Unit I

Metabolic Pathways in Higher Plants and their Determination

PCI Syllabus

Metabolic pathways in higher plants and their determination

- Brief study of basic metabolic pathways and formation of different secondary metabolites through these pathways- Shikimic acid pathway, Acetate pathways and Amino acid pathway.
- Study of utilization of radioactive isotopes in the investigation of Biogenetic studies

Book Chapter Content

- Introduction
- Basic Metabolic Pathways in Plants
- Shikimic Acid Pathway
- Biosynthesis of Amino Acids
- Acetate – Mevalonate Pathway
- Acetate Malonate Pathway
- Biosynthesis of Secondary Metabolites
  - Biosynthesis of Glycosides
  - Biosynthesis of Alkaloids
  - Biosynthesis of Isoprenoid Compounds
  - Biosynthesis of Triglycerides
  - Biosynthesis of Phenolic Compounds
- Stress Compounds
- Study of Utilization of Radioactive Isotopes in The Investigation of Biogenetic Studies
  - Tracer Techniques
  - Other Techniques to Investigate Biosynthetic Pathways

Introduction

Plant metabolism: It is defined as the complex physical and chemical events of photosynthesis, respiration, synthesis and degradation of organic compounds. Plant body is considered as a best biosynthetic laboratory than animal body for production of primary metabolites like sugars, amino acids and many secondary metabolites of pharmaceutical importance like glycosides, alkaloids, flavonoids, volatile oils, tannins, resins, enzymes, terpenes, color pigments etc.

Metabolism is considered as a sum of all biochemical processes and is distinguished into primary metabolism and secondary metabolism. The **primary metabolism** comprises of all the pathways necessary for survival of the cells, example photosynthesis, Calvin cycle, glycolysis, gluconeogenesis, Kreb's cycle etc.

Secondary metabolism produces a large number of specialized compounds (estimated around 200,000) which do not interfere in the growth and development of plants but are required for the plant to survive in its environment. Secondary metabolism is connected to primary metabolism by using building blocks and biosynthetic enzymes derived from primary metabolism. Primary metabolism governs all basic physiological processes that allow a plant to grow and set seeds, by translating the genetic code into proteins, carbohydrates, and amino acids. Specialized compounds from secondary metabolism are essential for communicating with other organisms through mutualistic (e.g. attraction of beneficial organisms such as pollinators) or antagonistic interactions (e.g. deterrent against herbivores and pathogens). They further assist in coping with abiotic stress such as increased UV-radiation. In any case, a good balance between products of primary and secondary metabolism is best for a plant's optimal growth and development as well as for its adjustment with often changing environmental conditions. Well known secondary metabolic compounds include alkaloids, glycosides, flavonoids, terpenoids etc. Humans use quite a lot of these compounds, or the plants from which they originate, for culinary, medicinal and nutraceutical purposes.

This unit comprises of overview of basic metabolic pathways and details about the secondary metabolites synthesis in plants.

Basic Metabolic Pathways in Plants

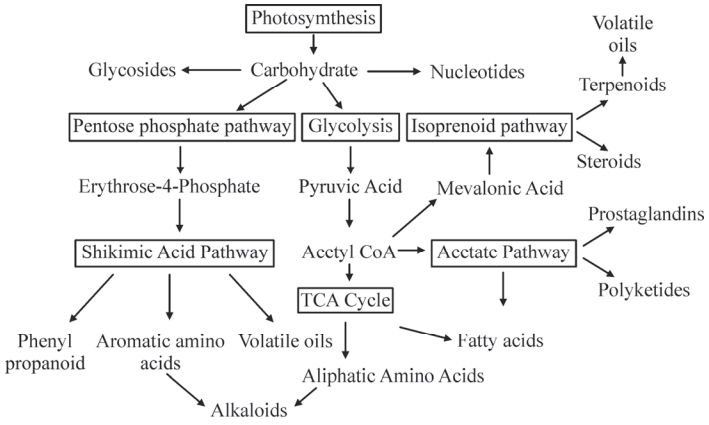


Fig. 1.1 Overview of biosynthesis of primary and secondary metabolites in plants

The products or byproducts of primary metabolism like carbohydrates, Acetyl COA, Shikimic acid and Mevalonic acid are used as building block or precursor for biosynthesis of secondary metabolites like alkaloids, glycosides, isoprenoids and many more. The important metabolic pathways are described here.

Shikimic Acid Pathway/Shikimate Pathway

The Shikimic acid pathway is a seven step metabolic pathway used by bacteria, archea, algae, fungi, some protozoans and plants for the biosynthesis of folates and aromatic amino acids *viz.* phenylalanine, tyrosine and tryptophan. This pathway is not found in animals and humans. Animlas and humans require these amino acids, hence the products of this pathway represents essential amino acids. The important steps involved in shikimic acid pathway are given below.

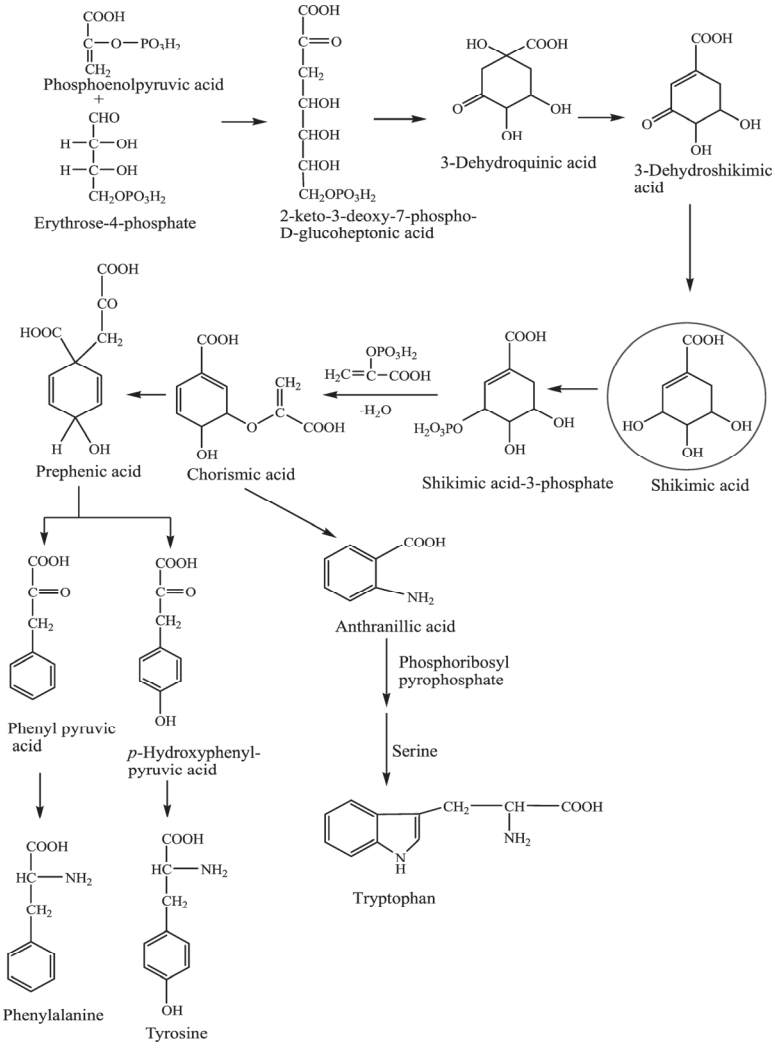


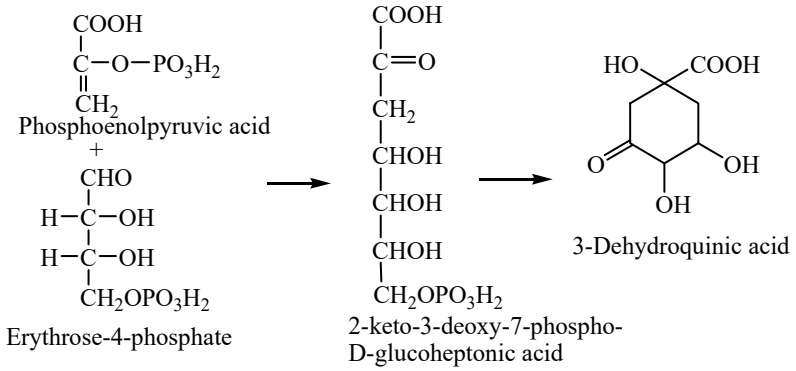
Fig 1.2 Shikimic acid pathway

Steps in Shikimic acid pathway

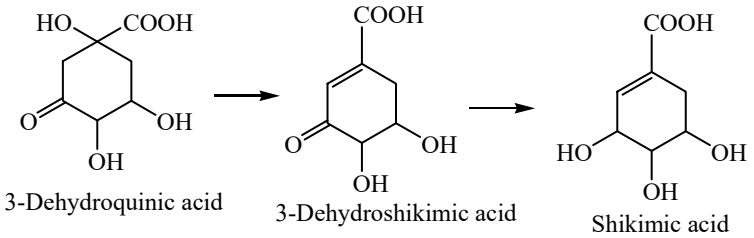
- Phosphoenolpyruvic acid and erythrose-4-phosphate react to form 2-keto-3-deoxy-7-phosphoglucoheptonic acid, in a reaction catalyzed by the enzyme *DAHP synthase*.

  2-keto-3-deoxy-7-phosphoglucoheptonic acid is then transformed to 3-dehydroquinic acid in a reaction catalyzed by *DHQ synthase*.

  Although this reaction requires nicotinamide adenine dinucleotide (NAD) as a cofactor, the enzymatic mechanism regenerates it, resulting in no net use of NAD.

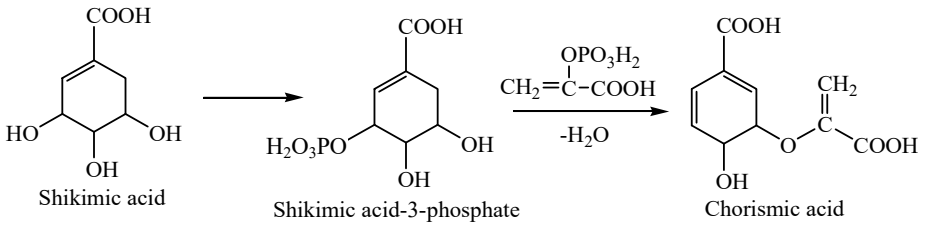


- 3-Dehydroquinic acid is dehydrated to 3-Dehydroshikimic acid by the enzyme 3-Dehydroquinate dehydratase, which is reduced to shikimic acid by the enzyme Shikimate dehydrogenase, which uses nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor.



