Name of Unit	Metabolic Pathways in Higher Plants and Their Determination
Subject /Course	Pharmacognosy and Photochemistry-II
Subject/Course ID	BP 504T
Class: B.Pharm. Semester	5th
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Learning Outcome of Module 01

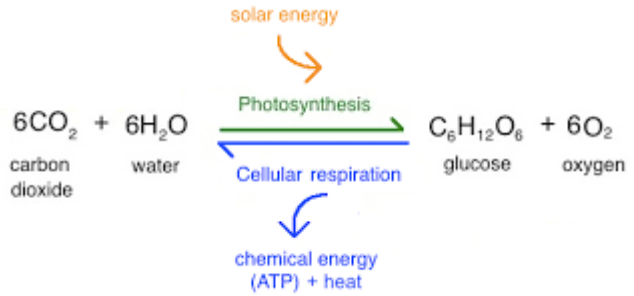
To outline the metabolic pathway in higher plants and their biogenetic studies.

Module Content

Topic
• The basic metabolic pathways producing secondary metabolites • Shikimic acid pathway • Shikimic acid pathway produced secondary metabolites • The Acetate (acetate-malonate) pathway producing Fatty acids • The Acetate-Mevalonte (Isoprenoid) pathway • The Acetate-Mevalonte (Isoprenoid) pathway produced secondary metabolites • The Amino acid Pathway • Radioactive isotopes in the investigation of biogenetic studies- Tracer techniques • Use of mutant strain 2. Use of Isolted organ 3. Grrafting method

THE METABOLIC PATHWAYS IN HIGHER PLANTS AND THEIR DETERMINATION

The plants are the chief source of crude drugs, especially the higher plants. The plants are living organisms. They are dependent on water and sunlight for their food and growth. Photosynthesis produces the important substrates for respiration (O_2) and building blocks (CH_2O)n as starting organic compounds for production of different primary and secondary metabolites.



Plant metabolism is the complex process involving all chemical reactions for maintaining the living state of the cells and the organism, synthesis of metabolites from simple reactants and breakdown of complex organic compounds.

Catabolism is the set of metabolic pathways that breaks down molecules into smaller units that are either oxidized to release energy or used in other anabolic reactions. Catabolism breaks down large molecules into smaller units.

Anabolism is the set of metabolic pathways that construct molecules from smaller units. These reactions require energy, known also as an endergonic process. Anabolism is the building-up aspect of metabolism, whereas catabolism is the breaking-down aspect. Anabolism is usually synonymous with biosynthesis.

The organic products produced in metabolism are called metabolites.

These are of two types.

Primary metabolites are the products of primary metabolism. They aredirectly involved in normal growth and development and thus are primarily needed.They are universally present in plant kingdom and are in abundance.eg.starch, cellulose, amino acids, organic acids, DNA, RNA.Most of the primary metabolites are the starting material for biosynthesis of secondary metabolites. Pharmacologically they are generally inert.

Secondary metabolites are derived from primary metabolites and as such are not essential for plant growth and development but may have specific functions such as attraction for pollination or defense against predators . They have limited distribution in certain genus or family(ies). e.g. Alkaloids (e.g. atropine and quinine),Terpenoids (e.g. menthol and citral),Gycosides(e.g. sennosides and digitoxin),Phenolics(e.g. tannic acid and gallic acid).

The series of chemical reactions producing metabolites regulated by specific enzymes are called

Metabolic Pathways.

Biosynthesis is a multi-step, enzyme-catalyzed process where substrates are converted into more complex products in living organisms. In biosynthesis, simple compounds are modified, converted into other compounds, or joined together to form macromolecules. This process often consists of metabolic pathways.

The building blocks are tiny chemical molecules produced from primary metabolites mostly from photosynthesis, glycolysis, and or Krebs cycle. The are very important in biosynthesis and production of secondary metabolites.These are considered to be intermediates, few important ones are acetyl CoA, shikimic acid , mevalonic acid derived from acetate ,shikimate and mevalonate respectively. The building blocks can be segregated based on the number of Carbon units

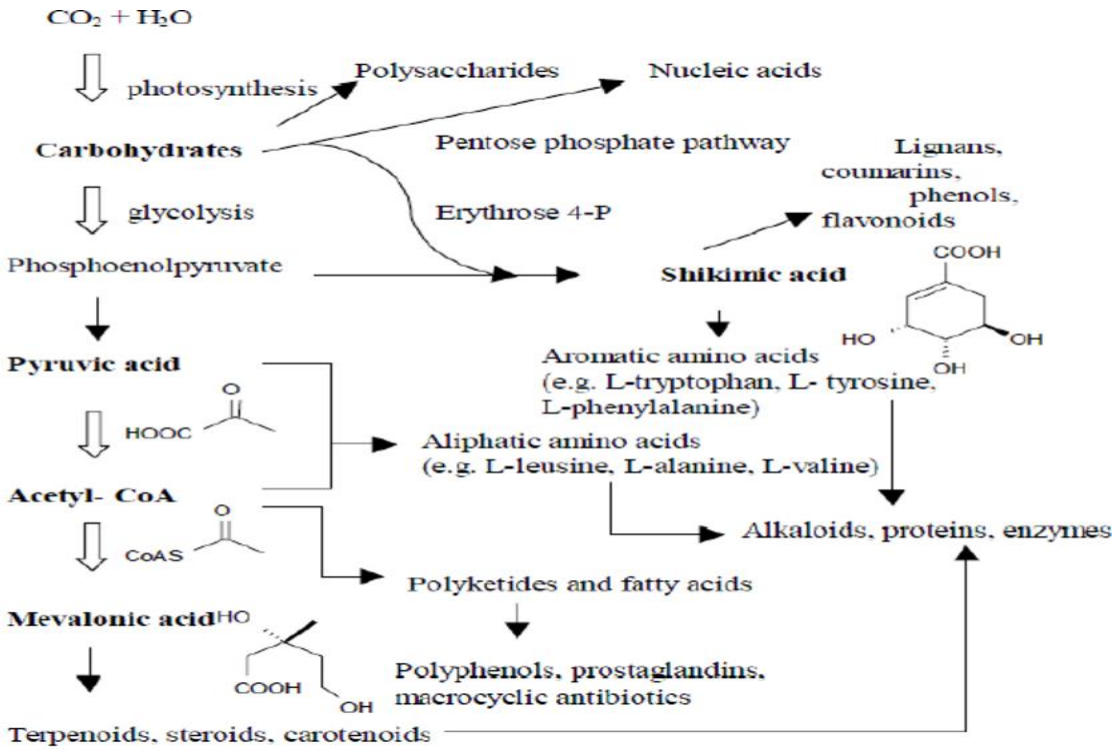
C1 derived from S methyl of L-methionine

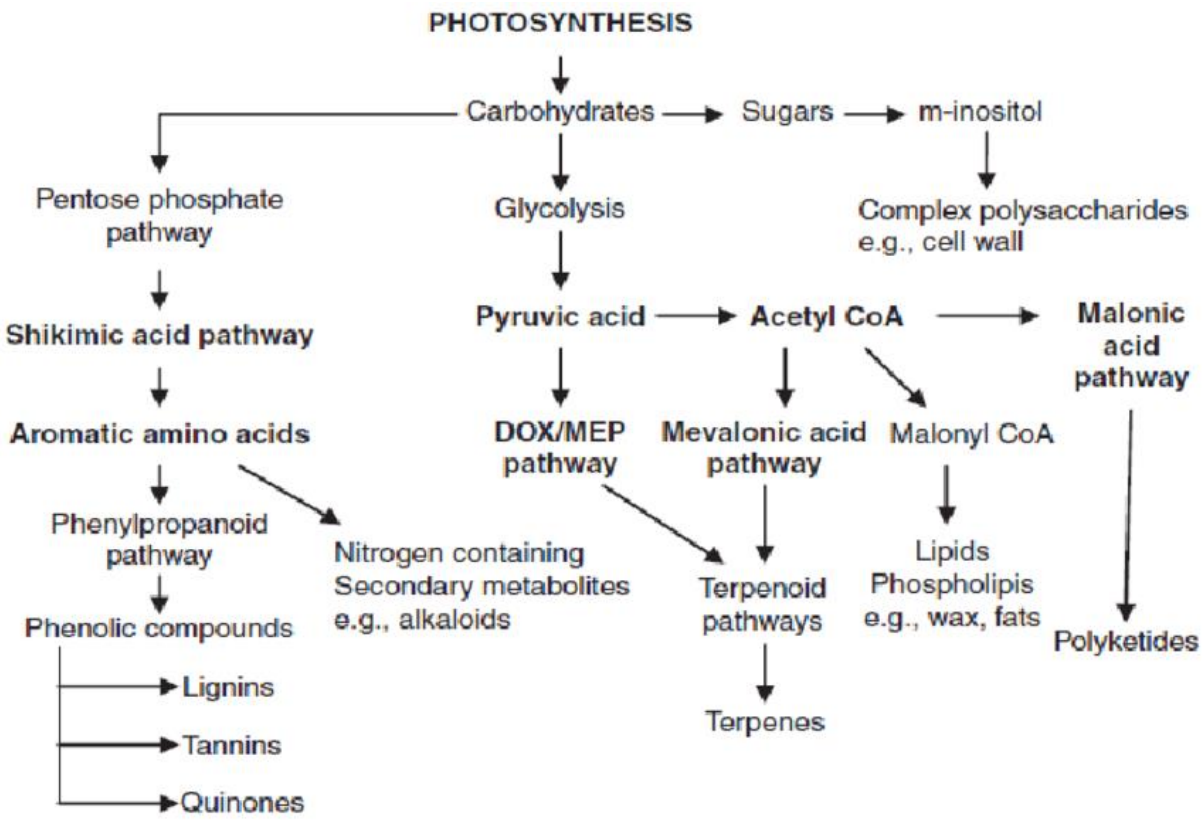
C2 derived from acetyl –CoA

C5 derived from isoprene units

C6-C3 units (phenyl propyl units) are derived from phenylalanine or tyrosine through shikimic acid pathway.

