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B.Pharm

Semester V

Subject Name: Pharmacognosy and Phytochemistry II
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CONTENTS

Metabolic pathways in higher plants and their determination

a) Brief study of basic metabolic pathways and formation of different secondary metabolites through these pathways- Shikimic acid pathway, Acetate pathways and Amino acid pathway.

The sum total of all the enzymatic mediated reactions occurring in the cell is collectively called metabolism. The reaction sequences occurring within organisms in an orderly and regulated way are known as metabolic pathways and the compounds formed during metabolism are called metabolites. During the process of metabolism many intermediate metabolites are formed which is used to denote the chemical pathways of metabolism.
Cellular metabolism has four functions:

1. To obtain chemical energy i.e ATP through degradation of energy reach biomolecules.
2. To transform biomolecules into building blocks or precursors needed for the synthesis of macromolecular cell components.
3. To assemble building blocks into proteins, nucleic acids, lipids and other cell components.
4. To form and degrade biomolecules required in the specialized functions of cells.

As a result of metabolic process in plants, plant synthesizes primary plant metabolites and secondary plant metabolites.

Primary plant metabolites-

These are considered as basic plant constituents like sugars, amino acids, coenzyme A, mevalonic acid and lipids. They are widely distributed in plants in large quantities; are directly involved in growth, development and reproduction; non-expensive to isolate from plant e.g starch from potato and do not possess biological or pharmacological action.

Secondary plant metabolites-

These are biosynthesized from the primary metabolites e.g- alkaloids, glycosides, tannins, flavonoids, terpenoids and volatile oils. They are more limited in distribution; restricted to taxonomic group; not directly involved in the growth and development; possess biological or pharmacological action on humans and animals; involved in chemical adaptation to environmental stress; serve as defensive, protective chemicals against microorganisms, insects and higher herbivorous animals; present in small quantities; generally expensive to isolate from plant compared to primary metabolites e.g- quinine form cinchona bark.

1.1 Basic Metabolic Pathway:

The building blocks for secondary metabolites are derived from primary metabolism as indicated below (Fig.1.1). This outlines how metabolites from photosynthesis, glycolysis, and the Krebs cycle are tapped off from energy-generating processes to provide biosynthetic intermediates. The most important building blocks used in the biosynthesis of secondary metabolites are derived from the intermediates acetyl coenzyme A (acetyl-CoA), shikimic acid, mevalonic acid, and 1-deoxyxylulose 5-phosphate. These are utilized respectively in the acetate, shikimate, mevalonate, and deoxyxylulose phosphate pathways.

In addition to acetyl-CoA, shikimic acid, mevalonic acid, and deoxyxylulose phosphate, other building blocks based on amino acids are frequently employed in natural product synthesis.

Living plants are solar-powered biochemical and biosynthetic laboratory which manufactures both primary and secondary metabolites from air, water, minerals and sunlight. The primary metabolites like sugars, amino acids & fatty acids that are needed for general growth & physiological development of plant which distributed in nature & also utilized as food by man. The secondary metabolites such as alkaloids, glycosides, Flavonoids, volatile oils etc. are biosynthetically derived from primary metabolites.

