UNIT 1

Metabolic Pathways in Higher Plants and their Determination

INTRODUCTION

The secondary metabolism is a biosynthetic source of several interesting compounds useful to chemical, food, agronomic, cosmetics, and pharmaceutical industries. The secondary pathways are not necessary for the survival of individual cells but benefit the plant as a whole. Another general characteristic of secondary metabolism is that found in a specific organism, or group of organisms, and is an expression of the individuality of species. The secondary metabolism provides chemical diversity to organic molecules with a low molecular weight that is related by the respective pathways; such organic molecules are called secondary metabolites. The secondary metabolites are often less than 1% of the total carbon in plant molecules. These organic molecules isolated from terrestrial plants are the most studied, and their syntheses have an important role in the protection against pathogens, unfavorable temperature and pH, saline stress, heavy metal stress, and UV radiation. Secondary metabolism reflects plant environments more closely than primary metabolism. There are three principal kinds of secondary metabolites biosynthesized by plants: Phenolic compounds, terpenoids/isoprenoids, alkaloids, and glucosinolates (nitrogen- or sulfur-containing molecules, respectively). Phenolic compounds are biosynthesized by the shikimate pathway and are abundant in plants. The shikimate pathway, in plants, is localized in the chloroplast. These aromatic molecules have important roles, as pigments, antioxidants, signaling agents, electron transport, communication, the structural element lignan, and as a defense.

A metabolic pathway is a linked series of chemical reactions occurring within a cell. The reactants, products, and intermediates of an enzymatic reaction are known as metabolites. Pathways are required for the maintenance of homeostasis within an organism and the flux of metabolites through a pathway is regulated depending on the needs of the cell and the availability of the substrate. The end product of a pathway may be used immediately, initiate another metabolic pathway, or be stored for later use. Metabolic pathways can be classified as:

- Energy utilizing (anabolic)
- Energy generating (catabolic)

Anabolic pathways: In contrast to catabolic pathways, anabolic pathways require energy input to construct macromolecules such as polypeptides, nucleic acids, proteins, polysaccharides, and lipids. An anabolic pathway is a biosynthetic pathway that combines smaller molecules to form larger and more complex ones.

Catabolic pathway: A catabolic pathway is an exergonic system that produces chemical energy in the form of ATP, GTP, NADH, NADPH, $FADH_2$, etc. from energy-containing sources such as carbohydrates, fats, and proteins. The end products are often carbon dioxide, water, and ammonia.

BIOSYNTHESIS

Biosynthesis is a process of forming larger organic compounds from small subunits within a living organism, i.e. anabolism. Biosynthesis is also known as anabolism since simple compounds are joined together to form macromolecules by enzymes. As an example, photosynthesis occurs inside the chloroplast. The light energy is converted into chemical energy during photosynthesis. The larger molecule glucose is biosynthesized from water and carbon dioxide by photosynthetic organisms (ATP, enzyme, cofactors).

Table 1.1 gives difference between synthesis and biosynthesis.

Table 1.1: The difference between synthesis and biosynthesis

Synthesis	***Biosynthesis***
Synthesis refers to the formation of macromolecules from small molecules artificially	Biosynthesis refers to the formation of larger organic compounds from small molecules within a living organism
Synthesis is artificial and chemical	Biosynthesis is biological and catalyzed by enzymes
Synthesis occurs outside living organisms.	Biosynthesis occurs within a living organism.

Types of Plants Metabolites

There are two types:
- Primary plant metabolites
- Secondary plant metabolites

Primary Plant Metabolites

A primary metabolite is a kind of metabolite that is directly involved in normal growth, development, and reproduction. It usually performs a physiological function in the organism (i.e. an intrinsic function). A primary metabolite is typically present in many organisms or cells. It is also referred to as a central metabolite, which has an even more restricted meaning (present in any autonomously growing cell or organism).

Properties

- It is a basic constituent.
- Simple and generally do not have any biological or pharmacological action.
- They are primarily utilized by plants for building their body and are responsible for the primary physiological functioning of the body.
  Examples: Starch, chlorophyll, aleurone grains, calcium oxalate crystals, cellulose

Secondary Plant Metabolites

Secondary metabolites are not essential as primary metabolites as these are not directly involved in the growth, development, and reproduction of organisms. They are organic

compounds that are not directly involved in the survival of plants but they produce some products which aid them in their normal growth and development. Secondary metabolites are compounds biosynthetically derived from primary metabolites but more limited in distribution in the plant kingdom, being restricted to a particular taxonomic group (species, genus, family, or closely related group of families).

Properties

- From the primary metabolites, secondary metabolites are synthesized.
- Generally, they have potent biological and/or pharmacological action.
- It is also known as active constituents
  Examples: Alkaloids, glycosides, tannins, resins, flavonoids, etc.

The building blocks for secondary metabolites are derived from primary metabolism as shown in Fig. 1.6. This outlines how metabolites from photosynthesis, glycolysis, and the TCA cycle (Krebs cycle) is tapped off from energy-generating processes to provide biosynthetic intermediates. The most significant 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-deoxy-xylulose 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 a solar-powered biochemical and biosynthetic laboratory that manufactures both primary and secondary metabolites from the air, water, minerals, and sunlight. The primary metabolites like sugars, amino acids and fatty acids are required for the general growth and physiological development of plants which are distributed in nature and also utilized as food by man. The secondary plant metabolites such as tannins, resins, alkaloids, glycosides, flavonoids, volatile oils, etc. are biosynthetically derived from primary plant metabolites.

SHIKIMIC ACID PATHWAY (SAP)

It is also known as aromatic biosynthesis since most of the products are aromatic. Commonly known as its anionic form shikimate, is a cyclohexene, a cyclitol, and a cyclohexane carboxylic acid. Its name comes from the Japanese flower shikimi the Japanese star anise, *Illicium anisatum*, from which it was first isolated in 1885 by Johan Fredrik Eykman. The elucidation of its structure was made nearly 50 years later. Shikimic acid is also the glycoside part of some hydrolyzable tannins. The shikimic acid pathway is a key intermediate from carbohydrate for the biosynthesis of C_6–C_3 units (phenyl propane derivative). The Shikimic acid pathway converts simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway to the aromatic amino acids (Fig. 1.1). The shikimate pathway is a 7-step metabolic route used by bacteria, fungi, algae, parasites, and plants for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) (Table 1.2).

Anthranilate synthase use chorismic acid → anthranilic acid → tryptophan chorismic mutase converts chorismic acid → prephenic acid.

Although there are only small differences in the sequence for the SAP in bacteria, fungi, and plants, there are considerable differences in the molecular organization of the pathway. This pathway is also important in the genesis of aromatic building blocks

of lignin and the formation of some tannins, vanillin, and phenyl propane units of flavones and coumarin (Fig. 1.1).