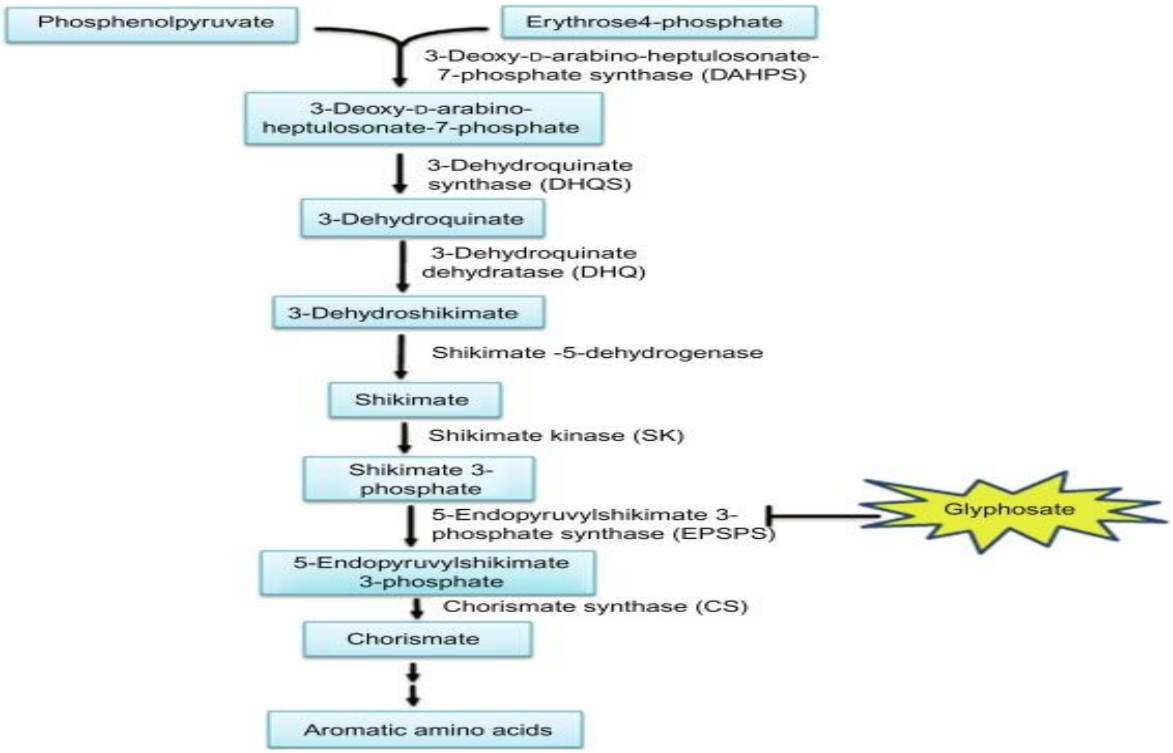
The Basic Metabolic Pathways Leading To Production of Secondary MetabolitesThrough Photosynthesis

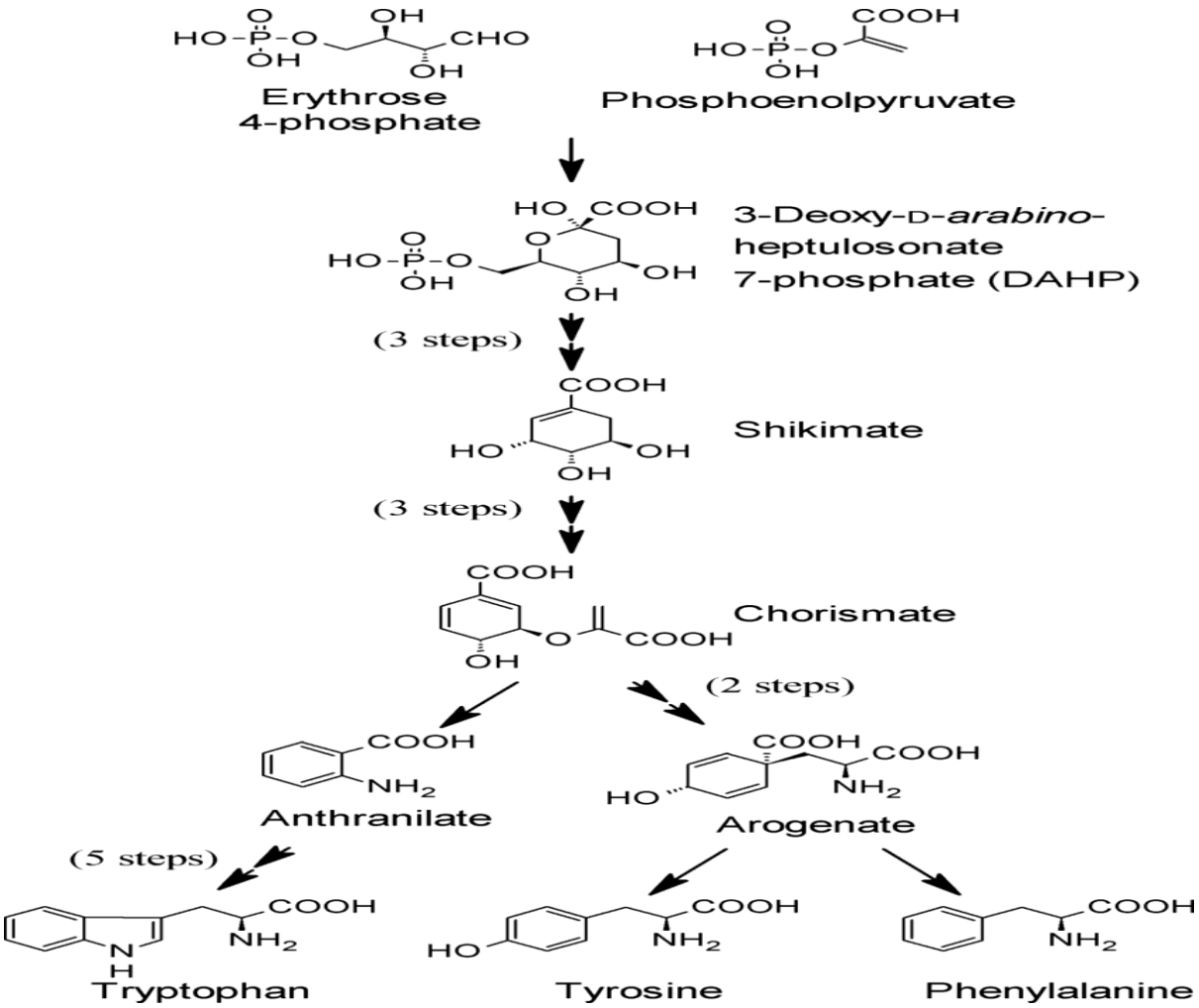




The Shikimic Acid Pathway

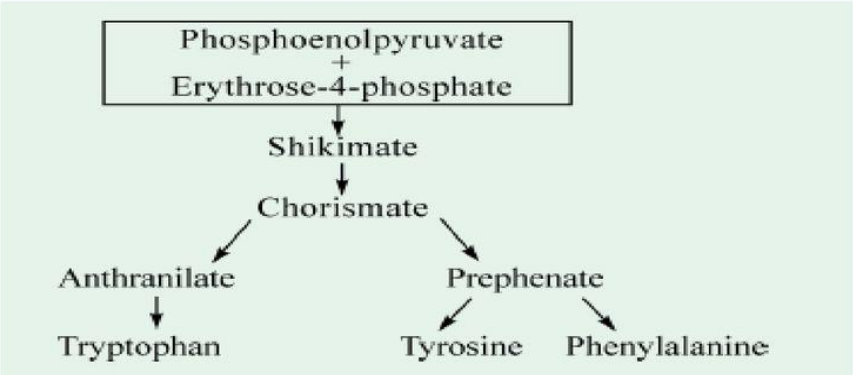
The Shikimate Pathway (shikimic acid pathway) is a seven-step metabolic **pathway** used by bacteria, archaea, fungi, algae, some protozoans, and plants for the biosynthesis of folates and aromatic amino **acids** (phenylalanine, tyrosine, and tryptophan).The pathway starts with two substrates, phosphoenol pyruvate and erythrose-4-phosphate, and ends with chorismate, a substrate for the three aromatic amino acids