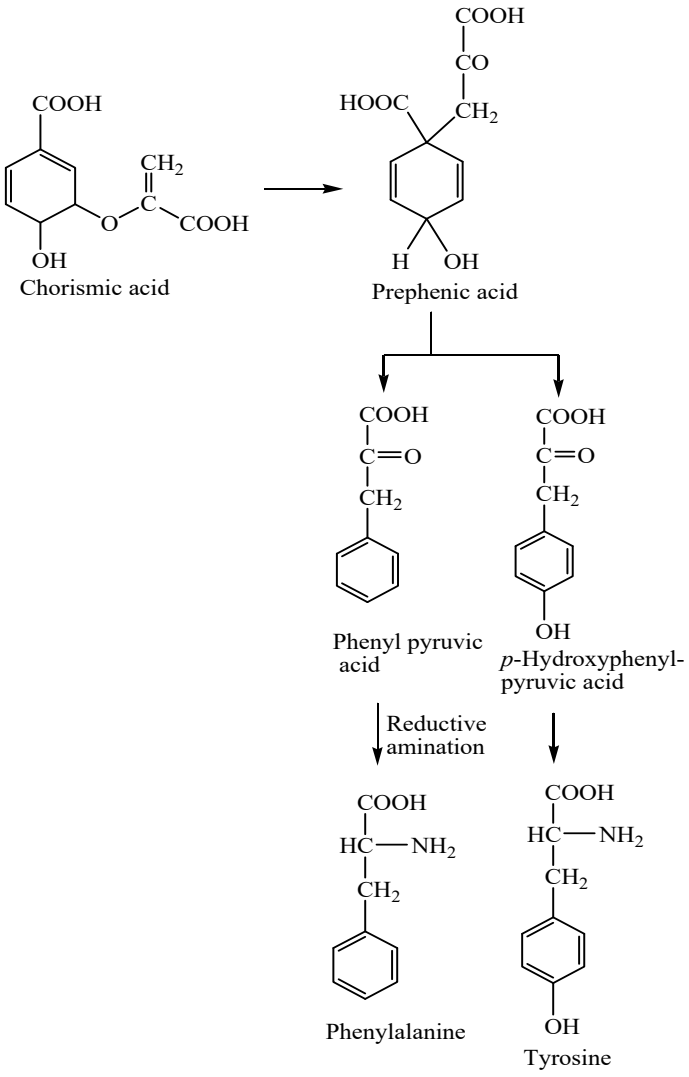
- The next enzyme involved is Shikimate kinase, an enzyme that catalyzes the ATP dependent phosphorylation of shikimic acid to form shikimate 3-phosphate. Shikimic acid 3-phosphate is then coupled with phosphoenol pyruvate to give 5-enolpyruvylshikimate-3-phosphate via the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase.

  Then 5-enolpyruvylshikimate-3-phosphate is transformed into chorismic acid by a chorismate synthase.

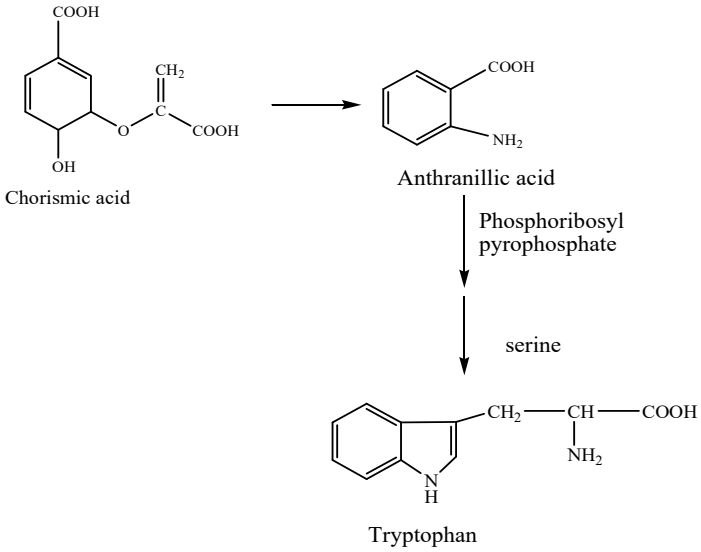


The formation of chorismic acid is the important step in the shikimic acid pathway as this compound can synthesize different types of intermediates.

- Prephenic acid is then synthesized by a Claisen rearrangement of chorismate by Chorismate mutase. The aromatic amino acids tyrosine and phenyl alanine are biosynthesized from prephenic acid through independent pathways.
- Prephenic acid is oxidatively decarboxylated with retention of the hydroxyl group by prephenate dehydrogenase to give *p*-hydroxyphenylpyruvic acid, which is transaminated using glutamate as the nitrogen source to give tyrosine and α-ketoglutarate.
- Similarly, prephenic acid undergoes aromatization to synthesize phenyl pyruvic acid followed by reductive transamination to synthesize phenyl alanine.



- In presence of glutamine, chorismic acid is converted to anthranilic acid. Later in conjugation with serine in presence of tryptophan synthase it converts into aromatic amino acid **Tryptophan**.



Importance of Shikimic Acid Pathway

Shikimic acid is a starting point of in the biosynthesis of some important secondary metabolites, as mentioned in the following examples.

1. Biosynthesis of glycosides *viz.*
   (a) Cyanogenetic glycosides- Prunacin, amygdalin
   (b) Isothiocyanate glycosides- Sinigrin
   (c) Coumarin Glycosides- Psoralen
   (d) Flavanoid glycoside- Quercetin, hesperidin
2. Biosynthesis of Alkaloids
   (a) Alkaloids derived from Tryptophan- Physostigmine, quinine
   (b) Alkaloids derived from Phenyl alanine, tyrosine and related amino acids- Ephedrine, papaverine etc.
3. Biosynthesis of lignin- Podophyllotoxin
4. Biosynthesis of Anthocyanins- Cyanidin
5. Biosynthesis of Phenyl Propanoids- Caffeic acid

Biosynthesis of Amino Acids

Amino acids are the precursors of some secondary metabolites particularly alkaloids. Plant synthesizes both the essential and non-essential amino acids. All amino acids are derived from

intermediates in Glycolysis, the Citric acid cycle or the Pentose Phosphate Pathway. Fig.1.3 represents the overview of amino acid synthesis in plants.

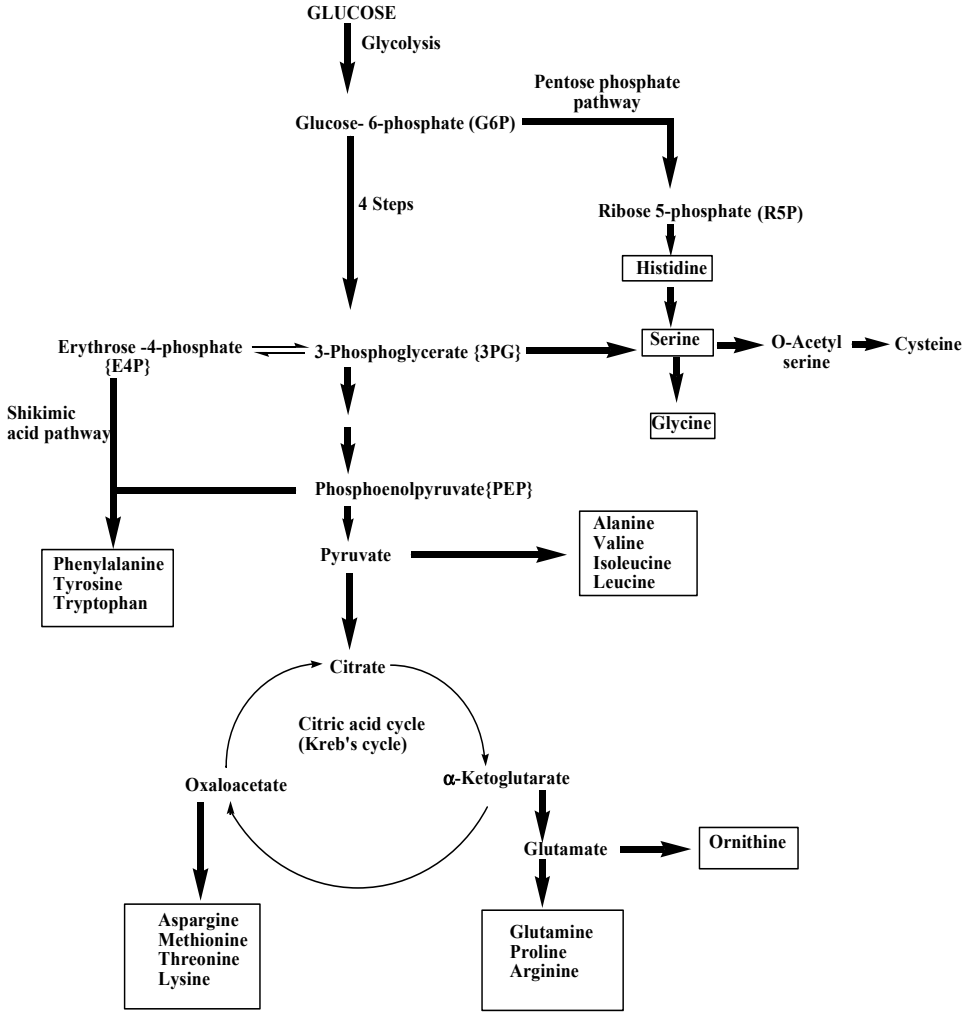


Fig. 1.3 Overview of Amino Acid synthesis

Biosynthesis of Phenylalanine, Tyrosine and Tryptophan (Aromatic amino acids)

The biosynthesis of Phenylalanine, Tyrosine and Tryptophan is well explained in Shikimic acid pathway.

Biosynthesis of Glutamate, Glutamine, Proline, Arginine and Ornithine

α- Ketoglutarate intermediate from Krebs cycle is involved in biosynthesis of several amino acids. The α-Ketoglutarate initially get converted into the amino acid glutamate in the presence of aminotransferase. This gutamate synthesizes the glutamine in presence of the enzyme

glutamine synthetase. L-glutamate also involved in the synthesis of proline. Ornithine is a non-protein amino acid formed mainly from L-glutamate in plants and synthesized from the urea cycle. Arginine get synthesized through ornithine. The biosynthesis of proline, arginine is a complex biochemical chain of reactions. The possible biosynthesis can be represented as in figure1.3 (A).

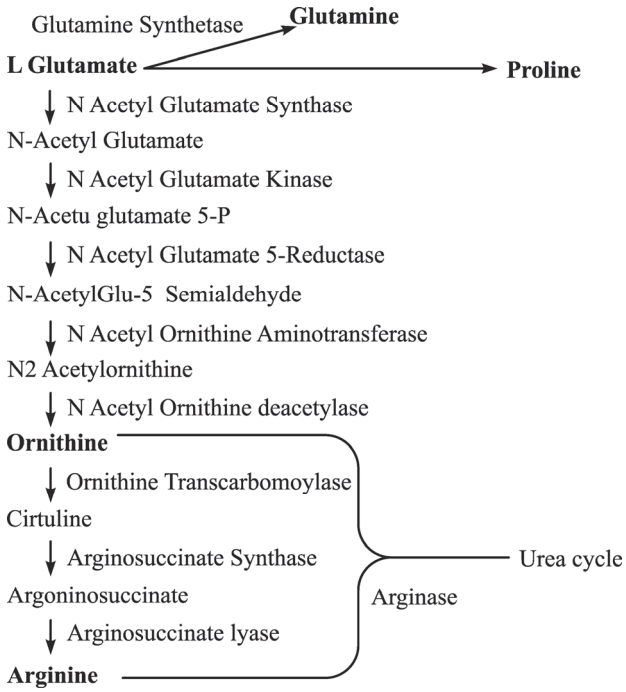


Fig. 1.3 (A) Overview of amino acid synthesis from L-Glutamate

Biosynthesis of Lysine, Aspargine, Methionine and Threonine

This is highly complex pathway which starts with intermediate of Kreb's cycle- Oxaloacetate. The oxaloacetate undergoes transamination to synthesize aspartate. In the presence of enzyme aspartokinase (catalyst) phsphorylation of aspartate takes place which initiates the conversion of aspartate to other amino acids. Lysine is synthesized from aspartate through diaminopimelate pathway. Aspartate also involves in biosynthesis of aspargine through aspargine synthetase enzyme. The intermediate aspartate 4-semialdehyde is produced which later involved in biosynthesis of methionine and threonine.