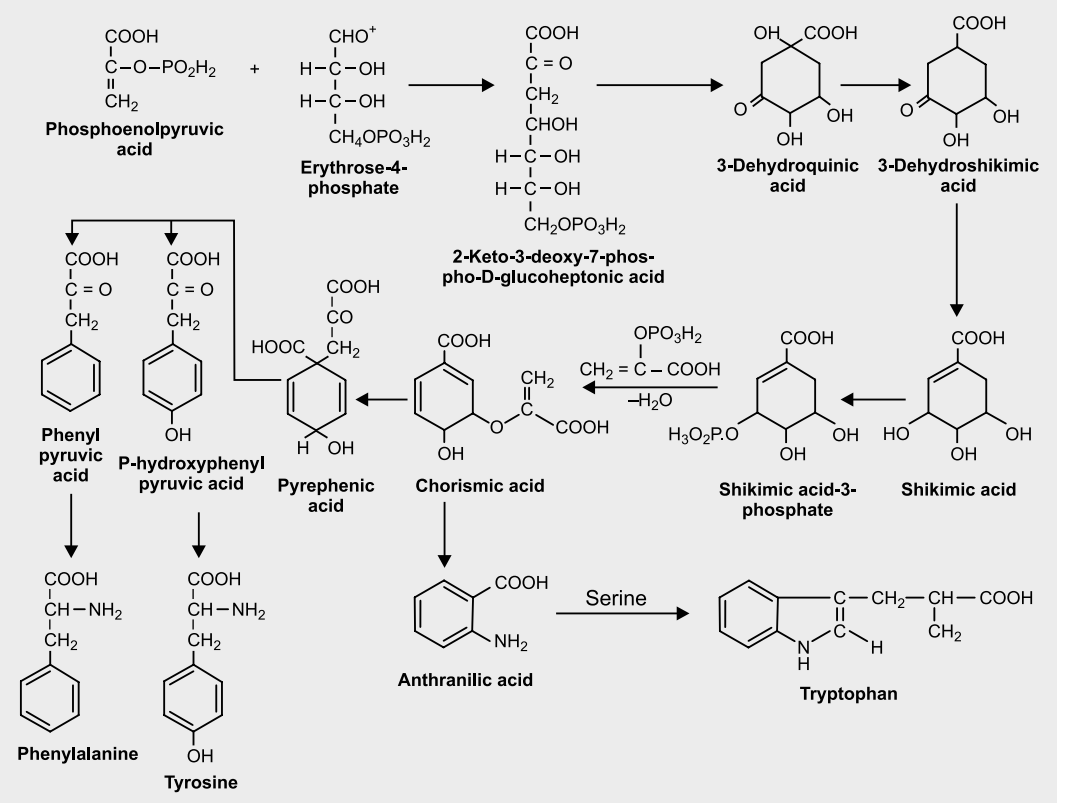

Fig. 1.1: Shikimic acid pathways

Table 1.2: Seven steps of the SAP

Reaction step	*Substrate*	*Enzyme/cofactor*	*Product*
1.	Phosphoenolpyruvate (PEP), erythrose-4-phosphate	3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHPS; Co^{2+}, Mg^{2+} or Mn^{2+}	3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP), Pi
2.	3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP)	3-Dehydroquinate synthase DHQS /Co^{2+}, NAD^+	3-Dehydroquinic acid (DHQ), Pi
3.	3-Dehydroquinic acid (DHQ)	3-Dehydroquinate dehydratase (DHQ dehydratase EC 4.2.1.10)	3-Dehydroshikimic acid (DHS), H_2O
4.	3-Dehydroshikimic acid (DHS), NADPH + H^+	Shikimate dehydrogenase (SDH; EC 1.1.1.25)	Shikimic acid, $NADP^+$

Contd.

Table 1.2: Seven steps of the SAP *(Contd.)*

Reaction step	***Substrate***	***Enzyme/cofactor***	***Product***
5.	Shikimic acid, ATP	Shikimate kinase enzyme (SK)	Shikimic acid 3-phosphate (S3P), ADP
6.	Shikimic acid 3-phosphate (S3P), PEP	5-*Enol*pyruvylshikimate 3-phosphate synthase also called aro A enzyme (EPSPS)	5-*Enol*pyruvylshikimate 3-phosphate (EPSP), Pi
7.	5-*Enol*pyruvylshikimate 3-phosphate (EPSP)	Chorismate synthase (CS; / $FMNH_2$	Chorismic acid, Pi

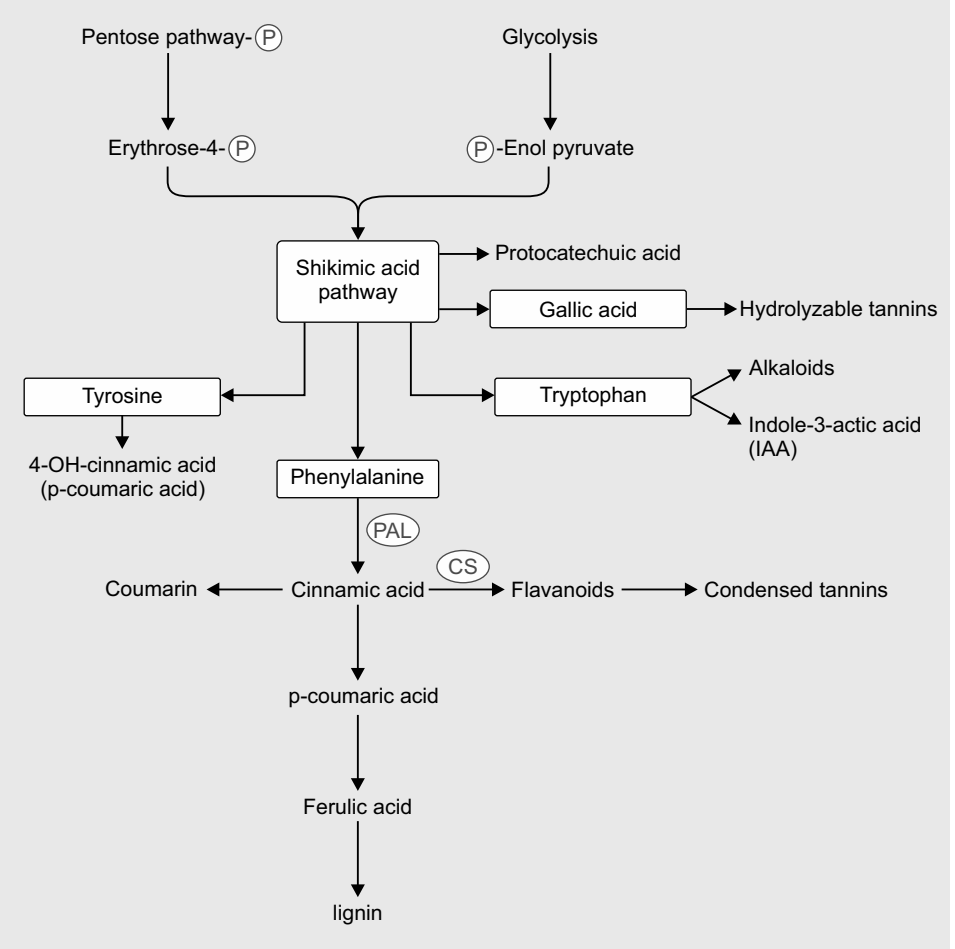

Fig. 1.2: The central role of the shikimic acid pathway in the synthesis of various primary and secondary metabolites. (PAL: Phenylalanine ammonia-lyase; CS: Chalcone synthase)