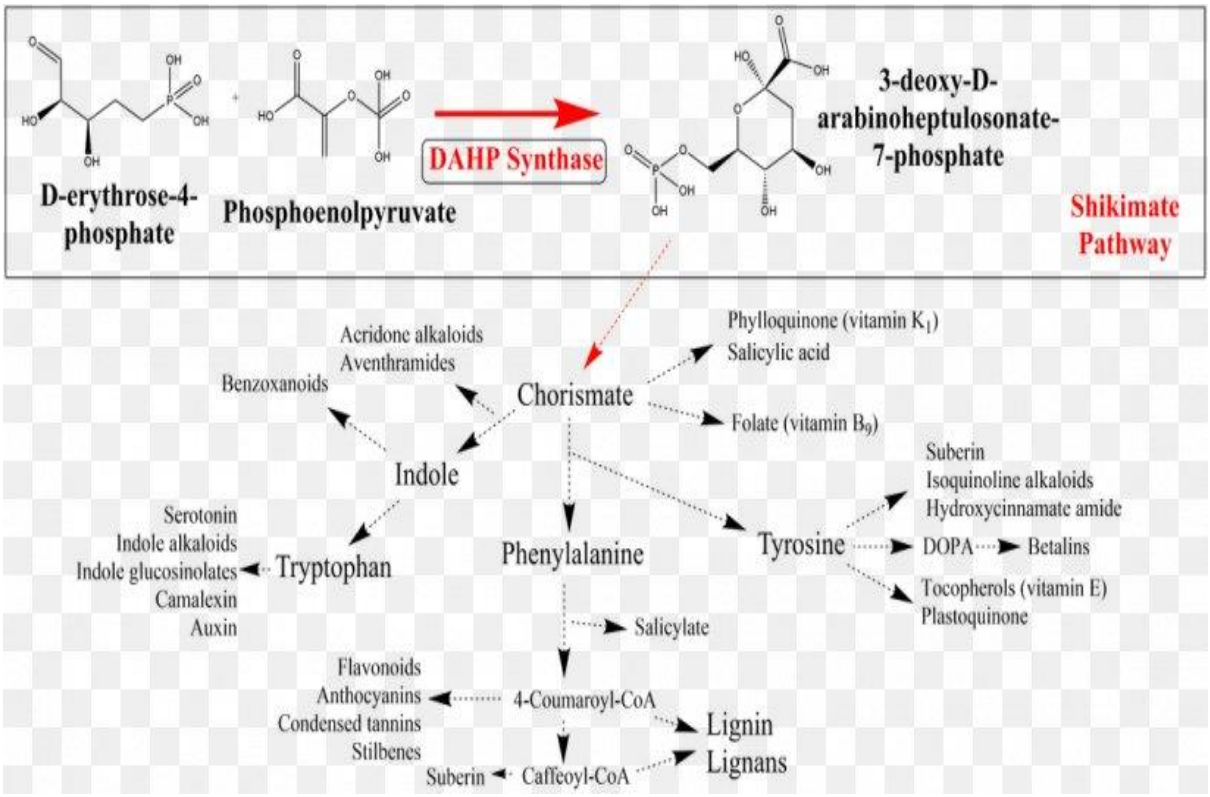


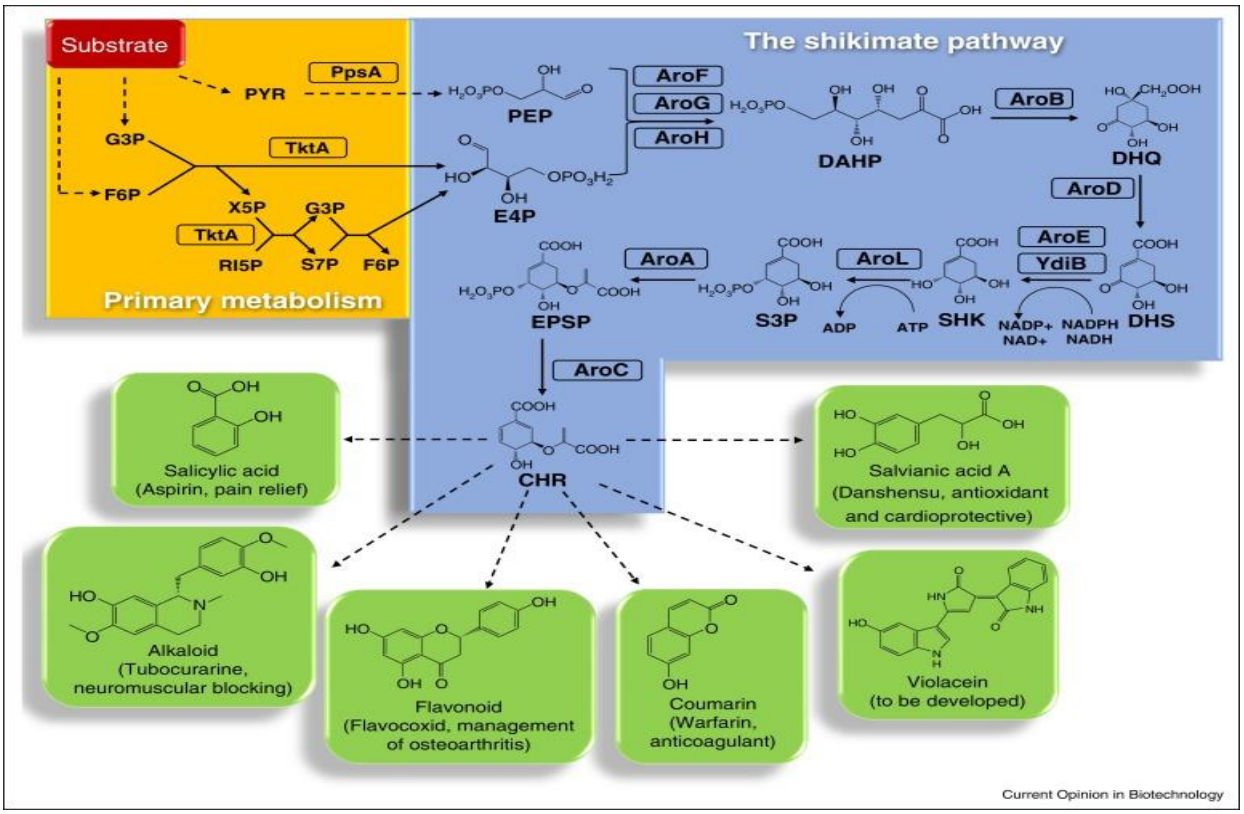
Biosynthesis of aromatic amino acids:

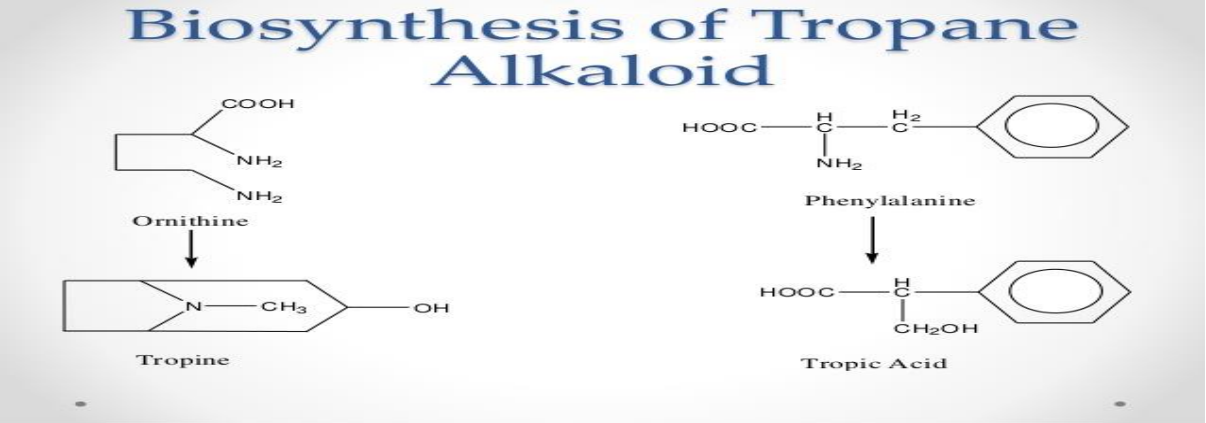
- Biosynthesis of aromatic amino acids starts with a common pathway, the **Shikimate pathway**.
- The biosynthesis begins with **Phosphoenolpyruvate** and **Erythrose-4- phosphate** to form Shikimate.
- Shikimate then goes on to form the branch point intermediate **Chorismate**.
- Chorismate can be converted into **anthranilate** (L-Trp) or **prephenate** (L-Phe and L-Tyr).