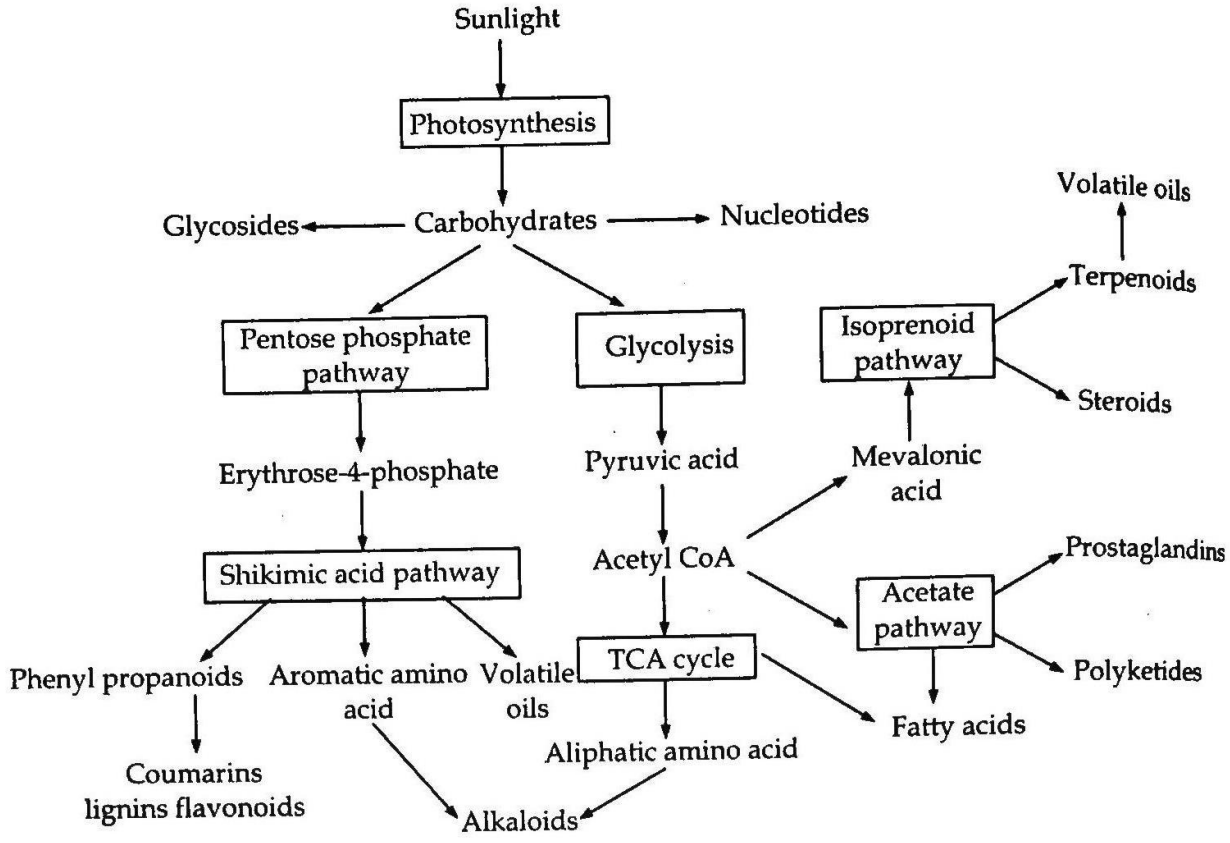

Fig.1.1 Basic Metabolic Pathway

1.2 Shikimic Acid Pathway:

The shikimate pathway provides an alternative route to aromatic compounds, particularly the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan. This pathway is employed by microorganisms and plants, but not by animals, and accordingly the aromatic amino acids feature among those essential amino acids for human beings, to be obtained from the diet.

Shikimic acid is named after the highly toxic Japanese shikimi (*Illicium anisatum*) flower from which it was first isolated.

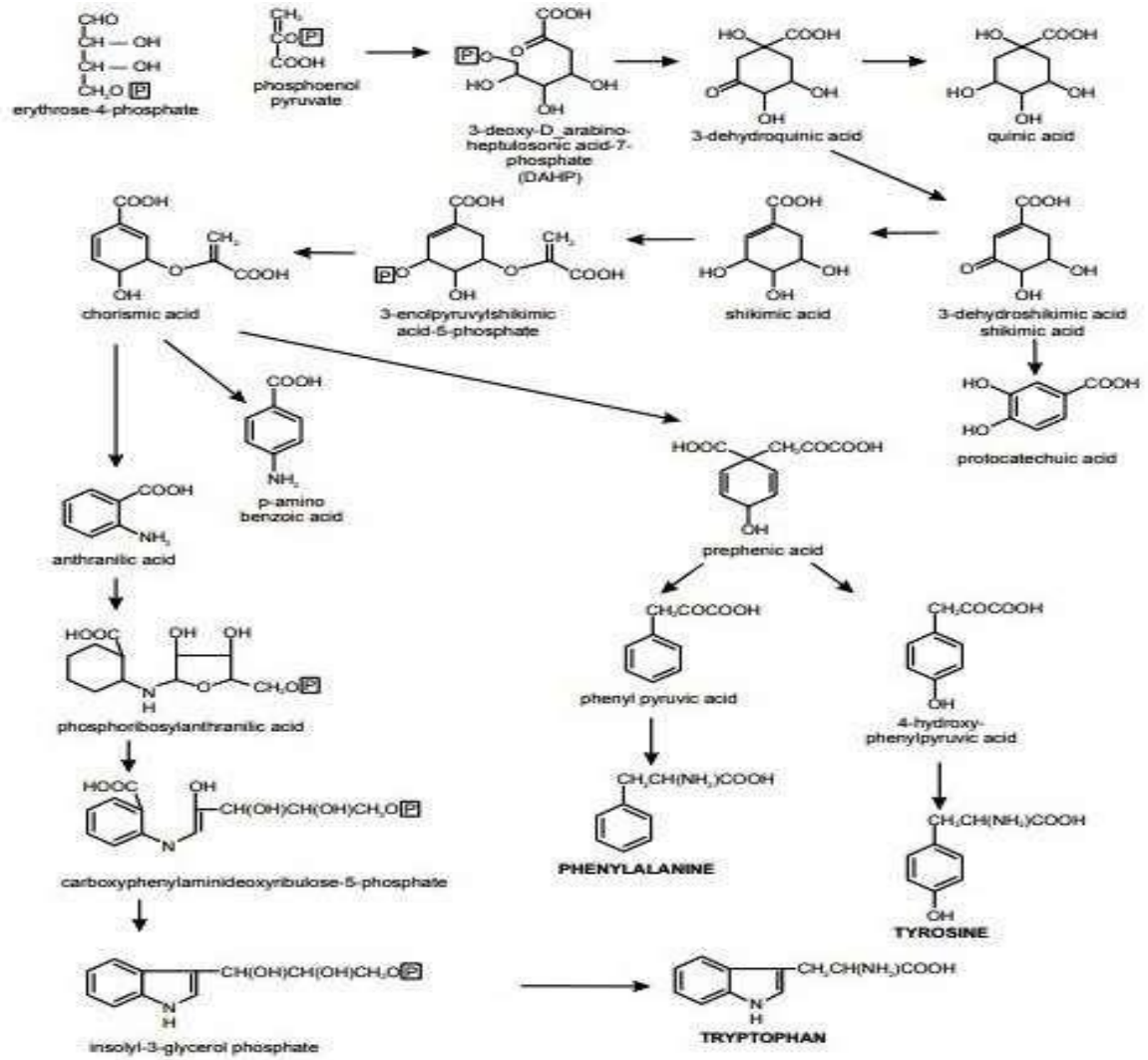

Fig.1.2 Shikimic acid Pathway

Shikimic acid pathway starts with the precursors, Erythrose 4-phosphate and Phosphoenolpyruvate coupling to form 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP), a reaction catalysed by phospho-2-oxo-3-deoxyheptonate aldolase. The enzyme, 3-

dehydroquinate synthase, catalysing the cyclization of DAHP to 3-dehydroquinic acid, requires cobalt (II) and nicotinamide adenine dinucleotide (NAD) as cofactors.