Steps of SAP

- The SAP consists of 7 sequential enzymatic steps and begins with an aldol-type condensation of two phosphorylated active compounds, the phosphoenolpyruvic acid (PEP), from the glycolytic pathway, and the carbohydrate D-erythrose-4-phosphate, from the pentose phosphate cycle, to give 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP). The seven enzymes that catalyze the pathway are known: 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS; EC 4.1.2.15, now EC 2.5.1.54), 3-dehydroquinate synthase (DHQS; EC 4.2.3.4), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQ/SDH; EC 4.2.1.10/EC 1.1.1.25), shikimate kinase (SK; EC 2.7.1.71), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS; EC 2.5.1.19), and chorismate synthase (CS; EC 4.2.3.5).
- In the 2nd reaction step, DAHP loses phosphate (Pi); the enolic-type product is cyclized through a second aldol-type reaction to produce 3-dehydroquinic acid (DHQ). The 3-dehydroquinate synthase (DHQS) catalyzes this cyclization in the SAP. The DHQ dehydrates to produce 3-dehydroshikimic acid (DHS) (3-dehydroquinate dehydratase); this compound has a conjugated double carbon-carbon. The protocatechuic and the gallic acids (C_6–C_1) are produced by branch-point reactions from DHS.
- The 4th step in the pathway is a reduction reaction of DHS with reduced nicotinamide adenine dinucleotide phosphate (NADPH).
- The 5th section of the pathway is the activation of shikimic acid with adenosine triphosphate (ATP) (shikimate kinase, SK) to make shikimic acid 3-phosphate (S3P).
- The 6th chemical reaction is the addition of PEP to S3P to generate 5-enolpyruvylshikimic acid 3-phosphate; the enzyme that catalyzes this reaction step, 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), has been extensively studied. The reason for this interest is because glyphosate [N-(phosphonomethyl)glycine] is a powerful inhibitor of EPSPS, so glyphosate has been used as a broad-spectrum systemic herbicide. It is an organophosphorus molecule, phosphonic acid, and glycine derivative that has a similar molecular structure to PEP.
- The last reaction step of the SAP is the production of chorismic acid from catalytic action on the chorismate synthase (CS). This reaction is a 1,4-trans elimination of Pi, to yield the conjugated molecule, chorismic acid.

Synthesis of Chorismic Acid

The seventh and last reaction step of the SAP is the 1,4-transelimination of the Pi group at C-3 from EPSPS to synthesize chorismic acid. This last step is catalyzed by chorismate synthase (CS; EC 4.2.3.5) that needs reduced flavin mononucleotide (FMNH2) as a cofactor that is not consumed. The FMNH2 transfers an electron to the substrate reversibly. Spectroscopic techniques and kinetic isotope effect studies suggest that a radical intermediate in a non-concerted mechanism is developed. Chorismic acid, the final molecule of the SAP, is a key branch point to post-chorismic acid pathways, to obtain L-Phe, L-Tyr, and L-Trp,. L-Phe is the substrate to phenylpropanoid and flavonoid pathways.

Role of SAP

- The starting point in the biosynthesis of some phenolics. Phenylalanine and tyrosine are the precursors used in the biosynthesis of phenylpropanoids. The phenylpropanoids are then used to produce flavonoids, coumarins, tannins, and lignins.
- Gallic acid is synthesized.
- Shikimic acid is a precursor for indole, indole derivatives, and aromatic amino acid tryptophan and tryptophan derivatives such as psychedelic compound trimethyltryptamine.
- Many alkaloids and other aromatic metabolites are produced.

Significances of SAP

Phenolic compounds are secondary metabolites found most abundantly in plants. These aromatic molecules have important roles, as pigments, antioxidants, signaling agents, the structural element lignan, and as a defense mechanism. The expression of phenolic compounds is promoted by biotic and abiotic stresses (e.g., herbivores, pathogens, unfavorable temperature, pH, saline stress, heavy metal stress, and UVB and UVA radiation). These compounds are formed via the SAP in higher plants and microorganisms. The enzymes responsible for the regulation of phenolic metabolism are known, and shikimic acid is a central metabolite. The shikimate pathway consists of seven reaction steps, beginning with an aldol-type condensation of phosphoenolpyruvic acid (PEP) from the glycolytic pathway, and D-erythrose-4-phosphate, from the pentose phosphate cycle, to produce 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP). A key branch-point compound is a chorismic acid, the final product of the SAP. The shikimic and chorismic acids are the common precursors for the synthesis of L-Phe, L-Tyr, and L-Trp and diverse phenolic compounds.

ACETATE PATHWAYS

The form in which acetate is used in most of its important biochemical reactions is acetyl coenzyme A (acetyl-CoA). Acetyl-CoA is formed by oxidative decarboxylation of the glycolytic pathway product pyruvic acid. The mevalonate pathway, also known as the isoprenoid pathway or HMG-CoA reductase pathway is an essential metabolic pathway present in eukaryotes, archaea, and some bacteria.

Polyketides are basically derived from from poly-β- keto chains, formed by coupling of acetic acid (C_2) units via condensation reactions $nCH_3COOH \rightarrow -[CH_2CO]n-$

Examples of such compounds are fatty acids, polyacetylenes, prostaglandins, macrolide antibiotics, and many aromatic compounds, e.g. anthraquinones and tetracyclines (Fig. 1.3). The formation of the poly-β-keto chain can be visualized as a series of Claisen reactions. Two molecules of acetyl-CoA could participate in a Claisen condensation giving acetoacetyl- CoA and this reaction could be repeated to generate a poly-β-keto ester of appropriate chain length. However, a study of the enzymes involved in fatty acid biosynthesis showed this simple rationalization could not be correct.

It is now known that fatty acid biosynthesis involves initial carboxylation of acetyl-CoA $\rightarrow$ malonyl-CoA, a reaction involving ATP, CO_2, and the coenzyme biotin as the carrier of CO_2. The conversion of acetyl-CoA into malonyl-CoA increases the acidity of the α-hydrogens, and thus provides a better nucleophile for the Claisen condensation.