Secondary Metabolites produced in Shikimic acid Pathway

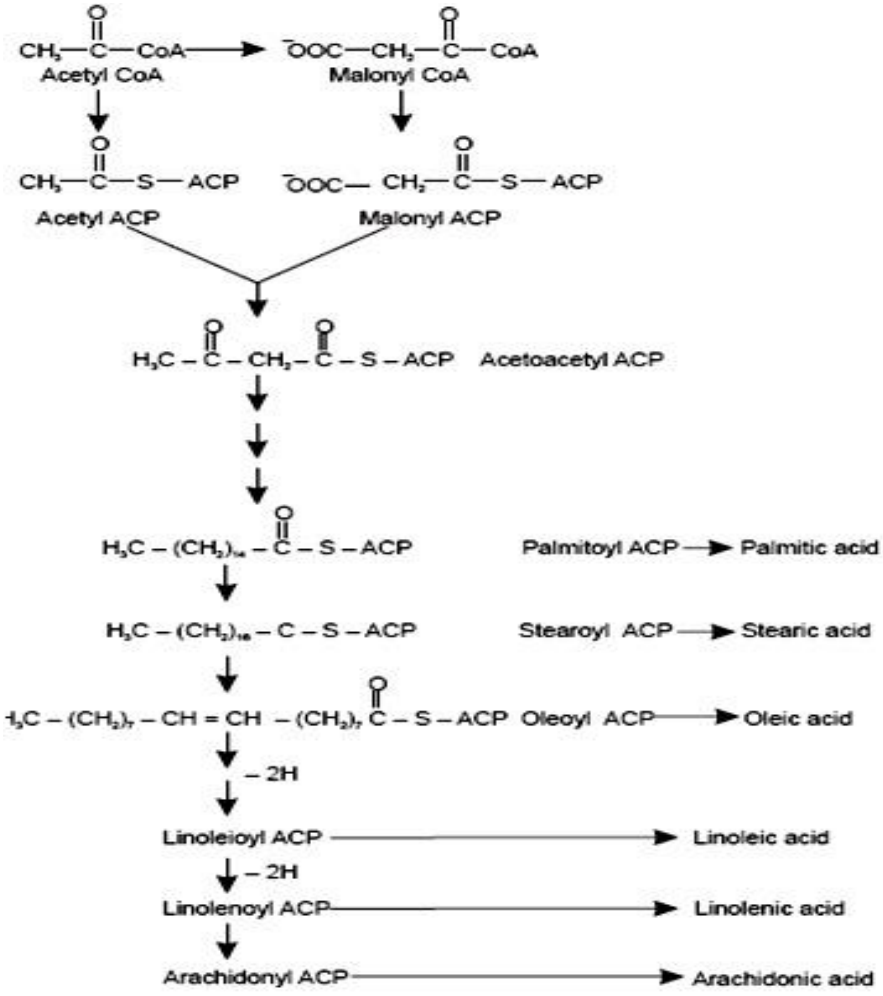






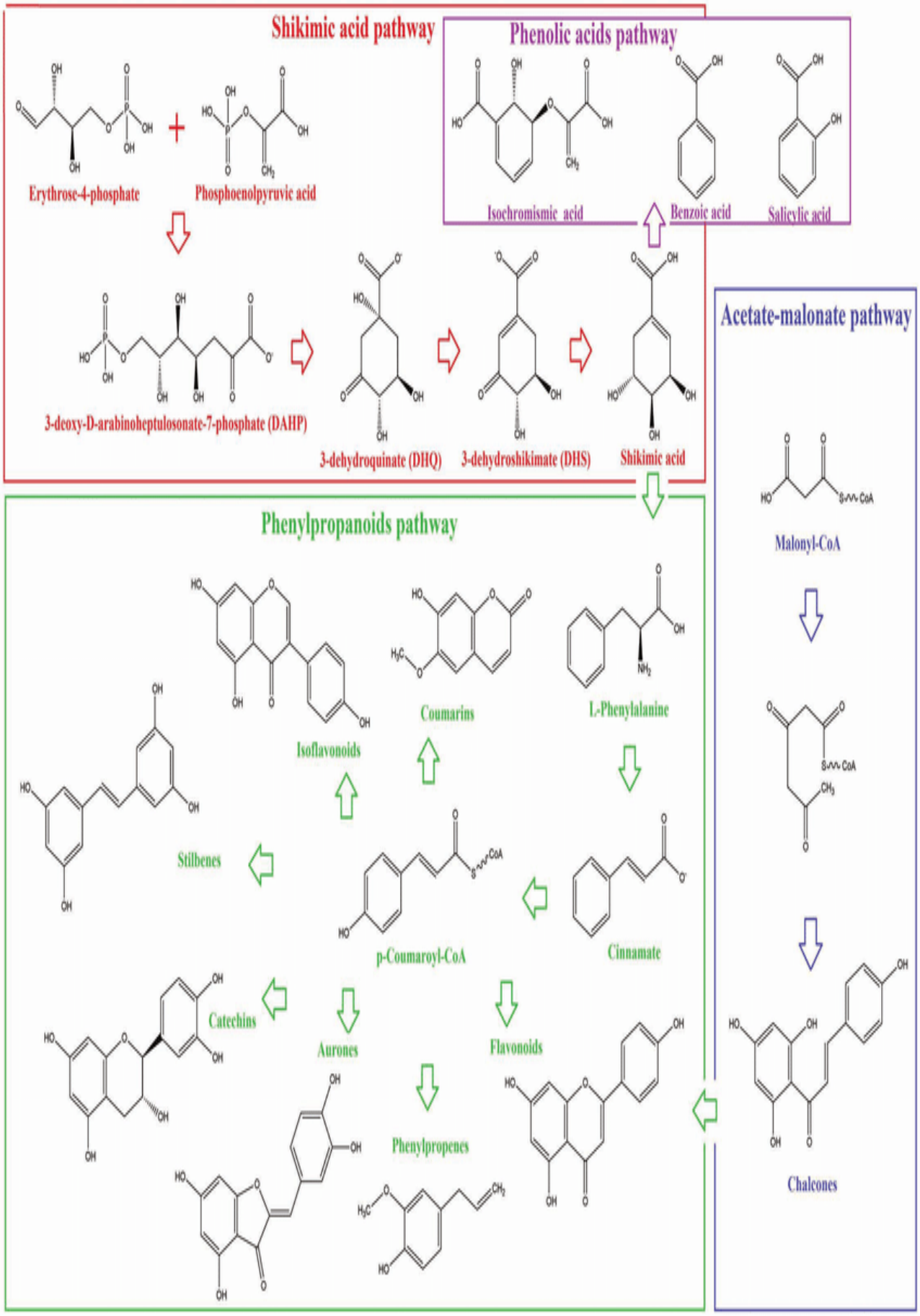
The Acetate –Malonyl pathway

The main products of the acetate-malonate pathway are the fatty acids, both those primary metabolites which occur universally and the more unusual compounds with a restricted distribution.It'salso known as the **acetate pathway** or **polyketide pathway**,which provides malonyl-CoA moieties for the C2 elongation reaction.