Biosynthesis of Serine, Glycine and Cysteine

Serine is the first amino acid produced from 3-phosphoglycerate which is originated from glycolysis through the enzyme phosphoglycerate dehydrogenase. The enzyme concentration in the cell is monitored by serine. Serine is then branched to synthesize other amino acids glycine and cysteine.

Biosyntheis of Alanine, Valine, Leucine and Isoleucine

Pyruvate is the key intermediate product of glycolysis which is involved in the biosynthesis of above amino acids. The few molecules of pyruvate is branched to synthesize alanine, valine, leucine and isoleucine and major part of pyruvate enters into Kreb's cycle.

Alanine is produced by the transamination of one molecule of pyruvate. Two molecules of pyruvates undergo condesation to produce α- acetolactic acid followed by α-keto-β-hydroxy valeric acid. The α-keto-β- hydroxy valeric acid is a intermediate to produce valine and leucine. It undergo reduction followed by transamination to synthesize valine. Similarly α-keto-β-hydroxy valeric acid undergoes acetate condensation and followed by sequence of reactions viz. reduction, dehydration and transamination to synthesize leucine

Isoleucine is produced by the same chain reaction as valine but starting with production of α-aceto-α-hydroxy propionic acid.

The Acetate – Mevalonate Pathway (Mevalonic Acid Pathway/Isoprenoid Pathway)

The Acetate mevalonate pathway is also known as isoprenoid pathways as it results in the isoprenoid synthesis via formation of isoprene units. HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase) enzyme plays an important role in the formation of Mevalonic acid hence pathway also known as HMG-CoA pathway. Acetic acid plays an important role in the biosynthesis of cholesterol, squalene and many steroidal compounds which are synthesized through acetate pathway. In 1950's the discovery of acetyl Coenzyme A confirmed the role of acetic acid in biogenetic pathways.

Acetyl coenzyme A from citric acid cycle undergoes condensation with another molecule of acetyl coenzyme A to form Acetoacetyl CoA followed by condensation with another acetyl coenzyme A molecule to form 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). HMG-CoA get reduced to mevalonic acid. This Mevalonic acid acts as a precursor in the synthesis of isoprenoid compounds. The 'active isoprene' C_5 units; isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) are the key intermediates synthesized by Mevalonic acid pathway. Both units yield Geranyl pyrophosphate (C_{10}- monoterpene). This Geranyl pyrophosphate again in association with IPP unit synthesizes Farnesyl pyrophosphate (C_{15} – sesquiterpene). Farnesyl pyrophosphate in further association with one IPP unit produces geranyl-geranyl pyrophosphate (C_{20} - diterpenes). This molecule further undergoes cyclization process to produce steroidal and penta cyclic triterpenoid skeleton. In this way acetate mevalonate pathway biosynthesizes wide range of monoterpenoids to pentacyclic triterpenoids.

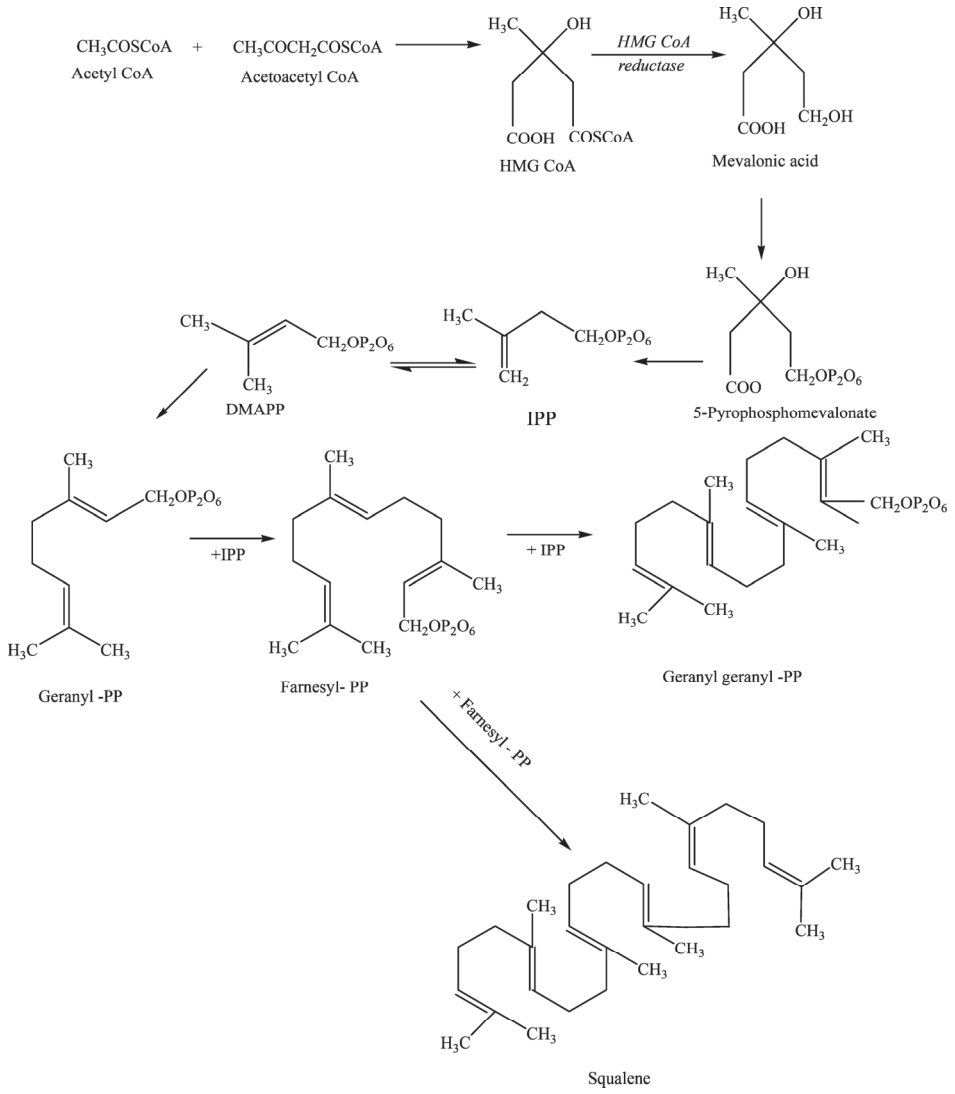


Fig. 1.4 The Acetate – Mevalonate Pathway

Acetate Malonate Pathway

The Acetate malonate pathway is mainly responsible for the synthesis of fatty acids which involves enzyme fatty acid synthase. It involves Acyl carrier protein (ACP) to yield fatty acid thioesters of ACP. These fatty acid thioesters form key intermediates in fatty acid synthesis. These C_2 acetyl CoA units further produce even number of fatty acids from butyric acid to arachidonic acid. Unsaturated acids are produced by subsequent direct dehydrogenation of saturated fatty acids. Enzymes involved in pathway plays an important role in governing position of newly introduced double bonds in the fatty acids.

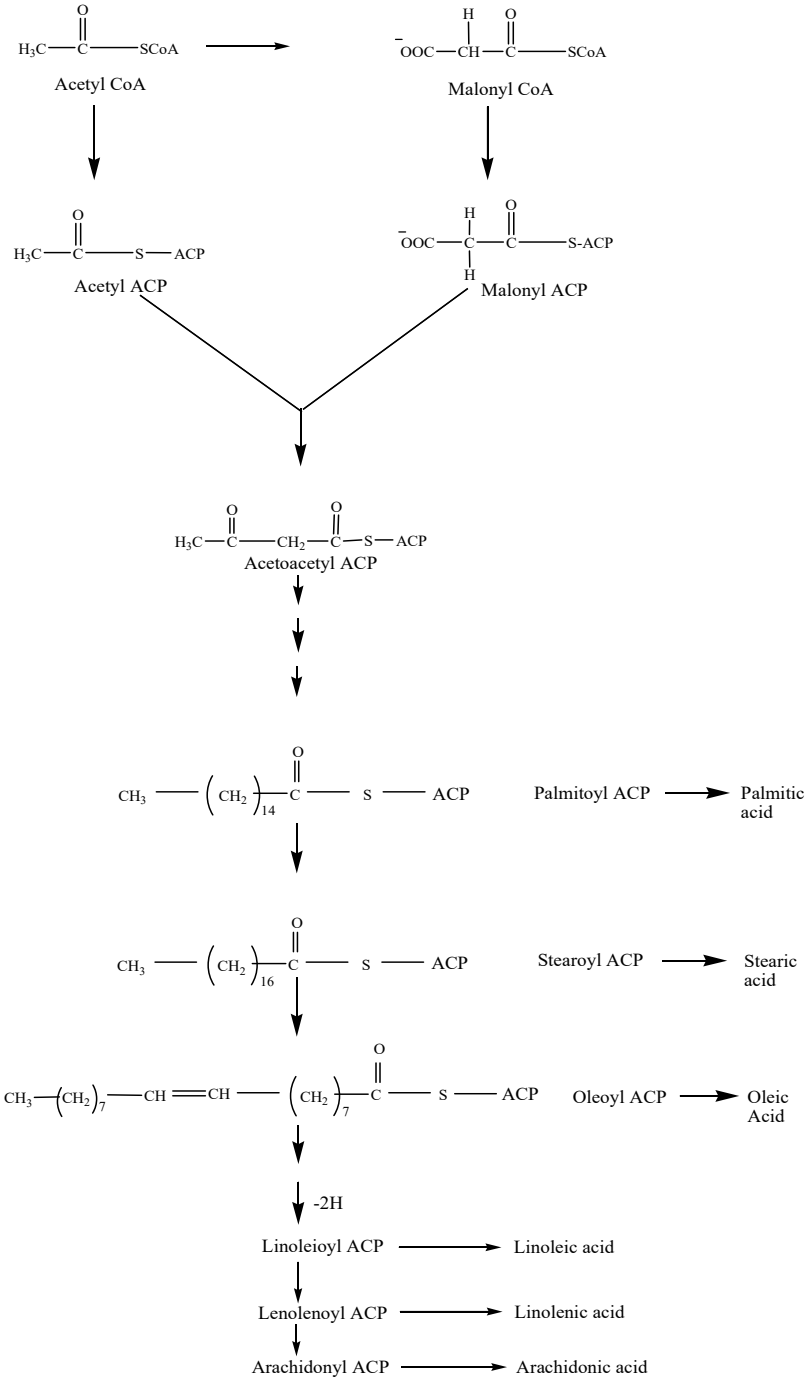


Fig. 1.5 The Acetate Malonate Pathway

Biosynthesis of Secondary Metabolites

Biosynthesis of Glycosides

The glycosides are the condesation products of Glycone (sugar) and Aglycone (non sugar) units. The reaction occurs in two parts; the first part of biosynthesis is the formation of a aglycone part and second part is coupling of aglycone unit with glycone moity.