Accordingly, the carboxylation step helps to activate the α-carbon and facilitate Claisen condensation, and the carboxyl is immediately removed on completion of this task. An alternative rationalization is that decarboxylation of the malonyl ester is used to generate the acetyl enolate anion without any requirement for a strong base. The chain extension process (mentioned in Fig. 1.3) continues for aromatics, generating a highly reactive poly-β- keto chain, which has to be stabilized by association with groups on the enzyme surface until chain assembly is complete and cyclization reactions occur. Conversely, for fatty acids, the carbonyl groups are reduced before attachment to the next malonate group.

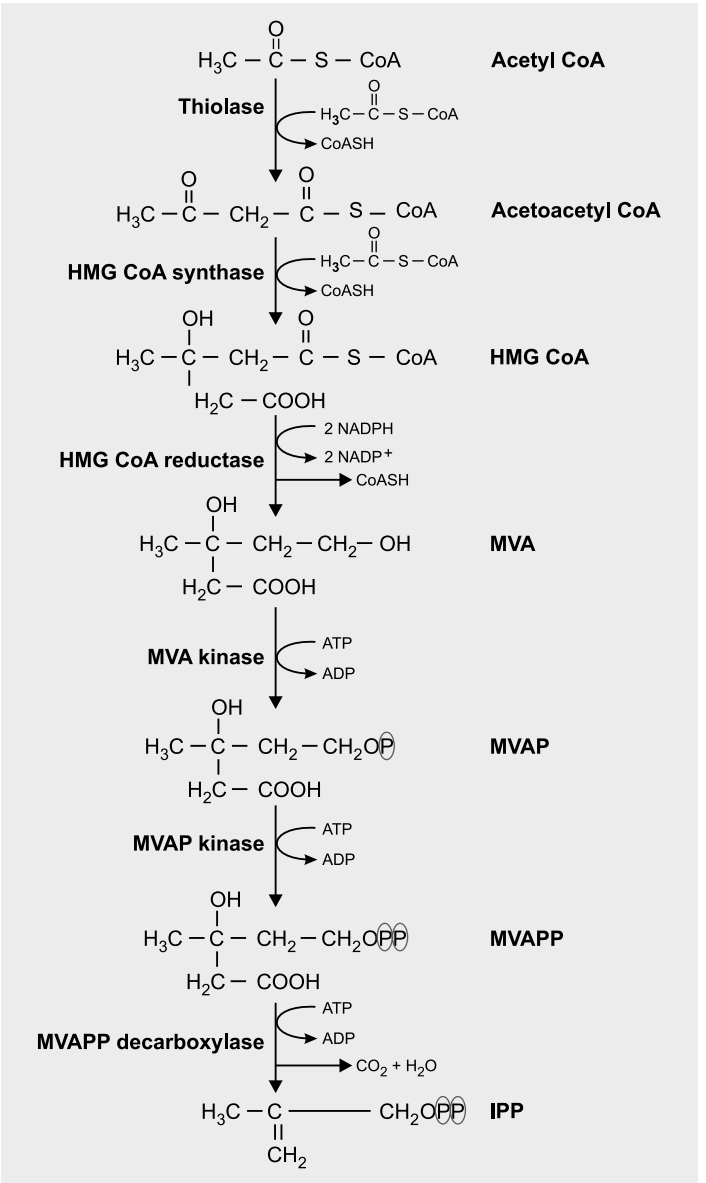

Fig. 1.3: The acetate/mevalonate pathway for the formation of IPP, the basic five-carbon unit of terpenoid biosynthesis. Synthesis of each IPP unit requires three molecules of acetyl-CoA

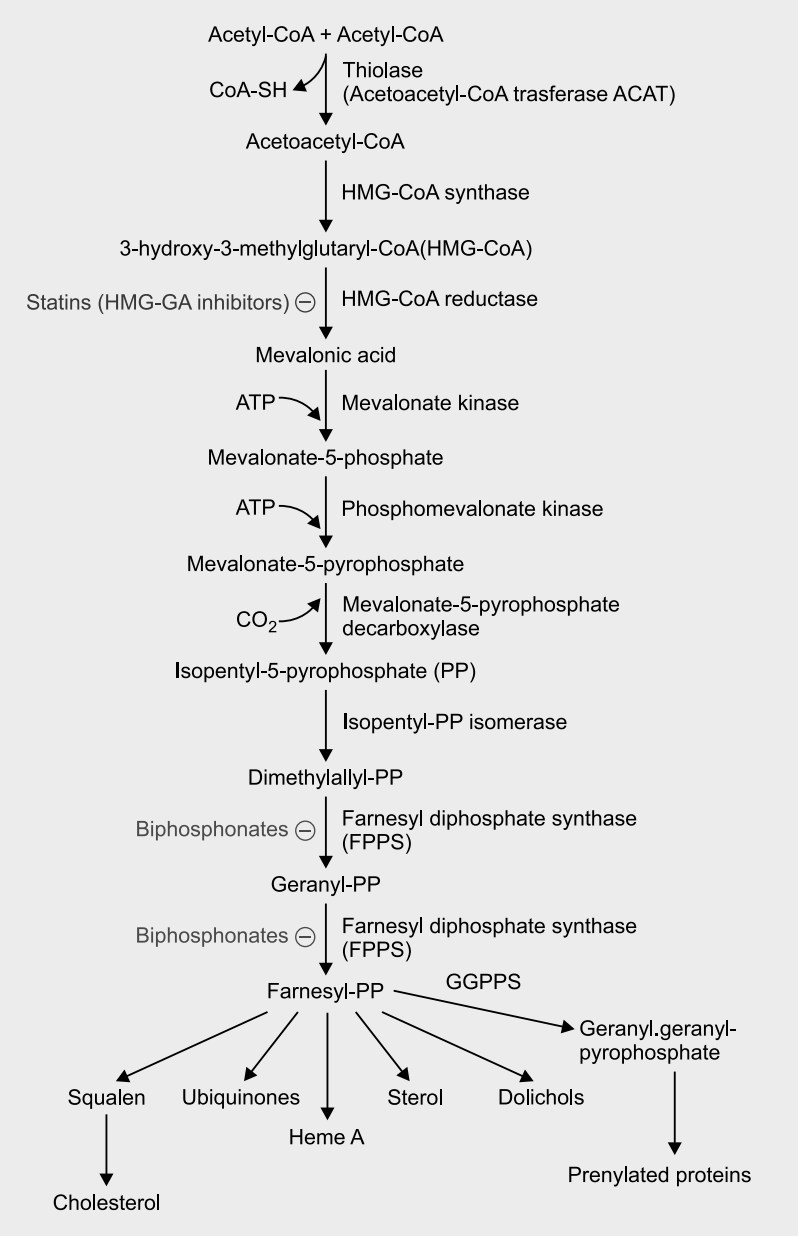

Fig. 1.4: The role of the acetate/mevalonate pathway in the synthesis of various secondary metabolites.