The acetate –malonylcpathway for biosynthesis of fatty acids

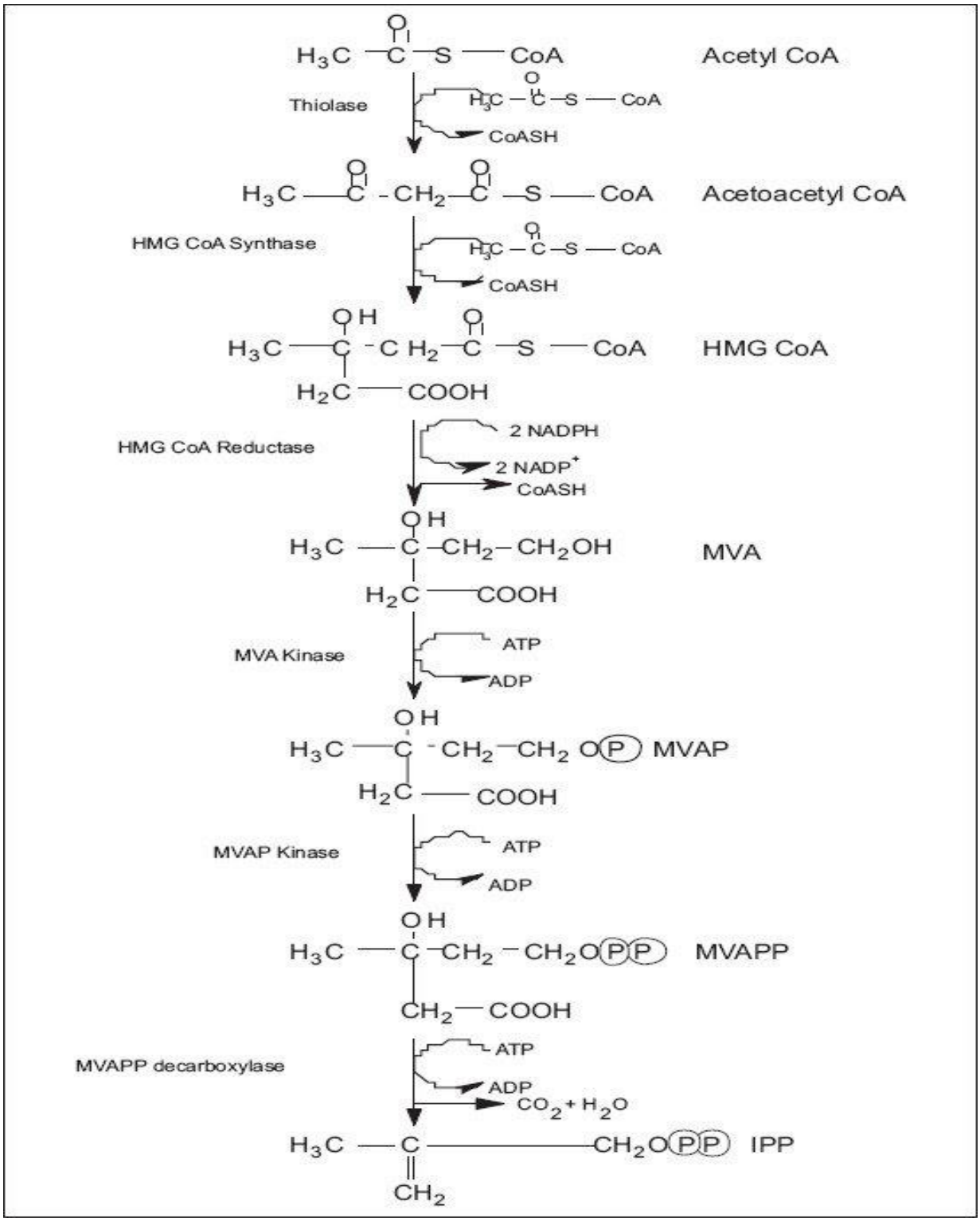
Biosynthesis of Phenolics through Shikimate and Acetate-Malonate Pathway



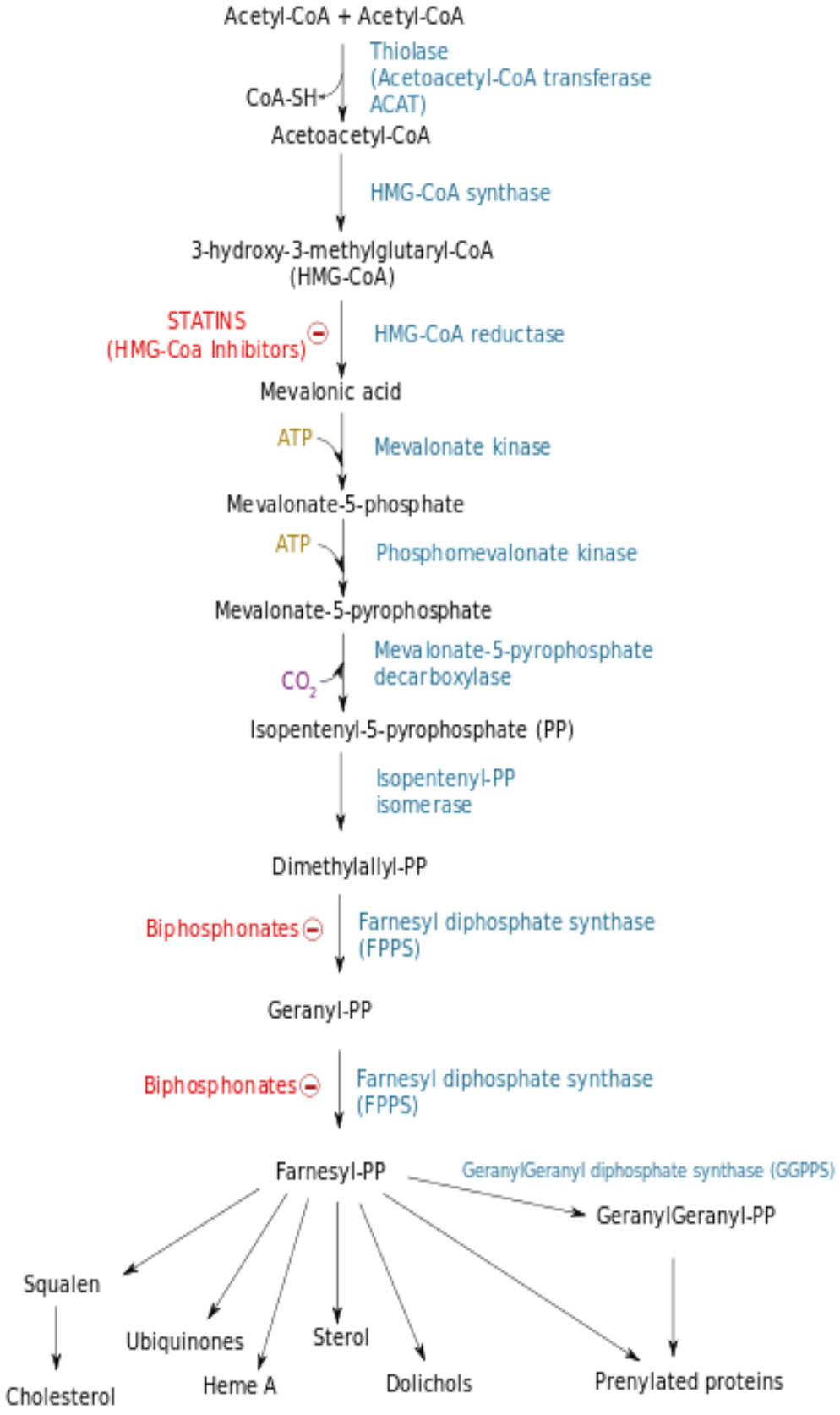
Acetate Mevalonate Pathway

Acetate** Mevalonate **Pathway or Isoprenoid

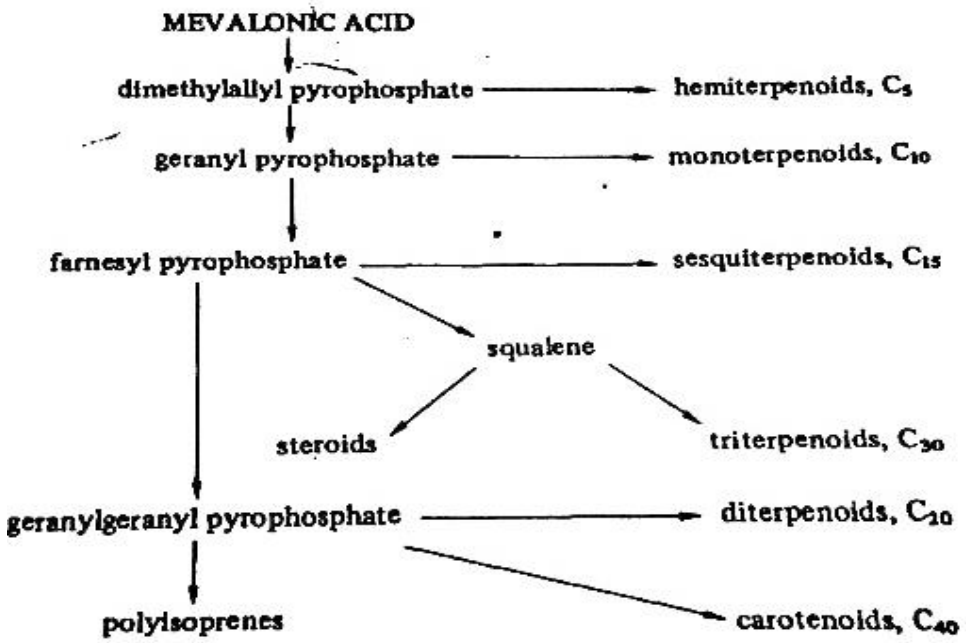
Pathway: Isoprenoid represent functionally and structurally the most diverse group metabolite based on C5. The mevalonate **pathway** begins with acetyl-CoA and ends with the production of IPP and DMAPP.



