (-28 parts per thousand). Thus table sugar (i.e purified sucrose) manufactured from each of these two plants can be distinguished by carbon isotope ratio analysis even though they are chemically identical.

Insensitivity of net photosynthesis to O_2 concentration at and below natural levels

Photosynthesis in C_3 plants is generally inhibited at O_2 levels above atmospheric concentrations (21%) while it is insensitive to O_2 concentrations even upto 100 percent, in C_4 plants. When the concentration of O_2 in the external atmosphere varies from very low levels to 21 percent or more, net photosynthesis in a C_4 plant remains substantially unchanged (Figure 8.10) where CO_2 concentration is held at 300 ppm and light conditions are optimal.

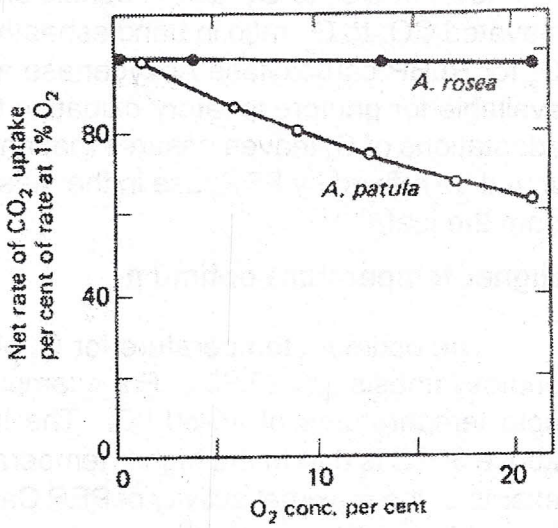


Figure 8.10 The effect of O_2 concentration on the rate of net photosynthetic uptake for a C_3 plant *Atriplex rosea* and a C_4 plant *Atriplex rosea*.

On the other hand, the rate of net photosynthesis in a C_3 plant is considerably reduced at 21 percent O_2, compared to the rate at about 2 percent. This inhibitory effect of 21 percent O_2 in C_3 plants is produced rapidly and is fully reversible, that is it disappears completely when the O_2 concentration decreases from 21 to 2 percent. This inhibition of photosynthesis by O_2 was discovered by the German biochemist O.Warburg in 1920 and is now referred to as the **Warburg effect**. The principal reason of Warburg effect is that CO_2 and O_2 compete for the same reactive site on the enzyme RuBP carboxylase which fixes the CO_2 in the Calvin cycle. This enzyme not only reacts with CO_2 but also with O_2. Thus O_2 is a competitive inhibitor with respect to CO_2 in the carboxylase reaction and CO_2 is a competitive inhibitor with respect to O_2 in the oxygenase reaction.

$$\text{RuBP} + CO_2 \xrightarrow{\text{RuBPCase}} \text{(2 3-phosphoglycerate}$$

$$\text{RuBP} + O_2 \xrightarrow{\text{RuBP oxygenase}} \text{2-phophoglycolate + 3-phosphoglycerate}$$

Further, the RuBP oxygenase generated glycolic acid formed after the dephosphorylation of 2-phosphoglycolate, serves as a substrate for the photorespiration and results in photorespiratory CO_2 release. Because of the lack of Warburg effect as well as photorespiratory CO_2 release, C_4 plants are more productive than do C_3 plants.

Lack of photorespiration

C_4 plants have low rates of photorespiration and high rates of net photosynthensis. This is because that mosophyll cells in C_4 plants function as a bicochemical pump, transporting CO_2 from the external atmosphere to the bundle sheath cells resulting in the build up of high level of CO_2 to O_2 ratio in bundle sheathcells than it is in the external atmosphere. An elevated CO_2 to O_2 ratio in bundlesheath cells permits CO_2 to compete more effectively with O_2 for RuBP carboxylase / oxygenase and thereby decreases the quantity of glycolic acid available for photorespiratory oxidation to O_2. In addition, the anatomical and biochemical adaptations of C_4 leaves ensures that any photorespired CO_2 release in bundle-sheath cells would be refixed by PEPcase in the mesophyll cells, before it has the opportunity to escape from the leaf.

Higher temperature optimum

The optimum temperature for C_4 photosynthesis is higher (30-45 °C) compared to C_3 photosynthesis (20-25 °C). For example, **Tidestromia oblongifolia**, a C_4 plant withstand upto temperatures of 45-50 °C. The high activity of C_4 photosynthesis at temperatures above 30 °C is due to the higher temperature stability of some of the C_4 cycle enzymes. For example, the maximal activity of PEP Case is in the range 30-35 °C compared with 20-25 °C for Rubisco. As a result, the quantum yield of photosynthesis in C_3 plants tends to decline with increasing leaf temperature while the quanturn of yield of C_4 plants remains essentially constant. The decline in quantum yield in C_3 plants is due to decreased carboxylation activity of Rubisco at the higher temperatures. This is because, the solubility of gasses in solution decreases with increasing temperature, but the solubility of CO_2 is affected more than the solubility of O_2. Consequently, higher temperatures increasingly favor oxygenation by Rubisco.

On the other hand, the C_4 photosynthesis decreases considerably on lowering the temperatures (below 12 to 15°C) and this has been attributed to the cold sensitivity of pyruvate phosphate dikinase which is essential for the regeneration of PEP in all types of C_4 plants.

Transpiration ratio

C_4 plants are characterized by low transpiration ratios when compared to C_3 plants. That is plants with C_4 photosynthesis generally transpire less water per molecule of CO_2 fixed. Transpiration ratios for C_4 plants are typically in the range of 200 to 350 while for C_3 plants this range is in between 500 and 1000. The low transpiration ratio for C_4 plants reflects their capacity to maintain high rates of photosynthesis while effectively conserving water.

The C_4 syndrome therefore confers a definite advantage to the C_4 plants particularly at situations where high temperature, high light and low water are prevailed.

8.8 Crassulacean Acid Metabolism (CAM)

Crassulacean acid metabolism (CAM) is another CO_2 concentrating mechanism traditionally illustrated by succulent plants from arid, semi-desert regions whereby nocturnal stomatal opening, CO_2 uptake, and malic acid storage are associated with reduced transpiration and improved water economy. CAM is so named because it was originally studied most extensively in the family crassulaceae. It is not only restricted to the family crassulaceae (**Crassula**, **kalanchoe**, **Sedum**) but also found in 23 different families of flowering plants including cactaceae, agavaceae, orchidaceae, portulacaceae, bromeliaceae, euphorbiaceae, liliaceae, aizoaceae, cucurbitaceae, asteraceae, asclepiadaceae, one family of ferns, the polypodiaceae and in the primitive plant *Welwitschia*. With the exception of crassulaceae and cactaceae, most families, however, are not exclusively CAM. Most families will have C_3 representatives as well and some are known to contain all three photosynthetic patterns: C_3, C_4 and CAM. There are only a few CAM plants classified as domestic crops, these include pineapple, agave, vanilla and pickly pear.

Anatomically CAM plants differ from C_4 and C_3 plants in having a succulent habit. The leaves and frequently stems and petioles of CAM plants are fleshy or succulent. The cells in these tissues contain large water-filled vacuoles in addition to chloroplasts and other organelles. As far as is known, all CAM plants possess the succulent habit. Although succulence appears to be prerequisite for CAM, but all succulents do not exhibit CAM (halophytes such as Salicornia and Borrichia).

Another important characteristic feature of CAM plants is their inverted stomatal cycle. They open their stomata during the cool, dessert nights and close them during the hot, dry days. This pattern of stomatal opening and closing by CAM plants is the reverse of that displayed by other plants, where the stomata are open during the day and closed at night. The CAM plant pattern of stomatal behavior thus permits these plants to achieve high degree of water conservation which is essential for the survival of CAM plants in extremely dry, or xerophytic habitats.

The inverted stomatal cycle of CAM plants also permits them to display a diurnal pattern of organic acid formation. During the night the total malate content of leaves increases and pH of the leaf cell sap decreases whereas during the day the malate content decreases and the pH of leaf cell sap increases. This diurnal pattern of acidification at night and deacification during the day is accompanied by the changes in the leaf storage carbohydrate that fluctuate inversely with malate levels.

Path of carbon

Nocturnal stomatal opening allows the CO_2 to enter into the leaf cells from the surrounding atmosphere. As in C_4 plants, the phosphoenol pyruvate carboxylase catalyzes the addition of CO_2 as HCO_3^- to the phosphoenol pyruvate (PEP) to form oxaloacetate. The oxaloacetate is then reduced to malate by NAD dependent malate dehydrogenase. The malate accumulates and is stored in the large vacuoles The phosphoenolpyruvate shown in figure 8.11 originates from the breakdown of starch and other sugars by the glycolytic pathway.

With the onset of day, the stomata closed and preventing loss of water and further uptake of CO_2. In this condition, the stored malate is retrieved from the vacuoleinto the cytosol where it is decarboxylated to CO_2 and pyruvate by NAD malic enzyme (in case of crassulaceae). Because the stomata are closed, the internally released CO_2 cannot escape from the leaf and instead it diffuses into the chloroplast where it is converted to carbohydrate by the Calvin cycle. The fate of pyruvate resulting from decarboxylation is uncertain, but the weight of evidence is that it is reduced to triose phosphate, which in turn can be converted back to glucose or starch by reversal of glycolysis.

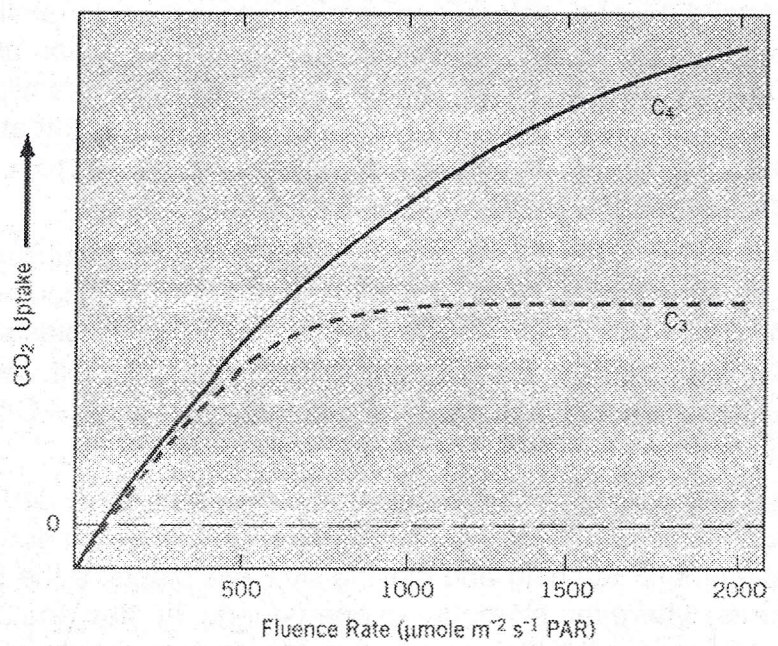


Figure 8.11 Crassulacean acid metabolism. Above: curves illustrating stomatal opening, CO_2 uptake, and changing acid content of cell vacuoles over a 24 hour period. Stomata open in the dark to admit CO_2 and close during the day to conserve water. Below, right; while the stomata are closed during the day, stored CO_2 is released to be assimilated via the calvin cycle.

The enzyme PEP carboxylase which is central to CAM pathway exists in two forms: active and inactive. The active form that operates at night is insensitive to malate while the inactive form that can not operates during the day time is inhibited by low concentrations of malate. The two forms are interconverted via phosphorylation by specific protein kinase (Figure 8.12). The night active form which is insensitive to malate is present in phosphorylated form to synthesize malate whereas inactive day form which is sensitive to malate is present in dephosphorylated form during the decarboxylation of malate.

It should be noted that CO_2 assimilation in both C_4 and CAM plants involves the Calvin cycle and the C_4 cycle. In both cases, the C_4 cycle produced C_4- acids are subsequently decarboxylated to provide CO_2 for the Calvin cycle. However, in C_4 plants the two cycles

are separated by different leaf cells: the C_4 enzymes in mesophyll cells and C_3 enzymes in bundle sheath cells. In CAM plants, both pathways are found in the same mesophyll cells, but the operation of the two pathways is separated in time. In addition, there is no closed cycle of carbon intermediates in CAM as there is in C_4 plants.

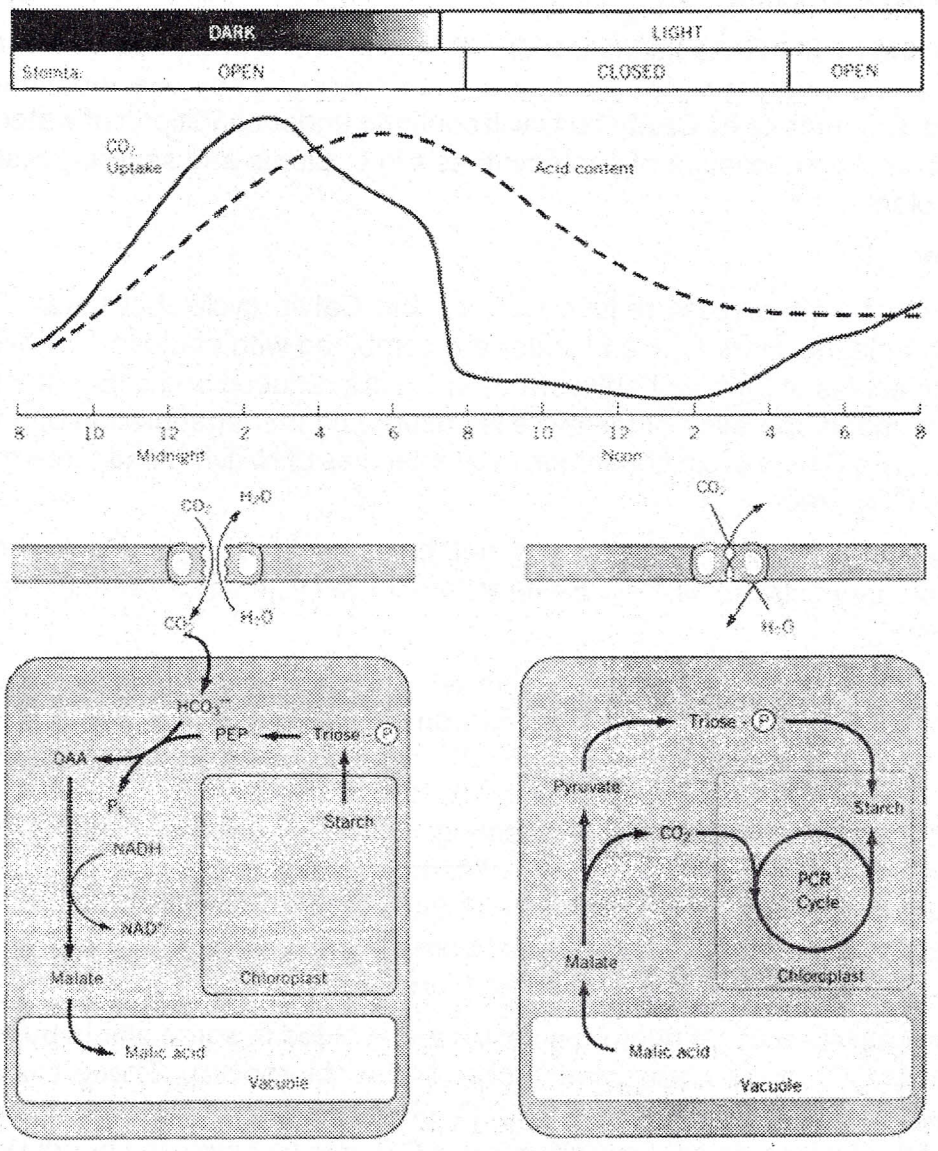


Figure 8.12 Diurnal regulation of CAM phosphoenolpyrurate (PEP) carboxylase by phosphorylation and dephosphorylation of its specific serine residue.

Ecological significance of CAM

Many CAM plants are true desert plants, growing in shallow, sandy soils with little available water. Nocturnal stomatal opening in them allows CO_2 uptake during periods where

evaporative water loss are at minimum. Then during the day light hours when the stomata are closed to reduce water loss, photosynthesis can proceed by using the reservoir of stored CO_2. This interpretation is supported by the transpiration of CAM plants. Typically a CAM plant loses 50 to 100g of water for every gram CO_2 gained, compared with values of 250 to 300 and 400 to 500 g for C_4 and C_3 plants, respectively. Thus CAM plants have competitive advantage on dry environments.

Although the CAM mechanism enables plants to improve water use efficiency, its rates of photosynthesis are very low that are only one half that of C_3 and one-third that of C_4. On the other hand, CO_2 uptake by CAM plants will continue under conditions of water stress that would cause complete cessation of photosynthesis in C_3 plants and severely restrict carbon uptake by C_4 plants.

8.9 Summary

Photosynthetic eukaryotes reduce CO_2 via the Calvin cycle that takes place in the stroma of chloroplasts. Here CO_2 and water are combined with ribulose-1, 5-bisphosphate to form two molecules of 3-phosphoglycerate, which are reduced and converted to carbohydrate. The continued operation of the cycle is ensured by the regeneration of ribulose-1, 5-bisphosphate. The Clavin cycle consumes two molecules of NADPH and three molecules of ATP for every CO_2 fixed.

Photosynthesis, like all other complex metabolic reactions, is subject to regulation. In this case the primary activator is light. Several key Calvin cycle enzymes, including Rubisco, are light activated.

Plants that utilize the Calvin cycle exclusively for carbon fixation also exhibit a competing process of light and oxygen dependent carbon dioxide evolution called photorespiration or photosynthetic carbon oxidation cycle (PCO). The PCO cycle also begins with Rubisco, which in the presence O_2, catalyzes the oxidation to form one molecule of 3-PGA plus one 2-carbon molecule, phosphoglycolate. Phosphoglycolate is subsequently metabolized in a series of reactions that result in the release of carbon dioxide and recovery of the remaining carbon by the Calvin cycle. The role of PCO cycle is not yet clear, although it has been suggested that it helps protect the chloroplast from photooxidative damage during periods of moisture stress, when stomata are closed and the carbon dioxide supply is cut off.

The dissipative effects of photorespiration are avoided in some plants by mechanisms that concentrate CO_2 at the carboxylation sites in the chloroplast. These mechanisms include C_4 – pathway and CAM metabolism. C_4 plants exhibit a division of labour between mesophyll cells, which contain C_4-cycle to reduce CO_2 into C_4 acids and bundle sheath cells, which contain Calvin cycle that actually fix carbon. A set of characteristic features collectively called C_4-syndrome allow C_4 plants to maintain higher rates of photosynthesis at lower carbondioxide levels, high light intensity and higher O_2 concentrations.

Crassulacean acid metabolism (CAM) is also a means of maintaining higher rates of photosynthesis in habitats with little available water. CAM plants exhibit an inverted stomatal cycle, opening for CO_2 uptake at night and closing during the day. The CO_2 is stored

as malate, again through the action of PEP carboxylase. Decarboxylation during the day furnishes the necessary CO_2 for photosynthesis.

8.10 Model questions

1. Discuss how C_3 photosynthesis will be more effective in C_4 plants.
2. Describe the main differences between C_3 and C_4 plants.
3. What is kranz syndrome in photosynthesis? Explain how this phenomenon confers greater photosynthetic efficiency in such plants?
4. Write short notes on :
   a. Kranz anatomy and its significance
   b. Path of carbon in CAM plants
   c. Photorespiration

8.11 Reference Books

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Dr. G. Rosaiah

LESSON: 9

RESPIRATION I : GLYCOLYSIS, PENTOSE PHOSPHATE PATHWAY AND CITRIC ACID CYCLE

9.0 Objective

In this lesson an overview of plant respiration, oxidative breakdown of respiratory substrate by glycolysis and citric acid cycle, alternative pentose phosphate pathway for glucose break down, and use of glycolytic and cictric acid cycle intermediates in the biosynthesis of cellular constituents are discussed.

9.1 Introduction

Living organisms, be it man, plant or microbe require a continued free energy for performance of mechanical work, the active transport of molecules against concentration gradients and the biosynthesis of complex molecules. The ultimate source of most biological energy is the visible sun light. Light energy is trapped and converted to chemical energy primarily as the reduced organic compounds by the green plants during photosynthesis. The chemical energy of organic compounds such as carbohydrates, lipids and proteins is released and stored as usable energy (kinetic energy) upon their oxidation in the process of respiration. In respiration these organic compounds are oxidized in a controlled manner with the release of free energy and its incorporation into a form called adenosine triphosphate (ATP). ATP is the cell's practical form of energy currency that can be readily utilized for the maintenance and development of the plant.

There are two types of respiration. One of these in which oxygen acts as the final electron ceptor is known as aerobic respiration. During this process respiratory substrates are oxidized com-

pletely resulting CO_2, H_2O and ATP as an end products. All higher plants carry out aerobic respiration. The second type that takes place in the absence of oxygen and causing incomplete breakdown of respiratory substrates to ethyl alcohol (or lactic acid), CO_2 and energy is known as anaerobic respiration. Generally anaerobic respiration takes place in the deep seated tissues, in germinating seeds, fruits and microorganisms.

9.2 Overview of plant respiration

Plants lead predominantly aerobic life. They obtain the energy needed to drive various cellular activities and carbon skeltons essential in the construction of cellular constituents by oxidizing photoassimilates. The most readily utilized photosynthate in the respiration is a six carbon sugar glucose. The summary equation for aerobic respiration with glucose as a substrate is as follows

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 12\ H_2O - 2880\ kJ \text{ or } 686\ k.cal/mol^{-1}$$

This equation is the reversal of photosynthesis.

$$6CO_2 + 12\ H_2O \xrightarrow[\text{chloroplast}]{nh\nu} C_6H_{12}O_6 + 6O_2 + 6H_2O$$

The photosynthetic equation is an endergonic reaction in which light energy is used to reduce the CO_2 to carbohydrate, with water as the ultimate source of electrons, while the respiratory equation should be understood as an exergonic reaction since energy is released by the oxidation of glucose to carbon dioxide, with water as an end product. Further, both the processes differ in the nature of enzymes involved and in their occurrence in different organelles in the cell. It is generally agreed that the rate of photosynthesis is at least 20 times higher than that of respiration. and is confined to chloroplast containing cells while respiration is carried out by all living cells in the plant.

The break down of glucose during respiration releases large amount of free energy i.e. nearly 2880 kJ (686 k. cal) per mole (180g) of glucose. If this total amount of energy is released all at once in a single step of oxidation, the cellular structures would be damaged (incineration). In order to prevent such damage, plants have evolved a mechanism of multistep process in which glucose is oxidized in a series of reactions that include phosphorylation, hydration, oxidation, decarboxylation, isomerization, group transfer, and cleavage reactions. These reactions as a whole can take place in three stages (Figure 9.1).

1) **Glycolysis**
2) **The tricarboxylic acid cycle** and
3) **The electron transport chain**

Glycolysis is the common pathway by which glucose is degraded to pyruvic acid in a series of reactions catalyzed by a group of soluble cytosolic enzymes in the living cells.

Pyruvic acid produced by glycolysis is oxidized completely to CO_2 with concomitant formation of reducing power NADH in a sequence of reactions referred to as the **citric acid cycle** (CCA). Except succinate dehydrogenase reaction all other CCA cycle reactions catalyzed by enzymes are located in the aqueous matrix of the mitochondrion. Succinate dehydrogenase is localized on the inner mitochondrial membrane.

NADH oxidation by O_2 is the final stage in the respiratory metabolism of glucose. In this stage, NADH is oxidized via a series of electron transport proteins referred to as the **electron transport chain**. This system is located in the inner mitochondrial membrane where it catalyzes the transfer of electrons from NADH to O_2. This electron transfer is accompanied by the conservation of free energy as ATP.

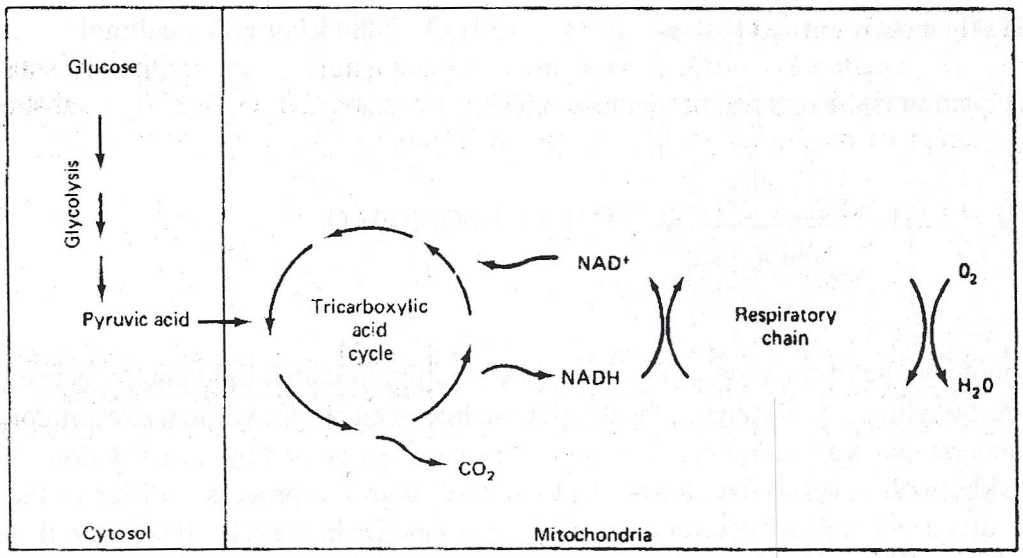


Figure 9.1 The three major stages of biological oxidation in plant cells.

Although the main function of respiration is to provide cellular source of energy, but it also supplies the carbon skeltons to be used for the synthesis of nucleic acids, proteins, cellulose and other cellular molecules. Accordingly not all the carbon that enters the respiratory pathway ends up as CO_2.

8.3 Respiratory substrates

A respiratory substrate is any organic plant constituent that can be partially broken down or oxidized completely to CO_2 and H_2O in a respiratory pathway. Carbohydrates are the principal respiratory substrates in plants and commonest among them are starch and sucrose. Starch is a polymer of glucose and serves as a glucose storage reserve in plants. Glucose is released from starch through hydrolysis by the enzyme amylase. Sucrose is a soluble disaccharide sugar and is converted into glucose and fructose upon hydrolysis by invertase enzyme. In addition to carbohydrates other substances such as fats, organic acids and to some extent proteins serve as respiratory substrates in some plant organs under certain physiological conditions.

Fats are storage reserves in certain seeds such as castor bean, groundnut and mustard. At the time of seed germination major portion of fat is converted into sucrose which is subsequently absorbed and respired by the growing embryo.

Under certain circumstances, organic acids may also be used as respiratory substrates in some plant tissues. For example, succulent plants that accumulate malic acid in their leaves when kept continuously in the dark for about 2 to 3 days respire malic acid completely to CO_2 and H_2O via a CCA cycle.

Proteins and amino acid derivatives serve normally as respiratory substrates only in seeds rich in proteins. The vegetative cells consume protein only during starving conditions. Otherwise proteins are seldom respired.

9.4 Respiratory quotient (RQ)

Respiratory Quotient is the parameter indicating the nature of the respiratory substrate. It is obtained by measuring the relative amounts of O_2 consumed and CO_2 evolved by a substrate which is under oxidation process according to the following formula:

$$RQ = \frac{\text{Moles of } CO_2 \text{ evolved}}{\text{Moles of } O_2 \text{ consumed}}$$

The magnitude of the RQ of a tissue depends on the oxidation state of the substrate utilized in respiration. When carbohydrate is being respired, the volume of CO_2 evolved is equal to the volume of O_2 absorbed as is evident from the equation given below. In this case the RQ is found to be unity.

$$C_6H_{12}O_6 + 6\,O_2 \longrightarrow 6\,CO_2 + 6\,H_2O$$

$$RQ = 6\,CO_2 / 6\,O_2 = 1$$

Organic acids are more oxidized than carbohydrates. When organic acids are used as respiratory substrate the RQ value may be more than one.

$$C_4H_6O_5 + 3\,O_2 \longrightarrow 4\,CO_2 + 3\,H_2O$$

$$RQ = 4\,CO_2 / 3\,O_2 = 1.33$$

Further, lipids and proteins are poorer in oxygen i.e. they are highly reduced. The proportion of oxygen to carbon in them is very low compared to carbohydrate. Hence they consume more oxygen for complete oxidation. In such cases the RQ value may be less than one.

$$C_{51}H_{98}O_6 + 145\,O_2 \longrightarrow 102\,CO_2 + 98\,H_2O$$

$$RQ = \frac{102\,CO_2}{145\,O_2} = 0.7$$

RQ values therefore provide information regarding the nature of the respiratory substrate being used.

9.5 Glycolysis

Glycolysis (from the Greek glykos – Sweet, lysis = Splitting) is the first stage in the respiratory metabolism of carbohydrates. It consists of a series ten enzymatic reactions that convert glucose into pyruvate with the generation of 2 mol of ATP per mol of glucose. This pathway takes place in the cytoplasm of all living organisms. The component reactions and enzymes of this pathway was first elucidated during early part of the twentieth century in yeast and mammalian skeletal muscle, largely by Embden, Meyerhof and Parnas. Hence it is also called EMP pathway in recognition of these three German biochemists.

The pathway as a whole may be divided into two parts. The first five of its reactions constitute the *preparatory phase* in which glucose is phosphorylated and cleaved to yield two molecules of trioses, an aldo triose glyceraldehyde-3-phosphate and keto triose dihydroxy acetone phosphate. This stage of glycolysis utilizes two ATP. The remaining five reactions of the pathway are referred to as *payoff phase*. In this phase two molecules of triose are converted to pyruvate with concomitant generation of four ATP. Glycolysis therefore has a net "profit" of two ATP per glucose (payoff phase minus preparatory phase). The overall reaction is

$$\text{Glucose} + 2\ NAD^+ + 2\ ADP + 2Pi \longrightarrow 2\ NADH + 2\ \text{pyruvate} + 2\ ATP + 2\ H_2O + 2\ H^+$$

The individual reactions of the glycolytic pathway are shown in figure 9.2. In plants the pathway usually beings with glucose as a substrate derived from various complex carbohydrates as shown in the figure 9.2a.

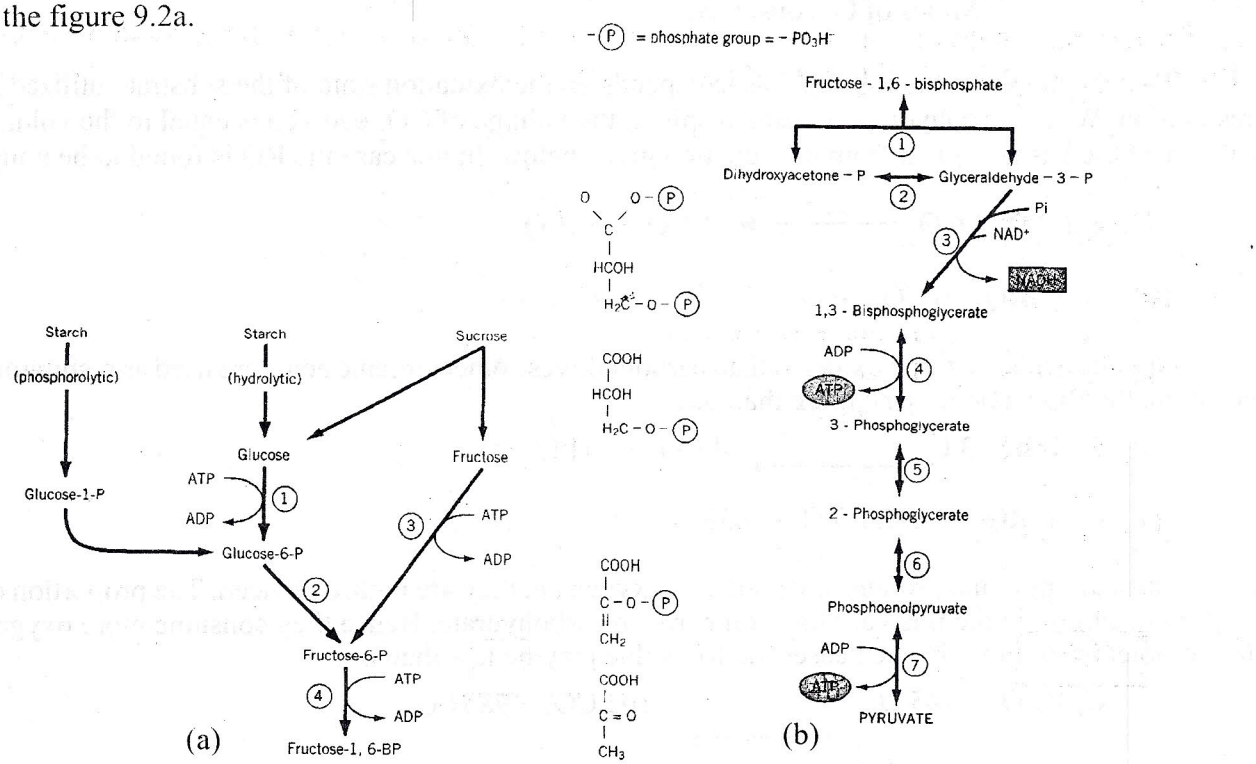
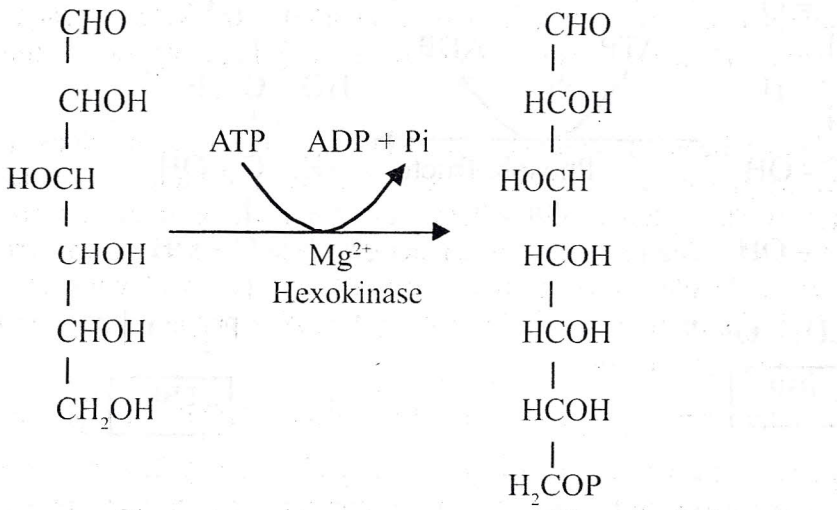


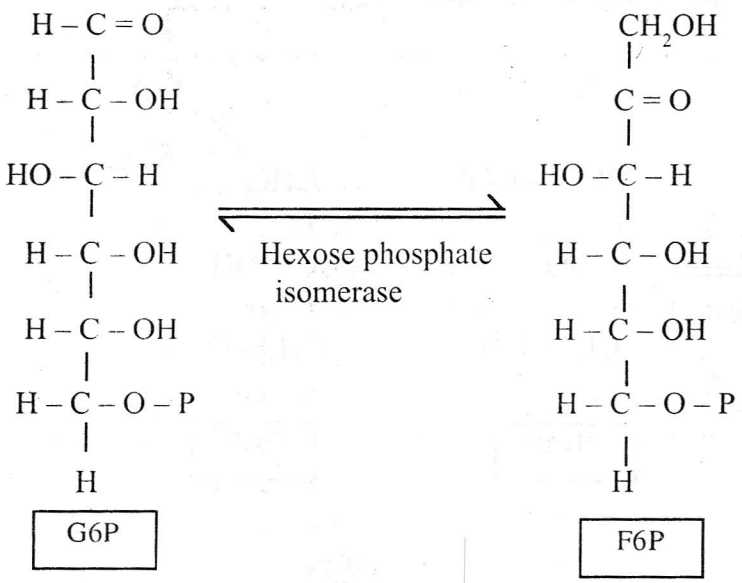
Figure 9.2 Reactions of glycolysis. (a) The conversion of storage carbohydrate to fructose-1, 6-bisphosphate (FBP). Enzymes are 1, hexokinase; 2, hexosephosphate isomerase; 3, fructokinase; 4, phosphofructokinase. All reactions are reversible, except for the conversion of F6P to FBP. (b) The conversion of FBP to pyruvate. Enzymes are ', aldolase; 2, triosephosphate isomerase; 3, glyceraldehydephosphate dehydrogenase, 4, phosphoglycerate kinase; 5, phosphoglycerate mutase; 6, enolase, 7, pyruvate kinase.

1) In the first step of glycolysis, glucose is primed for subsequent reactions by its phosphorylation at C-6 to yield glucose-6-phosphate. ATP is the phosphate donor. This reaction which is irreversible under intracellular conditions is catalysed by **hexokinase**. Hexokinase requires Mg^{2+} for its activity.

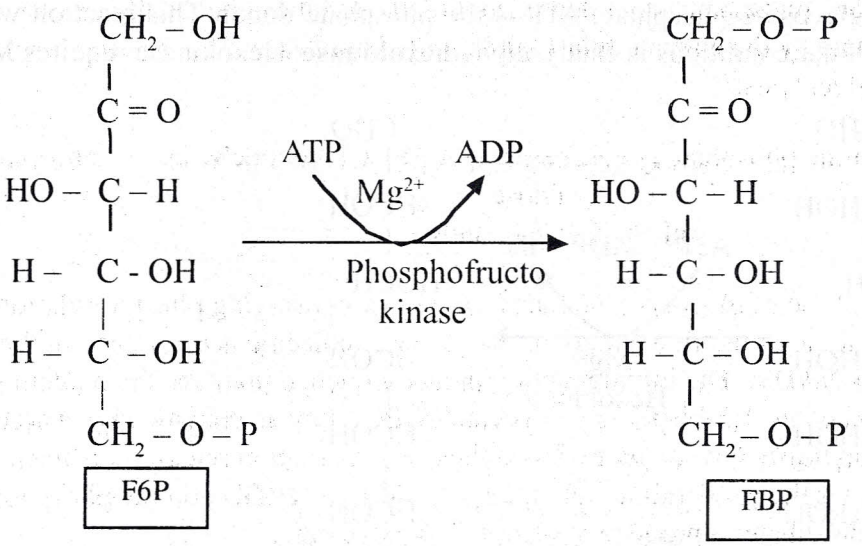


Glucose Glucose -6-phosphate

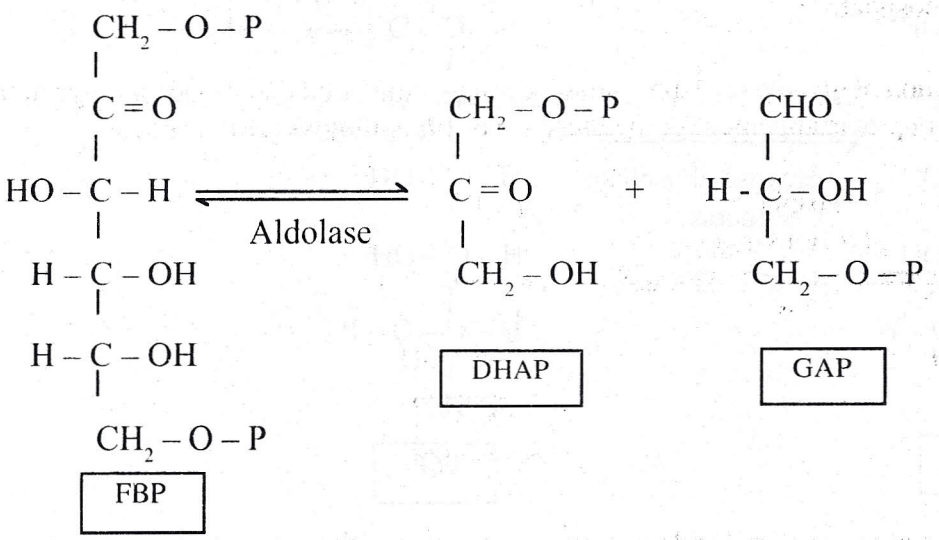
2) The next step in glycolysis is the isomerization of glucose-6-phosphate to fructose-6-phsophate. This reaction is catalyzed by **hexose phosphate isomerase**.



3) A second phosphorylation reaction follows the isomerization in which fructose-6- phosphate is phosphorylated by ATP to form fructose-1,6-bisphosphate. This compound was formerly known as fructose-1,6-diphosphate. Bisphosphate means two separate phodphate groups, whereas diphosphate means two separate phosphate groups joined by an anhydride bond. Hence, the name fructose-1,6-bisphosphate should be used. This reaction is irreversible and is catalyzed by **phosphofructo kinase**.



4) Fructose-1,6-bisphosphate is then cleaved to form two different triose phosphates namely glyceraldehyde-3-phosphate (GAP) and dihyroxyacetone phosphate (DHAP). The enzyme **fructose-1,6-bisphosphate aldolase** often simply called aldolase catalyses this reversible aldol condensation. Further, this is the "lysis" step that gives the process its name.

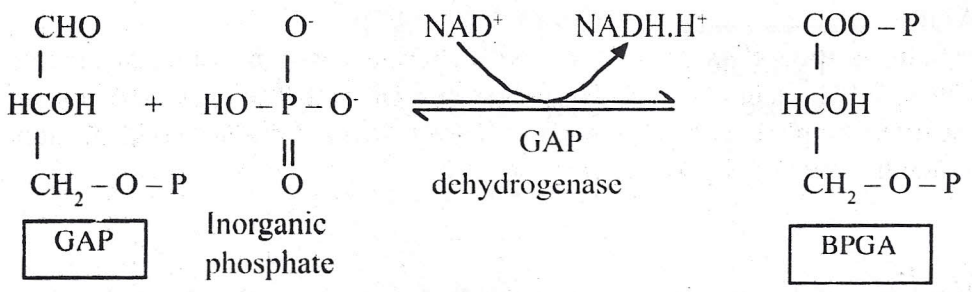


5) Glyceraldehyde -3-phosphate is directly used in the subsequent reactions of glycolysis, whereas dihydroxyacetone phosphate is not degraded directly and it can be readily converted into glyceraldehyde-3-phosphate by the enzyme **triose phosphate isomerase**. This reaction completes the preparatory phase of glycolysis.

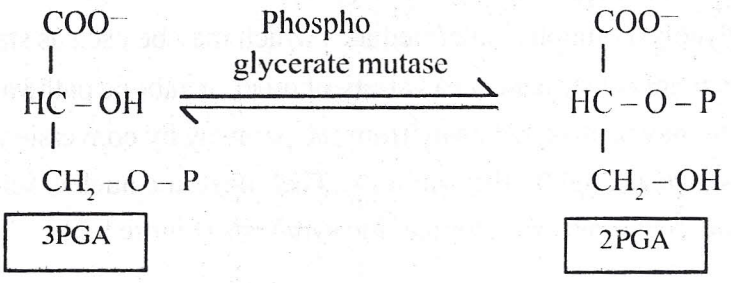
Dihydroxy acetone phosphate $\rightleftharpoons$ Glyceraldehyde - 3 - phosphate

Triose phosphate isomerase

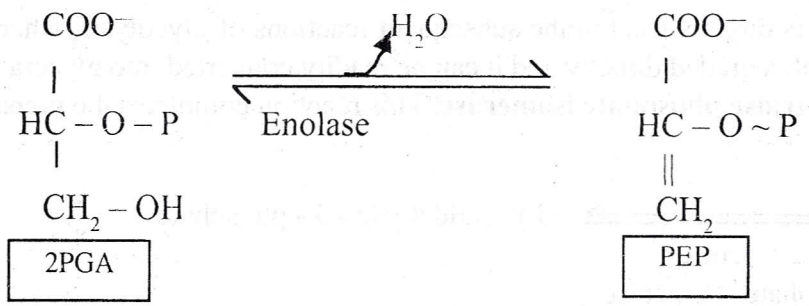
6) The payoff phase of glycolysis includes the energy conserving phosphorylation and oxidation steps in which some of the free energy of the glucose molecule is conserved in the form of ATP and reducing power NADH. The initial reaction in this sequence involves the oxidation of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate (BPGA) by an enzyme **glyceraldehyde-3-phosphate dehydrogenase**. Glyceraldehydes-3-phosphate oxidation releases sufficient amount of free energy that allows the concomitant reduction of NAD^+ to NADH and the phosphorylation of glyceraldehyde-3-phosphate to produce 1,3-bisphosphoglycerate.



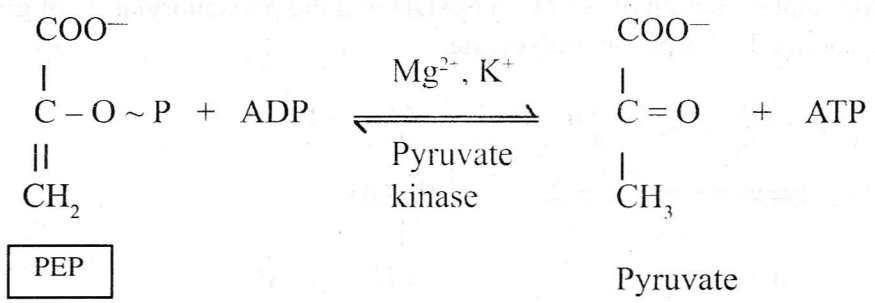
8) In the next reaction of glycolysis, 3-phosphoglycerate is converted into 2-phosphoglycerate (2PGA) by a phosphate group rearrangement in the presence of **phosphoglycerate mutase.**



9) 2-phosphoglycerate loses a molecule of water in presence of the enzyme **enolase** and is converted to phosphoenol pyruvate (PEP).



10) The last step in glycolysis is the transfer of the energy rich phosphate group from phospho-enolpyruvate to ADP. This reaction as catalyzed by **pyruvate kinase** results in the formation ATP and pyruvate.



Pyruvate is an end product of the glycolysis. In the overall process one molecule of glucose is converted into two molecules of pyruvate with 2 ATP and 2 NADH as the net energy yield.

9.5.1 Role of glycolysis in biosynthetic approach

In addition to energy release, the glycolysis supplies intermediates which may be used as starting materials for the synthesis of complex plant constituents in a variety of other metabolic pathways. For example, glyceraldehyde-3-phosphate may be diverted away from the pathway by conversion to glycerol. In the same way glucose-6-phosphate is used for the synthesis of cellulose and nucleic acids, phosphenolpyruvate for aromatic aminoacids and pyruvate for alanine synthesis (Figure 9.3).

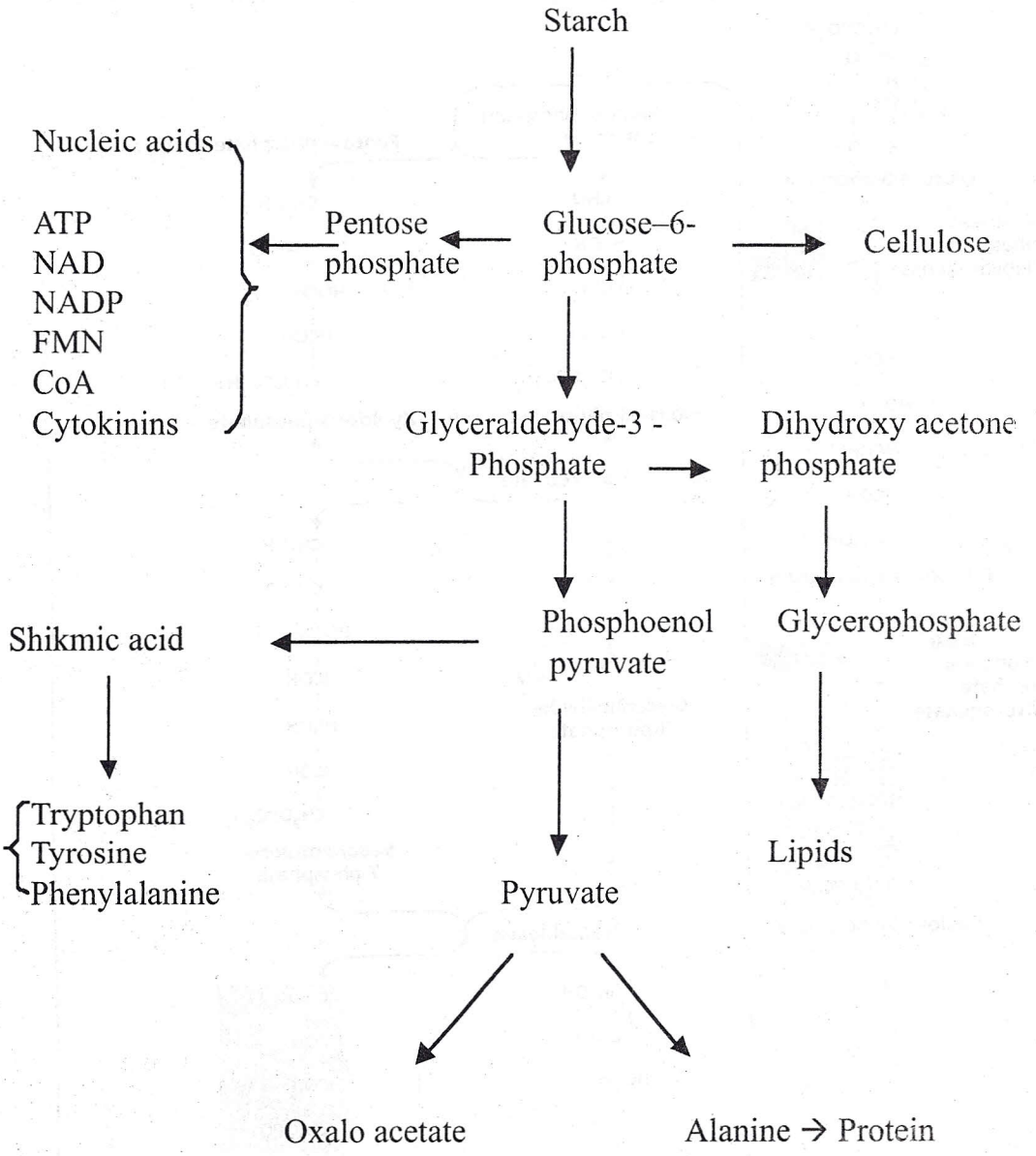


Figure 9.3 The role of glycolysis intermediates in biosynthesis.

9.6 The pentose phosphate pathway

Many hydrogenation reactions in biosynthetic pathways require NADPH as reducing agent and not NADH. As in animals and microorganisms, plants generate additional NADPH and precursors for nucleotide biosynthesis by an alternate route of glucose metabolism called **hexose monophosphate pathway, oxidative pentose phosphate pathway** or **phosphogluconic acid pathway.** It has been estimated that about 20 to 30 percent of the glucose being degraded flows into this pathway.

Figure 9.4 summarizes all of the reactions of the oxidative pentose phosphate pathway.

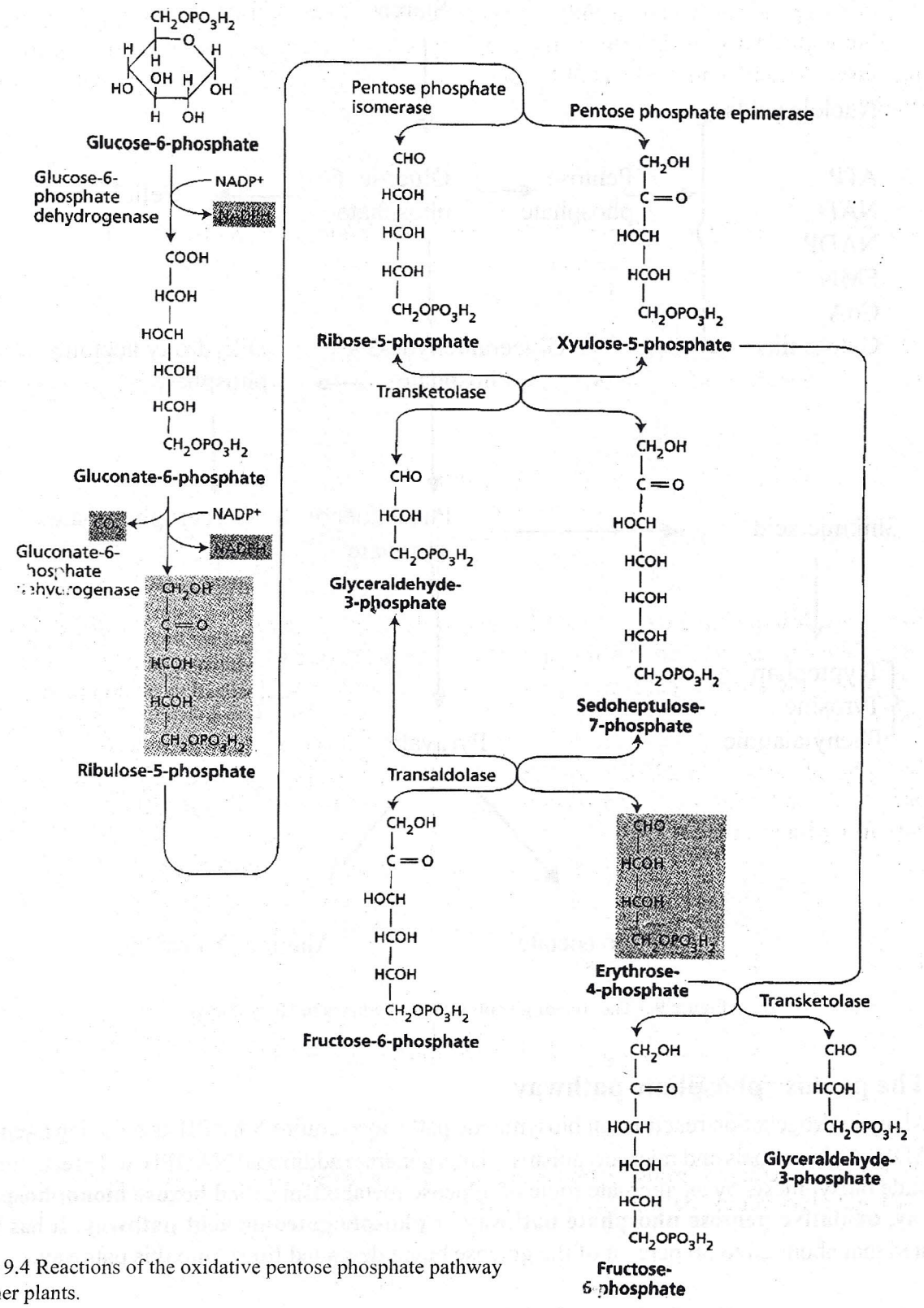
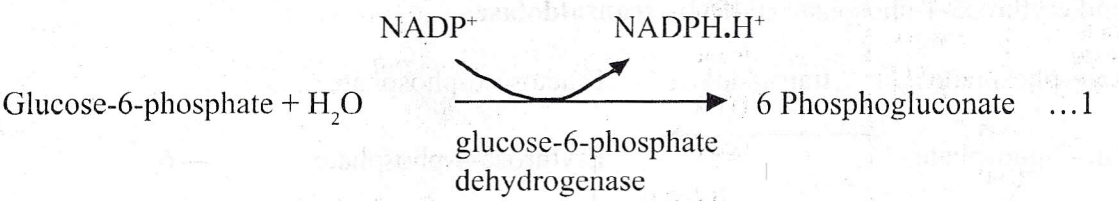
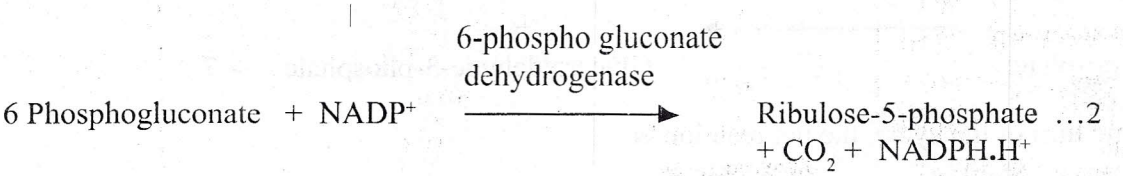


Figure 9.4 Reactions of the oxidative pentose phosphate pathway in higher plants.

1) The oxidative pentose phosphate pathway begins with the oxidation of glucose-6-phosphate to 6-phosphogluconate. This initial step catalyzed by $NADP^+$ - dependent **glucose-6-phosphate dehyrogenase** is sensitive to the level of $NADP^+$. That is the level of NADPH in the cell determines the operation of this cycle.

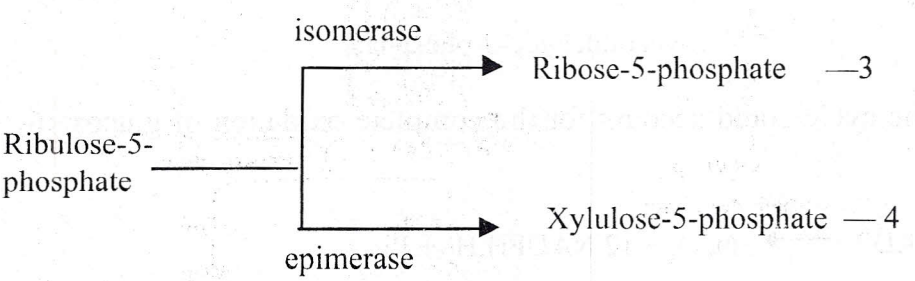


2) In the second reaction, 6-phosphogluconate undergoes oxidative decarboxylation catalyzed by an another $NADP^+$ specific **6-phosphogluconate dehydrogenase** to yield ribulose-5-phosphate and CO_2.



Both these reactions are oxidative and irreversible. Subsequent reactions of the pathway that include several isomerizations and group transfers are non-oxidative and reversible which convert the ribulose-5-phosphate into the pathway end products such as glyceraldehyde-3-phosphate and fructose-6-phosphate.

Ribulose-5-phosphate is maintained in equilibrium with two other phosphorylated pentoses ribose-5-phosphate (R5P) and xylulose-5-phosphate (Xu5P) by the action of **phosphoriboisomerase** and **phosphopentoepimerase**, respectively.



Transketolase converts ribose-5-phosphate and xylulose-5-phosphate to sedoheptulose-7-phosphate (S7P) and glyceraldehyde-3-phosphate (GAP).

Ribose-5-phosphate + Xylulose-5-phosphate $\xrightarrow{\text{transketolase}}$ Sedoheptulose-7-phosphate + Glyceraldehyde-3-phosphate — 5

Sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate are converted to fructose-6-phosphate (F6P) and erythrose-4-phosphate (E4P) by **transaldolase**.

Sedoheptulose-7-phosphate + Glyceraldehyde-3-phosphate $\xrightarrow{\text{transaldolase}}$ Fructose-6-phosphate + Erythrose-4-phosphate — 6

Finally, erythrose-4-phosphate reacts with xylulose-5-phosphate in the presence of **transketolase** to form fructose-6-phosphate and glyceraldehyde-3-phosphate.

Erythrose-4-phosphate + Xylulose-5-phosphate $\xrightarrow{\text{transketolase}}$ Fructose-6-phosphate + Glyceraldehyde-3-phosphate — 7

After one turn of the cycle, the net reaction is

Glucose-6-phosphate $\xrightarrow{2NADP^+ \rightarrow 2NADPH.H^+}$ Ribulose-5-phosphate + CO_2

Three turns of the pathway are required to produce one triose phosphate.

3 Glucose-6-phosphate $\xrightarrow{6NADP^+ \rightarrow 6NADPH.H^+}$ $3CO_2$ + 2 Fructose-6-phosphate + Glyceraldehyde-3-phosphate

Repetitive action of the cycle could account for the complete oxidation of glucose-6-phosphate.

Glucose-6-phosphate + 12 $NADP^+$ ⟶ $6CO_2$ + 12 $NADPH.H^+$ + Pi

Complete oxidation of glucose-6-phosphate by this pathway depends on the cells demand for NADPH requirement. Otherwise both fructose-6-phosphate and glyceraldehyde-3-phosphate can be metabolized via glycolysis.

9.7 The fate of pyruvic acid

Based on the presence or absence of oxygen, pyruvate produced in the glycolysis or indirectly through the oxidative pentose phosphate pathway may be oxidized to different metabolic products in

cells of higher plants. In aerobic tissues it is normally oxidized to CO_2 and H_2O. However, under some circumstances, for example, waterlogged or flooded soils, plant tissues may be subjected to hypoxic (low) or anoxic (zero) concentrations of O_2 and in such cases pyruvate is converted into CO_2 and ethyl alcohol or lactic acid.

Under anaerobic conditions both CCA cycle and electron transport chain can not function. As a result the ratio of NAD^+ to NADH in anaerobic tissues becomes very low. That is most of the cells NAD is present in reduced NADH. The continued oxidation of glucose however, requires oxidized NAD. In anaerobic cells, it is generated by forming ethanol or lactate.

The anaerobic breakdown of glucose to CO_2 and ethanol or lactate is called fermentation. Fermentation that converts pyruvate into ethanol is called **alcohol fermentation** or into lactate is referred to as **lactic acid fermentation**. In alcohol fermentation, the glycolysis product pyruvate is converted into ethanol and CO_2 through the action of **pyruvate decarboxylase** and **alcohol dehydrogenase** (ADH), while in lactic acid fermentation it is changed to lactate by the action of **lactate dehydrogenase** (LDH) as shown in the Figure 9.5.

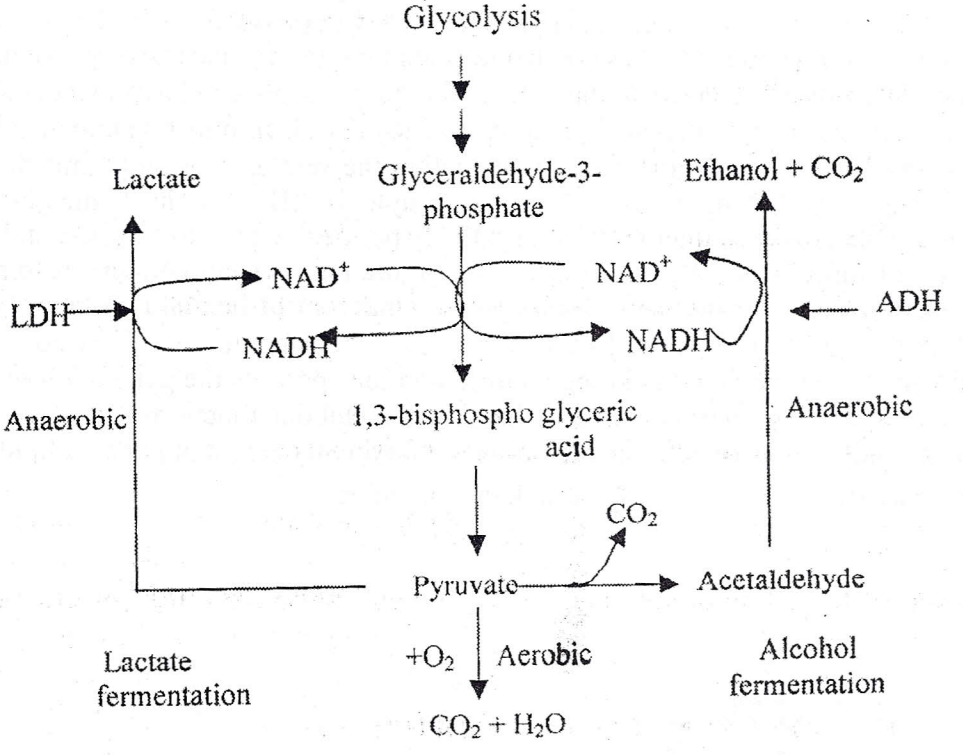


Figure 9.5 Fate of pyruvate in oxygenic and anoxygenic conditions. ADH, Alcohol dehydrogenase; LDH, lactate dehydrogenase.

9.8 Aerobic oxidation of pyruvate

In the previous sections we saw how plant cells obtain energy from the breakdown of glucose to pyruvate by glycolysis and oxidative pentose phosphate pathway or by fermentations in the absence

of oxygen. Under aerobic conditions, most plant cells can oxidize pyruvate completely to CO_2 and water through a cyclic pathway that consists of a series of dicarboxylic and tricarboxylic acids. The cyclic sequence of reactions in which these intermediate acids are consumed and regenerated continually was described by the German born British biochemist Hans A.Krebs (1937) in pigeon breast muscle. This cycle is then known as the **tricarboxylic acid cycle** (TCA) or the **citric acid cycle** (CCA) (because a tricarboxylic acid citrate is one of the intermediates) or **Krebs cycle** after its discoverer. Krebs was awarded the Noble prize in medicine and physiology in 1953 for his outstanding contribution in elucidating this central metabolic process. This cycle accounts for the major portion of carbohydrate (also fatty acids and amino acids) oxidation and generates numerous biosynthetic precursors. The citric acid cycle is therefore amphibolic, that is it operates both catabolically and anabolically.

9.8.1 Structure of mitochondria

The mitochondrion is the site of pyruvate oxidative metabolism. The break down of pyruvate to CO_2 via the citric acid cycle and the oxidation of NADH by electron transport chain for the formation of ATP are carried out within this cellular organelle.

Plant mitochondria vary in size and shape depending on their source and metabolic state. The actively growing plant tissues and guard cells for example are usually rich in mitochondria. They are typically ellipsoids of 0.5 to 1.0 μm in diameter and upto 3 μm in length (about the size of a bacterium, Figure 9.6). The mitochondrion is bounded by a smooth outer membrane and an extensively invaginated inner membrane. The space present in between the outer and inner membranes of a mitochondrion is called intermembrane space. The membranes are lipoproteinaceous. The outer mitochondrial membrane contains large nonspecific channels formed by proteins called the **porins**. Porins permit the free diffusion of molecules and ions having a molecular mass of upto 10 kDa. The inner membrane is relatively rich in proteins than the outer membrane and is freely permeable only to O_2, CO_2, and H_2O. It shows a dense mass of infoldings called **cristae**. This membranous system contains respiratory electron transport chain proteins and numerous transport proteins that control the passage of metabolites such as ATP, ADP, pyruvate, phosphate and other ions across the membrane. This controlled impermeability of the inner membrane to most metabolites and ions permits the generation of ionic gradients across this barrier and also the compartmentalization of metabolic functions between cytosol and mitochondria. The lipid fraction of both the membranes contain 80 percent of phospholipids, the major one being phosphotidyl choline or phosphatidylethanol amine.

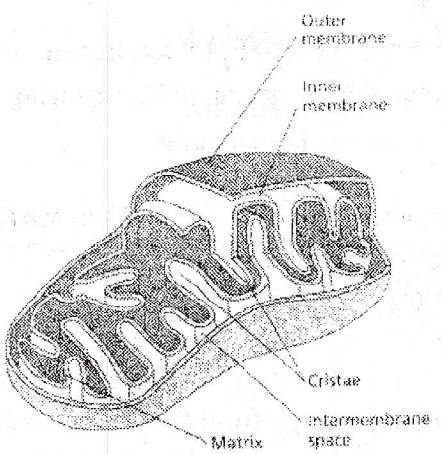


Figure 9.6 Three dimensional representation of a mitochondrion, showing the invaginations of the inner membrane that give rise to the cristae, as well as the location of the matrix and intermembrane spaces.

The inner mitochondrial compartment consists of a gellike substance called **matrix**. Matrix contains enzymes of citric acid cycle as well as substrates, nucleotide cofactors, and inorganic ions. The matrix also contains the mitochondrial genetic machinery such as DNA, RNA and ribosomes that generate several mitochondrial proteins.

9.8.2 Oxidative decarboxylation of pyruvate

Pyruvate formed in the initial phase of glucose metabolism then enters the mitochondrial matrix via a pyruvate translocation protein located in the inner mitochondrial membrane. Once inside the mitochondrial matrix the pyruvate before entering into the citric acid cycle undergoes oxidative decarboxylation by the enzyme **pyruvate dehydrogenase** complex to produce acetyl CoA, NADH and CO_2. Pyruvate dehydrogenase complex is a multienzyme. This complex consists of three enzymes: the **pyruvate dehydrogenase** (E_1), **dihydrolipoyl transacetylase** (E_2) and **dihydrolipoyl dehydrogenase** (E_3). They are present in the complex in multiple copies. The oxidation of pyruvate by this complex involves five cofactors such as thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD^+), coenzyme (CoA) and lipoic acid. Pyruvate dehydrogenase complex with the help of these cofactors catalyses a series of five inter linked reactions that fall into three processes such as decarboxylation, oxidation and conjugation to acetyl CoA. The five reactions catalysed by the pyruvate dehydrogenase complex are (Figure 9.7):

1) Pyruvate dehydrogenase (E_1), a TPP containing enzyme, decarboxylates pyruvate with the enzyme bound intermediate formation of hydroxyethyl-TPP.

2) E_1 also catalyses the oxidation of hydroxyethyl-TPP by the transfer of two electrons and the acetyl group from hydroxyethyl-TPP to the oxidized lipoamide E_2 to form the acetyl dihydrolipoamide.

3) Dihydrolipoyl transacetylase (E_2) catalyzes the transfer of the acetyl group to CoA to form acetyl CoA and fully reduced dihydrolipoamide – E_2.

4) Dihydrolipoyl dehydrogenase (E_3) oxidizes dihydrolipoamide – E_2 by the transfer of two hydrogen atoms from the dihydrolipoamide to the FAD, the prosthetic group of E_3, restoring the oxidized form of the lipoamide – E_2.

5) In the last step, the reduced $FADH_2$ group on E_3 transfers a hydride ion ($:H^-$) to NAD^+, forming NADH. The enzyme complex is now ready for another catalytic cycle.

