TEJASVI NAVADHITAMASTU

"Let our (the teacher and the taught) learning be radiant"

Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose

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E –content

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Topic: Instrumentation and Analytical Techniques
Subtopic: **TRACER TECHNIQUE, RADIOACTIVE DECAY AND MEASUREMENTS**
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TRACER TECHNIQUE

- Living plants considered as biosynthetic laboratory ⟶ primary as well as secondary metabolite.
- Different biosynthetic pathway: -
  - » Shikmic acid pathway
  - » Mevalonic acid pathway
  - » Acetate pathway
- Various intermediate and steps are involved in biosynthetic pathway in plants can be investigated by means of following techniques: -
  - » Tracer technique
  - » Use of isolated organ
  - » Grafting methods
  - » Use of mutant strain

- ***Definition*: -** 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.

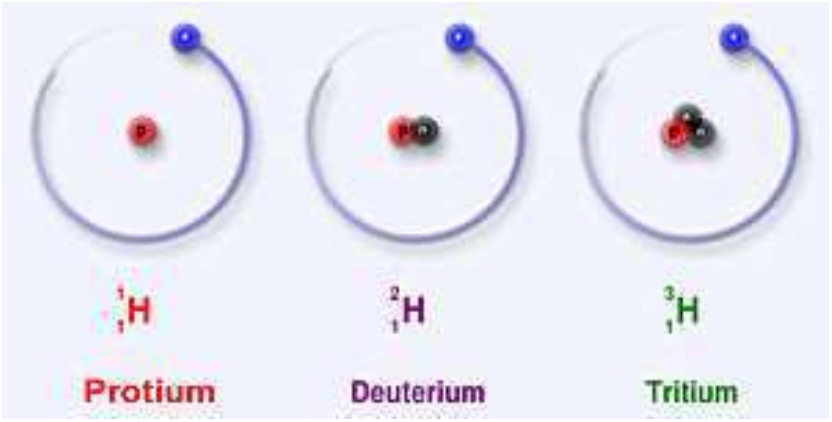
- In this technique different isotope, mainly the radioactive isotopes which are incorporated into presumed precursor of plant metabolites and are used as marker in biogenic experiments.

- The labelled compound can be prepared by use of two types of isotopes.
  - » Radioactive isotopes.
  - » Stable isotopes.
- **Radioactive isotopes: -** [e.g. ^{1}H, ^{14}C, ^{24}Na, ^{42}K, ^{35}S, ^{35}P, ^{131}I 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.
  - ^{3}H compound is commercially available.
- **Stable isotopes: -** [e.g. ^{2}H, ^{13}C, ^{15}N, ^{18}O]
  - Used for labelling compounds as possible intermediates in biosynthetic pathways.
  - Usual method of detection are: – MASS spectroscopy [^{15}N, ^{18}O]
  - NMR spectroscopy [^{2}H, ^{13}C]

Isotopes – equal *Z [* **atomic number** of a chemical element (also known as its **proton number**) is the number of protons found in the nucleus of an atom of that element*]*

Isobars – equal *A [*The **mass number** (**A**), also called **atomic mass number** or **nucleon number**, is the total number of protons and neutrons (together known as nucleons) in an atomic nucleus.*]*

Isotones – equal *N [*The **neutron number**, symbol **N**, is the number of neutrons in a nuclide.*]*



carbon-12, carbon-13 and carbon-14 are three isotopes of the element carbon with mass numbers 12, 13 and 14 respectively. The atomic number of carbon is 6, which means that every carbon atom has 6 protons, so that the neutron numbers of these isotopes are 6, 7 and 8 respectively.

RADIOACTIVE DECAY

Radioactive decay is the process in which an unstable atomic nucleus loses energy by emitting ionizing particles and radiation transforming the parent nuclide atom into a different atom called daughter nuclide .

