UNIT-II

Part- (A and B)

METABOLIC PATHWAY IN HIGHER PLANTS AND THEIR DETERMINATION

Points to be covered in this topic

(a) **Brief study of basic metabolic pathway and formation of different secondary metabolites through various pathways**

a) Shikimic acid pathway
b) Acetate-mevalonate pathway
c) Amino acid pathway

(b) **Study of utilization of Radioactive isotopes In the investigation of Biogenetic studies**

a) Tracer technique
b) Determination of nature of metabolites in various biochemical reactions

❑ Brief study of basic metabolic pathway and formation of different secondary metabolites through various 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**
- The compounds formed during metabolism are called **metabolites**

❖ 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

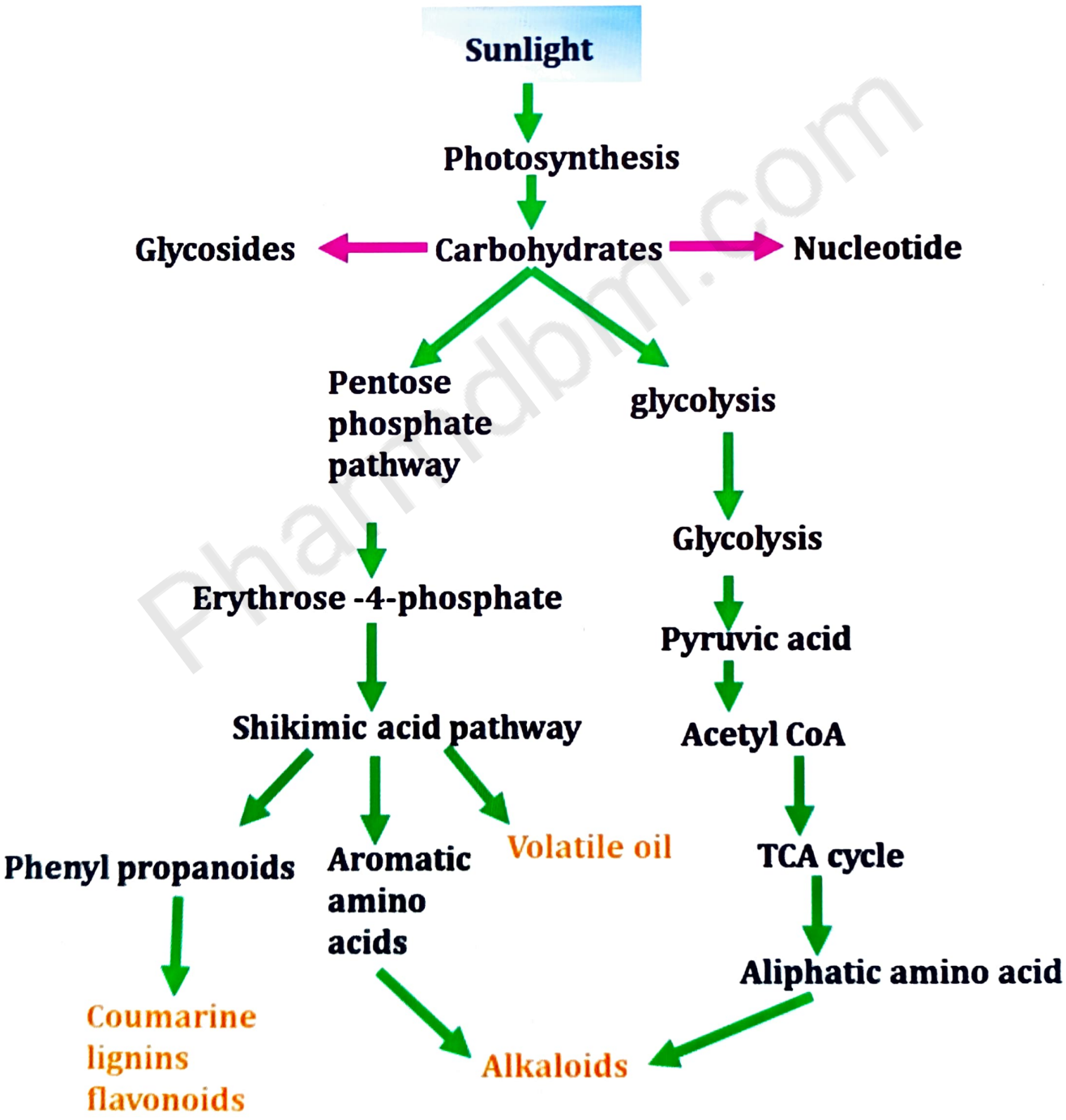
Metabolites are compounds synthesized by plants for both

- Essential functions such as → **Growth and development (primary metabolite)**
- Specific functions such as → **Pollinator attraction or defense against herbivory (secondary metabolite)**

PRIMARY METABOLITE	SECONDARY METABOLITE
• Involve directly in **growth, development and reproduction in living organism** • Produce in **large quantity** **EX-** **sugars, amino acids, coenzyme A, Mevalonic acid and lipids**	• **Not directly** involve in **growth, development and reproduction** • **End product** of **primary** metabolism • Produce in **small quantities** and their **extraction is difficult** **EX-** **Alkaloids, Glycosides, Tannins, Flavonoids, Terpenoids and Volatile oils**

❖ Basic metabolic pathway

- 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 deoxy xylulose phosphate pathways**