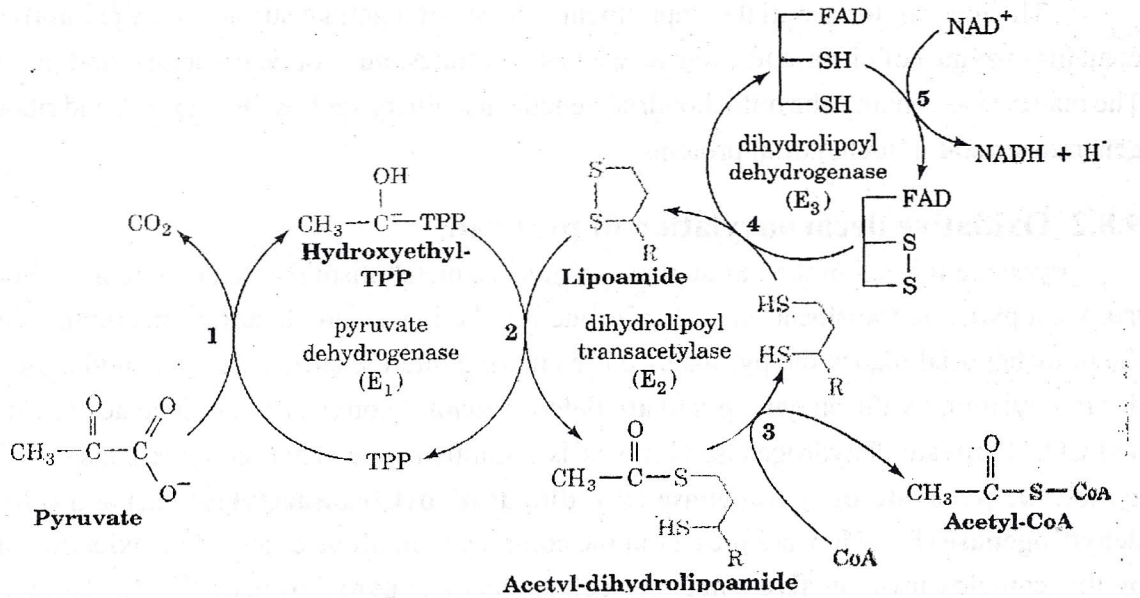


Figure 9.7 The five reactions of the pyruvate dehydrogenase multienzyme complex.

E_1 = TPP containing pyruvate dehydrogenase catalyzes reactions 1 and 2
E_2 = Lipoamide containing dihydrolipoyl dehydrogenase catalyzes reaction 3
E_3 = FAD containing dihydrolipoyl dehydrogenase catalyzes reactions 4 and 5

9.8.3 Citric acid cycle (CCA)

The overall pattern of the citric acid cycle is shown in Figure 9.8.

1) The citric acid cycle starts with the condensation of acetyl CoA with a four carbon oxaloacetate to form citrate, a six carbon tricarboxylic acid. This reaction which is an aldol condensation followed by a hydrolysis is catalyzed by **citrate synthase**. Oxaloacetate first condenses with acetyl CoA to form citryl CoA, which is then hydrolyzed to citrate and CoA.

$$\text{Oxaloacetate} + \text{Acetyl-CoA} + H_2O \xrightarrow{\text{Citrate synthase}} \text{Citrate} + \text{CoA} + H^+$$

2) Citrate is then isomerized to isocitrate via cis-aconitate. This isomerization reaction is accomplished by a dehydration step followed by hydration step in the presence of **aconitase**.

$$\text{Citrate} \underset{H_2O}{\rightleftharpoons} \text{Cis-aconitate} \underset{H_2O}{\rightleftharpoons} \text{Isocitrate}$$

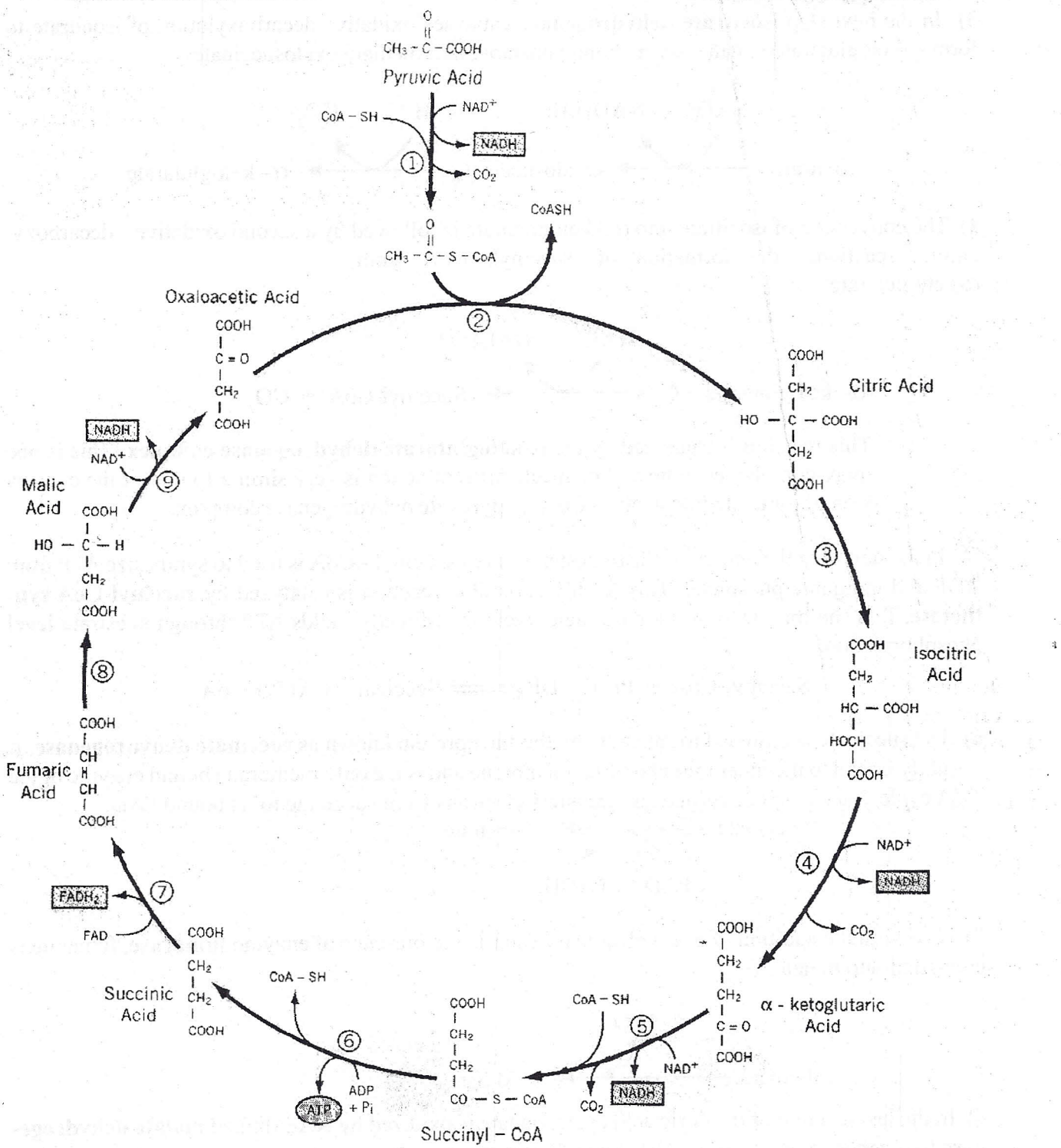
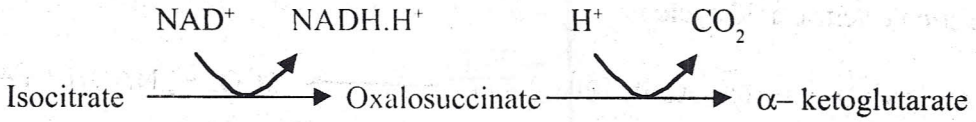
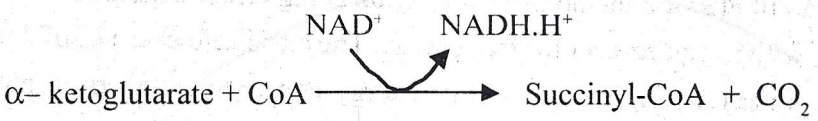


Figure 9.8 The reactions of the citric acid cycle. The citric acid cycle completes the oxidation of pyruvate to carbon dioxide. Energy of glucose is stored as NADH and $FADH_2$.

3) In the next step **isocitrate dehydrogenase** catalyses oxidative decarboxylation of isocitrate to form _–ketoglutarate via an enzyme bound unstable intermediate oxalosuccinate.



4) The conversion of isocitrate into α – ketoglutarate is followed by a second oxidative decarboxylation reaction, the formation of succinyl CoA from α-ketoglutarate.

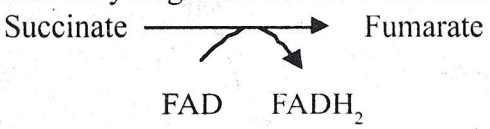


This reaction is catalyzed by the **α-ketoglutarate dehydrogenase complex**. This is one more complex enzyme and its mechanism of action is very similar to that of the conversion of pyruvate into acetyl - CoA by pyruvate dehydrogenase complex.

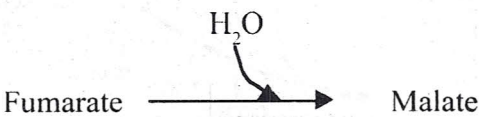
5) In the next step the energy of the thioester bond of succinyl - CoA is used to synthesize ATP from ADP and inorganic phosphate. This readily reversible reaction is catalyzed by **succinyl-CoA synthetase**. It is the only step in the citric acid cycle that directly yields ATP through substrate level phosphorylation.

$$\text{Succinyl-CoA} + \text{Pi} + \text{ADP} \rightleftharpoons \text{Succinate} + \text{ATP} + \text{CoA}$$

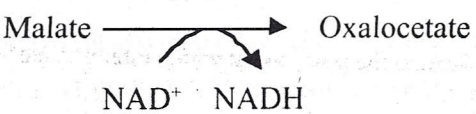
6) The succinate is oxidized to fumarate by the flavoprotein known as **succinate dehydrogenase**. It is tightly bound to the inner mitochondrial membrane and is the only membrane bound enzyme of the TCA cycle. Succinate dehydrogenase transfers electrons from succinate to its bound FAD.



7) Now with the addition of one molecule of water in the presence of enzyme **fumarase**, fumarate is converted into malate.



8) In the last reaction of the citric acid cycle, malate is oxidized by NAD-linked **malate dehydrogenase** to regenerate oxaloacetate, the cycles starting intermediate.



As a whole the citric acid cycle consists of eight enzyme catalyzed reactions and results in the formation of two CO_2, three NADH, one $FADH_2$ and one ATP for each acetyl CoA molecule oxidized. The net reaction of citric acid cycle is

$$\text{Acetyl CoA} + 3NAD^+ + FAD + ADP + Pi + 2H_2O \longrightarrow 2CO_2 + 3NADH + FADH_2 + ATP + 2H^+ + CoA$$

9.8.4 The amphibolic nature of citric acid cycle

Ordinarily one can thinks of a metabolic pathway as being either catabolic with the release of free energy or anabolic with a requirement for free energy. The citric acid cycle is, of course, catabolic because it involves degradation and is a major free energy conserving system in most organisms. However, several biosynthetic pathways utilize citric acid cycle intermediates as starting materials. The cycle is therefore amphibolic (both catabolic and anabolic, Figure 9.9).

The citric acid cycle intermediate oxalocetate for instance, is the starting material for the biosynthesis of an amino acid asparate and few other amino acids are synthesized from as parate. Another intermediate, α-ketoglutarate is used to synthesize glutamate. Other intermediates of the cycle such as succinyl-CoA and acetyl-CoA are used as precursors for the biosynthesis of porphyrins and lipids, respectively.

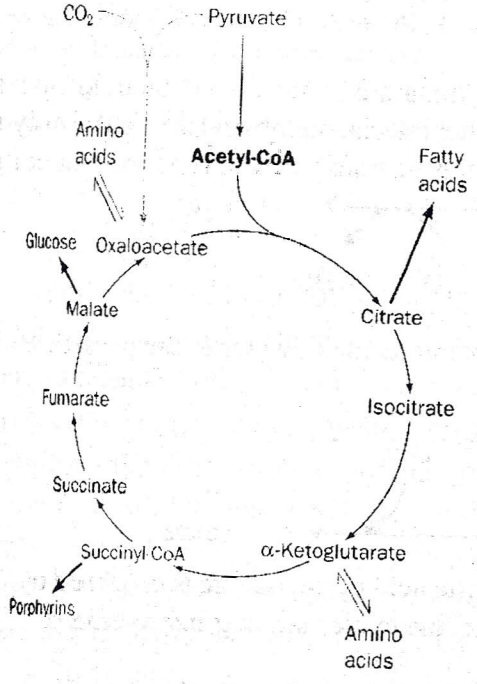


Figure 9.9 A diagram of the TCA cycle indicating the positions at which intermediates are drawn off for use in anabolic pathways.

9.8.5 Anaplerotic reactions

When intermediates of the TCA cycle are drawn off for use in synthetic reactions as discussed above, the level of cycle intermediates particularly oxalocetate will be affected adversely and the rate of cycle slows down. Moreover, all synthetic reactions in addition to carbon also require energy in the form of reducing potential and ATP. The cells therefore maintain a dynamic balance between the synthetic reactions and the TCA cycle intermediates by having replenishment mechanisms called **anaplerotic reactions** (filling up, Greek ana, up + plerotikos, to fill).

The main anaplerotic reactions of plant cells are **PEP carboxylase** and **malate dehydrogenase**. PEP carboxylase is a cytosolic enzyme present not only in the C_4 plants but also in other plants at some level. It catalyses the addition of CO_2 as bicarbonate to phosphoenol pyruvate to form oxaloacetate. Here, the PEP is derived from glycolysis. Cytosolic malaye dehydrogenase catalyzes the reduction of oxaloacetate on to malate.

$$PEP + HCO_3^- \rightleftharpoons \text{Oxaloacetate} \xrightarrow[NAD^+]{NADH} \text{Malate}$$

The malate then enters the matrix of mitochondrion by the malate translocator protein of inner membrane where it is reoxidized to oxaloacetate by the catalytic action of a mitochondrial malate dehydrogenase.

$$\text{Malate} + NAD^+ \rightleftharpoons \text{Oxaloacetate} + NADH$$

Byanaplerotic reactions, plant cells therefore maintain a steady balance of the citric acid cycle intermediates.

9.9 Summary

Respiration is a oxidation-reduction process in which photoassimilates are oxidized to form ATP and to supply carbon skeletons needed in the growth and development of the cell. Plant cells are aerobic and respiration of these cells takes place in three stages: Glycolysis, the citric acid cycle and the electron transport chain. In glycolysis, glucose is degraded to pyruvate with the concomitant net generation of two ATP in the cytoplasm. Key intermediate of this pathway is fructose-1,6-bisphosphate. In the conversion of glucose to pyruvate ATP is required for the hexokinase and phosphofructokinse reactions and ATP is produced in the 3-phospho glycerate kinase and pyuvate kinase reactions. Some of the 3-carbon sugars of this pathway serve as precursors to produce triglycerides and amino acids. In addition to glycolysis, plant cells contain alternate route of glucose metabolism called oxidative pentose phosphate pathway in order to fulfill the requirements of the growing cells for NADPH and five carbon sugars of nucleotide biosynthesis. In this pathway glucose-6-phosphate and 6-phosphogluconate dehydrogenases are NADPH yielding reactions. The first pentose phosphate formed

is ribulose-5-phosphate and it can be isomerized to yield ribose-5-phosphate and xylulose-5-phosphate. Pentose phosphates can be converted back into hexose phsophates by the non-oxidative transketolase and transaldolase reactions. Pyuvate, the end product of glycolysis is reduced to ethanol in the absence of oxygen or oxidized to acetyl CoA for its utilization as a substrate of citric acid cycle in the presence of O_2. It is produced in the pyruvate dehydrogenase multienzyme complex reaction. Aerobic oxidation of pyruvate takes in the mitochondria. Mitochondrion is a spherical or rod shaped organelle. It is bounded by a permeable outer membrane and impermeable highly invaginated inner membrane which encloses the matrix.

Acetyl CoA produced as a result of pyruvate dehydrogenase complex reaction is subsequently oxidized through a series of eight citric acid cycle enzymes. The oxidation of acetate yields NADH in the isocitrate, α ketoglutarate and malate dehydrogenase reactions, and $FADH_2$ in the succinate dehydrogenase reaction. One ATP is formed in the conversion of succinyl CoA to succinate by a mechanism called substrate level phsophorylation.

Several anabolic pathways utilize citric acid cycle intermediates as starting materials. These essential substrates are replaced by anaplerotic reactions of which the major one is synthesis of oxaloacetate from phosphoenol pyruvate and CO_2 by PEP carboxylase.

9.10 Model Questions

1. Explain the relation between CCA cycle and respiration.
2. Describe the process of glycolysis and how it is linked to the production of energy-rich compounds.
3. Describe the fate of pyruvic acid in aerobic respiration.
4. Describe the pentose phosphate pathway and its significance in plants.

9.11 Reference Books

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Dr. G. ROSAIAH.

LESSON: 10

RESPIRATION II : ELECTRON TRANSPORT AND OXIDATIVE PHSOPHORYLATION

10.0 Objective

In this lesson, the oxidation of energy rich coenzymes NADH and $FADH_2$ by components of electron transport chain, generation of proton motive force, synthesis of ATP by ATPase through oxidative phosphorylation are discussed.

10.1 Introduction

The main function of respiration is to release and convert the chemical energy of photoassimilates into a usable ATP form. As we have seen, that glucose oxidation by glycolysis and citric acid cycle synthesized only a net of four ATP molecules. The energy that is stored as ATP by these two processes represents a small fraction of the total amount of energy (2880 kJ/mole) contained in the glucose molecule. That is much of the hexose energy after glycolysis and citric acid cycle has been retained in the form of reducing power NADH and $FADH_2$. The NADH and $FADH_2$ are energy rich molecules because each contains a pair of electrons having a high transfer potential. The 12 electron pairs that are released from each molecule of glucose during its oxidation to CO_2 are to form 10 NADH and 2 $FADH_2$.

$$C_6H_{12}O_6 + 6\,H_2O \longrightarrow 6CO_2 + 24\,H^+ + 24\,e^-$$

NADH and $FADH_2$ are temporary storage places of electrons. Further, the continued oxidation of glucose by glycolysis and citric acid cycle requires NAD^+ and FAD be generated from NADH and $FADH_2$, respectively. The reoxidation of NADH and $FADH_2$ to NAD^+ and FAD occurs by transfer of electrons to O_2 by what is known as **electron transport chain.** It is located in the inner mitochondrial membrane.

Electron transport chain consists of a number of membrane bound proteins and electron carriers that undergo rapid and reversible oxidation and reduction during electron transport from reduced coenzymes to ultimate electron accepter O_2.

Before the detailed process of electron transport discussed, a mention should be required about what is oxidation and reduction.

10.2 Oxidation reduction

The conservation of energy from chemical reactions of photosynthesis and respiration involves oxidation-reduction or redox reactions. Chemically an oxidation is defined as the removal of an electron or electrons from a substrate. A reduction is defined as the addition of an electron or electrons to a substance. In biochemistry, oxidations and reductions does not simply involve the transfer of just electrons but also the whole hydrogen atoms. A hydrogen atom (H) consists of an electron plus a proton. When the electron is removed the hydrogen atom becomes a proton or hydrogen ion (H^+).

Oxidation-reduction reactions involve electrons being donated by an electron donor and being accepted by an acceptor. For example, NADH, can release electrons and hydrogen ions and become oxidized.

$$NADH.H^+ \longrightarrow 2e^- + 2H^+ \quad -1$$

However, electrons can not exist alone in solution, they must be part of atoms or molecules. The equation as written above tells us chemical information but does not itself represent a real reaction. The above reaction is only a half reaction, a term that implies the need for a second half reaction. This is because for any oxidation to occur, a subsequent reduction must also occur. For example, the oxidation of NADH can be coupled to the reduction of O_2 in a second reaction:

$$\tfrac{1}{2}\,O_2 + 2e^- + 2H^+ \longrightarrow H_2O \quad -2$$

This half reaction, which is a reduction, when coupled to the oxidation of NADH, yields the following overall balanced reaction:

$$NADH.H^+ + \tfrac{1}{2}\,O_2 \longrightarrow NAD^+ + H_2O \quad -3$$

In reactions of this type, we will refer to the substance oxidized, in this case, NADH, as the electron donor and the substance reduced, in this case O_2, as the electron acceptor. In biological oxidations and reductions therefore one of the half reaction must be an electron donor while the other must be an electron acceptor. Further a reduced/oxidized pair of an half reaction such as NADH/NAD^+ or O_2/H_2O is known as **redox couple.**

10.2.1 Reduction potentials (E_0')

The tendency of a substance to become oxidized or to become reduced is expressed as reduction potential (E_0'). This potential is measured electrically with reference to a standard hydrogen electrode. This electrode potential has been determined to be -0.420V (or -420 mV) at pH 7 and 30°C. The redox potential value of a redox couple of interest can then be determined with reference to this standard hydrogen half cell value. Spontaneously or in the presence of the appropriate catalysts an oxidation - reduction systems (OR) with negative reduction potentials reduce H^+ to H_2. OR systems with a positive reduction potentials are reduced by H_2. Using these convensions, at pH 7 the reduction potential (E_0) of $NADH + H^+ \longrightarrow NAD^+ + 2e^- + 2H^+$ is - 0.315 volts (electron donating half reaction)

and that of $\frac{1}{2}O_2 + 2e^- + 2H^+ \longrightarrow H_2O$ is + 0.815 V (electron accepting half reaction). Most molecules can be either electron donors or electron acceptors under different circumstances, depending on what other substances they react with.

In oxidation-reduction reactions, the redox couple having more negative reduction potential (like $NAD^+/NADH.H^+$ = - 0.315 V) donates electrons to the redox couple with more positive potential (like O_2/H_2O = + 0.815 V). The reduction potential value of a redox couple when determined at standard set of biochemical conditions is (Temperature 298 K; partial pressure of gases each 1 atm or 101.3 kPa; concentration of solutes each 1M) called standard reduction potential (E_0').

10.2.2 Reduction potential differences ($\Delta E_0'$)

The reduction potential difference of a redox reaction involving any two half-reactions can be determined by substracting the donor potential value from the acceptor potential value according to the following formula:

$\Delta E_0' = E_0' - E_0'$

(e^- acceptor) - (e^- donor)

$\Delta E_0'$ = Reduction potential difference

For example, the potential difference of two half reactions of a redox reaction involving the oxidation of NADH by O_2 is

$\frac{1}{2}O_2 + NADH + H^+ \rightleftharpoons H_2O + NAD^+$

$NADH \rightleftharpoons NAD^+ + 2e^- + H^+ \qquad E_0' = -0.315$ V (donor)

$\frac{1}{2}O_2 + 2H^+ + 2e^- \rightleftharpoons H_2O \qquad E_0' = 0.815$ V (acceptor)

$\Delta E_0' = 0.815 - (-0.315) = 1.30$ V.

Further, the potential difference of any oxidation-reduction reaction can then be used to calculate the standard free energy release ($\_G_0'$) associated with that reaction by the following equation:

$\Delta G^{o'} = -n\ f\ \Delta E_0'$

$\Delta G^{o'}$ = Standard free energy change

n = the number of electrons transferred

f = faraday, the electrical charge of one mole of electrons (1 f = 96,494 C. mol^{-1})

Thus for NADH oxidation:

$\Delta G^{o'} = -$ (2 x 96,494 x 1.13 V)

$= -218$ kJ.mol^{-1}

This means that the oxidation of 1 mole of NADH by O_2 (transfer of two electrons) under standard biochemical conditions is associated with the release of 218 kJ of free energy.

In respiration, this overall free energy change of NADH oxidation by O_2 is split up into three smaller packets by the components of electron transport chain. Each packet of energy released is then coupled with ATP synthesis in a process called **oxidative phosphorylation**.

10.3 Electron transport chain components and electron transport

Electron transport chain located in the inner mitochondrial membrane, consists of a series of four protein complexes known as **complex-I, complex-II, complex-III** and **complex-IV**. These complexes transfer electrons from NADH (and FADH2) to O_2 to generate ATP (Figure 10.1).

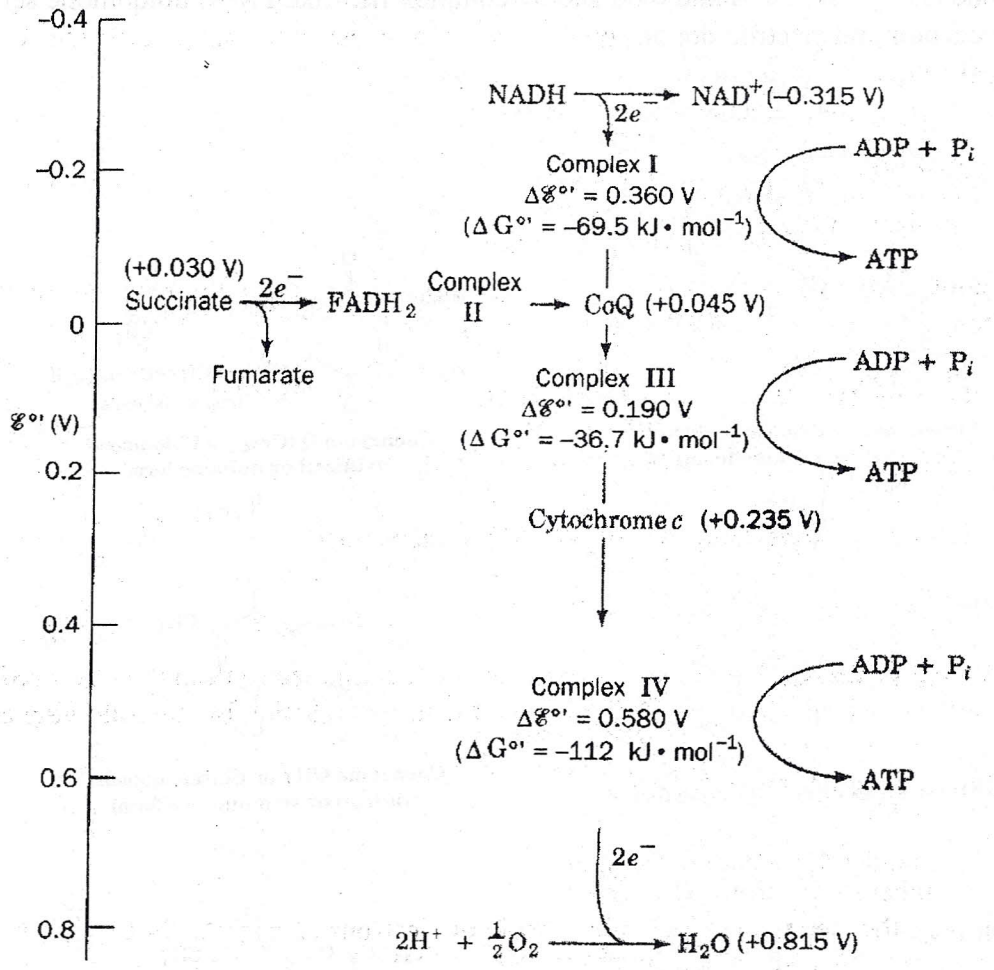

Figure 10.1 Mitochondrial electron transport chain. Four protein complexes showing transfer of electron pairs from lower (NADH or $FADH_2$) to higher standard reduction potentials (H_2O).

Complex - I: Complex I known as **NADH - ubiquinone oxidoreductase**, catalyzes oxidation of NADH by coenzyme Q (CoQ).

$NADH + CoQ\ (oxidized) \longrightarrow NAD^+ + CoQ\ (reduced)$

This complex contains one molecule of protein bound redox active flavin mononucleotide (FMN) and several nonheme iron-sulfur clusters. It passes electrons from NADH to CoQ, also called ubiquinone (UQ), so named because of its ubiquity in respiring organisms. The flavin protein of this complex accepts hydrogen atoms and donates electrons to iron-sulfur clusters by releasing two protons into the cytoplasm. We will consider what happens to the two protons later.

Ubiquinone is a fat soluble benzoquinone involved in electron transport. It can accept one electron to become the semiquinone radical ($QH^{\cdot}$) or two electrons to form ubiquinol (QH_2) as shown in Figure 10.2 . It is a mobile electron carrier between complex I (or complex II) and complex III. Ubiquinone is reduced by complex I and oxidized by complex III. Like FMN, ubiquinone serves as hydrogen atom acceptor and electron donor.

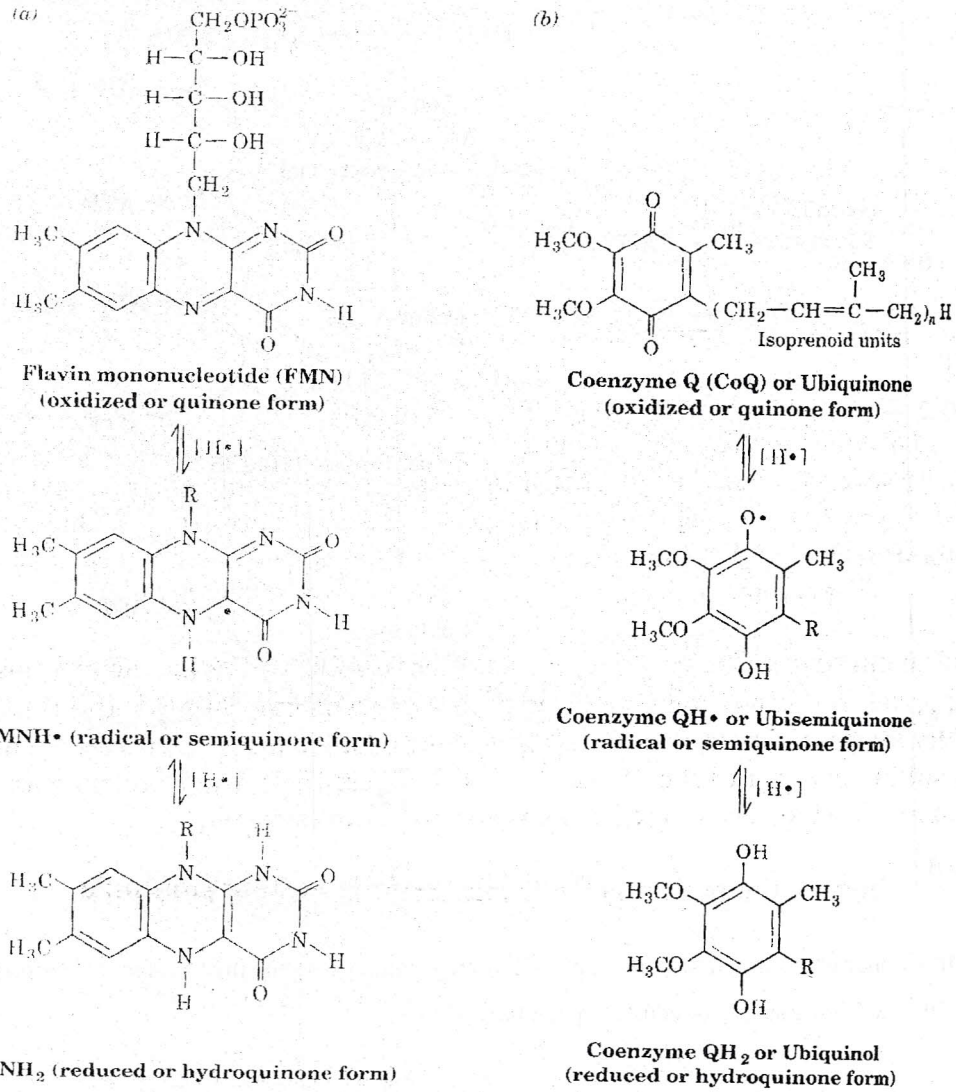

Figure 10.2 The Oxidation states of (a) FMN and (b) CoQ. Both coenzymes form stable semiquinone free radical states.

Complex II: This is the membrane bound citric acid cycle enzyme called **succinate dehydrogenase** or **succinate ubiquinone oxidoreductase**. It catalyzes the oxidation of succinate by CoQ via an enzyme bound FAD.

$$FADH_2 + CoQ\ (ox) \longrightarrow FAD + CoQ\ (red)$$

It contains tightly bound flavin adenine dinucleotide (FAD) and several non-heme iron-sulfur proteins. Electrons are believed to pass from succinate to FAD, then through the Fe-S centers to ubiquinone.

Complex III: Complex III is also named as **ubiquinone - cytochrome-c reductase,** (cytochrome bc_1 complex) catalyzes oxidation of ubiquinol by cytochrome c. That is it passes electrons from reduced ubiquinol to cytochrome c.

$$\text{Ubiquinol} + \text{Cyrochrome c (ox)} \longrightarrow \text{Ubiquinone} + \text{Cytochrome c (red)}$$

This complex contains cytochromes b and c_1 and one iron-sulfur cluster.

Cytochromes are redox active hemoproteins. They contain iron containing porphyrin ring called heme. The heme groups of these proteins undergo oxidation and reduction through loss or gain of a single electron by the iron atom.

$$\text{Cytochrome-}Fe^{2+} \rightleftharpoons \text{Cytochrome} - Fe^{3+} + e^-$$

There are three main groups of cytochromes designated as cytochrome a, cytochrome b and cytochrome c. Different cytochromes within each group are identified on the basis of differences in light absorption spectra. They are designated by numbers (for example cyt. a_1, cyt. a_3, cyt. c_1) or alphabets (cyt.b_k, cyt. b_T) or by light absorption maxima (cyt. b_{562} and cyt b_{566}). One cytochrome can transfer electrons to another that has more positive reduction potential and can itself accept electrons from a quinone or cytochrome molecule with a less positive reduction potential.

Iron-sulfur clusters are known to occur as prosthetic groups of iron-sulfur proteins. In the iron-sulfur proteins, the iron is not bound to heme group as it is in cytochromes. But it is bound to the inorganic sulfur atoms or the sulfur atoms of cysteine residues in the protein. The two most common types of iron-sulfur clusters are [2Fe-2S] and [4Fe-4S] (Figure 10.3). These proteins participate in one electron transfers, in which one of the Fe atoms is oxidized or reduced.

$$\text{Iron - sulfur protein-}Fe^{3+} + e^- \rightleftharpoons \text{Iron-sulfur protein-}Fe^{2+}$$

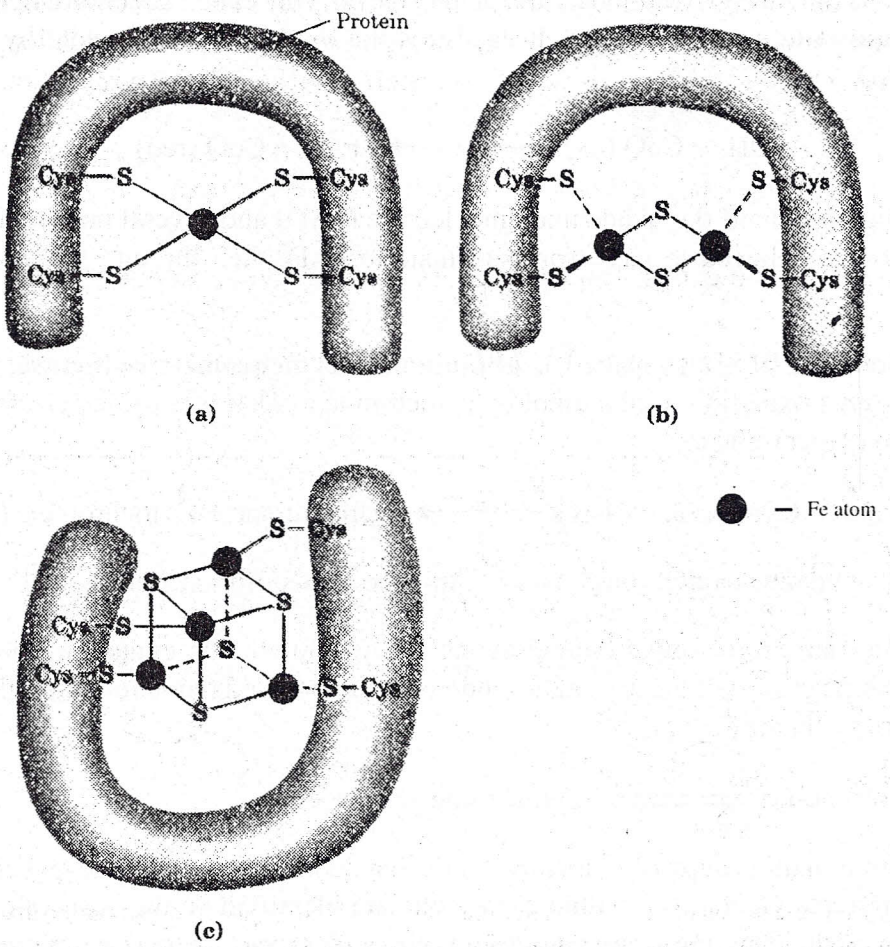

Figure 10.3 Iron Sulfur proteins. a) Fe-s, b) 2 Fe-2S and c) 4Fe-4S iron-sulfur cluster in Fe-S proteins. Note that only the inorganic S atoms are counted in these designations. For example, in the 2Fe-2S center (b), each Fe ion is actually surrounded by four S atoms.

Complex III is arranged asymmetrically in the inner mitochondrial membrane. Both cytochrome c_1 and the non-heme iron sulfur proteins are located on the membranes outer surface while cytochrome b is a transmembrane protein.

Complex III oxidizes the ubiquinol with the concomitant reduction of cytochrome c, a peripheral membrane protein located on the outer side of the membrane. Cytochrome c is a one electron carrier and mediates the electron transport between complex III and complex IV.

Complex IV: Complex IV also called **cytochrome oxidase**, catalyzes oxidation of reduced cytochrome c by O_2. Oxygen is the terminal electron acceptor of the electron transport chain.

$$\text{Cytochrome c (red)} + \tfrac{1}{2}O_2 + 2H^+ \longrightarrow \text{Cytochrome c (ox)} + H_2O$$

This complex contains cytochromes a and a_3 and two copper ions. This complex enzyme without generating incompletely reduced intermediates, catalyzes the one electron oxidations of four consecutive reduced cytochrome c molecules with the concomitant four electron reduction of one O_2 to $2H_2O$ mole cules.

$$4\ Cyt\ c^{2+} + 4H^+ + O_2 \longrightarrow 4\ Cyt\ c^{3+} + 2H_2O$$

The organization of electron transport chain in the inner mitochondrial membrane is shown in Figure 10.4.

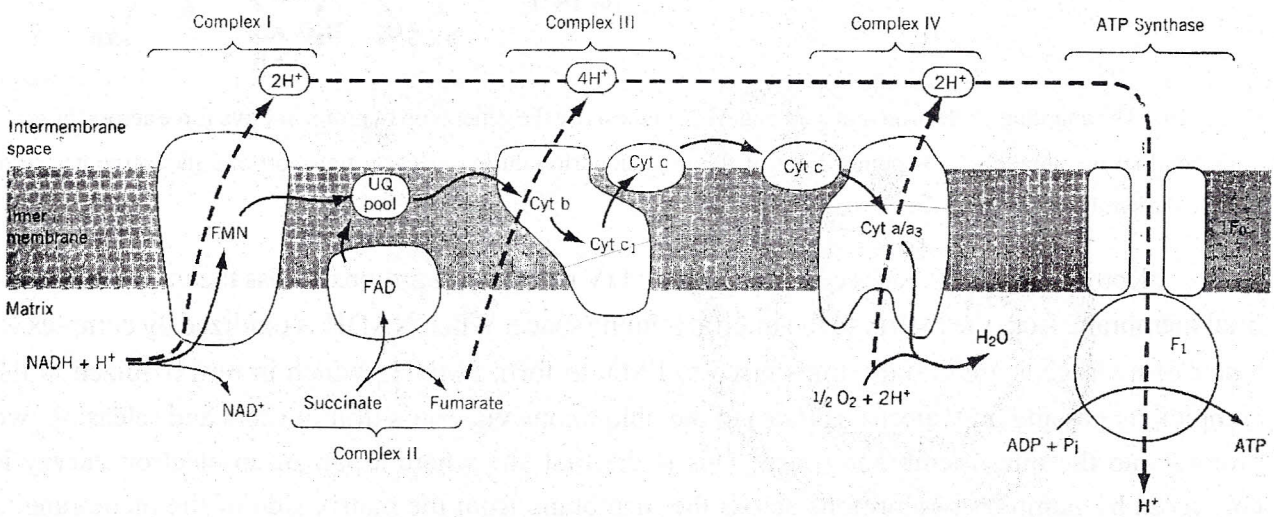

Figure 10.4 A schematic representation of the electron transport chain and proton pumping sites in the inner membrane of a plant mitochondrion. Solid arrow indicates the path of electrons from NADH or succinate to O_2. Energy conserved in the proton gradient is used to drive ATP synthesis through $F_o - F_1$ ATPase.

10.4 Proton motive force (Pmf)

During electron transport, the electrons from NADH to O_2 are passed from higher energy state to lower energy state down a potential gradient. This energetically downhill migration of an electron is accompanied by the release of free energy and is stored primarily in the form of electrochemical H^+ gradient called **proton motive force** across the inner mitochondrial membrane. The electrochemical potential of this gradient is then used to synthesize ATP in accordance with Mitchell's **chemiosmotic hypothesis**.

The electron transport chain complexes and carriers discussed earlier are oriented in the membrane in such a way that a separation of protons from electrons occurs across the membrane during the transport process. Hydrogen atoms, removed from hydrogen atom carriers such as NADH are separated into electrons and protons. The electrons are transported through complex I, ubiquinone, complex III, cytochrome c and complex IV to O_2, while protons are released into the inter membrane space. This results in the slight acidification of the membrane on its outer side (Figure 10.5).

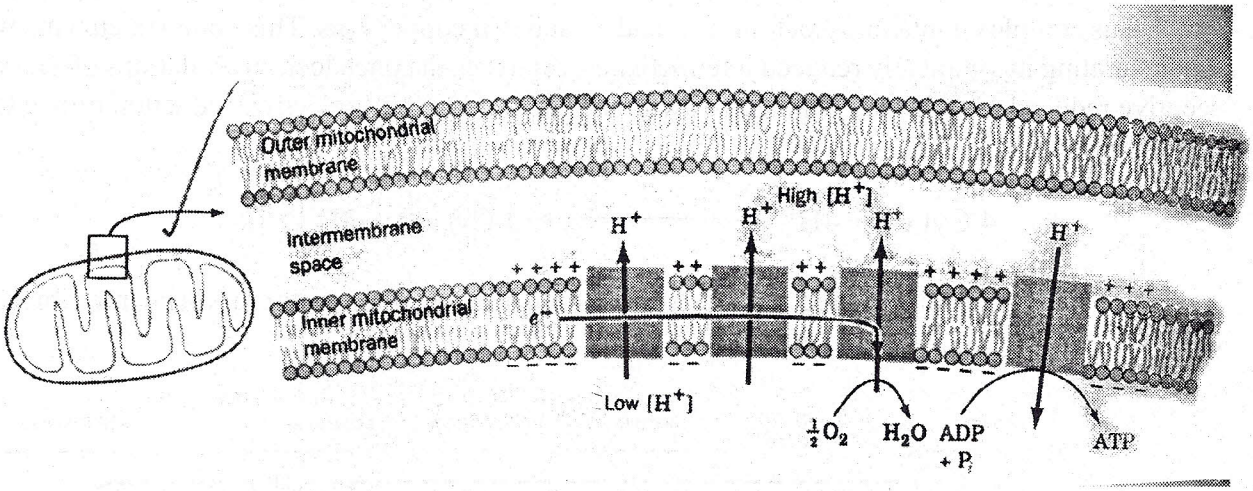

Figure 10.5 The coupling of electron transport and ATP synthesis by the generation of proton motive force across the inner mitochondrial membrane. H^+ is pumped out of the mitochondrion during electron transport and its exergonic return powers the synthesis of ATP.

Electron transport causes complex I, III and IV to transport protons across the inner mitochondrial membrane from the matrix to the intermembrane space. When NADH is oxidized by complex II, a pair of hydrogens are initially transferred to FMN to form $FMNH_2$, which in turn oxidized in the complex by passing only electrons (two) to the ubiquinone via iron-sulfur clusters and releasing two protons into the inter membrane space. This is the first site where a part of an electron energy is conserved by pumping two protons across the membrane from the matrix side of the membrane (a region of low H^+) to the cytoplasmic side of the membrane (a region of high H^+).

The second site of proton pumping in the electron transport chain involves oxidation of ubiquinol by complex III. Reduced ubiquinone (QH_2) donates electrons one at a time to the complex III in a sequence of steps called **Q cycle**. This is an electron transport cycle in complex III in which semiquinone anion ($QH^{\cdot}$) is fully reduced to QH_2 by taking one electron from complex I (or complex II) and a proton from the matrix (Figure 10.6). Fully reduced ubiquinone then diffuses laterally through the membrane until it encounters a complex III on the cytosolic side of the membrane. There one electron is transferred to a cytochrome b and a proton into the intermembrane space. A second proton is translocated when the resulting semiquinone reduces cytochrome c_1 on the cytosolic side. The electron then passes from cytochrome c_1 to peripheral cychrome c. The oxidized ubiquinone (Q) then diffuses to the matrix side of the membrane where it is reduced to semiquinone ($QH^{\cdot}$) by cytochrome b with concomitant absorption of matric H^+. Thus for each electron passing from ubiquinone to cytochrome c, two protons are translocated from the matrix into the intermembrane space. If this scheme is correct, then each pair of electrons passing through complex III contributes four protons to the gradient.

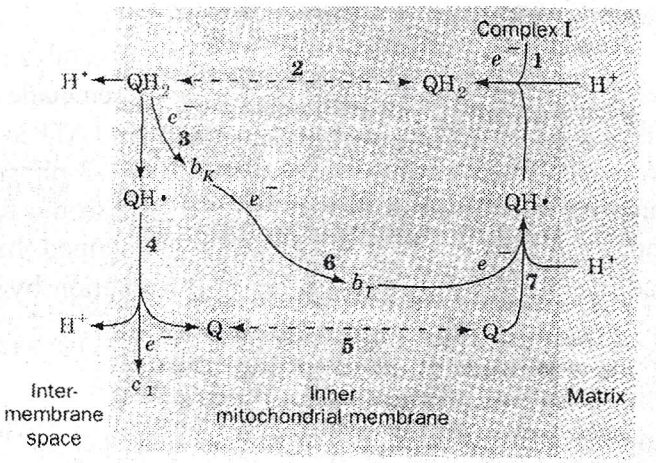

Figure 10.6 Oxidation of ubiquinol by complex III mediated Q cycle.

Although there is a considerable amount of controversy regarding the number of protons translocated by complex IV, the weight of evidence however supports that it catalyzes the transport of two protons for each pair of electrons transferred from cytochrome c to O_2. It appears that approximately a total of eight protons are translocated per molecule of NADH oxidized by electron transport chain.

Complex II does not translocate protons across the membrane. The reason is that the free energy released in this redox reaction is very small (-2.9 kJ mol^{-1}). It is not sufficient to synthesize ATP. This complex functions only to inject the electrons from succinate (via FAD) into the electron transport chain at the CoQ level. When these electrons are passed through complex III and IV, approximately six protons are translocated across the membrane from inside to outside for each molecule of $FADH_2$ oxidized.

Further, when O_2 is reduced to H_2O, it requires H^+ from the matrix to complete the reaction. These protons originate from the dissociation of water into H^+ and OH^-. The use of H^+ in the reduction of O_2 to H_2O and the translocation of H^+ by electron transport chain causes a net accumulation of OH^- on the inside and net accumulation of H^+ on the outerside. Because of their charge, both H^+ and OH^- ions can not freely diffuse through the membrane so that equilibrium can not be restored normally. Consequently a voltage or potential difference will develop across the membrane because of an unequal distribution of anionic and cationic charges and this is called **electrochemcial** or **transmembrane potential**. The net result is the generation of a pH gradient and electrochemical potential across the membrane with the inside of the matrix electrically negative and alkaline, and the outside of the membrane electrically positive and acidic. This pH gradient and electrochemical potential cause the membrane to be energized. The energized state of the membrane is expressed as the **proton motive force**. The proton motive force produced as a result of electron transport process can be used to drive the formation of high energy phosphate bonds in ATP. This idea of proton gradient driving ATP synthesis was first proposed in 1961 by the English Scientist Peter Mitchell and is called chemiosmotic theory. He later received the Nobel prize for this important contribution.

10.5 Oxidative phosphorylation

ATP synthesis is an endergonic process. Its synthesis as catalyzed by ATP synthase occurs in coupling with the electron transport chain. Great efforts have been made in many laboratories over the years to understand the mecahnism of electron transport coupled ATP synthesis. For sometime it was believed that an energy rich intermediate like glyceraldehyde -3-phosphate is formed in the redox reactions. Such an intermediate could subsequently give rise to the formation of ATP from ADP. Experimental evidence for such a mechanism could not be obtained. In 1961 Mitchell proposed the most widely accepted chemiosmotic hypothesis of ATP formation by electron transport generated proton motive force.

The chemiosmotic theory presumes that:

1) The inner mitochondrial membrane is impermeable to ions such as H^+, OH^-, K^+ and Cl^- whose free diffusion would discharge an electrochemical gradient.

2) The electron transport chain is localized in the membrane in such a way that a pH gradient and membrane potential are formed by vectoral extraction and excretion of protons during electron transport from inside to outside of the membrane.

3) ATP synthase is so ingeniously constructed that it can take advantage of the pH gradient and membrane potential to couple ADP and Pi into ATP.

4) In the presence of protonophores such as 2,4 dinitrophenol or carbonylcyanide p-trifluoromethoxy phenyl hydrazone, the membrane becomes leaky to protons and no pH gradient is formed but electron transport will be continued without ATP formation.

The proton motive force represents the energy storage that can be used by the ATP synthase complex to form ATP. The enzyme ATP synthase is the most complex structure in the inner mitochondrial membrane. It consists of two parts, F_0 and F_1. F_0 is a water insoluble membrane integrated protein composed of several types of subunits (4 to 5) that contains a pore through which protons can pass from the cytoplasm into the mitochondrial matrix. F_1 is water soluble perpheral multisubunit headpiece located on the matrix side of the membrane. Five different subnits make up the F_1 part namely alpha (α), beta (β), gama (γ), delta (δ), and epsilon (ε). Three alpha and three beta subunits form a hexameric structure that is connected to F_0 via the γ, δ and ε subunits by a stalk. F_1's catalytic site for ATP synthesis is contained on the beta subunit of this alpha, beta, gama, delta epsilon multimer (Figure 10.7).

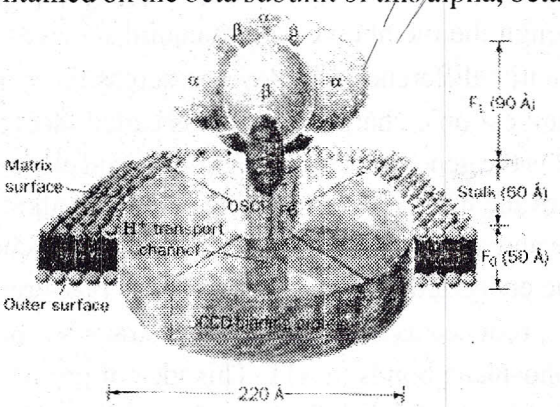

Figure 10.7 An interpretive drawing of ATP synthase indicating the postulated positions of its component subunits.

The F_1/F_0 ATPase is the smallest known biological motor. The chemical mechanism of how this complex synthesizes the ATP is not yet understood in detail. However a mechanism proposed by Paul Boyer suggested that an energy dependent conformational change occurs in the catalytic subunits of ATPase during exergonic proton gradient dissipation. These activated subunits some how drives ATP synthesis from ADP and Pi during their relaxation back to the deactivated conformation. Recently, a "rotational model" for the catalytic mechanism of ATP synthesis has been proposed by Abrahams et al (1994) from X-ray structures of the mammalian mitochondrial F_1 particles. According to this model, the flow of protons through F_0 generates a torque that is transmitted to F_1 by the gama subnuit of this complex. This torque is caused by the rotation of gama within the F_1 complex. In essence gama is a rotating shalft that rotates alternatively against the alpha and beta subunits and mediates the rotational energy convention between the proton influx (into the matrix via F_0) and ATP synthesis at the surface of the F_1 complex. In this system gama is rotor and F_1 is the stator of the ATPase motor.

In respiration, the ATPase catalyzed ATP synthesis is called oxidative phosphorylation. The number of ATP synthesized depends on the nature of the electron donor and is largely worked out by Green, Racker, Lehninger, Chance and others. It is now known that approximately three ADP can be phosphoryled per two electrons transferred from NADH to oxygen. The number of ADP phosphorylated per atom oxygen is frequently expressed as the **P:O ratio**. In the mitochondrial electron transport this ratio is 3 for NADH and 2 for $FADH_2$ as electron donors. ATP is formed at the expense of the electron transport generated proton motive force. For each molecule of ATP syntehsized two to four protons enter the matrix through ATPase, the most likely number is three. This is the mecahnism of electron transport phosphorylation. It now can be said that ATP is synthesized by a chemiosmotic mechanism.

The overall equation for glucose oxidation by plant cell is:

$$\text{Glucose} + 10\ NAD^+ + 2\ FAD + 4\ ADP + 4\ Pi \rightarrow 10\ NADH + 10\ H^+ + 2\ FADH_2 + 4\ ATP + 6\ CO_2.$$

The oxidation of 10 NADH and 2 $FADH_2$ via the electron transport chain then yields approximately 30 and 4 ATP, respectively. It can be said that a total of 38 ATP are produced from each molecule of glucose. The ATP syntehsized in mitochondrion is then used to drive the different energy consuming reactions within and outside the organelle.

10.6 Summary

Respiratory electron transport and oxidative phosphorylation is a biological process through which the NADH and $FADH_2$ produced by glucose oxidation are oxidized with concomitant formation of ATP. The process takes place in the inner mitochondrial membrane. It is a rapid and reversible oxidation-reduction process associated with the standard free energy change of -218 kJ per each molecule of NADH oxidized by O_2. The electrons released by oxidation of NADH and $FADH_2$ can pass through electron transport chain which consists of four complexes linked by mobile electron carriers. Complexes I, III and IV participate in the oxidation of NADH and complexes II, III and IV are involved in the oxidation of $FADH_2$. Electron transport causes complexes I, III and IV to accumulate protons in III and IV the intermembrane space so as to generate proton motive force across the

membrane. Proton motive force represents stored free enrgy in the form of the H^+ gradient and is used to drive ATP synthesis by a mecahnism called chemiosmotic hypothesis proposed by Mitchell.

The formation of ATP is catalyzed by the F_o/F_1 ATPase. It synthesises ATP by coupling this process to the exergonic transport of protons back into the mitochondrial matrix. The ratio of moles of ATP produced per mole of coenzyme oxidized or per mole M oxygen atom reduced to H_2O, called the P:O ratio is three for NADH oxidation and two for $FADH_2$ oxidation. Each molecule of ATP synthesized requires the translocation of at least three protons by the ATPase enzyme.

9.7 Model Questions

1. Describe the role of electron carriers in electron transport chain.
2. Discuss the mechanism of oxidative phosphorylation
3. Write Short notes on :
   (a) Oxidation – reduction
   (b) Proton motive force
   (c) Coenzyme Q
   (d) ATPase

9.8 Reference Books

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Dr.G.ROSAIAH

LESSON: 11

NITROGEN METABOLISM : N_2 FIXATION

11.0 Objective

In this lesson sources of nitrogen available to plants, nitrogen cycle, N_2 fixers, establishment of *rhizobium* – legume symbiosis, nodule formation and biochemistry of nitrogen fixation are discussed.

11.1 Introduction

Like carbon, hydrogen and oxygen, nitrogen is essential to the structure and welfare of the plant. It is an essential constituent of many biomolecules such as proteins, nucleic acids, hormones, vitamins, alkaloids, chlorophyll and certain phospholipids.

The atmosphere contains about 79 percent of molecular nitrogen by volume. This shows that the entire plant world is said to be submerged in a sea of nitrogen. However, this form of nitrogen is not directly available for use by higher plants. The reason is that the triple bonded structure $N \equiv N$ is extremely stable and chemically very unreactive. It undergoes reaction with other substances at high temperature or pressure beyond the ranges to be found in living organisms. With the exception of few microorganisms capable of fixing molecular nitrogen, plants however, meet their nitrogen supply by absorbing it as nitrate (NO_3^-) or ammonium (NH_4^+) salts from the soil.

11.2 Sources of nitrogen

There are four types of nitrogen sources namely (1) nitrate nitrogen, (2) ammonia nitrogen, (3) organic nitrogen and (4) molecular nitrogen.

1) Nitrate nitrogen (NO_3^-) : Nitrate is the most oxidized form of nitrogen present in the soil as a result of fixation by physical or biological means, addition of fertilizers and decomposition of organic wastes. It is readily available source of nitrogen to the plants. Within the plant body nitrate is reduced to ammonia before entering the metabolism.

2) Ammonia nitrogen (NH_4^+): This is a reduced form of nitrogen found in the soil. Some plants can uptake NH_4^+ directly from the soil through their roots along with nitrate ions. Ammonia is directly incorporated into the organic molecules. Ammonical form of nitrogen in soil arises either due to introduction of fertilizers or microbial action upon dead and decaying organisms.

3) Organic nitrogen: The soil may also contains organic form of nitrogen such as proteins or partially degraded proteins, urea, and amino acids. These are generally formed due to the decomposition of dead and decaying organisms by the activity of microorganisms. Roots of higher plants take up these organic nitrogenous compounds for their direct incorporation into cellular constituents.

4) Molecular nitrogen (N_2): Molecular nitrogen is the source of nitrogen available to a few prokaryotes called nitrogen fixing bacteria. Prokaryotes absorb nitrogen and reduce it to ammonia in a process called **biological nitrogen fixation**.

11.3 Nitrogen cycle

Nitrogen is a very mobile element. It moves between the soil and the atmosphere via living organisms. This cyclic process known as **nitrogen cycle** is essential to maintain the nitrogen balance in the atmosphere. N_2 cycle involves processes like **dinitrogen fixation, ammonification, nitrification** and **denitrification** (Figure 11.1).

The process of reducing dinitrogen (N_2) to ammonia (NH_3) or nitrate (NO_3^-) is known as dinitrogen fixation. It occurs by industrial and natural processes. Natural processes are both physical and biological. These processes as suggested by Sprent and Sprent (1990) and Vitousek et al (1997) causes annual N_2 fixation of about 200-250 million metric tons.

Industrially ammonia is synthesized by reacting N_2 with H_2 at very high temperature of about 300 to 400^{0}C and pressure of about 350 bars. This nitrogen fixation reaction is called Haber-Botch process. According to FAO/UNIDO/World Bank working group on fertilizers (1996) that the industrial production of nitrogen has been estimated to be more than 80×10^{12} g yr^{-1} (about 30 percent of the total N_2 fixed) and most of it is destined for use as a agricultural fertilizers.

In addition to the fixation of N_2 by industrial proceses, it appears that approximately 5 to 10 percent of the dinitrogen fixed annually occurs as a result of lightning strokes. Lightning strokes and ultraviolet radiations provide necessary energy to combine water vapor, O_2 and N_2 in the atmosphere to form fixed nitrogen, the nitric acid (NHO_3^-). Nitric acid is brought into the soil by rain water.

The remaining 60 to 65 percent of the total N_2 fixed occurs by the activity of soil microorganisms. This process is known as biological nitrogen fixation. Both free living and symbiotic soil bacteria and cyanobacteria fix N_2 into ammonium by this process.

Once fixed into ammonium or nitrate, nitrogen primarily enters the nitrogenous building blocks of proteins, nucleic acids and other macromolecules of plants and then into animals and microorganisms.

Animal wastes and remains of life after death by decomposition causes nitrogen to be returned to the soil. Decomposition carried out by a group of bacteria and fungi results in the conversion of unabsorbable organic nitrogen into the absorbable NH_3. This soil contained process is called **ammonification**. Most of the ammonium formed by ammonification is then transformed into the nitrate via nitrite. This soil activity involves a group of bacteria called **nitrifying bacteria.** The process in which nitrate is produced from ammonia by the activity of nitrifying bacteria is called **nitrification.** There are two groups of nitrifying bacteria called *Nitrosomonas* group and *Nitrobacter* group. Bacteria of the first group that includes *Nitrosomonas, Nitrosococcus* etc. oxidize ammonia to nitrite. Nitrite is further oxidized to nitrate by **Nitrobacter** group of organisms. The energy liberated during these processes is utilized for the assimilation of CO_2 into carbohydrates by nitrifying bacteria.

Plants can readily absorb nitrate from the soil. Nitrate can also serves as a source of terminal electron acceptor for another group of soil bacteria known as **denitrifiers.** Denitrifiers, for example, *Thiobacillus denitrificans and Pseudomonas denitrificans* anaerobically reduce nitrate to dinitrogen in their electron transport process. This process known as **denitrification** according to the most estimates accounted for to be the loss of about 93 to 150 million metric tons of dinitrogen per year into the atmosphere.

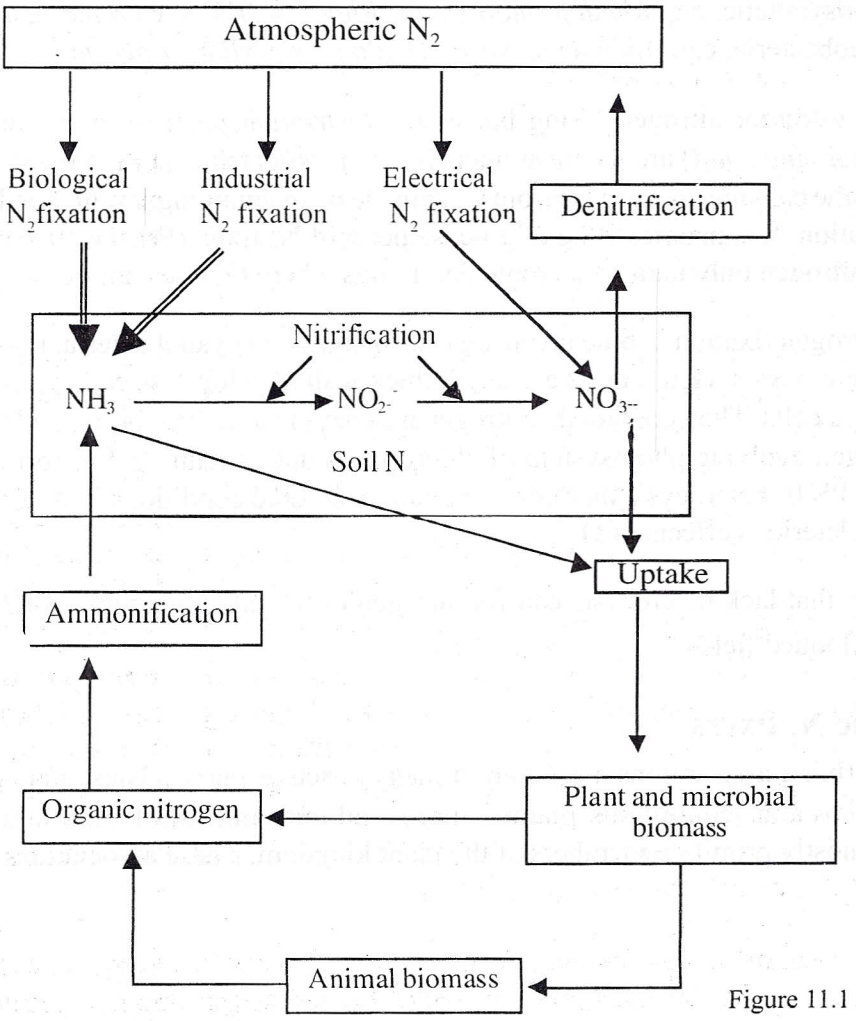

Figure 11.1 Nitrogen cycle.

11.4 Biological nitrogen fixation

Atmospheric dinitrogen fixation by some free living and symbotic soil bacteria is known as biological nitrogen fixation (BNF). The organisms capable of reducing dinitrogen to ammonia are known as **diazotrophs** or **nitrogen fixers**. Nitrogen fixation is a domain of nitrogen fixers because they contain an enzyme complex called the **dinitrogenase**. This enzyme complex catalyzes the reduction of dinitrogen to ammonia in both free living and symbiotic diazotrophs. Nitrogenase is a very oxygen sensitive enzyme. It undergoes irreversible damage with increasing O_2 concentrations.

11.4.1 Free living nitrogen fixers

Nitrogen fixation by free living microorganisms is called asymbiotic nitrogen fixation. The asymbiotic nitrogen fixers can be classified as

1) aerobic e.g. *Azotobacter, Beijerinckia,*
2) facultative anaerobes, e.g. *Klebsiella, Enterobacter,*
3) obligate anaerobes, e.g. *Clostridium*, *Bacillus,*
4) microaerobic, e.g. *Aquaspirillum,*
5) photosynthetic, e.g. *Rhodopseudomonas*, *Rhodospirillum*, *Chromatium* and
6) cyanobacteria, e.g. *Anabaena, Nostoc*, *Calothrix, Cylindrosperma.*

Among the different nitrogen fixing bacteria, *Clostridium pasteurianum* and *Azotobacter* (*A.chroococum* and *A.vinelandii*) are the most intensively investigated genera. Aerobic N_2 fixers like *Azotobacter* protect the enzyme nitrogenase from O_2 damage by maintaining low O_2 conditions through high rates of respiration. It consumes 100g of glucose per g of N_2 fixed (Burris, 1976). Other organisms generally fix nitrogen only under anaerobic conditions where O_2 does not pose a problem.

In general, nitrogen fixation in blue green algae also known as cyanobacteria, is associated with forms possessing heterocysts. Heterocysts are large, thick walled colourless cells present along with the normal vegetative cells. They contain the nitrogenase enzyme to reduce N_2 into NH_3. Heterocysts lack PS II, the oxygen evolving photosystem of chloroplasts but contain PS I to form ATP. Due to lack of O_2 evolving PS II, heterocysts therefore maintain anaerobic conditions in order to protect the nitrogenase from deleterious effects of O_2.

Cyanobacteria that lack heterocysts can fix nitrogen only under anaerobic conditions such as those that occur in flooded fields.

11.4.2 Symbiotic N_2 fixers

Symbotic N_2 fixing associations are found in many vascular plants. These may exist between species of *Rhizobium* and leguminous plants, microsymbiont and non-leguminous plants, and cyanobacteria and mostly primitive members of the plant kingdom. These associations can be represented as follows :

1) *Rhizobium* – legume associations

Legumes + *Rhizobium leguminosarum* : Biovars – *trifolii, phaseoli, viciae, melilotic, loti.*

2) *Bradyrhizobium* + legume associations.

Legumes + *Bradyrhizobium japonium, B.lupini.*

3) *Azorhizobium* + Sesbane associations (form modules both on roots and stems)

4) Microsymbiont - nonleguminous associations

Parasponia + *B. parasponia*

Psychotria sps + *Klebsiella* (nodules located on leaves)

Rhizobium, Bradyrhizobium, and *Azorhizobium* are collectively referred to as **rhizobia**.

5) *Actinomycete* – non-leguminous associations

Non-legumes (*Alnus, Casuarina, Myrica*, certain members of Rosaceae) + *Frankia* (filamentous bacterium). The nodulated roots of these plants containing this N_2 fixer are called **actinorhizal roots**.

Except *Psychotria* sps + *Klebsiella* associations, all other symbiotic associations produce multicellular structures called **nodules** on the noots.

6) *Azotobacter paspali, Azospirillum* + tropical grasses associations. These associations may come under associative symbiosis. In associative symbiosis there exists some sort of interdependence between the host and the microsymbiont, although both the partners can grow satisfactorily apart.

7) Cyanobacterial associations

a) With angiosperms	*Gunnera* + *Nostoc*
b) With gymnosperms	Cycad (*Macrozamia communis*) + *Nostoc* or *Anabaena*
c) With pteridophytes	*Azolla* (fern) + *Anabaena azollae*
d) With bryophytes	*Anthoceros* + *Nostoc*
e) Lickens	Symbiosis of fungi (ascomycetes and basidiomycetes) with green algae and cyanobacteria.

Among cyanobacterial associations, *Azolla* (a water fern) and *Anabaena azollae* association is especially important in rice fields. The molecular N_2 fixed by the algal partner in this association leaks out of the fern plant and thereby supplies the nitrogen needs of the rice plants. This association can fix

as much as 0.5 Kg of atmospheric N_2 per hectare per day.

11.4.3 Rhizobium and root nodulation

Nitrogen fixation by the legume *rhizobium* symbiosis is of considerable agricultural importance. A typical legume *rhizobium* association will fix 25 to 60 Kg N ha^{-1} annually, while nonsymbiotic organisms fix less than 5 Kg N ha^{-1} (Sprent and Sprent 1990). Leguminous plants are classified into three major botanical sub-families of the family leguminoseae: the ceasalpinoideae, the mimosodeae and the papilionoideae. This family includes nearly 700 genera and 17,000 species of leguminous plants of which 500 genera and approximately 10,000 species are belong to the subfamily papilionoideae. So far only 20 percent of leguminous plants have been investigated for nodulation of which 90 percent are known to contain root modules.

Legume – *Rhizobium* symbiotic N_2 fixation takes place in the special organs of the plant host called root nodules. Nodules contain symbiotically synthesized an oxygen binding hemeprotein called **leghemoglobin**. It is a monomeric protein with a molecular mass of 15 to 17 kDa. The globin portion of this protein is synthesized by the plant while the heme is synthesized by the *Rhizobioum*. Leghemoglobin is present in the cytoplasm of infected nodule cells and gives the nodules a pink colour. This protein has a high affinity for O_2 at the low O_2 tensions that exist in the interior of the nodule and release O_2 at even lower concentrations within the bacteroid, where O_2 is accepted by a bacteroid respiratory chain oxidase. Thus leghemoglobin delivers O_2 to bacteroids at concentrations necessary for their aerobic respiration, but harmless to their N_2 fixing nitrogenase enzyme system.

The establishment of legume - *Rhizobium* symbiosis and subsequent nodule formation is a complex process that involves a sequence of multiple physiological and biochemical interactions between the bacteria and the host roots. The development of symbiosis requires a coordinated expression of number of plant and bacterial genes. Further, the interaction of a particular legume species with its respective *Rhizobium* symbiont is known to be fairly specific.

The process of root hair infection by rhizobia consists of several specific events such as migration and multiplication of rhizobia in the rhizosphere, mutual recognition of plant host and *Rhiozobium* species, rhizobial adherance to root hairs, root hair curling, root hair infection, root nodulation, and transformation of vegetative bacteria into enlarged pleomorphic bacteroids which fix nitrogen (Figure 11.2).