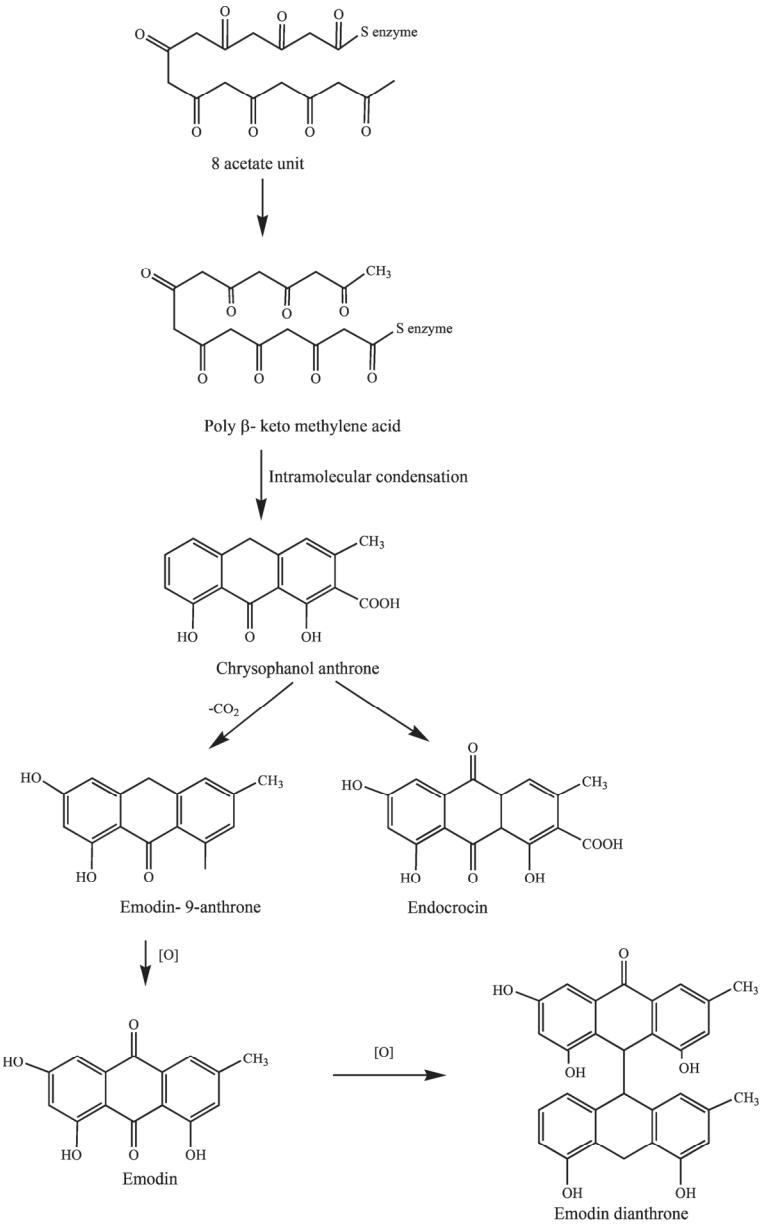
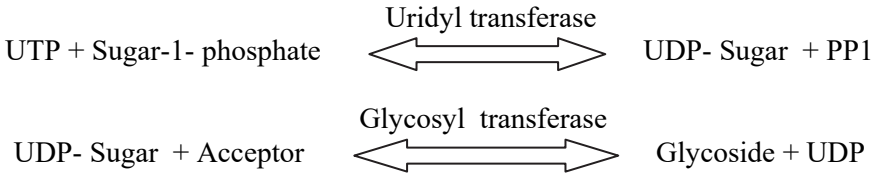


Fig. 1.6 Biosynthesis of Emodin and other Anthraquinone derivatives

The glycoside formation involves the transfer of uridyl group from uridine triphosphate (UTP) to sugar-1-phosphate in the presence of enzyme uridyl transferases to produce sugar-uridine diphosphate complex. In subsequent reaction this sugar nucleotide complex reacts with acceptor (Aglycone) units which leads to glycoside production.



A. *Biosynthesis of Anthracene Glycosides*: The knowledge of biosynthesis of anthracene aglycone has been established from the (Fig. 1.6) studies with microorganisms specifically *Penicillium islandicum.*

***Synthesis of Emodin and other related derivatives*:** An intermediate poly β-ketomethylene acid is considered to be formed from 8-acetate unit which undergoes intermolecular condensation gives rise to emodin and other related anthraquinone derivatives.

***Synthesis of Alizarin*:** The another pathway for production of anthraquinone glycoside is through the Shikimic acid – mevalonic acid mediators. This pathway can be observed in Rubiaceae family plants, eg: Biosynthesis of Alizarin from *Rubia tinctorum* (Rubiaceae).

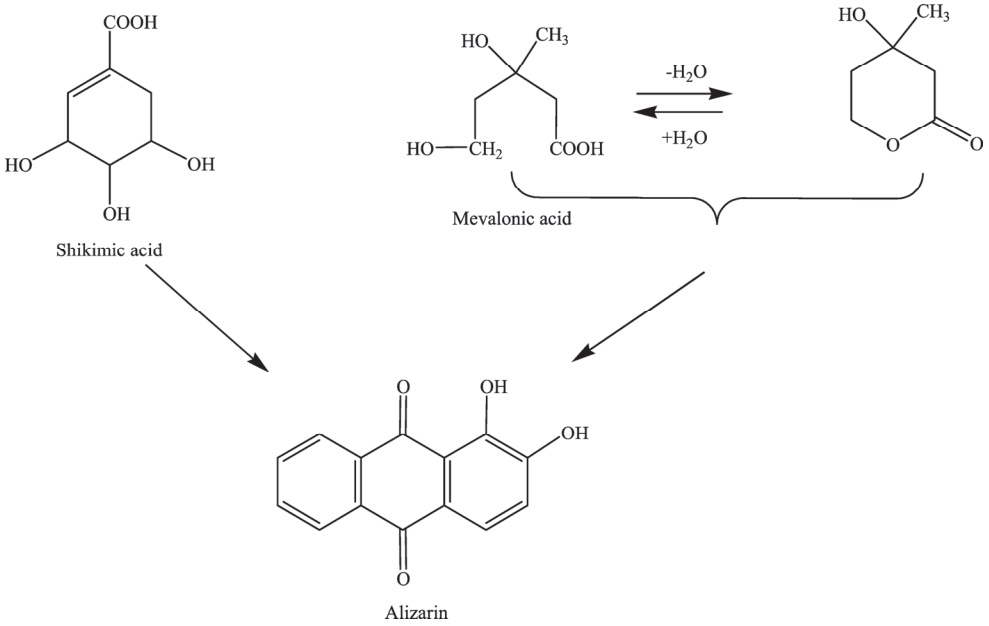


Fig. 1.7 Biosynthesis of Alizarin from *Rubia tinctorum* (Rubiaceae)

B. *Biosynthesis of Cyanogenetic Glycosides*: The aglycone units of cyanogentic glycosides are the phenylpropanoid compounds derived from aromatic amino acids phenylalanine and tyrosine obtained from Shikimic acid pathway, eg; Prunacin and Dhurrin.

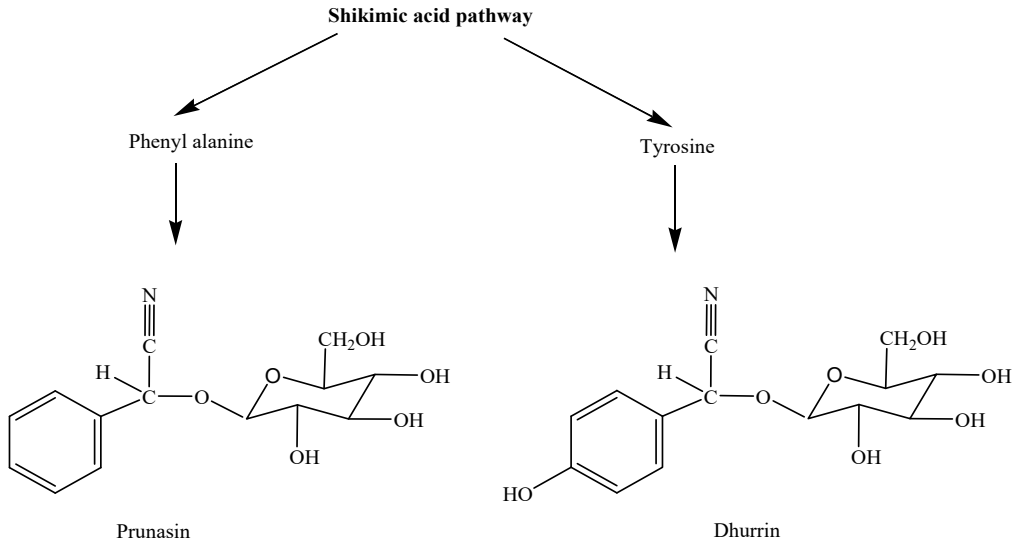


Fig. 1.8 Biosynthesis of Cyanogenetic Glycosides

C. ***Biosynthesis of Isothiocyanate aglycone*****:** The aglycones of isothiocyanate may consist of either aliphatic derivative biosynthesized via acetate pathway or aromatic derivatives produced biosynthetically via Shikimic acid route.

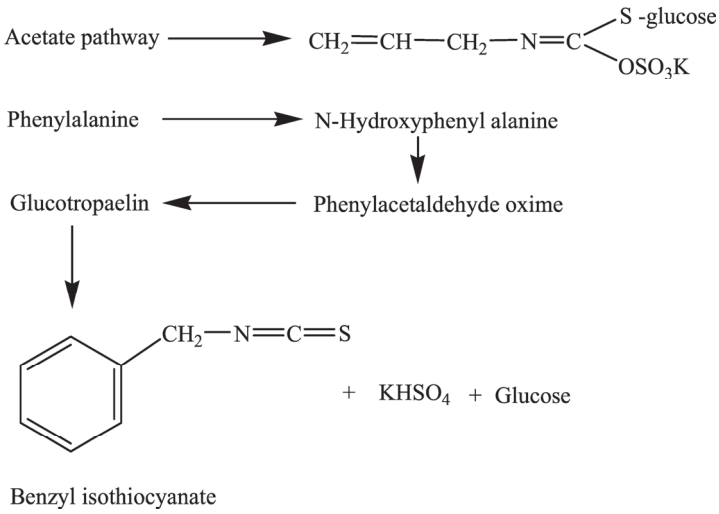


Fig. 1.9 Biosynthesis of Isothiocyanate Aglycone

D. ***Biosynthesis of Flavonoid Aglycone*****:** The aglycones of Flavonol glycosides are derived from both acetate metabolism and Shikimic acid athway. The A ring arises by head –to-tail condensation of two malonyl Co-A units and Acetyl Co-A unit. The B ring and C_3 unit comes from a C_6-C_3 precursor, which may be cinnamic acid.