Significance of Acetate Pathway

Flavonoids are secondary metabolites involved in several physiological responses to the environment, such as defense against herbivores. Flavonoid uptake in the diet is also associated with health benefits for humans (*Homo sapiens*), mostly due to their activity as antioxidants. The basic backbone structure of flavonoids is synthesized by a combination of the phenylpropanoid and polyketide (or acetate) pathways, the former

providing *p*-coumaroyl-CoA from Phe while the latter provides malonyl-CoA for C2 chain elongation by chalcone synthase.

Polyketides are metabolites built primarily from combinations of acetate units. The biosynthesis of saturated and unsaturated fatty acids is covered, together with prostaglandins, thromboxanes, and leukotrienes. Cyclization of polyketides to give aromatic structures is then rationalized in terms of aldol and Claisen reactions. More complex structures formed via pathways involving alkylation reactions, phenolic oxidative coupling, oxidative cleavage of aromatic rings, and employing starter groups other than acetate are developed. The use of extender groups other than malonate gives rise to macrolides and polyethers, whilst further cyclization of polyketide structures may be achieved through Diels–Alder reactions. The acetate pathway operates with the involvement of acyl carrier protein (ACP) to yield fatty acyl thioesters of ACP. These acyl thioesters form important intermediates in fatty acid synthesis.

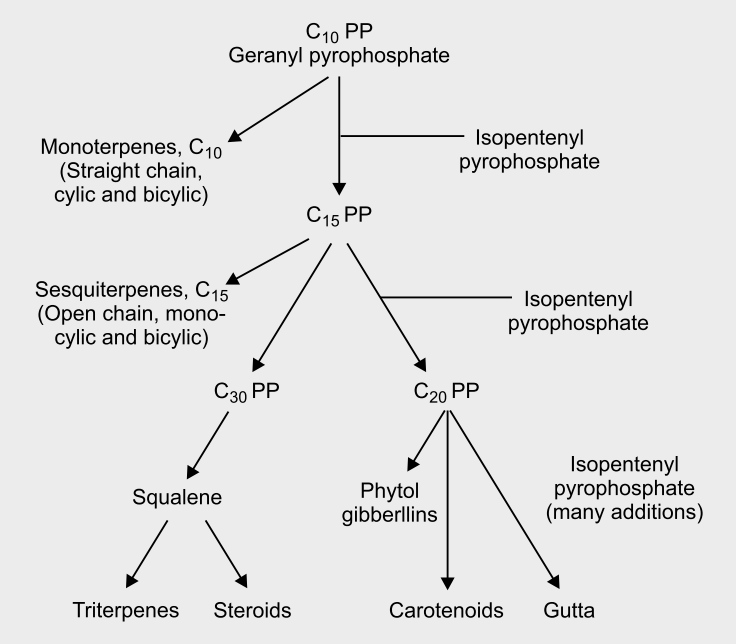

Fig. 1.5: The role of the acetate pathway in the synthesis of various secondary metabolites

Acetate-Malonate Pathway

The biosynthesis of fatty acids is catalyzed by the enzyme fatty acid synthase. Acetyl-CoA and malonyl-CoA are converted into enzyme-bound thioesters, the malonyl ester using an acyl carrier protein (ACP). The Claisen reaction follows giving acetoacetyl-ACP (β-keto acyl- ACP; R=H), which is reduced stereospecifically to the corresponding β-hydroxy ester, consuming NADPH in the reaction. Then follow the elimination of water giving the E (trans) α, β-unsaturated ester. Reduction of the double bond again utilizes NADPH and generates a saturated acyl-ACP (fatty acyl-ACP; R=H) which is two carbons longer than the starting material. Fatty acyl-ACP condensing again with malonyl-ACP, and going through successive reduction, dehydration, and reduction steps, gradually increasing the chain length by two carbons for each cycle, until the

required chain length is obtained. At this point, the fatty acyl chain can be released as a fatty acyl-CoA or as a free acid. The chain length elaborated is probably controlled by the specificity of the thioesterase enzymes that subsequently catalyze release from the enzyme. The combination of one acetate (thioester-ACP) starter unit with seven malonates would give the C16 fatty acid, palmitic acid, and with eight malonates the C18 fatty acid, stearic acid. The two carbons at the head of the chain (methyl end) are provided by acetate, not malonate, whilst the remainder are derived from malonate, which itself is produced by carboxylation of acetate. This means that all carbons in the fatty acid originate from acetate, but malonate will only provide the C2 chain extension units and not the C2 starter group. The linear combination of acetate C2 units explains why the common fatty acids are straight-chained and possess an even number of carbon atoms. Natural fatty acids may contain from four to 30, or even more, carbon atoms, the most abundant being those with 16 or 18 carbons. The rarer fatty acids containing an odd number of carbon atoms typically originate from the incorporation of a different starter unit, e.g. propionic acid, or can arise by loss of one carbon from an even-numbered acid. Biosynthesis of unsaturated fatty acids is occurring by more than one biosynthetic route, but in most organisms, the common mechanism is by desaturation of the corresponding alkanoic acid, with further desaturation. Mevalonic acid is itself formed from three molecules of acetyl-CoA, but the mevalonate pathway channels acetate into a different series of compounds than does the acetate pathway. Three molecules of acetyl-coenzyme A are used to form mevalonic acid. Two molecules of acetyl Co-A combine initially in a Claisen condensation to give acetoacetyl-CoA, and a third is incorporated via a stereospecific aldol addition giving the branched-chain ester β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). This third acetyl-CoA molecule appears to be bound to the enzyme via a thiol group, and this linkage is subsequently hydrolyzed to form the free acid group of HMG-CoA. In the acetate pathway, acetoacetic acid thioester (bound to the acyl carrier protein) would have been formed using the more nucleophilic thioester of malonic acid. The mevalonate pathway does not use malonyl derivatives and it thus deviates from the acetate pathway at the very first step. In the second step, acetoacetyl-CoA is a more acidic substrate and might act as the nucleophile rather than the third acetyl-CoA molecule. The enzyme thus achieves what is a less favorable reaction, e.g. the conversion of HMG-CoA into MVA involves a two-step reduction of the thioester group to primary alcohol and provides an essentially irreversible and rate-limiting transformation. The six-carbon compound MVA is transformed into the five-carbon phosphorylated isoprene units in a series of reactions, beginning with phosphorylation of the primary alcohol group. Two different ATP-dependent enzymes are involved, resulting in mevalonic acid diphosphate, and decarboxylation/dehydration then follows to give IPP (isopentyl pyrophosphate). IPP is isomerized to the other isoprene unit, DMAPP (3, 3-dimethyl allyl pyrophosphate), by an isomerase enzyme (Figs 1.2 and 1.4). This conversion generates a reactive electrophile and, therefore, a good alkylating agent. DMAPP possesses a good leaving group, the diphosphate. These two main intermediates IPP and DMAPP set the 'active isoprene' unit as a basic building block of isoprenoid compounds. Both of these units yield geranyl pyrophosphate (C10-monoterpenes) which further in association with IPP produces farnesyl pyrophosphate (C15-sesquiterpenes) (Fig. 1.4). Farnesyl pyrophosphate with one more unit of IPP develops into geranylgeranyl pyrophosphate (C20-diterpenes). The farnesyl pyrophosphate multiplies with its unit to produce squalene, and its subsequent cyclization gives rise to cyclopentanoperhydrophenanthrene skeleton

containing steroidal compounds like cholesterols and other groups like triterpenoids. The acetate mevalonate pathway thus works through IPP and DMAPP via squalene to produce two different skeleton containing compounds, that is, steroids and triterpenoids (Fig. 1.4). It also produces a vast array of monoterpenoids, sesquiterpenoids, diterpenoids, carotenoids, polyprenols, and also the compounds like glycosides and alkaloids in association with other pathways.