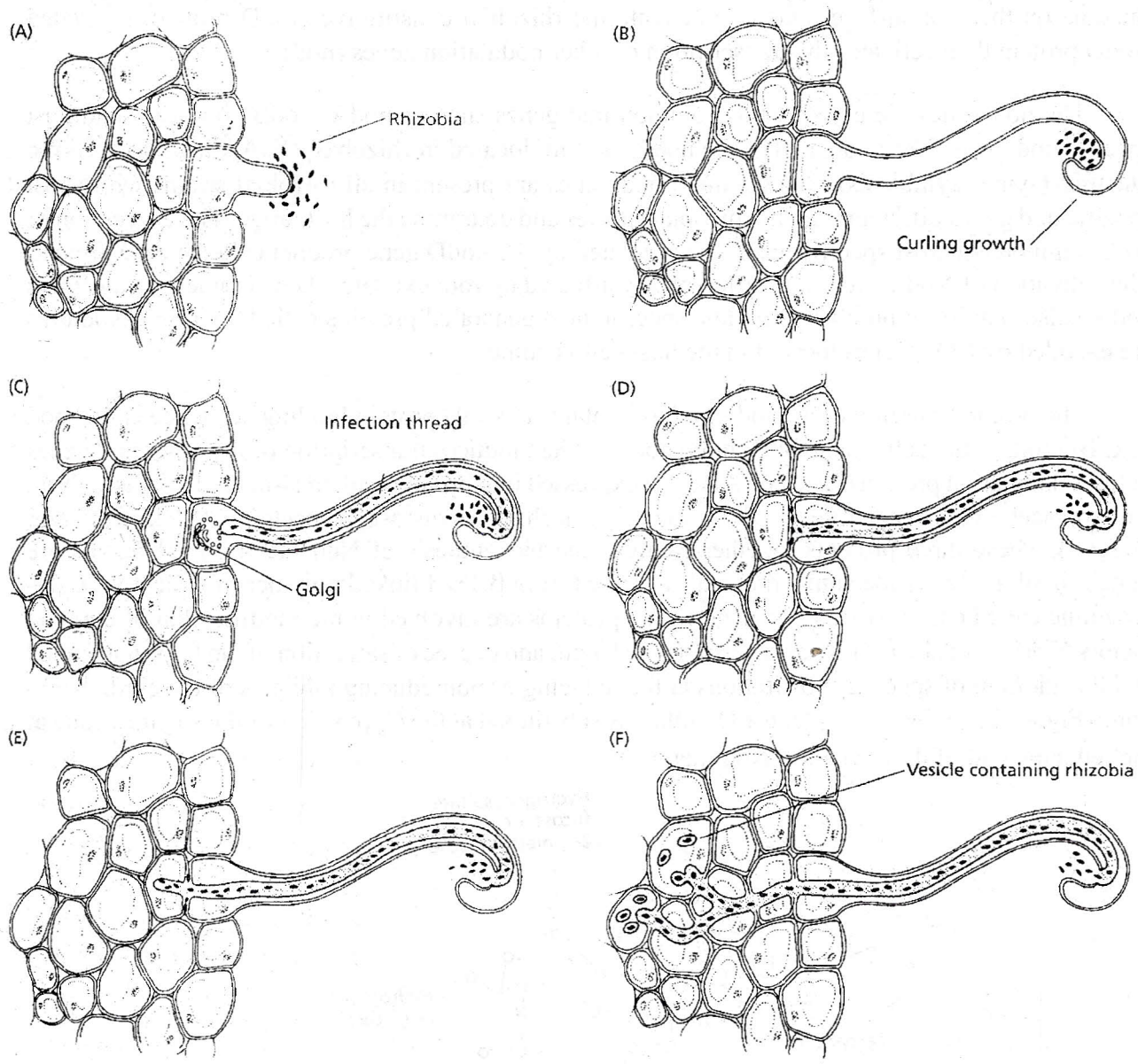

Figure 11.2 The infection process. (A) Rhizobia bind to an emerging root hair in response to chemical attractants sent by the plant. (B) In response to nod factors produced by the bacteria, the root hair exhibits abnormal curling growth. (C) Localized degradation of the rot hair wall and formation of infection thread. (D,E,F) Infection thread enters the cortex and releases the membrane enclosed bacteroids into the cortical cells.

Rhizobia are gram negative free living, aerobic, rod shaped, saprophytic soil bacteria. Migration of rhizobia in the rhizosphere soil surrounding to host roots is the first step in the establishment of symbiotic relationship. It is a chemotactic response (movement toward a chemical substance) mediated by chemical stimulants secreted by the roots. These chemical attractants are root exudates that contain amino acids, sugars, flavonoids and betains. They cause a large increase in the number of

rhizobia on the root surfaces and also activate the rhizobial constitutive NodD protein. Activated NodD protein then activates the transcription of other nodulation genes (nod).

The nod genes are classified as common nod genes such as nodA, nodB and nodC and host specific nod genes like nod EFGH. The nod genes are located in rhizobial DNA known as the **sym plasmid** (sym – symbiosis). The common nod genes are present in all rhizobial strains while host specific nod genes differ among the rhizobial species and determine the host range. The expression of both common and host specific genes are regulated by the nodD gene product called NodD protein. The activation of NodD protein is differentially affected by root exudates. Development of an active nodule also requires a number of nodule specific host generated proteins called nodulins. Nodulins are encoded by NOD genes located in the host cell genome.

The promoter region of all nod operons contains a NodD protein binding sequence called nod box. Binding of the activated NodD to the nod box then induces transcription of the other nod genes to form nodulation proteins. The nod ABC are expressed into proteins called N-acyltransferase, chitin – oligo-saccharide deacetylase and the chitin oligosaccharide synthase, respectively (Stokkermans et al 1995). These three proteins together catalyze the biosynthesis of Nod factors. Nod factors are lipochitin oligosaccharide signal molecules derived from β 1→4 linked polymer of N-acetyl-D-glucosamine called chitin. Host specific nod gene proteins are involved in the modification of the Nod factors (Carlson et al 1995). They determine the length and degree of saturation of the fatty acid added and the addition of specific substitutions at the reducing or nonreducing moieties of the chitin backbone (Figure 11.3). For example, the O-sulfate is substituted at the C_6 position of the sugar residue at the reducing end of the *R.meliloti* Nod factor.

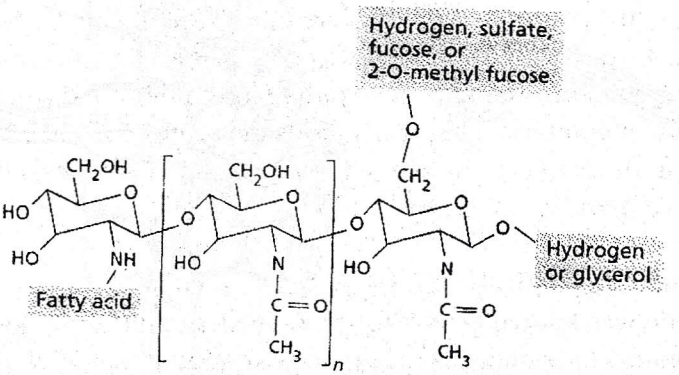

Figure 11.3 Basic Structure of the Nod factors containing lipochitin oligosaccharide back bone. The fatty acid chain typically has 16-18 carbons. The number of repeated middle sections (n) is usually 2 to 3.

Nod factors are secreted into the soil solution by the rhizobia. They induce several morphological changes of the host roots as a prerequisite for rhizobial invasion. These changes include the production of short and thicker roots with more number of root hairs, and elongation, branching and curling of the root hairs.

The recognition and attachment of a particular *Rhizobium* to its plant host is medicated by two classes of molecules called lectins and complex polysaccharides. Lectins are small, non-enzymatic

sugar binding proteins synthesized by the host plant under the direction of host specific Nod factors. The host plant lectins located on the root surface recogniz complex carbohydrate receptors present on the compatible *Rhizobium* cell surface and thereby bind the bacteria to the root. The protein part of a lectin molecule contains specific site that determines its sugar binding specifities. In addition to lectins, a calcium binding protein called *rhicadhesin*, located on the rhizobial cell surface and other physicochemical factors may also have a role in attachment.

As a result of these early events, the rhizobia become enclosed in the small compartment formed by the curling at the root hair tip. The cell wall is degraded at a bacteria localized site by hydrolytic enzymes such as pectinase, hemicellulase and cullulase. They may be released by the infected rhizobia. This event causes the bacterial cells to reach the outer surface of the plasma membrane. The portion of the plasma membrane where the bacterial cells are localized then undergoes an invagination to form a tubular internal structure called an **infection thread**. The infection thread grows down the root hair from the root surface into the root cortex. It is elongated by the fusion of Golgi-derived membrane vesicles at the site of infection. The infection thread continues to grow beyond the root hair cell and penetrates the cortex of root. As the infection thread moves through the root hair into the cortex, the bacteria continue to multiply. Within the cortex, extensive branching of the thread occurs which results in the infection of many cortical cells by the same thread.

In the meantime, the mitogenic signals from the infected bacteria stimulate the localized cell division in the root certex. This signal may involves the synthesis of plant hormone ethylene. It determines the position of the primary nodule meristem in the cortex. The diploid cortical cells of this nodule primordium divide and become tetraploid from which the nodule will develop. When the infection thread reaches the cells of this specialized organ, the tip of the thread buds off to release the bacteria enclosed versicles. Once inside the nodular cortical cells, the shizobia stop dividing and transformed into swollen, irregular cells called **bacteroids.** The membrane surrounding the bacteroids is nowcalled the peribacteroid membrane. These bacteroids then can synthesize N_2 fixing nitrogenase complex with accompanying other metabolic changes, for example, synthesis of protective protein called leghemoglobin in the cytoplasm of the host plant.

11.5 Biochemistry of nitrogen fixation

Prior to the 1960's little was known about the biochemistry and enzymology of N_2 fixation. In 1960 Carnahan and collaborators announced the first successful reduction of N_2 to ammonia by a cell extract of *Clostridium pasteurianum*. The enzyme system which catalyses this reaction is called nitrogenase. Later on enzyme system capable of N_2 fixation were extracted from other free living organisms like aerobic bacterium *Azotobacter* and also from symbiotic organisms such as bacteroids in legume root nodules and root nodules of actinorhizal roots.

11.5.1 Components of nitrogenase enzyme

Nitrogenase is a complex enzyme. It is also known as dinitrogen oxidoreductase. The enzyme irrespective of is source, is composed of two oxygen sensitive non heme iron sulfur proteins. They are designated as **Fe protein** (also known as component II, or azoferredoxin or dinitrogenase reductase) and **MoFe protein** (component I or molybdo ferredoxin or dinitrogenase).

The Fe protein is a smaller homo dimer made up of two identical α – subunits. Its molecular weight varies from 55.5 to 72.6 kDa depending on the bacterial species. This protein contains four iron ions and four sulfur ions as a single 4Fe-4S cluster. This cluster may lie between its two subunits or in association with just one of them. The Feprotein serves as one electron donor and a specific reductase of MoFe protein. It has two binding sites for MgATP and one for MgADP. This protein is extremely oxygen sensitive and has a half life ($t_{\frac{1}{2}}$) in air of 30-45 seconds.

The MoFe protein is a heteromeric tetramer consisting of two α– subunits and two β – subunits. The total molecular mass of the MoFe protein from different organisms ranges from 180 kDa to 270 kDa. However, in most of the N_2 fixers the molecular weight of it is around 220 kDa. The MoFe protein contains two iron molybdenum sulfur cofactors (Fe-Mo-S cofactor) and variable number of 4Fe-4S clusters. This protein binds and reduces N_2 to NH_3. The MoFe protein is also oxygen sensitive, but less sensitive than Fe protein with an half life in air of 10 seconds.

11.6 Mechanism of nitrogenase reaction

Nitrogen fixation by nitrogenase requires a source of reducing power, an ATP generating system, and an anaerobic environment.

11.6.1 Sources of reducing power

The reducing power is supplied to the niotrogenase in the form of reduced ferredoxin or flavodoxin. The reduced ferredoxin that can functions as a reductant in different N_2 fixers is generated by the pyruvate ferredoxin- oxidoreductase or hydrogenase reactions (strict anaerobes), by non-cyclic photophosphorylation (cyanobacteria, green bacteria) or by reverse electron transport (purple bacteria). Aerobic N_2 fixing organisms contain ferredoxin and / or falvodoxin and these carriers are reduced with NADH or NADPH as H-donors.

11.6.2 Generation of ATP

In aerobic free living N_2 fixers and in nodule bacteroids, the ATP is produced from ADP and Pi by the oxidation of respiratory substrates through glycolysis, citric acid cycle and the electron transport chain.

In anaerobic organisms, for example in *Clostridium* the ATP is generated via "phosphoroclastic" cleavage of pyruvate to acetate

$$\text{Pyruvate} + \text{CoA} + \text{Fd}_{(ox)} \xrightarrow[\text{oxidoreductase}]{\text{Pyruvate – ferredoxin}} \text{Acetyl CoA} + \text{CO}_2 + \text{Fd}_{(red)}$$

$$\text{Acetyl CoA} + \text{Pi} \xrightarrow{\text{transacetylase}} \text{Acetyl phosphate} + \text{CoA}$$

$$\text{Acetyl phosphate} + \text{ADP} \xrightarrow{\text{acetokinase}} \text{Acetate} + \text{ATP}$$

ATP in the photosynthetic cyanobacteria is synthesized via photophosphorylations (Figure 11.4).

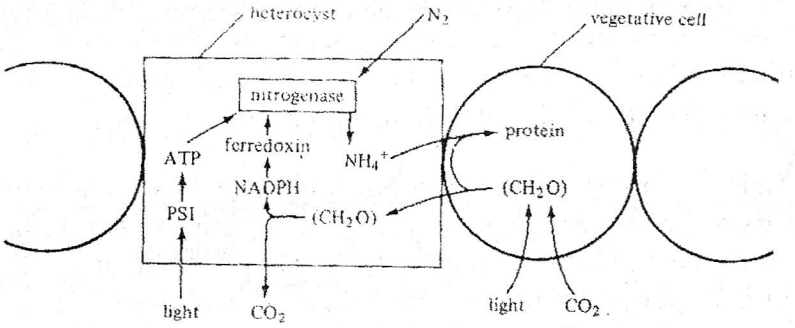

Figure 11.4 Nitrogen fixation in heterocysts. Adjacent vegetative cells provide metabolites for generation of NADPH. ATP is synthesized in the heterocysts by cyclic photophosphorylation.

11.6.3 Anaerobic conditions

Nitrogenase is rapidly inactivated by O_2 so that the enzyme must be protected from this reactive substance. Cyanobacteria provide protection by carrying out nitrogen fixation in heterocysts, bacteroids by having symbiotically synthesized leghemoglobin in the cytoplasm of infected cells and other aerobes protect the enzyme with increased level of respiratory activity as mentioned earlier.

11.6.4 Nitrogenase catalysis

The N_2 fixation process by nitrogenase begins with the transfer of electrons from the reduced ferredoxin or flavodoxin to the Fe protein. Two molecules of ATP (as MgATP complexes) bind to the reduced Fe protein and are hydrolyzed as the electron is passed from the Fe protein to the MoFe protein. ATP hydrolysis is thought to cause a conformational change in the protein which results in lowering of its redox potential by about 100 mV i.e. from about 300 mV to 400mV. In other words the ATP binding causes the Fe protein to be present in super reduced state. The MgATP activated reduced Fe protein then bound to the MoFe protein, forming a redox active nitrogenase complex. Two binding sites for Fe protein exist on the MoFe protein. When the complex is formed, the electron is then transferred from 4Fe-4S cluster of the Fe protein to Fe-Mo-S cofactor of the MoFe protein with the concomitant hydrolysis of bound ATP (Figure 11.5). A minimum of 2 MgATP molecules are hydrolyzed for each electron transferred from Fe protein to MoFe protein.

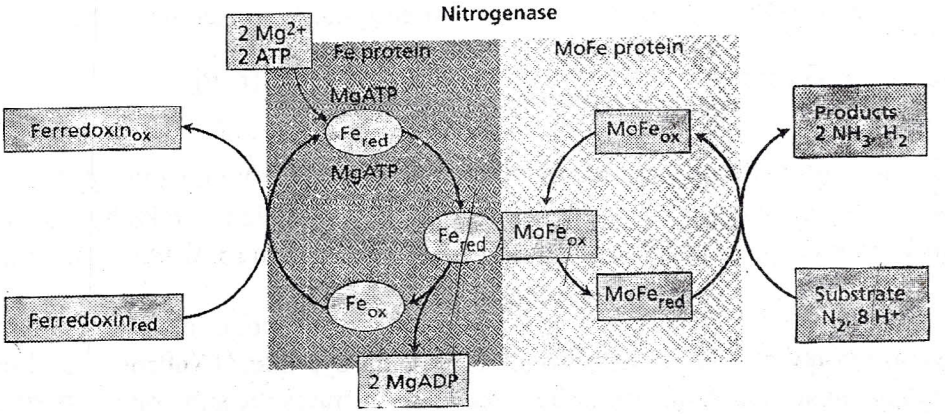

Figure 11.5 The reaction catalyzed by nitrogenase.

After each electron transfer the complex dissociates, and the electron from the Fe atoms in MoFe protein is utilized to reduce the bound dinitrogen to NH_3. The reduction of dinitrogen which takes place on the MoFe protein actually occurs in three discrete steps, each involving an electron pair (Figure 11.6).

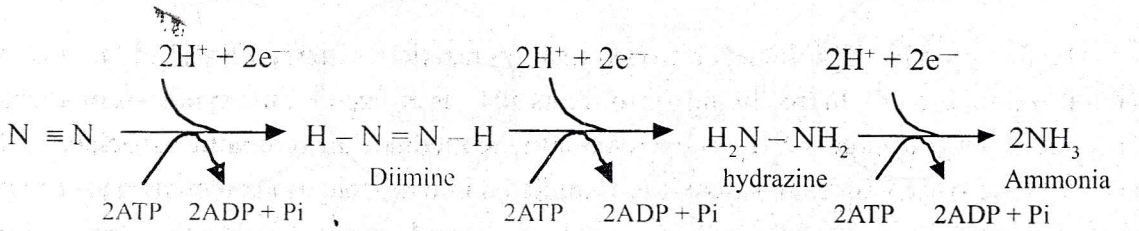

Figure 11.6 Reduction of dinitrogen to ammonia by nitrogenase in three discreate electron transfer steps.

Actually only one electron is transferred from Fe protein to MoFe protein at a time so that a number of (at least 6 times) these electron transfer steps will be required before the final reduced products are released from the enzyme. The transfer of six electrons requires a total of 12 ATP per molecule of N_2 fixed.

In fact the mechanism of dinitrogen fixation to NH_3 by nitrogenase occurs with concomitant evolution of molecular hydrogen. This is an inherent property of the enzyme nitrogenase. Under normal conditions, for two moles of ammonia formed one mole of H_2 is evolved. Therefore nitrogen reduction is expensive and requires at least 8 electrons and 16 ATP molecule per molecule of N_2 fixed (Figure 11.7).

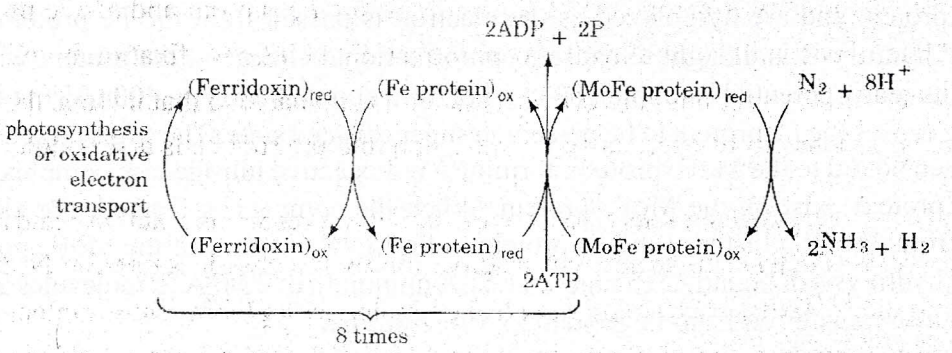

Figure 11.7 The flow of electrons in the nitrogenase-catalyzed reduction of N_2.

$$N_2 + 8e^- + 16\ ATP \longrightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

The production of H_2 along with nitrogen fixation is a energy consuming wasteful process. Some rhozobia, however contain an oxygen dependent enzyme called uptake hydrogenase that enables the organism to regain its lost energy by coupling H_2 oxidation to ATP production.

Nitrogenase has broad substrate specificity and catalyses the reduction of a variety of substrates such as dinitrogen, H^+, acetylene, azide, nitrogen oxide and cyanide. Dinitrogen and protons from water are the natural substrates. Apart from these natural substrates the substrate of particular interest

is acetylene, which is reduced to ethylene. The rate of reduction of acetylene to ethylene is used to estimate nitrogenase activity by gas chromatography.

$$HC \equiv CH + 2H^{+} + 2e \longrightarrow H_2C = CH_2$$

The first product of biological nitrogen fixation reaction is ammonium (NH_4^+). It is excreted and/or utilized for growth. In free living diazotrophs NH_4^+ is utilized for the synthesis of amino acids, and in some strains the excess NH_4^+ is excreted into the medium. In symbiotic associations, NH_4^+ is excreted into the host cytoplasm, where it is assimilated into organic molecules such as amino acids, amides or ureides, which are transported via xylem sap and made available for plant growth. The primary route of fixed ammonia transport seems to be the synthesis of glutamine by the glutamine synthetase: glutamine oxoglutarate aminotransferase (GS:GOGAT). In some legumes however, substances such as allantoin and allantoic acid synthesized from fixed nitrogen can act as nitrogen transporting organic compounds to other parts of the plant.

11.7 Summary

Atmospheric dinitrogen is a relatively inert gas that must first be reduced to a metabolically useful form, NH_3, by nitrogen fixation. Nitrogen fixation is found only among prokaryotic organisms. Nitrogen fixing bacteria are both free living and symbiotic anaerobic, aerobic and photosynthetic organisms. These organisms called N_2 fixers contain an enzyme complex nitrogenase. The catalytically active enzyme complex is composed of wo proteins, the Fe protein and MoFe protein. It can catalyzes the reduction of molecular nitrogen to ammonia. Symbiotic N_2 fixation involves complex genetic and biochemical interactions between host plant root and bacteria that include the recognition of specific signals, attachment of bacteria and its invasion into the root cells and nodule formation.

Nitrogenase is a very oxygen sensitive enzyme. Increased respiratory activity and heretrocysts formation are used as mechanisms to prevent inactivation by oxygen. In symbolic N_2 fixation, the transport of O_2 to the N_2 fixing bacteroids is controlled by leghemoglobin. Once molecular nitrogen has been reduced to ammonia, the ammonia can be incorporated into organic compounds.

11.8 Model Questions

1. What organisms fix atmospheric nitrogen? Describe the mechanism of nitrogen fixation.
2. Give an account of the process of rhizobial infection and nodule development in legume roots.
3. Write briefly on the following :
   (a) N_2 cycle
   (b) Leghemoglobin
   (c) Components of nitrogenase

11.9 Reference Books

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5. Bacterial Metabilism – G.Gottschalk. Springler – Verlag, New York.
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Dr.G.ROSAIAH

LESSON: 12

NITROGEN METABOLISM : NITROGEN UPTAKE, ASSIMILATION AND PROTEIN SYNTHESIS

12.0 Objective

In this lesson assimilation of fixed and absorbed nitrogen into amino acids by GS-GOGAT, reductive amination and transamination, nitrate reductase system for the assimilation of nitrate, relationship between nitrogen metasolism and photosynthesis, protein classification and mechanism of protein synthesis are discussed.

12.1 Introduction

In the previous lesson we have seen how atmospheric dinitrogen is fixed into ammonia by both free living and symbiotic microorganisms. Plants that can not fix dinitrogen usually meet their nitrogen requirements by absorbing it from the soil either as NO^-_3 or ammonium ion (NH^+_4). In most soils, ammonia is generally converted to nitrate by the nitrifying bacteria as described in the earlier lesson. However, the activity of nitrifying bacteria in both acidic and anaerobic conditions is almost all zero so that most of the soil nitrogen can be accumulated as ammonium. In such cases some plants, particularly members of the family Ericaceae take advantage of this nitrogen source for the synthesis of nitrogen compounds. Ammonium ions may be utilized directly in the synthesis of amino acids. In nitrate assimilation, the nitrogen in NO^-_3 is converted to a higher energy form in nitrite (NO^-_2) then to

yet higher energy form in ammonium (NH_4^+) and finally into the amide nitrogen of glutamine. This process consumes the energy equivalent to 12 ATPs per nitrogen (Bloom et al. 1992). In this chapter we will see first the assimilation of ammonium nitrogen and then nitrate nitrogen.

12.2 Assimilation of Ammonium

Reduced nitrogen in the form of NH_4^+ generated either by fixation or by uptake from the soil can be assimilated, first into amino acids and then into other nitrogen containing biomolecules. Two amino acids, glutamate and glutamine, provide the critical entry point. The amino groups of most other amino acids are derived from glutamate via transamination reactions. The amide nitrogen of glutamine is the source of amino groups in a wide range of biosynthetic processes.

12.2.1 GS – GOGAT Pathway

The biosynthetic pathways to glutamate and glutamine are simple and appears to be similar in all forms of life. The primary pathway that has been worked out largely in the legume root nodules with labelled $^{13}N_2$ or $^{15}N_2$ involves sequential action of two enzymes, **glutamine synthetase** (GS) and **glutamine : 2-OXO glutarate amino transferase** (GOGAT). The later enzyme in short is called **glutamate synthase**. The pathway by which ammonium is assimilated into glutamine through the action of these two enzyme systems is called **GS: GOGAT cycle** or **glutamate synthase cycle** (Figure 12.1). The two enzymes of this pathway remain repressed in bacteroids and induced in host cells.

Glutamate synthase cycle begins with the glutamine synthetase catalyzed reaction in which glutamate and NH_4^+ can react to yield glutamine.

$$\text{Glutamate} + NH_4^+ + ATP \xrightarrow[Mg^{2+}]{GS} \text{Glutamine} + ADP + P_i$$

This reaction is an endergonic process needing ATP and Mg^{2+}. In plants this enzyme is known to occur in the cytosol, root plastids and shoot chloroplasts. The cytosolic forms are expressed in order to transport the absorbed or fixed ammonium. This enzyme is composed of eight identical subunits with a molecular mass of 350 kDa.

In the presence of a reducing source the glutamine transfers its amide group to α – ketoglutarate (also called 2–oxoglutarate). This reaction is catalyzed by glutamate synthase (GOGAT).

$$\text{Glutamine} + \alpha - \text{ketoglutarate} + NADH \longrightarrow 2 \text{ glutamate} + NAD^+$$

The α – ketoglutarate and NADH used up in this reaction are derived from the respiratory metabolism of photosynthates. Plants contain two types of GOGAT. One form that accepts electrons from NADH is present in the cytoplasm of infected cells and plastids of non-photosynthetic tissues. Here, it functions in the synthesis of glutamate from glutamine and α-ketoglutarate to export fixed nitrogen from the roots to the shoots. The other form that accepts electrons from reduced ferridoxin is located in the chloroplasts where it may be involved in the incorporation of NH_4^+ (produced from nitrate) into glutamate.

The net result of GS – GOGAT pathway in nodulated and non nodulated roots is the formation of one extra glutamine. This is available for export to the shoots where it supplies the amino group to synthesize other amino acids.

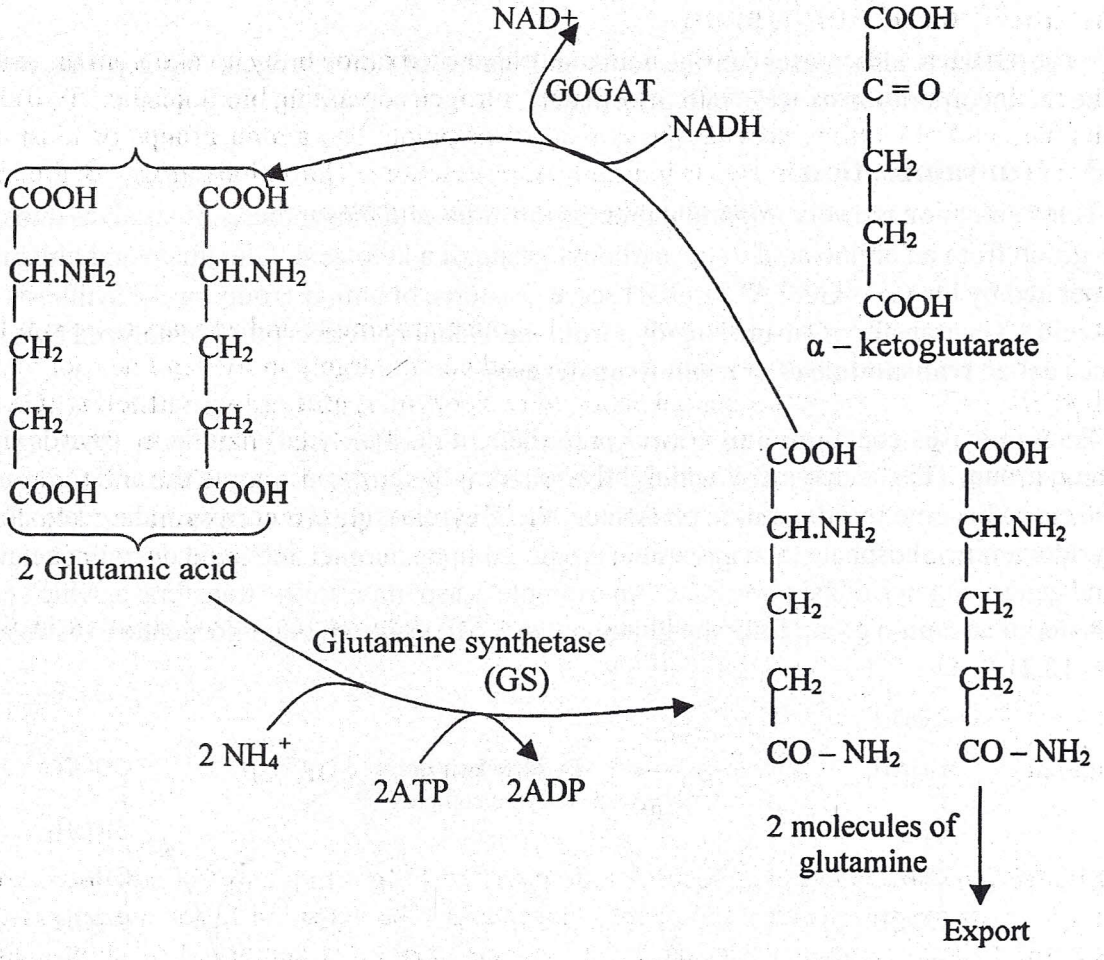


Figure 12.1 Assimilation ofammonium by the GS – GOGAT pathway. Two molecules of glutamine are formed by the addition of ammonium to two molecules of glutamate catalyzed by the enzyme glutamine synthetase (GS). One molecule of glutamine is available for export to the host plant while the second molecule undergoes a transamination with α-ketoglutarate to form two molecules of glutamate in the presence of glutamate synthase (GOGAT).

12.2.2 Reductive amination

Ammonia can also be assimilated through an alternative route known as reductive amination. In this process the ammonium is directly added to α – ketogutarate by the enzyme **glutamate dehydrogenase** (GDH) to form glutamate.

$$\alpha - \text{ketoglutarate} + NH_4^+ + NAD(P)H \longleftrightarrow \text{Glutamate} + H_2O + NAD(P)^+$$

The K_m of GDH for ammonium ion is very low (10 mM), that is it has low affinity for NH_4^+ and consequently not capable of assimilating low levels of NH_4 normally prevalent during nitrogen fixation by bacteroids in root nodules. This low level is due to the high affinity of glutamine synthetase for NH_4T in nodules.

The GDH is also present in the roots and leaves of non nitrogen fixing plants in order to catalyze the incorporation of NH_4^+ into glutamate.

12.2.3 Transamination

Transamination is a very important process in amino acid biosynthesis. It involves transfer of an amino group from an amino acid to the carbonyl group of a keto acid. Glutamate and glutamine that are generated by the GS – GOGAT or GDH serve as source of amino group for the synthesis of other amino acids. The transfer of an amino group from these donors to acceptor is catalyzed by a group of enzymes called **transaminases** or **aminotransferases**.

Transaminases contain the pyridoxal phosphate, a derivative of vitamin b_6 (pyridoxine) as a prosthetic group. This coenzyme which tightly bound to the enzyme accepts the amino group of the amino acid to become pyridoxamine phosphate, thereby releasing the corresponding ketoacid product. Pyridoxamine phosphate then passes the amino group to another keto acid, forming a new amino acid and generating pyridoxal phosphate. An example is aspartate amino transferase, which catalyzes the transfer of an amino group from the glutamate to the boxyl atom of oxaloacetate to form aspartate (Figure 12.2).

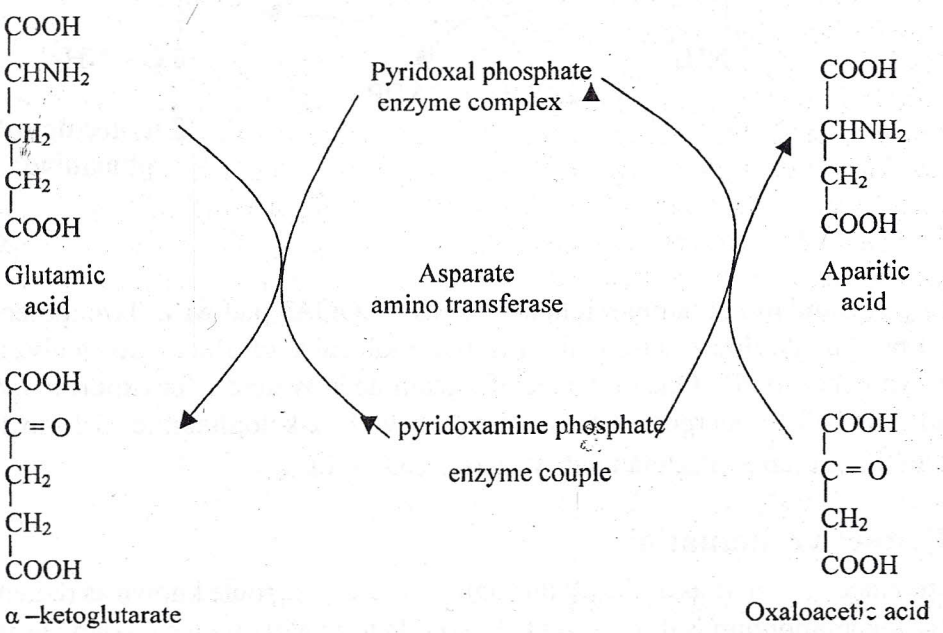


Figure 12.2 Sythesis of asparitic acid by transamination.

Figure 12.2 Sythesis of asparitic acid by transamination.

Transamination from glutamate and glutamine to a variety of ketoacids therefore accounts for the synthesis of a number of other amino acids. Transaminases are found in the cytoplasm, chloroplasts, mitochondria, glyoxysomes and peroxisomes.

Some nodulated plants for example pea and clover transport their fixed nitrogen in the form of aspargine. Synthesis of aspargine requires two reactions, the **aspartate amino transferase** reaction and **asparagine synthetase** reaction. First one is asparate amino transferase reaction in which the amino group from glutamate is ransferred to oxaloacetate to yield asparate.

The second step as catalyzed by asparagine synthetase, the amide nitrogen of glutamine is transferred to asparate to form asparagine

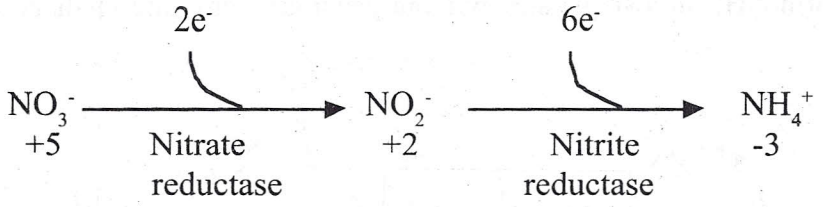
Glutamate + Oxaloacetate ⟶ Asparate + _ – ketoglutarate

Glutamine + Asparate + ATP ⟶ Glutamate + Asparagine + ADP + Pi

12.3 Assimilation of nitrate

Nitrate is the more abundant form of nitrogen in well aerated, humid and warm soils. This form of nitrogen produced by the activity of ammonifying and nitrifying bacteria is most readily absorbed by the plants. Absorption of nitrate by plant roots from the soil is a carrier mediated, energy dependent active process. The carrier protein involved in the uptake of nitrate is inducible and is induced only in the presence of nitrate in the soil. This is supported by the observations that uptake of nitrate is prevented by inhibitors of respiration, uncouplers of oxidative phosphorylation, inhibitors of protein synthesis, low temperature and anaerobic conditions.

Nitrate absorbed by the plants is not directly used for the biosynthesis of nitrogen containing biomolecules. It needs to be reduced to ammonia before it can be incorporated into the organic compounds. The oxidation state of nitrate is +5 and that of ammonia is -3. The reduction of nitrate to ammonia hence entails transfer of 8 electrons which takes place in two separate enzymic steps. The first step is catalyzed by **nitrate reductase** (NR). In this reaction, the nitrate by accepting two electrons is reduced to nitrite. The second step of this process is the reduction of nitrite to ammonia. The enzyme **nitrite reductase** (NiR) catalyzes this reaction. It involves transfer of six electrons.



Nitrate reductase is a ubiquitous substrate inducible cytosolic enzyme. The enzyme isolated from higher plants is composed of two identical subunits, each with a molecular mass of 115 kDa. Each subunit contains three prosthetic groups such as FAD, cytochrome b and molybdenum (Mo). Nitrate reductase being a molybdoprotein requires molybdenum as an essential cofactor for its activity. Molybdenum was found to be essential for plants grown on nitrate but not with ammonium as the nitrogen source. One symptom of molybdenum deficiency in plants is the accumulation of large amounts of nitrate due to decreased activity of the nitrate reductase.

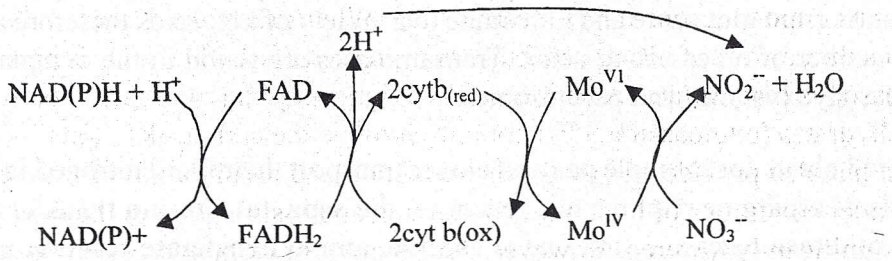


Figure 12.3 The prosthetic groups of nitrate reductase and the sequence of reactions according to Guerrero et al (1981). NAD(P)H denotes that both coenzymes NADH and NADPH may function as H donors.

In general, NR from higher plants utilize NADH as an electron donor. However, the recent observations confirmed the existence of this enzyme in two forms in various tissues of the higher plants. One of these is specific for NADH (NADH : nitrate oxidoreductase, EC. 1.6.6.1) and the other functions with either NADH or NADPH as an electron donor (NAD(P)H: Nitrate oxidoreductase, EC. 1.6.6.2).

The NAD(P)H generated either by glycolysis or HMP pathway supplies the H atoms to reduce FAD of nitrate reductase. Cytochrome b transfers electrons from $FADH_2$ to Mo which reacts directly with nitrate to form nitrite (Figure 12.3).

Nitrate reductase activity is regulated by its phosphorylation and dephosphorylation by specific NR protein kinase and phosphatase. The enzyme becomes non functional when it is present in phosphorylated condition in the dark. This inactivation occurs due to the binding of a inhibitor protein to phosphorylated NR. The enzyme regains its activity in the light period by a release of inhibitor protein and subsequent dephophorylation by phosphatase enzyme.

Nitrite formed by the action of NR is further reduced to ammonia in plastids of roots or chloroplasts of leaves by nitrite reductase. Nitrite reductase from higher plants appears to be composed of a single polypeptide chain having a molecular weight of 61 to 64 kDa. This enzyme contains an iron sulfur cluster (4Fe – 4S) and a siroheme (= Fe – S – heme) as prosthetic groups. It functions with ferredoxin as an electron donor. Reduced ferredoxin is derived either from photosynthetic electron transport in the chloroplasts or from NADPH generated by HMP pathway in non-green tissues.

Nitrite reductase catalyzes the transfer of six electrons from the reduced ferredoxin to the nitrite via its prosthetic groups to form NH_4^+ in a single step without generating any intermediary adduct (Figure 12.4).

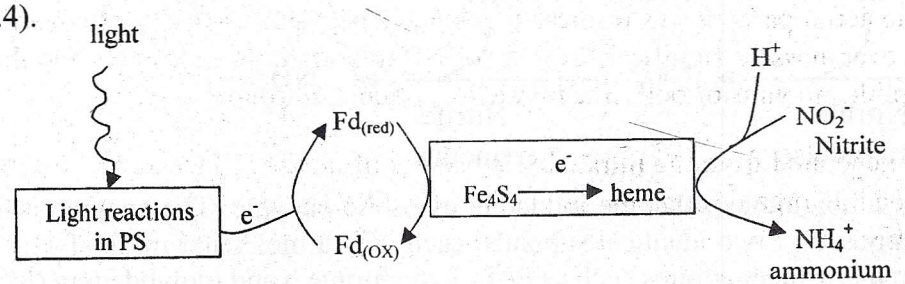


Figure 12.4 Model for coupling photosynthetic electron flow, via ferridoxin, to the reduction of nitrite by nitrite reductase. The enzyme contains two prosthetic groups, Fe_4 S_4 and heme, which participate in the reduction of nitrite to ammonium.

Light plays an important role in NO_3^- assimilation. When green plants are transferred from light to dark conditions the activity of nitrate reductase is depressed even when NO_3^- is present in adequate amounts. The reason is that nitrate reduction shows a tight coupling with the carbohydrate levels of the plant. Carbohydrates (photoassimilates) not only provide the carbon skeletons for the incorporation of ammonia but also provide energy for the reduction and assimilation of nitrate and ammonia (Figure 12.5). Under conditions of high nitrate reduction and assimilation in the dark, carbohydrates in the plant are significantly lowered. However, the lowering of carbohydrate levels in the light conditions is not as impressive because of the compensating effects of photosynthesis.

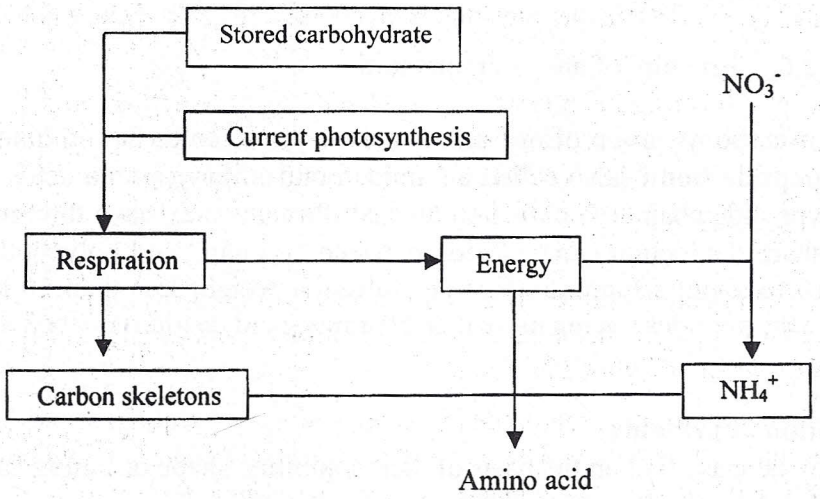


Figure 12.5 Relationship between the nitrate reduction and the carbohydrate content of the plant.

Nitrate absorbed by the roots may be reduced there itself, accumulated in vacuoles or transported to the aerial parts through the xylem stream. The relative proportion of nitrate reduced in roots and shoots varies with the plant species. Xylem sap of many woody plants contain only traces of nitrate and nitrogen predominantly occurs in organic form. That is plants belonging to this group such as bush berry (*Vaccinium angustifolium*), cran berry (*Vaccinium macrocarpon*), several species of *Rhododendron, Lupinus, Rhaphanus* and *Pisum etc*, reduce bulk of the absorbed nitrate in the root system. Their leaves generally have a very low activity of NR. In plants like *Xanthium pennsylvanium, X.borago* and different species of *Gossypium* and *Cucumis*, most of the absorbed nitrate (95 – 99%) is transported to the aerial parts. Roots of these plants have negligible activity of NR. Majority of the crop plants, however, possess significant activity of NR in both roots and shoots and their xylem sap contain considerable amounts of both free nitrate and organic nitrogen.

Ammonia generated from the nitrate by the action of nitrate reductase and nitrite reductase is finally assimilated into organic compounds via the GS – GOGAT or GDH or transamination systems as described earlier.

12.4 Protein classification and synthesis

Proteins are highly complex organic molecules present in cells of all forms of life. They provide structure, catalyze cellular reactions, and carry out a number of other functions. They are made up of twenty basic structural units called amino acids. An amino acid consists of an amino group, a carboxyl

group, a hydrogen atom, and a distinctive R group, all of which are bonded to an α – carbon. This carbon atom is named α (alpha) because it is adjacent to the carboxyl (acidic) group (Figure 12.6). The R group is referred to as a side chain that varies among different amino acids.

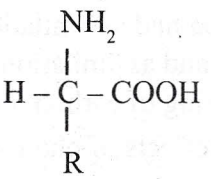


Figure 12.6 Structure of an _ – amino acid.

In proteins, the _ – carboxyl group of one amino acid is joined to the α – amino group of another amino acid by a **peptide bond** (also called an amide bond). Many amino acids joined by peptide bonds form a polypeptide chain, which is unbranched. Proteins may have thousands of amino acid units. In fact no sharp distinction can be made between "protein" and polypeptide. Generally, the peptides having 10 to about 50 amino acids or molecular weight less than 10 kDa are known as polypeptides while the peptides having more than 50 amino acid residues or above 10 kDa are known as proteins.

12.4.1 Classification of proteins

Proteins may be classified on the basis of their solubility, shape or biological function. Generally they are classified as simple, conjugated and derived proteins.

Simple proteins: The simple proteins yield only α – amino acids upon their hydrolysis. They are further classified into various groups on the basis of their decreasing solubility in aqueous solution. They are albumins, globulins, glutelins, prolamines, albuminoids, histones and protamines.

Albumins: These proteins are soluble in water and neutral salt solutions. Albumins are coagulated by heat. Examples are plasma albumin, serum albumin, ovalbumin in egg white, lactalbumin in milk.

Globulins: Globulins are insoluble in water but soluble in dilute solutions of neutral salts. Like albumins, these are also coagulated by heat. Further, albumins and globulins may be differentiated on the basis of the concentration of salt required to precipitate them from solution. Globulins are precipitated by lower concentrations of ammonium sulphate or sodium sulphate and such concentrations of these salts does not cause albumins to precipitate. Examples are edestin in hemp seed, ovaglobulin in egg white and plasma globulin.

Glutelins: These are insoluble in water and dilute solutions of neutral salts. However, glutelins are soluble in acids and bases. Heat causes coagulation of these proteins. Examples are glutemin in wheat and oryzenin in rice.

Prolamines: These are insoluble in water and absolute alcohol but soluble in 60 to 80 percent ethyl alcohol. They are not coagulated by heat. Zein in corn, gliadin in wheat, hordein in barley, and secalin in rye are example of this group.

Albuminoids (sclero-proteins): This group of proteins are soluble only after prolonged boiling with concentrated acid solutions. Keratin in hair, feathers, nails and horn, collagen in tendons, skin and bone, fibroin in silk are known albuminoids.

Histones: These are soluble in water and dilute acids, but insoluble in ammonia. These are non coagulated by heat. Examples are nucleohistones of nucleic acids.

Protamines: These are water, dilute acid, and ammonia soluble proteins. They contain relatively large number of basic diamino acids. Examples are sturin and salmin in the sperm of fish.

Conjugated proteins

The conjugated proteins contain non-protein portion in addition to simple protein group. On hydrolysis they yield non-proteinaceous substances and α – amino acids. Depending on the nature of the non-protein part which is called prosthetic group, conjugated proteins are further classified as (1) glycoproteins, contain carbohydrates, (2) phospho proteins, contain phosphoric acid, (3) lipoproteins, contain phospholipids, (4) chromoproteins, contain metals and (5) nucleoproteins contain nucleic acids as prosthetic groups.

Most of the conjugated proteins may be hydrolyzed to proteins free from other structural components i.e. simple proteins. Thus all the proteins may be classified into two broad groups: the soluble group and the insoluble group. The soluble group that includes the albumins, globulins, glutelins, prolamines, protamines and histones are soluble in aqueous media or some other common solvents. Scleroproteins also known as fibrous proteins are insoluble group of proteins because they are not dissolved in common solvents.

Derived proteins

This class of proteins are derived from the natural proteins by the action of physical, chemical or enzymatic agents. The derived proteins are further classified on the basis of cleavage product into primary derived proteins and secondary derived proteins.

Primary derived proteins: These are derivatives of proteins in which size of the protein molecule is not materially altered. These may be of three types.

a) **Proteans:** Proteans are the first products produced by the action of acids, enzymes or water on proteins and are insoluble in water. For example edestan is derived from edestin.

b) **Meta Proteins:** Metaproteins are produced by the further action of acid or alkali on proteins. These are insoluble in water, but soluble in dilute acid or alkali.

c) **Coagulated proteins:** These are insoluble protein products produced by the action of heat or alcohol on protein. Coagulated egg white is an example of this type.

Secondary derived proteins: These are derivatives of proteins formed as a result of hydrolysis. The molecules are smaller than those of the original protein. These may be of mainly three types.

a) **Proteoses:** These are soluble in water, not coagulated by heat, precipitated by saturating their solution with ammonium sulphate.

b) **Peptones:** These are soluble in water, not coagulated by heat and not precipitated by ammonium sulphate.

c) **Polypeptides:** Polypeptides are combination of two or more amino acids.

Alternatively proteins may be classified into two broad groups on the basis of their secondary structure, the **fibrous proteins** and **globular proteins**.

Fibrous proteins have the polypeptide chains arranged in long strands or sheets. These proteins usually consist of a single type of secondary structure. They provide protection, support, shape and form to the organisms. Collagen, fibroin, keratin, elastin are examples of fibrous proteins. Unlike fibrous proteins, the globular proteins contain the polypeptide chains that are folded into a globular or spherical shape. These are structurally complex, often containing several types of secondary structure. Most enzymes and peptide hormones are globular proteins.

Functionally proteins are classified as enzymes, transport proteins, nutrient and storage proteins, contractile or motile proteins, structural proteins, defence proteins, regulatory proteins and other proteins.

12.5 Mechanism of protein synthesis

Proteins are the end products of most information pathways. A typical cell requires thousands of different proteins at any given moment. These must be synthesized in response to the cell's current need, transported to the appropriate cellular location, and degraded when the need has passed. Protein synthesis is a complex biochemical process that in eukaryotic cells requires the participation of twenty amino acid precursors, ribosomes, 20 or more enzymes to activate the amino acids, different types of tRNAs, a dozen or more auxiliary enzymes and other specific protein factors for the initiation, elongation, and termination of polypeptides and number of other enzymes for the final processing of different kinds of proteins. In this section protein synthesis in both prokaryotes and eukaryotes is discussed.

Amino acids are the basic structural units of proteins. They are composed into a long chain protein polymers by the direction of DNA. A segment of DNA that contains the information required for the synthesis of a functional protein is referred to as a gene. A typical cell's DNA contains thousands of genes each encodes a distinct protein. A gene that contains the information as sequence of nitrogen bases in the form of codons can determine the amino acid sequence of a polypeptide. The mechanism of protein synthesis is broadly divided into two steps: the **transcription** and the **translation**.

12.5.1 Transcription

The conversion of genetic information of a segment of DNA into an RNA strand with a base sequence complementary to one of the DNA strands by the DNA directed RNA polymerase is called

transcription. Transcription generates three kinds of RNA such as messenger RNA (mRNA), that bears the message for protein synthesis, transfer RNA (tRNA) that carries amino acids during protein synthesis and ribosomal RNA that can be associated with the proteins to form the protein synthetic machine, the ribosome.

mRNA: Messenger RNA is a molecule of single stranded RNA synthesized under the direction of DNA by the enzyme RNA polymerase. The portion of DNA that is to be transcribed starts unwinding in the presence of an enzymes **helicase, topoisomerase** and proteins called **single stranded DNA binding proteins** (SSBS). One of the separated strands acts as a template or sense strand and the base sequence of this strand is transcribed into the mRNA sequence. Ribonucleotide triphosphates such as ATP, GTP, CTP and UTP are used to produce an RNA copy of the DNA sequence.

$$n\,[ATP, GTP, CTP, UTP] \xrightarrow[\text{DNA template}]{\text{RNA polymerase}} RNA + n\,PP_i$$

RNA synthesis proceeds in a 5′ to 3′ direction with new nucleotides being added to the 3′ end of the growing chain.

Prokaryotic mRNA is transcribed and translated in a single cellular compartment. It contains the message for several proteins so that it is **polycistronic**. In contrast, eukaryotic mRNA is normally monogenic (monocistronic) and is synthesized in the nucleoplasm by RNA polymerase II. Further the primary RNA transcript that is produced by the action of RNA polymerase II from the DNA template strand is a long complex structure called **heterogenous nuclear RNA** (hn RNA). This hn RNA is converted into mRNA by post-transcriptional modifications carriedout by an enzyme polyadenylate polymerase. The sized eukaryotic mRNA contains 3′ poly A and 5′ methylguanosine cap. The processed mRNA then passes out of the nucleus into the cytoplasm where its information is translated into the protein.

The message for protein synthesis resides in the base sequence of mRNA. The information for selecting a specific amino acid out of twenty and get them tagged to form a protein molecule is controlled by three bases together called triplets or codons. The mRNA contains four different types of bases that can form 64 different types of codons. Each codon specifies a particular amino acid. These 64 codons comprise a codon dictionary of an amino acids called genetic code.

The genetic code is defined as the information system that specifies insertion of an amino acid in a growing polypeptide chain. It provides relation between the sequence of bases of mRNA and the sequence of amino acids in protein. Of the 64 triplets that can be formed from 4 bases, three triplets such as UAA, UGA and UAG do not code for any amino acid and are termed as chain termination codons or nonsense or stop codons. The remaining 61 codons direct amino acid incorporation into a protein. These codons are called sense codons. Among the sense codons the AUG is the polypeptide chain initiator which codes an amino acid methionine.

12.5.2 Translation

The mechanism by which the mRNA base sequence is converted into the amino acid sequence of a polypeptide chain is called translation. Translation consists of the following 4 basic steps:

a) Formation of aminoacyl tRNA
b) Chain initiation
c) Chain elongation and
d) Chain termination

a) Formation of aminoacyl tRNA: The first step of protein synthesis is an amino acid activation, a process in which an amino acid is attached to a specific transfer RNA molecule to form aminoacyl tRNA. It occurs in two steps. In the first step, the amino acid is activated by reaction with ATP to yield aminoacyladenylate enzyme complex and pyrophosphate. In the second step amino acid from aminoacyladenylate enzyme complex is transferred to specific tRNA to from aminoacyl tRNA and AMP. Both these steps are catalysed by a single enzyme called **aminoacyl tRNA synthetase**.

$$\text{Amino acid} + \text{ATP} + \text{E} \longrightarrow \text{Aminoacyladenylate-E-complex} + \text{PPi}$$

$$\text{Aminoacyldenylate-E-complex} + \text{tRNA} \longrightarrow \text{Aminoacyl tRNA} + \text{AMP}$$

There are at least 20 aminoacyl tRNA synthetases which are highly selective in recognition of the amino acid to be activated and its prospective tRNA acceptor. Each aminoacyl tRNA synthetase is able to pick up "its" amino acid out of the twenty present in the cytoplasm and transfer it to the correct tRNA. The protein synthetic machinery recognizes only the anticodon of the aminoacyl tRNA and can not tell whether correct amino acid is attached. Formation of specific aminoacyl tRNA complex is the function of a aminoacyl tRNA synthetase. For that some some aminoacyl tRNA synthetases will even proof read just like DNA polymerases do. If any wrong aminoacyl tRNA is formed, aminoacyl tRNA synthetases will hydrolyze that incorrect amino acid from the tRNA in order to prevent the formation of incorrect product. Synthetases recognize their tRNA partner primarily on the basis of its anticodon and amino acid acceptor stem.

b) tRNA: Transfer RNA is the smallest species of RNA in the cell. The base sequence of a yeast alanine tRNA molecule was first determined by Robert Holley in 1965. The tRNA molecules consist of about 73-90 ribonucleotides. All tRNAs, in addition to the usual bases A, G, C and U contain unusual bases such as inosine, pseudouridine, dihydrouridine, ribothymadine and methylated derivatives of guanosine and inosine. Almost all known tRNAs can be drawn in a clover leaf form (Figure 12.7). Each tRNA molecule contains five arms named for their structure or function. They are D arm, anticodon arm, variable arm, TψC or T arm and amino acid acceptor arm. The anitcodon arm contains the codon triplet which is complementary to the mRNA codon triplet. The amino acid acceptor stem holds the activated amino acid at its 3′ terminal - OH group. The 3′ end of all tRNAs has the same C – C – A sequence with a free hydroxyl group. Each amino acid is specified by one to three tRNAs.

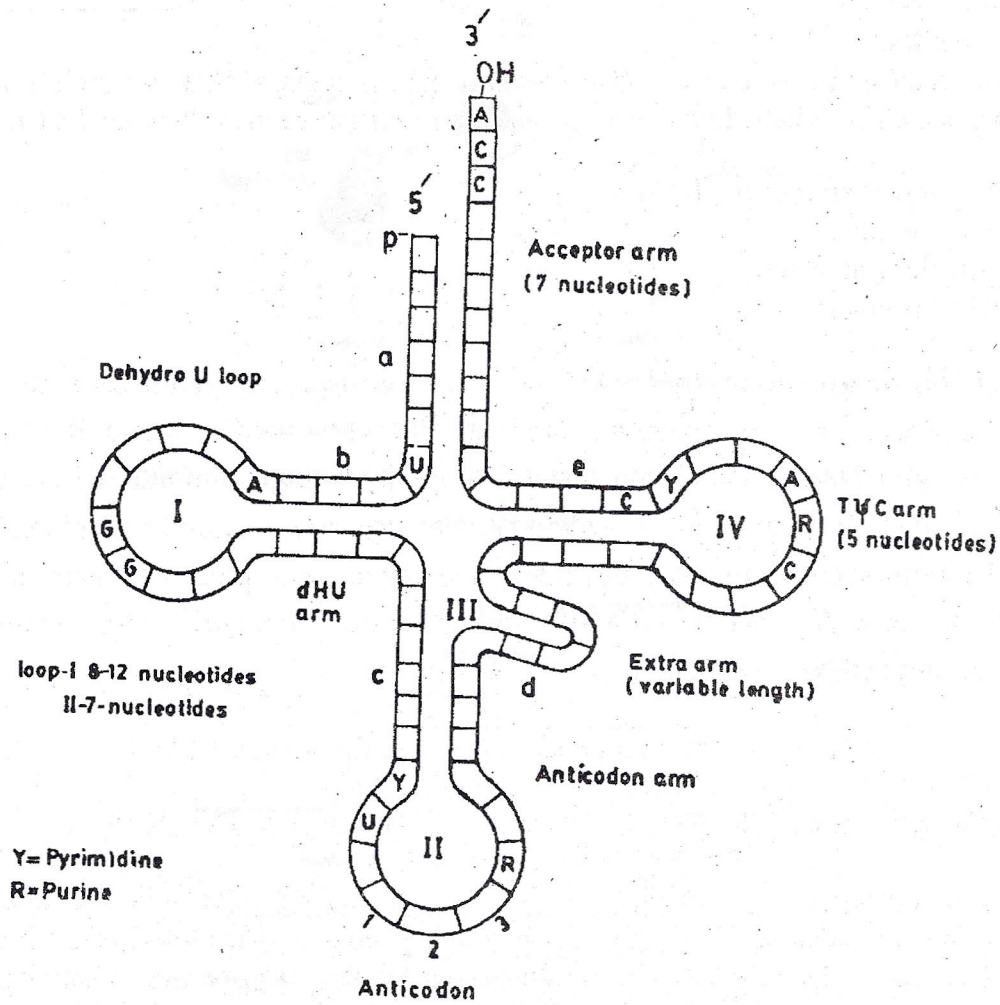


Figure 12.7 Common features of transfer RNA.

d) Ribosomes: The actual catalytic machinery for polypeptide synthesis is present in the ribosomes. Ribosomes are ribonucleo protein molecules made up of two interacting but separable subunits. There are two classes of ribosomes designated as 70S and 80S. The 70S type of ribosomes found in prokaryotes and mitochondria and chloroplasts of eukaryotes can be dissociated into a larger 50S subunit and a smaller 30S subunit. The 30S subunit contains 21 different proteins (labeled as S_1 to S_{21}) and a 16S RNA molecule. The 50S subunit contains 34 different polypeptides (labeled as L_1 to L_{34}) and a 23S and 5S type of RNA molecules (Figure 12.8 and 12.9). The 80S type of ribosomes were found in the cytoplasm of eukaryotes. They are composed of 40S and 60S type of small and large subunits, respectively (Figure 12.9).

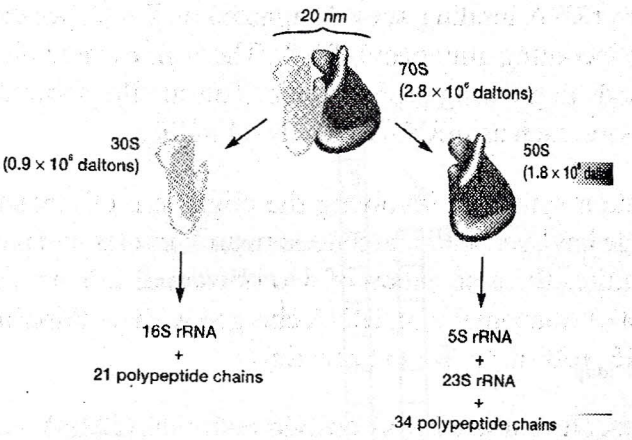


Figure 12.8 The structure of the 70S prokaryotic ribosome.

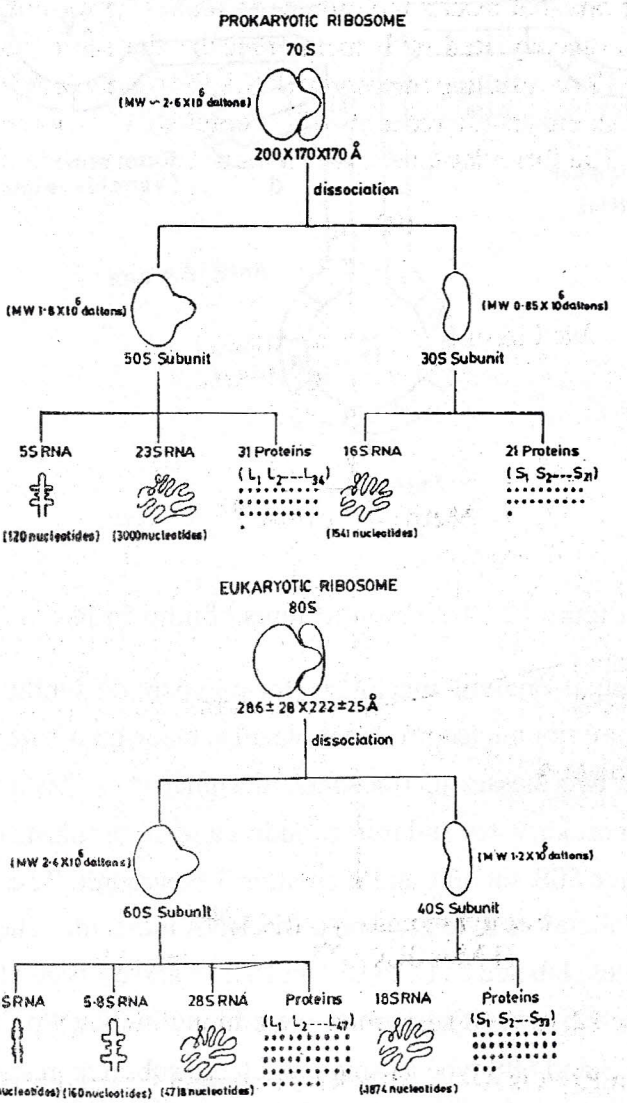


Figure 12.9 Components of prokaryotic and eukaryotic ribosomes

The larger subunit contains two tRNA binding sites designated as P site, which binds the peptidyl tRNA and the A site to bind the incoming aminoacyl tRNA. The peptidyl transferase of P site catalyses the formation of peptide bonds in growing protein chain. *The smaller subunit is mainly involved* in ribosomal recognition processes such as mRNA and tRNA binding.

d) Initiation: Initiation of protein synthesis involving the alignment of ribosome and mRNA for correct translation of the message has been well understood in prokaryotes and partly in higher plants. It is a complex process that requires the association of two ribosomal subunits, proper alignment of mRNA on the smaller (30s or 40s) ribosomal unit, tRNA charged with methionine and protein initiation factors called IF_1, IF_2 and IF_3 (eIF-n, 'e' for eukaryotic).

Protein synthesis in prokaryotes starts with N-formylmethionine (f Met). A special tRNA brings formylmethionine to the ribosome to initiate protein synthesis. This initiator tRNA abbreviated as $tRNA_f^{Met}$ is different from the one that inserts methionine in internal positions ($tRNA_m^{Met}$). In E.coli, uncharged $tRNA_f^{Met}$ is first aminoacylated with methionine by the same aminoacyl – tRNA synthetase that changes $tRNA_m^{Met}$. The resulting methionyl $tRNA_f^{Met}$ is specifically N-formylated to yield formylmethionyl $tRNA_f^{Met}$ in an enzymatic reaction which employs N^{10} formyltetrahydrofolate as its formyl donor (Figure 12.10). The formylation enzyme does not recognize methionyl $tRNA_m^{Met}$.

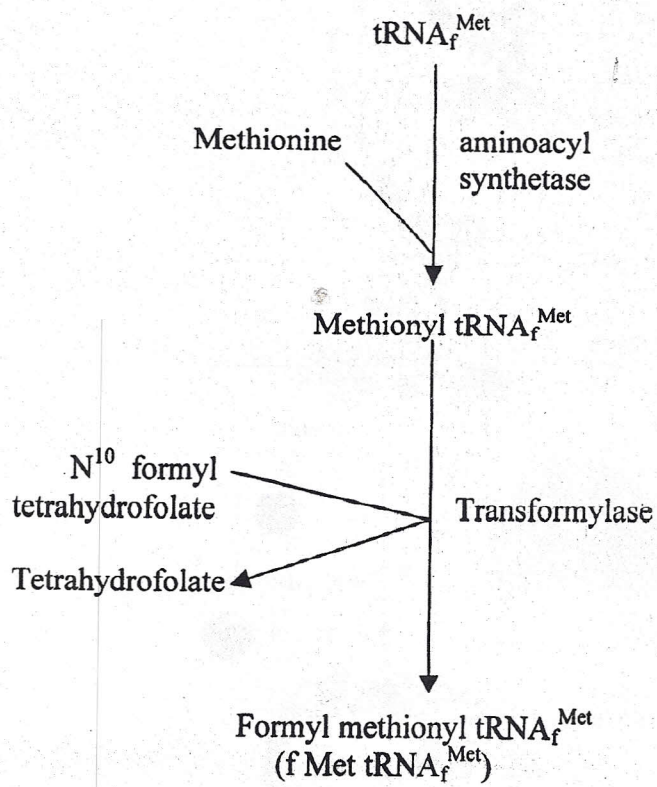


Figure 12.10 Synthesis formyl methionyl $tRNA_f^{Met}$.

The chain initiating codon of mRNA is AUG. All mRNAs contain first codon AUG, hence it is called chain initiatior codon. Actually intact ribosomes do not directly bind mRNA so as to initiate polypeptide synthesis. As shown in the Figure12.11, upon completing a cycle of polypeptide synthesis, the

30S and 50S subunits remain associated as inactive 70S ribosome. It is dissociated into subunits by IF-3 and IF-1. Now, GTP, mRNA and a complex of IF-2 with fmet – $tRNA_f^{Met}$ bind to the 30S subunit in unknown order. In the next step, the 50S subunit is bound and the 70S initiation complex is formed. This binding is accompanied by the hydrolysis of GTP to GDP and Pi. Initiation results in the formation of an fMet- $tRNA_f^{Met}$. mRNA ribosome complex in which the fMet- $tRNA_f^{Met}$ occupies the ribosomes 'P' site while its 'A' site is ready to accept an incoming aminoacyl tRNA.