The dehydroquinic acid, on reduction gives quinic acid. By dehydration of 3-dehydroquinic acid forms 3-dehydroshikimic acid, which forms shikimic acid followed by reduction. The enzymes catalysing the dehydration of dehydroquinic acid are of two kinds. Form 1, associated with shikimate dehydrogenase, is independent of shikimate concentration, while form 2 is specifically activated by shikimate.

It has been suggested that the two forms provide a control in the utilization of dehydroquinic acid producing either shikimic acid or protocatechuic acid.

After phosphorylation, catalysed by shikimate kinase, shikimic acid adds on enol pyruvate to form 3-enolpyruvylshikimic acid-5-phosphate. This reaction is catalysed by enolpyruvylshikimate phosphate synthase, whereas conversion to chorismic acid is catalysed by chorismate synthase.

The formation of chorismic acid is an important branch point in the shikimic acid pathway as this compound can undergo three different types of conversion. In the presence of glutamine, chorismic acid is converted to anthranilic acid, whereas chorismate mutase catalyses the formation of prephenic acid. Chorismic acid is also converted into p-aminobenzoic acid.

Then after anthranilic acid is converted first to phosphoribosyl anthranilic acid and then to carboxyphenylaminodeoxyribulose-5-phosphate, these reactions being catalysed by anthranilate phosphoribosyl transferase and phosphoribosyl anthranilate isomerase, respectively. Ring closure to form indolyl-3-glycerol phosphate is catalysed by indolyl-glycerol phosphate synthase. The enzyme catalysing the final reaction, that is, tryptophan synthase consists of two components; component A catalyses the dissociation of indolylglycerol phosphate to indole and glyceraldehydes-3-phosphate, whereas component B catalyses the direct condensation of indole with serine to form tryptophan.

Tyrosine and phenylalanine are both biosynthesized from prephanic acid, but by independent pathways, which act as a precursor for the biosynthesis of phenylpropanoids. The henylpropanoids are then used to produce flavonoids, coumarins, lignin and tannins.

In the formation of tyrosine, prephanic acid is first aromatized to 4-hydroxyphenylpyruvic acid, a reaction catalysed by prephenate dehydrogenase. Transamination, catalysed by tyrosine aminotransferase, then gives tyrosine.

The biosynthesis of phenylalanine involves first the aromatization of prephanic acid to phenyl pyruvic acid, a reaction catalysed by prephenate dehydratase, and then transamination catalysed by phenylalanine aminotransferase, which gives phenylalanine.

1.3 Acetate pathways:

Acetate-Mevalonate Pathway:

Since a long time it was believed that acetic acid is involved in the synthesis of cholesterol, squalene and rubber-like compounds. The discovery of acetyl coenzyme A further supported the role of acetic acid in biogenetic pathways. Later, mevalonic acid was found to be associated with the acetate.

- The pathway begins with acetyl CoA molecule produced from pyruvic acid, which is the end product of glycolysis.
- First 2 molecules of acetyl CoA forms acetoacetyl CoA through Claisen condensation.
- 3rd molecule of acetyl CoA forms β-hydroxy β-methylglutaryl-CoA by aldol addition.
- Next on reduction gives rise to mevalonic acid, which is the main precursor for biosynthesis of terpenoids.
- Mevalonic acid on ATP mediated phosphorylation gives mevalonic acid diphosphate which on decarboxylation gives the 1st isoprene unit, isopentyl pyrophosphate (IPP).
- By the isomerase enzyme, the IPP gives 2nd isoprene unit Dimethyl allyl pyrophosphate (DMAPP)
- Electrophilic addition of IPP with DMAPP via enzyme prenyl transferase yield C_{10} unit, geranyl pyrophosphate (GPP), which is the precursor for synthesis of monoterpenes.
- Combinations of another IPP unit with GPP give rise to form farnesyl pyrophosphate (FPP), C_{15} unit which acts as a precursor for the synthesis of sesquiterpene.
- Further addition of IPP unit gives C_{20} geranyl geraniol pyrophosphate (GGPP) to produce a range of Diterpenes.
- On further addition of IPP unit gives C_{25} geranyl farnesyl pyrophosphate called Sesterterpenes.
- The tail to tail addition of two FPP units yields C_{30} unit, triterpene. Similarly 2 units of GGPP yield C_{40} unit, tetraterpene.

The acetate mevalonate pathway thus works through IPP and DMAPP via squalene to produce two different skeleton containing compounds, that is, steroids and triterpenoids. It also produces vast range of monoterpenoids, sesquiterpenoids, diterpenoids, carotenoids, polyprenols, and also the compounds like glycosides and alkaloids in association with other pathways (Fig.1.3).