Significances

- Numerous compounds classed as terpenoids or steroids are biosynthesized by the acetate-mevalonate pathway.
- The mevalonate pathway is an important metabolic pathway that plays a key role in multiple cellular processes by synthesizing sterol isoprenoids, such as cholesterol, and non-sterol isoprenoids, such as dolichol, heme-A, isopentenyl tRNA, and ubiquinone.

AMINO ACID PATHWAY

They arise at various levels of EMP pathway (glycolysis) and TCA (Krabs cycle) systems. N appears to enter the metabolism of the organism by reductive amination of alpha ketoacids: pyruvic, oxalacetic and alpha-ketoglutaric acids give alanine, aspartic acid, and glutamic acid respectively. By transamination with other appropriate acids, alanine, aspartic acid, and glutamic acid serve as alpha amino donors in the formation of other amino acids. Proline, hydroxyproline, ornithine, and arginine: These amino acids are of importance in the secondary metabolism of some plants in that they are precursors of several alkaloids. Their formation is complex. N acetyl derives are involved in the formation of ornithine. Arginine is produced from ornithine through the urea cycle.

Plants can synthesize all 20 of the amino acids. Whereas humans cannot synthesize some of them. These 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 non-essential amino acids are synthesized by simple pathways, whereas the biosynthesis of the essential amino acids is complex. All 3 aromatic amino acids are derived from the SAP.

All amino acids are derived from intermediates in glycolysis, the TCA cycle, or the pentose phosphate pathway (HMP pathway). Nitrogen enters these pathways by way of glutamate and glutamine. Mammals can synthesize only about half of them generally those with simple pathways.

α-Ketoglutarate gives Rise to Glutamate, Glutamine, Proline, and Arginine

Proline is a cyclized derivative of glutamate. ATP reacts with the side-chain carboxyl group of glutamate to form an acyl phosphate, which is reduced by NADPH or NADH to glutamate γ-semialdehyde. This intermediate undergoes rapid spontaneous cyclization and is then reduced further to yield proline.

Arginine is synthesized from glutamate via ornithine and the urea cycle in animals. The pathways to proline and arginine are somewhat different in mammals. Proline can be synthesized by the pathway above mentioned, but it is also formed from arginine obtained from dietary or tissue protein. Arginase, a urea cycle enzyme, converts arginine to ornithine and urea. The ornithine is converted to glutamate γ-semialdehyde by the enzyme ornithine γ-aminotransferase. The semialdehyde cyclizes, which is then

converted to proline. The pathway for arginine synthesis is different in mammals. When arginine from dietary intake or protein turnover is insufficient for protein synthesis, the ornithine γaminotransferase reaction operates in the direction of ornithine formation. Ornithine is then converted to citrulline and arginine in the urea cycle.

Serine, Glycine, and Cysteine are Derived from 3-phosphoglycerate

The major pathway for the formation of serine is the same in all organisms. The hydroxyl group of 3-phosphoglycerate is oxidized by a dehydrogenase (using NAD) to yield 3-phosphohydroxypyruvate. Transamination from glutamate yields 3-phosphoserine, which is hydrolyzed to free serine by phosphoserine phosphatase. Serine (three carbons) is the precursor of glycine (two carbons) through the removal of a carbon atom by serine hydroxymethyltransferase. The overall reaction, which is reversible, also requires pyridoxal phosphate. Together with cysteine and cystine, these amino acids arise at the triose level of metabolism. Alanine, valine, and leucine isoleucine: Produced similarly to valine but the precursor is α-aceto α-hydroxy propionic acid instead of alpha aceto lactic acid.

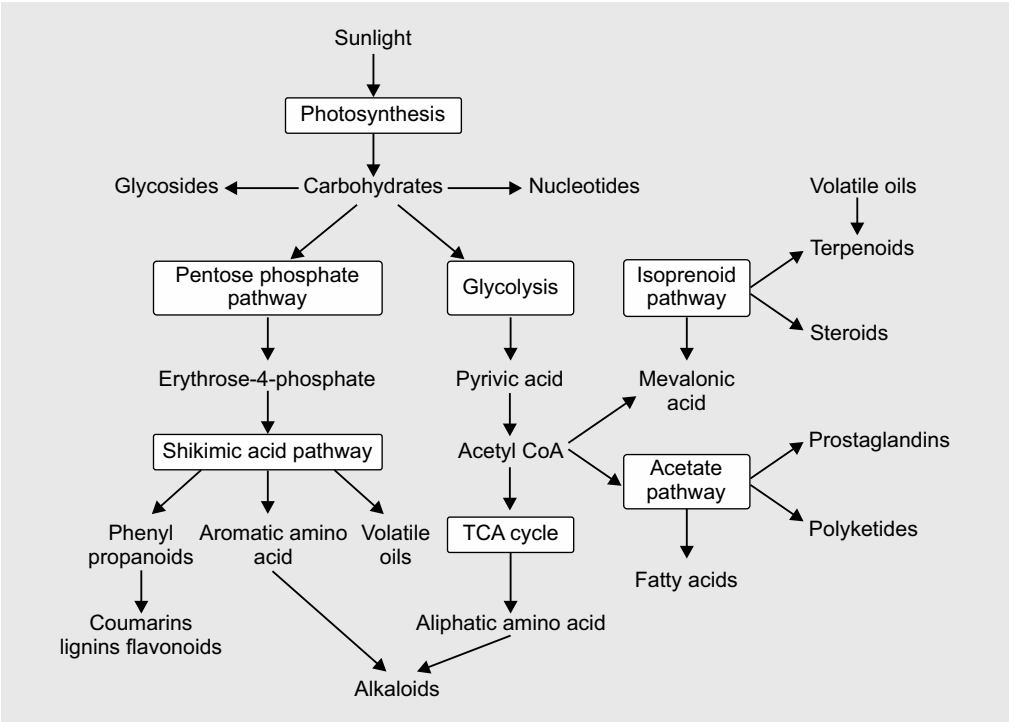

Fig. 1.6: The basic metabolic pathway

Lysine

- It is derived from aspartate involving a pathway utilizing 2, 3-dihydropicolinic acid and diaminopimelic acid.
- It is the precursor of some alkaloids of *Nicotiana, Lupinus,* and *Punica*.
- *Lysine:* $H_2N\text{-}(CH_2)_4\text{-}CH(NH_2)\text{-}COOH$