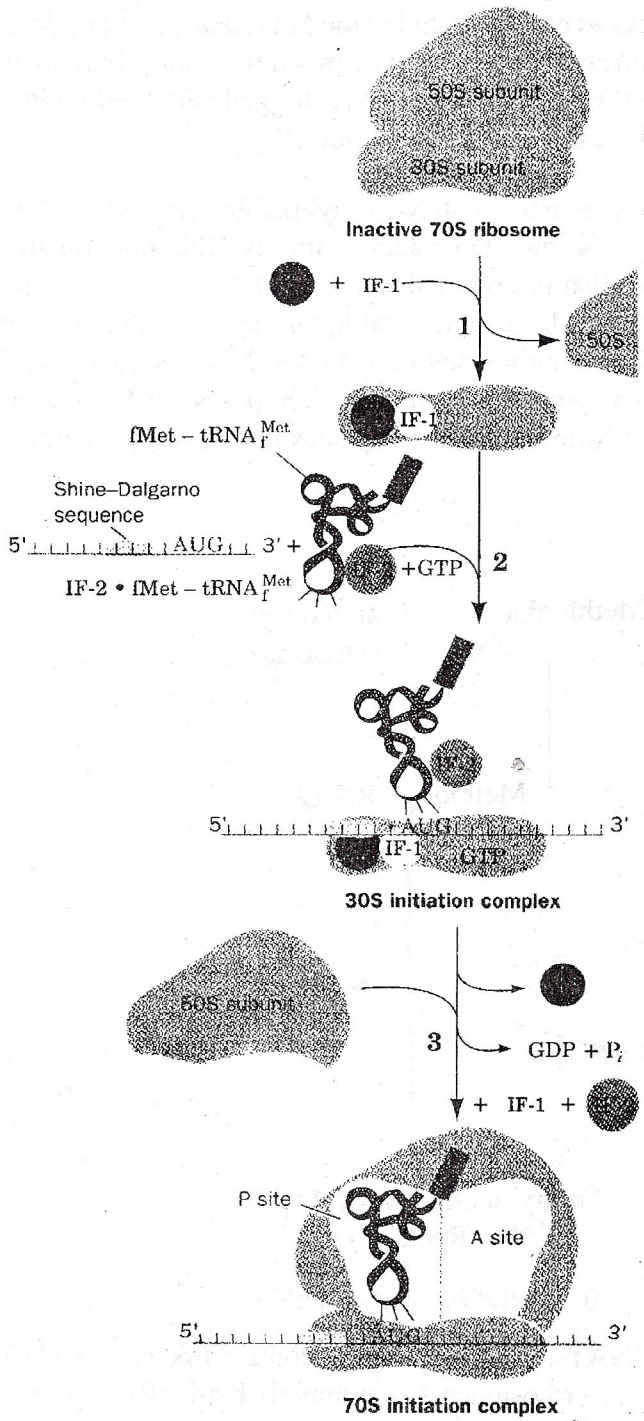


Figure 12.11 The initiation pathway in *E.coli* ribosomes.

Eukaryotic initiation takes place with the binding of special initiator Met- $tRNA_i^{Met}$ (here the subunit 'i' stands for eukaryotic initiator tRNA) to 40S subunit followed by attachment of the mRNA, GTP and 50s subunit. Like prokaryotic initiation, here methionine is not formylated. Moreover, 6 initiation factors designated as eIF-1 to eIF-6 are involved in the formation of eukaryotic initiation complex.

e) Chain elongation: The elongation of polypeptide chain takes place in three steps. In the first step, the aminocyl tRNA as aminoacyl tRNA. EF.Tu.GTP binds to the ribosome 'A site containing proper mRNA codon. This binding is assisted by EF-Tu (elongation factor Tu) and GTP hydrolysis to GDP and Pi. In the remainder of this step which serves to regenerate the EF-Tu.GTP complex, GDP is displaced from EF.Tu.GDP by the elongation factor EF-Ts, which in turn is displaced by GTP (Figure 12.12).

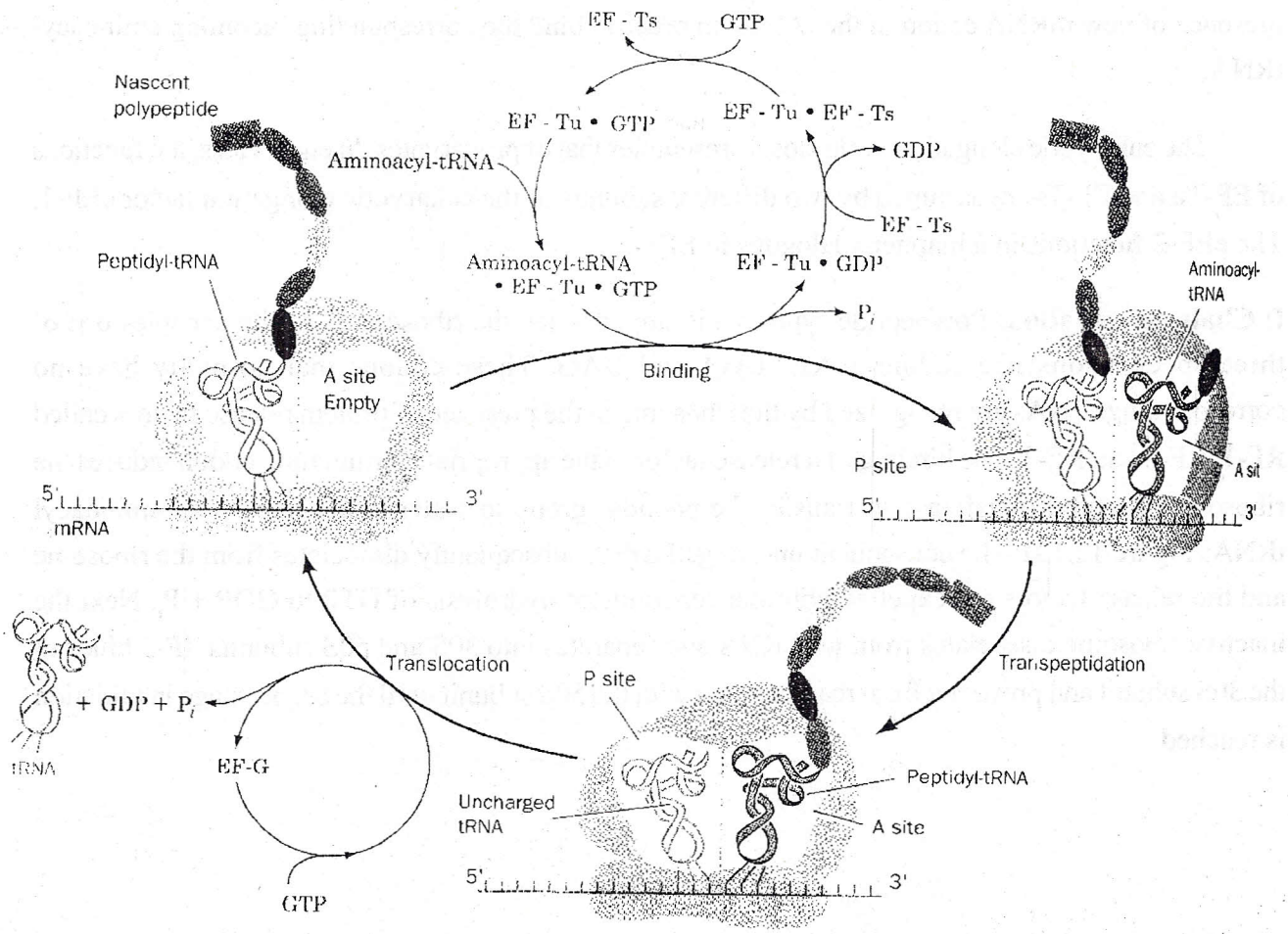


Figure 12.12 The elongation cycle in *E.coli* ribosomes. Eucaryotic elongation follows a similar cycle but EF-Tu and EF-Ts are replaced by a single multisubunit protein, eEF-1, and EF-G is replaced by eEF-2.

In the second step of the elongation cycle, a peptide bond is formed between the α – amino group of 'A' site amino acyl tRNA and the α – carboxyl group of 'P' site peptidyl tRNA. This reaction known as transpeptidation and results in the synthesis of new peptide bond, involves transfer of peptidyl group from `P' site peptidyl – tRNA to an amino acid on `A' site aminooxyl-tRNA in the presence of **peptidyl** transferase **peptidyl transferase**. As a result, the peptide chain grows by one amino acid Ribosomes read mRNAs in 5′ → 3′ direction and polypeptide synthesis proceeds from N-terminus to C-terminus, that is incoming amino acids must be appended to a growing polypeptide's C-terminus.

In the last step of the elongation cycle, the now uncharged P-site tRNA (at first $tRNA_f^{Met}$ but subsequently a non initiator tRNA) leaves the P site, and the newly lengthened peptidyl tRNA together with its bound mRNA moves form the 'A' site to the 'P' site in a process called **translocation**. Translocation requires the participation of EF-G and hydrolysis of GTP. This process then allows the presence of new mRNA codon in the 'A' site in order to bind the corresponding incoming aminoacyl tRNA.

The eukaryotic elongation cycle closely resembles that of prokaryotes. In eukaryotes, the functions of EF-Tu and EF-Ts are assumed by two different subunits of the eukaryotic elongation factor eEF-1. The eEF-2 functions in a manner analogous to EF.G.

f) Chain termination: Polypeptide synthesis is stoped when the ribosomal 'A' site occupies one of three special nonsense codons UAA, UGA and UAG. These codons that normally have no corresponding tRNAs are recognized by the ribosome in the presence of protein release factors called RF-1, RF-2 and RF-3. The binding of a release factor to the appropriate termination codon induces the ribosomal peptidyl transferase to transfer the peptidyl group to water rather than to an aminoacyl tRNA (Figure 12.13). The consequent unchanged tRNA subsequently dissociates from the ribosome and the release factors are expelled with the concomitant hydrolysis of GTP to $GD^P + P_i$. Next the inactive ribosome dissociates from its mRNA and separates into 30S and 50S subunits. IF-3 binds to the 30S subunit and prevent it from reassociating with the 50S subunit until the proper stage in initiation is reached.

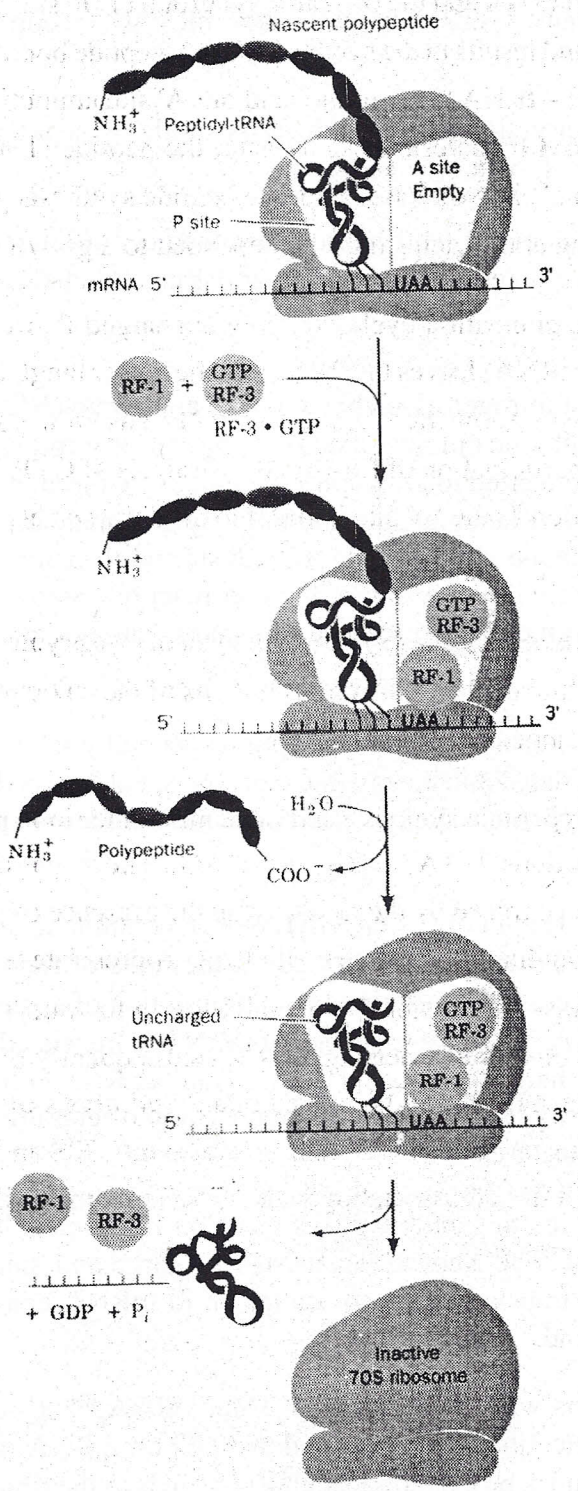

Nascent polypeptide
NH3+
Peptidyl-tRNA
A site
Empty
P site
mRNA 5'
UAA
3'
RF-1
+
GTP
RF-3
RF-3 • GTP
NH3+
GTP
RF-3
RF-1
5'
UAA
3'
H2O
NH3+
Polypeptide
COO−
Uncharged
tRNA
GTP
RF-3
RF-1
5'
UAA
3'
RF-1
RF-3
+ GDP + Pi
Inactive
70S ribosome

Figure 12.13 The termination sequence in *E.coli* ribosomes. RF-1 recognizes the termination codons UAA and UAG, whereas FF-2 recognizes UAA and UGA. Eucaryotic termination follows an analogous pathway but requires only a single release factor, eRF, that recognizes all three termination codons.

The termination of eukaryotic protein synthesis is similar except that only one release factor, eRF together with GTP appears to be involved.

The linear polypeptide chain just synthesized then undergoes different post-transcriptional modifications that includes secondary, tertiary and quaternary structural changes.

12.6 Summary

The principal sources of nitrogen available to plants are nitrate (NO^-_3) and ammonium (NH_4^+). Ammonium derived from symbiotic nitrogen fixation or by root absorption from the soil or generated through nitrate reduction is converted to glutamine and glutamate through the action of GS-GOGAT. Glutamate is also formed from α-ketoglutarate and NH_4^+ by glutamate dehydrogenase. Once assimilated into glutamine or glutamate nitrogen may be transferred to many other organic compounds through various reactions, including the transamination reaction. Some legumes incorporate their fixed ammonium into asparagine in the presence of asparate aminotransferase and aspargine synthetase.

Plants that do not form nitrogen fixing associations generally take up nitrogen in the form of nitrate. Before nitrate is assimilated into the organic molecules, it is reduced to nitrite in the cytosol by the enzyme nitrate reductase. Nitrite is then reduced to ammonium in root plastids or chloroplasts via the enzyme nitrite reductase.

Amino acids formed as a result of GS-GOGAT, reductive amination or transamination are used for the synthesis of proteins. Proteins are classified on the basis of their solubility or structure or function. Protein synthesis occurs by transcription and translation of a gene. A DNA segment called gene is transcribed into mRNA. The base sequence of mRNA is translated into an amino acid of the protein. Translation requires charged aminoacyl tRNA. Transfer RNA consist of 70 – 95 nucleotides that can be arranged in the clover leaf secondary structure. Amino acids are appended to their cognate tRNAs by specific tRNA synthetases.

The actual process of protein synthesis takes place on ribosomes that serve as workbenches, with mRNA as the blue print. The ribosome consists of a large and small subunit which contain rRNAs and polypeptides. In translation ribosomes attach to mRNA and synthesize a polypeptide beginning at the N-terminal end.

Protein synthesis begins with the binding of fMet- $tRNA_f^{Met}$ (prokaryotes) or an initiator methionyl $tRNA_i^{Met}$ (eukaryotes) to an initiator codon AUG on mRNA ribosomal complex. In the elongation of polypeptide chain, a proper aminoacyl tRNA binds to the ribosomal 'A' site with the aid of EF-Tu and GTP. Peptidyl transferase then catalyzes the transpeptidation reaction by to lengthen polypeptide chain. During translocation, the peptidyl tRNA moves to the P site and the ribosome

travels along the mRNA one codon. Protein synthesis was terminated when the release factors and the chain termination codons are interacted at the rib Bomal A site. Newly formed polypeptide then undergoes variety of post transcriptional modifications to form a functional protein.

12.7 Model Questions

1. Write a detailed account of nitrogen assimilation in plants.
2. "Ribosome is the site of protein synthesis". Explain.
3. What are proteins? Give protein classification on the basis of their solubility.
4. Write short notes on :

(a) GS-GOGAT
(b) Transamination
(c) tRNA

12.8 Reference books

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G.ROSAIAH.

LESSON-13

LIPIDS

13.0 OBJECTIVES

After completion of this lesson you will learn:

1) The naturally occurring biomolecules, that have saponifiable esters of long-chain fatty acids are called lipids.
2) Different classes of membrane lipids and the structure of membranes.
3) In fats and oils, stress is given on properties and distribution and importance of storage lipids or triacylglycerols.
4) Classification of lipids based on various aspects.
5) Biosynthesis of both saturated and unsaturated fatty acids.
6) Lipid catabolism by lipid degrading enzymes and β-oxidation

CONTENTS

13.1 INTRODUCTION

Lipids are naturally occurring biomolecules comprising a large and diverse group of compounds that are saponifiable esters of long-chain fatty acids. These are organic non-polar compounds that are totally or nearly insoluble in water. These are soluble in organic solvents like ether, chloroform or benzene. Lipids occur in plants and animals and are utilized by human beings.

By virtue of the presence of both polar (ionic) and non-polar groups, they are 'amphiphilic' in nature. Sphingo-myelins and cerebrosides are insoluble in ether and phosphatides in acetone. Moreover, all lipids are not esters or potential esters of fatty acids, as

indicated by amide nature of the fatty acid moiety in cerebrosides and presence of ether linkages in many lipids.

The main biological functions of lipids are:-

1) These are major structural elements of cell membranes of all living systems.
2) They are major reserve source of energy.
3) They are carriers of fat soluble vitamins A, D, E and K.
4) They are metabolic fuels and emulsifying agents.
5) Many hormones found in animals are lipids or derivatives of lipids.
6) They provide essential fatty acids such as linoleic and linolenic.
7) They provide precursors that is linoleic acid for biosynthesis of postaglandins, which is essential for maintaining various body functions.
8) Insulating materials against atmospheric heat and cold.
9) Protect organs against physical shock.
10) Protective coatings of lipids prevent excessive loss of water and infection.

The most abundant kinds of lipids are the fats or triacylglycerols which are major fuels for most organisms. There is another class, polar lipids, are major components of cell membrane, the "containers" in which metabolic reactions occur. Membranes enclose cells from the environment and make possible internal compartmentalization of metabolic activities within cells. Membranes also contain enzymes and transport systems.

Fatty acids are characteristic building block components of most of the lipids. Fatty acids are long-chain organic acids having 4 to 24 carbon atoms with a single carboxyl group and a long, non-polar hydrocarbon tail. This gives most lipids their water-insoluble and oily or greasy nature (Fig. 13.1).

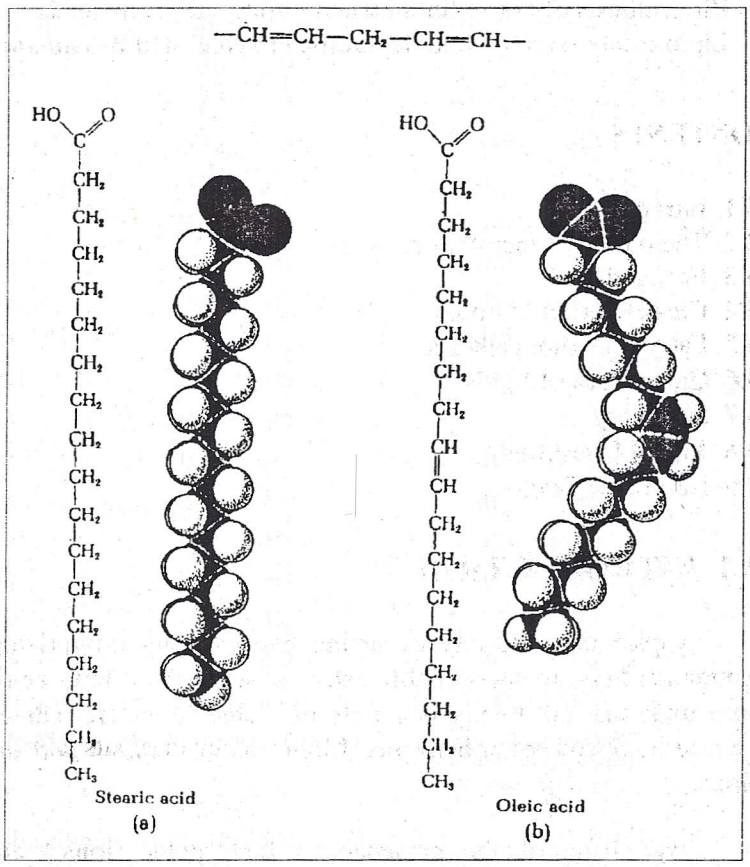


Fig. 13.1 Common fatty acids shown as structural formulae and as space-filling models.

There are several classes of membrane lipids. These are called polar lipids, as they have one or more highly polar 'head' groups and hydrocarbon tails. The most abundant membrane lipids are phospholipids. Phospholipids serve as structural elements of membranes. These lipids contain phosphorous in the form of phosphoric acid groups. So these are called phosphoglycerides (Fig. 13.2). Phosphoglycerides contain two fatty acid molecules esterified to the first and second hydroxyl groups of glycerol. The third hydroxyl group of glycerol forms an ester linkage with phosphoric acid.

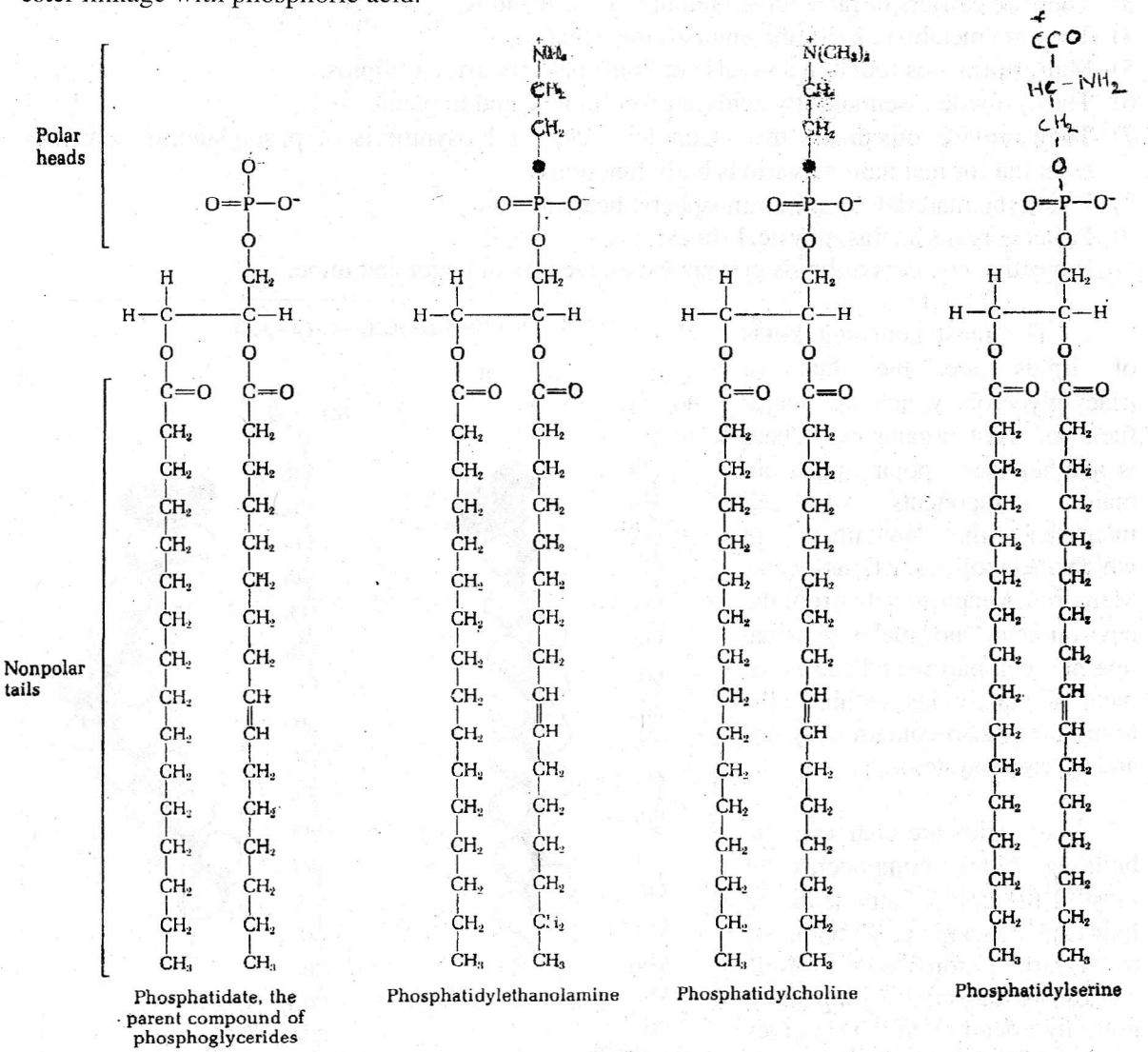
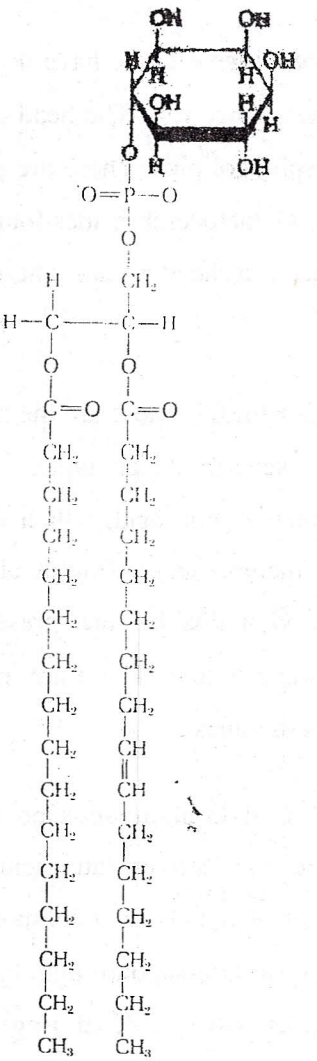


Fig. 13.2 The common phosphoglycerides.

There are several different classes of *phosphoglycerides.* These differ in their head alcohol groups. All phosphoglycerides contain two non-polar tails, *contributed by their long-chain fatty acids.* One fatty acid is saturated and the other is unsaturated. The unsaturated fatty is esterified to the middle or 2-hydroxyl of the glycerol. Phosphoglycerides are named according to the alcohol in polar heads (Fig. 13.2).



Phosphatidylinositol

Cardiolipin: found in the mitochondrial membrane differs from the rest of phosphoglycerides. It is a double phosphoglyceride (Fig. 13.3).

Membrane lipids are amphipathic, which means phosphoglycerides have a polar hydrophilic head and hydrophobic, non-polar tails. Whereas the storage lipids (triglycerol) and waxes are not.

Sphingolipids are also important components of membranes. These also have a polar head and two non-polar tails, but they contain no glycerol. Sphingolipids are composed of one molecule of a long chain fatty acid, one molecule of long-chain amino alcohol sphingosine and a polar head alcohol.

There are three subclasses of sphingolipids, sphingomyelins, cerebrosides and gangliosides.

Sphingomyelins:- These are the simplest and most abundant sphingolipids. They contain (Fig. 13.4) phosphocholine or phosphoethanolamine as their polar head groups. As they have phosphorus, they may also be classed as phospholipids together with phosphoglycerides. These are present in most membranes of animal cells, the myelin sheath surrounding certain nerve cells.

Fig. 13.3 Cardiolipin, a "double" phosphoglyceride, is present in large amounts in mitochondrial and bacterial membranes.

Cerebrosides:- These have no phosphorous and have no electric charge since their polar head groups are neutral. The head consists of one or more sugar units, so cerebrosides often called glycosphingolipids. These are glycolipids, a generic name for lipids having sugar groups (Fig. 13.5). Galactocerebrosides found in the cell membranes of the brain. Glucoceribrosides contain D-glucose as head group. These are present in the cell membranes of non-neutral tissues.

Gangliosides:- These are the most complex sphingolipids, contain very large polar heads made up of several sugar units. One or more terminal sugar units of gangliosides in N-acetylneuraminic acid, which is also called as sialic acid. It has a negative charge at pH 7. Some membrane glycolipids also contain N-acetylneuraminic acid in the oligosaccharide side chain. Gangliosides are present in brain membranes and lesser amounts in non-neutral membrane tissues. These are important components of specific receptor sites on the surface of cell membranes.

Cells contain non-saponifiable lipids in addition to saponifiable lipids. Non-saponifiable lipids do not contain fatty acids and thus cannot form soap, whereas saponifiable lipids yield soaps of their fatty acid components on hydrolysis by heating with alkali. There are two major classes of non-saponifiable lipids, steroids and terpenes. Steroids are complex fat-soluble molecules with four fused rings. Sterols are the steroids that contain steroid alcohols. Plant cell membranes contain stigmasterol. These are having a double bond between 22 and 23 carbons (Fig. 13.6).

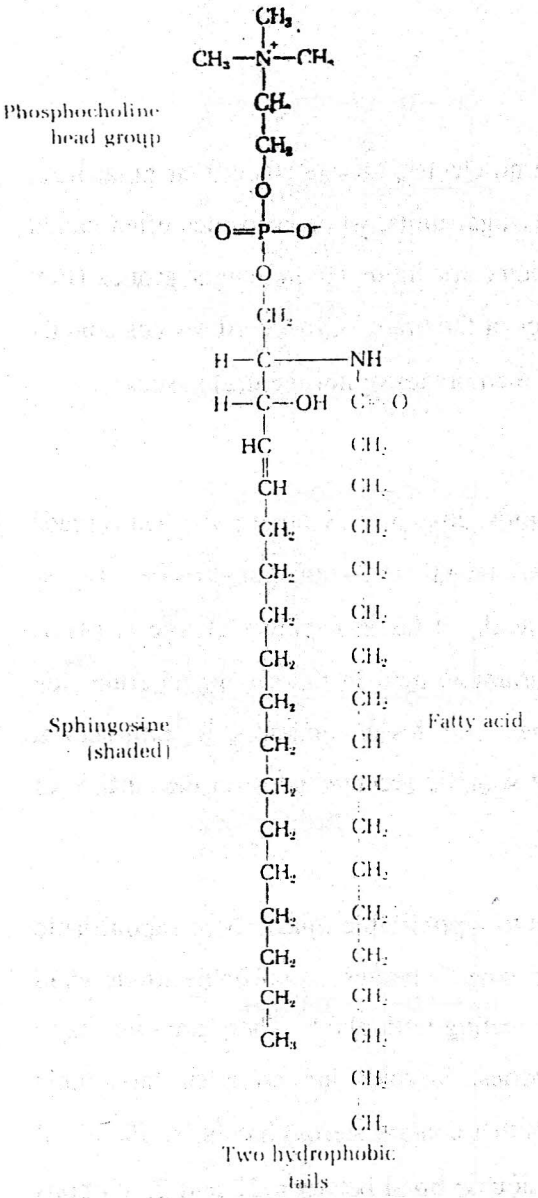


Fig. 13.4 Structure of sphingomyelin.

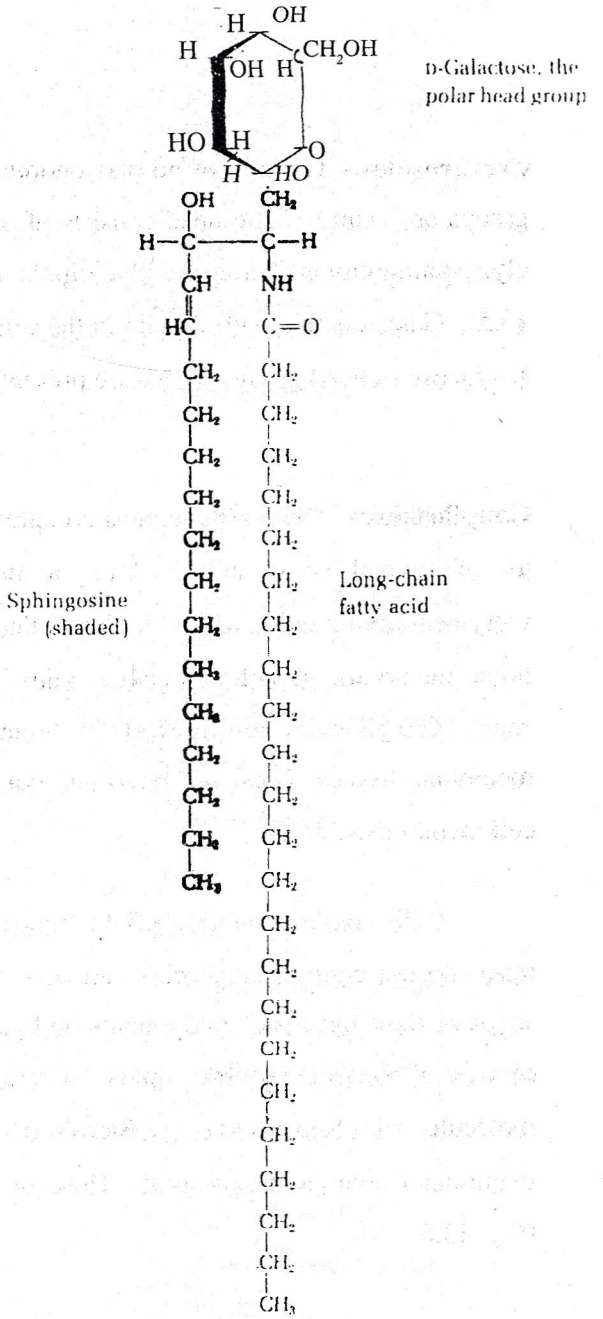
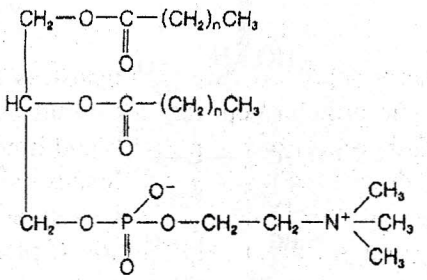
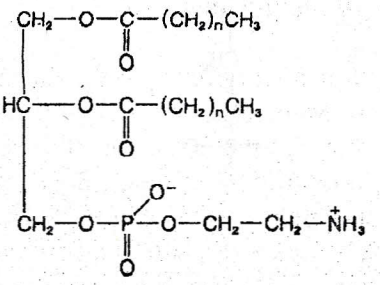


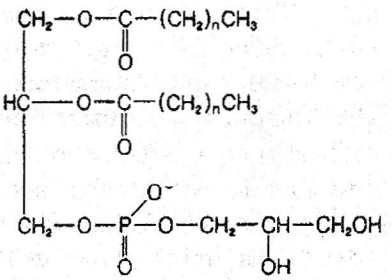
Fig. 13.5 A galactocerebroside. The fatty acid component of cerebrosides usually has carbon atoms.



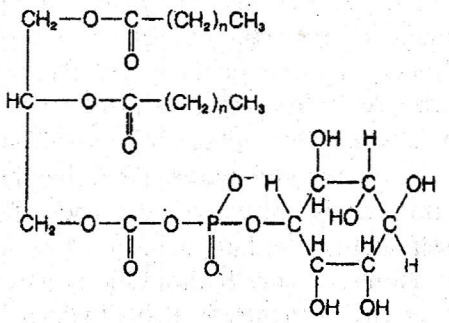
phosphatidyl choline



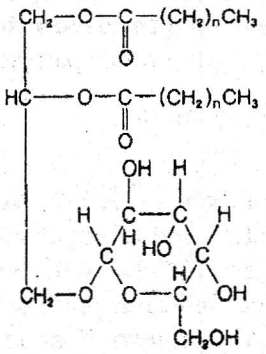
phosphatidylethanolamine



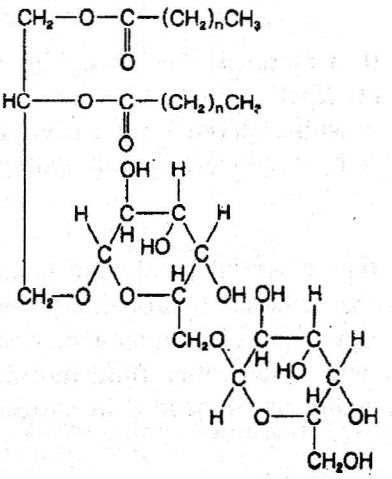
phosphatidylglycerol



phosphatidylinositol



monogalactosyldiglyceride



digalactosyldiglyceride

Fig. 13.6 Structures of membrane phospholipids and glycolipids

The nature of membranes

Electron micrographs show that most biological membranes are similar, regardless of their location. Membranes consist of proteins and lipids. The proteins representing about one half of two-thirds of the membrane dry weight. The kinds and proportions of proteins and lipids vary with the kind of membrane and physiological state of the cell. So some differences exist between plasmalemma, tonoplast, endoplasmic reticulum and membranes of dictyosomes, chloroplasts, nulcei, mitochondria, peroxysomes and glyoxysomes. The principal lipids of plant membranes are phospholipids, glycolipids and sterols.

The four most abundant phospholipids are phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl inositol. The two most abundant glycolipids are monogalactosyldiglyceride, with one galactose sugar and digalactosyl-diglycereride, with two galactoses (Fig. 13.6). The glycolipids are found mainly in chloroplast membranes, in which phospholipids are much less abundant. The structures of all these lipids have some common features. First, they have a 3-carbon glycerol backbone to which two long-chain fatty acids are esterified. These fatty acids have 16 or 18 carbons with double bonds betweeen them. The fatty acids are hydrophobic (water fearing), the glycerol backbone with its oxygen atoms is more hydrophilic (water loving). The final part of these lipids, shown at the bottom of each structure is also a hydrophilic portion, because it is either electrically charged or has numerous oxygens, with which water can associate by hydrogen bonding. Molecules with hydrophobic and hydrophilic regions are said to be amphipathic molecules.

In all membranes, the hydrophilic parts of lipids dissolve in water at either surface, and the fatty acids repelled by water extend toward the interior part of the membrane from each side and associate with each other by van der Waals forces. Sterols have a long hydrophobic part rich in carbon and hydrogen and a small hydrophilic part.

Another essential membrane component is Ca^{2+}. The function of Ca^{2+} is not well understood, but it bonds hydrophilic portions of phospholipids to each other and to negatively charged groups of proteins on the membrane surfaces. In 1972, S.J. Singer and G.N. Nicholson proposed a new model called **fluid mosaic model** (Fig. 13.7). This model indicates that some protein molecules are imbedded in the fluid bilayer of lipids such that "the proteins float in a lipid sea".

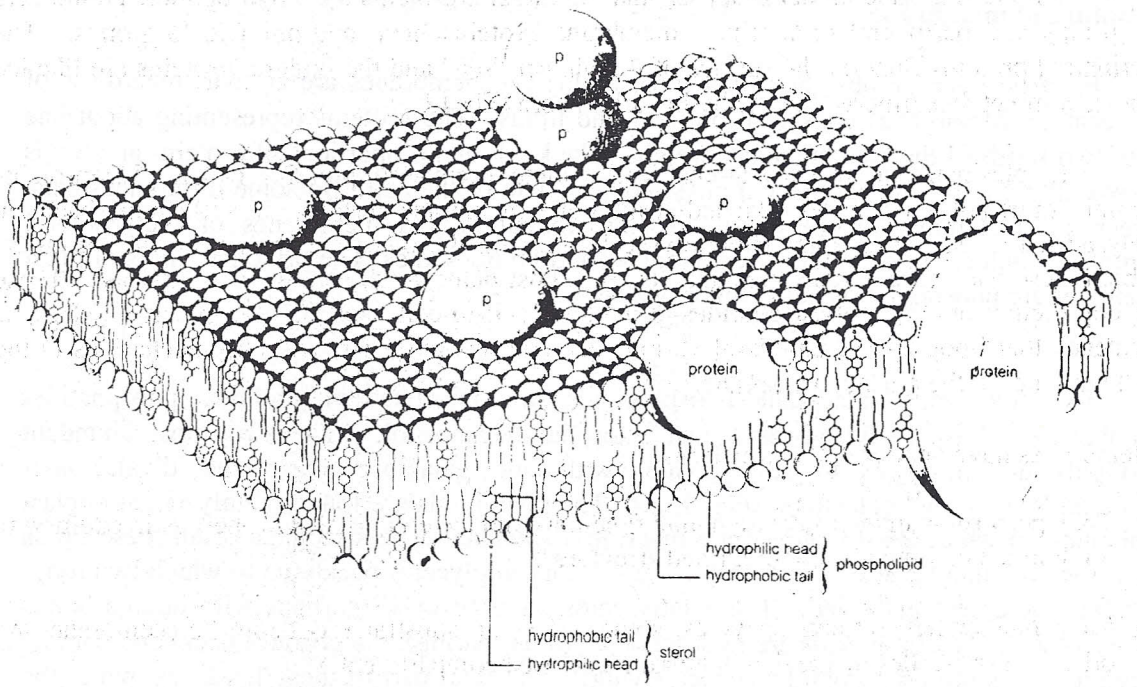


Fig. 13.7 The fluid mosaic model of membrane structure

The matrix is a polar lipid bilayer. The bilayer is fluid because the hydrophobic tails of its polar lipid consist of a mixture of saturated and unsaturated fatty acids. This is fluid at the normal temperature of the cell.

The lipids are truly fluid, a phospholipid molecular can move laterally in one-half of the bilayer of a bacterial membrane from one end of the cell to the other in one second. Some of the hydrophobic proteins penetrate deeply into the lipid-rich interior. These are called **integral proteins**, are bound tightly within the membrane and can be removed only with certain detergents that break hydrogen bonds between all components of the membrane. Some proteins extend all the way through the bilayer. These are called **peripheral proteins**, are loosely bound to one or the other side of the membrane surface and can be removed with dilute salt solutions or with detergents (Fig. 13.8).

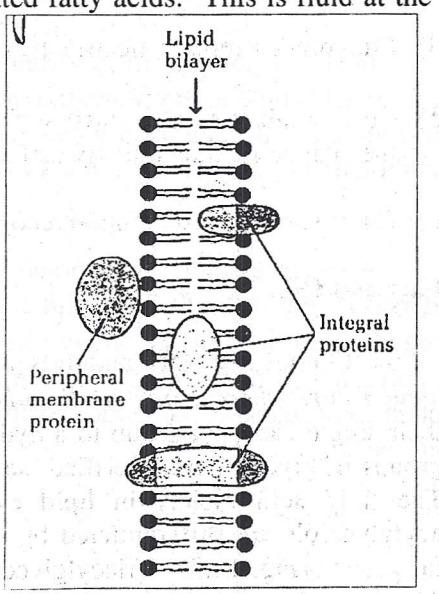


Fig. 13.8 Membrane proteins.

The fluid mosaic model proposes that the integral proteins have hydrophobic amino acid R groups and peripheral or extrinsic membrane proteins have hydrophobic R groups. The peripheral proteins float on the surface of the bilayer "sea" and the integral proteins are like ice bergs, completely surmerged in the hydrocarbon core (Fig.13.7).

In plasmamembranes of animal cells, some peripheral proteins called glycoproteins contain short polysaccharides attached at the outer membrane surface. In pathogenic bacteria, polysaccharids bound to lipids (lipopolysaccharides) in the outer membrane are the means by which these bacteria can recognize and attach to host plant in which they cause disease. These glycoproteins and lipopolysaccharides provide a cell-to-cell recognition process. There is evidence that lipopolysaccharides of various bacteria recognize specific polysaccharides in the cell wall, not in the plasma membrane.

Membranes have very complex functions:

1) They perform many complex dynamic functions and have biological properties in addition to holding the cell together, static, fixed structures.

2) Most membranes contain enzymes, some acting on substrates outside the membrane and others on substrates inside the membrane-enclosed compartment.

3) Membranes contain transport systems, to translocate certain specific organic nutrient molecules. It helps to preserve the steady-state constancy of the internal medium of cells by regulating the flow of materials in and out of cells.

4) Membranes contain electrically charged surface groups.

5) Cell membranes also have self-sealing characteristics. If they are punctured or disrupted mechanically, they quickly and automatically reseal.

6) Outer surfaces also contain recognition sites to recognize molecular signals.

Fats and Oils

Chemically, fats and oils are very similar compounds, but *fats are solids at room temperature whereas oils are liquids*. Both are composed of long-chain *fatty acids* esterified by their single carboxyl group to a hydroxyl of the 3-carbon alcohol *glycerol*. All three hydroxyl groups of glycerol are esterified, so fats and oils are often called triglycerides/triacylglycerols. The fatty acid moiety in lipid esters is known as acyl group. The various classes of acylglycerols are differentiated by the number of glycerol-OH groups that are esterified with fatty acids (Fig. 13.9). Triacylglycerols are the major storage and transport form of fatty acids. Although the triacylglycerols are considered a single species, they represent a family of molecules with varying fatty acid composition, e.g, palmitic acid + 2 oleic acid, 2 palmitic acid

+ linoleic acid, palmitic acid + stearic acid + oleic acid. Such triacylglycerols with different fatty acids are known as mixed triacylglycerols.

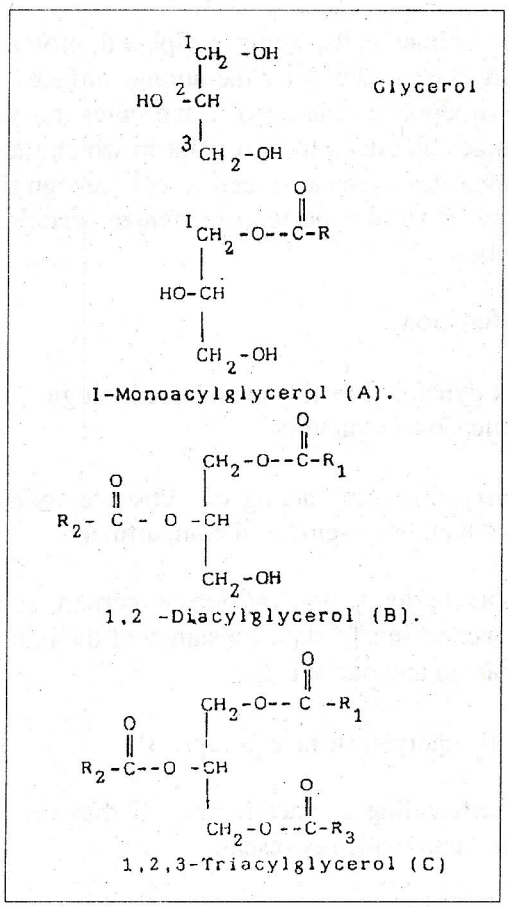


Fig. 13.9 Three classes of acylglycerols

1. Monoacylglycerols (A)
2. Diacylglycerols (B)
3. Triacylglycerols (C)

Properties of triacylglycerols (natural fats):

Triacylglycerols may be either liquid or non-crystalline solids at room temperature. Pure fats and oils are colourless, odourless and tasteless. Any colour or smell is due to the presence of impurities dissolved in them.

1) Hydrolysis of fats:- By heating fats with water, at high temperature and pressure, glycerol and fatty acids are obtained. In human body lipases enzymes present in pancreatic juices are responsible for hydrolysis of dietary fats (Fig. 13.10).

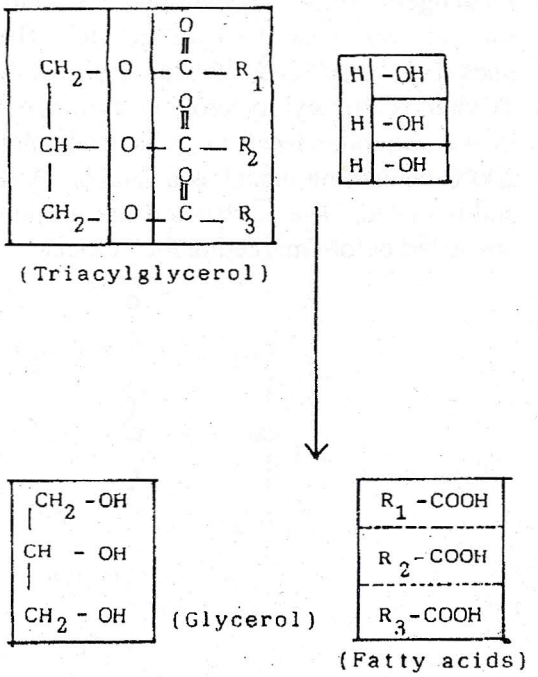


Fig. 13.10. Hydrolysis of Triacylglycerols (Fats) into glycerol and respective constituent fatty acids

2) Saponification:- Hydrolysis of triacylglycerols by alkali is known as saponification, because one of the products of hydrolysis is soap (Fig. 13.11). The number of milligrams of (Potassium hydroxyde (KOH) required to saponify the free and combined fatty acids in one gram of a given fat is known as saponification number. High number indicates that the fat is made up of a low molecular weight fatty acids and *vice versa.*

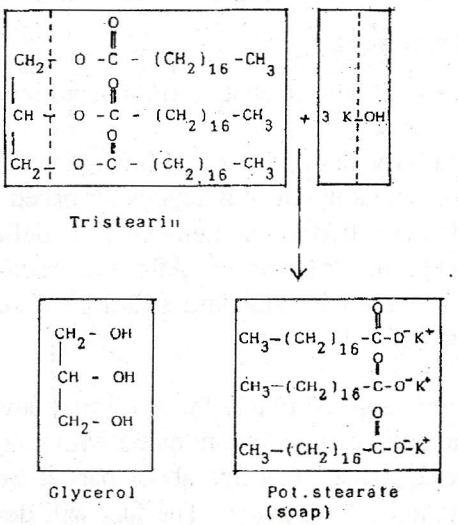


Fig. 13.11 Saponification of fats

3) **Hydrogenation:-** The unsaturated double bonds present in the fatty acid moiety of the triacylglycerols can be hydrogenated. The unsaturated bonds are converted into saturated ones (Fig. 13.12) and oils are solidified. The process is known as hardening of oils and fats. Triolein is a triacylglycerol of unsaturated oleic acid. The unsaturated double bonds present in this compound can be converted into saturated ones by passing H_2 under pressure at 200°C containing nickel as a catalyst. After completion of the reaction, the nickel is filtered and removed. The hardened product known as *vegetable ghee* is formed. Vitamin A and D are added before marketing the product.

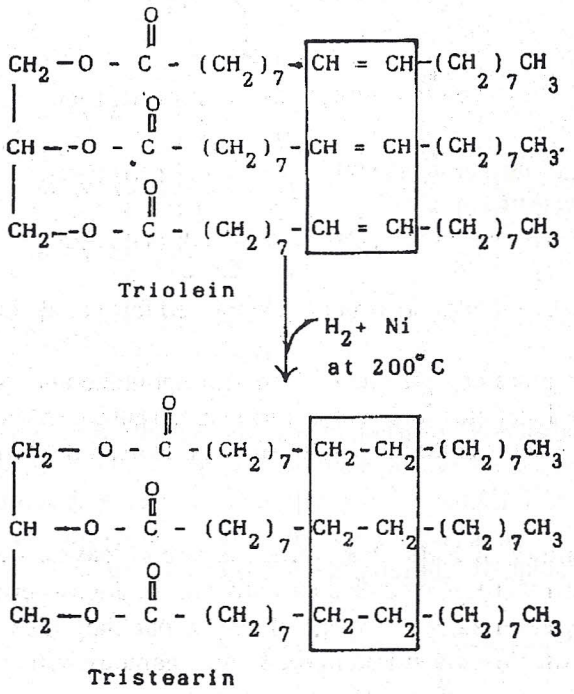


Fig. 13.12 Hydrogenation of oils and fats

4) **Halogenation:-** Unsaturated fatty acids in fats and oils react with halogens by adding them at double bonds (Fig. 13.13). The amount of halogens absorbed is an index of the degree of unsaturation. The index value is called iodine number. It is defined as the number of grams of iodine that will add to 100 grams of fat or oil. After the reaction is completed, the unused halogen is determined by titration with standard solution of sodium thiosulphate and the quantity of iodine used is determined.

5) **Rancidity:-** The term rancid is applied to any fat or oil that develops a disagreeable odour. The natural fats from animal sources are contaminated with enzyme lipases. The action of enzymes and also atmospheric moisture brings about partial hydrolysis of fats with some degree of oxidation of unsaturated fatty acids. The fats will develop a characteristic smell and taste. To prevent rancidity, antioxidants like vitamin E, phenol and vitamin C are added to fats and oils. These have affinity for O_2, so rancidity is prevented.

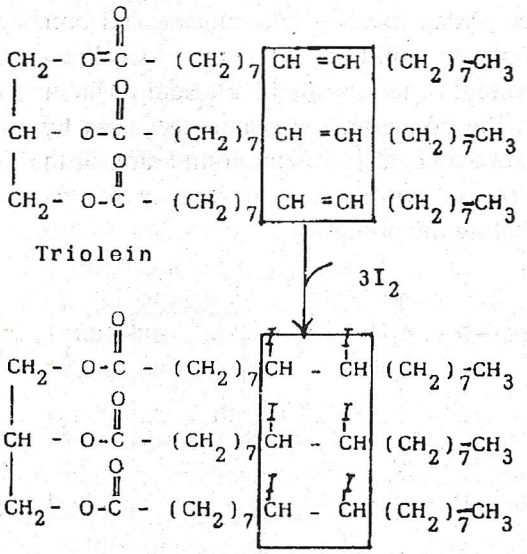


Fig. 13.13 Halogenation of unsaturated fatty acids in fats

6) **Oxidation:-** The unsaturated fatty acids undergo spontaneous oxidation at double bond forming aldehydes and ketones and resins form thin transparent coating on the surface to which oil is applied. This is called drying of oils and is used in manufacturing of paints and varnishes.

Distribution and importance of Fats: Fat storage is rare in leaves, stems, and roots but occur in many seeds and some fruits (e.g. avocados and olives). In angiosperms, fats are concentrated in the endosperm or cotyledon storage tissues of seeds, but they also occur in the embryonic axis. In gymnosperm seeds, they are stored in the female gametophyte.

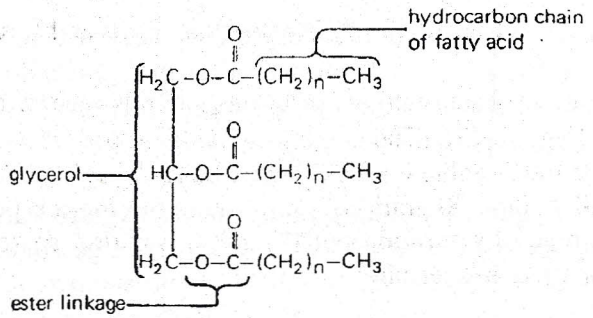


Fig. 13.14. The general structure of a fat or oil, both of which are triglycerides

Compared with carbohydrates, fats contain larger amounts of carbon and hydrogen and less oxygen, and when they are respired, more O_2 is used per unit weight. As a result, more

ATP is formed, demonstrating that greater amounts of energy can be stored in a small volume as fats than as carbohydrates. Most small seeds contain fats as the primary storage materials. When these fats are respired, enough energy is released to allow establishment of the seedling. Larger seeds contain much starch and only small amounts of fats, but seeds of conifers and those in nuts are usually fat rich. Fats are always stored in specialized bodies in the cytoplasm. These bodies have been called lipid bodies, **spherosomes**, and **oleosomes** (Latin, *oleo*, oil).

The main origin of oleosomes is apparently from two sources, the endoplasmic reticulum (ER) and plastids. In each case fats apparently accumulate between the two layers of phospholipids and glycolipids present in the outer membrane of the plastid envelope or the ER membrane. This accumulation causes separation of the lipid bilayer into two halves with fats forcing them apart until a distinct oleosome swells and pinches off.

Formation of Fats: It is now clear that fats stored in seeds and fruits are not transported there from leaves but are synthesized *in situ* from sucrose or other translocated sugar. Although leaves produce various fatty acids present in lipids of their membranes, they seldom synthesize fats. Furthermore, both fatty acids and fats are too insoluble in H_2O to be translocated in phloem or xylem.

Conversion of carbohydrates to fats requires production of the fatty acids and of the glycerol backbone to which the fatty acids become esterified. The glycerol unit (L-α-glycerophosphate) arises by reduction of dihydroxyacetone phosphate produced in glycolysis. The fatty acids are formed by multiple condensations of acetate units in acetyl CoA. Most of the reactions of fatty-acid synthesis occur only in chloroplasts of leaves and proplastids of seeds and roots. The fatty acids synthesized in those organelles are mainly palmitic and oleic.

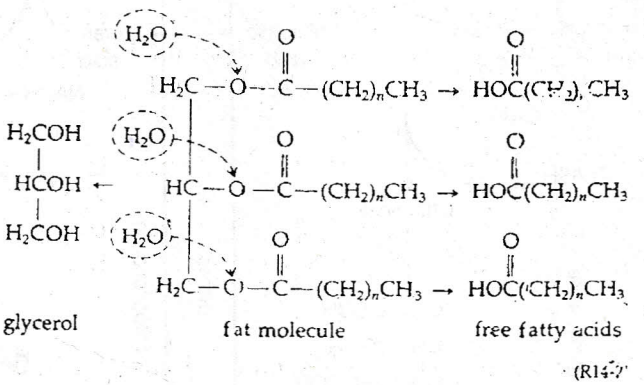
$$8 \text{ acetyl CoA} + 7 \text{ ATP}^{3-} + 14 \text{ NADPH} + 14 \text{ H}^{+} \rightarrow \text{palmityl CoA} + 7 \text{ CoA} + 7 \text{ ADP}^{2-} + 7\text{H}_2\text{PO}_4^{-} + 14 \text{ NADP}^{+} + 7\text{H}_2\text{O}$$

CoA is hydrolyzed away when palmitic or other fatty acid is combined with glycerol during formation of fats or membrane lipids. This emphasizes that conversion of acetate units into fatty acids is energy expensive, because almost two pairs of electrons (2 NADPH) and one ATP are needed for each acetyl group present.

Conversion of Fats to Sugars: β-oxidation and the Glyoxylate Pathway: Breakdown of fats stored in oleosomes of seeds and fruits releases relatively large amounts of energy. For seeds, this energy is necessary to drive early seedling development before photosynthesis begins. Because fats cannot be translocated to the growing roots and shoot, they must be converted to more-mobile molecules, usually sucrose. Conversion of fats to sugars occurs largely in fat-rich seeds and fungal spores and in some bacteria, not in humans or other animals. Most of the reactions necessary to convert fats to sugars occur in microbodies called **glyoxysomes**. Structurally, glyoxysomes are almost identical to peroxisomes of photosynthetic cells, but many of the enzymes they contain are different, they are formed as proglyoxysomes (small glyoxysome precursors) in the cotyledons of developing seeds, then during germination and

early seedling development they mature into fully functional glyoxysomes (Trelease, 1984). They persist only until the fats are digested, then they disappear. In cotyledons that emerge above ground and become photosynthetic, they are replaced by peroxisomes (Beevers, 1979).

The breakdown of fats begins with action of lipases, which hydrolyzes the ester bonds and releases the three fatty acids and glycerol.



Lipids and Other Natural products

The glycerol resulting from lipase action is converted with ATP to α-glycerolphosphate; this molecule is then oxidized by NAD^+ to dihydroxyacetone phosphate, most of which is converted to sugars by reversal of glycolysis. The fatty acids are taken into the matrix of the glyoxysome where they are first oxidized to acetyl CoA units and NADH by a metabolic pathway called β-oxidation, because the beta-carbon is oxidized.

palmitate + ATP^{3-} + 7 NAD^+ + 7 FAD + 7 H_2O + 8 CoASH → 8 acetyl CoA + AMP^{2-} + $pyrophosphate^{2-}$ + 7 NADH + 7 H^+ + 7 $FADH_2$

In fat-rich seeds the potential energy in $FADH_2$ appears to be totally wasted. This waste occurs because of the absence of an electron-transport system in the glyoxysomes and because glyoxysomes contain an enzyme that transfers H atoms of $FADH_2$ directly to O_2, forming H_2O_2. Each H_2O_2 is then degraded to ½ O_2 and H_2O by catalase. Glyoxysomes can process both NADH and acetyl CoA released in β-oxidation, yet they require the help of mitochondria and the cytosol to form sugars, reactions that occur in the glyoxysomes are called the **glyoxylate pathway**, namely, conversion of acetate units of acetyl CoA to succinic and malic acids (Fig. 13.14).

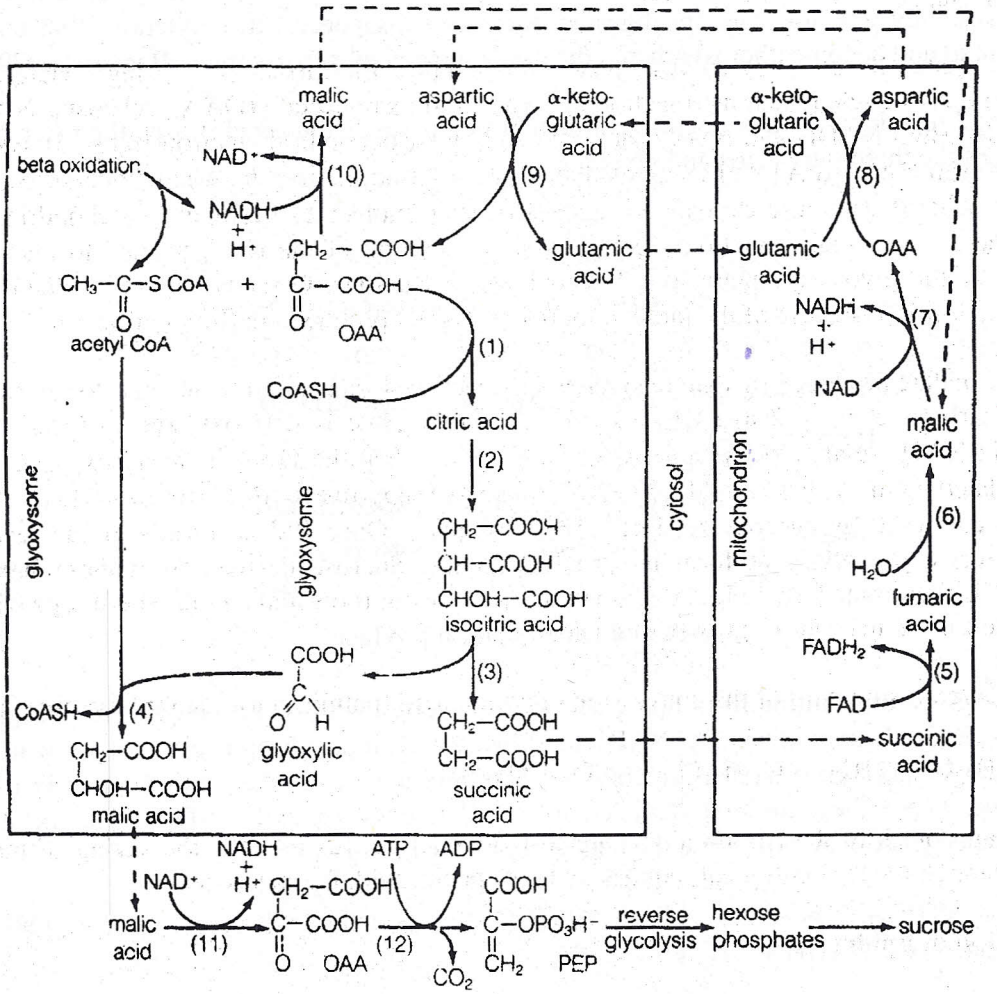


Fig. 13.14 Cooperation of glyoxysomes, cytosol, and mitochondria in converting fatty acids of reserve fats to sucrose by the glyoxylate pathway

Raction 1:- Acetyl CoA reacts with oxaloacetic acid and form citric acid.

Reaction 2:- Isocitric acid is formed from citric acid.

Reaction 3:- After isocitric acid (6 carbons) is formed, it undergoes cleavage by isocitrate lyase and succinate (4 carbons) and glyoxylate (2 carbons) are produced.

Reaction 4:- The glyoxylate reacts with another acetyl CoA to form malate and free coenzyme A in presence of malate synthetase enzyme. This malate is transported to the cytosol where it is converted to sugars.

Succinate (Reaction 3) moves to the mitochondria for further processing. Here it is oxidized to kreb's cycle reactions (reaction 5, 6 and 7) to oxaloacetate (OAA), releasing NADH and $FADH_2$. Both NADH and $FADH_2$ are oxidized by mitochondrial electron-transport system with O_2 to form H_2O 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 reaciton 1 of the glyoxylate pathway. Without these reactions, OAA is converted mainly to malate (reaction 10) because of the large amounts of NADH 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. 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, PEP **carboxykinase**. 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 fatty acid (palmitic) to a sugar (sucrose) is

$$C_{16}H_{32}O_2 + 11O_2 \rightarrow C_{12}H_{22}O_{11} + 4CO_2 + 5H_2O$$

Although one-fourth of the carbon atoms are lost from fatty acids as CO_2, the saving of three-fourths is enough for the ecological requirements of species with fat-rich seeds.

Classification of lipids:

Unlike polysaccharides and proteins, lipids have no repeating monomeric units. They have been classified on the basis whether they are saponifiable or non-saponifiable. Alkaline hydrolysis of an ester to form salt and acid is called saponification. The saponifiable lipids are further classified on the basis of their hydrolytic products.

Saponifiable lipids:- Saponifiable lipids are of two types: (1) Simple lipids, and (2) Compound lipids.

(1) Simple lipids

(a) Fats and Oils: These are esters of fatty acids with alcohol glycerol. Lipids from animal sources are solids and are called animal fats and are liquid from plants and are called vegetable

oils. This difference in melting point is due to the presence of degree of unsaturation of constituent fatty acids.

(b) Waxes: These are esters of fatty acids with long-chain alcohols.

(2) Compound lipids: These lipids contain fatty acids, alcohols and some other radicals. Thse are further divided into phospholipids, glycolipids, sulpholipids, gangliosides and lipoproteins.

(a) Phospholipids: These lipids contain alcohols, phosphoric acid and a nitrogenous base. Based on alcohol these are further subdivided into Glycerophospholipids and Sphingophospholipids.

1) **Glycerophospholipids:** Glycerol is the alcohol present in them. Phosphoric acid is ester linked to nitrogenous alcohol. Example: phosphatidic acid, lecithins, cephalins and plasmalogans.

2) **Sphignophospholipids:** Sphingosine is the alcohol in these lipids. Fatty acids, phosphoric acid and nitrogenous base are also present. These are the lipids that contain both sphingosine and phosphoric acid.

b) Glycolipids or cerebrosides: These lipids have alcohol sphingosine, fatty acid, a hexose and sulphated hexose.

c) Sulpholipids: These contain alcohol sphingosine, fatty acid and sulphated hexose.

d) Gangliosides: These lipids contain sphingosine, long chain fatty acid, glucose, galactose, hexosamine and neuraminic acid.

e) Lipoprotein: These are complexes of lipids and protein.

f) Lipopolysaccharides: These are complexes of lipids and Polysaccharides.

3) **Derived or associated lipids:-** These are non-saponifiable and include hydrolytic products of saponifiable lipids. These are derived from steroids, prostaglandins, fat soluble vitamins and terpenes.

Fatty acids are also classified according to R chain. The fats and oils contain fatty acids and glycerol. Fatty acids are monocarboxylic acids. More than 70 fatty acids have been identified in nature. They may be saturated or unsaturated and they are straight chain or cyclic. In majority of fatty acids 16-18 carbon atoms are present. The naturally occurring fatty acids below 8 carbon atoms are liquids at room temperature and are volatile.

1) Saturated fatty acids:- The alkyl R chain does not contain double bonds. The general formula C_nH_{2n+1} COOH represents all saturated fatty acids. Acetic acid, lauric acid, myristic acid, palmetic acid and stearic acid are some of the fatty acids in mammalian tissues.

2) Unsaturated fatty acids:- In unsaturated fatty acids, the alkyl R group has one or more than one double bond. Fatty acids with one double band are known as monounsaturated and fatty acids with two or more double bonds are known as polyunsaturated fatty acids.

Nomenclature of fatty acids:

a) Numbering of fatty acids from carboxyl end:- This is also called Δ numbering. The C of COOH is designated as No.1.

b) n or omega (ω) numbering of fatty acids:- In this system, the numbering starts from the C-atom farthest from the –COOH group.

c) Greek lettering system:- The C-atom adjacent to the COOH group is called alfa (α) c-atom, the next one beta (β), then gamma (γ) and delta (δ). The farthest is called omega (ω) at the terminal end (Table 13.1 and 13.2)

Table 13.1 Some monoenoic and polyenoic fatty acids present in mammalian tissues

Name	Formula	No. of C atoms, double bonds and position of double bond(s)
1. Palmitoleic acid	$CH_3 (CH_2)_5$-CH=CH $(CH_2)_7COOH$	16:1 Δ 9
2. Oleic acid	$CH_3 (CH_2)_7$-CH=CH$(CH_2)_7COOH$	18:1 Δ 9
3. Linoleic acid	$CH_3 (CH_2)_3 (CH_2CH=CH)_2 (CH_2)_7COOH$	18:2 Δ 9, 12
4.Linolenic acid	$CH_3 (CH_2$-CH=CH$)_3 (CH_2)_7COOH$	18:3 Δ 9, 12, 15
5. Arachidonic acid	$CH_3 (CH_2)_3 (CH_2CH=CH)_4 (CH_2)_3COOH$	20:4 Δ 5, 18, 11, 14

Δ is the number of first C atom of double bond from – COOH end

Table 13.2 Nomenclature of fatty acids

Omega (ω) terminal →	CH_3-	CH_2-	CH_2-	CH_2-	CH_2-	CH_2-	CH_2-	CH_2-	CH_2-	COOH ←	Carboxyl (C) terminal
	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	
Δ Numbering →	10	9	8	7	6	5	4	3	2	1	
	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	
n. or Omega (ω) →	1	2	3	4	5	6	7	8	9	10	
numbering	↓	↓					↓	↓	↓		
Greek lettering →	ω	ω^{-1}					γ	β	α		
system											

4) Melting points of fatty acids:- Melting points increase with an increase in their chain length in case of saturated fatty acids and the melting points decrease with increase in the degree of unsaturation in case of unsaturated fatty acids (Table 13.3).

Table 13.3 Melting points of some saturated and unsaturated fatty acids found in animal tissues

Fatty acids	No. of C-atoms	No. of double bonds	M.P.
1. Lauric acid	12	0	44°C
2. Palmitic acid	16	0	63°C
3. Stearic acid	18	0	70°C
4. Oleic acid	18	1	13°C
5. Linoleic acid	18	2	-5°C
6. Linolenic acid	18	3	-11°C
7. Arachidonic acid	20	4	-50°C

5) Solubility of fatty acids:- Solubility of saturated fatty acids decreases with the increase of the chain length and in case of unsaturated fatty acids solubility increases with the increase of double bonds.

6) Isomerism in fatty acids:- In saturated fatty acids, the presence of double bond prevents the rotation of groups around double bond (Fig. 13.15).

7) Essential fatty acids:- Certain unsaturated fatty acids are not synthesized biologically. They must be present in our diet. These are known as essential fatty acids. These are found in the structural lipids of the cell. They are concerned with the integrity of the mitochondrial membrane and occur in high concentration in reproductive organs.