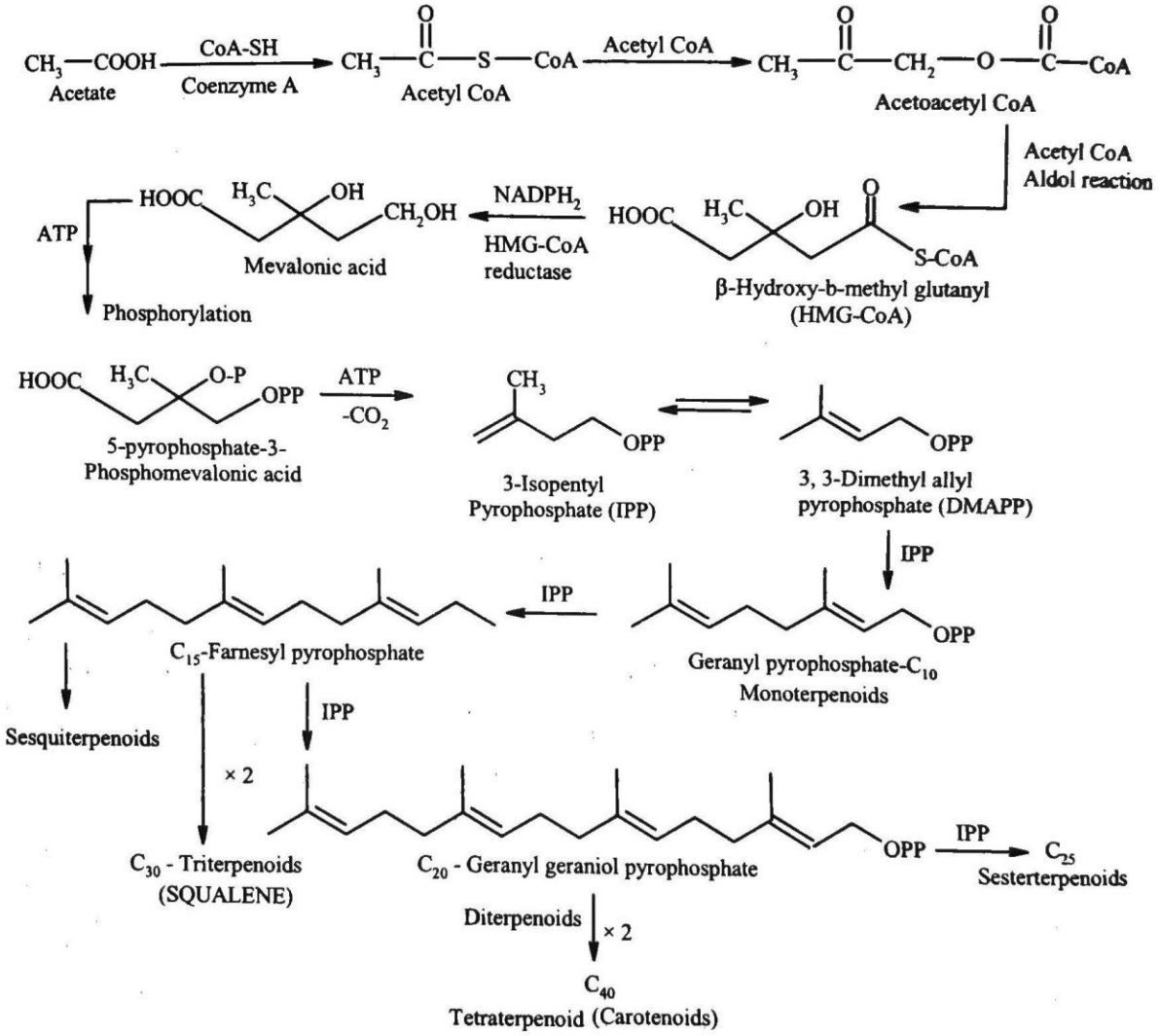

Fig.1.3 Acetate-mevalonate pathway

Acetate-Malonate Pathway:

Acetate pathway operates with the involvement of acyl carrier protein (ACP) to yield fatty acyl thioesters of ACP. These acyl thioesters forms the important intermediates in fatty acid synthesis. These C2 acetyl CoA units at the later stage produces even number of fatty acids from n-tetranoic (butyric) to n-ecosanoic (arachidic acid). The synthesis of fatty acids is thus explained by the reactions given below (Fig.1.4). Unsaturated fatty acids are produced by subsequent direct dehydrogenation of saturated fatty acids. Enzymes play important role in governing the position of newly introduced double bonds in the fatty acids.