Basic metabolic pathway

SHIKIMIC ACID PATHWAY

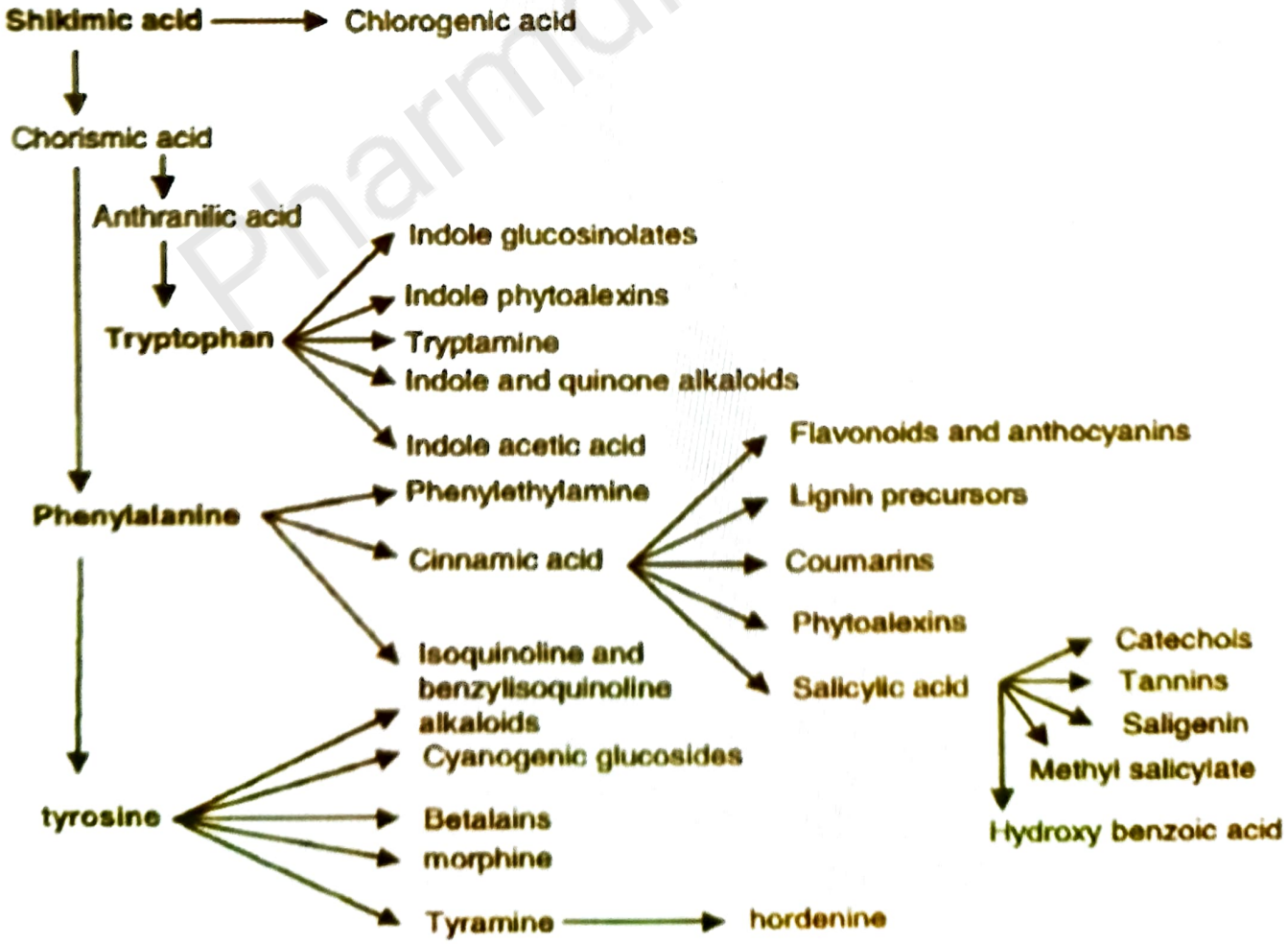
- **Metabolic pathway** for **biosynthesis of aromatic amino acid (phenylalanine, tyrosine, tryptophane)**
- Shikimic acid is key intermediate from carbohydrate for **biosynthesis of C_6-C_3** unit (phenyl propane derivative)
  EX- Tyrosine and phenyl alanine
- Shikimic acid name is derived from shikimi, japense flower (star anise, ***Illicium anisatum***)
- Occur in **plant, bacteria, fungi** but **not in animal**

Biosynthetic precursor -

(starting material)

- ✓ **Phosphoenol pyruvate (glycolysis)**
- ✓ **Erythrose- 4 – phosphate** (pentose phosphate pathway)
- ✓ Nitrogen from other animal acid – **glutamate , glycine, serine**

- **Phenylalanine and tyrosine** form the basis of **C_6-C_3 phenylpropane** units **found in many natural products**, for example, **cinnamic acids, coumarins, lignans, and flavonoids,** and along with **tryptophan are precursors of a wide range of alkaloid structures**