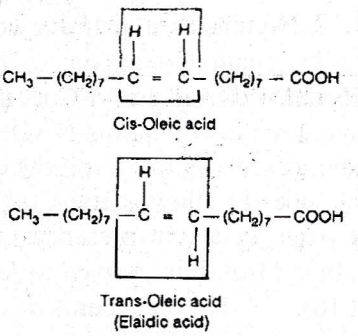


Fig. 13.15 Cis-Trans isomerism of fatty acids. Trans isomer is not utilized by organisms

Fatty acid biosynthesis

Fatty acids are long-chain single carboxyl groups containing organic acids, mostly of even carbon numbers. These are building units of majority of lipids. They are either saturated or unsaturated with 1, 2, 3 or more double bonds. All saturated acids upto 10 carbon atoms are liquid.

Fatty acid biosynthesis

Fatty acids are long-chain single carboxyl groups containing organic acids, mostly of even carbon numbers. These are building units of majority of lipids. They are either saturated or unsaturated with 1, 2, 3 or more double bonds. All saturated acids upto 10 carbon atoms are liquid & volatile. Higher saturated acids are solid and their melting points increase with increase in chain length. Unsaturated fatty acids are liquid at room temperature. Some common fatty acids found in plant lipids are given in table 13.4.

Table 13.4 Essential fatty acids

Name	Formula	No. of C atoms, double bonds and position of double bond(s)
1. Linoleic acid	$CH_3 (CH_2)_3 (CH_2CH{=}CH)_2 (CH_2)_7COOH$	18:2 Δ 9, 12
2. Linolenic acid	$CH_3 (CH_2CH{=}CH)_3 (CH_2)_7COOH$	18:3 Δ 9, 12, 15
3. Archidonic acid	$CH_3 (CH_2)_3 (CH_2CH{=}CH)_4 (CH_2)_3COOH$	20:4 Δ 5, 8, 11, 14

A. Saturated

Fatty acids are synthesized in cytosol. They can be synthesized from any substance that forms acetate like amino acids, carbohydrate, ethyl alcohol and fatty acids themselves.

The major product of fatty acid synthesis is palmitic acid. This is a C_{16} acid. Higher length fatty acids are synthesized by chain elongation. Chain elongation takes place by sequential addition of 2-carbon units derived from acetyl-CoA through activated donor malonyl-ACP (Acyl Carrier Protein). Biosynthesis also requires NADPH, ATP & bicarbonate (HCO_3) and acetyl CoA. Nearly all metabolic acetyl-CoA is produced in mitochondria by pyruvate oxidation. Mitochondrial membrane doesn't allow acetyl-CoA to diffuse to cytosol. So it first forms citrate. Citrate reacts with oxaloacetate in presence of citrate synthase and can pass through mitochondrial membrane. In cytosol, it is cleaved to release acetyl-CoA in presence of ATP and citrate lyase (Fig. 13.16). The regenerated oxaloacetate cannot enter into mitochondria. This is reduced to malate by NADPH & cytosolic malate dehydrogenase. Malate converts to pyruvate by $NADP^+$ - malic enzyme and readily passes to mitochondria. Oxaloacetate is synthesized from pyruvate in mitochondria by pyruvate carboxylase. During this process one NADPH is regenerated and diffuses into the cytosol.

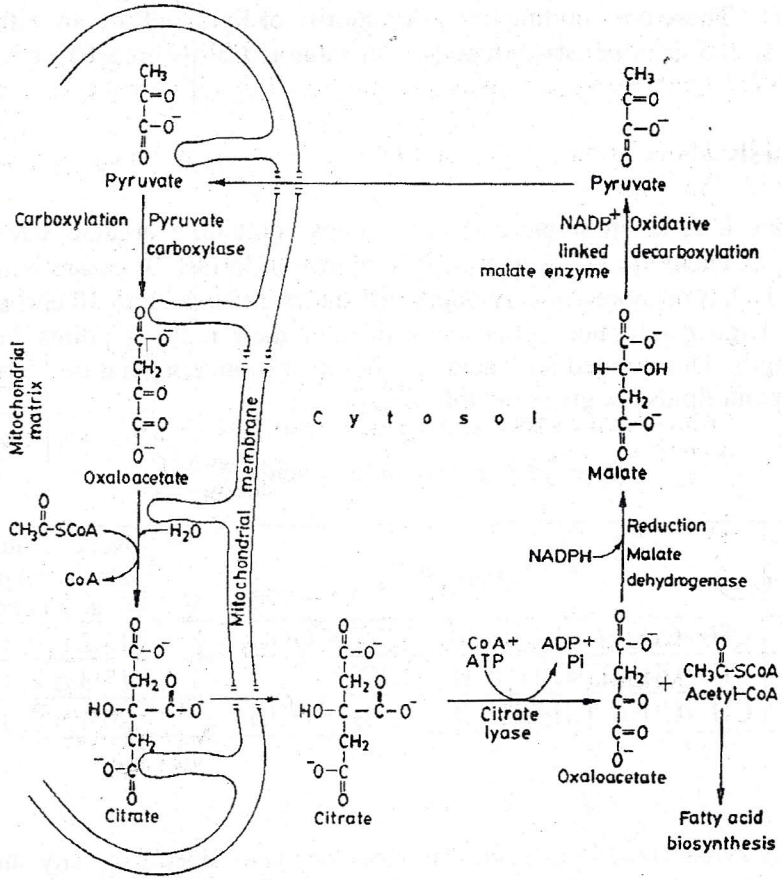
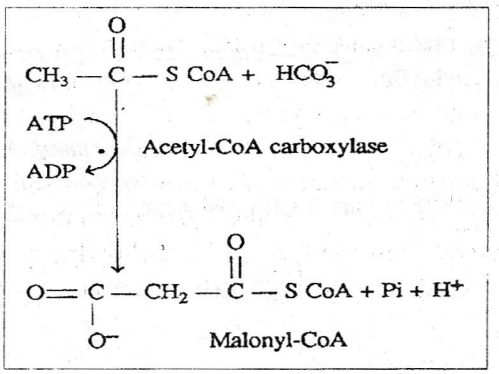


Fig. 13.16. Diffusion of acetyl-CoA through mitochondrial membrane

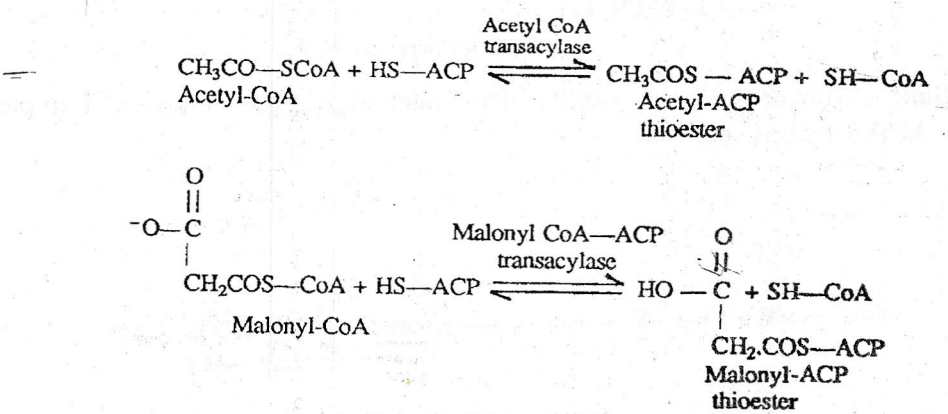
Malonyl-CoA, a 3-carbon compound is biological precursor for 2-carbon additions during fatty acid synthesis. Biosynthesis of malonyl-CoA itself is an important initial step in fatty acid biosynthesis. Irreversible carboxylation of acetyl-CoA to malonyl-CoA in presence of carbonate and ATP is catalysed by the enzyme acetyl-CoA carboxylase.



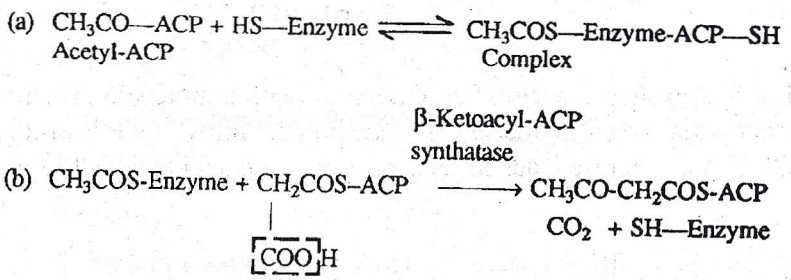
In plant tissues like leaves, which do not have storage triacylglycerols, the primary source of fatty acids is chloroplast and further modification takes place in ER. In tissue with triglycerols (oil seeds) fatty acids are synthesized in proplastids and further modification occurs in ER.

The fatty acid backbone is built up by addition of 2 carbon atoms in each step, through the following sequence of reactions:

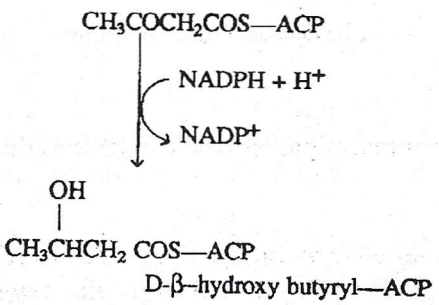
1) **Transacylation:** One molecule of acetyl-CoA or malonyl-CoA, reacts with ACP (Acyl Carrier Protein) to form either acetyl-CoA or malonyl-CoA – ACP in presence of transacylases.



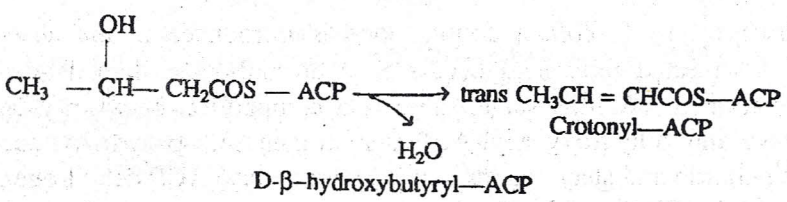
2) Condensation: One molecule of malonyl-ACP & one molecule of acetyl - ACP condense in the presence of β-ketoacyl-ACP synthase irreversibly to form acetoacetyl-ACP. Out of four carbons of acetoacetyl-ACP, two originate from acetyl-CoA & two from malonyl-CoA. The third carbon of malonyl-CoA is released as CO_2 during decarboxylation.



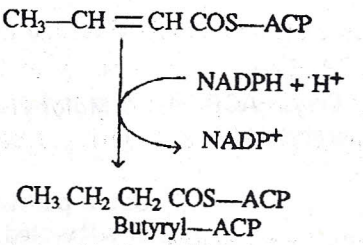
3) Reduction:- β-ketoacetyl-ACP formed by condensation is reduced to D-β-hydroxy butyrol-ACP in presence of NADPH and β-ketoacyl-ACP reductase enzyme.



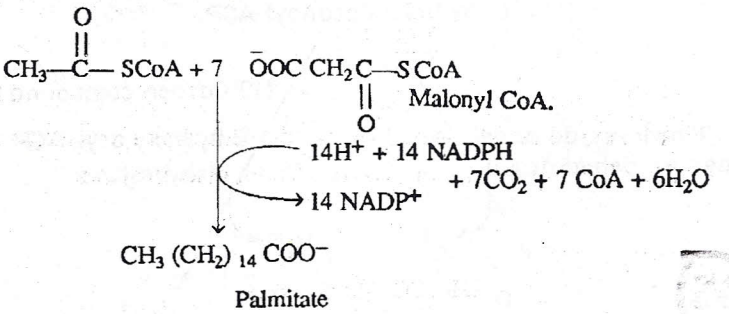
Dehydration: D-β-hydroxy butyryl-ACP is dehydrated to α, β-trans-enoyl-ACP in presence of β-hydroxy ACP-dehydrase enzyme.



4) Reduction:- Reduction of crotonyl-ACP leads to the formation of butyryl-ACP in presence of NADPH and enoyl-ACP reductase.



In this way repeated 7 cycle of condensation with a molecule of malonyl-CoA, followed by reduction, dehydration and reduction, a 16-carbon palmitoyl-ACP is produced. Hydrolysis of palmitoyl-ACP by thioesterase leads to release of palmitic acid and ACP-SH.



B. Unsaturated:

1) Monoenic:- In higher plants/animals double bond can be introduced into long-chain saturated acyl-CoA.

Ex:- Stearoyl-CoA can be converted into oleoyl-CoA. A cis Δ^9 double bond is introduced through an oxidase and uses NADPH or NADH and molecular oxygen.

$$\text{Stearoyl-CoA} + \text{NADH} + H^+ + O_2 \rightarrow \text{Oleoyl-CoA} + NAD^+ + 2H_2O$$

A double bond can be introduced either by anaerobic pathway or aerobic pathway.

Anaerobic pathway:- In *E. coli*, a double bond is introduced in the absence of oxygen. Biosynthesis of a saturated fatty acid occurs in a normal way till a 10-carbon β-hydroxy decanoyl-ACP is formed. At this stage, there is a competition between β-hydroxydecanoyl thioester dehydrase and β-hydroxy acyl-ACP dehydrogenase. β-hydroxy decanoyl thioester dehydrase yields palmitic and stearic acids and β-hydroxy acyl-ACP dehydrogenase yields β, γ-unsaturated fatty actyl-ACP (Fig. 13.17).

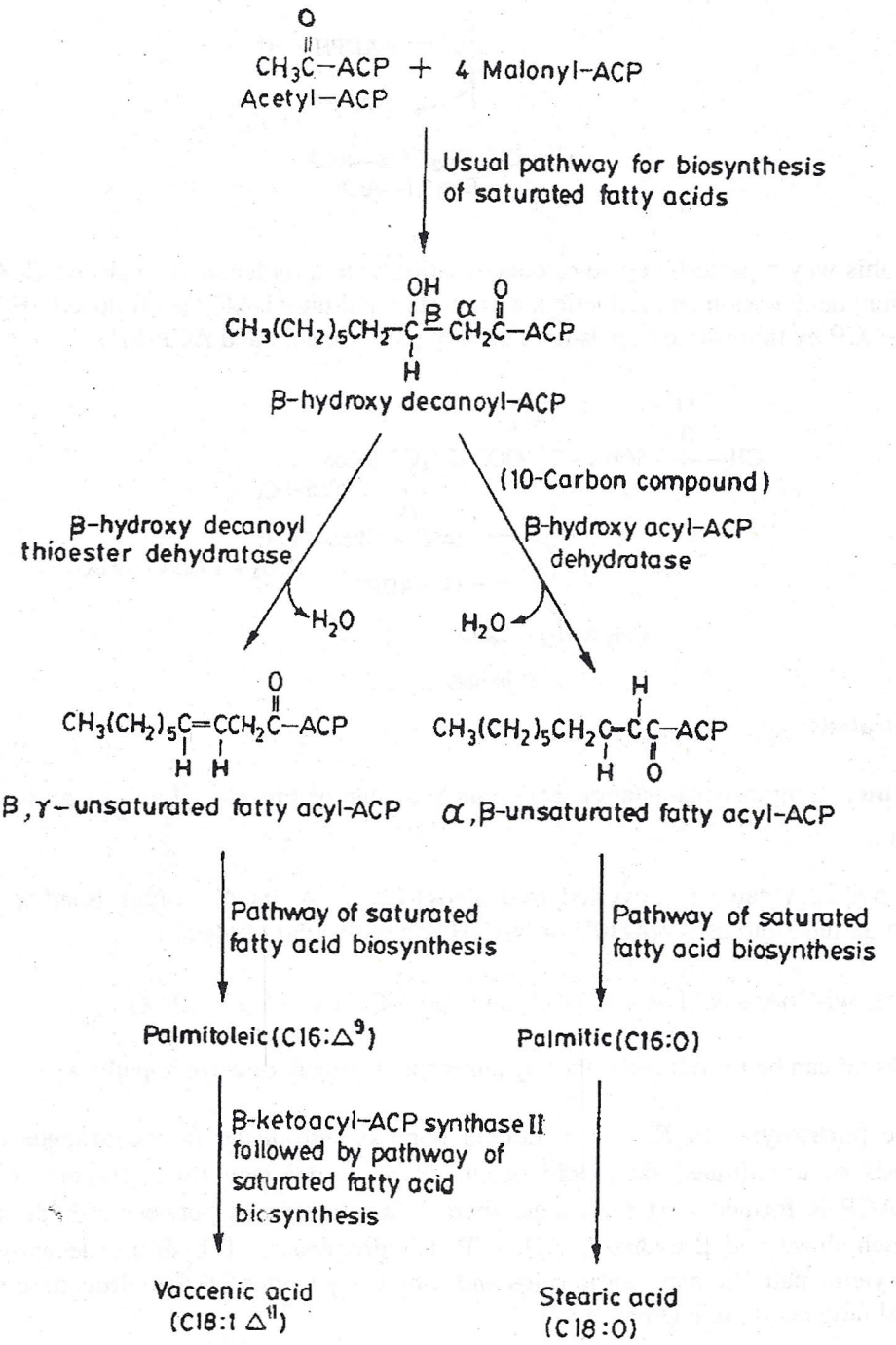


Fig. 13.17 Anaerobic pathway of fatty acid biosynthesis

(1) Aerobic pathway:- In eukaryotes, double bond is introduced after complete synthesis of saturated fatty acids. Cytochrome b_5 reductase transfers electrons from NADH to cytochrome b_5 by means of flavin (F). Fe^{3+} is reduced to Fe^{2+}. Stearoyl-CoA desaturase uses two electrons from cytochrome b_5 along with one atom of oxygen to form a cis-bouble bond in 9, 10 positions of oleic acid.

(2) Polyenoic:- Eukaryotes synthesize polyunsaturated fatty acids (PUFs) eg. linoleic and linolenic acids. Though they are the most abundant fatty acids, their biosynthesis is not well defined. The major site of polyenoic acids is chloroplast lamellar membrane. Aerobic mechanism is the main mechanism for the synthesis of unsaturated acids in plants.

(3) Hydroxyl:- In presence of NADPH and O_2, ω-hydroxylation occurs followed by in-chain hydroxylation involving cofactors. Hydroxy fatty acids are also formed as intermediates in oxidation or by specific hydroxylation reactions. D-2-hydroxy palmitate is synthesized from D-2-hydroperoxypalmitate intermediate of α-oxidative degradation (Fig. 13.18).

(4) Cyclic:- Some families like Malvaceae and Sterculaceae contain cyclopropane fatty acids in their seed oils. Methylene group is added from S-adenosyl methionine across the double bond of a monoenoic fatty acid, which is a part of phospholipid. Cyclopropane fatty acids become unsaturated and result in formation of cyclopropene acids. α-oxidation cleaves carboxyl-carbon forming malvalic acid from sterculic acid.

(5) Chain termination:- In plants, the specific acyl-ACP hydrolases cleaves specific acyl-ACP to free acid and after activation to acyl-CoA derivative, it is incorporated into complex lipid. Stearoyl-ACP desaturase and oleoyl-ACP hydrolase are involved in chain termination at C_{18} level in plants.

$$\text{Steroyl} - \text{ACP} \xrightarrow[\text{desaturase}]{\text{Stearoyl-ACP}} \text{Steroyl-ACP} \xrightarrow[\text{hydrolase}]{\text{Oleoyl-ACP}} \text{Oleoyl-ACP} \rightarrow \text{free oleic acid}$$

(6) Regulation:- There are several mechanisms to control the rate of fatty acid metabolism. The control factors are concentration and availability of primary substrates, their products and regulation, covalent and non-covalent factors, changes in activators and inhibitors and regulation of biosynthetic enzymes. The supply of malonyl-CoA determines the rate of fatty acid synthesis and degradation. In plant tissue, a high molecular weight heat-labile inhibitor of carboxyl-transferase enzyme is present which regulates the *in vivo* activity. An increase in the availability of acetyl-CoA results in increased lipogenesis.

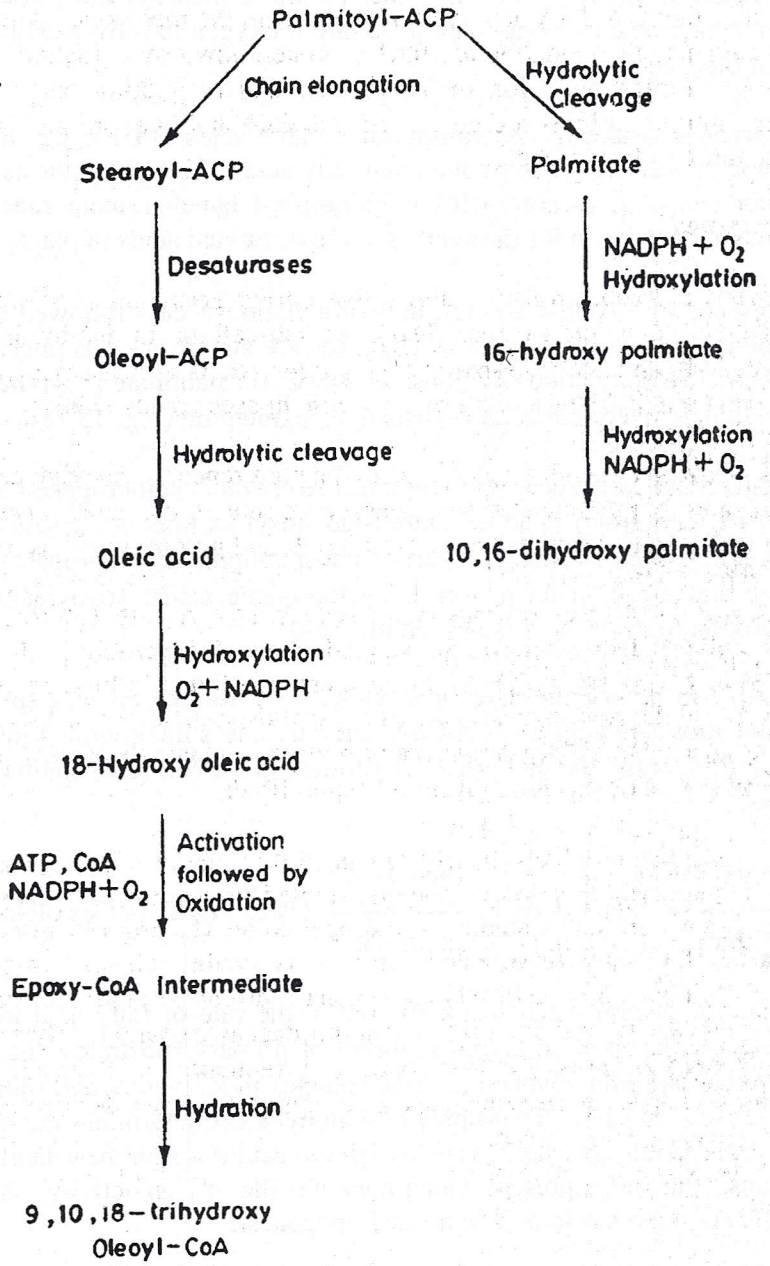


Fig. 13.18 Biosynthesis of hydroxy fatty acids

Catabolism of lipids

Lipid mobilization is mediated by some important enzymes. This is controlled by hormones. Plant hormones like GA, IAA and cytokinins regulate the metabolic switching. The fatty acids obtained by enzymatic degradation are further broken down by oxidation. This step provides not only energy through operation of TCA cycle and respiration but also carbon skeleton to the growing embryo. Lipids are converted into carbohydrates through glyoxalate cycle, as a by-pass for a portion of TCA cycle.

(A) Lipid degrading enzymes

1. **Lipases:-** Lipases are widespread in nature and active during germination of seeds. Two types of lipases, alkaline and acidic lipases. These are sub-cellular in distribution and are known to be membrane-bound. The primary acyl groups (α, α^1) in triacylglycerols are cleaved by alkaline lipases although sn-1 and sn-3 specific lipases are also known.

   Acyl hydrolases, cleave mono- and diacylglycerols in preference to triacylglycerols. The activity of these enzymes is prominent during senescence and in damaged tissues. They catalyse cleavage of acyl groups from glycerophospholipids and glycosyl acylglycerols.

2. **Phospholipases:-** Out of the 4 types of phospholipases (A, [A_1, A_2], B, C and D) known, phospholipase-D is widely distributed in plants and showed specificity. It catalyses transphosphatidylation. It is a very active degradative enzyme found in high proportions in storage tissue and leaves. Phospholipase B attacks lysophospholipid formed by action of phospholipase A. The other phospholipids are not important in plants, because non-specific acyl hydrolases are capable of deacylating glycerophospho-lipids.

3. **Lipoxygenase:-** This enzyme is widely distributed in higher plants with high activity in leguminous seeds. It catalyses oxidation of polyunsaturated fatty acids to hydroperoxides. Lipoxygenase forms conjugated cis-pentadienyl hydroperoxides (having trans double bond next to hydroperoxide) from polyunsaturated fatty acids having cis-cis 1,4-pentadiene system. After aerobic reaction has occurred, lipoxygenase promotes anaerobic reaction of polyunsaturated fatty acids with hydroperoxide. The product is oxo-acids Hydroperoxide forms hydroxy compounds or ketols, vinyl ethers etc., by reduction, as their accumulation damages cell membrane. Lipoxygenase mediates scavenging of oxygen during germination of seeds.

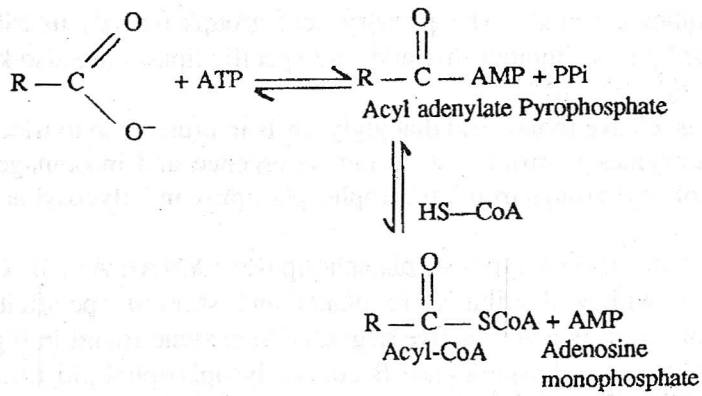
B) Fatty acid oxidation:

Fatty acids obtained by degradation of complex lipids are catabolized by various types of oxidation.

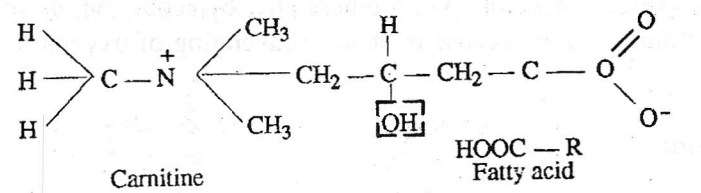
1. β-oxidation:

It occurs at β-carbon from carboxyl group. The cleavage splits into 2 carbons from carboxyl end as acetate, leaving behind fatty acid shortened by 2 carbons. Repeated β-oxidation removes acetate molecules till oxidation completes.

The first step in β-oxidation is activation of fatty acids by formation of acyl-CoA derivatives which occurs in outer mitochondrial membrane. It requires thiokinase, CoA, ATP & Mg^{2+} ions. The fatty acid reacts with ATP to form acyl-adenylate. This is then attacked by –SH group of CoA to form acyl-CoA.



The occurrence of fatty acid activation in out mitochondrial membrane and β-oxidation in mitochondrial membrane requires its transportation . Long-chain acyl-CoA cannot diffuse through inner mitochondiral membrane, although medium – chain (upto C10) fatty acyl-CoA can do so. Long-chain acyl-CoA reach mitochondrial matrix by diffusion through inner mitochondrial membrane, as carnitive derivative (acyl-carnitine). On the matrix side of membrane, acyl group is transferred back to CoA, and this reaction is catalysed by fatty acyl-CoA Carnitine-fatty acyltransferase enzyme.



Mitochondrial β-oxidation:- In the mitochondrial matrix activated fatty acid is degraded in 4 steps – oxidation, hydration, oxidation and thiolytic cleavage.

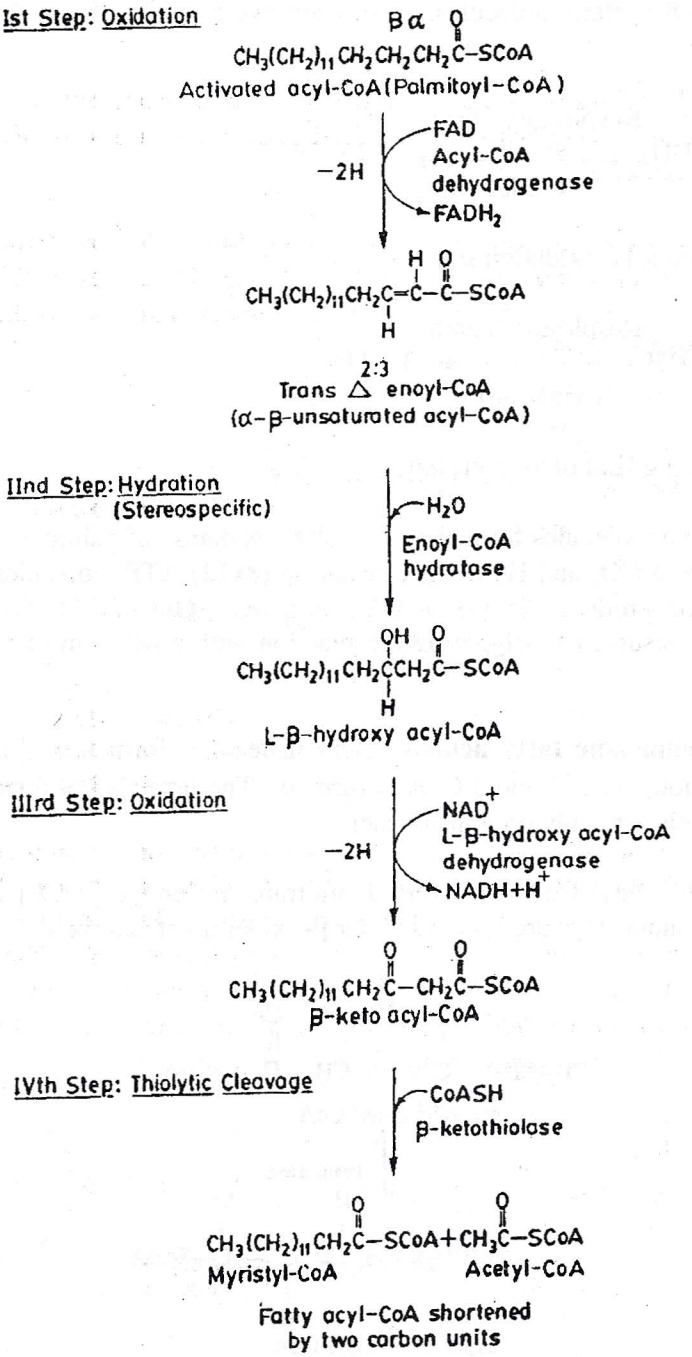


Fig. 13.19 β-oxidation of even carbon chain saturated fatty acids.

Such 7 successive 4-step cycles of β-oxidation results in complete breakdown of palmitate to yield 8 acetate molecules. In each cycle of β-oxidation 5 ATP molecules are formed as follows:

$$FADH_2 \xrightarrow[\text{(oxidation)}]{\text{Respiratory chain}} 2\ ATP$$

Formed during Ist oxidation step

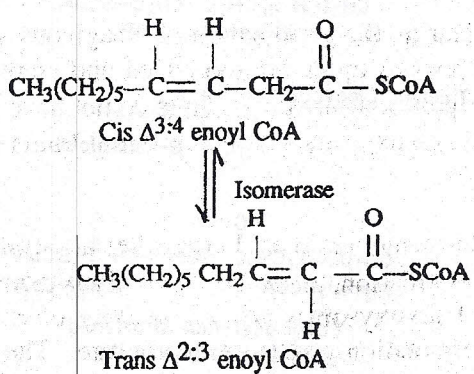
$$NADH \xrightarrow[\text{(oxidation)}]{\text{Respiratory chain}} 3\ ATP$$

Formed during IInd oxidation step

The 8 acetate molecules formed by complete oxidation of palmitate enter the TCA cycle, oxidise completely to CO_2 and H_2O. As a result 96 (8x12) ATP molecules and each cycle of 4 steps of β-oxidation produces 35 (7x5) ATPs as above. Out of 131 (96+35) ATP molecules produced, one is consumed in triggering the reaction with a net gain of energy in the form of 130 ATP molecules.

β-oxidation of monoenoic fatty acids:- Here instead of formationof trans $\Delta^{2:3}$ enoyl-CoA during first oxidation, cis $\Delta^{3:4}$ enoyl CoA is formed. The latter is not a substrate for acyl-CoA dehydrogenase. This acts only on trans isomer.

Hence is $\Delta^{3:4}$ enoyl-CoA is converted into trans $\Delta^{2:3}$ enoyl-CoA by an isomerase enzyme and then the subsequent steps are same as under β-oxidation of saturated fatty acids.

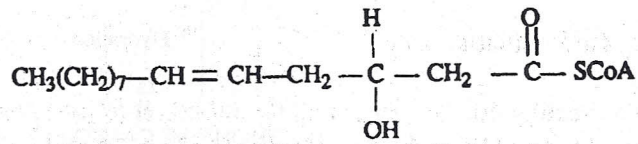


(From C_{18} fatty acid shortened by 4 carbons in two degradative cycles)

β-oxidation of Polyenoic acids

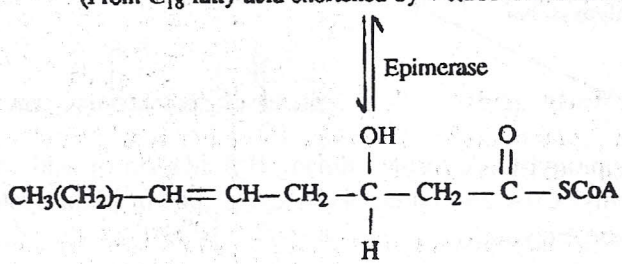
An activated C_{18} unsaturated fatty acid with cis Δ^6 and cis Δ^9 double bonds first undergoes 2 rounds of degradation as under saturated fatty acids. The resulting C_{14}-acyl-CoA (cis Δ^2, Δ^5 enol-CoA) is hydrated by enoyl-CoA hydrolase by the same enzyme which hydrates trans $\Delta^{2:3}$ double bond under saturated fatty acid β-oxidation. Hydration (2nd step) of cis $\Delta^{2:3}$ double bond yields D-isomer of β-hydroxy acyl-CoA which is not a substrate for β-hydroxy acyl-CoA dehydrogenase enzyme.

An accessory enzyme epimerase changes the configuration of hydroxyl group at 3rd carbon to form L-isomer from D-isomer. Subsequent degradation occurs as given under saturated fatty acids.



D-3-hydroxy cis Δ^5 enoyl-CoA (C_{14})

(From C_{18} fatty acid shortened by 4 carbons in two degradative cycles)



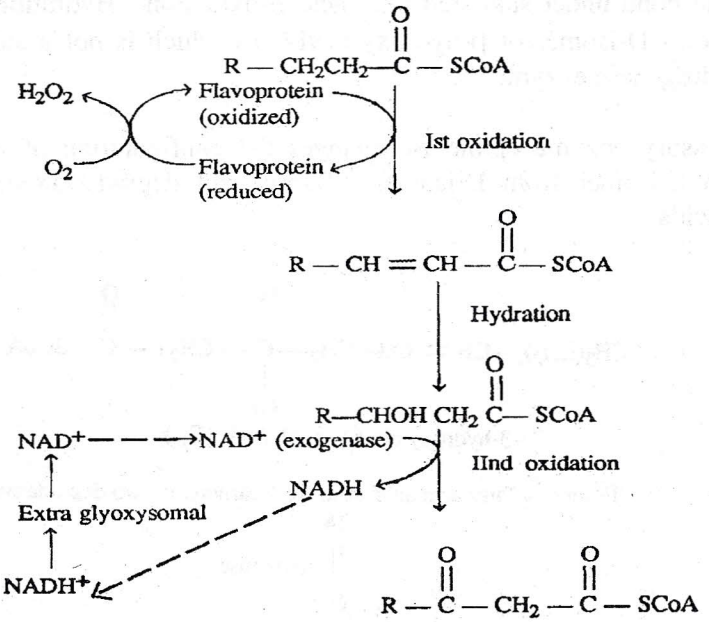
L-3 hydroxy cis Δ^5 enoyl CoA

Glyoxysomal β-oxidation:- During the germination of oleaginous seeds very little β-oxidation occurs in mitochondria. The reserve lipids are mobilized and converted into carbohydrates in specialized organelles called glyoxysomes. Since plants do not have any system for transporting fatty acids and triacylglycerols, glyoxysomes mobilize stored lipids into transportable sugars in germinating seeds.

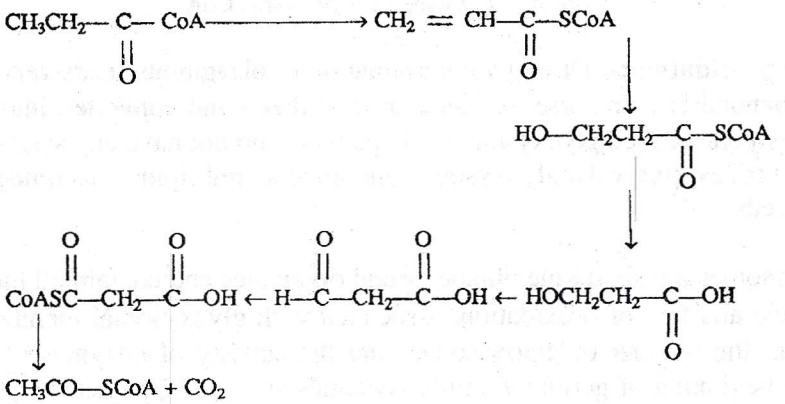
Glyoxysomes are single-membrane-bound organelles and contain all the five enzymes of glyoxylate cycle and that of β-oxidation, associated with glyoxysomal membrane. At the time of germination, the number of glyoxysomes and the activity of enzymes of glyoxylate cycle varies. In the beginning of germination glyoxysomes are rare. The activation of fatty acids to their acyl-CoA occurs in glyoxysomes. So glyoxysomes are the only intracellular site for β-oxidation differs from mitochondrial oxidation in two ways:

(a) Flavoprotein (reduced in acyl-CoA dehydrogenase step) is directly oxidized by molecular oxygen.

(b) Reoxidation of NADH formed during oxidation of α-hydroxyacyl-CoA cannot occur in glyoxysomes.



In animal tissues, propionyl-CoA formed during β-oxidation of odd-carbon-chain fatty acids is converted into succinyl-CoA and enters TCA cycle, but in plant system it involves a hydratase and 2-dehydrogenase enzyme.



13.7 SUMMARY

- Fatty acids are long-chain carboxylic acids that may have one or more double bonds that are usually cis. Their anions are amphiphilic molecules that form micelles in water.
- Fatty acids are rarely occur free in nature but are components of lipids.
- The most abundant class of lipids, the triacylglycerols or natural fats are non-polar molecules that constitute the major nutritional store of animals.
- The lipids that occur in membranes are the phospholipids, the sphingolipids and cholesterol.
- Sphingolipids such as cerebrosides and gangliosides have complex carbohydrate head groups that act as specific recognition markers in various biological processes.
- Biological membranes contain a high proportion of proteins. Integral proteins have non-polar surface regions that hydrophobically associate with the bilayer core peripheral proteins bind to integral proteins on the membrane surface by polar interactions.
- According to the fluid mosaic model of membrane structure, integral proteins resemble icebergs floating on a two-dimensional lipid sea.
- Though fats and oils are similar compounds, fats are solids and oils are liquids at room temperature.
- Triacylglycerols may be either liquid or non-crystalline solids at room temperature.
- Conversion of carbohydrates to fats requires production of the fatty acids and of the glycerol backbone to which the fatty acids become esterified.
- Breakdown of stored fats releases large amounts of energy. This step occurs in glyoxysomes and the process is known as β-oxidation or glyoxylate pathway, where the reactions occur.
- Lipids are classified based on whether they are saponifiable or non-saponifiable.
- Fatty acids are synthesized in cytosol. They can be synthesized from any substance that forms acetate like amino acids, carbohydrates, ethyl alcohol and fatty acids themselves.
- Lipid catabolism is mediated by enzymes like lipases, phospholipases and lipoxygenases.
- Fatty acids obtained by degradation of complex lipids are catabolized by various types of oxidations that include β-oxidation.

✈ β-oxidation occurs in mitochondria and glyoxysomes.

13.8 Questions

1. Write about classification of lipids.
2. What is glyoxylate pathway. Discuss about the various reactions that occur in the pathway?
3. Write short notes on:
   a) Properties of triacylglycerols
   b) Fluid-mosaic model of membranes
   c) Unsaturated fatty acid biosynthesis
4. Write an essay on fatty acid biosynthesis.
5. Describe briefly about mitochondrial and glyoxysomal β-oxidation.

13.9 Reference Books

1. **Plant Physiology**, 3rd ed., Frank B. Salisbury and Cleon W. Ross, CBS Publishers and Distributors.
2. **Text Book of Modern Biochemistry**, Volume 1, Mukhtar Ahmad, Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi.
3. **Recent Advances in Plant Biochemistry**, S.L. Mehta, M.L. Lodha and P.V. Sane, ICAR Publications, New Delhi.
4. **Biochemistry**, 2nd ed., Donald Voet and Judith G. Voet, John Wiley & Sons, Inc.
5. **Plant Physiology** L. Taiz and E. Zeiger. Sinduer Associates, Inc., Sunderland, Massachusetts.

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Dr. P. KIRANMAYEE

LESSON-14

PHOTOMORPHOGENESIS

14.0 OBJECTIVES

In this lesson, you will learn:

- The concept of light-regulated plant development or photomorphogenesis.
- The discovery of phytochrome and review the basic chemistry of this uniquely photoreversible pigment.
- The physiological effects of phytochrome, showing how it is involved in every aspect of development.
- How phytochrome can be used to monitor changes in the natural light environment.
- How phytochrome works at the molecular level.
- Responses of plants to blue light and UV-B radiation.

CONTENTS

14.1 INTRODUCTION

Everybody is familiar with the fact that the life of a green plant depends on light. During photosynthesis, light energy is converted into organic matter. The first scientific documents of photomorphogenesis was described by Julius von Sachs, the founder of modern experimental plant physiology (Fig. 14.1). Sachs noted that darkened seedlings or parts of older plants developed an irregular, misshapen appearance, characterized by a thin, elongated stem and rudimentary, yellow leaves. He described this syndrome as an "etiolation illness" which could never be cured but be alleviated, if another part of the plant was exposed to light.

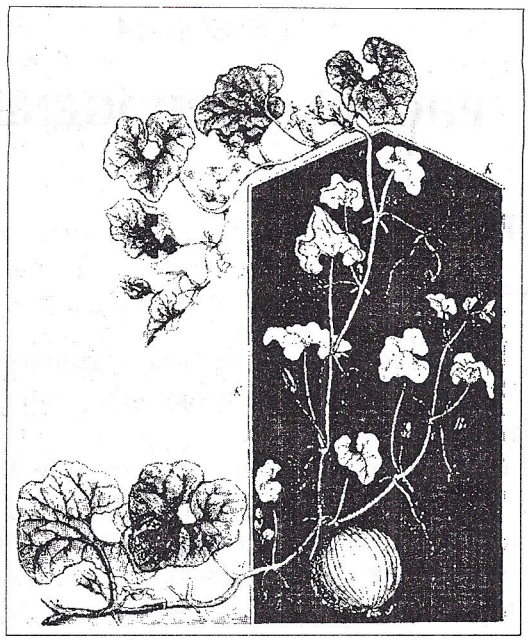


Fig. 14.1 Experimental arrangements used by Julius von Sachs to demonstrate the etiolation syndrome of a partly darkened plant.

According to today's meaning 'Photomorphogenesis' embraces all regulatory effects of light (visible and near-ultraviolet parts (300-800 nm) on the development of plants, independent of photosynthesis. The developmental effects of complete darkness is designated as 'skotomorphogenesis' or 'etiolation'.

Photomorphogenetic processes utilize radiant energy to "trigger" or initiate reactions that control or alter growth, development or differentiation photomorphogenetic reactions are initiated by low levels of radiant energy. In many plants, for example, photosynthesis may be driven by solar flux densities in the range of 1000 to 1200 Wm^{-2}, whereas photomorphogenetic reaction, such as seed germination, is triggered at flux densities of around 0.01 to 0.1 Wm^{-2}. Moreover, in photosynthesis radiant energy must be supplied continuously, whereas in photomorphogenesis a brief exposure to an appropriate radiation source may suffice to set the process in motion.

The photoreceptors used in photomorphogenesis differ from the photoreceptors of photosynthesis. Chlorophyll, phycobilin or rhodopsin pigments are present in large amounts in the cell and are densely packed in membranes specialized to convert light energy into chemical energy. In contrast, photomorphogenetic photoreceptors must be conceived as sensory pigments. The cellular level of these is extremely low and the function is not dependent on membranes.

Responses to red light: For many years, it was known that plants respond to low levels of irradiation by several different growth responses, such as etiolation and bending. It was observed that the seeds of some plant species did not germinate if maintained in the dark in a fully imbibed condition. If such seeds were given a brief exposure to light, germination proceeded in a normal manner.

In the 1930s, two scientists L.H. Flint of the U.S. Department of Agriculture & E.D. Mc Alister of the Smithsonian Institution, were studying the germination of light-sensitive seeds under light or different wavelengths obtained from a series of filters. They reported in 1935 that the seeds of Lettuce (*Lactuca sativa* cv. Grand Rapids) were promoted to germinate by light in the spectral region of 525 to 700 nm. The optimal promotive effect was noted around 660 nm, high referred to as red light. They also found that, far-red light i.e. 700 to 820 nm, has no promotive influence on the germination of lettuce seeds.

Dry seeds of Grand Rapids lettuce do not germinate, nor dry seeds respond to light. Seeds were allowed to imbibe water from water-soaked blotters in petri dishes for 16 hours in complete darkness. If left in darkness for a further 32 hours, a few seeds germinated. All the seeds germinated when 16 hour dark imbibed seeds were exposed to a brief flash of white light or sunlight and returned to darkness for an additional 32 hours. If the dark period was interrupted at the end of 16 hours with varying wavelengths rather than white light or sunlight, seed germination was promoted by red light (660 nm). Far-red light did not promote germination. Seed germination was inhibited, if seeds promoted to germinate by red light were given an immediate exposure to far-red light (730 nm). The promotion of seed germination in red light (660-680 nm) and the inhibition of seed germination in far-red light are reversible.

Most photomorphogenic responses in higher plants appear to be under control of one of three signal transducting photoreceptors:

(1) phytochrome, which absorbs in the red (R) and far-red (FR) regions of the spectrum.

(2) a blue and UV-A-absorbing receptors, cryptochrome.

(3) one or more UV-B-receptors.

Phytochrome: It is now well established that the ubiquitous chromo-protein called phytochrome plays a critical role in almost every stage of plant development. Its existence was predicted on the basis of a simple physiological observation, seed germination and growth of etiolated seedlings exhibited photoreversible responses to red and far-red light.

In 1950s, H.A. Borthwick, a botonist, and S.B. Hendricks, a physical chemist and their colleagues began a study of action spectra for such a phenomenon as germination of photosensitive lettuce seeds, pea stem elongation and photoperiodic control of flowering. One exciting observation was the similarity of action spectra, with peaks in the red an far-red. This was the discovery of photoreversibility – a response potentiated by red light could be negated if

the red light treatment were followed immediately with far-red light. In 1960, they proposed that the seeds contain a pigment and named the pigment as **Phytochrome**.

This pigment exists in two forms: a red absorbing form called Pr and a far red-absorbing form called Pfr (Fig. 14.2).

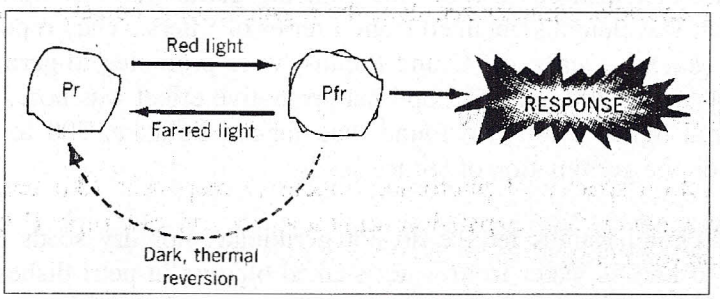


Fig. 14.2 The photoreversible pigment system

In an experiment, groups of seeds were imbibed with water in darkness for three hours before subjected to light treatments. The light treatments were either 1 minute' of red light or 4 minute' of far-red light. Following irradiation, the seeds were returned to darkness for 48 hours. The number of germinated seeds in each lot were counted (Table 14.1).

Table 14.1 Photoreversible control of germination

Irradiation	Germination (%)
R	88
R, Fr	22
F, Fr, R	84
F, Fr, R, Fr	18
R, Fr, R, Fr, R	72
R, Fr, R, Fr, R, Fr	22

When the R and FR treatments are alternated, the % germination appears to depend on whether R or FR was presented last.

Beltsville group was predicted several features of this hypothetical pigment system.

- because seeds and dark-grown seedling tissues responded initially to red light, the pigment was probably synthesized as the P_r form, which accumulated in darkness. P_r was stable and probably physiologically inactive.

- because treatment with red light initiated germination and other developmental events Pfr was probably the active form. Pfr was unstable and was either destroyed or could revert to P_r in darkness by a non-photochemical, temperature-dependent reaction.

- because the pigment could not be seen in dark-grown, chlorophyll-free tissue, it was at a very low concentration.

Borthwick and Hendricks summarized that, the pigment must be acting catalytically and was a protein.

Structure and Properties of Phytochrome

Chromophore: The action spectra of photomorphogenetic responses like seed germination, stem elongation, hook opening, leaf expansion, anthocyanin and chlorophyll synthesis show maxima in the red region (660 nm).

The chemical structure of phytochrome (Pr form) the tetrapyrrole chromophore is covalently linked to apoprotein via a thioester bond (-S-) to the vinyl group of ring A. The chromophore-binding crevice is hydrophobic. Additional hydrophobic site on protein moiety binds flavor (Fig. 14.3).

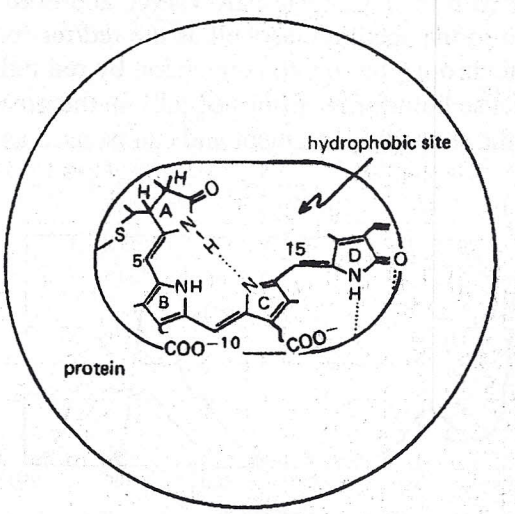
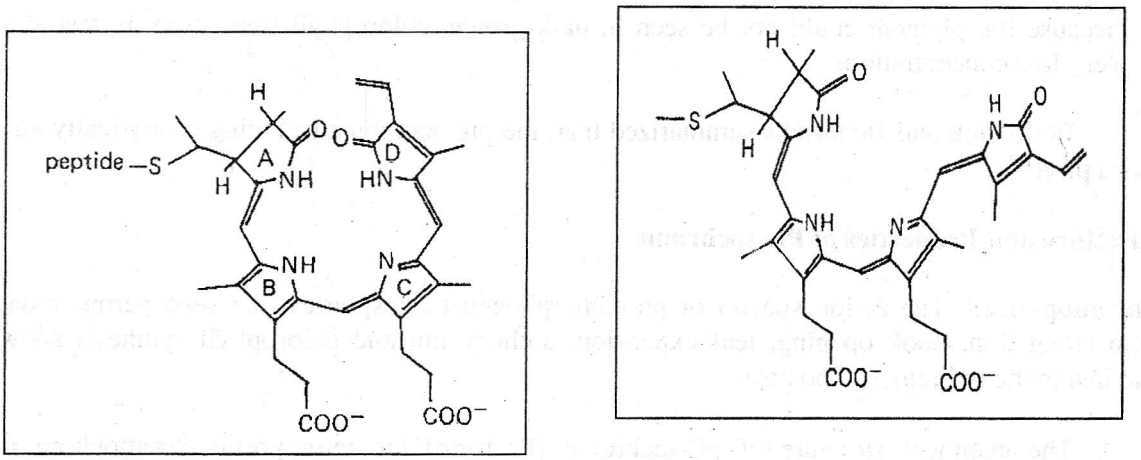


Fig. 14.3 Covalent linkage of chromophore to apoprotein

The cyclic Pr-chromopeptide can be photo-isomerized to a semi-extended chromopeptide in the presence of thiol, acts as nucleophilic catalyst.

The Pfr chromophore results from the photo-induced addition of amino acid residue to the ring A methene bridge (Fig. 14.4).



Pr Pfr

Fig. 14.4 The chromophore structures of Pr and Pfr chromopeptide

Spectroscopy: The absorption spectra of phytochrome are qualitatively similar to those of porphyrins and chlorophylls with their characteristic visible and Soret bands. Note differential absorption in the blue region of the spectrum as well as the red/far-red region. Some blue light effects are mediated by phytochrome, but photo conversion by red light is 50 to 100 times more effective than the blue. Because both forms absorb equally in the green region (500 to 550 nm), green light does not change the state of the pigment and can be used as a safe light (Fig. 14.5).

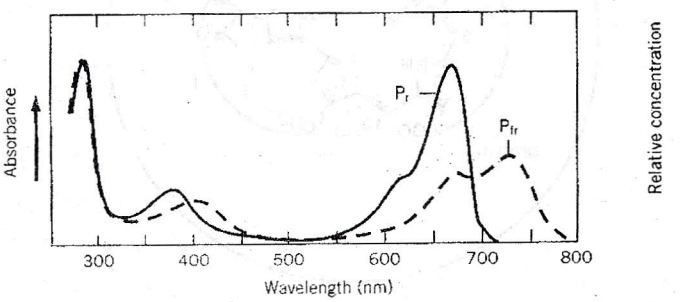


Fig. 14.5 Absorption spectra of purified phytochrome

Phytochrome is believed to exist *in vivo* as a dimer with one chromophore per monomer. It appears that crude extracts of all plants contain a Pr-specific protease that cleaves the protein into several fragments. Isolation of intact, or native, protein can be optimized by first converting the pigment to the Pfr form, adding protease inhibitors and working rapidly at ice

temperature. Molecular mass estimates for native monomer range from 120 kDa to 127 kDa (maize).

The molecular mass of oat phytochrome is 124 kDa. A polypeptide map containing 1128 aminoacids has been deduced from DNA nucleotide sequence analysis. The chromophore is attached at cysteine-321, part of a unique 11-amino acid sequence at the NH_2 terminal end of the protein (Fig. 14.6). Chemical data indicates that the chromophore is housed within a cavity in the folded protein, shields the chromophore from the external aqueous environment.

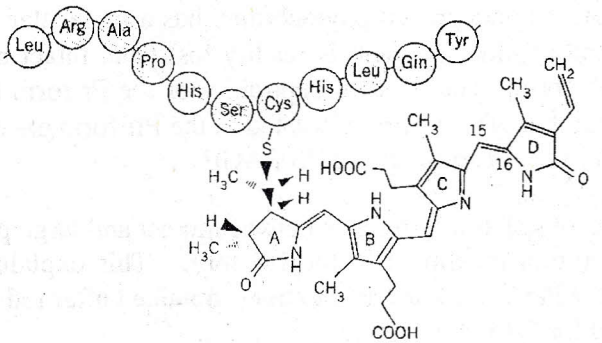


Fig. 14.6 Structure of the phytochrome chromophore and its binding to the apoprotein.

As noted earlier, Pr is biologically inactive and that formation of Pfr initiates an active physiological response. The exact nature of the phototransformation between the two forms is not clear, though, both the chromophore and the apoproein are believed to undergo confirmational changes (Fig. 14.7).

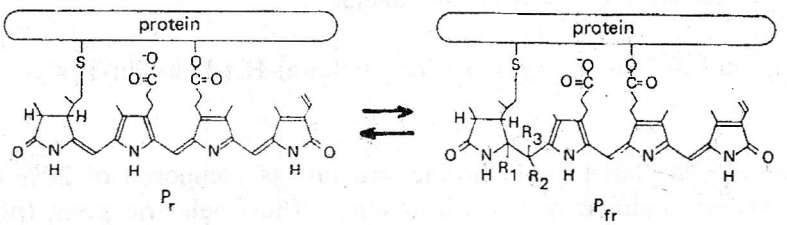


Fig. 14.7 A mechanism of the phototransformation of phytochrome based on the addition of the 4-5 double bond.

The principal difference between the Pr chromophore and the Pfr chromophore appears to be a *cis-trans* isomerization of the methine bridge between rings C and D. The absorption of red light provides the energy required to overcome a high activation energy for rotation around the double bond, which is not normally achieved at ambient temperature.

Protein moiety

Siegelman & Firer (1964) reported a molecular weight of 90,000-1,50,000 for rye phytochrome. Many different values for the molecular weight of phytochrome have appeared in the literature. Three factors seem to have contributed to this confusion:

(1) different plant sources,
(2) proteolytic degradation during isolation,
(3) the tendency of phytochrome to aggregate.

It appears that intact / undegraded phytochrome has a molecular weight of 1,24,000 per monomer. A 6000-dalton peptide fragment is readily lost from intact phytochrome, when the phytochrome is in the Pr form. Thus, the susceptibility of the Pr form to proteolysis accounts for the earliest observation that phytochrome isolated in the Pfr form exhibits a higher molecular weight than that isolated in the Pr form upon SDS PAGE.

The most significant spectral difference between intact and large phytochromes is the red shift of the visible absorption maximum in the Pfr form. This explains why the absorbance maximum at > 730 nm for Pfr *in vivo* or crude extracts obtained after red irradiation of tissues is longer than that obtained for Pfr *in vitro*.

Amino acid composition and sequence

Immuno affinity – purified large oat phytochrome of greater than 98% purity that exists in solution as a dimer of its 118,000 molecular weight monomers contains about 35% non-polar amino acid residues, with 115 carboxylic amino acids per monomer (Table 14.2). The following phytochrome sequence has recently been elucidated.

Leu-Arg-Ala-Pro-His-Ser-Cys (-S-Chromophore)-His-Leu-Gln-Tyr

Protein structure: The large phytochrome structure is composed of 20% α-helix, 30% β-pleated sheet and 50% random coil confirmations. The isoelectric point (pI) on the surface charges on large phytochrome ranges from 5.8 to 7.0 depending on the source and proteolytic modification of *in vitro*.

Table 14.2 Amino acid compositions of large phytochrome expressed as residues (rounded to the nearest integer) per ~ 120,000 – dalton subunit.

Amino acid	Rye phytochrome[a]	Oat phytochrome[b]	Oat phytochrome[c]
Lys	58	64	63
His	28	34	33
Arg	47	51	50
Asp	104	118	112
Thr	46	38	44
Ser	75	73	80
Glu	128	122	122
Pro	88	45	44
Gly	77	72	77
Ala	110	93	93
Cys	26	27	16
Val	89	79	81
Met	32	26	31
Ile	54	51	52
Leu	111	119	114
Tyr	23	23	21
Phe	43	45	43
Trp	--	8	9
Total residues	**1139**	**1088**	**1085**

[a]Data from Rice and Briggs (1973).
[b]Data from Hunt and Pratt (1980).
[c]Data from Roux *et al.* (1982).

Phytochromes in green plants

Phytochrome in green plants is different from phytochrome in etiolated tissues. The form of phytochrome expressed in etiolated tissues is only one of five gene products. Four other gene products are expressed at low levels in both dark and light grown tissues. If Pfr is degraded as rapidly in green plants as it is in eliotated tissues, how do plants under continuous illumination maintain adequate levels of phytochrome? It has long been argued (on the basis of both physiological and *in vivo* spectrophotometric studies) that some properties of phytochrome in light-grown green plants differed from those of etiolated seedlings.

Phytochrome from light-grown *Avena* tissue is smaller (118 kDa) and has a shorter Pr absorption maximum (652 nm) compared with the pigment from etiolated tissue (124 kDa; 666

nm). Phytochrome is neither immunoprecipitated nor recognized on immunoblots by antibodies raised against phytochrome from etiolated seedlings. The kinetics of photoconversion (Pr to Pfr and back to Pr) appear to be similar, but Pfr in light-grown tissue has a longer half-life. The half life of Pfr in treated seedlings grown under continuous light is about 8 hours compared with 1.0 to 1.5 hours in etiolated seedlings.

The labile form of phytochrome that accumulates in dark-grown seedlings is called as Type I phytochrome, the more stable form found in green seedlings is called as Type II phytochrome. Recent studies using recombinant DNA techniques have shown there are multiple forms of Type II phytochrome, encoded by a small family of differentially regulated genes.

The best characaterized family of genes has been isolated from *Arabidopsis thaliana*. There are five phytochrome genes, *phyA*, *phyB*, *phyC*, *phyD* and *phyE*. The gene *phyA* is expressed in dark-grown tissue. This encodes the Labile Type I form of phytochrome (PHY A). This accumultes in dark. Transcription of *phyA* is inhibited by PfrA (far-red absorbing form of PHY A), so PHY A does not accumulate in the light. PHY A protein is rapidly degraded. The remaining Type II phytochrome genes (*phyB, phyC, phyD* & *phyE*) are expressed at low levels in both light and darkness. Their products (PHY B-E) are light stable.

Finally, the accepted dogma of phytochrome is that Pr is biologically inactive and formation of Pfr initiates active developmental responses. Recently, it has been shown that the normal vertical growth habit of *Arabidopsis* seedlings is reduced when grown in red light. On the other hand, mutants that lack photochemically functional phytochrome and are unable to produce Pfr, exhibit a normally erect habit regardless of light treatment. Liscum and Hangarter have concluded that the normal erect growth habit occurs when phytochrome B is in the Pr form. It is assumed that the erect habit is the "active response".

Physiological effects of phytochrome

Phytochrome mediated effects are conveniently grouped into three categories on the basis of their energy requirements. The classical red, far-red photoreversible responses discovered by Hendricks & Borthwick are known as low fluence responses (LFRs). Photon-fluence requirements for LRFs are in the range of 10^{-1} to 10^{2} µmol m^{-2} of red light. Very fluence responses (VLFRs) are induced by much lower light levels, 10^{-6} to 10^{-3} µmol m^{-2} red light. High irradiance reactions (HIRs) require continuous irradiation. Photoinhibition of seed germination appears to be an example of a high irradiance reaction (Fig. 14.8).

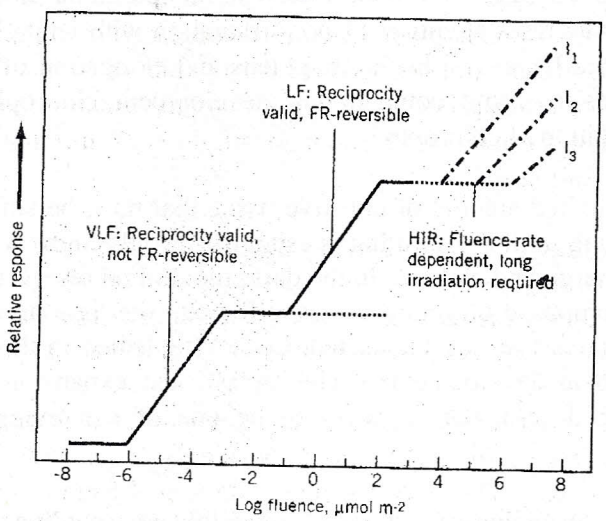


Fig. 14.8 The three categories of Phytochrome

1) Low Fluence Phytochrome Responses

Seed Germination: The germination of most seeds is influenced by light. This includes the majority of non-agricultural species are known as positively photoblastic and germination inhibited by light are negatively photoblastic.

Soil attenuates light very quickly. A 1 mm thickness of fine soil, passes less than 1% of the light at wavelengths longer than 70 nm. So most light-requiring seeds need not be burried very deeply for germination. Some seeds eg: *Sinapsis arvensis* require very little Pfr to stimulte germination and may exhibit germination when covered with upto 8 mm of soil. Suppression of germination in negatively photoblastic seeds like oats (*Avena fatua*) requires long-term exposures at high fluence rates.

Seedling development: Plants grown in darkness take an unusual appearance. Generally, the stems of dicot seedlings are very long and spindly with a pronounced recurve just below the leaves. The leaves undergo limited development and remain small and clapsing, as though they were in the embryo. Chlorophyll is absent and the seedlings appear white or yellow in colour.

In monocots like grass seedlings continue to elongate and remain tightly rolled up. The first internode or mesocotyl of grass seedlings elongates excessively in the dark and the coleoptile grows (which is a modified leaf) slowly arrested chloroplast development and low activities of enzymes. This general condition exhibited by dark-grown plants is called **etiolation**.

In dicotyledonous seedlings, hypocotyl elongation, plumular hook opening and leaf expansion have received the most attention. Upon irradiation with white light, the growth rate of the hypocotyl slows, the hypocotyl hook straightens and elongation of epicotyl accelerates. Light stimulates the leaves to unfold, complete leaf development, chloroplast development and this proceeds the accumulation of chlorophyll.

A seed carries a limited amount of nutritive tissue that must be sufficient to support the development of the seedling until the seedling is established in the light and photosynthesis can take over the supply of energy and carbon. In the dark, the limited reserves of a seed are helped to extend the plumule, composed of young leaves will reach the light and be able to carry out photosynthesis before the reserves are exhausted. Once established in the light, the remaining reserves may be invested in development of chloroplast, leaf expansion etc. So the role of phytochrome in seedling development appears to be one of conveying information to the seedlings.

In addition to morphological changes in etiolated seedlings, other changes at morphological, biochemical and biophysical are also modulated by phytochrome (Table 14.3).

Table 14.3 Selected example of phytochrome-mediated responses

Photoperiodic floral induction
Nyctinastic leaf movements
Phototropic sensitivity
Seed germination
Stem elongation
Plumular hook opening
Leaf and cotyledon expansion
Chloroplast development
Chlorophyll and carotenoid synthesis
Anthocyanin synthesis
Enzyme activation
Protein synthesis
mRNA transcription
Chloroplast phototactic movement
Surface potential (root tips)
Transmembrane potential

In most LFR experiments, the level of the response with FR, either alone or in sequence with R, is typically higher than dark controls. FR never establishes complete photoreversibility.

Bioelectric Potential and Ion Distribution: The response time for most phytochrome-mediated developmental effects is measured in hours or even days. But there are some responses which are measured in minutes or seconds. Most of these responses appear to related to membrane based activities like bioelectric potential or ion flux. T. Tanada observed that dark-grown barley root lip would float freely in a glass beaker with a specially prepared negatively charged surface. Within 30 minutes following a brief red irradiation the root tips would adhere to the surface. A subsequent far-red treatment would release the root tips from the glass. Adhesion and release was correlated with phytochrome-induced changes in the surface potential of the root tips. A brief red treatment generated a positive surface potential, attracting the tips to the negatively charged surface. A far-red treatment generated a negative surface potential, causing the tips to detach.

Phytochrome modulated transmembrane potentials have been reported, but in most cases red light induces a depolarization of the membrane within 5 to 10 seconds following a red light treatment. A subsequent far-red treatment causes a slow return to normal polarity or small hyperpolarization.

One of the oldest and most detailed studies of membrane-based phytochrome effects is chloroplast rotation in *Mougeotia*, a filamentous green alga. It contains a single flat chloroplast that is capable of rotating around its long axis so that either its face or its edge is oriented toward incident light (Fig. 14.9).

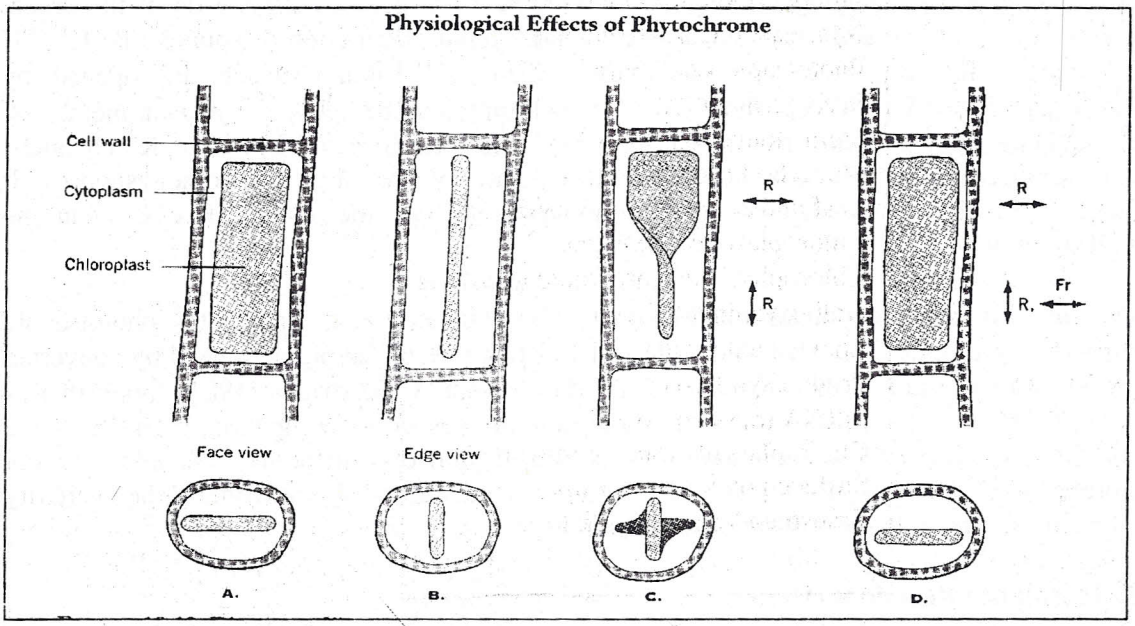


Fig. 14.9 Diagram of Haupt's experiments with *Mougeotia* chloroplasts

Reorientation of the chloroplast is mediated by phytochrome: Red light is most effective and it is far-red reversible. W. Haupt employed plane-polarized light and microbeams of red and far-red light to irradiate specific locations in the cell. He found that the phytochrome responsible for chloroplast rotation was located not in the chloroplast itself but in the cortical cytoplasm, that is, the region of cytoplasm lying inside the plasma membrane. Reorientation of the chloroplast in plane-polarized light was dependent on the direction of polarization relative to the long axis of the cell. Polarized light was most effective when its electrical vector was parallel to the long axis of the cell. Far-red was most effective at reversing the red effect when polarized in a plane at right angles to the plane of red light. These results suggest that the phytochrome molecule assumes a particular orientation in the cytoplasm and that the orientation of Pfr is normal to the orientation of Pr.