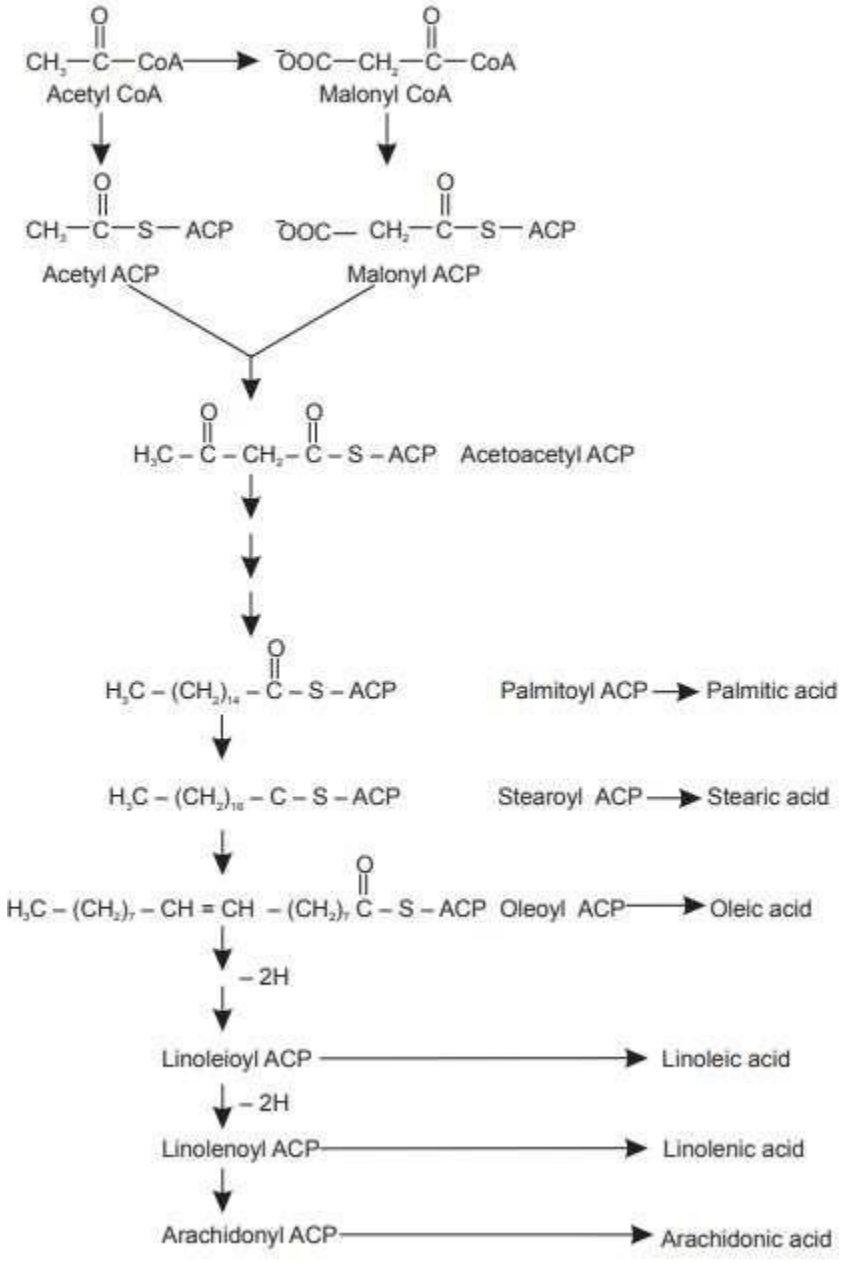

Fig.1.4 Acetate-malonate pathway

1.4 Amino acid pathway:

Plants and bacteria can synthesize all 20 of the amino acids. Whereas humans cannot synthesize 9 of them. These 9 amino acids must come from our diets and are called essential amino acids. The essential amino acids are Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, and Valine. The 11 amino acids are called non-essential amino acids like Alanine, Arginine, Aspargine, Aspartate, Cysteine, Glutamate, Glutamine, Glycine, Proline, Serine and Tyrosine. The non-essential amino acids are synthesized by simple pathways, whereas biosynthesis of the essential amino acids are complex. All 3 aromatic amino acids are derived from shikimate pathway.

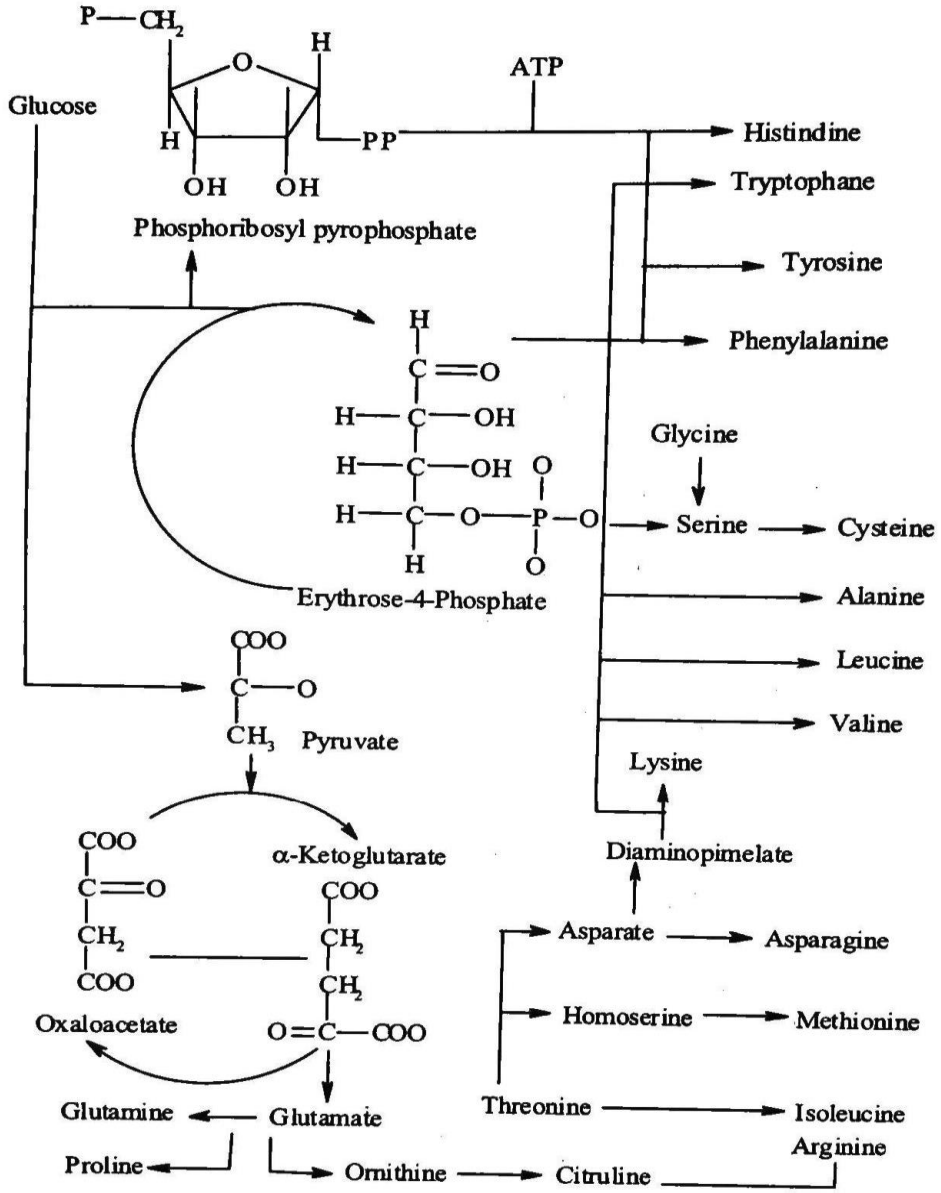

Fig.1.5 Amino Acid pathway

b) Study of utilization of radioactive isotopes in the investigation of Biogenetic studies.