Mevalonate pathway

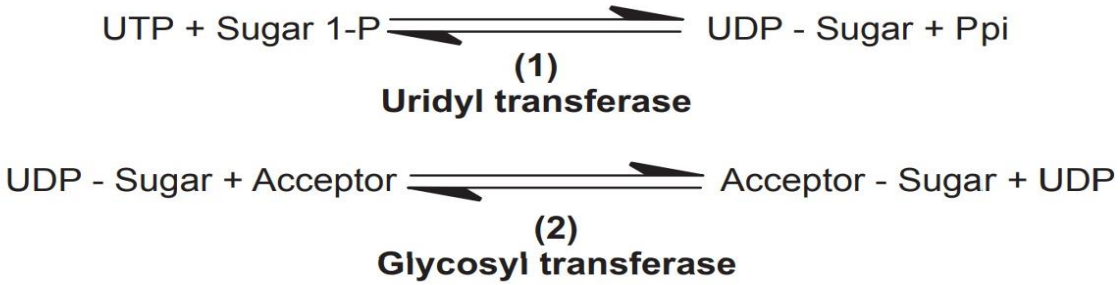


Compounds Biosynthesised by the Acetate- mevalonate Pathway

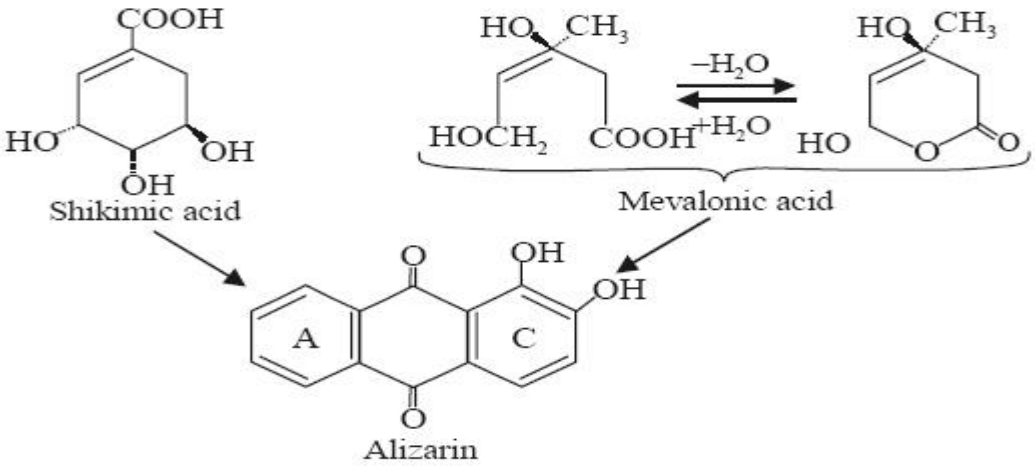


BIOSYNTHESIS OF GLYCOSIDES

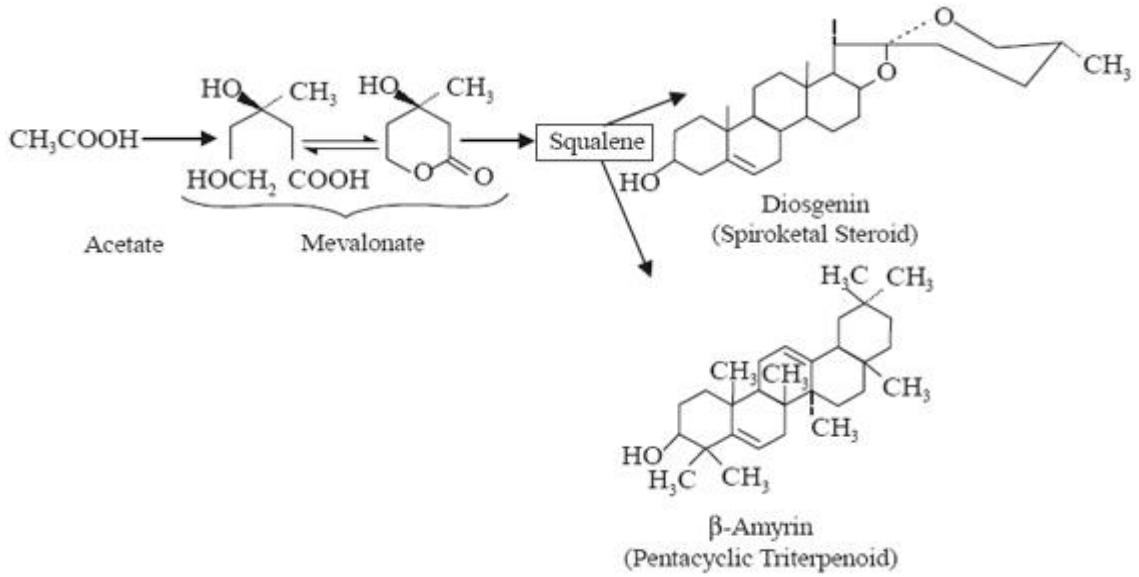
It's two step process :