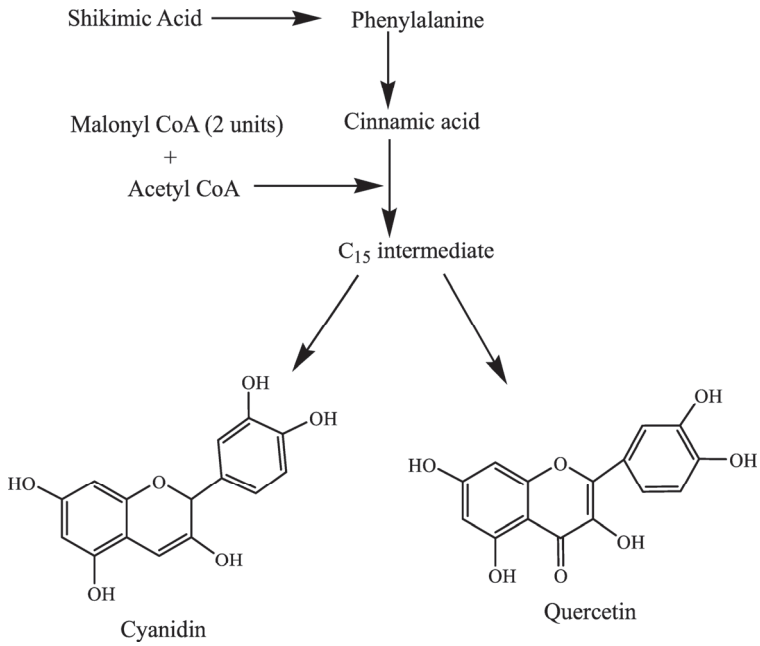


Fig. 1.10 Biosynthesis of Flavonoid Aglycone

E. ***Biosynthesis of Lactone, Phenol, Alcohol and aldehyde Glycoside*****:** The aromatic nuclei of alcohol, aldehyde, lactone and phenol glycosides are derived from C_6-C_3 precursor formed via Shikimc acid pathway.

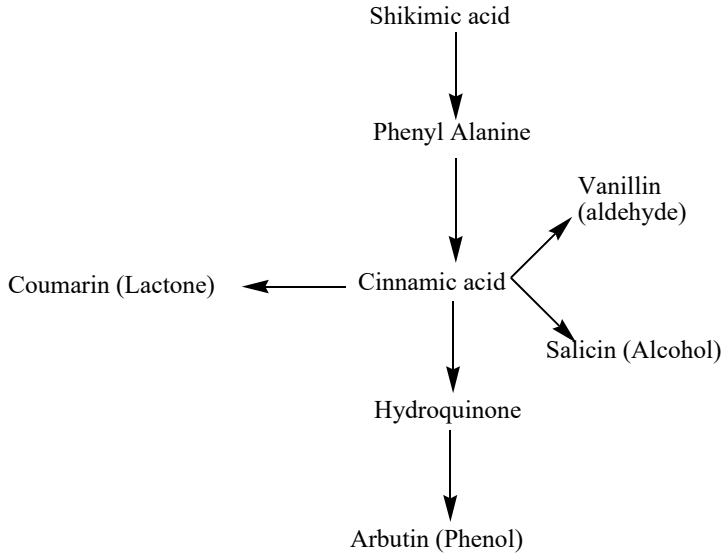


Fig. 1.11 Biosynthesis of Lactone, Phenol, Alcohol and aldehyde Glycoside

F. Biosynthesis of Aglycones of Cardiac Glycosides and Saponins: The aglycones of cardio active glycosides are steroidal in nature. They are the derivatives of cyclopentanophenanthrene ring which contains the following.

An unsaturated lactone ring attached to C 17 ,

A 14-α Hydroxyl group and

A cis- juncture of rings C & D

The steroidal biosynthesis is relatively similar to the cholesterol production via Acetate, Mevalonate, isopentenyl pyrophosphate followed by squaline pathway. The biosynthesis of cholesterol involves cyclization of aliphatic triterpene-squalene. Eg: Biosynthesis of Digitoxigenin, Digoxigenin and Gitoxigenin.

Similarly neutral sapogenins (saponin glycosides) are derivatives of steroids while acid sapogenins possess triterpenoids. Both the sapogenins are biosynthesized by similar pathway till the production of triterpenoid hydrocarbon squalene. This further branches to give cyclic triterpenoid in one direction and steroids in another direction. Figure 1.12 shows the brief outline of biosynthesis of certain steroidal compounds viz. squalene, cholesterol, digitoxigenin, scillarennin etc.

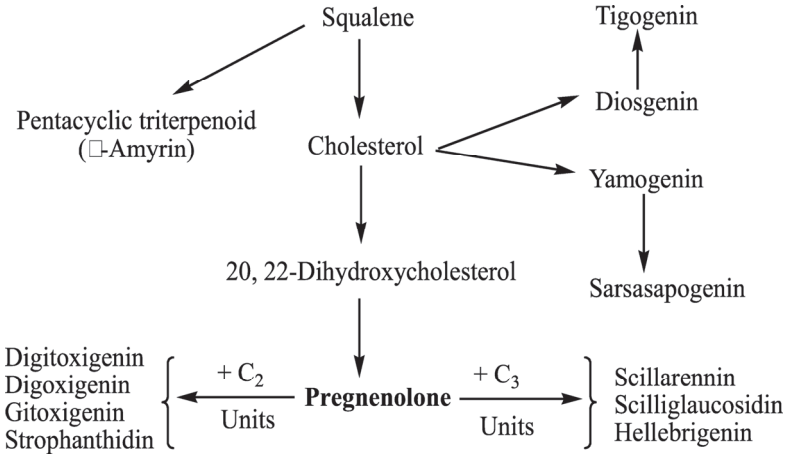


Fig. 1.12 Biosynthesis of steroidal aglycone

Biosynthesis of Alkaloids

Alkaloids are the nitrogen containing compounds; some derived from purines / pyrimidines and majority are produced from amino acids. Ornithine produces pyrrolidine and tropane alkaloids while lysine gives piperidine, quinolizidine, and indolizidine alkaloids. Similarly, nicotinic acid gives rise to pyridine alkaloids and tyrosine produces phenyl ethylamines and simple tetra hydroisoquinoline alkaloids. Pharmacologically important alkaloids are discussed below;

A. *Alkaloids derived from ornithine*: Ornithine is a precursor of the cyclic pyrrolidines *viz*. alkaloids of tobacco (nicotine, nornicotine) and other plants of Solanaceae family. The

amino acid ornithine on decarboxylation produces putrescine and proline- the basic unit of tropane, ecgonine and nicotine group of alkaloids (pyrolidine ring).

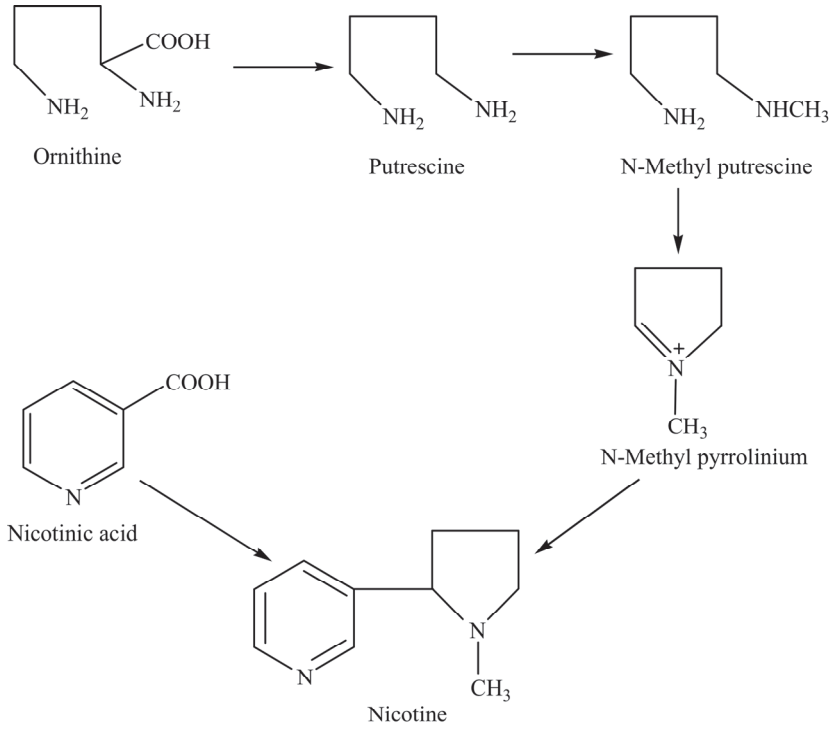


Fig. 1.13 Biosynthesis of Nicotine

B. ***Alkaloids derived from lysine*****:** Lysine is a precursor for piperidine which forms the basic skeleton for many alkaloids. Lysine and its derivatives are responsible for the biosynthesis of some of the bitter principles *viz*. lupine, lupanine, anabasine and other related compounds.

3L-lysine ⟶ 3-cadaverine ⟶ 17-oxosparteine ⟶ lupanine

Fig. 1.14 Biosynthesis of Lupanine

C. ***Alkaloids derived from phenyl alanine, tyrosine and related amino acids*****:** The amino acids phenyl alanine, tyrosine and related amino acids and their respective decarboxylation products serve as a precursor for many alkaloids. Tyrosine is considered to be a precursor for the huge family containing alkaloids. The first essential intermediate is dopamine; dopamine is the precursor in the biosynthesis of papaverine, berberine, and morphine.

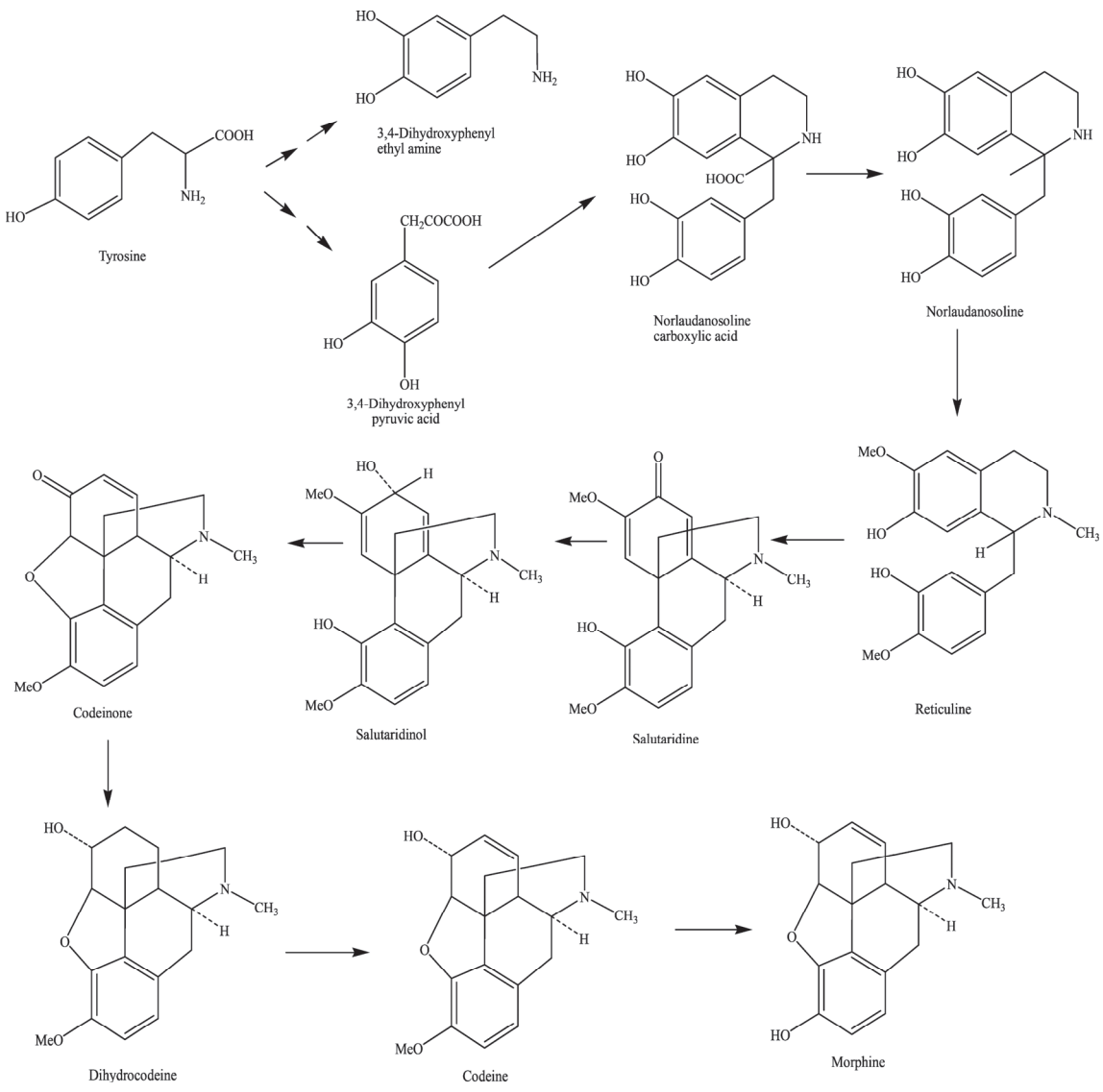


Fig. 1.15 Biosynthesis of Morphine

D. Alkaloids derived from tryptophan: Important group of alkaloid derived from tryptophan is indole alkaloids. Tryptophan and its decarboxylation product tryptamine, serve as a precursor for biosynthesis of a large number of indole alkloids.The biosynthesis of quinine and related alkaloids in cinchona takes place through the tranformation of indole to quinine.