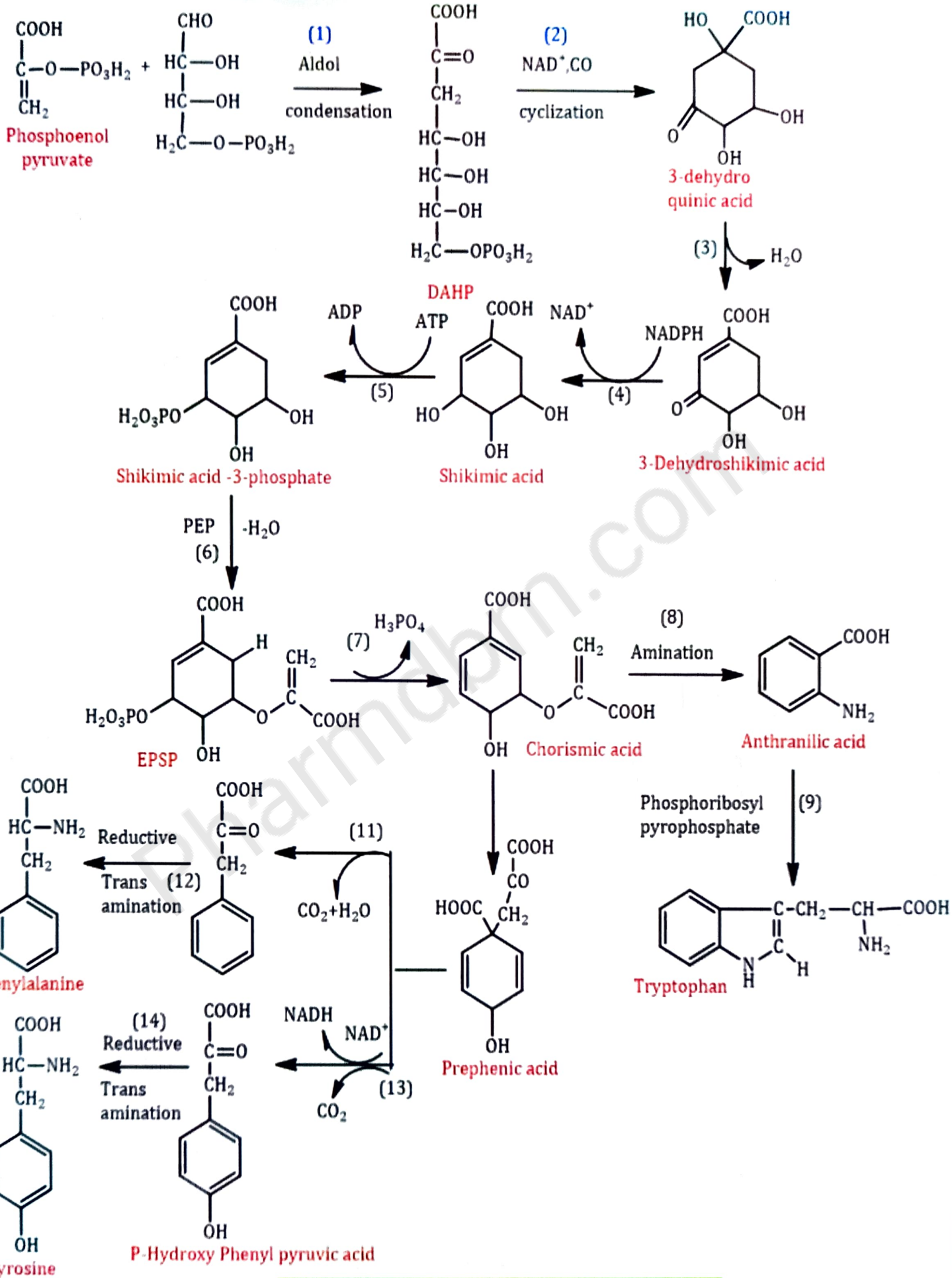
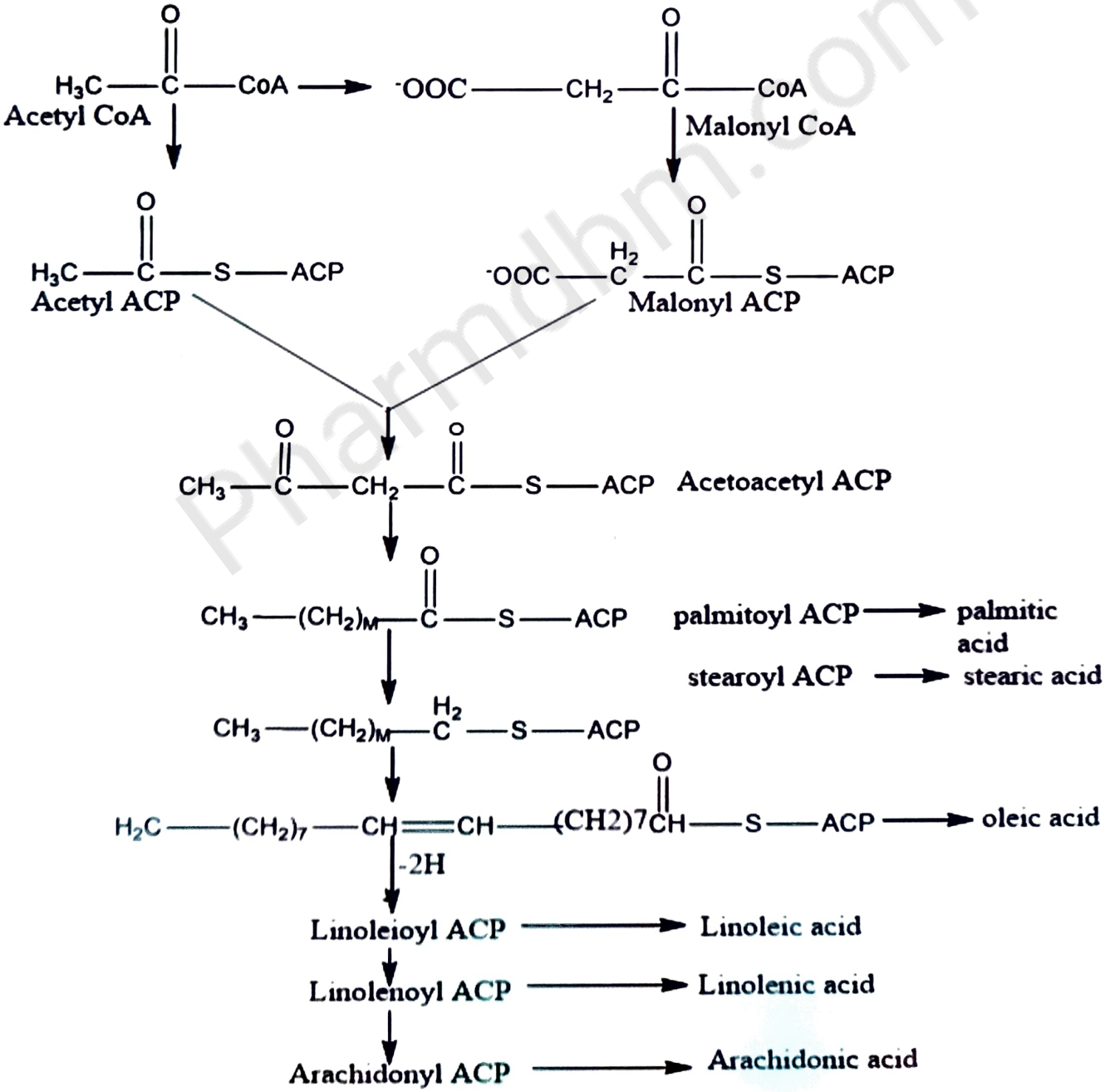