There are 5 techniques used for the investigation of biosynthetic pathway of primary and secondary metabolites.

1. Tracer technique 2. Use of isolated organ and tissues 3.Grafting method
4. Use of Mutant strains 5.Enzymatic studies

Out of the above 5 methods, in Tracer technique method radioactive isotopes are used for the investigation of biogenetic studies.

2.1 Tracer technique:

Tracer techniques utilizes radioactive isotope labelled compound to find out or to trace the different intermediates and various steps in biosynthetic pathways in plants, at a given rate & time. When the labelled compounds are administered to the plants, they become a part of metabolic pool (Collective term for all the substances involved in the metabolic process in a biological system) and undergo reaction characteristics.

Elements existed with identical chemical properties or same atomic no. but different atomic weights/ mass no. are called isotopes. In other words, isotopes are atoms of same element whose nuclei contain same no. of protons but different no. of neutrons.

Stable isotopes- They are stable and do not emit radiation, e.g- ^{2}H, ^{13}C, ^{15}N, ^{18}O

Radioactive isotopes- They are unstable and emit radiations. The phenomenon of emitting radiation is called radioactivity and such isotopes are called radioactive isotopes.

Significance of tracer techniques-

- Applicable for living systems. Wide ranges of isotopes are available.
- High sensitivity
- More effective
- Simple administration and isolation.
- Shows accurate results when enough metabolic time & technique is used.
- Position & Quantity of compound containing tracer isotope ^{14}C marked glucose is used for glucose determination in the biological system.
- For different studies, different tracers can be used. For studies on nitrogen and amino acid, Labelled nitrogen gives specific information than carbon.
- Biosynthetic pathway can be traced by incorporating radioactive isotopes into the precursor or starting material. e.g- By incorporation of ^{14}C to phenyl alanine, the biosynthesis of cyanogenetic glycosides, prunacin can be traced. Location and quantity can be determined in biological system.

Different trace elements used for different studies-

1. For studies on protein, alkaloids and amino acid, nitrogen atom gives more specific information than carbon.
2. For studies on glycosidic linkage- O, N, S and C atom.
3. For studies on terpenoids- O atom.

2.2 Steps involved in tracer techniques-

1. Preparation of labelled compound
2. Incorporation of labelled compound
3. Separation and isolation of labelled compound
4. Determination of nature of metabolites in various biochemical fractions.

1. Preparation of labelled compound-

In biological investigation, the use of bioactive isotopes enables the metabolism of compounds to be followed in living organisms for detection and estimation of soft and easily absorbed radiation from labelled compound.

- Labelled compounds may be prepared by use of radioactive isotopes and stable isotopes e,g- Radioactive isotopes- ^{14}C, ^{3}H, ^{32}P, ^{131}I
- Stable isotopes- ^{2}H, ^{15}N, ^{13}C, ^{18}O
- Radioactive carbon and hydrogen are mostly used in biological investigation.
- Radioactive isotopes having long half-life are used.

Criteria for selection of trace elements-

- Starting concentration of trace element must be sufficient to withstand dilution in the course of metabolism.
- Physical and chemical nature of compound must be known.
- Half-life should be sufficiently long.
- Should not damage the tissue system
- Should have low radiation energy.
- Instruments used to detect properties of metabolites are Scintillation chamber, GM counter, Autoradiography, NMR and MS- ionization technique.

2. Incorporation of labelled compound to tissue system

i) Root feeding ii) Stem feeding iii) Direct injection

iv) Infiltration v) Floating method vi) Spraying technique

i) Root feeding- In case roots are biosynthetic sites e.g- Tobacco. The plants are cultivated hydroponically to avoid microbial contamination.

ii) Stem feeding- Labelled compounds are administered through the cut ends of stem immersed in a solution. For latex containing plants this method is not suitable.

iii) Direct injection- This method is used in plants with hallow stem. e.g- Umbelliferae and capsule plants (opium poppy). Microsyringe is used to inject labelled compound solution.

iv) Infiltration (wick feeding)- A thread is drawn through the stem which is dipped into radioactive solution or a flap can be cut in stem and this dipped in the solution.

v) Floating method- When a small amount of material is available, this method is used. Leaf disc/chopped leaves are floated on labelled compound solution.

vi) Spraying technique- Compounds have been absorbed after being sprayed on leaves. e.g- steroids.

3. Separation and isolation of labelled compound-

- Different methods are used depending on nature of drug and its source.
- Soft tissue (Fresh)- Infusion, Maceration
- Hard tissue- Decoction and hot percolation
- Unorganized drug- Maceration with solvent
- Fat and oil- Non-polar solvent
- Alkaloids, Glycosides, Flavonoids- Slightly polar solvent
- Plant phenol- Polar solvent

Detection and assay of radioactivity labelled compound-

When radioactive tracers are used in biogenetic studies, adequate methods for the detection and estimation of the label are essential.