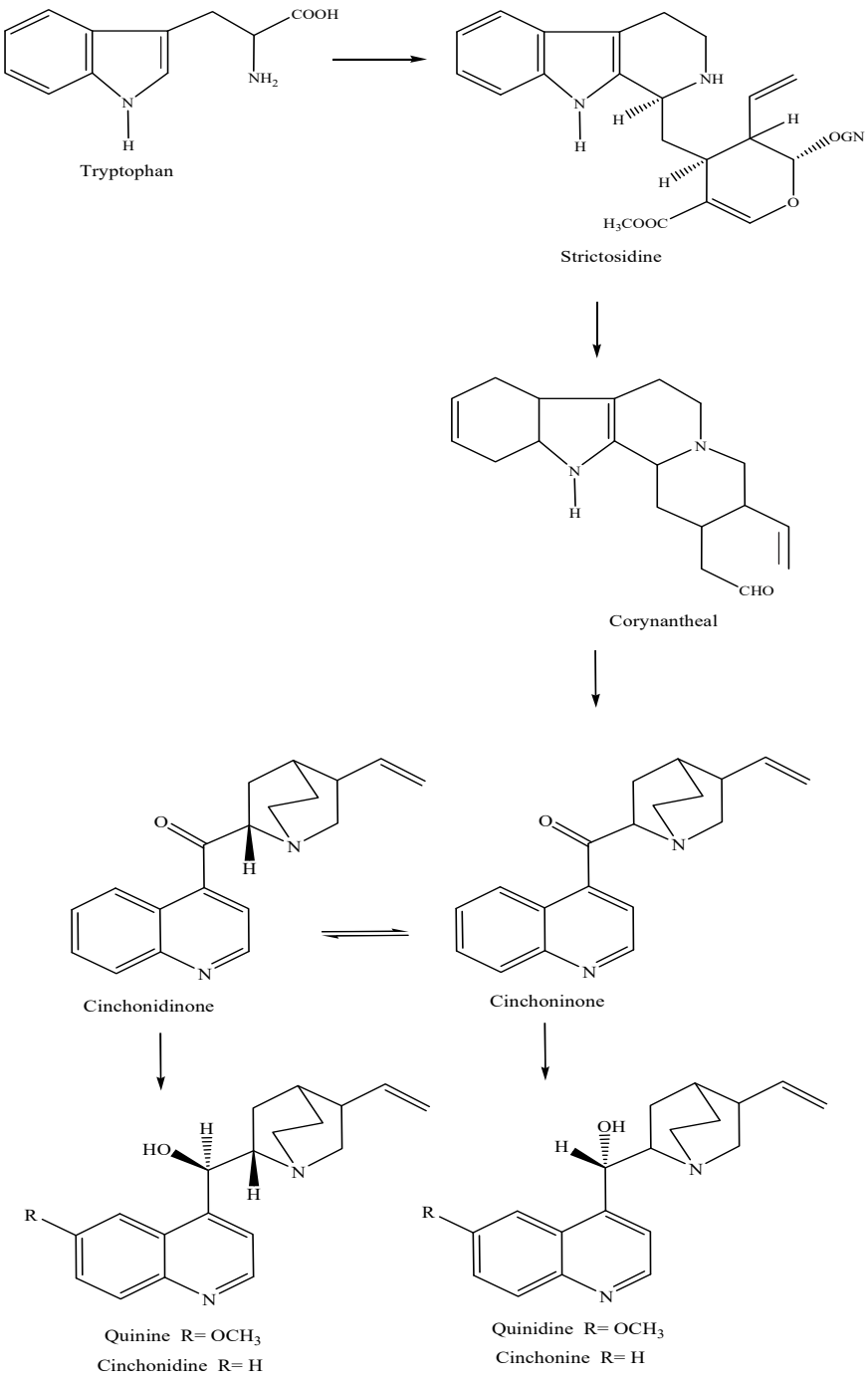


Fig 1.16 Biosynthesis of Cinchona alkaloids

Biosynthesis of Isoprenoid Compounds

Polymeric isoprene derivatives viz. steroids, carotenoids, gibberellic acid etc. though shows structural variation, are synthesized by very few pathways. To understand the biosynthesis of isoprenoid compound, acetate mevalonate pathway can be reffered.

Biosynthesis of Triglycerides (Fats and Fatty Acids)

Biosynthesis of triglycerides occurs in two stages; biosynthesis of fatty acid and formation of triglyceride. (Refer Acetate malonate pathway).

Biosynthesis of Phenolic Compounds

Most of the phenolic compounds are flavonoids. Flavonoids have basic skeleton from C_{15} body of flavones. Flavones occures both as colored and colorless nature. Some of the common phenolic compounds are coumarin, flavone, flavonol, anthocyanidines.

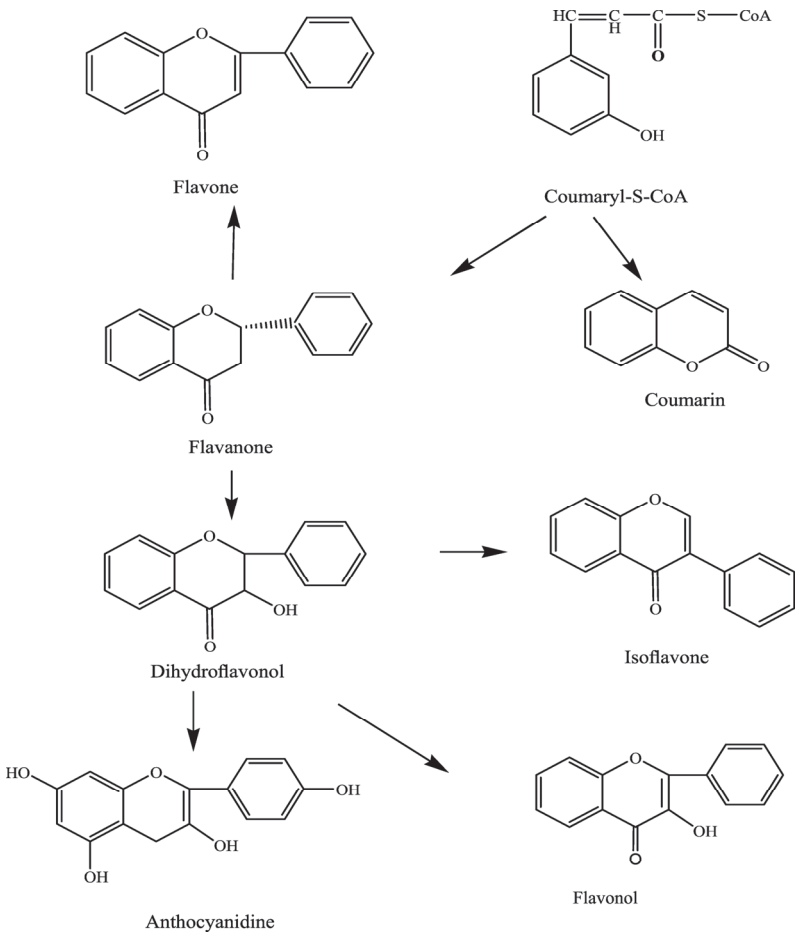


Fig. 1.17 Biosynthesis of Phenolic Compounds

Stress Compounds

These are the compounds which accumulate in the plants at higher than a normal level as a result of the biotic stress (attack by the pathogens, insects or herbivores) or abiotic stress (particularly contamination by heavy metals and different types of fertilizers) or due to injury or disturbances in normal metabolism (due to polymerization, oxidation or hydrolysis of natural substances). These are the products of primary or secondary metabolism. Several environmental and biological factors promote the synthesis of stress compounds these include mechanical wounding of plants, salinization, frost damage, desertification, drought, exposure to cold and salt. Chemically stress compounds are of different nature which includes phenols, resins, carbohydrates, hydroxycinnamic acid derivatives, coumarins, bicyclic sesquiterpenes, triterpenes and steroidal compounds. Stress compounds are also of pharmaceutical importance as they may be involved in various crude drugs formed pathologically *viz*. gums and oleoresins are potential drugs. Phytoalexins is one of the examples of stress compounds produced as an antifungal by plants after fungal infestation. The antifungal isoflavonoid pterocarpans produced by many species of Leguminosae family as a stress compound.

Study of Utilization of Radioactive Isotopes in the Investigation of Biogenetic Studies

Investigation of biogenetic pathways are generally carried out by using the radioactive isotopes.

Isotopes: Isotopes of a given element have nuclei with the same number of protons but different numbers of neutrons. They have different physical properties but the same chemical properties. Some isotopes are stable; however, radioisotopes are unstable and disintegrate, with the emission of three main types of radiation- alpha, beta and gamma radiations. A radioisotope, or any compound that contains a radioisotope, is said to be radiolabeled and is called a radionuclide. Each radioisotope has a characteristic rate of decay and pattern of radiation. For example, 14-C is a low energy beta emitter with a half-life of 5500 years. The labelled compounds are prepared using radioactive isotopes. The common radioactive isotopes in wide use are ^{14}C, ^{3}H, ^{35}S, ^{32}P, ^{36}Cl, ^{131}I, ^{60}Co. Different isotopes are used in different studies; nitrogen atom is used in the studies on proteins, alkaloids and amino acids; carbon and hydrogen are useful in biological investigations and metabolic pathways and oxygen is useful in studies on terpenoids.

Many methods have been used for the investigation of different metabolic pathways by using radiolabeled compounds and they are as follows

Tracer Techniques

Tracer techniques are the techniques which utilizes a labelled radioactive compound/ isotope to find out the different intermediates and various steps involved in biosynthetic pathways in a plant at a given time and rate. These compounds after administration in the plant become a part

of general metabolic pathway of the plant and undergo characteristic metabolic reactions associated with plant metabolism. The different types of tracer techniques are as follows.

- Use of isolated organs
- Grafting methods
- Use of mutant strain

Criteria for selection of tracer

- The physical and chemical nature of the presumed precursor of biochemical reaction must be known for proper labeling.
- The initial concentration of labeled compound must be sufficient to complete the targeted biogenetic pathway.
- The labeled isotope should be of a sufficiently longer half-life.
- The labeled compound should not damage the system into which it has been inserted.