Fig. SHIKIMIC ACID PATHWAY

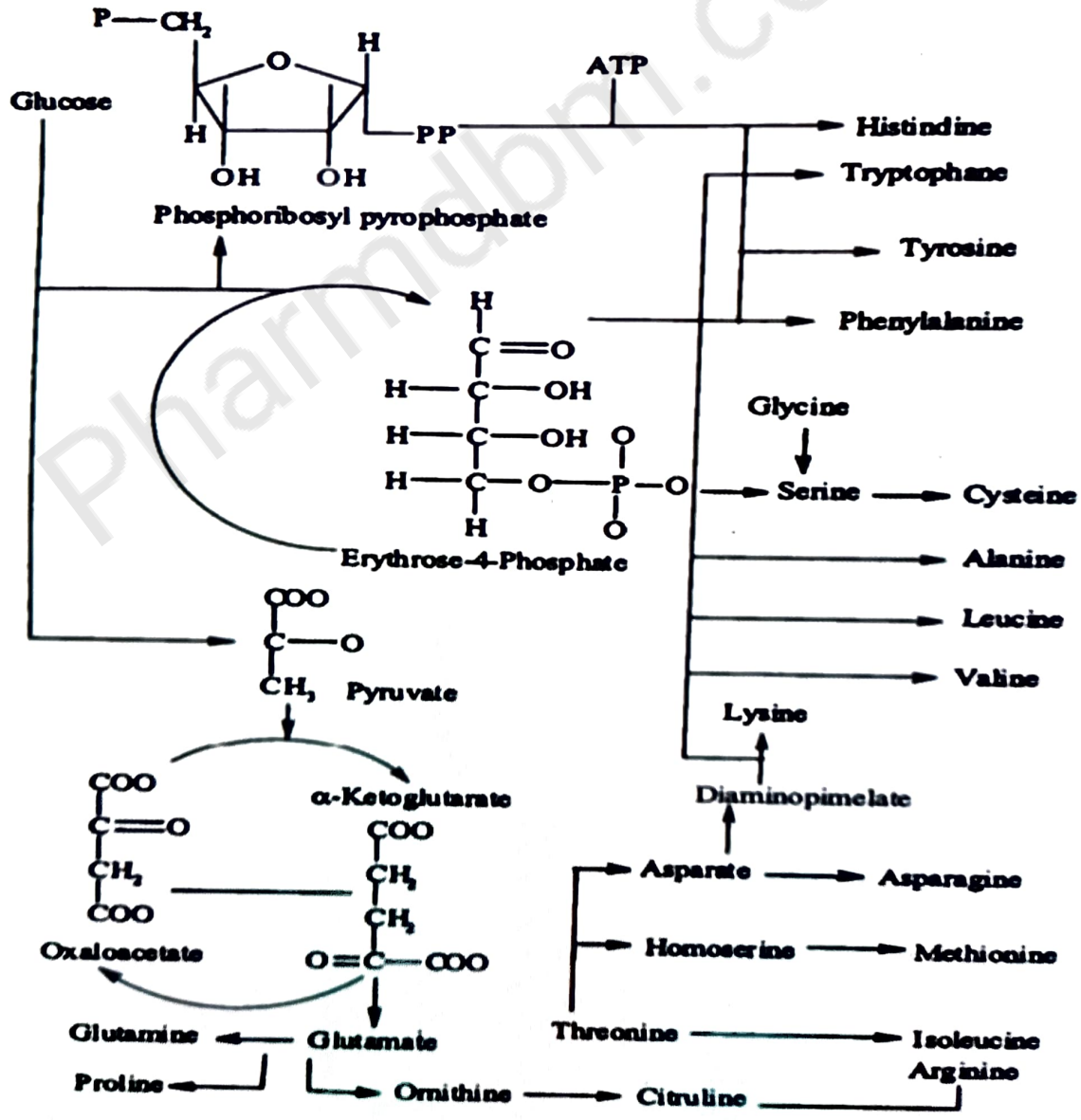
ACETATE-MEVALONATE PATHWAY

- 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 C_2 acetyl CoA units at the later stage produces even number of fatty acids from n-tetranoic (butyric) to n-ecosanoic (arachidic acid)
- Unsaturated fatty acids are produced by subsequent direct dehydrogenation of saturated fatty acids
- Enzymes play an important role in governing the position of newly introduced double bonds in the fatty acids



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. EX- 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**



