PLANT BIOSYNTHESIS

The living plant may be considered as a biosynthetic laboratory not only for primary metabolites like sugars, Amino acids and fatty acids and also for a multitude of secondary products of pharmaceutical significance such as glycosides, alkaloids, flavonoids, volatile oils etc.

Primary metabolites:

Substances that are widely distributed in nature occurring in one form or the other in virtually all organisms and are needed for general growth and physiological development because of their basic cell metabolism.

Secondary metabolites:

Biosynthetically derived from primary metabolites but are more limited in distribution, usually being restricted to a taxonomic group. They represent chemical adaptations to environmental stresses/ they serve as defensive, protective/offensive chemicals against microorganisms, higher animals etc.

In terms of cellular economy, secondary products are in general expensive to produce and accumulate.

Biosynthesis:

Biosynthesis is defined as building up of complex chemical compounds from simpler ones by a series of reactions catalysed by enzymes in cells, during the physiological processes of a living organism. Other than enzymes, the process require precursors like ATP, NADH, NADPH, FADPH, and FADH.

Generally compounds generated via biosynthesis are carbohydrates, proteins, vitamins, antibiotics, fats, alkaloids, gums etc.

Biogenesis: Development of life from preexisting life.

There are different biosynthetic pathways in plants like

- Shikmic acid pathway
- Mevalonic acid pathway
- Acetate pathway

Various steps and intermediates are involved in biosynthetic pathways. These can be investigated by means of following techniques.

- Tracer techniques
- 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 / 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 C^{14} 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 C^{14} 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: ^{10C}-^{11}C -8.8 Sec to 20mins

^{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.

Preparation of labeled compound:

- Many compounds which are most conveniently prepared from natural sources.
  Ex: By growing chlorella in an atmosphere containing $^{14}CO_2$, 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).

$$CH_3MgBr + {}^{14}CO_2 \rightarrow CH_3{}^{14}COOMgBr + H_2O$$

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

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.

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 oflabeled compound by various methods like

- Geiger muller counter
- Scintillation counter
- Auto radiography
- Gas ionization chamber
- Bernstein ballentine counter
- Mass spectrometer
- NMR spectrometer

Methods of tracer techniques:

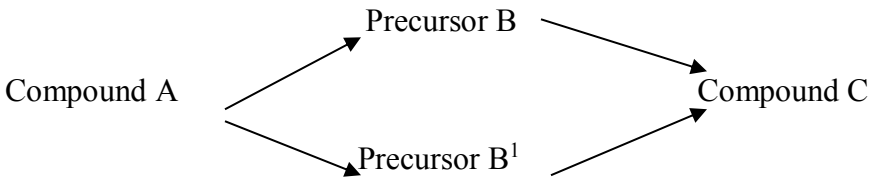
I. Competitive feeding
II. Precursor product sequence method
III. Sequential analysis method
IV. Isotope incorporation method

1. Competitive Feeding:

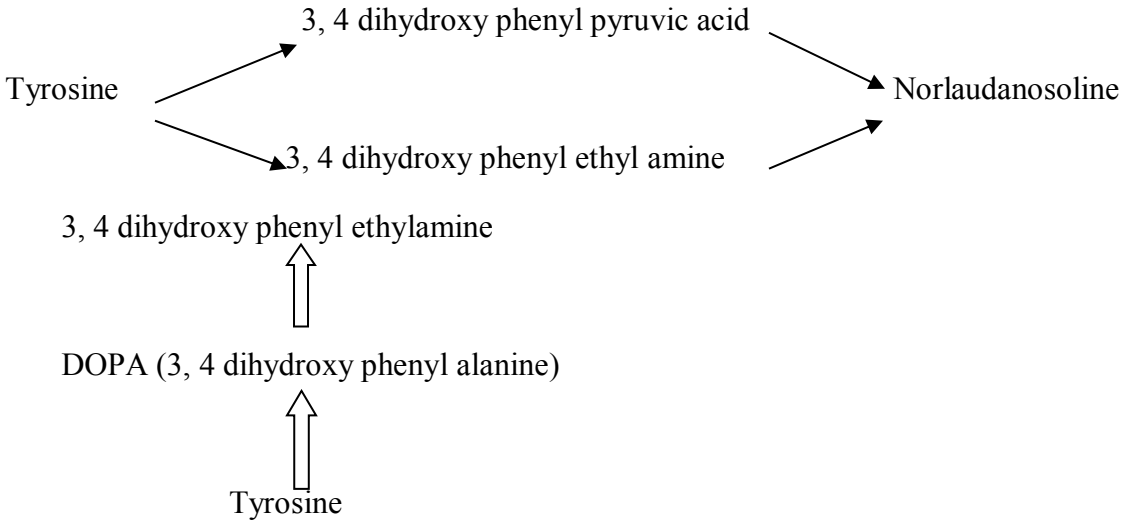
By this method, one can accurately determine the actual precursor involved in the biosynthesis of a particular metabolite.

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.

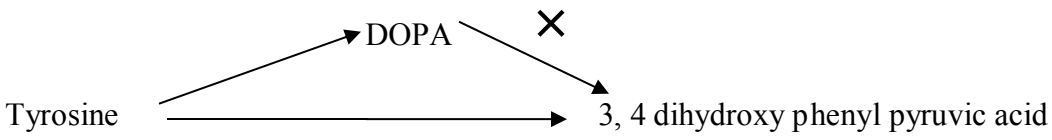
$A \rightarrow B \rightarrow C$ but not $A \rightarrow B1 \rightarrow C$



E.g. :



Similarly it was considered that 3, 4 dihydroxy phenyl pyruvic acid would also be synthesized through DOPA but by labeling experiments and competitive feeding it is confirmed that tyrosine directly gives 3, 4 dihydroxy phenyl pyruvic acid.