Biosynthesis of Anthraquinones

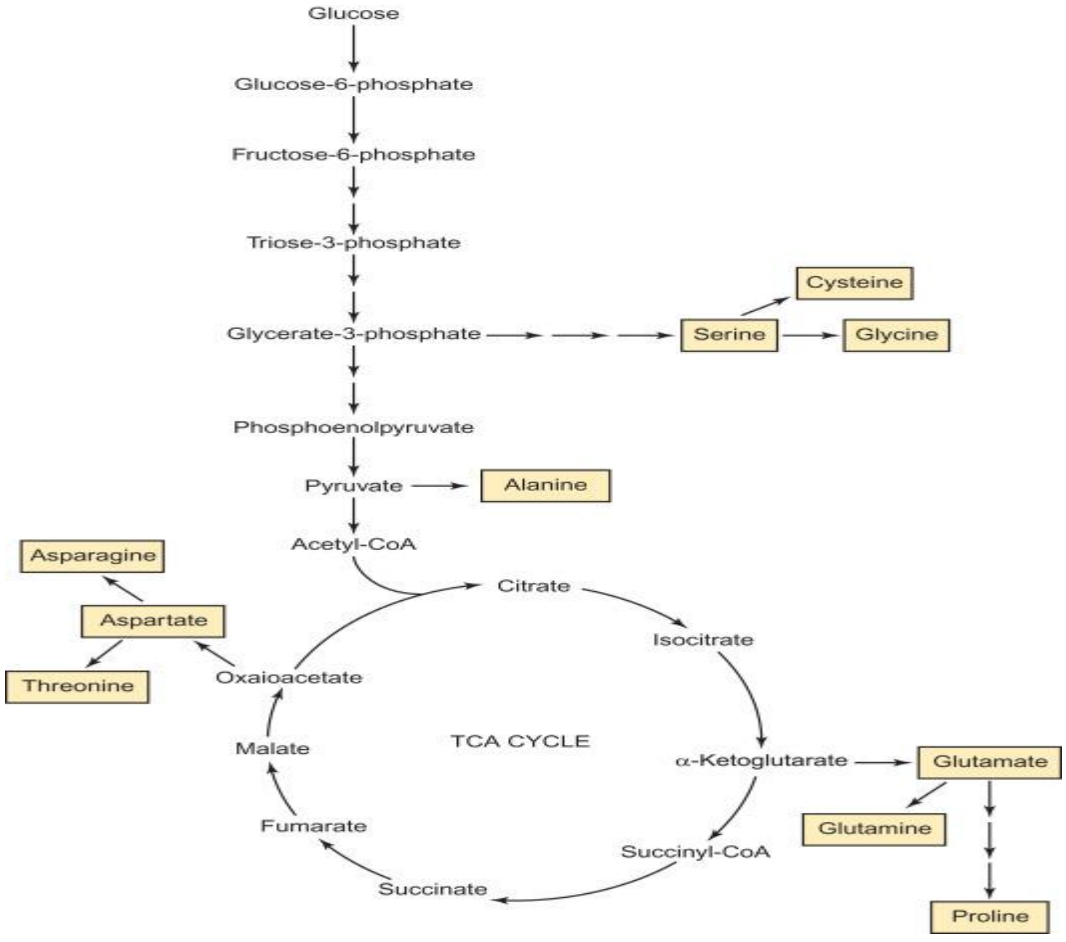


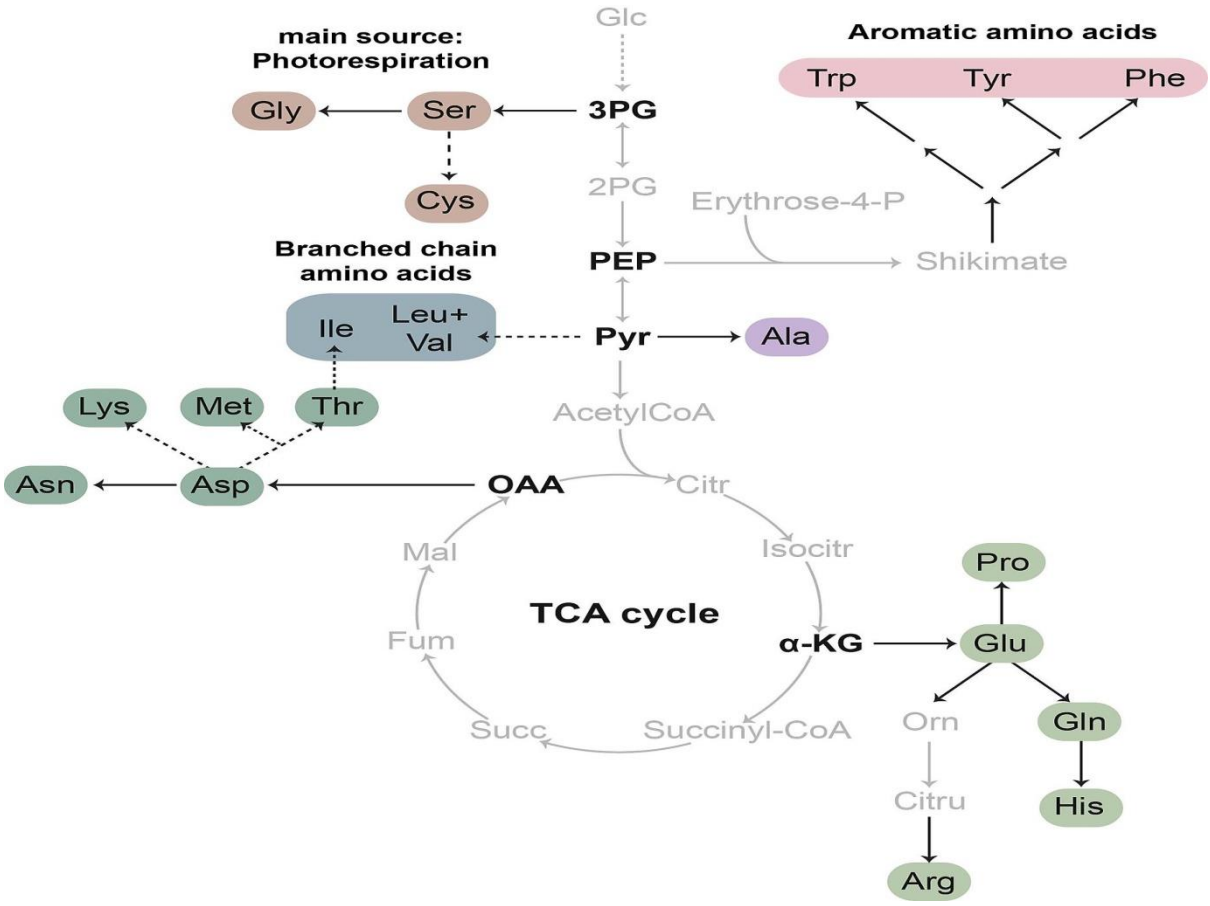
Biosynthesis of Steroids/Saponins



BIOSYNTHESIS OF AMINO ACIDS

All **amino acids** are derived from intermediates in glycolysis, the citric **acid** cycle, or the pentose phosphate **pathway**. Nitrogen enters these **pathways** by way of glutamate and glutamine. Some **pathways** are simple, others are complex:





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

Tracer technique is an effective tool to study these biosynthetic pathways. This **technique** makes use of different isotopes, mainly the radioactive isotopes, which are incorporated into the presumed precursors of plant metabolites and are used as markers in **biogenetic** experiments.

Types

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

Tracer techniques

It can be defined as a technique which utilizes a labeled compound to find out or to trace different intermediates and various steps in biosynthetic pathways in plants at a rate and time. The labeled compound can be prepared by use of two types of isotopes.

* Radioactive isotopes

*Stable isotopes

Radioactive isotopes: Examples: C^{14}. P^{32},H^{1} ,Na^{24},S^{32},P^{35}

For biological investigations –carbon and hydrogen

Metabolic studies – Sulphur, phosphorus, alkali, alkaline earth metals

For studies on proteins and aminoacids – labeled nitrogen

Stable isotopes: Examples: H^{22},C^{13},N^{15},O^{18} This isotopes are scarcely available in nature. Used for labeling compounds as possible intermediates in biosynthetic pathways.Detected by mass spectroscopy and NMR spectroscopy.

Significance of tracer techniques:

1. Tracing of biosynthetic pathway: By incorporation of radioactive isotope into the precursors / starting material, the whole biosynthetic pathway can be traced Ex: By incorporation of radioactive isotope of carbon C14 in to phenylalanine, the biosynthesis of cyanogenetic glycoside prunasine can be traced.

2. Location and quantity of compound containing tracer: If location and quantity of glucose is determined in a biological system C14 labeled glucose may be used. The labeled glucose being chemically indistinguishable from native glucose, will mix completely with available glucose polls in the body of organism studied. Both location and quantity of glucose present in tissues can then be determined by radioactive assay.

3. Different tracers for different studies: For studies on proteins, alkaloids, aminoacids, labeled N_2 atom give more specific information than labeled carbon.

Criteria for tracer techniques:

The starting concentration must be sufficient so as to withstand resistance with dilution in course of metabolism.

The labeled compound must be involve in synthesis reaction.

The labeled compound must be harmless to system to which it is used.

Proper labeling is required. For proper labeling physical and chemical nature of compound must be known

The tracer should be highly pure.

The radioactive isotope with greater halflife period is preferred Ex: ^{10}C - ^{11}C -8.8 Sec to 20mins while^{14}C –about 5000 years so it is preferred.

Steps for tracer techniques:

1. Preparation of labeled compound.
2. Introduction of labeled into biological system.
3. Separation and determination of labeled compound in various biochemical fractions at a later time.

1. Preparation of labeled compound: Many compounds which are most conveniently prepared from natural sources. Ex: by growing chlorella in an atmosphere containing $^{14}CO2$, all the carbon compounds of the organisms become labeled as ^{14}C. The ^{3}H labeled compound are commercially

available. Tritium labeling is effected by catalytic exchange in aqueous media by halogenation of unsaturated compound with tritium gas. ^{3}H is pure beta emitter of low intensity and its radiation energy is lower than ^{14}C.

By use of organic synthesis Grignard reagent (CH_3MgBr).

$CH_3MgBr + 14CO2 \rightarrow CH_3\ ^{14}COOMgBr + H2O$

$CH_3\ ^{14}COOMgBr + H_2O \rightarrow CH_3\ ^{14}COOH + Mg\ (OH)\ Br$

2. Introduction of labeled compound into biological system:

Root feeding and stem feeding : Most common method. Selection of the plant part depends upon the site of biosynthesis of desired metabolites. Root biosynthesis - Tobacco alkaloids Stem biosynthesis - Latex (Euphorbiaceae)

Direct injection method : Hollow stems (Umbelliferae) Capsules (Opium poppy)

Wick feeding : To carry out feeding on plants rooted in soil/other support without disturbance to roots wick feeding is possible. In this method cotton strands are passed through the plant stem. The terminal ends of these cotton strands are immersed in the reagent labeled with radioisotope.

Floating method : When the small amount of material is available leaf discs chopped leaves are made to float on the substrate solution.

Spray method : This method is used for those reagents which are readily absorbed from the leaf surface. Eg : Steroids

The plant is exposed to the organic compound labeled with the radioisotope for a short period of time using one of the above techniques. The biosynthesis occurs sequentially and at each step radioactive products are formed. These products are isolated and identified.

3. **Separation and determination of labeled compound:** Separation of compound depends upon the type of plant material.

Soft and fresh tissue - Maceration, Infusion

Hard tissue - Decoction hot percolation

Unorganized drugs - Maceration

Different solvents are used depending upon the type of plant material.

Fat, oils, alkaloids, glycosides - nonpolar solvents

Flavonoids - slightly polar solvents

Phenols - polar solvents

Determination of labeled compound by various Detectectors like;

Geiger muller counter

Scintillation counter

Auto radiography ϖ Gas ionization chamber

Bernstein ballentine counter

Mass spectrometer

NMR spectrometer

Geiger – Muller counter

A Geiger counter (Geiger-Muller tube) is a device used for the detection and measurement of all types of radiation: alpha, beta and gamma radiation.Basically it consists of a pair of electrodes surrounded by a gas. The electrodes have a high voltage across them. The gas used is usually Helium or Argon. When radiation enters the tube it can ionize the gas. The ions (and electrons) are attracted to the electrodes and an electric current is produced.Ascaler counts the current pulses, and one obtains a "count" whenever radiation ionizes the gas.

Advantages

They are relatively inexpensive, durable, easily portable, detect all types of radiations

Disadvantages

a)They cannot differentiate which type of radiation is being detected.

b)They cannot be used to determine the exact energy of the detected radiation & have a very low efficiency.

Scintillation Counter

Scintillators are used in conventional film-screen radiography, many digital radiographic receptors, fluoroscopy, scintillation cameras, most CT scanners, and PET scanners. **Scintillation detectors** consist of a scintillator and a device, such as a PMT, that converts the light into an electrical signal. More sensitive than Geiger-Muller counter..Wide spread detection.