❑ 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**

TRACER TECHNIQUE

- It can be defined as technique which **utilizes a labelled compound** to find out or to **trace the different intermediates** and various steps in **biosynthetic pathways in plants, at a given rate & time**
- Labelled compound can be prepared by use of **two type of isotopes**

1. Radioactive isotopes :

[e.g. ^{1}H, ^{14}C, ^{24}Na, ^{42}K, ^{35}S, ^{35}P, decay with emission of radiation]

- For **biological investigation-carbon & hydrogen**
- For **metabolic studies- S, P, and alkali and alkaline earth metals** are used
- For studies on **protein, alkaloids, and amino acid - labelled nitrogen atom give more specific information**

2. Stable isotopes :

[e.g. ^{2}H, ^{13}C, ^{15}N, ^{18}O]

- Used for labeling compounds as possible intermediates in **biosynthetic pathways**
- Usual method of detection are : - **Mass spectroscopy [^{15}N, ^{18}O]**
- **NMR spectroscopy [^{2}H, ^{13}C]**

➢ 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 & 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

❖ 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**

Fig. Hydroponic farming

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 hollow stem**. e.g- Umbelliferae and capsule plants (opium poppy). **Micro syringe** 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

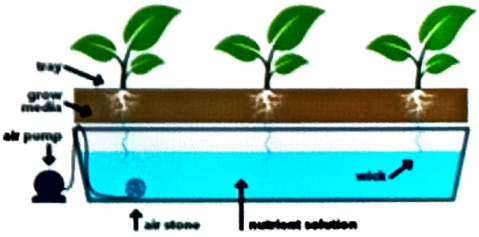


Fig. wick feeding

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

Fig. Infusion

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 3H, 14C labelled compounds Liquid scintillation counter is use
- Modern instruments are used for mixed radiation like 3H and 14C . This is possible because both are β-emitters and different radiation energy.
- Different instruments are used to determine nature of metabolites. e.g- GM Counter, Scintillation or liquid scintillation counter and ionization chamber

For stable isotopes -

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

4. Determination of nature of metabolites in various biochemical fractions

- Geiger-Muller counter
- Scintillation or liquid scintillation counter
- Ionization chamber
- Mass Spectrophotometer
- NMR Spectrophotometer
- Autoradiography

➢ 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**
- A scaler 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**

Fig. Geiger counter

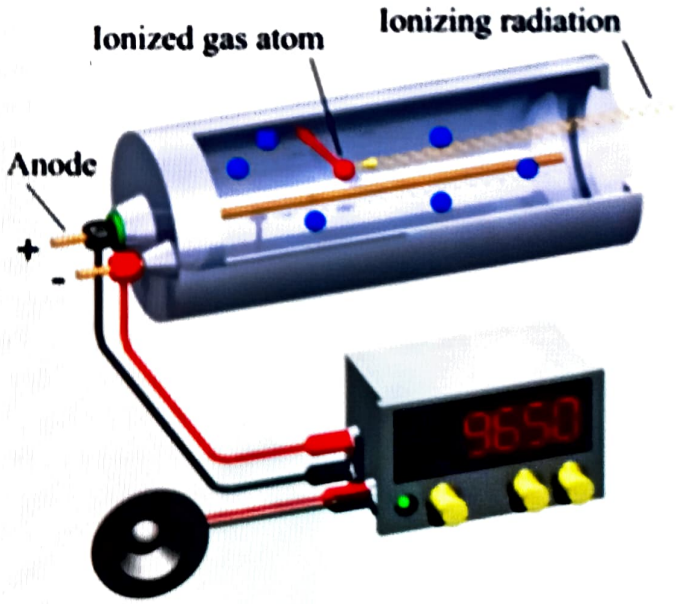


Fig. Geiger counter

➢ Ionization chamber

- These detector collect all the charges created by direct ionization within the gas through the application of electric field.
- If **gas is air and walls of chamber are of a material whose effective atomic number is similar to air, the amount of current produced is proportional to the exposure rate**
- **Air-filled ion chambers** are used in portable survey meters, for performing **QA testing of diagnostic and therapeutic x ray machines, and are the detectors in most x-ray machine phototimer**
- Having low intrinsic efficiencies because of **low densities of gases** and **low atomic numbers** of most gases