STUDY OF THE UTILIZATION OF RADIOACTIVE ISOTOPES IN THE INVESTIGATION OF BIOGENETIC STUDIES

A radioactive tracer (RT), radiotracer, or radioactive tag is a chemical compound in which one or more atoms have been replaced by a radionuclide so that, due to its radioactive decay, it can be used to investigate the mechanism of chemical reactions by following the path that the radioisotope takes from the reactants to the products. Radio labeling or radio monitoring is therefore the radioactive component of an isotope tag. Hydrogen (H), carbon (C), phosphorus (P), sulfur (S), and iodine radioisotopes have been used extensively to map the course of biochemical reactions. A RT may also be used to track the distribution of a material within a natural system, such as a cell or tissue, or as a stream tracer to track fluid flow. RT are also used to determine the location of fractures caused by fractures Produced by hydraulic fracturing in the production of natural gas. RT forms the basis of a wide range of imaging devices, such as PET scans, SPECT scans, and technetium scans. Radiocarbon dating uses the naturally occurring carbon-14 isotope as an isotope tag. The isotopes of the chemical element vary only in weight. For example, hydrogen isotopes can be written as 1H, 2H, and 3H, the mass number is superseded to the left. When the atomic nucleus of the isotope becomes unstable, the substances comprising the isotope are radioactive. Tritium is an example of a radioactive isotope. The idea behind the use of RT is that an atom in a chemical compound is replaced by another atom with the same chemical element. Nevertheless, the substituting atom is a radioactive isotope. This method is often referred to as nuclear marking. The effectiveness of this method is because radioactive decay is much more energetic than chemical reactions. Radioactive isotopes can therefore be present at low concentrations and measured by sensitive radiation detectors such as Geiger counters or scintillation counters. George de Hevesy received the 1943 Nobel Prize in Chemistry for his work on the use of isotopes as tracers in the study of chemical processes. There are two main ways in which RT are used. When a labeled chemical compound undergoes chemical reactions, one or more of the components will have a radioactive tag. Analysis of what happens to the radioactive isotope provides detailed information on the chemical reaction process. A radioactive compound is inserted into the living organism and the radioisotope provides a means of creating a picture showing how the compound and its reaction products are dispersed around the organism.

Living plants are known to be biosynthetic laboratories for the processing of both primary and secondary metabolites. Various intermediate steps are involved in the biosynthetic pathway in plants can be investigated using the following techniques:

1. Tracer technique
2. Use of isolated organ
3. Grafting methods
4. Use of mutant strain

Out of the above 4 methods, in the Tracer technique method, radioactive isotopes are used for the investigation of biogenetic studies.

Tracer Technique

In this technique, the radioactive substances are frequently used as tracers or tagged in various fields. Radio-isotope is added to the reactant and its movement is studied by measuring radioactivity in the draft plant. It can be defined as a technique that utilizes

a labeled compound to find out or to trace the different intermediates and various steps in biosynthetic pathways in plants, at a given rate and time. When these isotopes are added into the plant they become part of the metabolic pool and undergo reaction characteristic to the metabolism of that particular plant.

It can be described as a technique that uses a marked compound to identify or trace specific intermediates and different steps in biosynthetic pathways in plants at a given rate and time. This technique often uses marked compounds that, when introduced into the plant system become part of the general metabolic pool and undergoes reactions. This method was used specifically for the determination of primary metabolites and secondary metabolites in biosynthetic pathways. Metabolic products of plants: A living plant can produce two kinds of useful metabolites by absorbing food, water, sunlight, and minerals. *Primary metabolites:* These compounds are abundant in nature and are found in various forms in all types of species. They are important for the normal growth and development of the organism. *Secondary metabolites:* These are chemical compounds biosynthetically derived from principal metabolites such as carbohydrates, fats, proteins, mineral nutrients, vitamins, etc. Examples: Alkaloids, terpenoids, glycosides, flavonoids, tannins, and coumarins.

Significance of Tracer Technique

- 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 and technique is used.
- Position and 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 give specific information than carbon
- *Tracing of biosynthetic pathway:* For example, by incorporation of a radioactive isotope of ^{14}C into phenylalanine, the biosynthetic cyanogenetic glycoside prunasin, can be detected.
- *Location and quantity of compound containing tracer:* ^{14}C labeled glucose is used for determination of glucose in a biological system
- *Different tracers for different studies:* For studies on amino acids nitrogen gives more specific information than C.
  - *For glycosides:* O, N, S, C atoms
  - *For terpenoids:* O atoms

Basic Steps involved in Tracer Technique

- Preparation of labeled compound
- Incorporation of the labeled compound into tissue system
- Separation or isolation of labeled compound
- Determination of natural metabolites

Preparation of Labeled Compound

The labeled compound can be prepared by the use of two types of isotopes.

- *Radioactive isotopes:* Example: ^{1}H, ^{14}C, ^{24}Na, ^{42}K, ^{35}S, ^{35}P, ^{131}I decay with emission of radiation]
- *Stable isotopes:* Example: ^{2}H, ^{13}C, ^{15}N, ^{18}O

For biological investigation, carbon and hydrogen are mostly used. For specific purposes S, P, alkali, and alkaline earth metals are used. Natural C possesses 2 isotopes with masses no 12 and 13. A radioactive isotope of C has masses no 10, 11, and 14. For studies on protein, alkaloids, and amino acid labeled nitrogen atoms give more specific information. ^{3}H compound is commercially available.

Radioisotope may be

- *Generally labeled:* Average distribution of radioisotope in the various positions of the molecule is irregular
- *Uniformly labeled:* Average distribution of radioisotope in all possible positions of the molecule is regular
- *Specifically labeled:* When radioisotopes are localized in certain well-defined positions and well defined relative abundances
- *For example (1–^{14}C) phenylalanine:* ^{14}C is present on C1 of carboxylic end
- *(1,3–^{14}C) phenylalanine:* ^{14}C is present on C1 and C3 positions
- A labeled compound can be prepared by growing Cholera in $^{14}CO_2$ environment—all C compounds are ^{14}C labeled
- The tritium ^{3}H compound is commercially available. Tritium labeling is affected by the catalytical exchange in aqueous media by hydrogenation of unsaturated compounds with tritium gas
- Or by use of organic synthesis

Criteria for Tracer Element

- The starting concentration of tracer must be sufficient to withstand resistance with dilution in course of metabolism.
- *Proper labeling:* For proper labeling physical and chemical nature of the compound must be known.
- A labeled compound should involve in the synthesis reaction.
- Labeled should not damage the system to which it is used.