There must be a singal chain that links the phytochrome to the chloroplast. Calcium may function as a second messenger for phytochrome, perhaps interacting with the cytoskeleton to control chloroplast orientation. The uptake of Ca^{2+} into *Mougeotia* cells is stimulated by red light. Application of the calcium ionophore A23187 to specific sites on the cell wall stimulate the chloroplast to reorient, if calcium is available in the suspension medium.

A correlation between phytochrome and ion movements has been demonstrated in *nyctinastic* or sleep movements of leaves. Paired leaves or leaflets are generally horizontal during the day but fold together when darkened. Plants that show this behaviour have a bulbous zone called the *pulvinus* at the base of the leaf or leaflet. The pulvinus drives leaf movement by altering its shape by changing the volume of cells on the upper and lower side of the organ. Changes in the volume and shape of these cells is by rapid distribution of solutes like K^+, Cl^+ and malate. K^+ moves through electrically gated K^+ channels which are opened by phytochrome-driven H^+ efflux. The role of phytochrome may be activate a plasma membrane bound ATPase proton pump that inturn depolarizes the membrane to open the K^+ channels. Indeed, Ca^{+2}/calmodulin has been implicated as a mediator of several phytochrome responses. It remains to be demonstrated, that phytochrome promotes an increase of free Ca^{+2} in the cytoplasm of plant cells.

Very Low Fluence Responses (VLFRs): A low far-red fluence promotes phototrophic sensitivity as red light does. This indicates that less than 1% of the pigment need by converted to Pfr in order to saturate the response. D.F. Mandoli and W.R. Briggs (1981) found that as little as 0.01% Pfr is required to elicit inhibition of mesocotyl elongation. This extreme sensitivity to light makes the study of VLFRs technically difficult. VLFRs are not photoreversible. The evidence that a VLF response is mediated by phytochrome is the similarity of its action spectrum to the absorption spectrum of Pr.

High Irradiance Reactions (HIRs)

In the natural environment, plants are exposed to long periods of sunlight at relatively high fluence rates. Under such conditions, the photomorphogenic program achieves maximum expression and responses like leaf expansion and stem elongation. Such light-dependent

responses are known as high irradiance responses (HIRs). HIRs show the following characteristics:

(1) full expression of the response requires prolonged exposure to high irradiance,

(2) the magnitude of the response is a function of the fluence rate and duration,

(3) HIRs are not fully red, far-red photoreversible.

Like other responses of etiolated seedlings, the initiation of anthocyanin accumulation is a classic phytochrome-dependent LFR. The red, far-red photoreversibility is limited to brief irradiation when long-term irradiations are applied, the action peak for anthocyanin accumualtion is shifted to the far-red, with reduced effectiveness in the red.

Although the effectiveness of red and far-red light argues in favour of phytochrome as a photoreceptor, the unique characteristic of phytochrome reactions, photoreversibility is conspicuously absent from high irradiance reactions.

Based on variations in action spectra, at least three categories of HIRs can be recognized:

(a) action in the blue-UV-A, red and far-red.
(b) action in the blue-UV-A, red and far-red.
(c) action in the blue-UV-A, red and far-red.

In order to explain these differences, at least two photoreceptors must be involved. Phytochrome and a blue-UV-A receptor.

Hartmann presented seedlings with light of either 658 nm or 766 nm. These wave lengths were chosen because they are absorbed by Pr and Pfr. When presented separately, 658 and 766 nm light were ineffective at inhibiting hypocotyl elongation. When the wavelengths are presented simultaneously both could inhibit elongation as efficiently as 716 nm light. 766 nm light is (Hartman's thesis) ineffective because it converts phytochrome predominantly to the inactive Pr form. 658 nm light converts the pigment to the Pfr form that, is rapidly lost by degradation reactions. Light at 716 nm is effective because it establishes an intermediate level of Pfr, balancing the competing reaction of Pfr action and Pfr degradation.

In some systems, a separate blue-UV-A receptor interact with phytochrome in controlling the high irradiance reaction. A blue light-dependent inhibition of hypocotyl elongation in light-grown seedlings (*Cucurbita, Lactuca* and *Lycopersicum*) can be demonstrated by simultaneous irradiation with blue and white light. This causes inhibition of stem growth compared with controls receiving white light alone. But these conditions do not alter the ratio of Pfr to total phytochrome.

Finally, phytochrome does not induce anthocyanin biosynthesis in totally dark grown seedlings. Red, far-red photoreversible control is following a prolonged blue light treatment.

These results strongly support that a separate blue-UV-A photoreceptor my be operative in some HIRs and that it may act cooperatively with phytochrome.

Mechanism of phytochrome action

To answer a question, whether the phytochrome molecule is associated with membranes in the cell, there are two strategies for answering this question. The first strategy to be attempted was to fractionate cells and using dual-wavelength difference spectroscopy, assay for phytochrome in the various fractions. Using this approach, phytochrome has been reported in association with every fraction of the cell, including plastids, mitochondria, Endoplasmic reticulum and Plasma membrane as well as the soluble fraction.

The subcellular distribution of phytochrome has also been studied by immuno-cytochemistry. Material fixed for examination by either light or electron microscopy is probed with antibodies to phytochrome. The antibodies are labelled with the enzyme peroxidase or some other marker to make direct visualization of the Ag-Ab complex. In dark-grown at coleoptile parenchyma cells, phytochrome appears to be uniformly distributed throughout the cytosol, some of the pigment is associated with the plasma membrane, ER and the nuclear envelope.

Since isolated organelles have phytochrome associated with them, it should be a simple matter to test purified organelles for R/Fr photoreversible functions. Isolated mitochondria have been thoroughly documented and exhibit photoreversible NADP reduction calcium fluxes and ATPase activity.

The evidence indicates that phytochrome is not an intrinsic membrane protein. It may induce changes in membrane properties, by a loose association with the membrane or through an intermediate, but yet unidentified, signal chain.

Phytochrome and gene action:

In all the physiological events there is participation of phytochrome, suggests an involvement of gene expression. This gives rise to the expectation that changes in the level of specific gene products i.e. proteins and ultimately mRNA levels are subject to regulation by phytochrome.

Phytochrome regulation at the level of protein was first reported in 1960 by A. Marcus. He reported red far-red reversible control of glyceraldehyde-3-phosphate dehydrogenase activity in bean seedlings. Nine years later, M. Jaffe reported phytochrome-dependent increases in the RNA content of pea buds 24 hours after a red light treatment. In 1985, E.M. Tobin and J. Silverthorne were able to list nine identified and multiple unidentified proteins whose genes were expressed differently in light-grown and dark-grown plants. Seven of these genes have been shown to be regulated by phytochrome.

The majority of phytochrome-regulated genes studied so far are nuclear genes encoding from mRNAs of chloroplast proteins. Two have been studied extensively. The small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the light-harvesting chlorophyll a/b binding proteins (LHCP).

Nuclear runoff experiments have confirmed that phytochrome regulates the genes for both of these proteins at the transcriptional level. In these experiments, nuclei isolated immediately following the light treatment are incubated in the presence of a radiolabled RNA precursor (^{32}P uridine-triphosphate). Conditions are chosen such that only transcripts that were initiated prior to isolation of the nuclei will incorporate the label. The amount of RNA transcribed from a particular gene can then be measured by hybridizing the labelled transcripts to cDNA known to contain that gene. Results of these experiments confirm that Pfr acts to increase the rate of transcription of these two genes.

There are two examples of important genes whose transcription is negatively regulated by phytochrome. One negatively regulated gene encodes for NADPH-protochlorophyllide oxidoreductase. This enzyme catalyzes reduction of protochlorophyllide to chlorophyllide. The level of translatable mRNA decreases within an hour following a brief red pulse and remains low in continuous light. The effect of a red pulse is reversible with far-red. A decrease in mRNA would decrease in activity in light.

The second example of negatively regulated transcription is the phytochrome gene itself. 5 seconds of red light causes a rapid decline in translatable phytochrome mRNA in etiolated seedlings. After a 15-minutes lag period, the level of mRNA drops by 50% within the first hour and by more than 95% in two hours (Fig.14.10A). The decline in mRNA is far-red reversible though the level of Pfr established by far-red light alone is sufficient to induce loss of mRNA. It appears that phytochrome autoregulates transcription of its own mRNA by some form of feedback inhibition (Fig. 14.10B).

Phytochrome controls gene expression and that it can do so at the transcription level. Some studies have suggested that Pfr may activate another protein (a second messenger) that binds to certain DNA sequences called light-regulated elements (LREs) (Fig. 14.10A). Binding of the regulatory protein to the LRE stimulates transcription of the gene. In absence of this, protein transcription will not occur. LREs have been identified for two genes, one encodes the small sub-unit of Rubico (rbc S) and the other is the apoprotein of LHCP (cab). The LRE for the rbc S gene will impose light sensitivity on reporter genes that are not normally sensitive to light if the reporter gene is inserted into the chromosome in the region of the LRE.

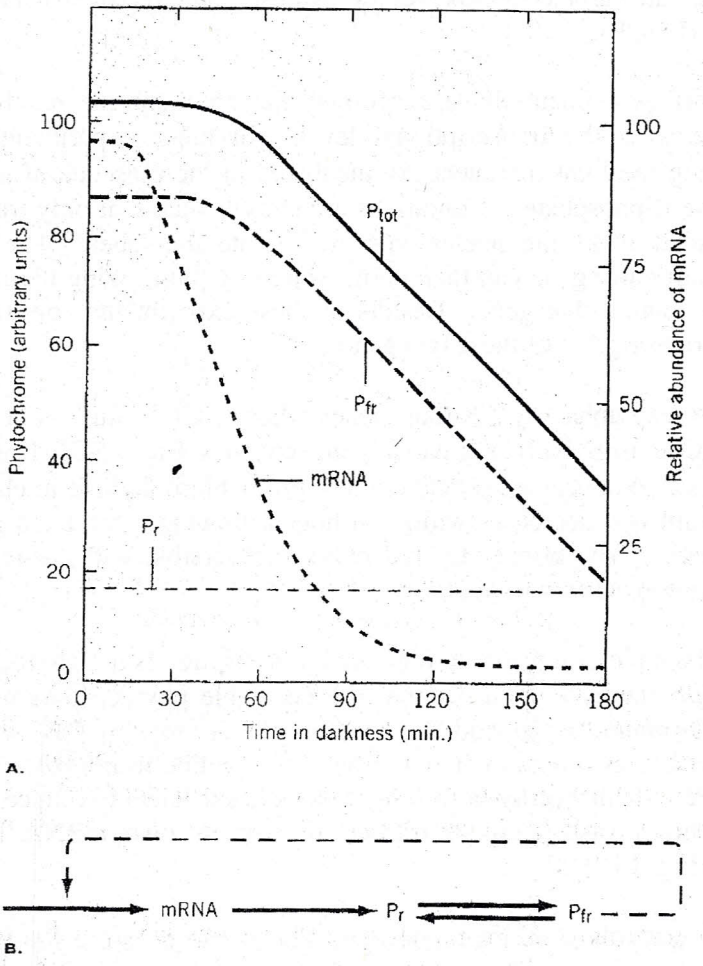


Fig. 14.10 A. Phytochrome-induced decline in phytochrome mRNA
B. The feedback inhibition of phytochrome mRNA by Pfr

In another interesting study, D. Ernst and D. Oesterheldt (1984) reported an increased transcription rate *in vitro* when phytochrome was added to **isolated** rye nuclei. This suggests the possibility of a direct interaction between phytochrome and the nucleus, obviating the need for an intervening protein or second messenger.

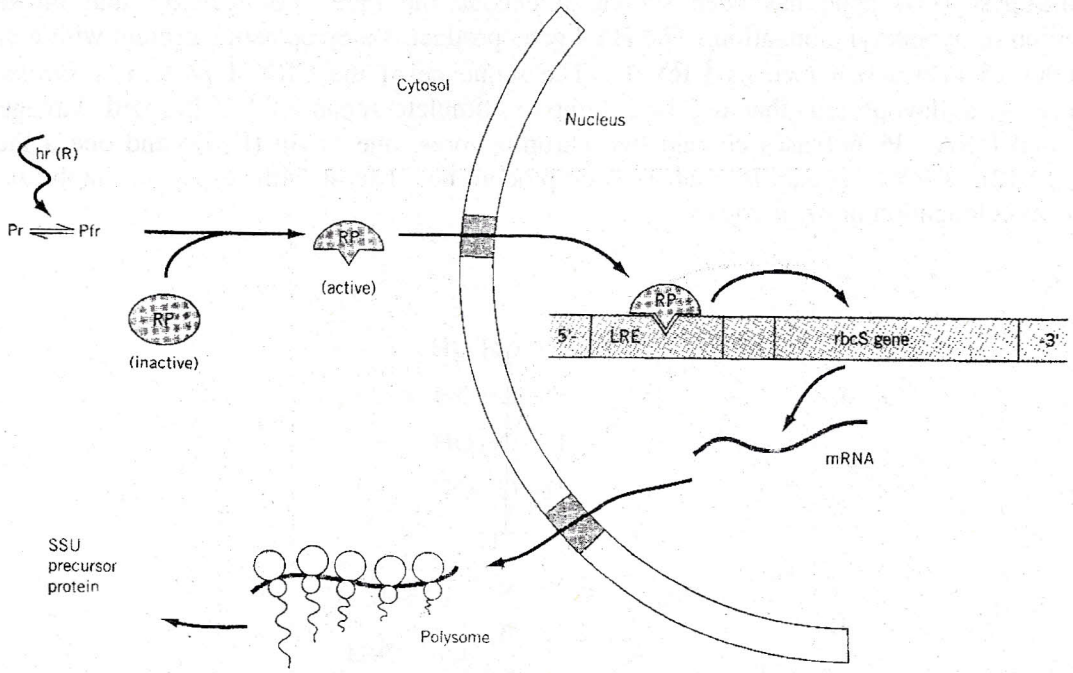


Fig. 14.11 Schematic model for phytochrome regulation of the Rubisco small subunit gene (rbcS)

There is still much to be learned about the molecular biology of development. The complex developmental events require the coordinated input of many gene products that must be expressed in the right tissues at the right time. When we understand something of these complex spatial and temporal interactions we will begin to understand and appreciate the true role of phytochrome in regulating plant responses to the natural radiation environment (Fig. 14.11).

Cryptochrome: A wide range of plant responses to blue and UV-A radiation have been known or suspected for a long time. These responses are prevalent in lower plants such as ferns, mosses and fungi. These responses also share similar action spectra with higher plant responses such as photoperiodism and hypocotyl elongation. The identity of the blue/UV-A photoreceptor has proven difficult to unravel, hence the name cryptochrome, which means hidden pigment. Cryptochrome includes carotenoids and flavins or both. Like carotenoids, flavins are ubiquitous in living organisms. The three most common are riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The flavins may occur free or complexed with proteins, they are called flavoprotein. Both FMN and FAD are important cofactors in cellular oxidation-reduction reactions.

Using a combination of genetic, photobiological and biochemical approaches, the **Arabidopsis HY4** gene has been shown to encode the blue photoreceptor that mediates inhibition of hypocotyl elongation. The **HY4** gene product is a cytoplasmic protein with a mass of about 75 kDa and named as **CRY 1**. The sequence of the CRY 1 protein is similar to photolyase, a flavoprotein that use blue light to stimulate repair of UV-induced damage to microbial DNA. Photolyases contain two chromophores, one flavin (FAD) and one a Pterin (Fig. 14.12). CRY1 appears to qualify as cryptochrome, at least with respect to inhibition of hypocotyl elongation in *Arabidopsis*.

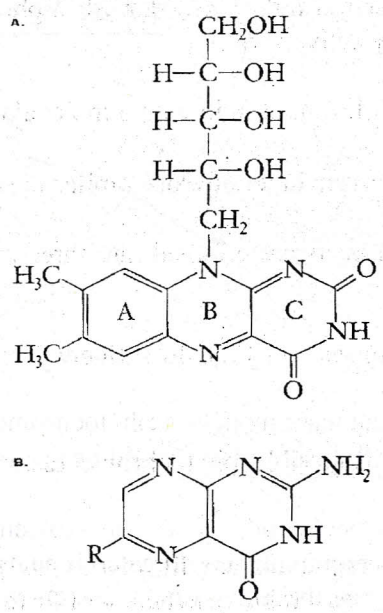


Fig. 14.12 The structure of Riboflavin (*A*) and Pterin (*B*).

14.4 Summary

- The phytochromes are a unique family of chromoproteins that play a critical role in almost every stage of plant development from seed germination to flowering.
- The existence of phytochrome was predicted on the basis of physiological experiments that demonstrated photoreversibility with red (660 nm) and far red (730 nm) light.
- The pigment exists in two forms: Pr absorbs maximally at 660 nm and Pfr absorbs at 730 nm. When Pr absorbs far-red light, it is converted to Pfr, when Pfr absorbs far-red light, it is converted back to Pr.

- In *Arabidopsis* there are five phytochrome genes encoding five species of phytochrome *(PHYA-E).*
- Phytochrome A (PHYA) accumulates in dark grown seedlings as PrA, which is stable and PfrA is unstable and is destroyed with a half-life of 1 to 1.5 hours. PHYB is expressed at low levels in both light and dark.
- PfrB is stable with a half-life of 8 hours or more.
- A mixture of red and far-red light will establish a photoequilibrium mixture of P_r and Pfr. Pfr is the physiologically active form.
- Phytochrome is a bluish chromoprotein with a molecular mass of about 124 kDa.
- A chromophore is an open chain tetrapyrole similar in structure to phycocyanin.
- Phytochrome-mediated effects are grouped into three categories on the basis of their energy requirements.
- Very low fluence responses (VLFR), low fluence responses (LFR) and high irradiance reactions (HIR).
- LFRs include the photoreversible phytochrome responses such as seed germination and deetiolation.
- VLFRs are not photoreversible and are difficult to study because they saturate at light levels below those that cause a measurable conversion of Pr to Pfr.
- HIRs require prolonged exposure to high irradiance, are time dependent and are not photoreversible.
- Phytochrome is the sensor that detects changes in red/far-red fluence ratio that occur under canopies and as end-of-day signals.
- The signal transduction pathway for phytochrome action is unknown.
- The identification of mutants deficient of one or more phytochrome is an important first step in deciphering this important regulatory system.

14.5 Model Questions

1. What is photomorphogenesis? Write the historical aspects of photomorphogenesis.
2. Write an essay on phytochrome.
3. Discuss about phytochromes in green plants.
4. Write short notes on:
   a) Cryptochrome
   b) Mechanism of Phytochrome action
   c) LFRs
5. Write an essay on physiological effects of phytochrome
6. Phytochrome and gene action.

14.6 Reference Books

1) **Introduction to Plant Physiology**, 2nd edition, William G. Hopkins, John Wiley & Sons, Inc.
2) **Advanced Plant Physiology,** Malcolm B. Wilkins, English Language Book Society/Longman.
3) **Plant Physiology** L. Taiz and E. Zeiger. Sinauer Associates, Inc., Sunderland, Massachussetts.

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Dr. P. KIRANMAYEE

LESSON-15

PLANT GROWTH REGULATORS

15.0 OBJECTIVES

In this lesson, you will learn:

- A discussion of the hormone concept in plants and some of the controversies that surround it.
- An introduction of the five major groups of plant hormones – auxins, gibberellins, cytokinins, abscisic acid and ethylene – followed by a description of their principal physiological roles.
- A brief description of a few hypothetical hormones, the polyamines and other biological active substances including brassinosteroids that can influence growth and development.
- Description of two general models of hormone function. The hormone-binding proteins and their role in signal perception and of second messengers in the signal transduction pathway.
- Biosynthesis, transport and metabolism of auxins, gibberellins, cytokinins, abscisic acid and ethylene.

15.1 INTRODUCTION

Multicellular plants are complex organisms and their development requires an extraordinary measure of coordination between cells. In order to coordinate their activities, cells must be able to communicate with each other, often at some distance. The principal means of intercellular communication are the hormomes, the chemical messengers that carry information between cells and so coordinate their growth and development. Plant hormones have been the subject of intensive investigation.

The Hormone concept in plants: There are numerous chemical substances, natural and synthetic, that profoundly influence the growth and differentiation of plant cells and organs. Their role in development has been studied for nearly a century, yet the concept of hormones in plants is steeped in controversy.

In 1905, the British Physician E.H. Starling introduced the term **hormone** (Gr.; to *excite* or *arose*) to describe these chemical messengers. The concept of hormones in plants may be traced back to observations of Duhamel du Monceau in 1758. He observed the formation of roots on the swellings that occur above girdle wounds around the stems of woody plants. Root-forming substances, produced in the leaves and migrating down the stem would account for the initiation of roots above the wound. It was Darwin's observations and experiments that ultimately led F.W. Went, to describe a hormone-like substance as the causative agent when plants grew toward the light. At about the same time, H. Fitting introduced the term *hormone* into the plant physiology literature.

Hormones are naturally occurring organic substances that, at low concentration, exert a profound influence on physiological processes. Like animal hormones, plant hormones are naturally occurring organic substances that influence physiological processes at low concentration. The site of synthesis of plant hormones, is not so clearly localized. Although some tissues or parts of tissues may be characterized by higher hormone levels than others, synthesis of plant hormones appears to be much more diffuse and cannot always be localized to discrete organs.

Whether plant hormones act in a concentration-dependent manner is a subject of continuing dispute among students of plant hormones. Some argue that plant cells respond to hormone concentration, as they do in animals, others argue that it is not the hormone concentration that is important, but changing sensitivity of the target cells to the hormone.

Another difficulty with plant hormones is the multiplicity of their effects. Each group of plant hormones is known to influence a wide variety of developmental events. Most of these events can be influenced by more than one hormone group (Table 15.1).

Table 15.1 The influence of plant growth hormone groups on different categories of development
(An x indicates a demonstrated effect of that hormone group on one or more aspects of that developmental category. The absence of an x does not mean that the hormone is ineffective, only that an effect has not been reported in the literature).

	Hormone Group				
	Auxins	**Gibberellin**	**Cytokinin**	**Abscisic acid**	**Ethylene**
Dormancy		x	x	x	x
Juvenility	x	x			
Extension Growth	x	x	x	x	x
Root development	x	x	x		x
Flowering	x	x	x	x	x
Fruit development	x	x	x	x	x
Senescence	x	x	x		x

The differences between animal and plant hormones led to some confusion in terminology. The *plant growth substances* is preferred by some-there is even an International Plant Growth Substance Society. Others argue that "**substances**" too vague and growth is influenced by these chemicals. A second term, *plant growth regulator* is preferred and it is used to denote synthetic compounds that exhibit hormonal activity.

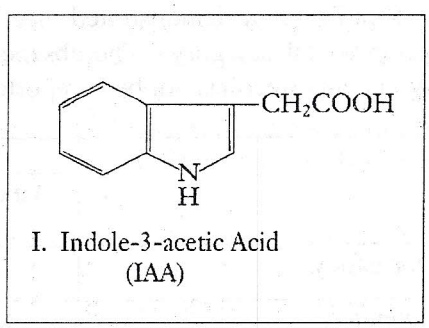
There are currently five recognized groups of plant hormones: auxins, gibberellins, cytokinins, abscisic acid (ABA) and ethylene. In addition to these hormones, two other groups appear to be active in regulating plant growth, the brassinosteroids and polyamines.

15.2 AUXINS

Auxins were the first plant hormones to be discovered. Auxins are synthesized in the stem and root apices and transported through the plant axis. They are characterized principally by their capacity to stimulate cell elongation in excised stem and coleoptile sections, but also influence a host of other developmental responses, including root initiation, vascular differentiation, tropic responses and the development of axillary buds, flowers and fruits.

15.2.1 Natural and synthetic auxins

The principal auxin in plants is indole-3-acetic acid (IAA) (Fig. 15.1). Indole-3-ethanol, indole-3-acetaldehyde and indole-3-acetonitrile are also naturally occurring auxins. All these serve as precursors to IAA and their activity is due to conversion to IAA in the tissue. The synthetic chemicals also express auxin like activity. One of these, indole butyric acid (IBA), was originally thought to be strictly synthetic, but recently IBA has been isolated from seeds and leaves of maize and other species (Epstein *et al.*, 1989). A chlorinated analog of IAA (4-chloroindolacetic acid, or 4-chloro IAA) has also been reported in extracts of legume seeds (Engvild, 1986) and phenylacetic acid (PAA) (Fig. 15.2A, B), a naturally occurring aromatic acid, has recently been reported to have auxin activity (Leuba and Le Toureau, 1990). It has not yet established, whether or not they are converted to IAA in the tissue before they become active.



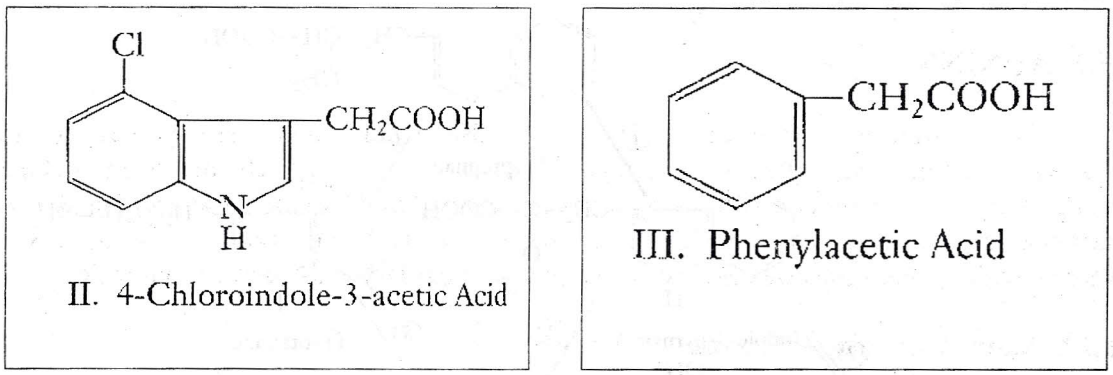
I. Indole-3-acetic Acid (IAA)

Fig. 15.1 The true auxin in plants, IAA

15.2.2 Biosynthesis and metabolism of auxins

The auxin IAA is ubiquitous in the plant. Highest concentrations of the hormone are detected in meristematic regions and actively growing organs like coleoptile apices, root tips, the apical buds of growing stems and germinating seeds. The major IAA synthesis sites are young, rapidly growing leaves, developing inflorescences and embryos following pollination and fertilization.

The amount of IAA present depends on the type and age of tissue and its state of growth. For example, in vegetative tissues, the amount of IAA is between 1 μg and 100 μg kg^{-1} fresh weight, but in seeds it appears to be much higher. The high level of hormone in seed helps in the growth of young seedling when the seed germinates.



(A) (B)

Fig. 15.2 A) 4-chloroindole-3-acetic acid; B) Phenylacetic acid

15.2.3 Biosynthesis of IAA

IAA is synthesized from the aromatic aminoacid, tryptophan. In 1930, K.V. Thimann first observed the synthesis of IAA in the mould *Rhizophus suinus*, which had been fed the amino acid tryptophan, which is converted to IAA. The synthesis of IAA is studied by feeding the plants tryptophan carrying a radioactive label, usually carbon (^{14}C) or tritium (^{3}H) and examined the radioactivity of isolated IAA or its intermediates.

- In most higher plants, synthesis of IAA occurs in three steps, conversion of tryptophan to indole-3-pyruvic acid (IPA) (Fig. 15.3). This transamination reaction is catalyzed by tryptophanamino transferase, this will remove amino groups from tryptophan, phenylalanine and tyrosine.

- The second step is the decarboxylation of IPA to form indole-3-acetaldehyde (IAAld) in presence of an enzyme indole-3-pyruvate decarboxylase.

- Final step is, IAAld is oxidized to IAA by a NAD-dependent indole-3-acetaldehyde dehydrogenase.

- IAAld may also be reversibly reduced to indole-3-ethanol. This exhibits auxin activity in bioassays of stem sections.

In *Arabidopsis* and in members of Brassicaceae, an alternative pathway is evidenced. IAA may also be formed from indole-3-acetonitrile (IAN), an indole derivative. IAN exhibits auxin activity, probably by conversion to IAA through the action of a nitrilase enzyme.

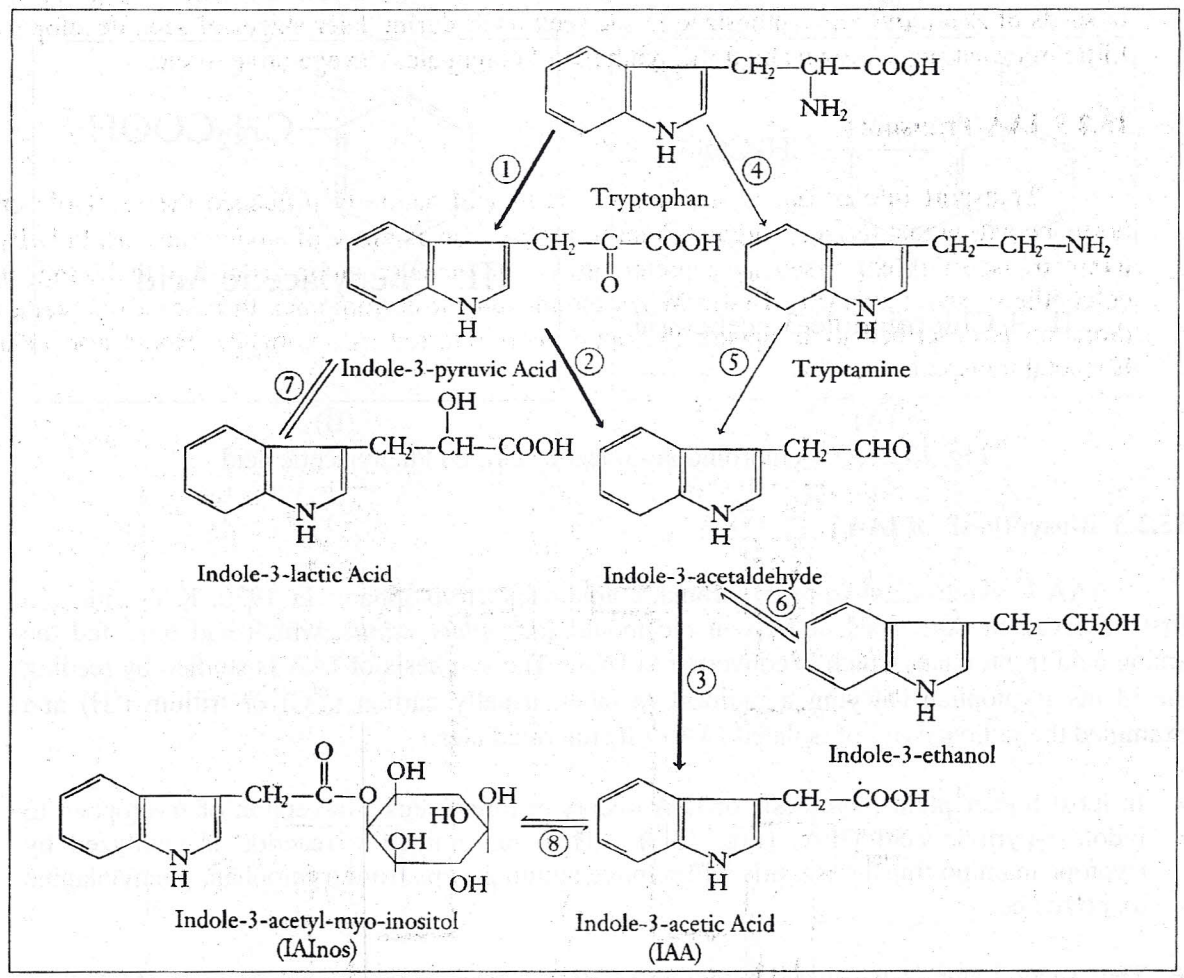


Fig. 15.3 The biosynthetic pathway of auxin (IAA)

15.2.4 IAA conjugates

Two populations of the hormone were recognized. One was free-moving, could be obtained by diffusion into Agar, the other is bound auxin, could be obtained by solvent extraction or by hydrolysis under alkaline conditions. The bound auxin is now recognized as IAA that has formed chemical conjugates like glycosyl esters or peptides.

IAA-conjugates are inactive but release free, active IAA upon solvent extraction, alkaline hydrolysis or *in vivo* enzymatic hydrolysis. In germinating seedlings, large pools of IAA conjugates appear to be important source of active hormone.

The site of synthesis of IAA conjugates are not well understood. The conjugates found in seeds of *Zea mays* are synthesized in the seed itself during later stages of seed development. Little information is known about the synthesis of conjugates in vegetative tissues.

15.2.5 IAA Transport

Transport into or out of a tissue or organ will naturally influence the level of active hormone within that tissue or organ. Polar transport is an example of auxin transport. Polarity in auxin transport is expressed as a preferential movement of auxin from top to bottom in a coleoptile or shoot axis (Fig 15.4). When the movement is from apex to base of the plant, the direction is described as basipetal; the opposite is referred as acropetal. Roots also exhibit basipetal transport of auxin.

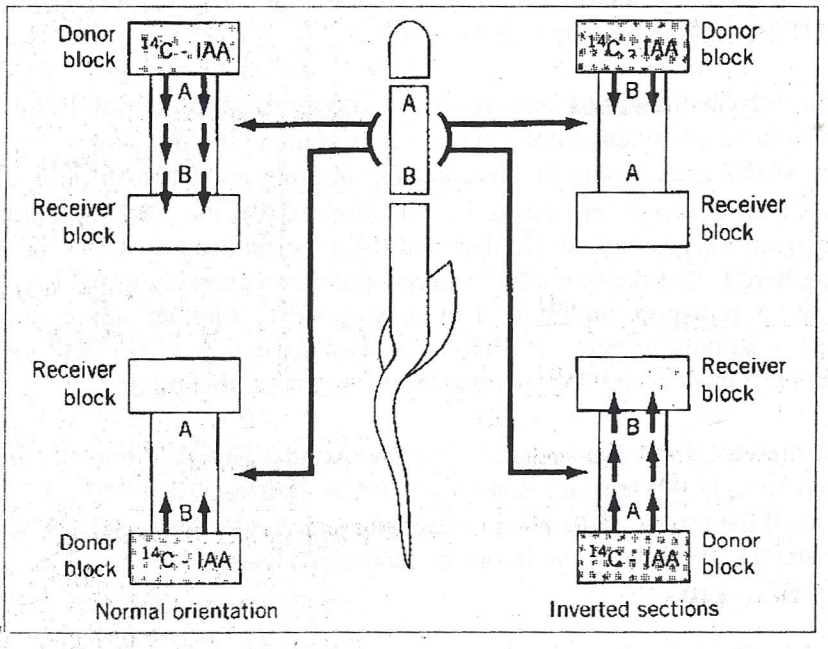


Fig. 15.4 Polarity in auxin transport in an oat coleoptile segment

How is polarity in auxin transport established? Many observations indicate the involvement of some form of carrier-mediated, active transport mechanism. First, it can be shown that polar transport is inhibited by respiratory poisons such as cyanide and 2,4-dinirophenol.

Second, certain chemicals called phytotropins, like 2,3,5-triiodobenzoic acid (TIBA), 9-hydroxyfluorine-9-carboxylic acid (HFCA or morphactin) and N-1-naphthylphthalmic acid (NPA).

Third, the uptake of radioactive IAA is partially inhibited by non-radioactive IAA. These results suggest that the labelled and unlabelled IAA compete for a limited number of carrier sites.

IAA is a weakly acidic, lipophilic molecule. Depending on the pH, IAA may exist either in the protonated (IAAH) or the unprotonated, anionic form (IAA^-). In cell wall space, where pH is about 5.0, any IAA^- will rapidly protonate to form IAAH. IAAH has a higher lipid solubility and penetrate more readily than IAA^-. It has been confirmed that the uptake of IAA into cells increases as the extracellular environment is made more acidic. Thus cells will take up auxin from the cell wall space as IAAH diffuses down its concentration gradient. In cytoplasm, where pH is about 7.0, IAAH will dissociate to IAA^- and H^+. The pH difference between the cell wall space and the cytoplasm serves to maintain the IAAH concentration gradient and encourage IAAH to continue moving into the cell.

More recently, inhibitors of polar transport are provided to be useful tools for exploring the role of auxins in developmental phenomena. One example is early stages in the formation of flower buds in *Arabidopsis*. *Pin*1 is a mutant of *Arabidopsis* that results in abnormal floral development. Polar transport of auxins is also affected by the *Pin*1 mutation. The polar transport of exogenously supplied ^{14}C-labelled IAA is reduced to 10% of normal. The interesting thing here is that the mutant phenotype could be generated in wild-type seedlings by applying the auxin transport inhibitors HFCA and NPA. Another auxin antagonist (2-[p-chlorophenoxyl] – isobutyric acid or (PIB) is known to inhibit auxin activity but does not interfere with polar transport and does not generate the mutant phenotype.

In another study, Liu and coworkers have shown that HFCA stimulates the formation of fused cotyledons in cultured mustard embryos. HFCA interferes with the normal initiation of two cotyledons and the transition from the axial symmetry of the early globular shaped embryo. Both of these studies show how inhibitors of auxin transport can be used as developmental probes.

15.2.6 Oxidation of IAA

IAA in aqueous solution is degraded by acids, ultraviolet, ionization radiation and visible light in presence of sensitizing pigments such as riboflavin. The most prevalent form of IAA degradation, appears due to oxygen and peroxide, either separately or in combination, in the presence of a suitable redox system.

An enzyme responsible for inactivating IAA was first isolated from plant extracts by Tang and Bonner (1947) and was called *IAA oxidase*. Oxidative decarboxylation of IAA is known to be catalyzed by peroxidases from a variety of plant sources. The oxidative

decarboxylation of IAA by peroxidase is now recognized by some physiologists to be synonymous with IAA oxidase.

The pathway for oxidative decarboxylation of IAA is shown in Figure 15.5. The major end products of oxidation by cell-free enzyme preparations are 3-hydroxy-methyl oxindole and 3-methyleneoxindole. IAA oxidase activity is higher in the older, non-growing tissues than it is in younger, actively growing tissues, which have a high auxin requirement. Oxidative break down is the only known means for irreversibly removing IAA from the active pool and may be very important in regulating IAA-mediated responses.

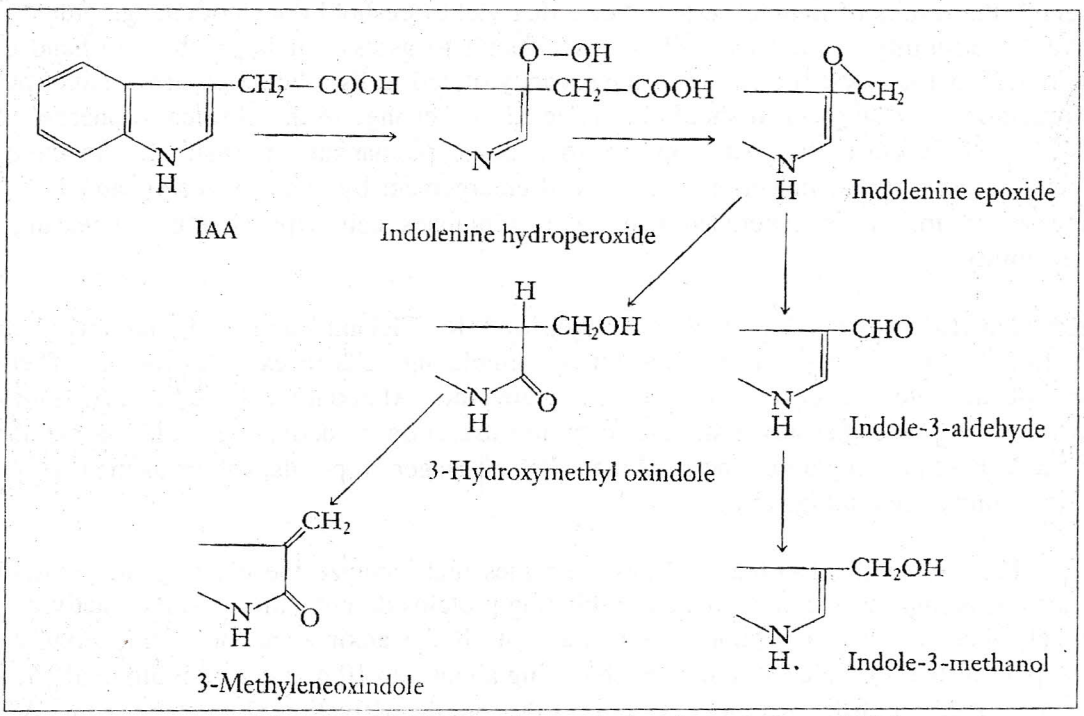


Fig. 15.5 Schematic pathway for oxidative degradation of IAA

15.2.7 Mechanism of action of auxins

The primary mechanism of hormone action in plants generally and auxin in particular continues to elude us more than half a century after its discovery. Although auxin appears to be involved in a wide range of growth and developmental responses, efforts to understand how auxin works have focused largely on the fundamental role of auxin in cell expansion.

Auxin and cell expansion: Cell expansion is the most studied hormonal response in plants. Two major theories have been proposed to account for auxin-induced cell expansion. In the 1960s, shortly after Watson and Crick's DNA structure and DNA-RNA-protein dogma, it was proposed that auxin activated the genes for certain proteins that were necessary for all growth.

In the meanwhile, Mitchell's chemiosmotic model for oxidative phosphorylation was gaining universal acceptance and interest was turning toward cellular membranes and the control of ion flux across membranes – and ATPase proton pumps in particular. A second theory, which attributed cell expansion to auxin-induced proton excretion, was the acid growth theory.

An increase in growth rate, would require an increase in wall extensibility (m), an increase in turgor pressure (P) or a decrease in yield threshold (Y). Direct measurements of P, using a micropressure probe, indicate that turgor pressure does not change during auxin-stimulated increase in the growh rate of pea stem sections. Although Y cannot be measured directly, the results of indirect tests indicate that yield threshold does not change either. That leaves extensibility, **m** and extensibility is difficult to assess. It is on the one hand a rate coefficient, but it is also a measure of the capacity of cell walls to undergo irreversible (plastic) deformation. A change in **m** should be reflected as a change in the physical properties of the wall, especially plasticity or its capacity to undergo permanent deformation. So there is a general agreement that induction of rapid cell enlargement by auxin is accompanied by large increases in **m**. It is concluded that auxin stimulates cell expansion by increasing wall extensibility.

Acid Growth theory: In 1970, D. Rayle and R. Cleland suggested that auxin causes acidification of the cell wall environment by stimulating cells to excrete protons. There the lower pH activates one or more wall loosening enzymes. At about the same time, A. Hanger, in Germany, proposed that auxin stimulated proton excretion by activating a plasma membrane-bound ATPase proton pump. The combined Cleland-Hager proposals, known as the acid growth theory, summarized in Fig. 15.6.

Plant membranes contain ATPase enzymes that catalyze the electrogenic transport of protons. It is important to note that auxin-binding proteins do not exhibit ATPase activity. It is unlikely that the plasma membrane ATPase is itself the auxin receptor. Still, auxin causes hyperpolarization of the cell membrane beginning about 8 to 10 minutes after auxin application. Hyperpolarization of the membrane would result from the activation of an electrogenic ATPase proton pump.

Auxin will also cause growing cells to excrete protons. This is an energy dependent process. Metabolic inhibitors and inhibitors of auxin induced growth will inhibit auxin-induced proton excretion. With **Avena** coleoptiles, the pH of the apoplastic solution drops from 5.7 to 4.7 within 8 to 10 minutes of auxin application. Acid solutions at a pH of 3, 4 are required to induce a rate of elongation comparable to optimum auxin concentrations. Auxin also activates phospholipase A_2 (PLA_2), a membrane bound enzyme that hydrolytically excises the fatty acid from the central (C_2) glycerol carbon of a phospholipid. The product is a free fatty acid plus a phospholipid with a single fatty acid, called a lysophospholipid (LPC). Activation of PLA_2 can be blocked by Ig G-antiABP (Auxin binding protein) and the products of PLA_2, both LPC and

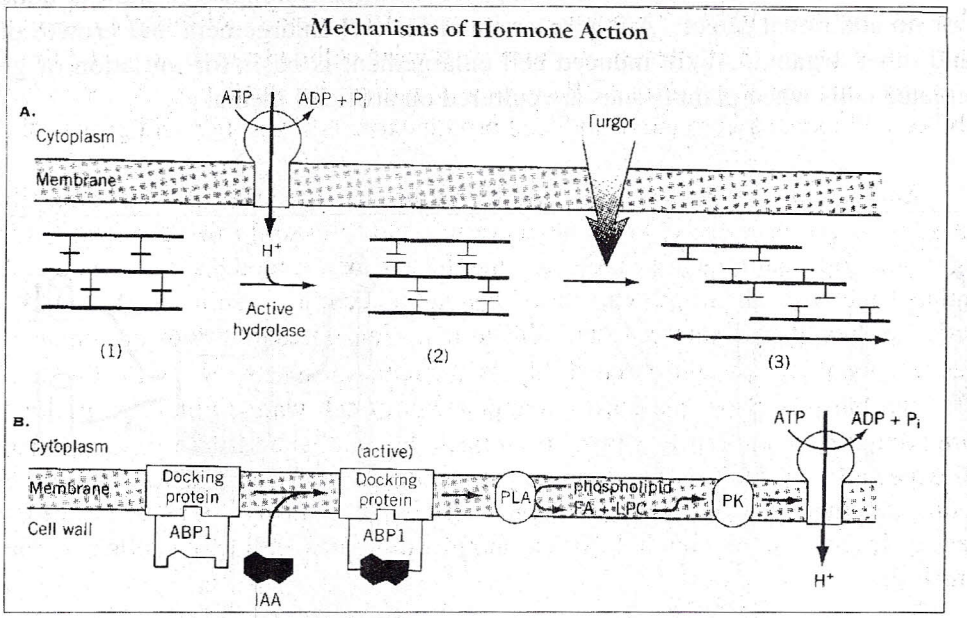


Fig. 15.6 The acid growth hypothesis for cell enlargement. (A) Cell wall polymers are extensively cross-linked with load-bearing bonds (1), which limits the capacity of the cell to expand. An ATPase proton pump located in the plasma membrane acidifies the cell wall space by pumping protons from the cytoplasm. The lower pH activates wall-loosening enzymes that cleave the load-bearing bonds (2). The force of turgor acting on the membrane and cell wall cause the polymers to auxin with activation of the ATPase proton pump. See text for details. Abbreviations: ABP1, auxin binding protein 1; PLA, phospholipase A_2; FA, fatty acids; LPC, lysophospholipid; PK, protein kinase. (B based on Macdonald, 1997).

fatty acids, stimulate proton secretion and elongation. These effects are inhibited by vanadate, which blocks the plasma membrane proton-ATPase. These data suggest that PLA_2 follows ABP1 in the chain and that lipids, both LPC and fatty acids, function as second messengers. Both the IAA and LPC effects on proton secretion and elongation can be blocked by protein kinase inhibitors, suggesting that the lipids activate the proton-ATPase by a phosphorylation-dependent mechanism.

15.2.8 Physiological action of Auxin

(a) Cell growth and differentiation: Auxin regulated cell elongation in **Avene** coleoptiles was the basis for its discovery. Auxin concentration-response curves show that response will be increased with increased concentrations of auxin till an optimum concentration is reached (Fig. 15.7). Concentration exceeding the optimum results in reduced growth. Growth responses often said to assay for unknown hormone concentrations, a technique known as bioassay. Intact stems and coleoptiles do not show a significant response to exogenous auxin application. High

endogenous auxin content of intact tissues support maximum elongation and the added auxin has little or no additional effect. Auxin is essential for cell enlargement and growth of leaves, flowers and other organs. Auxin-induced cell enlargement is basis for initiation of growth of undifferentiated cells when plant tissues are cultured on artificial media.

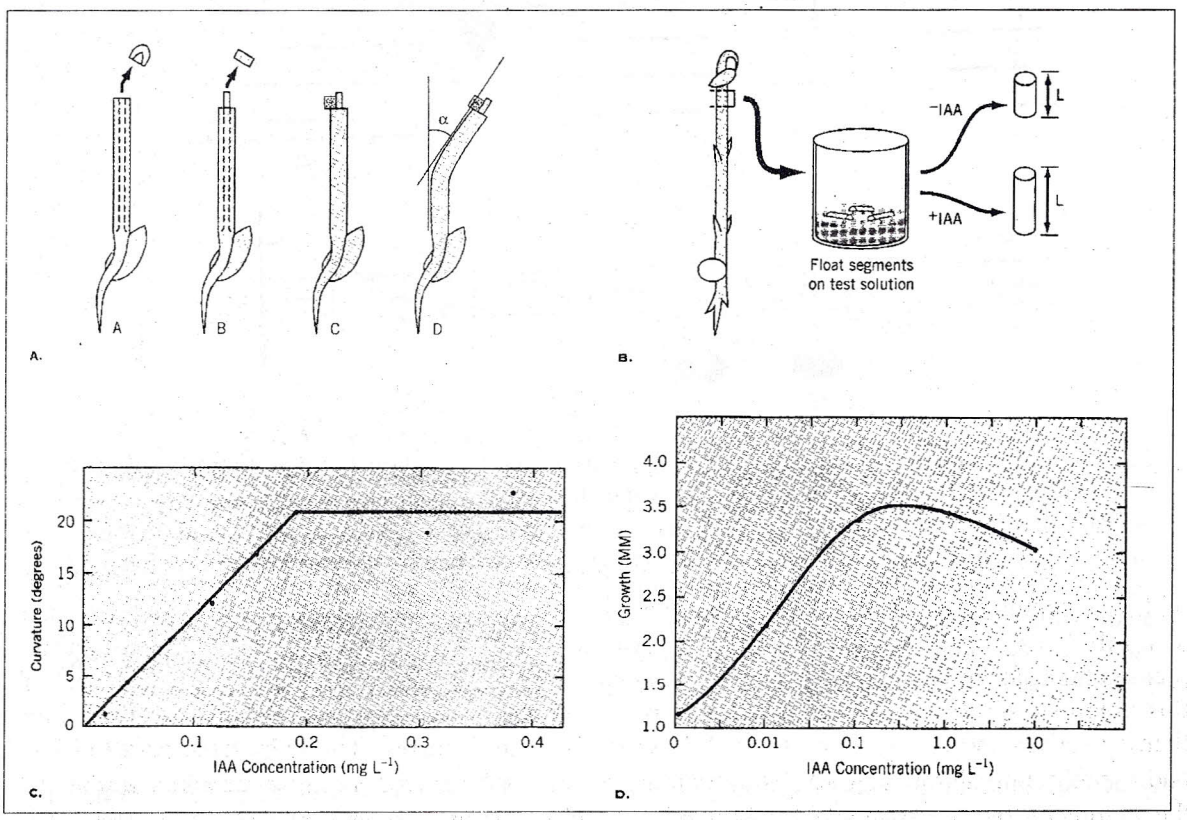


Fig. 15.7 Concentration response curves for two classic auxin responses

Auxins also induce vascular differentiation in young and rapidly developing leaves. W.P. Jacobs and coworkers found that regeneration of vascular tissues around wounds in *Coleus* (Lamiaceae) is also under the control of auxin. The extent of vascular regeneration is directly proportional to the auxin supply when exogenous auxin is substituted for the leaves, as leaves are the sources of auxin.

Auxin is also required for vascular differentiation in plant tissue culture. When buds (source of auxin), are implanted into clumps of undifferentiated callus tissue in culture, differentiation of callus parenchyma into vascular tissue occurs in regions adjacent to the implant. The same effect is achieved when agar wedges containing IAA and sugars are substituted for the implanted bud.

Shoot and root development: There are two types of buds, axillary buds and apical buds. In many plants, mitosis and cell expansion in the axillary bud is arrested at an early stage and the bud fails to grow. The removal of shoot apex stimulates the axillary bud to resume growth (Fig. 15.8). The apical but is able to exert a dominant influence that suppresses cell division and enlargement in the axillary bud. The phenomenon of coordinated bud development is known as **apical dominance**.

A B C

Fig. 15.8 Apical dominance in broadbean (*Vicia faba*).
A. Control plant, B. Removal of stem apex promotes axillary bud growth,
C. Dominance can be resotred by applying auxin

How does auxin from the shoot apex suppress axillary bud development? The most widely accepted theory is that the optimum auxin concentration for axillary bud growth is much lower than it is for the elongation of stems. Auxin flows out of the shoot apex to the base of the plant is thought to maintain an inhibitory concentration of auxin at the axillary bud. Removal of this auxin supply by decapitation reduces the supply of auxin in the region of the axillary and thereby relives the bud of inhibition. Inhibitors of auxin transport triiodobenzoic acid (TIBA) and naphthylphthalamic acid (NPA), stimulate release of buds from dominance when applied to the stem between the shoot apex and the bud. Lines of tomato that exhibit prolific branching (in absence of apical dominance) also fail to export radioactively labelled IAA from the shoot apex.

It is now clear that, cytokinins will antagonize the auxin effect. Application of cytokinins either to stem apex or to the axillary bud will release the bud from inhibition.

Other experiments have shown that, there is a relation between the inhibition of bud growth and Abscisic acid (ABA) content of the bud. It appears that the ABA content in the axillary bud is under control of IAA moving down from the shoot apex. Application of ABA to the shoot apex also releases axillary buds from inhibition. Ethylene production, stimulated by auxins, has also been implicated in axillary bud inhibition, but there is no conclusive evidences. The nature of other hormonal interaction with auxin is complex and has not been clearly defined.

Leaf Abscission: The process of shedding organs is known as abscission. Abscission occurs as a result of the development of a special layer of cells called the abscission layer. As the organ ages, the cell walls in the abscission layer weaken and separate. Abscission appears to be dependent on the concentrations of auxin on either side of the abscission layer. Auxin content is high in young and rapidly growing portions of a plant and declines as the organ ages and approaches senescence. This can be demonstrated by excising a leaf blade while leaving the petiole attached to the stem. If auxin is applied to the cut end, distal to the abscission layer, abscission of petiole will be delayed when compared with the controls.

Root elongation and development: Root elongation is sensitive to auxin. At low concentrations (10^{-8} M or less) IAA will promote the growth of excised root sections and intact roots. Higher concentrations (10^{-5} to 10^{-6} M) cause inhibition of root growth. This indicates that high auxin concentration stimulates production of ethylene. Removal of root tip or application of auxin antagonists promotes the growth of roots. Auxin inhibits root elongation, but high auxin concentrations will promote initiation of secondary roots. Removal of young leaves and buds (sources of auxin), reduce the number of secondary roots formed. Auxins also promote adventitious root formation on stems (Fig. 15.9).

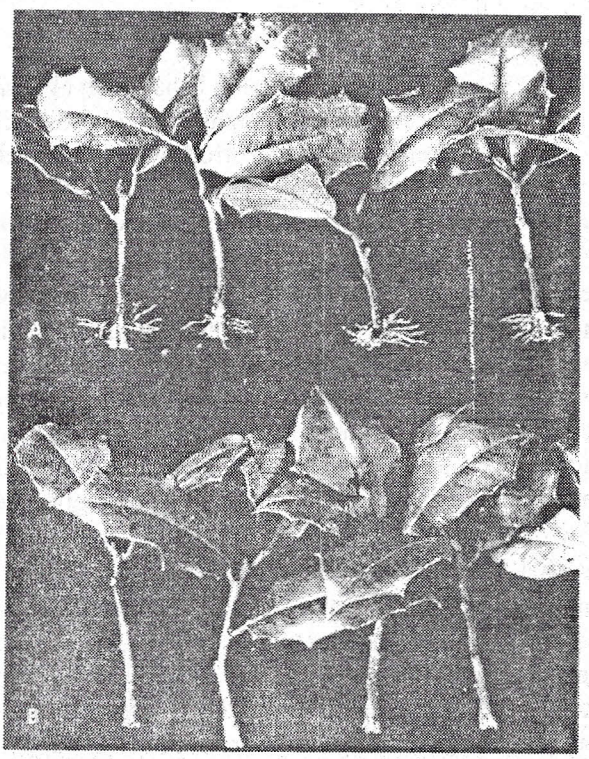


Fig. 15.9 Auxin stimulated adventitious root development
A. Treated with Indole Butyric acid
B. Untreated controls

Flower and fruit development: Auxin does not seem to play a major role in the initiation of flowers, since exogenous auxin tends to inhibit flower formation. But members of the family Bromeliaceae exhibit a strong stimulation of the flowering response following application of either auxin or ethylene.

The sex of imperfect flowers (monoecious or dioecious) is genetically determined. In the cucurbits the flower is bisexual in its early stages but one sex organ or the other aborts. Application of auxin during the bisexual stage ensure the formation of female flowers.

Fruit set, i.e., the initiation of ovary development, requires successful pollination and fertilization. During the mid-1930s, it was found that pollen was a rich source of auxin. The synthetic auxin, 4-chlorophenoxy-acetic acid is used to stimulate fruit set, when cool night temperatures would tend to reduce fruit set. Auxin induces parthenocarpy. J.P. Nitsch in the 1950s indicated that the developing seed was the source of auxin for continuing fruit development. Nitsch found that removal of seeds prevented further development of the fruit, but supplying the fruit with auxin restored normal development.

Auxins may also be used to control abscission of fruits. Auxins may cause early fruit drop or prevent premature fruit drop. This causes an increase in the size of those remaining on the tree, which terms as thinning of fruits. Later application of auxin delays abscission thus preventing premature fruit drop.

15.3 GIBBERELLINS

Gibberellins are produced both by fungi and higher plants. The exogenous application of gibberellins causes hyperelongation of intact stems. Gibberellins are also involved in seed germination and mobilization of endosperm reserves during early embryo growth and also flower and fruit development.

Gibberellins are the only hormones that can be defined on the basis of their chemical structure than the biological activity. The chemical family of this hormone is based on the *ent* **gibberellane** structure (Fig. 15.10). Based on the number of carbon atoms in the structure, gibberellins are known as C_{20}-gibberellins and C_{19}-gibberellins. The naturally occurring gibberellins have been chemically characterized are assigned as "A" number. GA_3, a C_{20} gibberellin also known as **gibberellic acid** (GA) was the first isolated and characterized gibberellin from fungal cultures. GA_1 and GA_{20} are C_{19}-GAs, the most important gibberellins in higher plants. A carboxyl group at carbon-7 in all GAs is required for biological activity. C_{19}-GAs are more biologically active than C_{20}-GAs. Those GAs with 3-β-hydroxylation, 3-β, 13-dihydroxylation or 1,2-unsaturation are more active and those with both 3-β-OH and 1,2-unsaturation exhibit the highest activity.

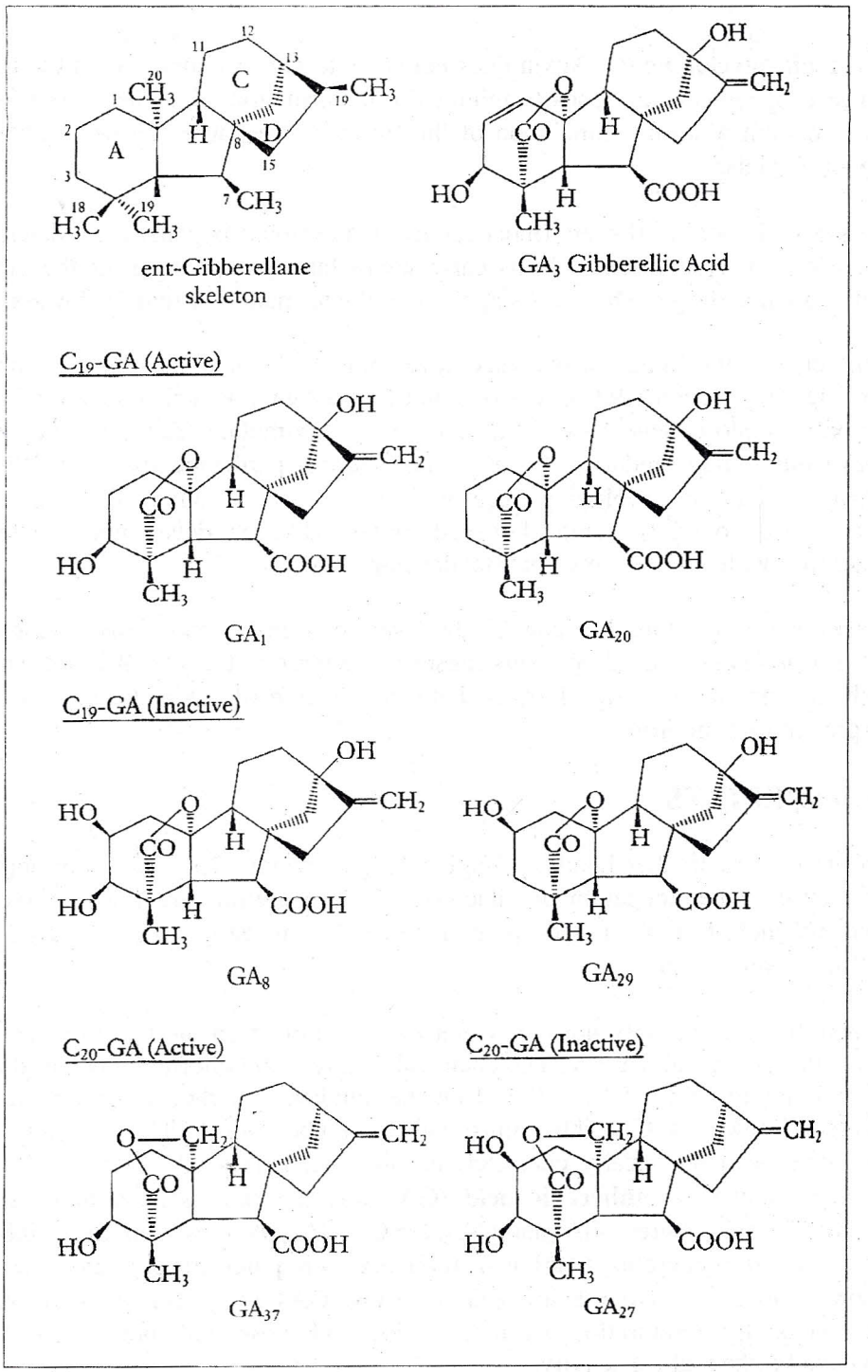


Fig. 15.10 The *ent*-gibberellane skeleton and chemical structures of selected active and inactive gibberellins

15.3.2 Gibberellin biosynthesis and metabolism

Developing seeds, fruits, the young leaves of developing apical buds and elongating shoots and the apical regions of roots are the sites of gibberellin biosynthesis. Immature seeds and fruits are prominent sites of gibberellin biosynthesis. As the seeds mature, metabolism appears to shift in favor of gibberellin-sugar conjugates.

Gibberellin biosynthesis: Gibberellins are diterpenoid acids related to naturally occurring terpenoids. Gibberellin synthesis may be considered in three stages. The first stage is the mevalonic acid pathway, which leads to other terpenoids. In this pathway, the 5-carbon (C_5) isoprenoid unit isopentenyl pyrophosphate (IPP) is synthesized from acetyl coenzyme A and used to build up the C_{20} geranylgeranyl-pyrophosphate (GGPP).

The second stage in the synthesis of gibberellins is the biosynthesis of ***ent***-kaurene from GGPP and its conversion to GA_{12}-7-aldehyde. GA_{12}-7-aldehyde is the precursor for all other known gibberellins. The third stage is the biosynthesis of all gibberellins from GA_{12}-7-aldehyde (Fig. 15.11).

The first two steps in the synthesis of gibberellins from GGPP involve the cyclization of GGPP first to copalylpyrophosphate, then to kaurene. Flowing cyclization, the carbon at 19 position on kaurene undergoes three successive oxidations in the sequence $CH_3 \rightarrow CH_2OH \rightarrow CHO \rightarrow COOH$ to form kaurenoic acid. The two cyclization steps and the oxidation kaurene to kaurenoic acid are inhibited by antigibberellin dwarfing agents like Amo-1618, CCC, phophon-D and ancymidol. The final two steps involve a hydroxylation of carbon-7 and contraction of the ring with extrusion of carbon-7 to form GA_{12}-7-aldehyde. Oxidation of aldehyde group on carbon-7 to carboxyl group gives GA_{12}. The C_{19}-GAs arise by subsequent oxidative elimination of carbon-20. Interconversions among GAs in pea seed and seedlings is presented in (Fig. 15.12). The 13-hydroxylation pathway (bold arrows), leading to GA_{20} and GA_1, is probably of widespread occurrence in higher plants.

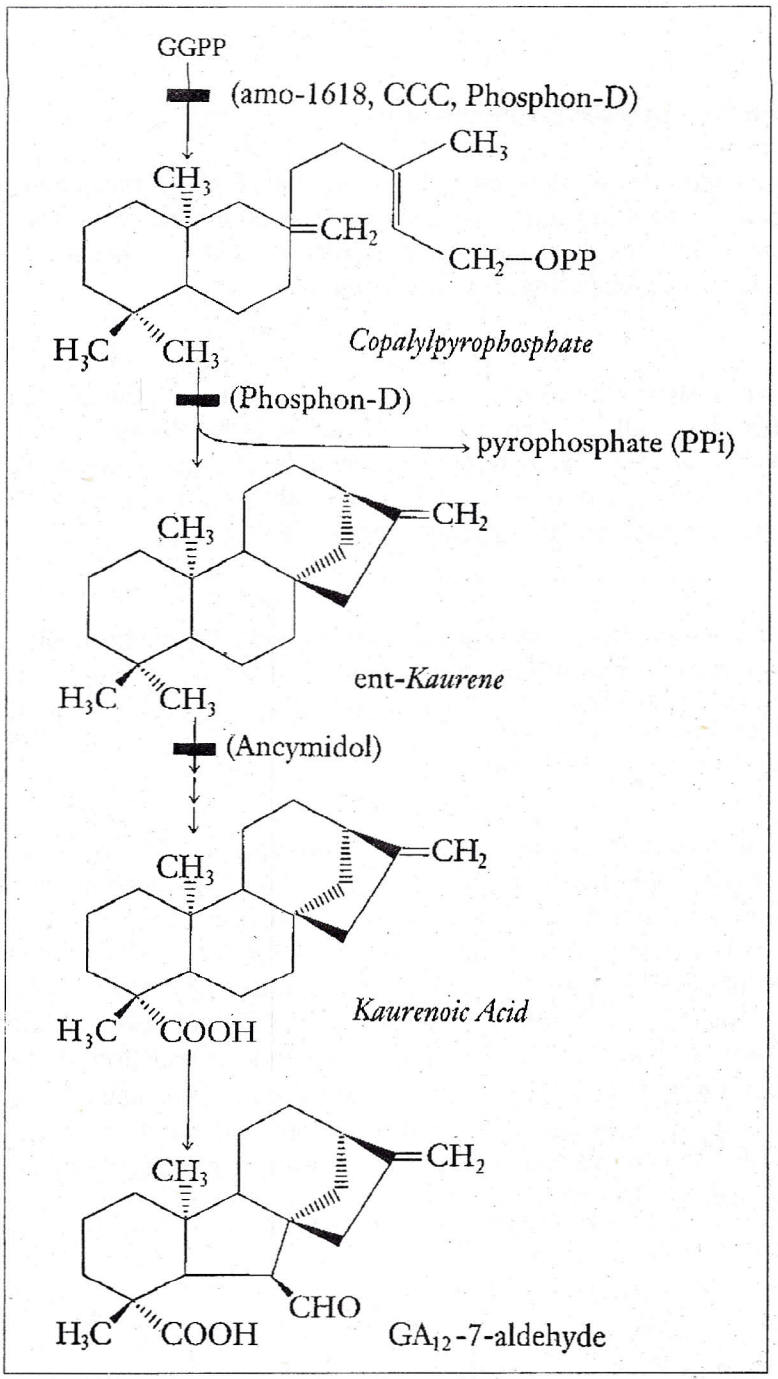


Fig. 15.11 Gibberellin biosynthesis

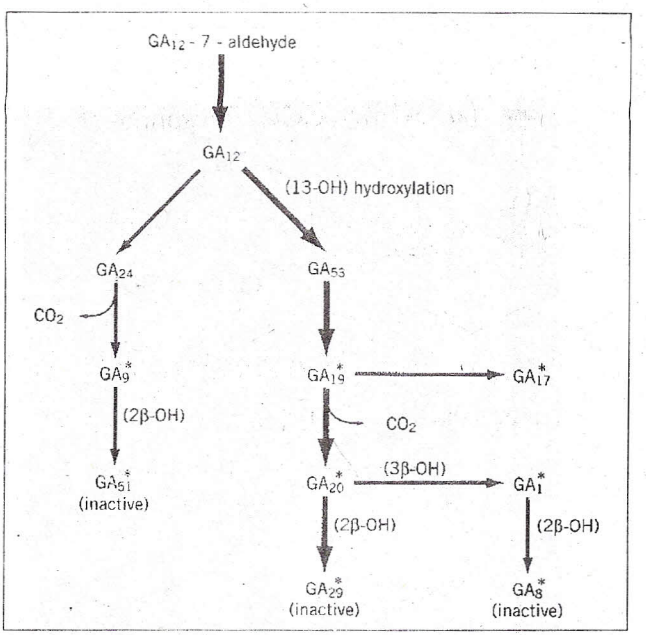


Fig. 15.12 Proposed pathways for gibberellin biosynthesis in pea

In immature, actively developing seeds, the principal free gibberellins are GA_1, GA_8. Small amounts of GA_4, GA_5, GA_6 (C_{19}GAs) and GA_{37} and GA_{38} (C_{20} GAs) are also found. Mature seeds contain GA_8-glucoside and small amounts of GA_1, GA_4, GA_{37} and GA_{38} glucosyl esters.