The three types of radiation

Use this table to find information about and to compare α, β and γ radiation

	Alpha (α)	Beta (β)	Gamma (γ)
Nature	It's a nucleus of helium ${}^{4}_{2}He$. Two protons and two neutrons	It's an electron e^-	It's an electromagnetic wave
Charge	+2	-1	0
Mass	Relatively large	Very small	No mass
Speed	Slow	Fast	Speed of light
Ionizing effect	Strong	Weak	Very weak
Most dangerous	When source is inside the body	When source is outside the body	When source is outside the body

HALF-LIFE

Half life of radio isotope is the time period required for radionuclide to decay to one half the amount originally present .

$$t1/2 = 0.693/\lambda.$$

λ is decay constant , a characteristic of a given isotope decaying in unit time

1 curie = *3.7x10^{10} radioactive decays per second* [exactly].

In the International System of Units (SI) the curie has been replaced by the becquerel (Bq), where

1 becquerel = 1 radioactive decay per second = 2.703x10^{-11} Ci.

- Bequerel is the unit of radioactivity is defined as one disintegration per second (1 d. p. s.).
- Frequently used units are curie , defined as the quantity of radioactive material in which the number of nuclear disintegrations per second is same as the 1gm of radium (3.7 X 10 ^{10} Bq).
- **Specific activity is defined as disintegration rate per unit mass of radioactive atoms.**

SIGNIFICANCE OF TRACER TECHNIQUE

- Tracing of Biosynthetic Pathway: - e.g. By incorporation of radioactive isotope of ^{14}C into phenylalanine, the biosynthetic cyanogenetic glycoside prunasin, can be detected.
- Location & Quantity of compound containing tracer: - ^{14}C labelled glucose is used for determination of glucose in biological system
- Different tracers for different studies: - For studies on nitrogen and amino acid. (Labelled nitrogen give specific information than carbon)
- Convenient and suitable technique

CRITERIA FOR TRACER TECHIQUE

- The starting concentration of tracer must be sufficient withstand resistance with dilution in course of metabolism.
- Proper Labelling: - for proper labelling physical & chemical nature of compound must be known.
- Labelled compound should involve in the synthesis reaction.
- Labelled should not damage the system to which it is used.

ADVANTAGES

- High sensitivity.
- Applicable o all living organism.
- Wide ranges of isotopes are available.
- More reliable, easily administration & isolation procedure.
- Gives accurate result, if proper metabolic time & technique applied.

LIMITATION

- Kinetic effect
- Chemical effect
- Radiation effect
- Radiochemical purity
- High concentration distorting the result.

REQUIREMENT FOR TRACER TECHNIQUE

- Preparation of labelled compound.
- Introduction of labelled compound into a biological system.
- Separation & determination of labelled compound in various biochemical fractions at later time.

PREPARATION OF LABELLED COMPOUND

- The labelled compound produce by growing chlorella in atmosphere of $^{14}CO_2$.

  ↓

  All carbon compounds ^{14}C labelled.

- The ^{3}H (tritium) labelled compound are commercially available. Tritium labelling is effected by catalytic exchange in aqueous media by hydrogenation of unsaturated compound with tritium gas. Tritium is pure β – emitter of low intensity & its radiation energy is lower than ^{14}C.
- By the use of organic synthesis: -

$$CH_3MgBr + {}^{14}CO_2 \longrightarrow CH_3{}^{14}COOHMgBr + H_2O$$

$$\downarrow$$

$$CH_3{}^{14}COOH + Mg(OH)Br$$

INTRODUCTION OF LABELLED COMPOUND

PRECAUTION: -

- The precursor should react at necessary site of synthesis in plant.
- Plant at the experiment time should synthesize the compound under investigation
- The dose given is for short period.

1. Root feeding
2. Stem feeding
3. Direct injection
4. Infiltration
5. Floating method
6. Spray technique

SEPARATION OF DETECTED COMPOUND

a) Geiger – Muller counter.
b) Liquid Scintillation counter.
c) Gas ionization chamber.
d) Bernstein – Bellentine counter.
e) Mass spectroscopy.
f) NMR eletrodemeter.
g) Autoradiography.

METHODS IN TRACER TECHNIQUE

1. **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.

Disadvantage: - 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.