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.

Disadvantages:

Sometimes radioactivity of isolated compound alone is not usually sufficient evident that the precursor fed during the studies is a direct precursor. It is due to the fact that the compounds may enter the general biogenetic pathways and get distributed randomly through the array of phytochemical constituents.

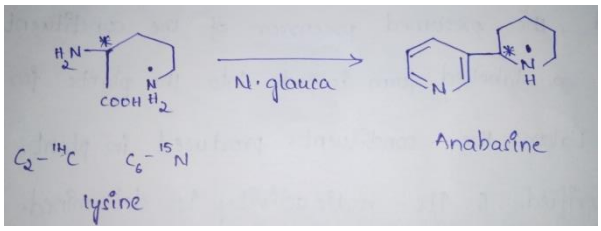
In such cases further proof can be obtained from studies by incorporating precursors from double and triple labeling experiments.

Eg : Anabasine

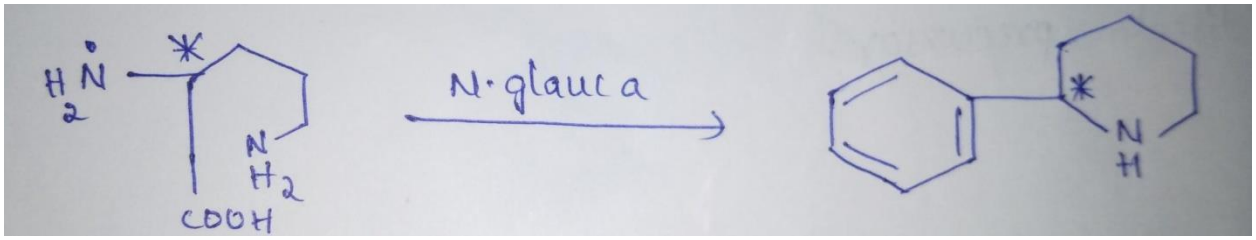
It is an alkaloid obtained from Nicotiana glauca.

Precursor - Lysine (which forms piperidine ring)

Radioactive labeled lysine (labeled with $_6C^{14}$ and $_7N^{15}$) at positions 2, 6 respectively was fed to Nicotiana glauca.



Biogenesis resulted in formation of anabasine alkaloid. With the help of radioactivity determination study, it was proved that radioactive N_2 and C at 6th and 2nd positions respectively.



Suppose in the same experiment when lysine was labeled with radioactive carbon C^{14} and nitrogen N^{15} at same position it was proved that only radioactive carbon and non-labeled N_2 was involved in the formation of piperidine ring.

Applications:

Stopping of hordenine production in barley seedlings after 15- 20 days of germination.

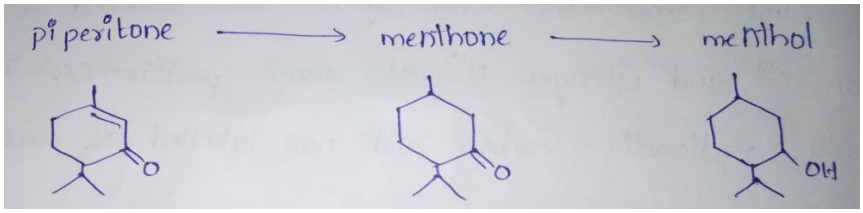
Applied in study of biosynthesis of morphine and erogot alkaloids.

3. Sequential analysis method:

The principle of this method of investigation is to grow plant in atmosphere of $^{14}CO_2$ and then analyze the plant metabolites at a given time intervals to obtain the sequence in which various related compounds become labeled.

Example:

Menthapiperita$^{14}CO_{2\ for}$ about 5 mins provided the evidence of probable biosynthetic sequences.



Applications:

$^{14}CO_2$ and sequential analysis has been very successfully used in elucidation of carbon in photosynthesis.

Determination of sequential formation of opium and tobacco alkaloids.

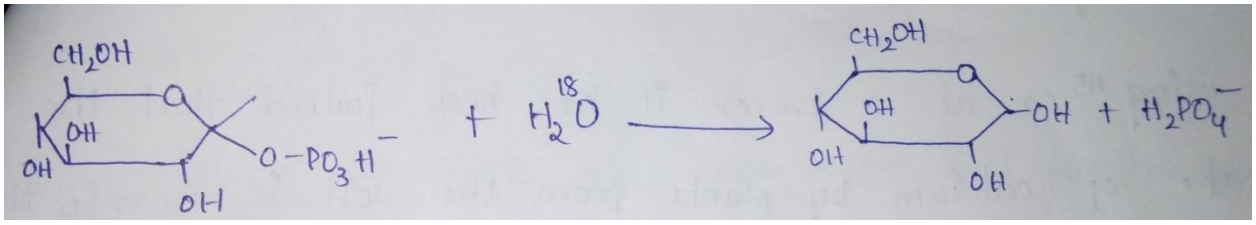
4. Isotope incorporation:

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 labeled mevalonic 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 labeled coumarin.
- 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.

II. <u>Isolated organs, tissues and cells:</u>

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.

III. 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.

IV. 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 Gibberellafugikuroi. 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 Clavicepspurpurea.

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.

METHODS OF TRACER TECHNIQUES