- For soft and easily absorbed radiation from ^{3}H, ^{14}C labelled compounds- Liquid scintillation counter.
- Modern instruments are used for mixed radiation like ^{3}H and ^{14}C. This is possible because both are β-emitters and different radiation energy.
- With all counters, the instrument is connected to a suitable rate meter which records the count over a given time.
- Different instruments are used to determine nature of metabolites. e.g- GM Counter, Scintillation or liquid scintillation counter and ionisation chamber.

For stable isotopes-

1. MS gives molecular peak depending on mass/charge ration.
2. NMR gives nature of carbon or proton
3. Autoradiography

Advantages-

1. Whole morphological parts like leaves or section, for which the resulting negative is viewed under microscope.
2. Radioactive compounds on paper/thin layer chromatography can be detected and amount of radioactivity in different spots determined by density measurement.

4. Determination of nature of metabolites in various biochemical fractions-

Depending on the nature of isotopes, various instruments are used to determine the chemical nature of intermediate and final product for radioactive isotopes.

- Geiger-Muller counter- It is a type of particle detector that measures ionizing radiation, e.g. alpha, beta particles or gamma rays, by ionization produced in low-pressure gas, usually helium, neon or argon with halogens added in the Geiger-Muller tube, which conducts electrical charges briefly when a particle or photon of radiation makes the gas conductive by ionization. This indictment has been detected in form of current pulse.
- Scintillation or liquid scintillation counter- A scintillation detector or scintillation counter is produced when the scintillation detector is coupled to an electronic light sensor such as a photomultiplier tube (PMT) or a photodiode. A scintillator is a material that exhibits scintillation- a luminescence property that is stimulated by ionizing radiation. Samples shall be dissolved or suspended in a "cocktail" containing a solvent (aromatic organics such as benzene or toluene), typically some form of a surfactant, and small amounts of scintillators.
- Ionization chamber- The ionization chamber is the simplest of all gas-filled radiation detectors and is commonly used for ionizing radiation, including x-rays, gamma rays and beta particles. Conventionally, the term "ionization chamber" is used solely to describe those detectors that collect all the charges caused by direct ionization of the gas using an electrical field.
- Mass Spectrophotometer- Mass spectrometry (MS) is an analytical technique used to measure the mass-to-charge ratio of charged particles. It is used to determine the mass of the particles, to determine the elemental composition of the sample or molecule, and to elucidate the chemical structures of the molecules, such as peptides and other chemical compounds.
- NMR Spectrophotometer- NMR spectroscopy is a research technique that exploits the magnetic properties of certain atomic nuclei to determine the physical and chemical properties of the atoms or molecules they contain. It relies on the phenomenon of

nuclear magnetic resonance and can provide detailed information on the structure, dynamics, reaction status and chemical environment of the molecules.

- Autoradiography- It is a tool for examining the distribution of radioactive material in a plant object, e.g. histological tissue, chromatography sheet. This method uses a photographic film or emulsion as an ionizing radiation detector. The specimen is in close contact with the emulsion for a period (exposure duration). In this technique, a sample containing a radiolabelled metabolite is placed in direct contact with suitable photosensitive material such as x-ray (photographic) film for a specific period. The pattern of delivery of radioactive substances can be elucidated with the aid of the autograph collected.

Methods of tracer techniques -

1. Precursor Product sequence-

The presumed precursor of the constituent under investigation on a labelled form is fed into the plant and after a suitable time the constituent is isolated, purified and radioactivity is determined.

Radioactivity of the isolated compound alone is not sufficient evidence that the particular compound fed is a direct precursor, because the compound may enter the general metabolic pathways and distributed randomly through a whole range of products.

Further evidence can be made by double and triple labelling experiments by using either different isotopes of specific labelling by one isotope at two or more positions in the molecule.

Application- Restricted synthesis of hyoscine, distinct from hyoscyamine in *Datura stramonium*. This method is applied to the biogenesis of morphine and ergot alkaloids.

Leete in his experiment used two doubly labelled lysines to determine which nitrogen of the lysine molecule was involved in the formation of the piperidine ring of anabasine in *Nicotiana glauca* (Fig.2.1).

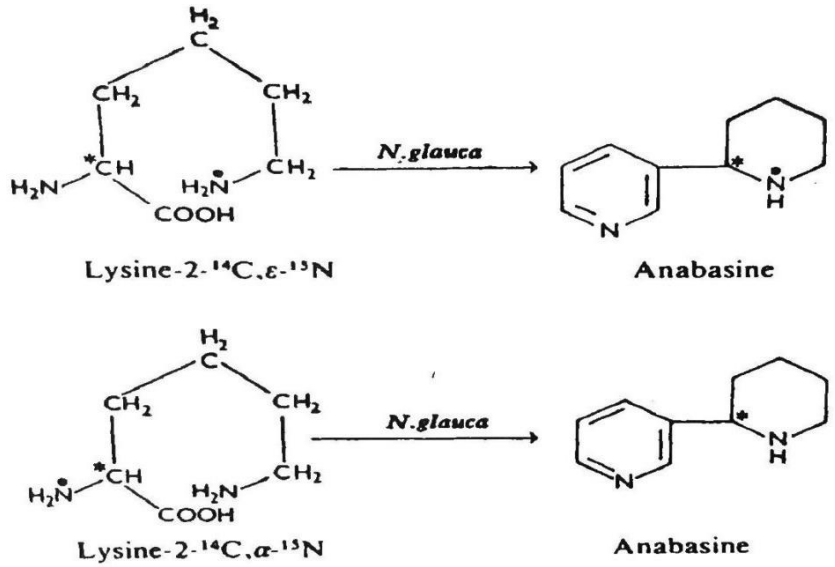

Fig.2.1

2. Competitive feeding-

If incorporation is obtained, it is necessary to consider whether this is the normal route of synthesis in plant not the subsidiary pathway. Competitive feeding can distinguish whether B or B' is the normal intermediate in the formation of C from A (Fig.2.2).