Gibberellins have been detected in both the phloem and xylem saps, studied by conducting the application of radioactively labelled GAs to stem or coleoptile sections. Transport is not to be polar but moves along with other phloem-translocated organic materials according to a source-sink relationship. Gibberellins synthesized in the root tips are distributed to the aerial portions of the plant through the xylem stream. It is not known that gibberellins are transported as free hormones or in the conjugated form.

15.3.3 Mechanism of Gibberellin action

1) **Gibberellin control of stem elongation:** Gibberellins act to stimulate both cell division and cell elongation in stems. In rosette plants, the rapid elongation is accompanied by cell divisions in the region just below the apical meristem and hyperelongation of daughter cells following gibberellin application.

   The gibberellin response in lettuce hypocatyls, is a reversal of blue and far-red light inhibition. Dwarf pea and cucumber stems are inhibited by red light (Fig. 15.13). This inhibition is also reversed by gibberellin.

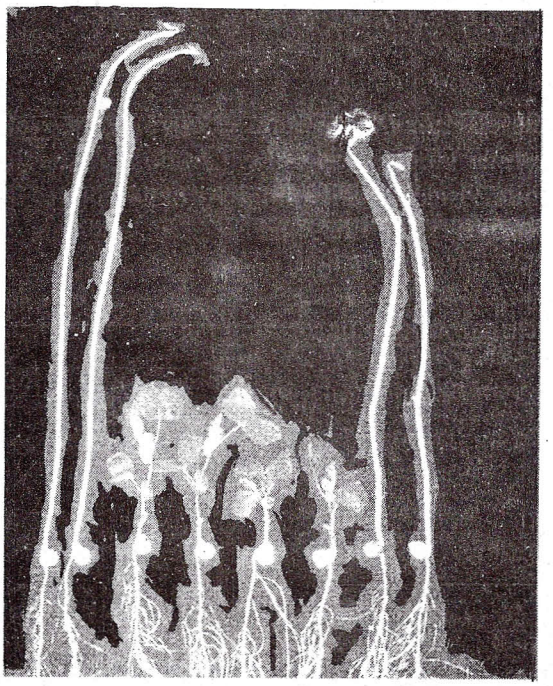


Fig. 15.13 Dwarf pea and cucumber stems are inhibited by GA

2) **GA control seed germination:** The α-amylase secreted from barley aleurone consists of multiple isozymes that fall into two major families characterized by their isoelectric points (P^I). The low group has pIs in the range of 4.5 to 4.85 and the high group has pIs in the range of 5.9 to 6.3. Within each group, the isozymes are quite similar but between the two groups there are major differences with respect to calcium requirement for secretion and sensitivity to EDTA and heavy metals. The two groups of isozymes are translated *in vitro* from two different mRNA populations encoded by two multigene families located on different chromosomes.

In addition to α-amylase, proteolytic enzymes (proteases), β-amylase and other starch degrading enzymes are involved in mobilizing the endosperm reserves (Fig. 15.14). GA-stimulated α-amylase synthesis is inhibited by inhibitors of transcription and that GA induces significant changes in RNA metabolism, especially mRNA. If GA acts to regulate gene expression, it clearly must regulate a large number of genes from several different families spread through out the genome.

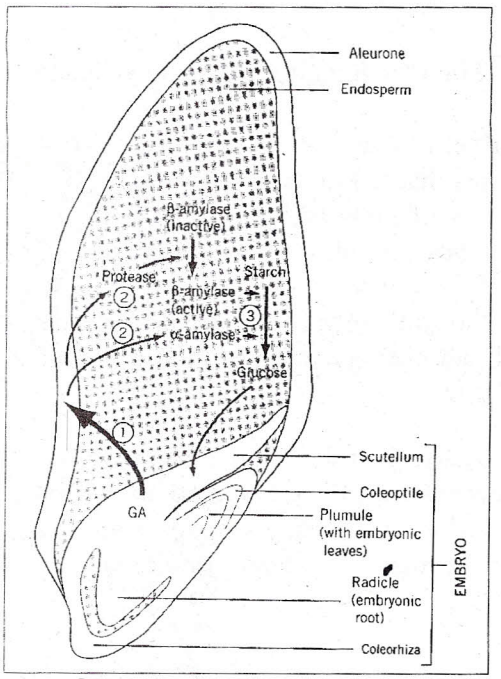


Fig. 15.14 Gibberellin induced release of enzymes and carbohydrate mobilization during germination.

Does GA regulate transcription of α-amylase mRNA? Based on evidence from several lines of investigation, it is clear that gibberellin dose regulate transcription of α-amylase mRNA. Both *in vivo* pulse labeling of protein and *in vitro* translation of protein from total aleurone RNA, followed by electrophoretic and autoradiographic analysis, show significant increase in the amount of α-amylase translated following the application of gibberellin (Fig. 15.15). Following gibberellin treatment α-amylase mRNA may comprise 20% of the total translatable mRNA. Finally, the rate of α-amylase synthesis following gibberellin treatment closely correlates with the rate of mRNA accumulation.

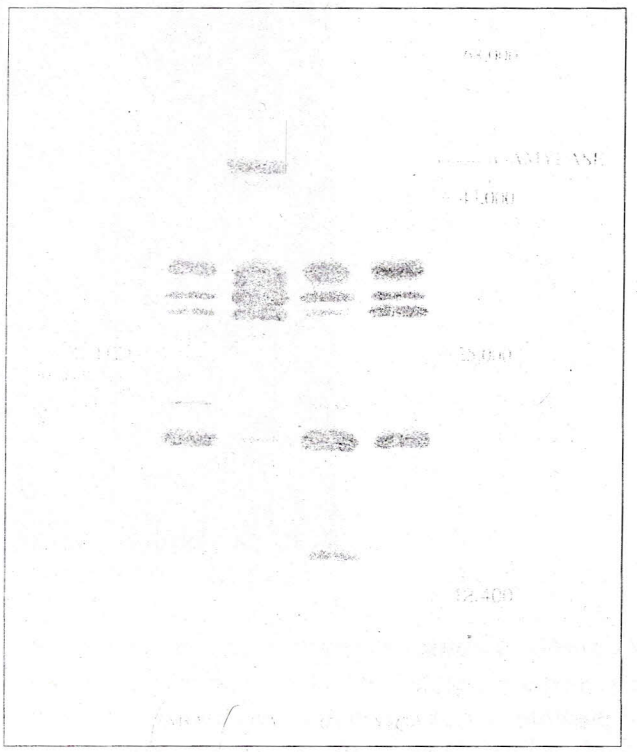


Fig.15.15 Hormonal control of α-analyse biosynthesis by barley alemon layers

15.3.4 The Physiological action of Gibberellins

1) Control of shoot elongation – Dwarf plants: It was excessive stem elongation in infected rice plants that led to the discovery of gibberellins and hyperelongation of stem tissue is one of the effects of gibberellins on higher plants. The relationship between dwarfing or internode length genes and gibberellins was pioneered by the work of B.O. Phinney on maize (*Zea mays*) and P.W. Brain and coworkers on garden pea (*Pisum sativum*). Application of exogenous gibberellin to the dwarf mutant of rice, bean, *Arabidopsis thaliana* and several others restore a normal, tall phenotypes (Fig. 15.16). Exogenous gibberellins have no effect on the genetically normal plant.

There are five mutants in maize, d_1, d_2, d_3, d_5 and an_1, exhibit the normal phenotype when treated with GA_3, but show no response to other hormones or growth regulators. While studies with dwarf plants have been instrumental in linking gibberellins. As with stem elongation, there are other dwarf mutants known that do not respond to application of gibberellin.

Fig. 15.16 The effect of GA on dwarf pea

2) Rosette Plants: A rosette is an extreme case of dwarfism. In this case, there is an absence of internodal elongation which is characterized by closely spaced leaves. The failure of internodal elongation may be genetic mutation or environmentally induced. Hyperelongation in rosette plants is brought about by the application of small amounts of gibberellins (Fig. 15.17).

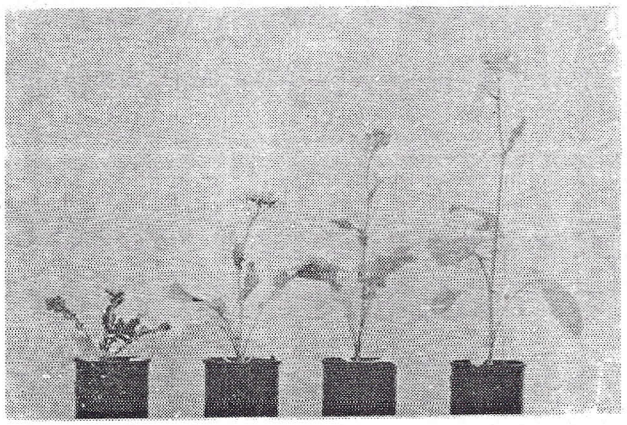


Fig. 15.17 Gibberellin – stimulated stem growth in a rosette genotype of *Brassica napus*

Environmentally limited rosette plants (Spinach, Cabbage) do not flower in the rosette form. Just before flowering these plants will undergo extensive internode elongation, known as bolting. Bolting is triggered either by photoperiodism or a combination of low temperature and photoperiod. Rosette plants can be induced to bolt by an exogenous application of GA.

Spinach contains six gibberellins, including GA_{19} and GA_{20} will cause bolting in spinach under short day conditions while GA_{19} is biologically inactive. J.A.D. Zeevaart and coworkers found that rosette plants of spinach contain high levels of the inactive form GA_{19} and low levels of the active GA_{20}. Upon transfer to long day conditions, the level of GA_{19} declined while the level of GA_{20} increased (Fig. 15.18). It may be concluded that GAs have a significant role in the control of stem elongation in rosette plants.

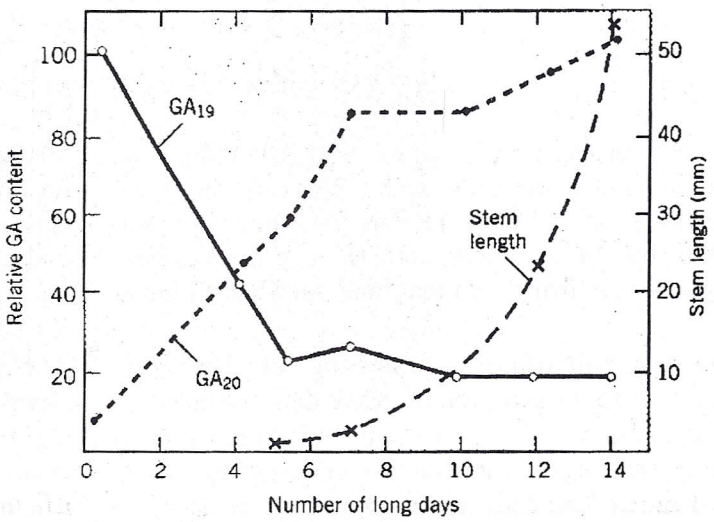


Fig. 15.18 Changes in gibberellin content accompanied by the transfer of spinach (*Spinacea oleraceae*) plants from short to long days exhibit extensive stem elongation.

The relationship between gibberellins and stem elongation in cold- requiring plants has not been studied as thoroughly as it has for photoperiodically sensitive plants. Exogenous application of GA_3 will substitute for the cold requirement in many plants and there is some evidence that gibberellin-like activity increases in plants following cold treatment.

Inhibition of stem growth: The growth of many stems can be reduced or inhibited by synthetic chemicals that block GA biosynthesis. These growth retardents or antigibberellins mimic the dwarfing genes by blocking specific steps in GA biosynthesis, thus reducing endogenous GA levels and suppressing internode elongation (Fig. 15.19).

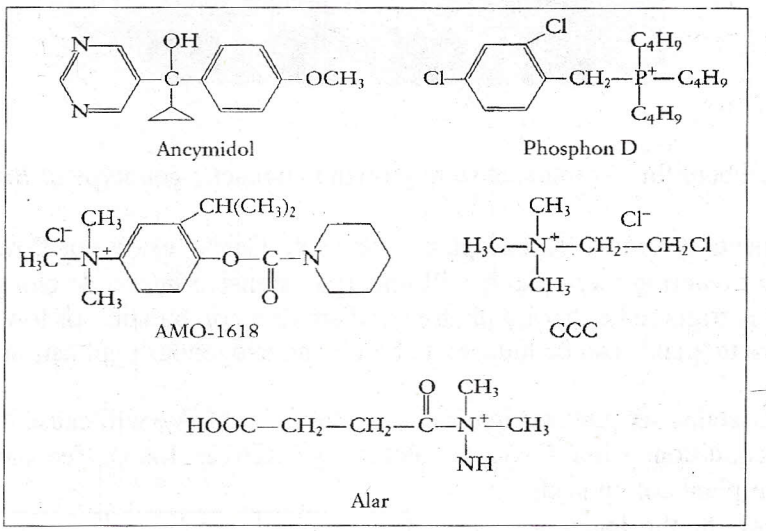


Fig. 15.19 Chemical structures of growth retardents

In some areas, wheat tends to "lodge" near harvest time, that is, it becomes top-heavy within grain and falls over. Spraying the plants with growth retardents produces a shorter, stiffer stem and thus prevents lodging. Growth retardents also need for pruning of vegetation. Alar has been widely used as a spray on cherries and apple. It enhances fruit colour and produces a firmer fruit that facilitates harvesting.

Seed germination: Germinating cereal grains secrete α-amylase and proteases which digest carbohydrates and protein. Cereal grains produce two half-seeds, with one half-seed containing the embryo and one without. The embryo-containing half-seed will proceed to secrete α-amylase, digest the starchy endosperm and germinate. The embryoless half-seed cannot germinate and does not produce elevated levels of α-amylase or any other hydrolytic enzymes required for germination. GA treatment will stimulate the embryoless half-seed to produce high levels of α-amylase (Fig. 15.20). GA stimulated α-amylase secretion can be blocked by inhibitors such as actinomycin D and cycloheximide, which inhibit RNA and protein synthesis. This indicates that gibberellin-stimulated *de novo* synthesis of α-amylase by the aleurone layer

is an early event in germination. seeds grow at high temperature, produce high levels of α-amylase in the absence of added GA.

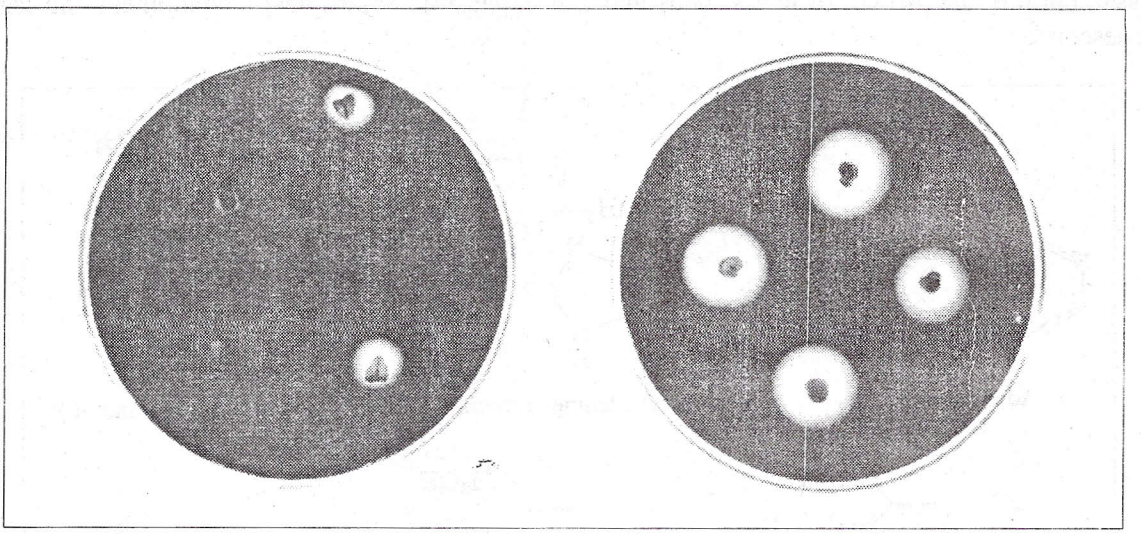


Fig. 15.20 Gibberellin – stimulated release of α-amylase from barley half seeds

Flowering: In the normal course of development, stem elongation appears to be a necessary prelude to flowering. Substitution of gibberellin for the long day or cold requirement seemed to indicate a role for gibberellin in the flowering process itself. Inhibitors of GA biosynthesis will suppress photoperiodic – induced stem elongation without interfering with flowering. It appears that stem elongation and flowering are separate. Though gibberellins do not play a role in flowering, they do influence the capacity of plants to flower as well as sexual characteristics of flowers and fruit development.

Many perennial plants must achieve a minimum stage of development before they are capable of flowering. Such plants are said to pass through a **juvenile phase**. The length of the juvenile phase can range from a few weeks to many years. In cases like *Cucumis* and *Cannabis sativa*, where auxins or ethylene promote femaleness in imperfect flowers, an application of gibberellins will promote formation of male flowers.

15.4 CYTOKININS

Cytokinins are N^6-substituted derivatives of the nitrogenous purine base ademine, characterized by their ability to stimulate cell diving in tissue culture. **Kinetin** (N_6-furfurylamino purine) was the first cytokinin to be discovered (Fig. 15.21). Kinetin does not occur naturally but was synthesized from herring sperm DNA.

The most wide spread naturally occurring cytokinin in higher plants is *Zeatin*. Zeaten is found with a ribose (the riboside) or ribose-phosphate (the ribotide) at the 9-position. In

addition to stimulate cell division, cytokinins also influence shoot and root differentiation in tissue culture, the growth of lateral buds and leaf expansion, chloroplast development and leaf senescence.

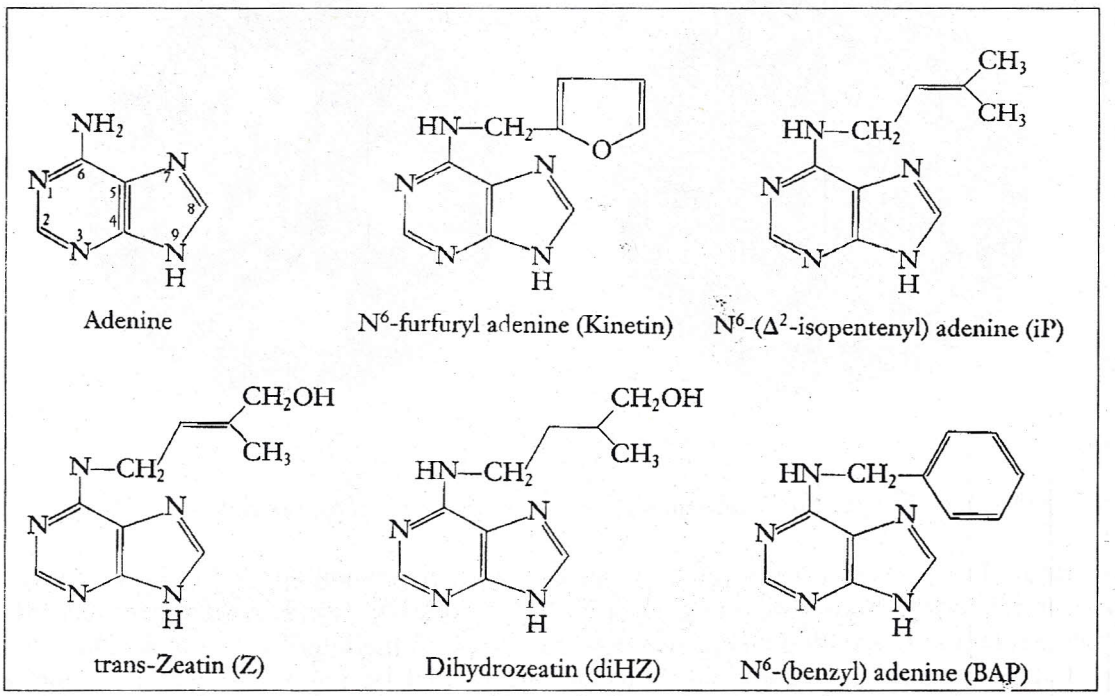


Fig. 15.21 The chemical structures of adenine and five adenine derivatives with cytokinin activity.

15.4.1 Cytokinin Biosynthesis and metabolism

A major site of cytokinin biosynthesis and high cytokinin levels in higher plants are the mitotically active root tip, in the xylem sap of root and in immature seeds and developing fruits.

15.4.2 Biosynthesis of cytokinins

Cytokinins are commonly found in the cell as modified bases in transfer RNA (tRNA) and methylated purines. These cytokinins are not incorporated during transcription of the tRNA, but are synthesized during post-transcriptional processing.

Enzymes direct *de novo* synthesis of cytokinins from adenosine-5' monophosphate have been isolated from the slime mould *Dictyostelium discoideum* tobacco callus tissue, and crown gall tissue (Fig. 15.22). This reaction is specific for the nucleotide; the enzyme will not add the

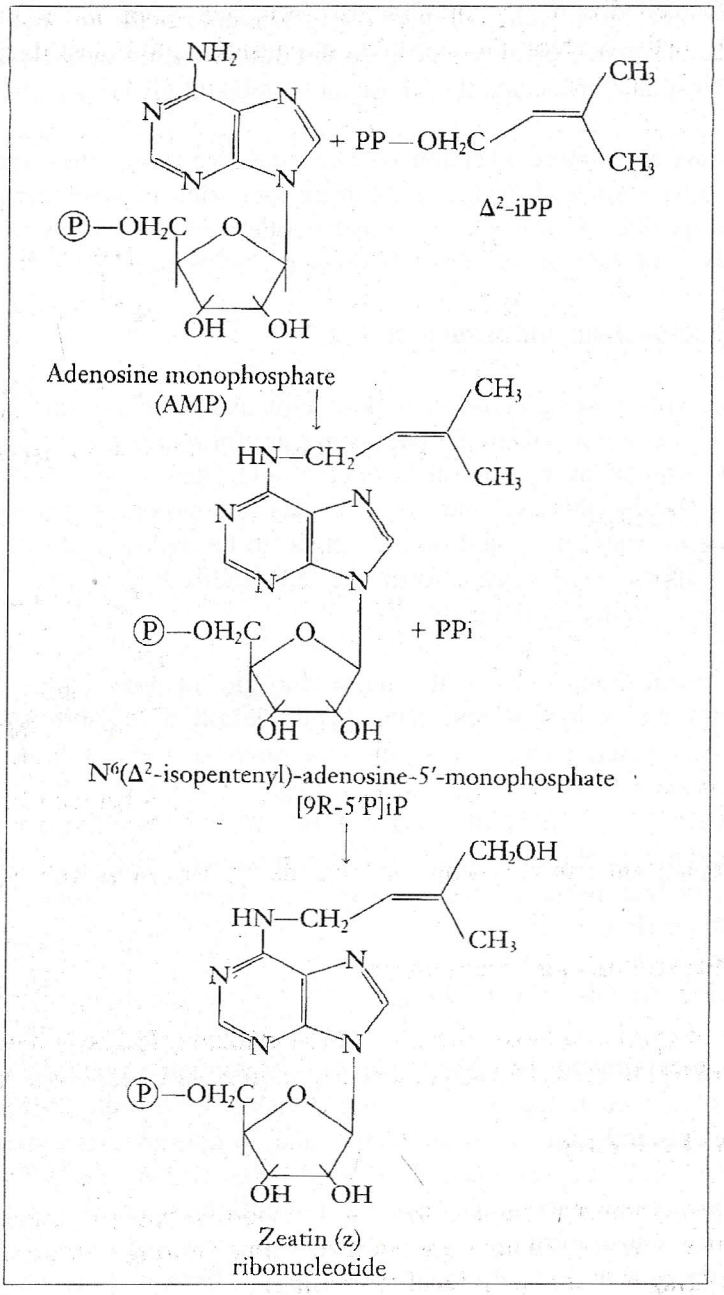


Fig. 15.22 The pathway for *de novo* cytokinin biosynthesis

isopentenyl group to either adenine or adenosine. The product, [9R-5'P] iP, is the precursor to all other naturally occurring cytokinins. Little [9R-5'P] iP accumulates in tissue and undergoes

a rapid hydroxylation of the side chain to give the comparable zeatin ribonucleotide. Reduction of the double bond in the side chain would give the dihydrozeatin derivative, while sequential hydrolysis of the phosphate group and the ribose moiety would give rise to zeatin.

Cytokinins undergo interconversion between the free base, ribosides and the ribotides when supplemented to tissues. Enzymes have been identified in wheatgerm that catalyze the conversion of iP to its riboside [(9R)iP] or to its ribotide ([9R-5'P] iP) as well as enzymes that catalyze the hydrolysis of the ribotides and ribosides to the free base (iP).

15.4.3 Cytokinin metabolism and transport

Conjugation with either glucose or amino acids and oxidation are two principle routes for regulating cytokinin activity. Glucose conjugates are formed at the nitrogen in position 7 or in 9 on the purine ring or as O-glucosides on the side chain (Fig. 15.23). The 7- and 9-glucosides are biologically inactive and O-glucosides are biologically very active. The N-glucosyl conjugates are very stable and do not appear to be hydrolyzed to give the active free base. O-glucosides appear to be storage forms that are readily hydrolyzed to yield biologically active cytokinins when needed by the plant.

Cytokinins form conjugates with the aminoacid alanine (Fig. 15.24). 9-Alanyl conjugates of zeatin and dihydrozeatin have been identified. These too are very stable conjugates that serve to inactivate the cytokinin in the same manner as N-glucosides.

A major route for removal of exogenously supplied cytokinins in many tissues is oxidation by the enzyme cytokinin oxidase. Cytokinin oxidase, partially purified from tobacco tissue, maize and crowngall tissue, cleaves the isopentenyl side chain from either Zeatin or iP or their ribosyl derivatives (Fig. 15.25).

15.4.4 The Physiological roles of cytokinins

Cell division and morphogenesis: Most mature, differentiated plant cells do not normally divide. Many cells may be induced to undergo division when cultured on artificial media containing vitamins mineral salts, a carbon source and an optimal concentration of hormones. On solid agar medium, cells derived from stem pith and cortex, cotyledon, leaf and other tissues will divide and enlarge to produce a mass of largely undifferentiated cells referred to as **Callus tissue**. Small lumps will also form in liquid culture on agitation and form a **cell free culture**. In both the cases, cell division and growth do not occur in the absence of cytokinin.

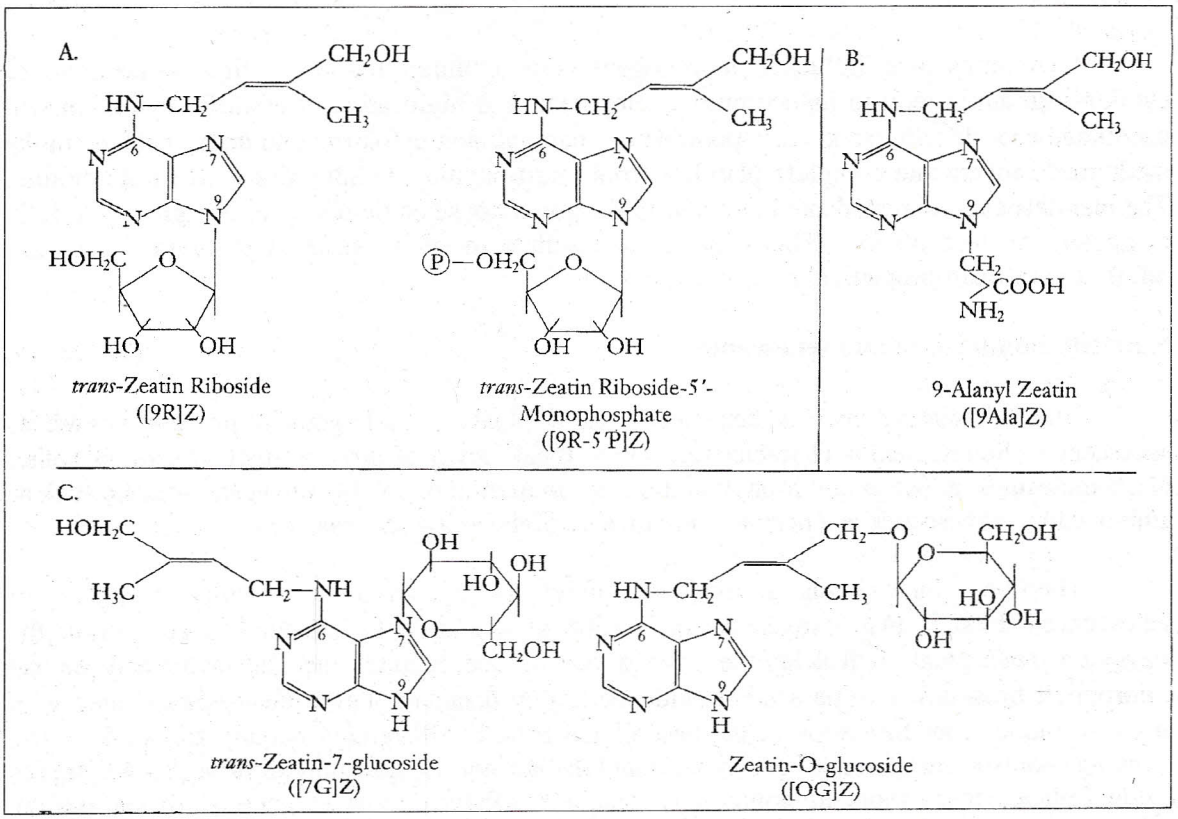


Fig. 15.23 Examples of Zeatin conjugates

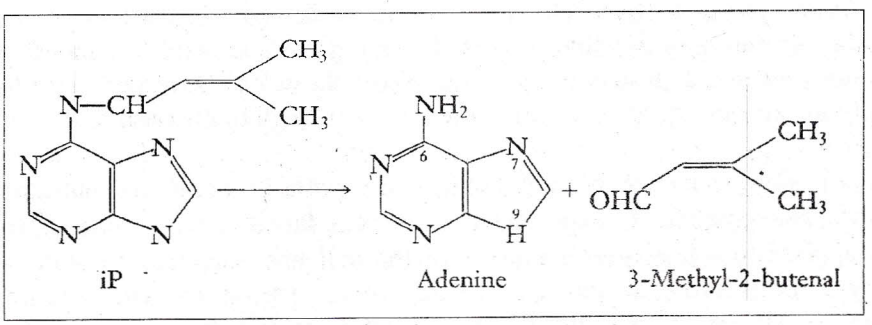


Fig. 15.24 Oxidative degradation of *iso*-pentenyl adenine (iP) by cytokinin oxidase

Cytokinins also influence morphogenesis in cultured tissues. High molar ratio of cytokinin to auxin tend to induce bud development, and high ratios of auxin to cytokinin will encourage root development. It is possible by manipulating cytokinin and auxin balance in the medium, to regenerate complete plantlets from undifferentiated callus tissue in sterile culture. The plantlets can be transplanted into soil in the green house or field where they grow into fully competent, mature plants. This capacity to regulate morphogenesis in cultured tissues has referred to as micropropagation.

Nutrient mobilization and senescence

When a mature leaf is detached from a plant, it undergoes a process known as senescence. Senescence is characterized by the break down of protein, nucleic acid and other macromolecules, a loss of chlorophyll and the accumulation of soluble nitrogen products such as amino acids. Senescence is a normal consequence of the aging process.

There are three kinds of evidences indicating a role for cytokinins in control of senescence. First is that, exogenous application of cytokinin to detached leaves and to the leaves on intact plants will delay the onset of senescence, maintain protein levels and prevent chlorophyll breakdown. The second evidence is that detached leaves have been treated with auxin to induce root formation at the base of the petiole will remain healthy for weeks. The growing root is a site of cytokinin synthesis and the hormone is transported through the xylem to the leaf blade. If the roots are continually removed as they form senescence of the leaf will be accelerated. When a mature plant begins its natural senescence, there is a sharp decrease in the level of cytokinins exported from the root. A third evidence comes from recent studies employing recombinant DNA techniques. Tobacco plants have been transformed with the *Agrobacterium* gene for cytokinin biosynthesis, designated *tmr*. The *tmr* gene encodes for the enzyme *iso-pentenyl-transferase*. The *tmr* gene was linked to a *heat shock promoter*. The heat shock promoter is active only when subjected to a high temperature treatment. By linking the *tmr* gene to the heat shock promoter, cytokinin biosynthesis can be turned on in the transformed plants simply by subjecting the plants to a brief period at high temperature.

There is some evidence that cytokinins exert a role in mobilizing nutrients (Fig. 15.25). In K. Mothes and coworkers' experiment, a nutrient labelled with radioactive carbon (^{14}C-glycine) is applied to a leaf after a portion of the leaf has been treated with cytokinin. The radioactivity is transported to and accumulated in the region of cytokinin treatment. It is unlikely that cytokinins act directly through stimulating protein synthesis since the mobilization of non-metabolites such as a-aminoisobutyric acid is directed by cytokinins equally well.

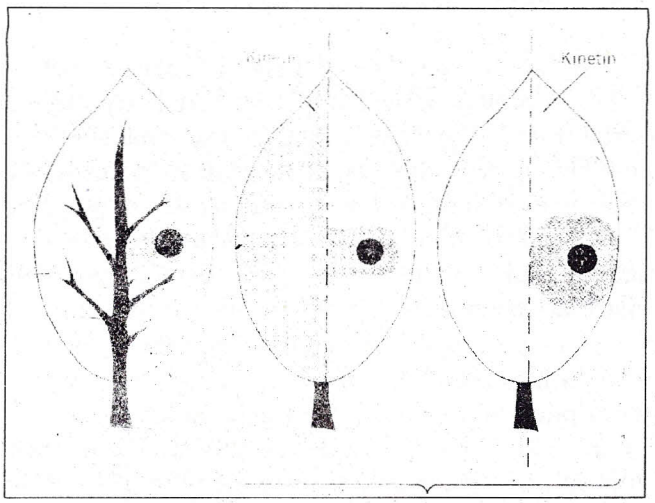


Fig. 15.25 Experiment demonstrating the role of cytokinin in nutrient mobilization

Other cytokinin effects: Cytokinin will stimulate cell enlargement in the cotyledons of cucumber and sunflower. The application of cytokinins will stimulate release of axillary buds from apical dominance, thus antagonizing the effect of auxins. This cytokinin – auxin antagonism is believed to account for the phenomenon of "Witch's broom", an example of extreme axillary bud release (Fig. 15.26). It is believed that the parasitism by fungi and bacteria stimulates an over production of cytokinin. The resulting release of apical dominance produces a dense mass of short branches.

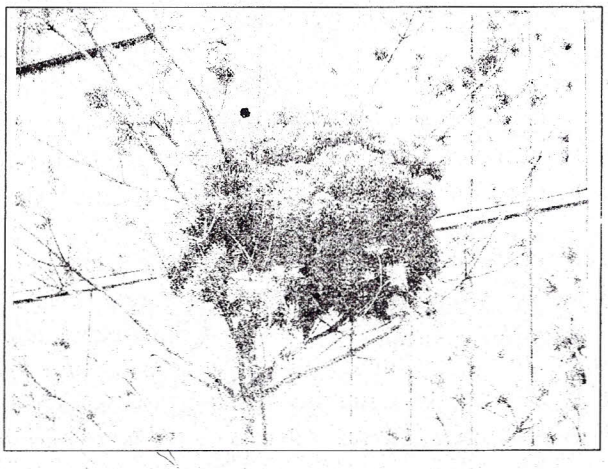


Fig. 15.26 Witch's broom on white pine (*Pinus strobus*)

15.4.5 Mechanism of cytokinin action

The action of cytokinin is poorly understood. There is some evidence that cytokinins have a role in regulating protein synthesis. In cultured soybean cells, cytokinins cause an increase in the overall rate of protein synthesis and change the pattern of proteins that incorporate ^{35}S-methionine. This activity is reflected in an increase in the polyribosome content of cultured cells following cytokinin treatment. An increase in polyribosomes might result from either an increase in the rate of transcription of mRNA or an increase in the stability of the mRNA. Either brief irradiation with low level red light or the addition of cytokinin to the medium will stimulate an increase in the abundance mRNA for both the small subunit of ribulose-1, 5-bis phosphate carboxylase-oxygenase and the principal chlorophyll a/b-binding polypeptide of the light-harvesting complex. E. Tobir and other colleagues were able to demonstrate that transcription of the mRNA is largely under control of red light while cytokinin seems to stimulate an increase in the abundance of mRNA. A reasonable interpretation of these results is that cytokinin acts post-transcriptionally to stabilize the mRNA.

15.5 ABSCISIC ACID

Unlike the previous three hormone classes, abscisic acid (ABA) is a single compound (Fig. 15.27). Two major areas of ABA action appear to be in the mobilization of reserves during seed development and germination and in the response to leaves to water stress. ABA is known to induce transport of photosynthate toward developing seeds and to promote the synthesis of storage protein. During germination, ABA antagonizes the promotory effect of gibberellin on α-amylase synthesis. Relatively large amounts of ABA are rapidly synthesized in the leaves in response to water stress, where it appears to play a major role in regulating stomatal opening and closing.

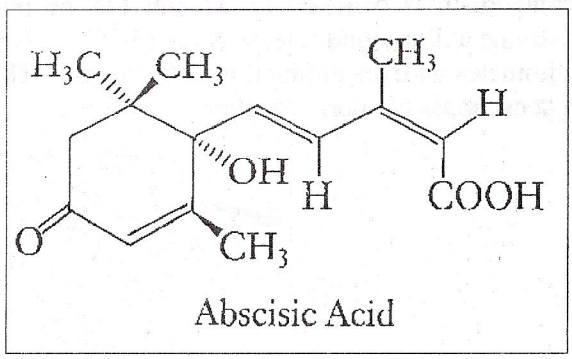


Fig. 15.27 The chemical structure of abscisic acid

15.5.1 Abscisic acid biosynthesis and metabolism

Abscisic acid is a 15-carbon isoprene derivative that appears to be synthesized by cleavage from a 40-carbon carotenoid precursor. ABA appears to occur in nature, green leaves and is synthesized in the chloroplasts. Stress induced ABA synthesis occurs in the chloroplasts and that the ABA migrates to other regions of the plant. Chloroplasts should probably be considered a major site of ABA synthesis.

ABA is a 15-carbon sesquiterpene and is also derived from mevalonic acid. Like other hormones ABA is present in very low concentrations, that is, 10 to 50 ngm/g fresh weight. Only

in water-stressed leaves, where the concentration may reach 400 ngm/g fresh weight, or in young developing seeds do ABA concentrations exceed these values.

There are two possible pathways for the ABA synthesis: (1) direct synthesis from a 15-carbon precursor, or (2) cleavage of a 40-carbon xanthophyll (Fig. 15.28). The intermediate for direct synthesis of ABA is the 15-carbon farnesylpyrophosphate.

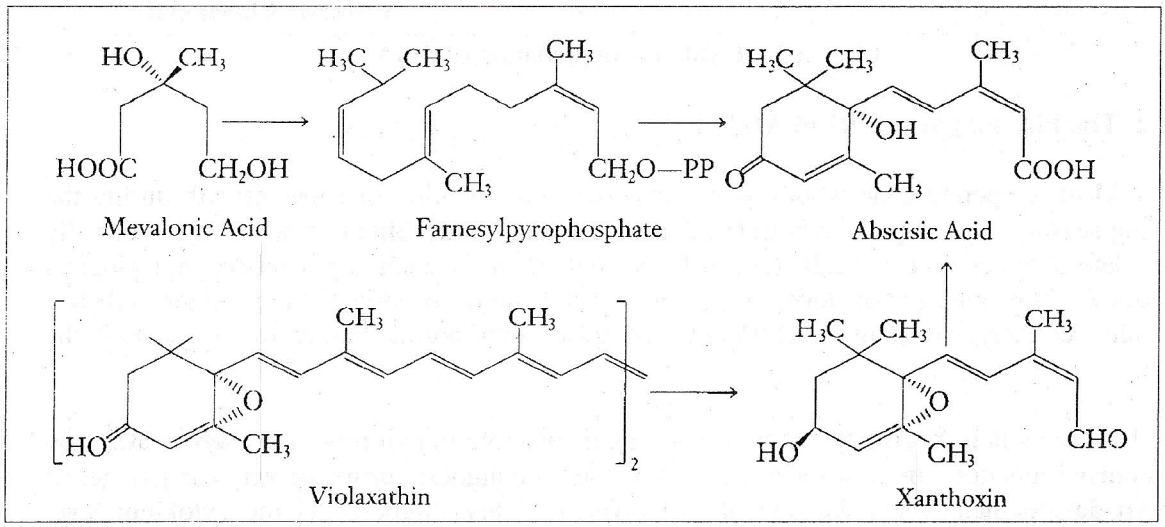


Fig. 15.28 Pathways for the biosynthesis of abscisic acid

First, the carbon skeleton of ABA and the position of the oxygen- containing substituents is very similar to that of violaxanthin. Second, it is known that violaxanthin can be degraded in the light *in vitro* to a 15-carbon derivative, xanthoxin, a natural constituent of plants. Third, a stoichiometric relationship between losses of violaxanthin and increases in ABA in stressed etiolated bean leaves.

ABA contains an asymmetric carbon atom. An asymmetric carbon atom gives rise to two different forms called enantiomers. There are two enantiomeric forms of ABA, designated R-ABA and S-ABA. ABA is rapidly metabolized when it is applied exogenously to plant tissues. A glucose ester of ABA has been found in low concentration in a variety of plants, but the principal metabolic route seems to be oxidation to phaseic acid (PA) and next reduction of the ketone group on the ring to form dihydrophaseic acid (DPA) (Fig. 15.29). At least some tissues appear to carry the metabolism further to form the 4'-glucoside of DPA. DPA and its glucoside are both metabolically inactive. PA, while inactive, is equally effective as a GA_3 antagonist in barley aleurone-α-amylase system.

Like gibberellins, ABA is found in both xylem and phloem fluids as well as in parenchyma cells outside the vascular tissue, and there is no evidence for a polarity in transport.

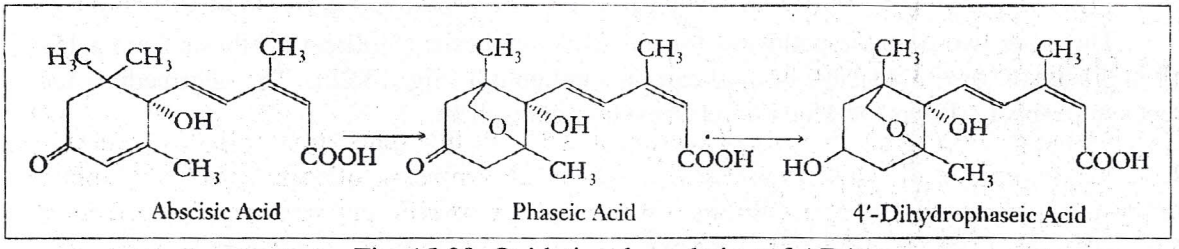


Fig. 15.29 Oxidative degradation of ABA

15.5.2 The Physiological roles of ABA

Most temperate zone woody plants experience a cessation of shoot growth during the growing season. This applies to both terminal and axillary buds. Shoot growth ceases normally in the late summer or early fall, terminal and axillary buds enter a period of physiological dormancy. The intensity of dormancy reaches a maximum in early to mid winter and then gradually declines, in response to chilling temperatures, until normal bud regrowth occurs in the spring.

It seems that ABA plays an important regulatory role in two processes, seed maturation and stomatal function. In most seeds, cytokinin levels are highest during the very early stages of embryo development when the rate of cell division is also highest. As the cytokinin level declines and the seed enters a period of rapid growth, both GA and IAA levels increase. In the early stages of embryogenesis, there is little or no detectable ABA. In the later stages of embryo development, as GA and IAA levels begin to decline, that ABA levels begin to rise. ABA levels peak during the maturation stage when seed volume and dry weight also reach a maximum. Maturation of the embryo is characterized by cessation of seed growth, accumulation of nutrient reserves and the development of tolerance to desiccation.

ABA serves to prevent **vivipary**, a precocious germination before the embryo reaches maturity or the seed is released from the fruit. Precocious germination will occur when the ABA concentration is reduced to 3 to 4 µg/g fresh weight of seed. A number of viviparous mutants in *Arabidopsis* and corn have reduced level of ABA. Vivipary can also be induced in corn by treatment of the developing ear at the appropriate time with fluridone, a chemical inhibitor of carotenoids. Carotenoids and ABA share early biosynthetic steps, fluridone inhibits ABA biosynthesis. Fluridone-induced vivipary can be alleviated by application of exogenous ABA. All of these results establish a strong connection between ABA and seed maturation and/or prevention of precocious germination.

In leaves of plants that have been grown to ensure minimum endogenous levels of ABA, exogenous ABA at concentrations of 10^{-3} to 10^{-4} M will induce complete stomatal closure. This appears to be a means for regulating water balance in the plant since the endogenous level of

ABA in leaves is generally very low if the plants are well watered. Subjecting leaves to water deficit, will induce as much as forty fold increase in the ABA level within as little as 30 minutes.

15.5.3 Mechanism of ABA action

On the basis of *in vivo* labeling experiments and cell-free translation of barley aleurone mRNA it is clear that ABA suppresses GA-induced synthesis of α-amylase and other hydrolases. It also promotes the synthesis of several ABA-specific polypeptide. The effect of ABA can be overcome by providing an excess of GA. ABA controls α-amylase at more than one level. Several studies have shown that ABA operates at the transcriptional level to suppress accumulation of GA-induced α-amylase mRNA, but an ABA-induced inhibitor of α-amylase activity has also been identified in mature starchy endosperm. Thus, it appears that ABA can prevent germination not only by suppressing transcription of α-amylase but also by inhibiting the activity of any enzyme that might be present in the endosperm.

15.6 ETHYLENE

Ethylene is a simple gaseous hydrocarbon with the chemical structure: $H_2C = CH_2$. Ethylene is not required for normal vegetative growth, although it can have a significant impact on the development of roots and shoots.

Ethylene is synthesized in response to stress and produced in large amounts by tissues undergoing senescence or ripening. Ethylene commonly used to enhance ripening in bananas and other fruits that are picked green for shipment. Ethylene is frequently produced when high concentrations of auxins are supplied to plant tissues. Many of the inhibitory responses to exogenously applied auxin appear to be due to auxin-stimulated ethylene release than auxin itself.

15.6.1 Ethylene biosynthesis and metabolism

Ethylene occurs in roots, stems, leaves, bulbs, tubers, fruits, seeds and so on. Ethylene production will vary from tissue to tissue within the organ, but is located in peripheral tissues.

M. Lieberman and L.W. Mapson (1964) first showed that methionine was converted to ethylene in a cell-free, non-enzymatic model system. These confirmed that plant tissues such as apple fruit converted L-[^{14}C]-methionine to [^{14}C]-ethylene and ethylene was derived from the third and fourth carbons of methionine. In 1977, when D. Adams and F. Yang demonstrated that S-adenosyl methionine (SAM) was an intermediate in the conversion of methionine to ethylene by apple tissue. In 1979, they further demonstrated the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC), a non-protein amino acid, in apple tissue fed L-[^{13}C]-methionine under anaerobic conditions.

There are three steps involved in ethylene biosynthesis (Fig.15.30). In the first step, an adenosine group is donated to methionine in the presence of methionine adenosyl transferase by a molecule of ATP, thus forming SAM. In the presence of ACC synthase, SAM is cleared to yield 5'-methylthio-adenosine (MTA) and ACC. The Ethylene-forming enzyme catalyzes the conversion of ACC to ethylene. This enzyme system has been studied in cells, protoplasts and intact vacuoles.

Ethylene production is promoted by IAA, wounding, and water stress. Induction of ACC synthase is blocked by inhibitors of both protein and RNA synthesis. This suggests that induction occurs at the transcriptional level. In *E. coli* carrying the cloned ACC synthase gene, the physical abundance of ACC synthase mRNA also increases in response to IAA and wounding. Control of ethylene production appears to be exercised through transcriptional regulation of the ACC synthase gene.

15.6.2 The physiological roles of Ethylene

Ethylene is known to effect plant growth and development. As a byproduct of hydrocarbon consumption, ethylene is also a common environmental pollutant that can play havoc with greenhouse cultures or laboratory experiments. The gas chromatograph has made possible quantitative analysis of ethylene at extremely low concentrations that could not otherwise be measured. Etheption (2-chloroethyl phosphonic acid) is a compound that, readily decomposes to produce ethylene, at physiological pH.

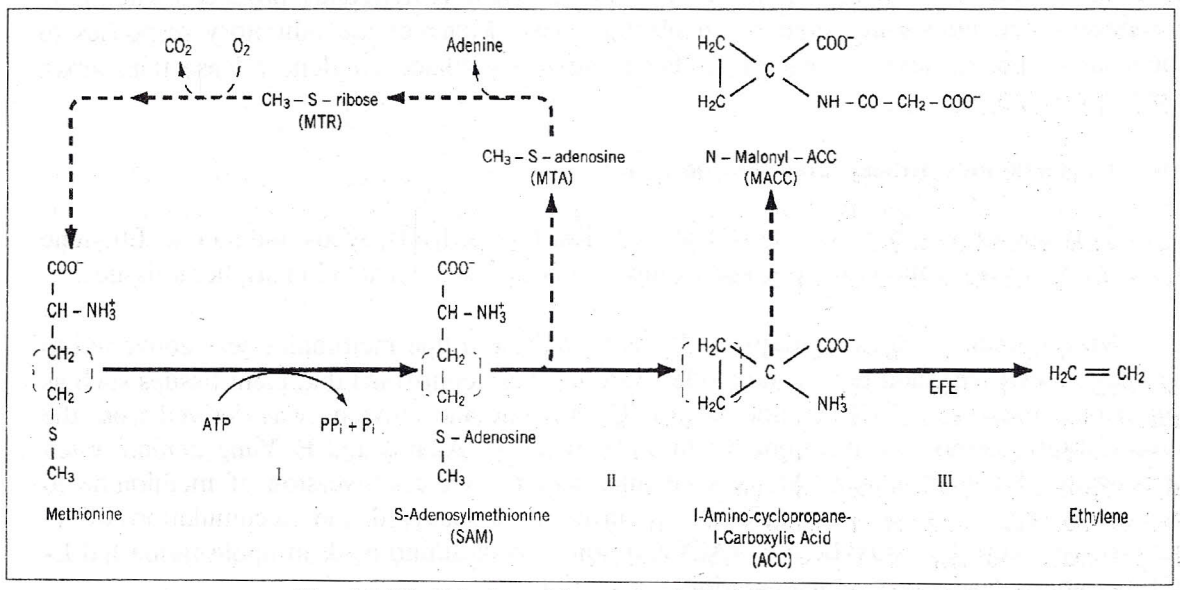


Fig. 15.30 Ethylene biosynthesis in higher plants

Vegetative development: Ethylene has been shown to stimulate elongation of stems, petioles, roots and floral structures of aquatic and semiaquatic plants. The effect is particularly noted in aquatic plants because submergence reduces gas dispersion and maintains higher internal ethylene levels. Ethylene promotes gibberellin synthesis in rice and the elongation effect can be blocked with antigibberellins, which suggests that gibberellin mediates the ethylene effect. Ethylene stimulates abnormal growth responses such as swelling of stem tissues and the downward curvature of leaves; promotion of seed germination, inhibition of bud break, reduced apical dominance and root initiation.

Fruit development: A variety of fruits release ethylene gas during the latter stages of the ripening process. The release is coincidence with a sharp rise in the respiratory rate measured by CO_2 evolution. Ethylene is autocatalytic, i.e., ethylene released by ripening fruits will in turn stimulate premature climacteric and ethylene production by other fruits stored nearby. As a consequence of the climacteric and ethylene production, a number of qualitative metabolic changes such as hydrolysis of starches to sugars, softening of the tissue through the action of cell wall degrading enzymes and synthesis of pigments and flavor components. The non-climacteric fruits such as citrus, will show enhanced ripening when exposed to ethylene gas. Ethylene stimulated ripening is of considerable economic importance. For long-term storage, apples are placed under conditions designed to minimize the production and accumulation of ethylene. These include low temperature and high ambient CO_2 concentration to suppress respiration or continuous exchange of air to prevent a build up of ethylene.

Flowering: Ethylene normally suppresses or delays flowering. Ethylene stimulates flowering in the family Bromeliaceae, eg., pineapple. This is known to be due to auxin-stimulated ethylene generation. Commercial growers now use ethephon and other ethylene releasing agents to stimulate uniform flowering in pineapple fields.

15.7 POLYAMINES

The term polyamine refers to a group of polyvalent compounds containing two or more amine groups. They were first observed as crystals in human semen (hence, spermine) by van Leeuwenhock, with the help of his primitive microscope, more than 300 years ago. Polyamines began to attract the attention of plant physiologists in the early 1970s.

Polyamines are derived biosynthetically from the amino acids arginine and lysine. In addition, spermidine and spermine biosynthesis involves S-adenosyl methionine, an intermediate in ethylne biosynthesis. In plant cells, polyamines occur conjugated with phenolic compounds such as hydroxy-cinnamic acid, coumaric acid or caffeic acid. At normal intracellular pH, polyamines are polycationic, that is, they carry multiple positive charges. Polyamines thus bind readily to nucleic acids, which are polyanionic and the phospholipids of the plasma membrane. It is possible that this binding character could effect the synthesis and/or activity of macromolecules and membrane permeability.

Thermophilic bacteria produce polyamines as a means of protecting against thermal inactivation of enzymes and spermine stabilizes DNA against thermal denaturation *in vitro*. Polyamines have been shown to be obligate growth factors for both prokaryote and eukaryote microorganisms and mammalian cells and stabilize oat protoplasts, influence cell division and embryogenesis in carrot tissue culture and delay senescence in some tissues. Whether polyamines can or should qualify as hormones may be an unprofitable effects indicate a role in plants that clearly warrants further study.

Hypothetical plant hormones: Studies on flowering clearly indicate the transmission of a diffusible chemical signal from leaf to the apex. The existence of a flowering hormone, called florigen and the hormone vernalin have been postulated to account for the effect of low temperature on the flowering behaviour of winter cereals and biennials. "Florigen" might also be a complex interaction between several regulatory molecules, rather than a single hormone.

Other biologically active substances: The active substances, which are a complex mixture of lipids, known as brassins or brassinosteroids, have been isolated from pollen of the rape plant, *Brassica napus* L. This stimulated elongation of bean second internodes. One brassinosteroid is brassinolide (Fig. 15.31). Like auxins, brassinolide is active in micromolar concentrations and stimulates elongation of hypocotyl and epicotyl tissue from a variety of legume seedlings and coleoptide tissue from wheat.

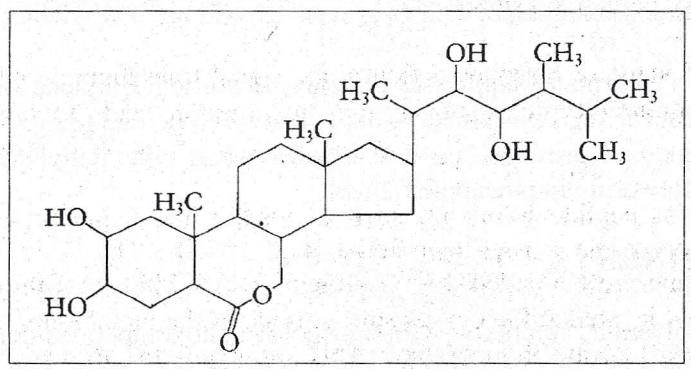


Fig. 15.31 Brassinosteroid

It operates synergistically with auxin to enhance the natural growth-promoting activity of that hormone. Two other biologically active compounds of interest are Coumarin and *trans*-cinnamic acid. Coumarin is known to inhibit auxin-induced elongation of *Avena* coleoptiles and other tissues *in vitro*, at low concentrations elongation may be promoted. Coumarin inhibits seed germination. Germination may proceed only after Coumarin levels in seed coat have been reduced by leaching. *Trans*-cinnamic acid also inhibits auxin activity in stem section assays and has been considered an antiauxin. The possibility that many of these biologically active substances may be used to increase yields has attracted attention in the fields of agriculture and horticulture.

15.8 BIOCHEMISTRY AND MODE OF ACTION OF HORMONES

The most challenging question about hormones is how to hormones bring about profound effects on the physiology of cell? Despite years of research into hormone action and the effects of hormones on plants, our understanding of hormone action at the molecular level is only just beginning. Experiments are thus designed to test whether those same principles might also apply to higher plants.

The sequence of events initiated by hormones can generally be resolved into three sequential stages: (1) the initial signal perception, (2) a signal transduction pathway, and (3) the final response. 1) Signal perception involves the reaction of the hormone with a receptor site. Plant hormones may diffuse from cell to cell either through plasmodesmata or through the apoplastic space. In either event, the cell destined to respond to the hormone, known as the target cell, must be capable of detecting the presence of the hormone molecule either in the cell or in the fluids immediately surrounding the cell. Detection is accomplished by interactions between the hormone and a cellular receptor that is both specific to the hormone molecule and characteristic of the target cell. Receptors are glycoproteins that bind reversibly with the hormone. As a result of binding the hormone, the receptor is induced to change its conformation and assumes an "activated" state. The formation of this active hormone-receptor complex completes the signal perception stage.

The second stage of hormone action is the signal transduction and amplification stage. The activated hormone-receptor complex sets into motion leads to the final, characteristic response.

Receptors for peptide hormones such as insulin and epinephrine are located on the extracellular surface of the plasma membrane (Fig. 15.32). The hormone receptor complex activates a membrane protein called the "G protein" which binds to a third membrane protein, adenylate cyclases – located at the cytoplasmic surface of the membrane. Binding of G protein to adenylate cyclase activate the enzyme. This stimulates the formation of cyclic adnosine monophosphate (cAMP) in the cytoplasm. The G protein may interact with an ion channel that controls the flow of calcium into the cell. The calcium will bind with one of a number of cytosolic calcium binding proteins, such as *Calmodulin*. The effect of either cAMP or the Ca^{+2}–calmodulin complex is to activate specific *protein kinases*.

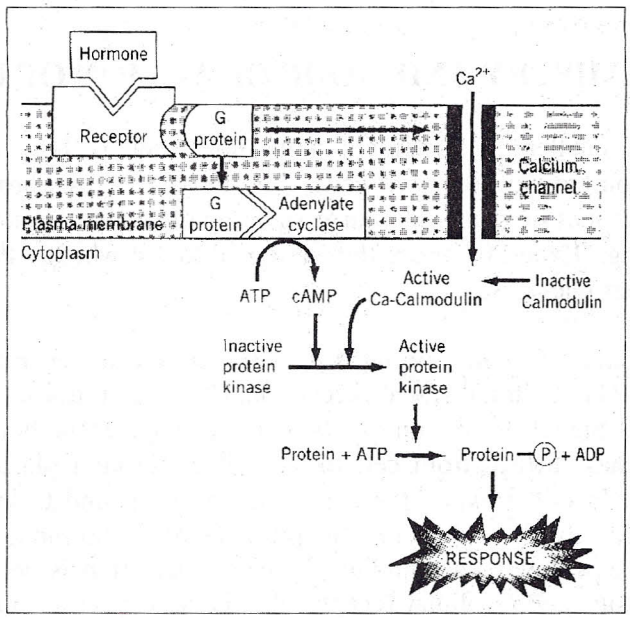


Fig. 15.32 A model for hormone action involving a plasma membrane-bound receptor

In this stage, the hormone is considered as first messenger, because it brings the original "message" to the cell surface. cAMP and calcium serve as second messengers. The function of second messenger is to relay information from the plasma membrane to the biochemical machinery inside the cell. Second messenger also provides for amplification of the original signal.

Steroid hormone receptors are located in the nucleus (Fig. 15.33). The hormone-receptor complex forms in the nucleus where it interacts with specific segments of DNA to either trigger or suppress transcription of mRNA.

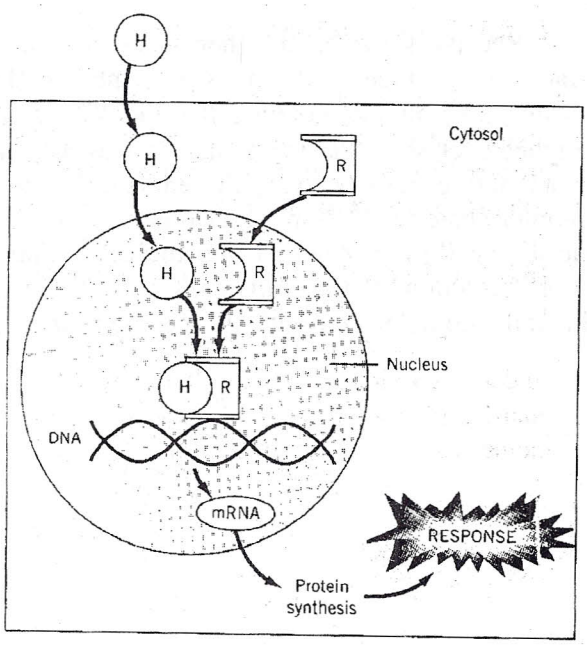


Fig. 15.33 A model for hormone action involving a nuclear hormone receptor

15.8.1 Hormone-binding proteins in plants

Proteins bind readily to small molecules in a nonspecific manner, especially when tissues are disrupted during isolation protocols. There are four accepted criteria to distinguish between nonspecific binding and hormone-binding properties.

First, binding must be specific. Second, the receptor should exhibit a high affinity for the hormone. Third, receptors can be saturated by increasing the concentration of hormone molecules. Fourth, the hormone must bind reversibly with the putative receptor.

15.8.2 Auxin binding protein

Auxin binding proteins (ABP) have been sought in two tissues, callus cultures of pith tissue (tobacco) and coleoptiles (maize). Three classes of IAA-binding proteins have been identified. Two are associated with membrane fractions (Plasma membrane) and one is found distributed between the cytoplasmic and nuclear fractions. One of the membrane-bound binding proteins has a low affinity for auxin but a high affinity for naphthylphthalamic acid (NPA), an auxin transport inhibitor. The second membrane-bound receptor has a moderate binding affinity for IAA but does not bind NPA.

The cytoplasmic-nuclear binding protein has a high affinity for IAA. Its location and high affinity for IAA suggest that the cytoplasmic – nuclear binding protein is capable of detecting low intracellular concentration of auxin. Libbenga and Mennes (1987) suggest that the cytoplasmic-nuclear auxin-binding proteins are receptors functioning similar to the steroid hormone receptors in animals.

15.8.3 Cytokinin-binding proteins

The most extensively characterized cytokinin-binding protein is CBF-1 protein (cytokinin binding factor). CBF-1 appears to be loosely associated with ribosomes since it is prepared by washing the ribosomal fraction with salt. This suggests that the CBF-1 cytokinin complex might have a role in regulating the protein translation process.

15.8.4 Gibberellin and Abscisic acid-binding proteins

There are no confirmed reports of high-affinity binding proteins for gibberellin, abscisic acid and ethylene. However, there is strong evidence for a high-affinity ABA-binding site on guard cell protoplasts. These sites are proteins located on the apoplastic surface of the plasma membrane.

15.8.5 Second messengers in plants

The most promising second messengers in plants are calcium and the phosphoinositides.

Calcium: Calcium controls many physiological processes in plants, including cell elongation and division, protoplasmic streaming, the secretion and activity of various enzymes, hormone action and tactic and tropic responses. Plants also contain several calcium-binding proteins. Among them calmodulin appears to be the dominant type.

For calcium to function effectively as a second messenger, the cytosolic Ca^{2+} concentration must be low and under metabolic control. Large amounts of calcium are stored in the Endoplasmic reticulum, the mitochondria and the large central vacuole. But the cytosolic Ca^{2+} concentration is kept low through the action of membrane-bound, calcium dependent ATPases. Activity of ATPase, the cytoplasmic Ca^{2+} concentration is under control of light and hormones (Fig. 15.34). In the cytosol, Ca^{2+} reacts with and forms a complex, $CaM.Ca^{2+}$. This serves to activate some enzymes. NAD kinases and protein kinases are stimulated by $CaM.Ca^{2+}$. NAD kinase catalyzes the phosphorylation of NAD to NADP in the presence of ATP. Similarly, many other enzymes are activated by protein kinase-catalyzed phosphorylation.

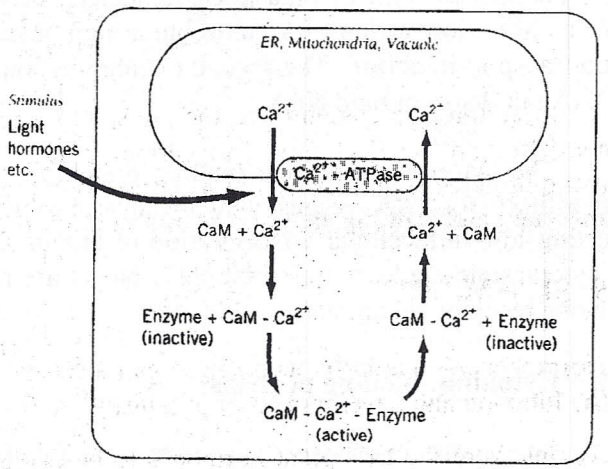


Fig. 15.34 Calcium as a second messager

Phosphoinositides: Another second messenger in plants is the inositol triphosphate system. In this system, the hormone receptor-complex activates a plasma membrane enzyme known as phospholipase C (Fig. 15.35). The hormone receptor complex may act through a G protein. Phospholipase C catalyzes the break down of phosphotidylinositol bisphosphate (PIP_2), a membrane phospholipid, to inositol triphosphate (IP_3) and diacylglycerol (DAG). Both IP_3 and DAG may function as second messengers. IP_3 moves into the cytoplasm where it stimulates the release of calcium from the vacuole. Note that IP_3 functions as a second messenger to mobilize yet another second messenger, calcium. At the same time, DAG activates a particular protein kinase called protein kinase C. Hydrolysis of PIP_2 by phospholipase C, an increase in IP_3 and DAG, transient increases in cytosolic calcium and activation of protein kinase C.

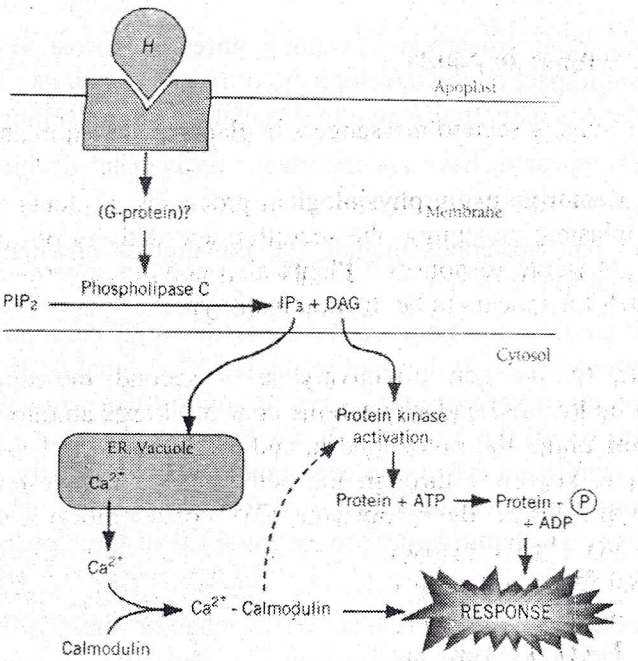


Fig. 15.35. A model for the inositol triphosphate system.

In addition to calcium and the phosphoinositides, there are other second messengers in plants. They are acetylcholine and certain lipids. The study of secondary messengers and hormone-initiated signal-transduction pathways in plant cells is in its infancy, but promises to be an important and exciting area of research in future.

15.9 SUMMARY

⇒ Hormones are naturally occurring organic substances. At low concentrations they effect on the function and development of organisms.

⇒ Auxins were the first plant hormones to be discovered. These are synthesized in the stem and root apices and transported through the plant axis. They are characterized by their capacity to stimulate cell elongation in excised stem and coleoptile sections, root initiation, vascular differentiation, tropic responses and the development of axillary buds, flowers and fruits.

⇒ Gibberellins are produced by both fungi and higher plants. The exogenous application of gibberellin causes hyperelongation of intact stems, involved in seed germination and mobilization of endosperm reserves during early embryo growth and flower and fruit development.

⇒ Cytokinins are N^6-substituted derivatives of the nitrogenous purine base adenine, characterized by their ability to stimulate cell division in tissue culture.

⇒ Abscisic acid is a terpenoid derivative involved in regulating seed germination, inducing storage protein synthesis and modulating water stress.

⇒ Ethylene is a gaseous hydrocarbon. It is not required for normal vegetative growth but it can have a significant impact on the development of roots and shoots. Its synthesis is stimulated by auxin. Ethylene is noted for its role in stimulating fruit development.

⇒ Brassinosteroids appear to have an important role in plant development and may soon be considered a distinct class of hormones.

⇒ Other possible plant hormones include the polyamines and the hypothetical hormones, florigen and vernalin.

⇒ The events initiated by hormones can be (1) the initial signal perception, (2) a signal transduction pathway, and (3) the final response. The signal transduction pathway involves one or more second messengers that serve to amplify the original signal. Plant hormones appear to qualify on all three counts. It has been known for the past 100 years that plant hormones have significant effect on development. More recently, proteins that could serve in the perception stage have been identified for auxin and cytokinin and ethylene. Lipid-based molecules and calcium appear to be involved in the signal transduction pathway in plants.

⇒ Auxin is synthesized from the amino acid tryptophan or released from storage as conjugate. In *Arabidopsis*, IAA is synthesized from glucosinolates via acetonitrile. IAA is transported in basipetal direction due to the location of specific auxin efflux carries near the base of each cell. Once IAA has accomplished its purposes, it can be removed by oxidation to inactive products.

⇒ Gibberellins are diterpenoids, related to carotenoids and other isoprene derivatives. The intermediates are geranyl-geranyl pyrophosphate and GA_{12}-7-aldehyde. Gibberellins appear to be transported in the phloem in response to source-sink relationships. Gibberellins can be inactivated by hydroxylation at the C_2 position or by conversion to inactive conjugates.

⇒ Cytokinins are synthesized by a condensation of an isopentenyl group with the amino group of adenosine monophosphate (AMP). Cytokinins will also form conjugates with sugars and are metabolized by oxidation.