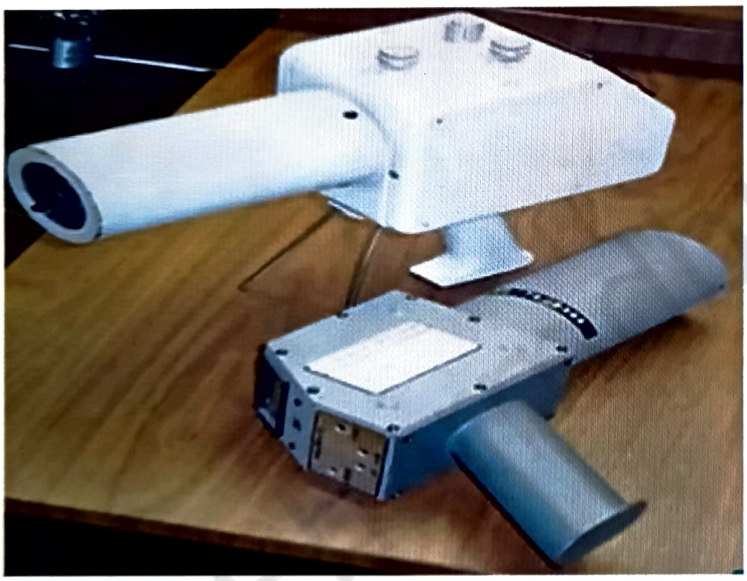


Fig. Ionization chamber

➢ 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

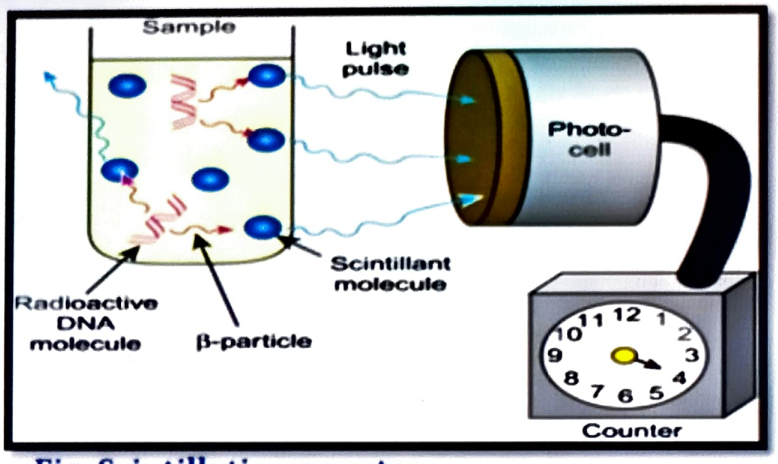


Fig. Scintillation counter

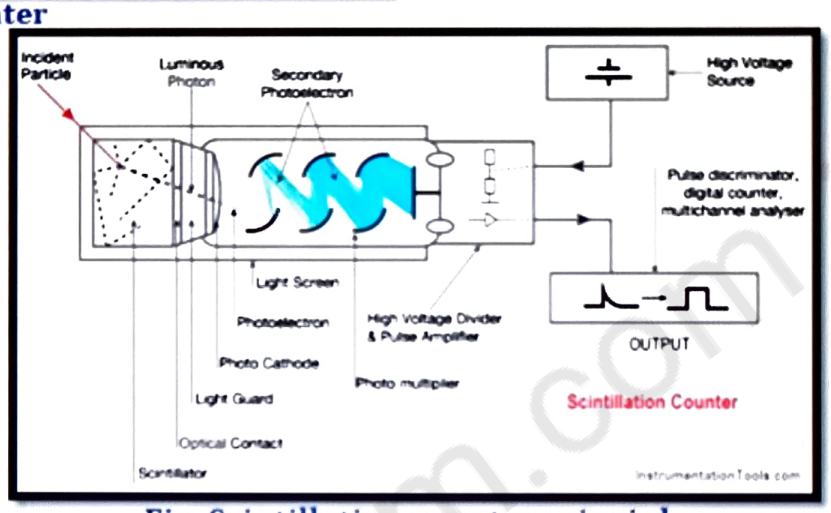


Fig. Scintillation counter principle

➢ 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 :**

✓ **Mass of the particles,**

✓ **Elemental composition of the sample** or molecule, and

✓ **Chemical structures of the molecules**, such as **peptides** and other chemical compounds