Steps involved in tracer techniques

1. Preparation of radio labeled compound.
2. Introduction of labeled compound into a biological system.
3. Separation of the labeled compound and determination of nature of metabolites in various biochemical fractions.

1. **Preparation of radio labeled compound:** The different radioactive isotopes are prepared by different methods. The radioactive ^{14}C can be prepared synthetically and naturally. The purity of ^{14}C used for biochemical investigations is highly important.^{14}C can be prepared by two methods;
   A. By the bombardment of ^{14}N with slow neutrons on the target material viz aluminum beryllium nitride in nuclear reactor. The disadvantage is that the labeled carbon produced by this method may contaminate with inorganic radioactive carbon compounds.
   B. The other method used is by growing algae *Chlorella* in an atmosphere containing $^{14}CO_2$. All the carbon compounds of the organism thus become labeled, possessing uniform labeling of each carbon atom.

   Tritium ^{3}H is effected by catalytic exchange (Platinum catalyst) in aqueous media, by irradiation of organic compounds with tritium gas and by hydrogenation of unsaturated compounds with tritium gas.
2. **Introduction of labeled compound into a biological system**

   Before the selection of method for introduction of radiolabeled compounds, the following precautions must be taken
   - The presumed precursor should introduce at specific site so that it takes part in the biochemical reaction.
   - The introduction time of labeled compound should be correct so that the presumed precursor should be available during the biosynthesis of the compound.

- The dose should be as minimum as possible to avoid the involvement of labeled compound in other biochemical reactions

The different methods for introduction of labeled compounds are available. These are:

***Root feeding*:** The plants in which the roots are site for biosynthetic reactions for production of targeted compounds, root feeding technique is used. e.g. Production of Datura and tobacco alkaloids. The plants are hydroponically cultivated to avoid microbial contamination along with labeled compound.

***Stem feeding*:** Substrate can be administered through cut ends of stem immersed in solution with necessary nutrients and labeled compounds. The stems which oozes latex after cutting are not suitable for this method.

***Direct injection*:** This is the easiest method for introduction of labeled compounds. Direct injection is possible for those plants which bears the hollow stems. e.g. Opium

***Infiltration*:** This method is suitable for the plants rooted in soil or with other support. The infiltration method enables to introduce the labeled compounds without disturbing the roots (wick feeding).

***Floating methods*:** This method is used when small amount of material is available. This technique is used in conjugation with vacuum infiltration to remove gases.

***Spray technique*:** The radiolabeled compound is mixed in water and then spread over the leaves. The compound gets absorbed in the leaves and participate in metabolic pathways. e.g steroids.

3. Separation of the labeled compound and determination of nature of metabolites in various biochemical fractions

***Separation of labeled compounds*:** For the successful separation of radiolabeled compound from the part of plants, different methods and solvents are used based on the nature of the part of the plant. For soft and fresh tissue maceration or infusion is used. For hard tissue decoction or percolation is used and for separation of labeled compound from unorganized drug maceration with adjustments is preferred.

Depending upon the nature of the presumed product, the solvent is selected for separation *viz*. for fats and oils, nonpolar solvents are used while for alkaloids, glycosides slightly polar solvents are used and for phenols polar solvents are used.

***Determination of labeled compounds*:** The different types of detectors are used for identification of radiolabeled compounds as mentioned below.

- Geiger – Muller counter
- Scintillators
- Autoradiography
- Mass spectroscopy
- NMR spectroscopy
- Gas ionization chamber

- Bernstein – Bellentine counter
- Radio paper chromatography

Important methods of determination of radiolabeled compounds are explained here.

Geiger – Muller Counter

The Geiger – Muller (GM) counter is an instrument used for measuring the ionizing radiation. It detects ionizing radiations *viz*. alpha particles, beta particles and gamma radiations using the ionization effect produced in a Geiger – Muller tube. A Geiger counter consists of a Geiger Muller tube with pair of electrodes, the sensing element which detects the radiation, processing units and results in display. Geiger Muller tube is filled with an inert gas such as helium, neon, or argon at low pressure and to this high voltage is applied. When radiation enters the tube, it ionizes the gas, the ions get attracted towards the electrode and an electric current is produced. A scale counts the current pulses and one can get the count whenever radiations ionizes the gas.

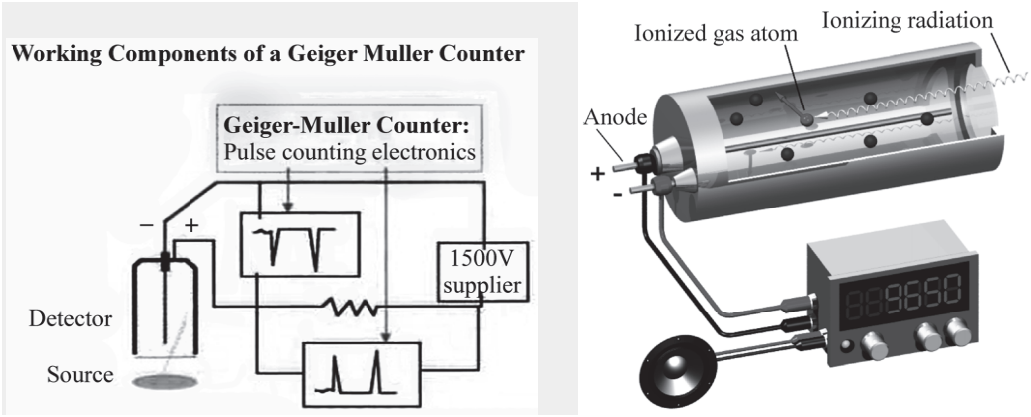


Fig. 1.18 Components of Geiger Muller counter

***Advantages*:** It is relatively economical, durable, easily portable and detects all types of radiations.

***Disadvantages*:** It cannot differentiate the type of radiation is being detected. It has less sensitivity and efficacy.

Scintillators

Scintillations literal meaning is luminescence and scintillators are materials which have property of luminescence, when excited by ionizing radiation. Scintillation counter is an instrument for detecting and measuring the ionizing radiations by using the excitation effect of incident radiation on a scintillator material and detecting the resultant light pulses. The scintillators detect the radiation with the help of photomultiplier tube that gives rise to an equivalent electric pulse. When an ionizing particle passes into the scintillator material, atoms are ionized along a track. The photon from the scintillation strikes a photocathode and emits an electron which

accelerated by a pulse and produce a voltage across the external resistance. This voltage is amplified and recorded by an electron counter.

***Applications*:** Scintillator counters are used to measure radiation in a variety of applications including handheld radiations survey meters, personal and environmental monitoring for radioactive contamination, medical imaging, radiometric assay, nuclear security, and nuclear plant safety.

***Advantages*:** It can accommodate samples of any type including liquids, solids, suspensions and gels.

- Easy sample preparation methods.
- It can count separately different isotopes in the same sample so dual labeling experiments can be carried out.
- Scintillators are highly automated, efficient and highly accurate.

***Disadvantages*:**

- It is very expensive.
- If high voltage applied to photomultiplier, electronic events occurs in the system that are independent of radioactivity known as photomultiplier noise.

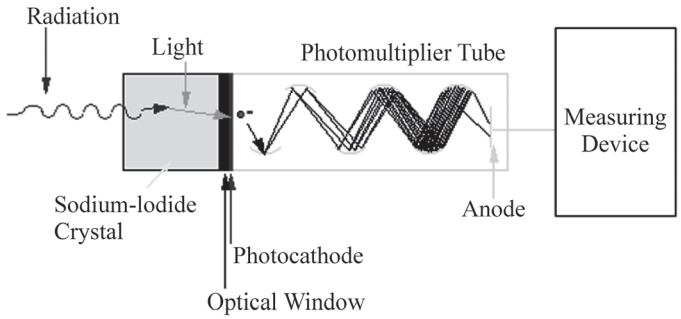


Fig 1.19 The photomultiplier Scintillator

Autoradiography

Autoradiography is the bio-analytical technique used to visualize the distribution of radioactive labeled substance with radioisotope in a biological sample. It is a method by which a radioactive material can be localized within a particular tissue, cell, cell organelles or even biomolecules. Autoradiography is based upon the ability of radioactive substance to expose the photographic film by ionizing it. In this technique a radioactive substance is put into direct contact with a thick layer of a photographic emulsion (thickness of 5-50 mm) having gelatin substances and silver halide crystals. It is then left in dark for several days for proper exposure. The silver halide crystals are exposed to the radiation which chemically converts silver halide into metallic silver (reduced) giving a dark color band. The resulting radiography is viewed by electron microscope, preflashed screen, intensifying screen, electrophoresis or digital scanners. It is a

very sensitive technique and is being used in a wide variety of biological experiments. Autoradiography, although used to locate the radioactive substances, it can also be used for quantitative estimation by using densitometer.

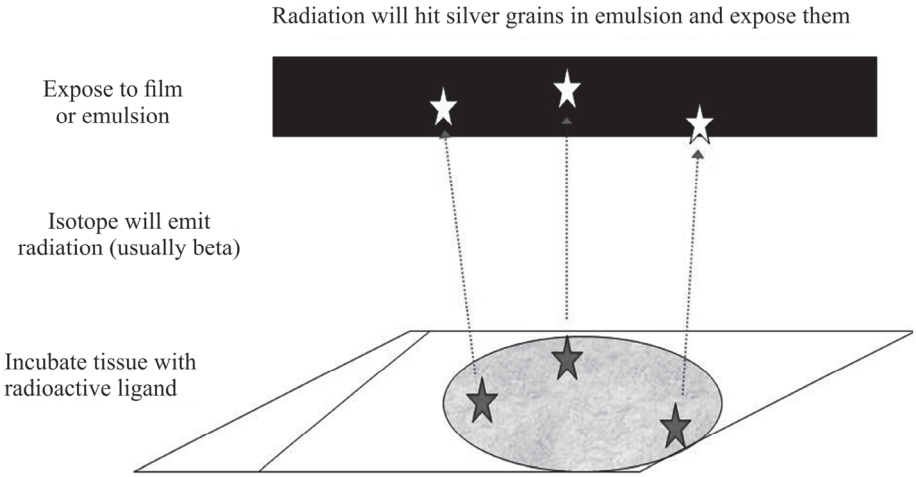


Fig. 1.20 Autoradiography

Applications

- To find and investigate the various properties of DNA.
- To find the location and amount of particular substance within a cell including cell organelle, metabolites etc.
- Tissue localization of radioactive substance.
- To find out the site and performance of targeted drug.
- To locate the metabolic activity site in the cell.

Mass Spectrophotometer

Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds.

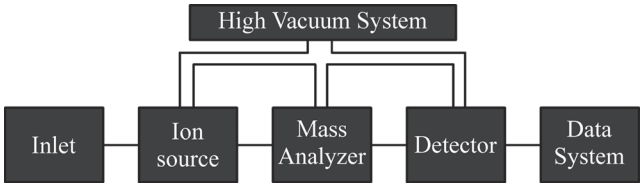


Fig 1.21 Block diagram of Mass Spectrophotometer

NMR Spectrophotometer

NMR spectroscopy is an analytical technique that measures the magnetic properties of certain atomic nuclei to determine physical and chemical properties of atoms or the molecules. It relies on the phenomenon of nuclear magnetic resonance and can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules.