Inactive B and B' are fed with labelled A to separate groups of plants and a control is performed by feeding labelled A only to another group. If the incorporation of activity into C is inhibited in the plants receiving B but is unaffected in the group receiving B' then we may conclude that the pathway from A to C probably proceeds via B.

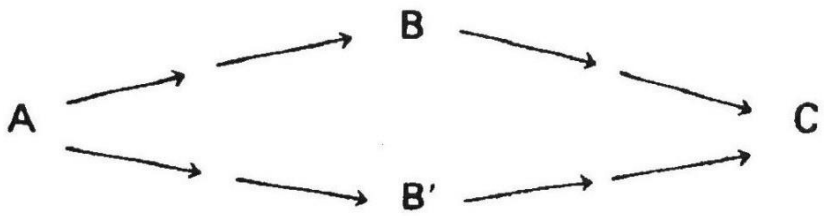

Fig.2.2

$A \rightarrow C$

$*A \rightarrow B \rightarrow C$

$*A \rightarrow B' \rightarrow C$

Tyrosine is the precursor for 3,4- dihydroxyphenylpyruvic acid and 3,4-dihydroxyphenylethylamine for the synthesis of Norlaudanosoline which produces morphine through reticuline. 3,4- dihydroxyphenylethylamine is produced from tyrosine via 3,4-dihydroxyphenylalanine (DOPA). It was considered that another precursor i.e 3,4-

dihydroxyphenylpyruvic acid would also be synthesized through DOPA but by labelling experiments and competitive feeding it has been confirmed that tyrosine directly gives 3,4-dihydroxyphenylpyruvic acid without any intermediate.

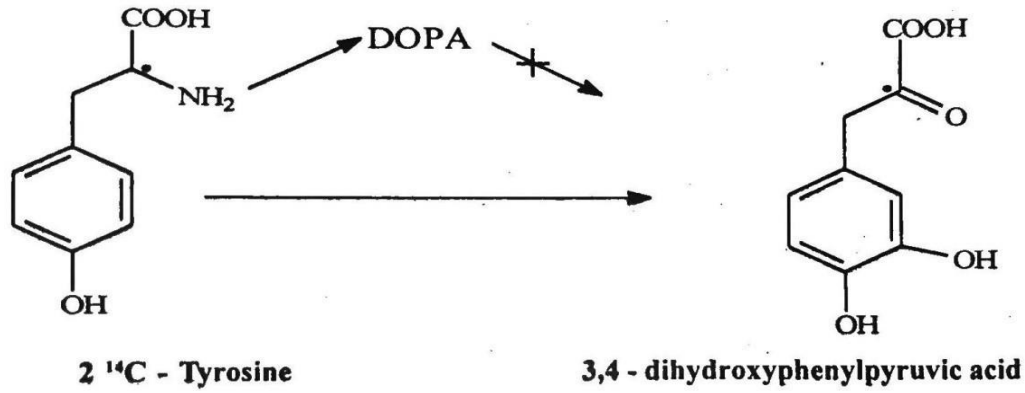

Fig. 2.3

Application- Used for elucidation of biogenesis of tropane alkaloid; biosynthesis of alkaloids of Conium maculactum using ^{14}C labelled compound.

3. <u>Sequential analysis</u>-

Principle of this method is to grow a plant in an atmosphere of $^{14}CO_2$ & then analyse the plant at given time interval to obtain the sequence in which various correlated compound become labelled.

Degradation of isolated radioactive compounds is important, because some units of molecule may become labelled more rapidly than others.

This method is used in the elucidation of path of carbon in photosynthesis.

Application- Exposure period to $^{14}CO_2$ as short as 5 min. have been used to obtain evidence of biosynthetic sequence as Piperitone → (-)-menthone → (-)-menthol in *Mentha piperita*

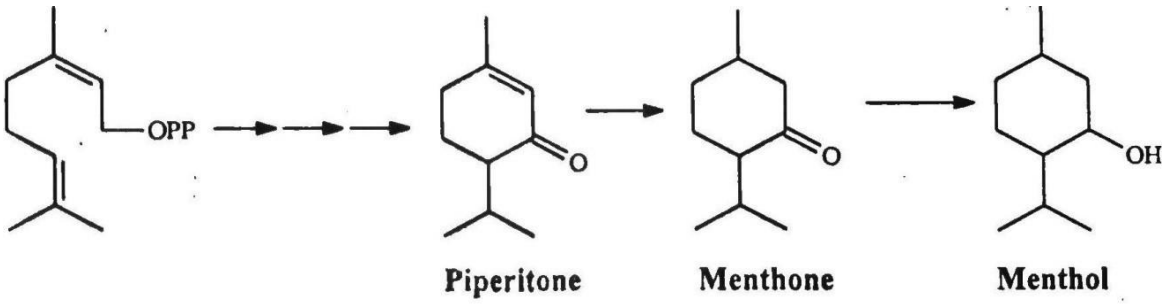

Fig.2.4 Biosynthetic sequence of Piperitone to Menthone to Menthol

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