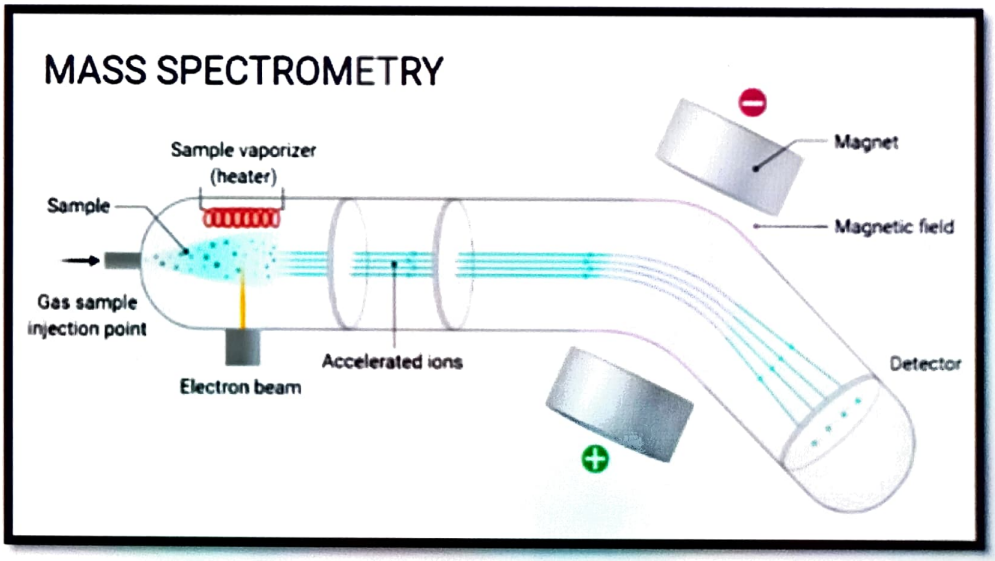


Fig. Mass Spectrophotometer

➢ 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

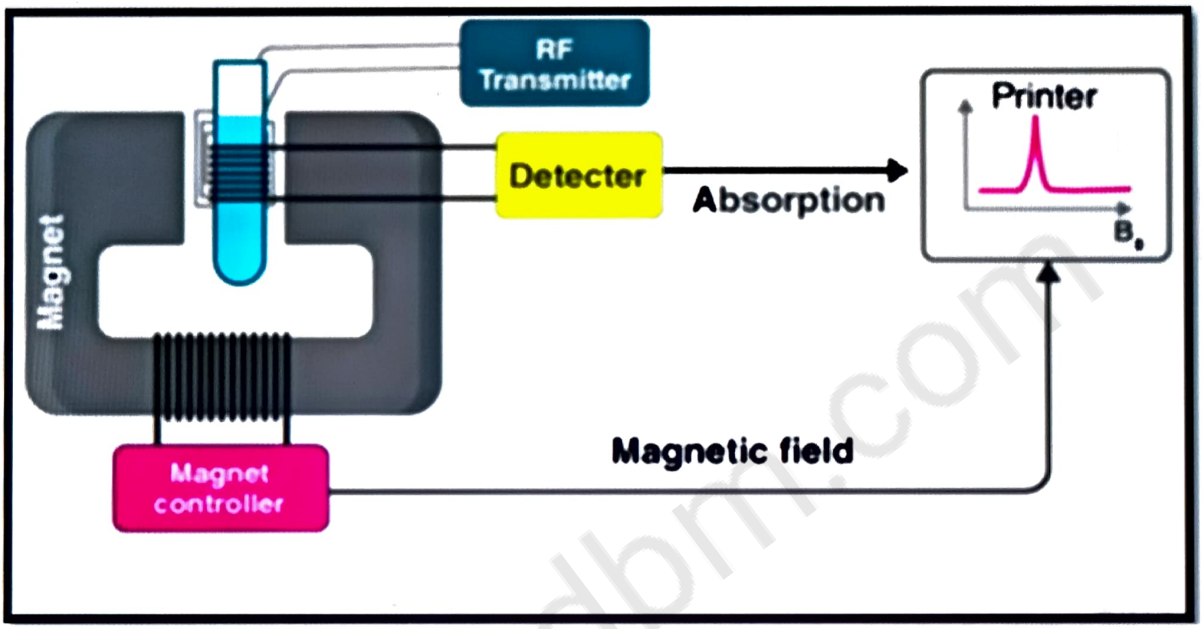
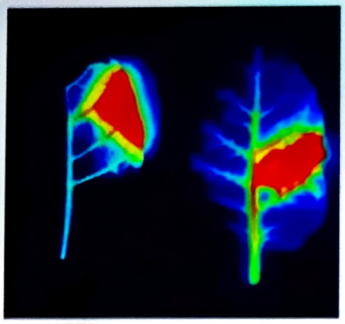


Fig. NMR Spectrophotometer instrumentation

➢ 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
- The specimen is in close contact with the emulsion for a period (exposure duration). In this technique, a sample containing a radio labelled 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



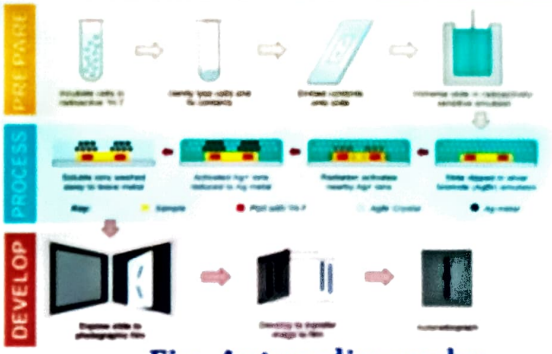


Fig. Autoradiography

❖ Methods of tracer techniques

➢ Precursor product sequence

In this technique, 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.**

Disadvantages:

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

Applications :

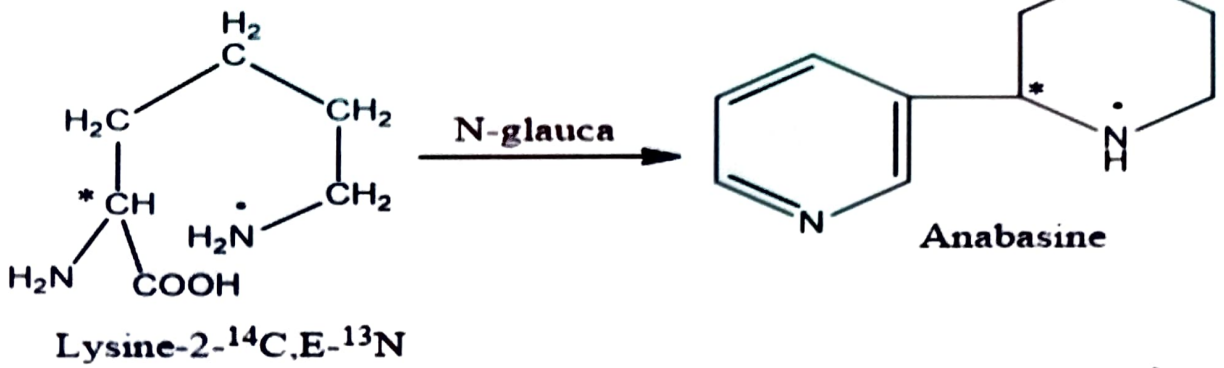
- Stopping of hordenine production in barley seedling after **15 - 20 days of germination**
- **Restricted synthesis of hyoscine**, distinct from hyoscyamine in ***Datura stramonium***
- This method is applied to the **biogenesis of morphine & ergot alkaloids**

➢ Double & multiple labelling

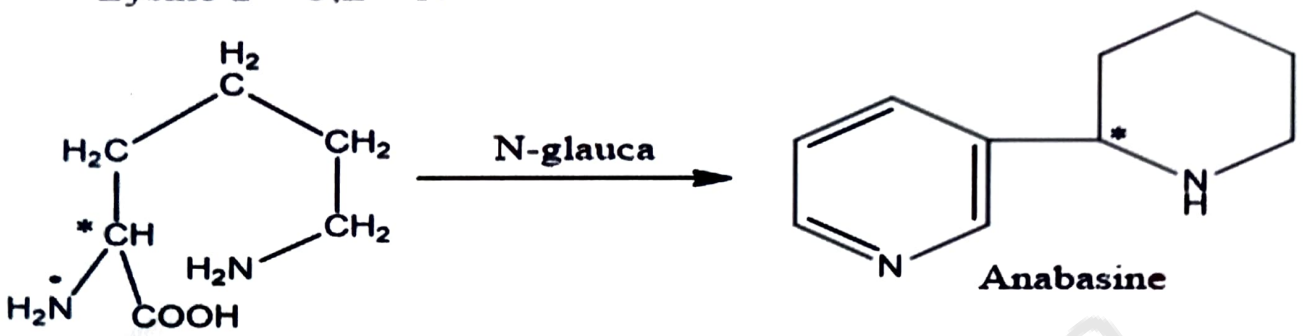
This **method give the evidence for nature of biochemical incorporation of precursor arises, double & triple labeling.**