Principal: Incident radiation interact with material. Atoms are raised to excited states. Excited states emit visible light: fluorescence Light strikes photosensitive a surface. Release of a photoelectron Multiplication

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.

Bernstein – Bellentine counter Their purpose was to measure the carbon-14 content of carbon dioxide, often as a means to date organic material (C-14 dating). The samples to be analyzed

would be combusted and the resulting carbon dioxide drawn into the tubes for counting. Methane was used as the counting gas (with which the carbon dioxide was mixed). The steel mounting box in the following photograph was convenient for holding and protecting the fragile glass tubes. It also provided some degree of shielding and it covered up the electrical connections.

Autoradiography Detecting radioactive compounds with a photographic emulsion (x-ray film).

Types:In-vivo autoradiography - receptors are labelled in intact living tissue by systemic administration of the radioligand (PET).

In-vitro autoradiography - slide-mounted tissue sections are incubated with radioligand so that receptors are labelled under very controlled conditions.

Uses :Map anatomical location of radiolabelled ligands to visualize and quantify receptors in tissue

Trace neurons by axonal transport of radioactively labelled amino acids, certain sugars, or transmitter substances

Measure DNA production (e.g., 3H-thymidine)

Advantages:Highly specific tool to pharmacologically characterize receptors intissue.

Provides location of receptor (etc) in tissue.

Enables characterization of receptors in different tissues.

Technically easy

Mass Spectrophotometer

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

NMR spectroscopy is a research technique that exploits the magneticproperties of certain atomic nuclei to determine physical and chemicalproperties of atoms or the molecules in which they are containe. It relies on the phenomenon of nuclear magnetic resonance and canprovide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules.

Determination methods of tracer techniques:

1. Competitive feeding
2. Precursor product sequence method
3. Sequential analysis method
4. Isotope incorporation method

1. Competitive Feeding By this method, one can accurately determine the actual precursor involved in the biosynthesis of a particular metabolite. Two precursors are then introduced into two separate groups of plants. If the radioactivity is observed in the group receiving precursor B and not in B1 receiving group, then the biosynthetic pathway for particular metabolite follows order. A → B → C but not A → B1 → C

   Applications: Used for elucidation of biogenesis of propane alkaloids, biosynthesis of alkaloids like connine, conhydrine (hemlock) can be studied.

2. Precursor product sequence method: In this method, the presumed precursor of the constituent under investigation on a labeled form is fed into the plants for suitable time period. Later the constituents produced in plant are isolated and purified and its radioactivity is determined.

   Applications: Stopping of hordenine production in barley seedlings after 15- 20 days of germination. Applied in study of biosynthesis of morphine and erogot alkaloids.

3. Sequential analysis method: The principle of this method of investigation is to grow plant in atmosphere of $^{14}CO2$ and then analyze the plant metabolites at a given time intervals to obtain the sequence in which various related compounds become labeled. Example: Menthapiperita$^{14}CO2$ for about 5 mins provided the evidence of probable biosynthetic sequences.

   Applications: $^{14}CO2$ and sequential analysis has been very successfully used in elucidation of carbon in photosynthesis. Determination of sequential formation of opium and tobacco alkaloids.

4. Isotope incorporation: This method provides information about the position of the bond cleavage and their formation during reaction. Example: the cleavage of glucose -1-phosphotase is catalysed by alkaline phosphatase. Reaction occurs with cleavage of either C-O bond/P-O bond. If the reaction is carried in presence of H_2O 18 enriched H_2O, the cleavage C-O cleavage path yields glucose containing one atom of ^{18}O. The P-O cleavage is characterized by phosphate containing one atom ^{18}O. During experimentation, the label invariably appears in inorganic phosphate identifying the P-O bond as the cleavage.

   General applications of tracer techniques:

   *Study of sequence cyclisation by use of ^{14}C, ^{3}H labeledmevalonic acid.

   *Inter relationship among 4-methyl sterol and 4, 4 dimethyl sterols by use of ^{14}C acetate.

   *Terpenoid biosynthesis by chloroplasts, isolated in organic solvent by use of two ^{14}C mevalonate.

   *Study of formation of scopoletin by use of labeled phenyl alanine.

   * Study of formation of cinnamic acid in pathway of coumarin from labeledcoumarin.

* Origin of carbon and nitrogen atoms of purine ring system by use of ^{14}C or ^{15}N labeled precursor.

*By using ^{45}Ca as a tracer it has been found that the uptake of calcium by plants from the soil is nearly the same both for CaO and $CaCO_3$ in acidic soils.

* By adding ammonium phosphate labeled with ^{32}P of known specific activity thus uptake of phosphorous is followed by measuring the radioactivity as label reaches first the lower parts of the plant then the upper parts, branches, leaves etc.

TYPES OF TRACER TECHNIQUES

Isolated organs, tissues and cells: Cultures of the organs, tissues and cells growing under controlled aseptic conditions can be used for feeding experiments. The radioactive tracers can be introduced by this process to the parenchymatous tissue of shoots, leaves, roots or other plant structures and the further analysis of such plant material can provide important information's about the incorporation of the labeled compounds for the determination of the sites of synthesis of particular compounds.

Isolated roots are also extensively used for the circulation of biogenetic pathways for tropane alkaloids in the roots of the solanaceous plants.

*The studies on the petal discs have been used for the elucidation of pathways for essential oil components such as rose oil.

*Isolated shoots, and leaves can be maintained in a suitable sterile medium for the studies on Nicotiana and Datura spp. In such types of studies on rooted leaves to get large organization of roots facilitates the study of the tobacco alkaloid, for their biogenetic sites which is generally considered to be roots.

Grafting: Grafting is an operation in which two cut surfaces of different but closely related plants placed so as to unite and further grow together.The major part of plant which is used for grafting is a stock.The portion that cut off from another plant is called as scion.In cases of plant propagation grafting has important place and plants like Cinchona, citrus, myristicaetc have been successfully grafted for the production of better quality drug.

Grafting has also considerable utility in the biosynthetic studies for elucidation of the pathways used for the biogenesis is of secondary metabolites. Solanaceous plants like Nicotiana, Datura have been intensively studied for the tobacco alkaloids and tropane alkaloids. Eg: The scions of tomato grafted onto the stock of Datura, shows the accumulation of tropane alkaloid. On the contrary when Datura scions are grafted on Lycopersicon, tomato stock, the production of tropane alkaloids does not occur as usual and shows only traces of

these alkaloids. The above experiments suggests the possibilities that the major site for the biogenesis of tropane alkaloid is roots and no other organ of Datura.

Mutant strains: Mutant strains of lower plants like fungi and microorganisms are produced in nature which lacks one or other enzyme because of which the normal metabolic pathways are gently affected. In such mutant strains metabolites are found at the intermediate stage and needs artificial supply of another intermediate. Such mutant strains can be used in the biosynthetic studies of the natural products.

The biogenetic pathways of the gibberellins are mostly similar in both higher plants and Gibberella fugikuroi. The mutant strainsGibberellacan be used to obtain variety of novel C^{20}isoprenoid compounds which are produced at level of geranyl pyrophosphate in mevalonic acid pathway. Very interesting results have been obtained by the studies on the ultraviolet induced strains of Claviceps purpurea.

These mutant strains can produce amino acids of diverse nature. When the rye plant is introduced with the spore culture of these mutant strains. The sclerocia produced demonstrate the blockages of biogenetic pathway of certain intermediates and there by the accumulation of specific alkaloids (ergot alkaloid) is blocked.

The blockage occurs after the formation of ChanoclavineI in mutant strains to such strains if agroclavine and other intermediates had been supplied artificially it indicated the reinstallation of normal pathway to produce final or specific alkaloid compounds completely.

Very Short Answer Questions

1. What are secondary metabolites?
2. What are the metabolic pathways?
3. Name Metabolic Pathways producing secondary metabolites.
4. What is biosynthesis ?
5. What are building blocks? Give examples.
6. Name amino acids produced in shikimate pathway.
7. Name secondary metabolites produced in shikimate pathway.
8. What is acetate-mevalonatepathway ?
9. Name secondary metabolites produced in acetate-mevalonate pathway.
10. What is acetate pathway ?
11. What is polyketidepathway ?
12. What are the chief products of acetate pathway ?
13. What are tracer techniques ?
14. Write in short Mutant strains in biogenetic studies .

5 Marks

1. Write a note on shikimic acid pathway.
2. Write a note on acetate pathway. Or discuss briefly acetate pathway
3. Discuss briefly Acetate-mevalonate pathway.
4. Discuss briefly Amino acids pathway.
5. Write a note on tracer techniques.

10 Marks

1. Discuss shikimic acid pathway in detail. Enumerate different secondary metabolites produced in this pathway.
2. Discuss acetate-mevalonate pathway in detail. Enumerate different secondary metabolites produced in this pathway.
3. Discuss utilization of radioactive isotopes in the investigation of biogenetic studies.