⇒ ABA is a 15-carbon isoprene derivative, synthesized by cleavage from a 40-carbon xanthophyll violaxanthin. ABA is inactivated by oxidation to phaseic acid and subsequent reduction to dihydrophaseic acid.

⇒ Ethylene is synthesized from the amino acid methionine. The principal intermediate is 1-aminocyclopropane-1-carboxylic acid (ACC). As there are limited amounts of methionine, the sulfur is salvaged and recycled during ethylene biosynthesis. Ethylene biosynthesis is controlled by transcriptional regulation of the rate-limiting enzyme, ACC synthase.

⇒ The mechanism of hormone action in plants has been studied in only two cases auxin control of cell elongation and gibberellin control of cereal grain seed germination.

⇒ In auxin control cell elongation, the first is an acid-stimulated loosening of cell wall polymers followed by turgor-induced expansion. Acid-induced expansion is complete within 30 to 60 minutes. Sustained cell expansion requires continued RNA and protein synthesis, in turn requires gene activation. Two categories of genes are known to be

activated by auxin, short-term responses that are activated within minutes of auxin application and long-term responses that appear after an hour or more.

⇒ Auxin is perceived on the outer surface of the plasma membrane by a complex of auxin-binding protein (ABP) and a transmembrane "docking protein".

⇒ Gibberellin stimulates seed germination by regulating gene transcription. Gene products include amylases and other enzymes responsible for degrading storage carbohydrate and mobilizing sugars for use by the developing embryo. The mechanisms for action of cytokinins and abscisic acid are poorly understood.

15.10 Model Questions

1. What are plant hormones? Explain about the physiological action of auxin.
2. Give a brief note on gibberellin biosynthesis, metabolism and transport.
3. Write short notes on:
   a) Hormone binding proteins in plants
   b) Oxidation of IAA
   c) Polyamines
4. Physiological functions played by gibberellin.
5. What are cytokinins and discuss briefly about the physiological roles played by cytokinins?
6. How do hormones work?
7. Give a detailed account on second messengers in plants.
8. Discuss briefly about the biosynthesis and metabolism of auxins.
9. Explain about biosynthesis, metabolism and transport of cytokinins.
10. Write an essay on biosynthesis, metabolism, transport and physiological roles played by Abscisic acid.
11. Biosynthetic pathways, metabolism and physiological roles played by ripening hormone.
12. Write short notes on:
    a) Brassinosteroids b) Acid growth theory c) Mechanism of action f cytokinin and ABA
13. Write an essay on auxin and cell expansion.
14. Discuss the mechanism of action of Gibberellins.

15.11 Reference Books

1) **Introduction to Plant Physiology**, 2nd edition, William G. Hopkins, John Wiley & Sons, Inc.
2) **Plant Physiology**. 3rd Ed., Salisbury and Ross, CBS Publishers and Distributors.
3) **Advanced Plant Physiology,** Malcolm B. Wilkins, English Language Book Society/Longman.
4) **Plant Physiology** L. Taiz and E. Zeiger Sinauver Associates Inc., Sunderland, Massachusetts.

P. Kiranmayee

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LESSON-16

PHOTOPERIODISM AND VERNALIZATION

16.0 OBJECTIVES

In this lesson, you will learn:

1) **Juvenility** : the early phase of growth during which flowering cannot be induced by any treatment. Physiological and morphological changes during juvenile phase.

2) **Photoperiodism**: including the distinction between short day plants, long day plants and other response types, the central role of the dark period, the nature of photoperiodic perception and a discussion of the elusive floral hormone.

3) Timing of biological processes by the internal biological clock.

4) Time measurement in photoperiodism, including the hour-glass hypothesis and the role of the biological clock.

5) Vernalization – the low temperature requirement for flowering in winter annuals and biennial plants.

6) The significance of photoperiodism in nature.

CONTENTS

16.1 INTRODUCTION

The development of most plants is seasonal with developmental processes and flowering occur at particular times of the year. They are immediate responses to environmental conditions like temperature, water supply or light intensity. In fact, seasonal changes in plants are rarely controlled in this way, but depend on certain characteristics of the environment.

It may be difficult for a layman to believe that plants can tell time. One example is the consistent flowering of various species at particular times of the year. Roses always bloom in the summer and chrysanthemums in the fall. In the northern latitudes, perennial plants sense the short days of autumn as a signal to induce bud dormancy, thus anticipating the unfavourable conditions of winter.

Photoperiodism and vernalization are two major mechanisms underlying such seasonal responses and the words indicate the characteristics of the inductive environmental signals. Photoperiodism combines the Greek words for light and length of time. It is the length of day that gives the most reliable indication of the advancing season and an organism's capacity to measure day length is known as photoperiodism. Flowering is either quantitatively or qualitatively depend on exposure to low temperature. This phenomenon is known as vernalization.

Photoperiodism is a response to the length of day and vernalization is an effect on flowering brought about by exposure to cold. These two mechanisms and their combinations enable plants to make seasonal adaptations.

Vernalization plays an additional role in seasonal flowering and is found in regions with winter temperatures that are unfavourable for growth. A requirement for exposure to cold for several weeks will prevent flowering until a winter has been experienced by the plant. Germination in late summer would not lead to immediate flowering in favourable photoperiods and vernalization becomes a device to ensure that the winter has passed before the onset of reproduction.

Juvenility, vernalization and photoperiodism are three important processes that determine when plants flower with respect to both ontogeny and season.

16.2 JUVENILITY

Juvenility is the name given to the early phase of growth during which flowering cannot be induced by any treatment. The juvenile duration varies widely. In woody plants, it lasts for several years (30-40 years), in herbaceous plants it is quite short, more than a few days or weeks in duration. In extreme cases, there is no juvenile phase since flower primordia are found in the seed. At the time of transition from juvenile to mature, physiological and morphological changes occur like leaf shape, thickness, leaf retention, thorniness, phyllotaxis, pigment content, rooting capacity etc. (Table 16.1).

Table 16.1 Juvenile and adult characters of English ivy (*Hedera helix* L.)

Juvenile character	**Adult character**
Three-or five-lobed palmate leaves	Entire, ovate leaves
Alternate phyllotaxy	Spiral
Anthocyanin pigmentation of young leaves and stem	No anthocyanin pigmentation
Stems pubescent	Stems glabrous
Climbing and plagiotropic growth habit	Orthotropic growth habit
Shoots show unlimited growth and lack terminal buds	Shoots show limited growth terminated by buds with scales
Absence of flowering	Presence of flowers

The transition from juvenile to adult appears to be associated with changes in the apex, the base of the plant may remain in the juvenile condition even after the transition to maturity has occurred at the apical meristem.

The attainment of maturity:

The transition to maturity is not necessarily accompanied by flowering. Even a fully matured tree may not flower if it is growing vigorously and irregular flowering is common in many trees. Lack of flowering does not necessarily indicate the juvenile condition.

It is generally agreed that size is important, it is not yet clear what component of size is critical for attaining of maturity. Two views have been examined. One is that a plant of sufficient size transmits one or more signals to the apex. The second is that the apical meristem behaves independently and undergoes the phase transition at a particular time.

Nutritional and hormonal factors:

The apex receives both nutrients and hormones from the rest of the plant. Factors like low light and high temperature reduce the supply of carbohydrates to the apex. This causes rejuvenation or prolong the juvenile phase in many plants. It is evident that, endogenous gibberellins may play a role in phase transition. Although many factors cause reversion to the juvenile condition, only time and / or size can bring about the transition to maturity.

The presence of roots close to the apex has been suggested to be important in maintaining juvenility. In ivy (*Hedera helix*), the aerial adventitious roots produced at the nodes of juvenile plants have a high concentration of extractable gibberellins and removing the roots decreased the amount of GA-like substances in the shoot apices.

16.3 PHOTOPERIODISM

In 1912, a French scientist J. Tournois found that both *Humulus* (hops) and *Cannabis* (hemp) plants flowered precociously during the winter in the greenhouse. Tournois eliminated temperature, humidity and light intensity. In 1914, he concluded that the shortening of day length or lengthening of night was responsible for early flowering. H. Klebs observed that, *Sempervivum funkii* grown as a vegetative rossette in the winter green house. By supplementing normal daylight with artificial light, Klebs was able to stimulate stem elongation and induce flowering. From this experiments, Klebs concluded that length of day triggered flowering in nature. W.W. Garner and H.A. Allard demonstrated the full impact of daylight on flowering and coin the term *Photoperiodism.*

Garner & Allard conducted experiments with a mutant cultivar of tobacco (*Nicotiana tobacum*), called Mary land Mammoth. In the field, these plants grew to be very tall with large leaves. During normal growing season, these plants would not flower. In green house, even very small plants flowered in the winter and early spring.

They are concentrated on soybean (*Glycine max*). When the cultivar Biloxi was sown over a three-month period from May to August, all the plants flowered within a three-week period in September. It appeared that all plants regardless of age, were simply awaiting some signal to initiate flowering.

Like Tournois, Garner and Allard eliminated environmental conditions and finally concluded that flowering was controlled by the length of day and night.

They went on to suggest that bird migration might also be keyed to day length. We now know that photoperiodic control is not limited to flowering, but is a basic regulatory component in many aspects of plant and animal behavior.

Photoperiodic response types:

Photoperiodic responses fall into three categories. They are short-day plants (SD plants), long-day plants (LD plants) and day-neutral plants (DNP).

Short day plants:-	*Chenopodium rubrum*
	Chrysanthemum sp.
	Cosmos sulphureus
	Euphorbia pulcherrima
	Xanthium strumarium
Long day plants:-	*Beta vulgaris*
	Raphanus sativus
	Triticum aestivum
Dry neutral plants:-	*Cucumis sativus*
	Helianthus annus
	Phaseolus vulgaris

Short-day plants are those that flower earlier in response to day lengths that are shorter than a value within a 24-hour cycle. Long-day plants respond to day lengths that are longer than a value, and day-neutral plants flower irrespective of day length.

In addition to these three basic categories, there are various species of the genus *Bryophyllum* are example of long-short-day plants (LSD plant). They will flower only if a certain number of short days are preceded by a certain number of long days. *Trifolium repens* is an example of short-long-day plant (SLD plant).

Intermediate – day length plants flower only in response to day lengths of intermediate length but remain vegetative when the day is either too long or too short. Another type of behaviour is amphophotoperiodism, flowering is delayed under intermediate day length (12 to 14 hours) but occurs rapidly under day lengths of 8 hours or 18 hours.

Photoperiodic induction

Many plants require more or less continuous exposure to the appropriate photoperiod in order to flower successfully. Others will proceed to flower even if, the plant is returned to unfavourable photoperiods. Such plants are called induced and the appropriate photoperiod is called an inductive treatment.

16.3.1 The central role of the dark period

Garner & Allard suggested that plants responded to the relative lengths of day and night. The term photoperiodism implies that plants measure the duration of day light. In fact, plants measure neither the relative length of day and night nor the length of the photoperiod. Instead, they measure the length of the dark period.

This was demonstrated by the experiments of K.C. Hamner and J. Bonner in 1938 under 24-hour cycles of light and dark, *Xanthium* flowered with dark periods longer than 8.5 hours but remained vegetative on schedules of 16 hours light and 8 hours dark (Fig. 16.1 A-H).

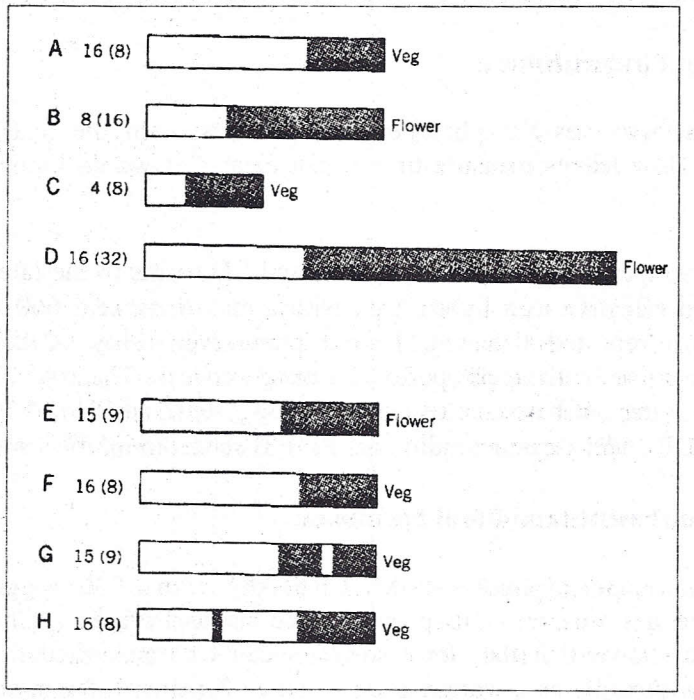

Fig. 16.1 The central role of dark period in *Xanthium strumarium*, a SD plant

The numbers in brackets indicate the length of dark period. Note that the plants flower whenever the dark period is uninterrupted for nine hours or more.

On schedules of 4 hours light-8 hours darkness, plants remained vegetative even though the 4-hour photoperiod is much shorter than the 15.5 – hour critical photoperiod (Fig. 16.1C). Schedules of 16 hours light – 32 hours darkness induced rapid flowering even though the photoperiod exceeded the critical daylength (Fig. 16.1D). The above results showed two conclusions:

(1) the relative length of day and night is not the determining factor in photoperiodism.

(2) it is the length of the dark period that is important.

The consistent feature of these experiments is that *Xanthium* will flower whenever the dark period exceeds 8.5 hours and will remain vegetative whenever the dark period is less than 8.5 hours.

The flowering effect of an inductive 9-hour dark period can be nullified by interrupting the dark period with a brief light-break (Fig. 16.1G), but a "dark interruption" of a long light period has no effect (Fig. 16.1H). At this point, it is clear that photoperiodism has little to do with daylength, rather it is a response to the duration and timing of light and dark periods.

16.3.2 The role of Phytochrome

If the leaf perceives the photoperiodic stimulus, then the leaf must be capable of measuring time. How leaves measure time is not clear, but we do know that phytochrome is involved.

Early action spectra on several SD plants and LD plants in the late 1940s indicated that red light was most effective as a light-break, with a maximum near 660 nm. H.A. Borthwick and his colleagues reported that red, far-red photoreversibility of the light-break clearly implicates phytochrome in the photoperiodic timing process. The role of phytochrome is far from clear at this point, but based on recent work suggested that PHY A is required to promote flowering of an LDP under certain conditions. PHY B seems to inhibit flowering.

16.3.3 Light requirements and floral hormones:

The Russian plant physiologist M. Chailakhyan in 1936 suggested that the floral stimulus might be a hormone. He proposed the name florigen. Chailakhyan and many investigators have shown that the floral stimulus can be transmitted through a graft union. When several *Xanthium* plants are approach-grafted in sequence, all can be brought to flower if only the first is induced by short days (Fig. 16.2).

In some of the early experiments, it was suggested that florigen, like auxin, could be transmitted through a non-living connection. Experiments involving anatomical studies proved that transmission of the stimulus occurred only when a tissue union had been established.

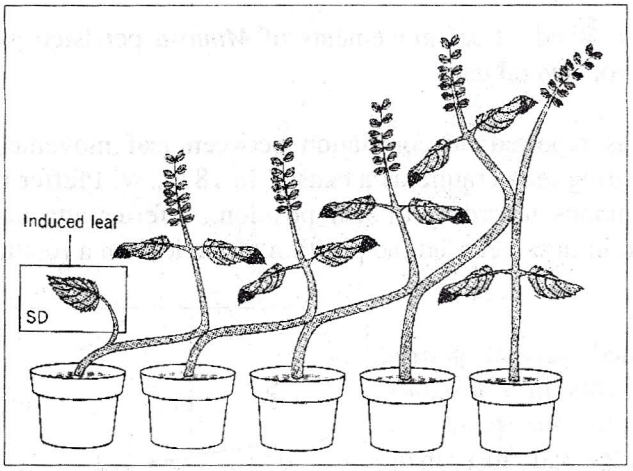

Fig. 16.2 Transmission of the floral stimulus in grafted plants

Auxins, cytokinins and ethylene have been reported to either enhance or suppress flowering in various species. Of these hormones, only gibberellins evoke flowering in a wide variety of species. Lang (1957) showed that repeated applications of dilute gibberellin solutions to the apex of annual LD plants elicited a flowering response under short days. But florigen and gibberellins are not equal.

Chailakhyan proposed that flowering is under control of two hormones. Gibberellin and a hypothetical substance, anthesin. Since gibberellin does not promote flowering in SD plants, he proposes that SD plants have sufficient gibberellin but lack anthesin. Synthesis of anthesin is stimulated by short days. LD plants, would have sufficient anthesin but lack adequate levels of gibberellin. Gibberellin synthesis is stimulated by long days.

Day-neutral plants would flower irrespective of day length because they are able to synthesize adequate levels of both the "hormones".

16.3.4 Temperature and Photoperiodism

In some cases, the flowering response is simply enhanced at certain temperatures while others respond at high or low temperatures.

Winter cereals will not normally flower during a single growing season, but must be planted in the fall in order to flower and produce a crop the following year. Spring strains will flower and produce grain in the same year they are planted. .

16.3.5 The Biological Clock

French astronomer M. De Mairan in 1729 raised that nyctinastic movements are due to exogenous control namely, the daily pattern of light and dark periods and internal or endogenous

time keeper might be involved. Leaf movements of *Mimosa* persisted even when the plants were placed in darkness for several days.

In 1863, J. Sachs reported no correlation between leaf movements and temperature fluctuations, thus eliminating temperature as a cause. In 1875, W. Pfeffer devised an apparatus for automatic and continuous recording of leaf position. Pfeffer attached a leaf, via a fine thread, to a stylus, which in turn recorded the position of the leaf on a rotating drum coated with carbon (Fig. 16.3).

Pfeffer contributed several papers devoted to leaf movements in *Phaseolus vulgaris* (Fig. 16.4). At one point, he showed that plants that had lost their rhythmic leaf movements will regain them if exposed to a new light-dark cycle. If the new cycle is inverted with respect to natural day and night, leaf movement will also be reversed. He concluded that persistent leaf movements under continuous light or darkness were a "learned" behaviour. In the end, Pfeffer was forced to conclude that leaf movements were an endogenous probably inherited behavior.

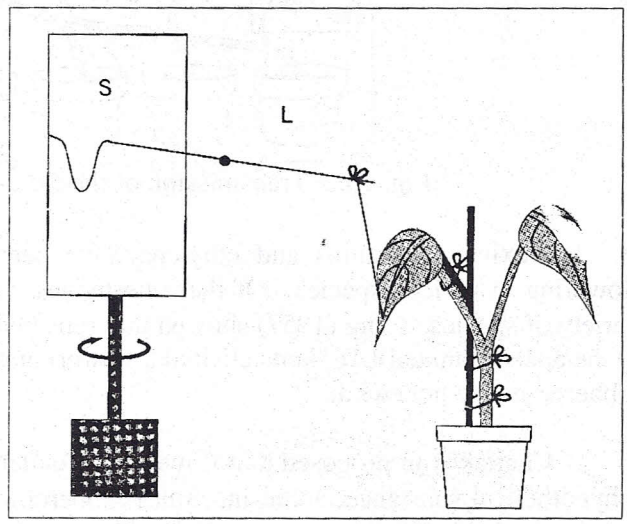

Fig. 16.3 Principle of the drum recording apparatus used by Bunning and others for recording of leaf movements.

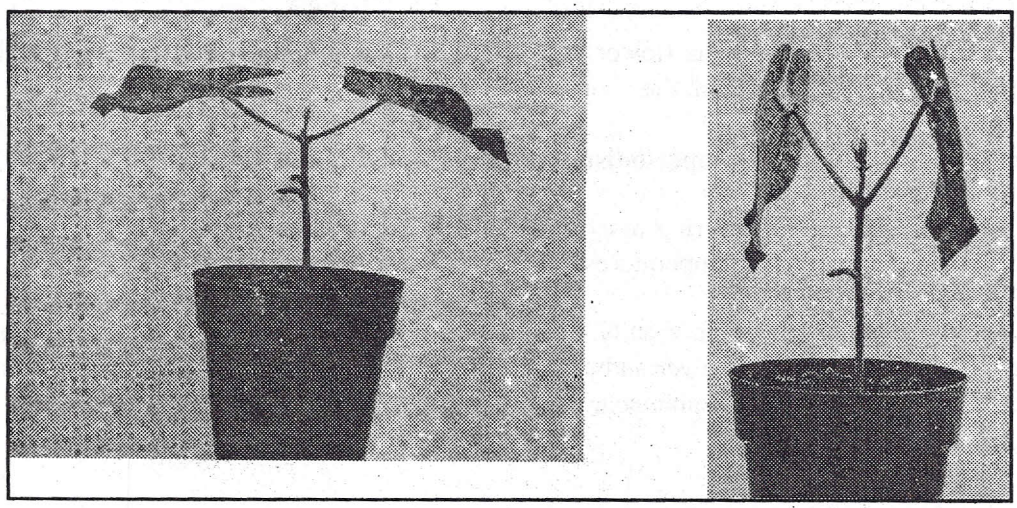

Fig. 16.4 Sleep movements of *Phaseolus vulgaris.*

A graphic plot of a biological rhythm against time describes a repeating pattern that resembles physical wave phenomena. Much of the terminology describes physical oscillations has been adopted to describe biological cycles. At the beginning, it is necessary to distinguish between simple periodic phenomena and endogenous rhythms. For example, photosynthetic carbon uptake describes a periodicity because it is light-driven and daylight is periodic over time. Photosynthesis is diurnal, in that it is active only during day light hours and is controlled by fluctuations in an external factor (light). The key to an endogenous rhythm is that it persists for several cycles, under constant conditions (constant light and constant darkness). The rhythmicity expressed under constant conditions is called as free-running.

The time required to complete a cycle is known as the period (T, tau) (Fig. 16.5A, B). Period is conveniently described as the time from peak to peak, but it applies equally well to any two comparable points in the repeating cycle. Constant rhythms are classified according to the length of their free-running period. Thus a circadian rhythm has a period of approximately 24 hours. Rhythms in metabolic activity with periods less than 24 hours (measured in minutes or hours). These are known as ultradian rhythms.

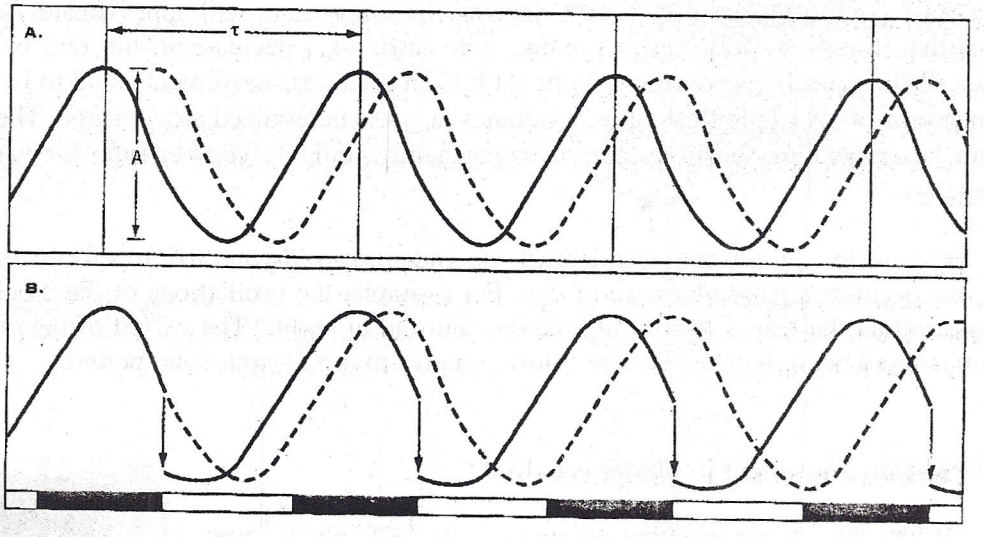

Fig. 16.5 Examples of circadian oscillators

Discussions of endogenous circadian rhythms are complicated by the fact that two time frames are involved solar time and circadian time. Solar time based on a normal 24 hour day, circadian time is based on the free-running period. One cycle is considered to be 24 hours long, regardless of its actual length in solar time. Each hour of circadian time is therefore $^{1}/_{24}$ of the free-running period. Thus if the free-running period is 30 hours, events that occur at 0, 15 and 30 hours of darkness will have occurred at circadian times CT : 0, CT : 12 and CT : 24 (Table 16.2).

The phase of the free-running cycle that corresponds to day in a normal light-dark environment is known as subjective day and that which corresponds to normal night is the subjective night.

Table 16.2. Example of circadian rhythmic phenomena in higher plants

Rhythm	**Organism**
Sleep movements	Many species
Stomatal opening	Banana, tobacco, *Vicia*
Stem growth	Tomato
CO_2 production	Orchid flowers
Gas uptake	Dry onion seeds
Membrane potential	Spinach leaves
mRNA expression	Pea

Most chemical reactions and growth and other biological responses respond to temperature (with a Q_{10} near 2) a 10°C increase in temperature will approximately double the rate of the process. A decrease in temperature leads to a decrease in the rate by the same amount. When seedlings are raised in the dark from seed, leaf movements tend to be small and unsynchronized. A single flash of light initiates larger synchronized movements. The circadian rhythm is temperature-sensitive, but some mechanism quickly compensates for variations in temperature.

The action of the biological clock or endogenous rhythms is to ensure that certain functions occur at a particular time of day. For example, the oscillations of the clock in beans determines that the leaves rise during the day and fall at night. The period of the endogenous rhythm is fixed but it may be "fast" or "slow" relative to the 24-hour solar period.

16.3.6 The measurement in photoperiodism

With the discovery that flowering in SD plants was determined by the length of a critical dark period, it seemed that the length of the critical dark period could be measured simply by the appearance and disappearance of some metabolites. Such an approach to timing was likened to an hourglass (Fig. 16.6) which, when the sand runs out, must be inverted to restart the timing process. This hypothesis is called "hourglass" hypothesis.

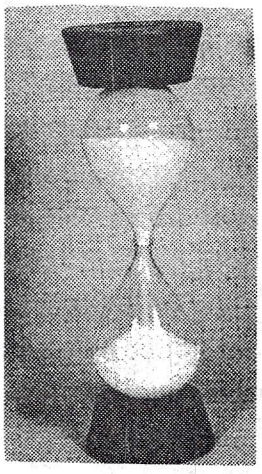

Fig. 16.6 An hourglass timer →

When the action spectra showed that phytochrome was the photoreceptor responsible for the light-break phenomenon. It is concluded that Pfr, present at the end of the photoperiod, was inhibitory to flowering. The length of the critical dark period represented the time required for Pfr to fall below some critical value long enough to allow for synthesis of florigen. The effectiveness of a red light break in the middle of an inductive long dark period was seen simply as raising the level of Pfr, thus restarting the timing process before sufficient florigen could be produced.

In 1936, Büning proposed that the rhythm was comprised of two phases, the photophile or light loving phase and the scotophile, the dark-loving phase, which altered about every 12 hours. According to Bünning's hypothesis, light falling on the plant during the photophile phase would promote flowering and light during the scotophile phase would inhibit flowering. When the plant is placed under continuous conditions, the photophile phase is equivalent to subjective day and the scotophile phase is equivalent to subjective night.

16.3.7 Genetic approach

If a mutant can be identified that influences timing at any level, the wild type gene can be isolated and its gene product analyzed for clues to its role in the timing mechanism. Flowering genes have been a part of plant breeding programmes for years. Because early flowering and insensitivity to photoperiod are most desired in crop species. Two crop species pea (*Pisum sativum*) and wheat (*Triticum aestivum*) have the genetics of photoperiodic processes received much attention. Both the crops are quantitative long-day plants. In peas, several genes that affect photoperiodic timing and the onset of flowering have been identified. **fsd** (flowering short days) is a recessive mutant that causes the plant to behave as a qualitative short-day plant. When the mutant is grafted to a wild type stock under long days, the mutant will flower.

Arabidopsis is a quantitative long-day plant with a photoperiod of 8 to 10 hours. Under long days, it flowers with 4 to 7 leaves in the rosette (about 3 weeks), under short days, flowering is delayed until 20 leaves (7 to 10 weeks). Flowering is also promoted by exposure to blue or far-red light. This reflects the role of phytochrome in photoperiodic phenomena. Here, flowering time refers not to the elapsed time (days to flowering) but to the number of rosette leaves produced before the flowering stem appears.

Mutants that affect phytochrome also influence flowering. The **hy1** mutant is defective in the synthesis of the phytochrome chromophore. In the absence of functional photoreceptor, **hy1** mutants show an elongated hypocotyl, flower earlier than wild type under both long and short days. Because they flower early under both conditions, the mutant shows a response to photoperiod.

There are certain other mutants like early flowering (elf 3) is an example of early flowering type and *Constans* (co) and *gigantea* (gi) are late flowering, which are day length-insensitive mutants. Both **co** and **gi** delay flowering under long days but no effect on flowering

time under short days. When wild type *CO* & *GI* are studied, the *GI* gene operates before the *CO* gene in the same pathway and that floral promotion under long days depends on the amount of *CO* mRNA transcribed.

The **elf3** mutant interacts with endogenous clock. **elf3** also effects leaf movement and transcription of the chlorophyll a/b binding protein (CAB). The rhythm for *CAB* expression is not lost if the entrained plants are shifted to continuous dark. This indicates that *ELF3* does not encode a component of the endogenous clock itself, but links the clock to the initial photoreception or light-on signal.

16.3.8 Photoperiodism in Nature

Photoperiodism reflects the need for plants to synchronize their life cycles to the time of year. Photoperiodism is more important to plants in the subtropical and temperate latitude where seasonal variations in day length are more pronounced. Many tropical plants respond to the small changes in day length that occur within 5 or 10 degrees of equator. This does not mean that the photoperiod response ties to a species to a particular latitude, since the critical photoperiod sets the upper (for SD plant) or lower (for LD plant) limits of daylength.

Photoperiodism helps to ensure that plants flower in their temporal niche. This helps the plants to reduce competition with others and ensures that the reproductive development is completed before the onslaught of unfavourable winter conditions. If flowering relied solely on plant size, non-uniform germination would be expected to spread flowering out in time. In cross-pollinating plants, flowering synchronized by photoperiod would serve to ensure maximum pollinating population or coordinate with the appearance of particular pollinating insects.

16.3.9 Influence of Temperature on Development

The temperature in tropical climates is relatively stable. Plants growing in temperate regions and closer to the poles show more or less variations in temperature on a daily and seasonal basis. Plants have evolved ways to incorporate this information in their developmental and survival stratagies. Plants use this information to ensure dormancy of buds, tubers and seeds and to modify their flowering behaviour. These are keys for survival over the conditions unfavourable to normal growth and development.

The temperature at which biological processes can occur is limited by the freezing point of water on the low side and the irreversible denaturation of proteins on the high side. The temperatures between these two extremes are called cardinal temperatures.

Living organisms are broadly classified according to their ability to withstand temperature. The plants that grow optimally at low temperature (0°C to 10°C) are called psychrophiles, e.g. algae, fungi and bacteria. The plants which grow optimally between 10°C to 30°C are mesophiles. Thermophiles can grow between 30°C to 65°C.

The temperature for the growth of plants is 0°C to 45°C. Temperature compatibility is very much species dependent. As a general rule, temperatures optimum for growth reflect the geographical region in which the species originated. The effects of temperature on physiology and metabolism influence plant distribution called **biogeography**. Temperature related metabolic effects not only limit distribution but have economic implications as well.

16.3.10 Temperature and flowering response

There is an interaction between temperature and photoperiod particularly with respect to flowering behaviour. In most cases, the interaction results in changes in the length of the photoperiod or a tendency toward day-length neutrality or an inability to flower though at high or low temperature extremes.

There are other plants in which flowering is either quantitatively or qualitatively dependent on exposure to low temperature. This phenomenon is known as **vernalization.** Low temperature treatments hasten flowering.

The term is a translation of the Russian *yarovizatsya*: both words combining the root for spring (Russian, **Yarov**; latin, **ver**) with a suffix means "to make" or "become". The word was coined by a Russian, T.D. Lysenko in 1920s. Vernalization reflects the ability of a cold treatment to make a winter cereal behave like a spring cereal with respect to its flowering behavior.

16.4 OCCURRENCE OF VERNALIZATION

Vernalization occurs in winter annuals and biennials. Typical winter annuals are called "winter cereals" (wheat, barley & rye). "Spring" cereals planted in the spring, come to flower and produce grains before the end of the growing season. Winter strains planted in the spring would fail to flower. Winter cereals are plated in the fall, they germinate and over winter as small seedlings, resume growth in the spring and are harvested about midsummer.

F.G. Gregory and O.N. Purns studied thoroughly about vernalization on the Petkus strain of rye in 1930s. The spring strain is a quantitative, long-day plant. Under short days, flower initiation does not occur until after about 22 leaves have been produced requiring about 4.5 months. Under long days, flowering in the spring strain is initiated after about seven leaves have been produced requiring about two months. The winter strain is not a LDP. These plants germinate at normal temperature, flowers slowly, requiring 4, 5 months, regardless of daylength. When the winter strains are planted in fall, receive an over winter low temperature treatment. When it resumes growth in the spring, the winter strain responds to photoperiod as the spring strain. Over wintering cold treatment can also be achieved by vernalizing the seed. This can be done by holding the germinated seed near 1°C for several weeks. This shows that the low temperature treatment does not alone promote early flower initiation, rather, the effect of vernalization is to render the seedling sensitive to photoperiod.

Biennials are monocarpic plants that flower and die in the second season. Ex: *Beta vulgaris*, Cabbages and related plants, carrots (*Daucus carota*), members of umbellifereae and some strains of black henbane. Biennials share with the winter annuals the property that subjecting the growing plant to a cold treatment stimulates a photoperiodic response.

Biennials grow as a rosette, with short internodes in the first season (Fig. 16.7). During winter, the leaves die back, but the apical meristem remains protected. New growth in the following spring is characterized by stem elongation called **bolting**, followed by flowering. The requirement for cold is qualitative/absolute. In the absence of a cold treatment, biennials are maintained in the non-flowering rosette habit indefinitely. As a rule, winter annuals or biennials, vernalizable plants tend to respond as long-day flowering plants, though some biennials are day-indifferent following vernalization. *Chrysanthemum* varieties require vernalization before responding as a quantitative SDP. As a perennial, *Chrysanthemum* requires vernalization on an annual basis.

Fig. 16.7 Vernalization and stem elongation in cabbage (*Brassica* sp.)

16.4.1 Effective temperature

The range of temperatures varies depending on the species and duration of exposure. In Petkus rye, the effective range is –5°C to +15°C and optimum between +1°C and +7°C. Flowering advances after as little as one to two weeks treatment at 1°C to 2°C and is maximum effect after about seven weeks at that temperature (Fig. 16.8).

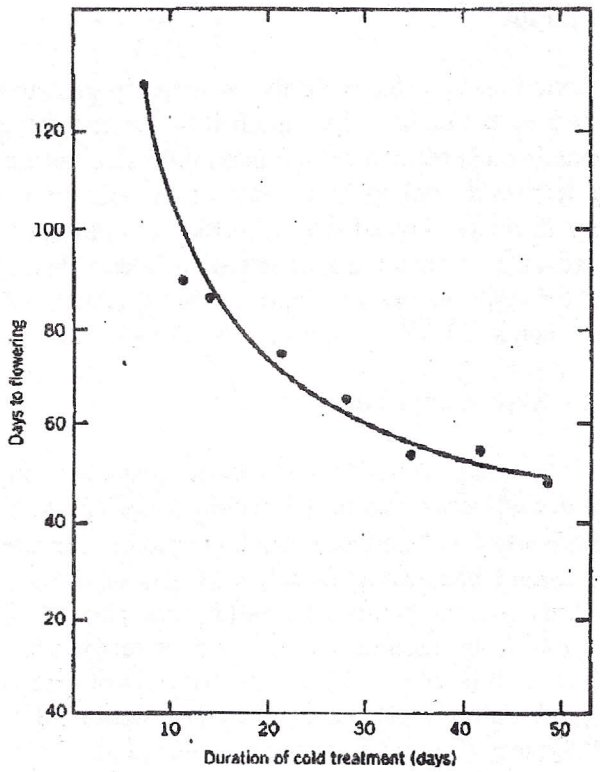

Fig. 16.8 Vernalization in Petkus rye (*Secale cereale*)

A longer exposure to lower temperatures within the effective range is required because the metabolic reactions leading to the vernalized state progress more slowly.

This figure (Fig. 16.8) shows that Petkus rye seeds were germinated in moist sand at 1°C for the time indicated. Cold treatments were scheduled so that all seeds were returned to the greenhouse at the same time. The number of days to flowering decreased with increasing length of the cold treatment.

The vernalized state is more or less permanent in most species giving rise to the concept of an induced state. All cold-requiring plants that have been studied are capable of being devernalized, a reverse state, if followed immediately by a high temperature treatment. Flowering in vernalized winter wheat can be nullified if the seedlings are held near 30°C for three to five days. For most plants there is a "neutral" temperature where neither vernalization nor devernalization occurs. For example, Petkus rye the neutral temperature is 15°C. Vernalized seeds can also be devernalized by drying them for several weeks or by maintaining the seeds under anaerobic conditions for a period of time following the cold treatment.

16.4.2 Perception of stimulus

A vernalization treatment is effective only on actively growing plants. Winter cereals may be vernalized as soon as the embryo has imbibed water and the germination process has been initiated. The biennials must reach a certain maximum size before they can be vernalized. This can be shown by localized cooling treatments or vernalization of moistened embryos. Early studies by Gregory & Purys showed that even the cultured apex of isolated rye embryo was susceptible to vernalization. Thus the induced state is established in a few meristematic cells can be maintained through out the development of the plant. Most biennials cannot be induced as seed. In these plants, it is the overwintering stem apex that perceives the stimulus.

16.4.3 The nature of the vernalization stimulus

Vernalization is an energy-depended metabolic process. Experiments with isolated embryos have been shown that vernalization treatments are effective only when the embryo is supplied with carbohydrate and O_2. A cold-induced, permanent change in the physiological or genetic state of the meristematic cells would be self-propagating, that is, it could be passed on to daughter calls by cell division. In plants like Petkus rye and *Chrysanthemum*, only tissue produced in a direct cell line from the induced meristem is vernalized. If the cold treatment is localized to a single apex, it will flower. All the buds that did not receive the cold treatment will remain vegetative. In graft union experiments, the transmission of "florigen" across a graft union which results in flowering in non-vernalized receptor plants. If a vernalized *Hyoscyamus* plant is grafted to an unvernalized plant, both will flower under long days. Transmission requires a successful graft union together with the flow of photoassimilate between the donor and receptor.

G. Melchers proposed the existence of transmissible vernalization stimulus called *Vernalin*. Like florigen, vernalin has resisted all attempts at isolation and remains a hypothetical substance. Adding to the complexity of vernalization is the apparent involvement of gibberellins in response to low temperature (Fig. 16.7). This was demonstrated by A. Lang (1957). He showed that repeated application of 10 µg of GA_3 to the apex would stimulate flowering in non-vernalized plants of *Hyoscyamus* and other biennials and maintained under short days. It has been shown that gibberellin levels tend to increase in response to low temperature treatments in several cold-requiring species. It is important to note that every situation in which gibberellin has substituted for long temperature or long days in promoting flowering involves bolting, the rapid elongation of stems from the rosette vegetative state. Less success has been achieved with gibberellins in caulescent LDP – whose stems are already elongated in the vegetative state. Following low temperature treatment, flower buds are seen at the time stem elongation begins. Following gibberellin treatment, the stem first elongates to produce a vegetative shoot. Flower buds do not appear till later. M. Chailakhyam suggested that vernalin is a gibberellin precursor. Vernalin would be accumulated in response to cold treatment in those plants requiring vernalization. But long days are required to complete its conversion to gibberellin.

16.5 SUMMARY

- Juvenility is the name given to the early phase of growth during which flowering can not be induced by any treatment.
- All living organisms can be broadly classified according to their ability to withstand temperature. Psychrophiles grow optimally at temperature of 0°C to 10°C, mesophiles at 10°C to 30°C and thermophiles at 30°C to 65°C. Most higher plants are mesophiles.
- Temperature is a principal factor in the distribution of plants. After experimentations, it is clear that temperature stability of principal metabolic pathway is a significant determinant in plant distribution.
- Plants also use temperature as cue in their developmental and survival strategies.
- Vernalization is the promotion of flowering by a period of low temperature.
- In case of winter annuals (cereals), vernalization changes the photoperiodic behaviour from daylength indifference to a quantitative long-day response. Biennials grow as rosette until vernalized.
- The flowering stem then bolts and responds as a long-day plant.
- A temperature 0°C to 5°C, applied to the actively growing apex of the plant for several weeks, is required for vernalization to be effective.

16.6 MODEL QUESTIONS

1. Write an essay on vernalization
2. Write short notes on the following:
   1) Juvenility
   2) The Biological Clock
   3) Genetic approach of photoperiodism
3. Give a brief account on photoperiodism

16.7 REFERENCE BOOKS

1) **Introduction to Plant Physiology**, 2nd edition, William G. Hopkins, John Wiley & Sons, Inc.
2) **Advanced Plant Physiology,** Malcolm B. Wilkins, English Language Book Society/Longman.
3) **Plant Physiology** L. Taiz and E. Zeiger Sinauer Associates, Inc., Sunderland, Massachusetts.

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Dr. P. KIRANMAYEE

Lesson – 17

STRESS PHYSIOLOGY

17.0 Objective

In this lesson, definition for stress, the effects of water deficits or drought on plants and how plants respond to water stress, heat-shock proteins, and hypersensitive reaction and systemic acquired resistance developed in response to biotic stress in plants are discussed.

17.1 Introduction

Plants are often subjected to various adverse environmental conditions throughout their lifetimes. Extremes in environmental parameters such as periods of prolonged drought and low soil moiture content; cold and hot temperatures; desiccating atmospheric conditions; prolonged periods of cloudiness; high wind velocities; excessive soil salinity; soil, water and air pollutants generated through human activities; snow; frost, and so forth, create stressful conditions for plants, which may have a significant impact on their physiology, development and survival. The study of plant responses with respect to these stressful environmental conditions is called the stress physiology. This branch of physiology helps to explain about the geographic distribution and the performance of plants in varying environmental conditions. Because stress invariably leads to reduced productivity, understanding stress responses are also important to make an attempts to breed stress-resistant cultivars that can withstand drought, salinity, and other yield limiting conditions.

17.2 Water is Stress ?

Terminology used to describe stressful environments and plant responses to these stresses has been the subject of controversy for some years. In 1972, Jacob Levitt (also Turner and Kramer, 1980) proposed a definition of biological stress derived from physical science. Physical scientists and engineers define stress as a force per unit area applied to

an object. In response to a stress an object develops a **strain** or change in dimension. This stress-strain relationship is readily described in mechanical examples, such as the stretching of an elastic band or the bending of a metal bar subjected to load. Levitt then suggested that **biological stress** is any change in environmental conditions that might reduce or adversely change a plant's growth or development and **biological strain** is the reduced or changed function. For example, when a plant is suddenly subjected to reduced light levels, its photosynthetic activity immediately reduced. According Levitt's definition we say that the reduced light levels are the stress and reduced photosynthesis is the strain.

Levitt went on to define **elastic biological strain** as those changes in an organism's function that return to the optimal level when biological stress has been removed. If the functions do not return to normal, the organism is said to exhibit **plastic biological strain**. In general, plant physiologists have emphasized in their studies such plastic strains as those caused by the stresses of frost, high temperatures, limited water or high salt concentrations. Elastic strains in plants such as the photosynthesis example just mentioned (if low light level does not persist too long, photosynthesis will resume at its original rate when the original light level has been restored) have been less studied by stress physiologists, although they must be extremely common.

Although Levitt has argued that the physical terminology can and should be applied to living organisms, the biological concept of stress, in practice, carries more general connotations. For example, at the ecosystem level, any external constraint that limits carbon gain (that is productivity) below the genetic potential of the plant may be considered a stress. This approach may have utility in particular situations, such as agriculture, where mathematical models can be used to estimate genetic potential under optimal environmental conditions. However, without a practical means for estimating genetic potential, it becomes far more difficult to judge the impact of stress according to this criterion.

When evaluating environmental stress another difficulty arises because that plant species are highly variable with respect to their optimum environments and their susceptibility to stressful conditions such as temperature, water potential, or salinity. An environment that is stressful for one plant may not be stressful for another.

Jones and Jones in 1989 defined the biological stress as an adverse force or influence that tends to inhibit normal systems from functioning. Even this definition has its problem of what is 'normal' or 'best' for a given plant. Clearly the answer to this question will often be highly subjective, depending on circumstances and judgements. So there is no single system of terminology that has received the support of all scientists working in the field of stress physiology.

17.3 Water stress : Plant responses and effects

Water stress may arise through either an excess of water or water deficit. The best known example of excess water is flooding or water logging. In such conditions, the plant tissues particularly root tissues may be subjected to low (hypoxic) or zero (anoxic) concentrations of ambient oxygen. Reduced oxygen in turn limits respiration, nutrient uptake, and other critical root functions. Stress due to water deficit is very common, so much so that the

correct term **water deficit stress** is usually shortened to simply **water stress**. Water stress in natural environments usually arises due to lack of rainfall. Water stress that may arise due to lack of the rainfall is called **drought stress**. Water stress that is produced as a result of excessive transpirational water loss is called **desiccation stress**.

Most plants have the capacity to resist stress, through either **stress avoidance** or **stress tolerance**. Plants such as the palms that grow at an Oasis or other plants such as mesquite (*Prosopis glandulosa*) and alfalfa (*Medicago sativa*) survive dry habitats as adult plants by extending their roots down to the water table. These plants are called **water spenders**. They never experience extremely negative water potentials so that they can certainly avoid the drought. Succulent species such as the cacti, century plant (*Agave americana*), and various other crassulacean acid metabolism plants (CAM) are **water savers**. They avoid the drought by storing enough water in their succulent tissues such as flesh leaves and stems and by closing stomata during the day light hours.

On the other hand, plants such as ferns and flowering plants survive desiccation of their protoplasm without injury through the drought tolerance. They retain the capacity for normal growth and development when the protoplasm is dehydrated. When the vegetative parts of most plants are intolerant of even moderate dehydration of their protoplasm, the foliages of ferns and angiosperms sometimes collectively known as resurrection plants, will survive air drying to as little as 7 percent water without injury. If tolerance increases as a result of exposure to prior stress, the plant is said to be **acclimated** (or hardened). Acclimation can be distinguished from **adaptation**, which usually refers to a genetically determined level of resistance acquired by a process of selection over many generations. Unfortunately, the term "adaptation" is also used in the literature to indicate acclimation. The morphological and physiological modifications associated with crassulacean acid metabolism plants are examples of adaptation while those modifications that are temporarily induced by slow drying and other stressful environments are examples of acclimation and are non heritable.

Some plants escape the drought stress altogether. The so called **ephemeral** desert plants are annuals that escape the drought by existing only as dormant seeds during the dry season. When enough rain falls to wet the soil to a considerable depth, they germinate, grow, and flower very quickly and thus complete their life cycle during a period of adequate moisture by forming dormant seeds before the onset of the dry season. In a similar manner, many arctic annuals rapidly complete their life cycle during the short arctic summer and survive over winter in the form of seeds. Because ephemerals never really experience the stress of drought or low temperature, they are known as **stress escapers**.

Water stress in plants is one of the major factors limiting crop production throughout the world. The physiological relationships associated with water stress therefore merit serious study. Water loss from plant tissues may cause a number of effects. It can lead to a disruption of membrane structure, reduction in photosynthetic activity, closure of stomata, alteration in enzyme activity, reduction in leaf expansion, shoot and root growth and so forth.

Plant membranes consist of a lipid bilayer interspersed with proteins and sterols. The physical properties of the lipids greatly influence the activities of the integral membrane proteins, including H^+-ATPases, carriers and channel forming proteins that regulate the transport of ions and other solutes, and of enzymes on which metabolism depends. Removal of water from the membranes disrupts their normal bilayer structure that is the membranes become very porous. When membranes are rehydrated, these proes permit large amounts of solute to be leaked between cellular compartments or from the cell into the extracellular space. Water stress within the bilayer may also displace membrane proteins, which, together with solute leakage, contributes to a loss of membrane selectivity and a loss of activity of membrane based enzymes.

Photosynthesis is particularly sensitive to water stress. It was extensively studied in chloroplasts of sunflower (*Helianthus annus*) leaves. Both electron transport activity and photophosphorylation are reduced in chloroplasts isolated from sunflower leaves with leaf water potentials below about –1.0 MPa. These effects reflect damage to the thylakoid membranes and ATP synthetase protein. Furthermore, the inhibitory effects of low water potential on photosynthesis are enhanced by high concentrations of magnesium. When sunflower plants were grown with different Mg^{2+} levels in the nutrient solution, the plants with the lower tissue Mg^{2+} concentrations maintained higher photosynthetic rates as leaves became dehydrated (Figure 17.1).

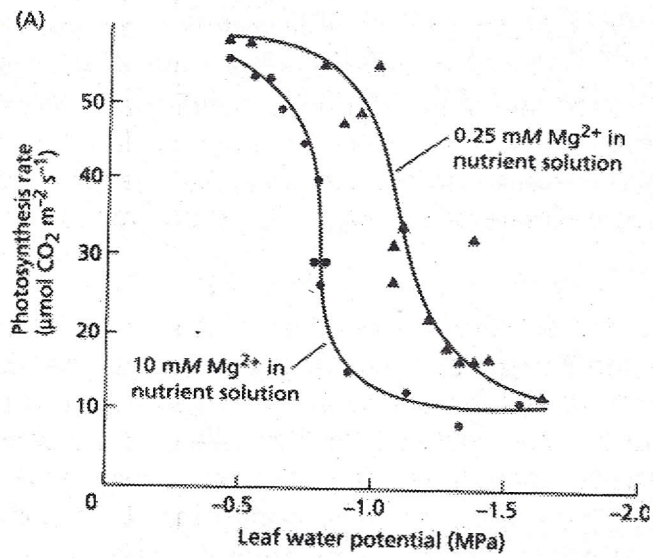

Figure 17.1 Photosynthetic rates (at CO_2 and light saturation) in water – stressed sunflower. The plants were grown with levels of Mg^{2+} in the nutrient solution chosen to produce different tissue Mg^{2+} concentrations. As water stress progressed, photosynthesis was affected much earlier in the plants that had higher Mg^{2+} levels solid curve).

Plants generally respond to acute water deficits by closing their stomata in order to match transpirational water loss through the leaf surfaces with the rate at which water can be resupplied by the roots. Unlike the surrounding epidermal cells, the surfaces of the guard cells are not protected with heavy cuticle. Consequently, guard cells themselves lose water

directly to the atmosphere. If the rate of evaporative water loss from the guard cells exceeds the rate of water regain from underlying mesophyll cells, a condition that arises due to a rapid drop in humidity or increase in temperature, the guard cells will become flaccid and the stomatal aperture will close. Such closure is called **hydropassive closure**. This closure does not require any metabolic energy. Stomatal closure is also regulated by **hydroactive process**. It is metabolically dependent and involves essentially a reversal of the ion fluxes that cause opening (see chapter 3). Hydroactive closure is triggered by water stress and involves the hormone abscisic acid (ABA). ABA is a normal constituent of leaves, which is synthesized at low rates in mesophyll cells and accumulates in the chloroplasts. In response to decreasing water potential in the leaf, stored ABA is released in to the apoplast where it is carried through the transpiration stream to the guard cells and initiates closure. The mechanism of plant response to water stress by increased ABA in its tissues is not yet clear.

According to the current model that ABA exists in two forms: the protonated form ABAH and the dissociated form ABA^-. The protonated (ABAH) form freely passes through most cell membranes whereas the dissociated form (ABA^-) does not cross membranes readily because of its negative charge. The ABAH tends to diffuse from cellular compartments with a low pH into compartments with a higher pH. In the light period, photosynthesis drives protons into the thylakoid lumen. As a result the stroma of the chloroplast becomes alkaline (pH 7.5 to 8.0) with respect to the cytosol of the mesophyll cell (pH 6.0 to 6.5). The resulting pH gradient between the stroma and the cytosol favors movement of ABAH into the chloroplast where it dissociates to impermeant ABA^-. In the dark, when photosynthesis ceases, protons leak back into the stroma, the pH gradient collapses, and ABAH moves back into the cytosol (Figure 17.2)

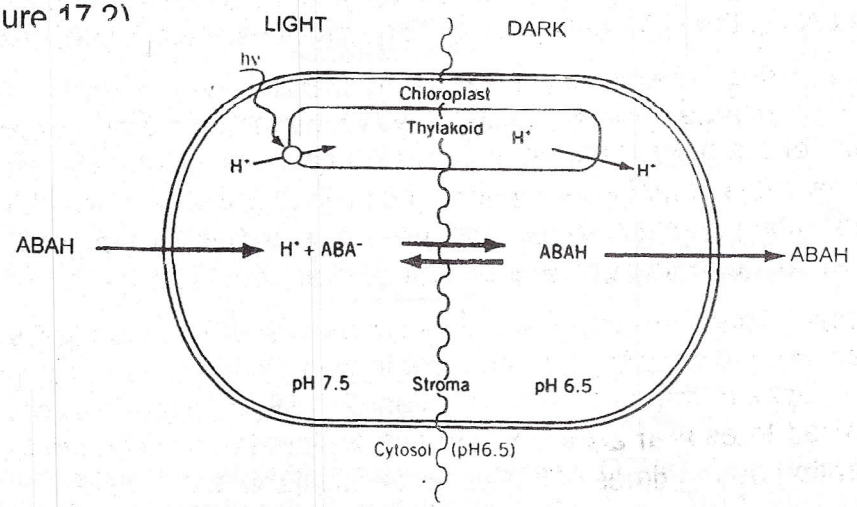

Figure 17.2 Accumulation of ABA in chloroplasts. Light stimulates proton uptake into the grana, making the stroma more alkaline. The increased alkalinity causes the weak acid ABAH to dissociated into H^+ and ABA^- anion. The concentration of ABAH in the stroma is lowered below the concentration in the cytosol, and the concentration difference drives the passive diffusion of ABAH across the chloroplast membrane. At the same time, the concentration of ABA^- in the stroma increases, but the chloroplast membrane is almost impermeable to the anion, which thus remains trapped. In the dark, protons leak back into the stroma, making the stroma acidic and ABAH moves back into cytosol.

Inhibition of photosynthetic electron transport and photophosphorylation in response to water stress would disrupt proton accumulation in the thylakoid lumen and lower the stroma pH. At the same time, there is an increase in the pH of the apoplast surrounding the mesophyll cells. The resulting pH gradient between the mesophyll cell and the apoplastic space stimulates a release of ABA from the mesophyll cells into the apoplast, where it can be carried in the transpiration stream to the guard cells to initiate stomatal closure.

In response to water stress, plants also exhibit another important mechanism known as **osmotic adjustment** or **osmoregulation.** Osmotic adjustment is a process by which water potential can be decreased without accompanying decrease in turgor by the accumulation of solutes. It develops slowly in response to tissue dehydration. Most of the adjustment can usually be accounted for by increases in concentration of a variety of solutes, including sugars, ions (K^+) and amino acids. One amino acid that appears to be particularly sensitive to water stress is **proline**. A sugar alcohol, **sorbitol** and **betaine** (N, N, N, trimethyl glycine) are other common solutes involved in osmotic adjustment. Accumulation of osmotic adjustment solutes in response to stress generates a more negative leaf water potential, thereby helping to maintain water movement into the leaf and, consequently, leaf turgor.

The most sensitive process to water stress is cell growth. When the turgor pressure in a plant cell falls, as happens in water stress, cell expansion is also decreased because of lack of pressure within the cell. There is thus a close correlation between decrease in cell size and the degree of water stress in plant tissues. A high turgor in plant tissues is sometimes only attained at night. This results in an enhanced growth rate at night as compared with during the day. In any case, the response of cellular growth to water stress appears as a slowing of shoot and root growth. This is usually followed by a reduction in cell-wall synthesis. Protein synthesis in the cell may be almost equally sensitive to water stress.

Leaf enlargement also depends on soil water availability. When leaf water potential dropped to –4 bars, leaf enlargement in sunflower was almost completely suppressed. In contrast, leaf enlargement in maize and soybean at –4 bars was 20 to 25 percent of the maximal rates (maximal rates of leaf enlargement occurred at about –1.5 to –2.5 bars in all three species). When water potential dropped below –4 bars, leaf enlargement continued to decrease in maize and soybean and became almost zero at about –15 bars.

Many mature plants, such as cotton, subjected to prolonged water stress will respond by accelerated senescene and abscisson of the older leaves. In the case of cotton, only the youngest leaves at the apex of the stem remain in cases of severe water stress. This process, sometimes referred to as **leaf area adjustment**, is another mechanism for reducing leaf area and transpiration during times of limited water availability.

In addition, many studies indicate that activities of certain enzymes, especially nitrate reductase, phenylalanine ammonia lyase (PAL) and a few others decrease quite sharply as water stress increases. A few enzymes such as α- amylase and ribonuclease, however, show increased activities. It was thought that such hydrolytic enzymes might break down starches and other materials to make the osmotic potential more negative, thereby resisting the drought (osmotic adjustment). Nitrogen fixation and reduction also drop with water stress.

17.4 Heat Shock Proteins (HSPs)

Each plant has its unique set of temperature requirements for growth and development. There is an optimum temperature with upper and lower limits at which each plant grows and develops most efficiently. As the temperature approaches these limits, growth diminishes, and beyond those limits there is no growth at all. Except in the relatively stable climates of tropical forests, temperatures frequently exceed these limits on a daily or seasonal basis, depending on the environment. Deserts, for example, are characteristically hot and dry during the day and experience low temperatures during the night. The upper temperature limits permiting survival of plants have long been of interest to biologists. Plants exhibit a wide range of responses to extremes of temperature and one such response to high temperature stress is the synthesis of stress proteins.

Exposure of most organisms to supraoptimal temperatures for brief periods suppresses the synthesis of most proteins and induces the synthesis of a new family of low molecular weight proteins known as **heat shock proteins (HSPs)**. The HSPs which are synthesized in response to high temperatures, help cells to withstand heat stress. Heat shock proteins were originally discovered in the fruit fly (*Drosophila melanogaster*) and later on they have been identified in other animals, including humans, as well as in plants and microorganisms. When cells or seedlings of soyabean for example, are suddenly shifted from 25 to 40 ^{o}C (just below the lethal temperature), synthesis of the commonly found set of mRNAs and proteins is suppressed, while transcription and translation of a set of 30 to 50 other proteins (HSPs) are enhanced. New HSP transcripts (mRNAs) can be detected 3 to 5 minutes after heat shock. They are either not present or present at very low levels in nonstressed tissues.

On the basis of molecular mass, heat shock proteins in higher plants are classified into three distinct classes. They are HSP 90, HSP70, and a heterogenous group of low molecule weight HSPs (LMW HSPs, that range in molecular mass from 17 to 28 kDa). Low molecular weight HSPs are more abundant in higher plants than in other organisms, and they show little homology with LMW HSPs in animals or microorganisms. However, some of the other HSPs are very similar in plants and animals. For instance, structural genes coding for a 70kDa HSP in maize, *Drosophila* and humans show a 75 percent homology. Another protein, **ubiquitin** (8 kDa) is also found in all eukaryotic organisms subjected to heat stress and is considered a HSP. Ubiquitin has an important role in marking proteins for proteolytic degradation. The HSPs are ubiquitous found throughout the cytoplasm as well as in nuclei, chloroplasts, endoplasmic reticulum, and mitochondria.

The functions of all the different HSPs are not yet known, but HSP 90, HSP 70, and HSP 60, (90, 70 and 60 kilodaltons respectively) and others act as molecular chaperones or chaperonins. Chaperones are a class of proteins normally present in the cell that direct the assembly of multimeric protein aggregates. For example, chloroplast contains a Rubisco-binding protein that helps to assemble the large and small subunits of Rubisco into a functional enzyme in normal conditions. It has been suggested that HSP 70 functions to prevent the disassembly and denaturation of multimeric aggregates during heat stress. At the same time, increased ubiquitin levels reflect an increased demand for removal of proteins damaged by the heat shock (Table 17.1).

TABLE 17.1 Principal heat shock proteins (HSP) found in plants and their probable functions. Families are designated by their typical molecular mass. The number and exact molecular mass of proteins in each family vary depending on plant species.

HSP Family	Probable Function
HSP 110	Unknown.
HSP 90	Protecting receptor proteins.
HSP 70	ATP-dependent protein assembly or disassembly reactions; preventing protein from denaturation or aggregation (molecular chaperone). Found in cytoplasm, mitochondria, and chloroplasts.
HSP 60	Molecular chaperone, directing the proper assembly of multi-subunit proteins. Found in cytoplasm, mitochondria, and chloroplasts.
LMW HSPs (17–28 kDa)	Function largely unknown. LMW (low molecular weight) HSPs reversibly form aggregates called "heat shock granules." Found in cytoplasm and chloroplasts.
Ubiquitin	An 8 kDa protein involved in targeting other proteins for proteolytic degradation.

Based on Vierling, 1990.

Cells or plants that have been induced to synthesize HSPs show improved thermal tolerance and can tolerate exposure to temperatures that were previously lethal. Some of the HSPs are not unique to high temperature stress. They are also induced by widely different environmental stresses or conditions, including water deficit, ABA treatment, wounding, low temperature, and salinity. Thus, the synthesis of new proteins appears to be a common response to stress.

17.5 The Hypersensitive Reaction (HR) and Systemic Acquired Resistance (SAR)

The plant responses to the biotic stress are collectively known as a **hypersensitive reaction.** The hypersensitive reaction is commonly activated by viruses, bacteria, fungi and nematodes. This reaction to these disease causing agents include changes in the composition and physical properties of cell walls, the biosynthesis of secondary metabolites and the formation of necrotic lesions at the site of invasion. An early event in any case is the activation of defense-related genes and synthesis of their products, called **pathogenesis-related (PR) proteins.** PR proteins include proteinase inhibitors that inactivate proteolytic enzymes secreted by the pathogen and lytic enzymes such as 1,3 – glucanase and chitinase that

degrade microbial cell walls. Genes that encode enzymes for the biosynthesis of isoflavonoids and other phytoalexins that limit the growth of pathogens are also activated in response to biotic stress. Lignin, callose, and suberin are accumulated in cell walls along with hydroxyproline-rich glycoproteins that are believed to provide structural support to the wall. These deposits strengthen the cell wall and render it less susceptible to attack by the invading pathogen. Further, the invaded cells initiate **programmed death,** a process that results in the formation of necrotic lesions at the infection site. Cell necrosis isolates the pathogen, slowing both its development and its spread throughout the plant.

Plant responses to biotic stress are genetically programmed. Both pathogens and plants carry genes that determine the nature of their interaction – whether disease will occur (virulent) or not occur (avirulent). According to the gene-for-gene hypothesis, pathogenic microorganisms carry avirulence *(avr)* genes and host plants carry corresponding resistance (R) genes. Disease occurs only when the pathogen lacks the *avr* genes or the plant carries recessive alleles at the R locus. A matching pair of pathogen *avr* genes and dominant plant R genes initiates a hypersensitive reaction. The *avr* genes encode enzymes for the production of substances called **elicitors** and R genes encode receptors that recognize elicitors. A number of elicitors that occur extracellularly in association with cell walls of fungi and bacteria evoke a hypersensitive response in host plants. For example, fungal elicitors include glucans, chitosan, and an unsaturated fatty acid arachidonic acid. Other elicitors include various polysaccharides, glycoproteins, and small peptides.

Recognision of elicitors by the plant cell takes place at the plasma membrane. In the vast majority of cases, the elicitor binding with the receptor at the plasma membrane, initiates one or more sequences of biochemical reactions called signal tranduction pathway that connects the stimulus to a cellular response. Typically, the end result of signal transduction pathways is to regulate transcription factors, which in turn regulate gene expression. Expression of defense response genes can be regulated by regulating intercellular Ca^{2+} levels. Thus, defense responses can be activated by stimulating Ca^{2+} uptake with Ca^{2+} ionophores or inhibited by blocking Ca^{2+} channels. The change in ion fluxes in turn causes differential gene activation to form many components of the hypersensitive reaction.

The hypersensitive reaction appears to be an early warning system, sending signals to other cells and tissues that prepare them to resist secondary infections. Initially it is limited to the few cells at the point of invasion, but over a period of time, the capacity to resist pathogens gradually becomes distributed throughout the entire plant. In effect, the plant reacts to the initial infection by slowly developing a general immune capacity. This phenomenon is known as **systemic acquired resistance (SAR).**

According to the currently available information, the development of SAR in plants involves an elevated levels of **salicylic acid** (Figure 17.3). When tobacco plants are incubated with TMV, the salicylic acid level rises as much as 20-fold in the inoculated leaves and 2-fold in the noninfected leaves. Then it was concluded that both salicylic acid and its acetyl derivative (aspirin), when applied to tobacco plants, induces pathogenesis related proteins to resist tobacco mosaic virus. When a pathogen infects a plant, it stimulates a localized

hypersensitive reaction (HR) and synthesis of salicylic acid in the plant. Salicylic acid is translocated through the phloem to other regions of the plant where it prevents secondary infection by other pathogens. Alternatively, salicylic acid may be converted to methylsalycylic acid (MSA), which is moderately volatile and may function as an air borne signal (Figure 17.4).

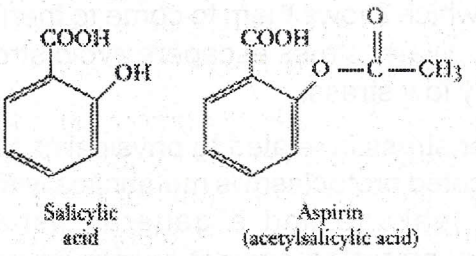

Figure 17.3 The chemical structure of salicylic acid and its commercial derivative acetylsalicylic acid (Aspirin).

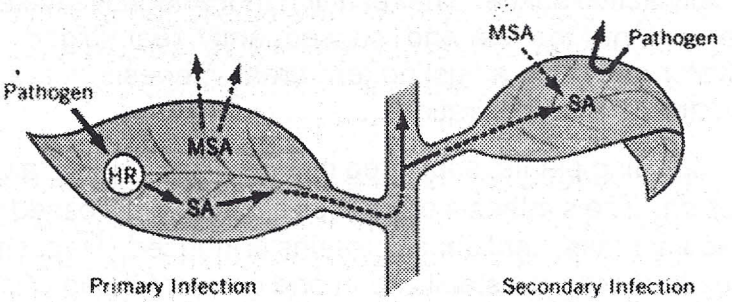

Figure 17.4 The possible role of salicylic acid in systemic acquired resistance (SAR). HR, hypersensitive reaction; SA, salicyclic acid; and MSA, methlsalysylic acid.

On the basis of recent experiments, it was suggested that jasmonates, especially jasmonic acid and its methyl ester (methyl jasmonate) also equally important in SAR processes of plants. Jasmonates have been studied extensively in the last decade and little is yet known about how they regulate gene expression. Jasmonic acid is synthesized from the unsaturated fatty acid, linolenic acid. Plant membranes are a rich source of linolenic acid in the form of phospholipids. It is thought that elicitors might bind with a receptor in the plasma membrane. The resulting elicitor-receptor complex, then activates a membrane bound phospholipase that release linolenic acid. The linolenic acid is oxidized to jasmonic acid which in turn acts to modulate PR gene expression.

17.6 Summary

Stress is usually defined as an external factor that exerts a disadvantageous influence on the plant. Under both natural and agricultural conditions, plants are exposed to

unfavorable environments that result in some degree of stress. Water deficit, heat stress and biotic stresses are some of the stressful conditions that restrict the plant growth and development.

Plants may respond to water stress in several ways. Water stress avoiders have mechanisms that isolate their cells from the stressful condition (CAM), while others tolerate stress by altering their metabolism which allows them to come to thermodynamic equilibrium with the stress but not suffer injury. Water stress escapers avoid stress by completing their life cycle during periods of relatively low stress.

Damage resulting from water stress is related to physical changes in the protoplasm and integrity of membranes. Desiccated protoplasm is metabolically dysfunctional and membrane damage leads to solute leakage and a general disruption of critical cell compartmentation. Most plants are protected against excessive desiccation by stomatal closure due to low turgor in the guard cells. Stomatal closure is triggered by decreasing water potential in the leaf. The hormone abscisic acid (ABA) appears to have a significant role in stomatal closure. Many plants cope with water stress by osmotic adjustment-an accumulation of osmotically active solute. The resulting more negative water potential helps to maintain water movement into the leaf and, consequently, leaf turgor. Yet other plants respond to prolonged water stress by adjusting leaf area. Senescence and abscission of older leaves reduce leaf area and transpiration.

Most organisms, including plants, subjected briefly to high temperatures, respond by changes in gene expression. The synthesis of most proteins is suppressed and synthesis of a new family of low molecular mass, heat shock proteins is induced. Heat shock proteins act as molecular chaperones that promote stabilization and correct folding of cell proteins.

Plants respond to biotic stress with a hypersensitive reaction. The hypersensitive reaction includes changes in the composition and increased strength of the cell wall, biosynthesis of phytoalexins, and necrotic lesions at the site of infection. These responses serve to isolate the potential pathogen and prevent its development and spread throughout the plant. Also associated with the hypersensitive reaction is the production of salicylic acid. Salicylic acid or its methyl ester may serve as a mobile signal, participating in systemic acquired resistance, a form of generalized immune response. Another possible signal agent is the jasmonic acid, a derivative of the fatty acid linolenic acid. The mechanisms of action of both salicylic acid and jasmonic acids are not yet clearly understood.

17.7 Model Questions

1. What is water stress ? Describe the effects and responses of plants to water stress.
2. Write short notes on
   a. Hypersensitive reaction
   b. Heat-shock proteins
   c. Systemic acquired resistance
   d. Stress hormone

17.8 Reference Books

1. Introductory plant physiology – G.R. Noggle and G.J.Fritz. Prentice Hall of India – New Delhi.
2. Plant Physiology – R.M. Devlin and F.H.Witham – CBS Publishers and distributors – New Delhi
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