Applications:

- This method is extensively applied to study the **biogenesis of plant secondary metabolite**
- Used for study of **morphine alkaloid. e.g., Leete,** use Doubly labeled lysine used to determine which hydrogen of lysine molecule was involved in formation of **piperidine ring** of anabasine in ***Nicotina glauca***



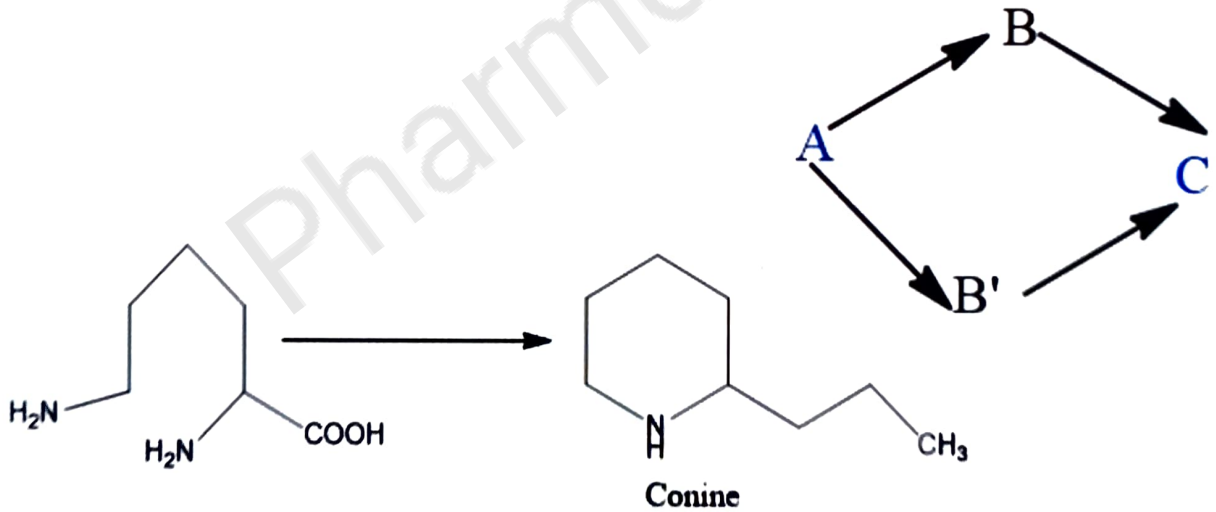
Lysine-2-^{14}C,E-^{13}N

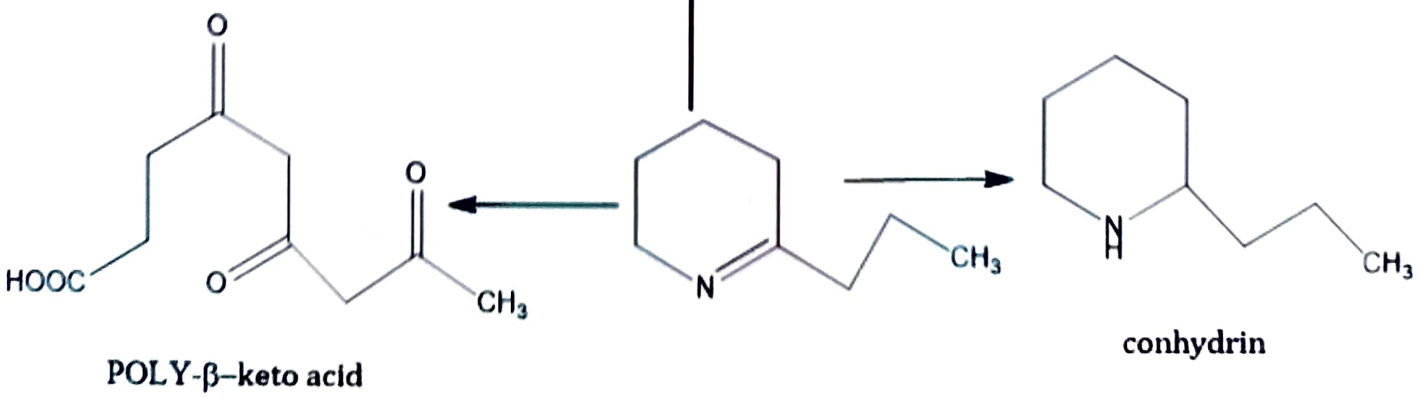


Lysine-2-^{14}C,α–^{15}N

➢ Competitive feeding

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





➢ Sequential analysis

The principle of this method of investigation is to grow plant in atmosphere of $^{14}CO_2$, & then analyze the plant at given time interval to obtain the sequence in which various correlated compound become labelled

Application :

- **$^{14}CO_2$, & sequential analysis has been very successfully used in** elucidation of carbon in photosynthesis
- **Determination of sequential formation of opium, hemlock and tobacco alkaloids**
- Exposure as less as 5 min $^{14}CO_2$, is used in detecting biosynthetic sequence as -
- **Piperitone ---------- (-) Menthone ---------- (-) Menthol in *mentha piperita***