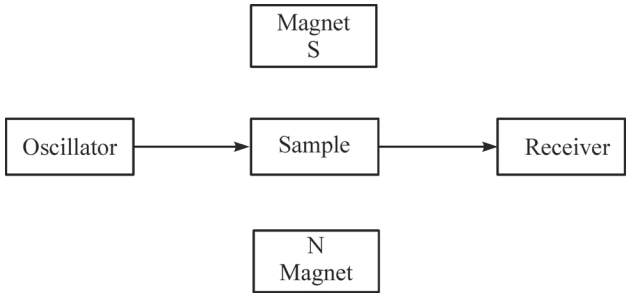


Fig. 1.22 Simple block diagram of NMR spectrophotometer

Other Techniques to Investigate Biosynthetic Pathways

Precursor Product Sequence

This is the most used technique for the elucidation of biosynthetic pathways in plants by using labeled compounds. In this technique, a presumed precursor of the constituent under investigation on a labeled form is fed into the plant and after a suitable time the constituent is isolated, purified and radioactivity is determined. Generally, the radioactivity of the isolated compound is not sufficient evidence to confirm the precursor –product sequence because labeled compound may enter the general metabolic pathways of the plants and from there may get randomly distributed through a whole range of biochemical reactions. If this happens degradation of isolated compound and determination of the activity of the fragment may not be able to give specific precursor product sequence. Therefore, for investigation of correct precursor product sequence, double or triple labeling either of different isotopes or specific labeling by one isotope at two or more positions in the molecule is employed. The double-triple labeling is widely used technique for the investigation of secondary metabolites in the plants. e.g. The best experimental example is incorporation of doubly labeled lysine into anabasine. It is known that lysine is precursor for anabasine. The double labeled lysine for the investigation of biogenesis of anabasine confirms which nitrogen of lysine molecule is involved in formation of pyridine ring of anabasine in *Nicotiana glauca*. In the experiment lysine labeled with C_2-^{15}N and C_6-^{15}N introduced, in the final product C_6-^{15}N retained instead of C_2- ^{15}N.

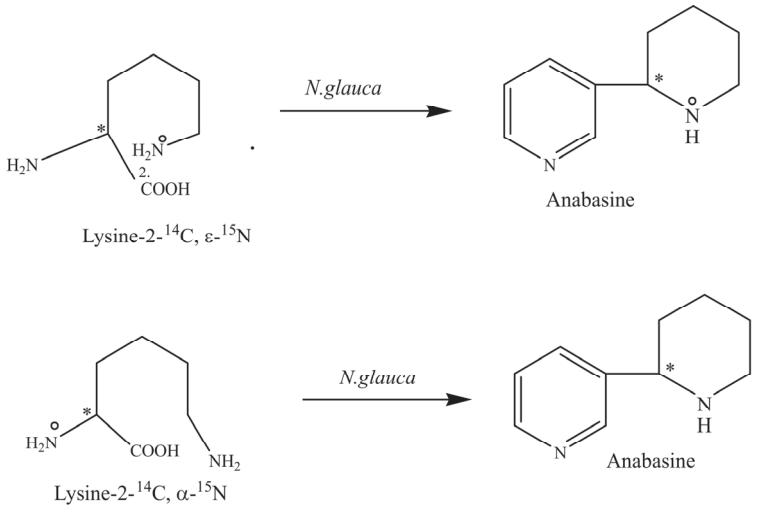


Fig. 1.23 Incorporation of doubly labeled lysine into anabasine

Applications

- Stopping of hordenine production in barley seedling after days of germination.
- Restricted synthesis of hyoscine, distinct from hyoscyamine in *Datura stramonium.*
- This method is applied to the biogenesis of Morphine & Ergot alkaloids.

Competitive Feeding

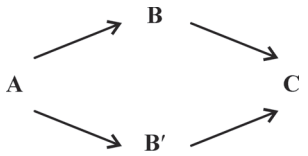


Fig. 1.24 Competitive feeding

In this technique, the labeled compounds used to determine precursor and exact intermediates. In plants, the biosynthesis of phytoconstituents is a very complex process which involves the many possibilities / probabilities for their precursors and intermediates and final products. It is always difficult and confusing task to predict them. Competitive feeding technique has great value in distinguishing the normal intermediate in the formation of phytoconstituents. The given example can explain the role of competitive feeding technique in identifying the intermediates.

In figure 1.24, A is the precursor and C is end product which synthesized through either B or B' as intermediates. The plant fed with labeled A act as control. Two other plants are fed with radiolabeled A simultaneously with radioactive B' in one plant and inactive B in another plant. If the incorporation or presence of labeled compounds into C is inhibited in the plants receiving B but is unaffected in the plant receiving B'; then we may confirm that the pathway from A to C proceeds via B.

Applications

- This method is used for elucidation of biogenesis of tropane alkaloids.

Sequential Analysis

This is another simple method for investigation of biogenetic pathways by using $^{14}CO_2$. In this technique, plant is grown in an atmosphere of $^{14}CO_2$ and plant parts are subjected to analysis at given time interval. This analysis provides the information about the sequence in which the various compounds become labeled. Most of the biosynthetic pathways are lengthy and complex, from the results of analysis certain biosynthetic pathways can be confirmed and some can be rejected.

This method has been very successfully implemented in the elucidation of the path of carbon in photosynthesis and also in the determination of sequential formation of alkaloids from opium, hemlock and tobacco. In one experiment very short period of 5 minutes exposure to $^{14}CO_2$ is used to investigate the biosynthetic sequence piperitone, menthone, menthol in *Mentha piperita*.

Use of Stable Isotopes

The stable isotopes ^{2}H, ^{13}C, ^{15}N and ^{18}O, which have a low natural occurrence can be used in similar way as radioactive elements for labeling compounds to be used as possible intermediates in biosynthetic pathways. The intermediate analysis can be done by using techniques like Mass spectroscopy and NMR spectroscopy.

Use of Isolated Organs Tissues or Cells

The cultivation of isolated organs, tissues and cells in suitable media is one of the techniques to investigate the biosynthetic pathway. It eliminates interference from other parts of the plant which may produce secondary changes in the metabolites. It is useful for the determination of the site of synthesis of particular compounds. Furthermore, it facilitates the easy introduction of labeled compound to parenchyma cells of tissue organ such as shoots, roots, petals, leaves etc.

Isolated part of the plants when placed in suitable solution or water, will have normal metabolism after separation from the plant this is the basis of this method. The isolated parts are grown on sterile nutrient media in aseptic condition. The radiolabeled compounds are added to the culture to study the biogenetic pathway. This method has the advantage that the nutrient solution or water medium does not requires sugar as sufficient starch is synthesized in the leaf and consequently bacterial and fungal growth in the nutrient solution is minimized. The isolated shoot end is placed in suitable nutrient media containing radiolabeled compound while isolated roots/ leaves are dipped in the media containing radiolabeled compounds. By using this technique tropane alkaloid are produced in the roots of Solanaceae family plants and number of precursors are incorporated to investigate the biogenetic pathway of alkaloids.

Use of Grafts

Grafting technique is particularly used for the determination of the sites of primary and secondary metabolism in plants. Grafting is an art of joining two different plant parts together in such a manner that they unite and continue their growth as a single plant. The upper portion of the one plant (stem/bud) known as scion is joined with stock/rootstock (stem/root/branch) of another plant in such a way that their tissue joined together to grow further.

Alkaloid formation by grafted plants has been studied extensively in Nicotiana and Datura. In this method tomato scions grafted onto Datura stocks accumulate tropane alkaloids whereas Datura scions on tomato stocks contain only small amount of tropane alkaloids. This indicates that site of alkaloids biosynthesis is Datura roots.

Grafting has its own limitations viz., grafting is possible only in dicots, possible between two different genera in the same family, and possible between two different species in the same genus. There are several techniques available for grafting; Scion attached method (inarching, saddle grafting or tongue grafting), Scion detached methods (veneer grafting, wedge grafting, whip and tongue grafting, softwood grafting), methods of grafting on established trees (side grafting, crown grafting) and methods of renovation (bridge grafting).

Use of Mutant Strains

Mutant strains are the species which are produced due to change in genetic characters. This results in the physical and physiological changes. Either physical methods (through radiation) or chemical methods (through Colchicines) are employed to produce a mutant strain. Most of the time mutants are produced due to lack of particular enzyme, enzyme deficiency results in metabolic blockage at particular stage. At this stage this microorganism may accumulate with an intermediate before blockage and need artificial supply another intermediate which may arises after block. Such organisms are useful to study some biogenetic pathways. A mutant of *Lactobacillus* is source of Mevalonic acid, an important intermediate in isoprenoid compound pathway. In case of higher plants, the production of mutant strain is not successful to study the biogenetic pathways.

Applications of Tracer Techniques

Tracer techniques are importantly used to investigate the biogenetic pathways in plants to synthesize secondary metabolite. These secondary metabolites have medicinal importance in the treatment of various diseases. By knowing the biogenetic pathways one can utilize this knowledge to synthesize the important secondary metabolite by chemically or by plant tissue culture techniques. Some of the important achievements of tracer techniques are enumerated below.

- To study squalene cyclization by using radiolabeled carbon and hydrogen (^{14}C, ^{3}H) in labeled mevalonic acid.
- To study the interrelationship between 4 methyl sterols and 4,4 dimethyl sterols by using radiolabeled carbon acetate.
- Use of 2-^{14}C labeled mevalonate to study the biosynthesis of terpenoids via chloroplast isolated in organic solvents.
- Investigation of cinnamic acid in coumarin pathway by using radiolabeled coumarin.
- To confirm the origin of carbon and nitrogen atoms of purine by using ^{14}C or ^{15}N precursor.
- Use of ^{14}C labeled carbon dioxide to determine the sequence of formation of compounds in carbon fixation pathway in photosynthesis by using algae Chlorella.

Probable Questions

Long answer questions

1. Explain Shikimic acid pathway and its significance in detail.
2. Write in detail about amino acid synthesis.
3. Explain the biosynthetic pathway of terpenoids and steroids.
4. Describe in detail about precursor product sequence and competitive feeding.
5. Write a note on Acetate hypothesis.
6. List the basic metabolic pathways and explain Isoprenoid biosynthesis.
7. Write an account of biogenetic pathways of alkaloids from different amino acids.
8. Give a detailed view of biosynthesis of glycosides.
9. Explain in detail the use of tracer technique in biosynthetic pathway.
10. Explain any two detectors used in tracer technique

Short answer questions

1. Explain the biosynthesis of aromatic amino acid.
2. Explain biosynthesis of anthraquinone glycosides.
3. Explain biosynthesis of isoprenoids**.**
4. Explain the biosynthesis of triglycerides.
5. Write an isotopes and their detection methods (detectors).
6. Write a note on Sequential analysis.
7. Write a note on Geiger -Muller technique.
8. Write a note on Scintillators and its applications in the study of biosynthetic pathways.

Very short answer questions

1. What are stress compounds?
2. Define radiolabeled isotopes.
3. Enlist the amino acids produced through Shikimic acid pathway.
4. Enlist the methods used for introduction of labeled compounds in biosynthetic pathways
5. Explain grafting method.
6. Explain biosynthesis of phenolic compounds.
7. Explain biosynthesis of flavonoid aglycone.
8. Enlist the pharmaceutically important secondary metabolites with examples.
9. Write applications of tracer techniques.
10. Write the building blocks for synthesis of anthraquinone glycoside and aromatic amino acids.