Incorporation of the Labeled Compound into Tissue System

Incorporation of the labeled compound into the tissue system is by following methods

- Root feeding
- Stem feeding
- Direct injection
- Wick feeding
- Floating method
- Spraying technique
- *Root feeding:* Plants are cultivated hydroponically to avoid microbial contamination, e.g. tobacco
- *Stem feeding*
  - The substrate can be administered through cut stems immersed into the solution containing the radioisotope
  - Not useful for latex-containing plants
  - *Direct injection:* For plants with hollow stem
- *Infiltration/Wick feeding*
  - For feeding the plant root in the soil or any other support without disturbing it

- A thread dipped in the solution containing radioisotope is drowned through the stem
- A flap cut on the stem can be dipped into the solution containing

- *Floating method:* The plant material is allowed to float on the solution containing the radioisotope
- *Spraying technique:* Solution containing radioisotope is sprayed on the plant part
- *Separation/isolation of the labeled compounds*
  - *For soft fresh tissue:* Infusion, maceration
  - *For hard tissue:* Hot percolation, decoction
  - *For unorganized drug:* Maceration with choice of solvent

  Fractional crystallization, partition chromatography are also used

Choice of Solvent for Extraction

- *Fat and oil:* Nonpolar solvent
- *An alkaloid, glycoside, and flavanoid:* Slightly polar solvent
- *Plant phenol:* Polar solvent

The usual method of detection is:

- Mass spectroscopy,
- Autoradiography
- Scintillation counter
- Gieger Muller counter
- Gas ionization chamber
- NMR
- Radio paper chromatography

Detection and Assay of the Radioactively Labeled Compound

- The metabolite is isolated, purified, and analyzed for isotopic content
- If $A \rightarrow B \rightarrow C \ldots X \rightarrow Y \rightarrow Z$
- The relation between precursor and metabolite is established

Methods in Tracer Technique

Precursor Product Sequence

In this, the 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.

Disadvantage: The radioactivity of isolated compounds alone is not usually sufficient evidence that the particular compound fed is a direct precursor, because the substance may enter the general metabolic pathway and from there may become randomly distributed through a whole range of products.

Application: This method is applied to the biogenesis of morphine and ergot alkaloids

Double and Multiple Labeling

In this method, specifically labeled precursor and their subsequent degradation of recover product are more employed.

Application

- This method is extensively applied to study the biogenesis of plant secondary metabolites.
- Used for the study of morphine alkaloid, e.g. Leete used doubly labeled lysine to determine which hydrogen of lysine molecule was involved in the formation of piperidine ring of anabasine in *Nicotiana glauca* (*N. glauca)*

Competitive Feeding

It may clarify the relative position of two intermediates

Application

- This method is used for elucidation of the biogenesis of propane alkaloids.
- Biosynthesis of hemlock alkaloids (conline, conhydrine, etc.) e.g. biosynthesis of alkaloids of *Conium maculactum* (hemlock) using ^{14}C labeled compounds.

Isotope Incorporation

This method provides information about the position of bond cleavage and its formation during the reaction, e.g. Glucose-1-phosphate cleavage is catalyzed by alkaline phosphatase. This reaction occurs with cleavage of either the C–O bond or the P–O bond.

Sequential Analysis

The principle of this method of investigation is to grow the plant in atmosphere $^{14}CO_2$ of and then analyze the plant at a given time interval to obtain the sequence in which various and sequential analysis has been very successfully used in the elucidation of carbon in correlated compound become labeled.

Application

- $^{14}CO_2$ photosynthesis.
- Determination of sequential formation of opium hemlock and tobacco alkaloids.
- Exposure as less as 5 min of $^{14}CO_2$, is used in detecting biosynthetic sequence as Piperitone- (-) Menthone- (-) Menthol in *Mentha piperita*.

Application of Tracer Technique

- Study of squalene cyclization by use of ^{14}C, ^{3}H labeled mevalonic acid.
- Interrelationship among 4-methyl sterols and 4, 4 dimethyl sterols, by use of ^{14}C acetate.
- Terpenoid biosynthesis by chloroplast isolated in an organic solvent, by use of 2–^{14}C mevalonate.
- Study the formation of cinnamic acid in the pathway of coumarin from labeled coumarin.
- Origin of carbon and nitrogen atoms of purine ring system by use of ^{14}C or ^{15}N labeled precursor.
- Study of the formation of scopoletin by use of labeled phenylalanine.
- By use of ^{45}Ca as a tracer, found that the uptake of calcium by plants from the soil. (CaO and $CaCO_2$).
- By adding ammonium phosphate labeled with ^{32}P of known specific activity the uptake of phosphorus is followed by measuring the radioactivity as the label reaches first in the lower part of the plant than the upper part, i.e. branches, leaves, etc.

Use of Isolated Organ

This method is focused on the use of isolated sections of plants (e.g. stem roots, etc.). This method is useful for the determination of the site of the synthesis of a particular compound. For exmaple: Roots and leaves for the study of Nicotiana and Datura, Petal disk for the study of rose oil, tropane alkaloids developed at the root of the Solanaceae family

Grafting Methods

This method is used for the study of the production of alkaloids by Grafted Plants. For exmaple: Tomato scions grafted on Datura accumulate alkaloids, while Datura scion grafted on tomatoes contained a very small amount of alkaloids. This suggests that the main site for the formation of Datura alkaloids is root.

Use of Mutant Strains

- The mutant strains of alkaloids are produced with a lack of certain enzymes.
- For exmaple: Gibberella mutant is produced to produce isoprenoids. *Lactobacillus acidophillus* is used for the mevalonic acid pathway for isoprenoid biosynthesis
- *Enzymatic study:* Enzymatic block can be applied through mutation to understand biogenesis.
- Mutant doesnot have the enzyme required for growing on minimum media
- Can be easily identified and isolated.

QUESTIONS

Short Questions

1. Define metabolism.
2. Name the basic metabolic pathways.
3. Write about the significance of the Shikimic acid pathway.
4. Classify metabolism with examples.
5. Give notes on tracer technique for investigation of biogenetic studies.
6. Give a note on the amino acid pathway.
7. Define biogenesis
8. Name the various methods for investigation of biogenetic pathway.
9. Write the differences between synthesis and biogenesis examples.
10. Write the significance of the acetate pathway.
11. What are radioactive isotopes?

Long Questions

1. Describe in detail about shikimic acid pathway.
2. Discuss the amino acid pathway.
3. Describe acetate pathways.
4. Mention basic steps involved in the tracer technique.
5. Write in detail about different techniques for investigation of biogenetic pathway.
6. Discuss the significance of tracer technique.
7. Illustrate the importance of SAP.
8. Interpret the significance of acetate pathway.
9. Explain the application of tracer technique.