Application: -

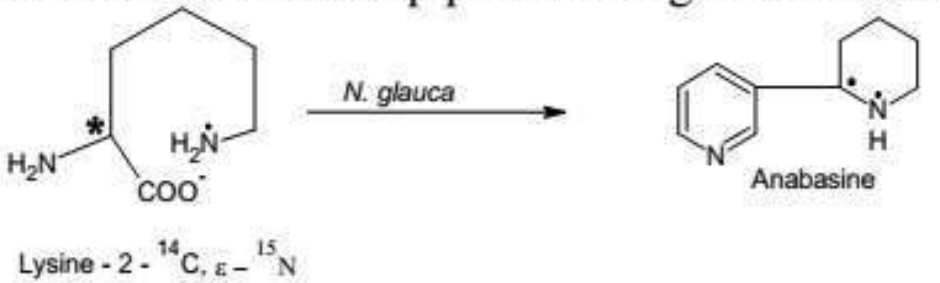
- Stopping of hordenine production in barley seedling after 15 – 20 days of germination.
- Restricted synthesis of hyoscine, distinct from hyoscyamine in Datura *stramonium*.
- This method is applied to the biogenesis of morphine & ergot alkaloids

2. **DOUBLE & MULTIPLE LABELLING**: - This method give the evidence for nature of biochemical incorporation of precursor arises double & triple labelling. In this method specifically labelled precursor and their subsequent degradation of recover product are more employed.

Application: -

- This method is extensively applied to study the biogenesis of plant secondary metabolite.
- Used for study of morphine alkaloid.

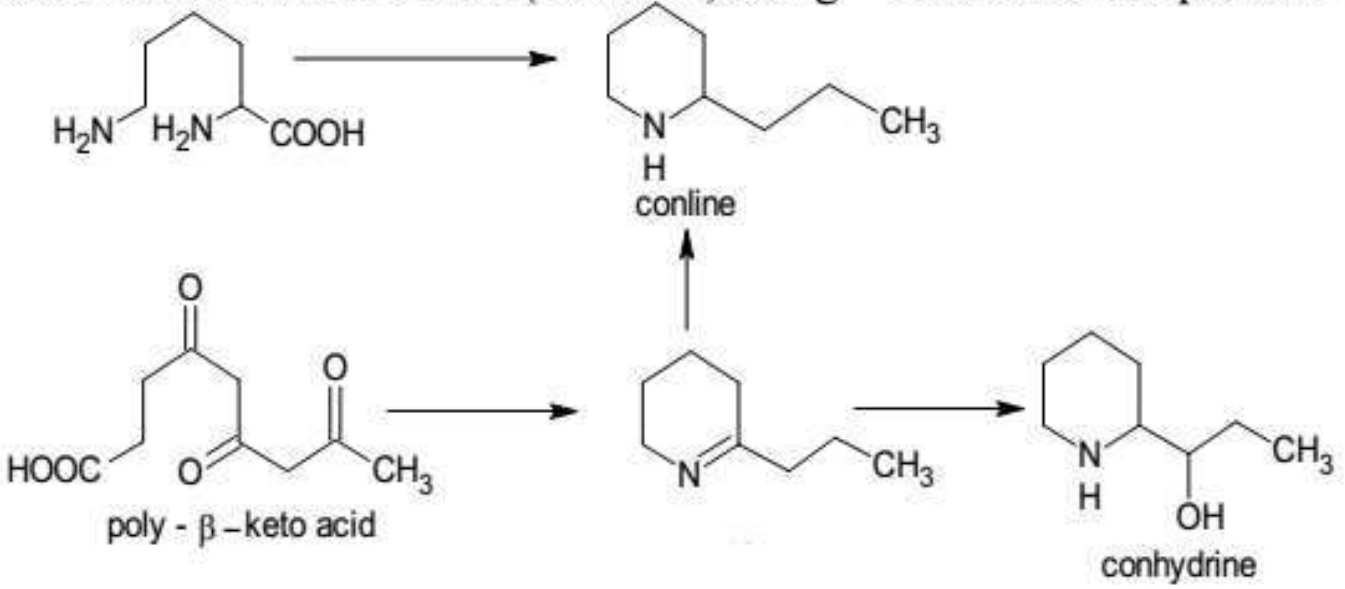
E.g. Leete, use Doubly labeled lysine used to determine which hydrogen of lysine molecule was involved in formation of piperidine ring of anabasine in Nicotina *glauca*.



3. **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.

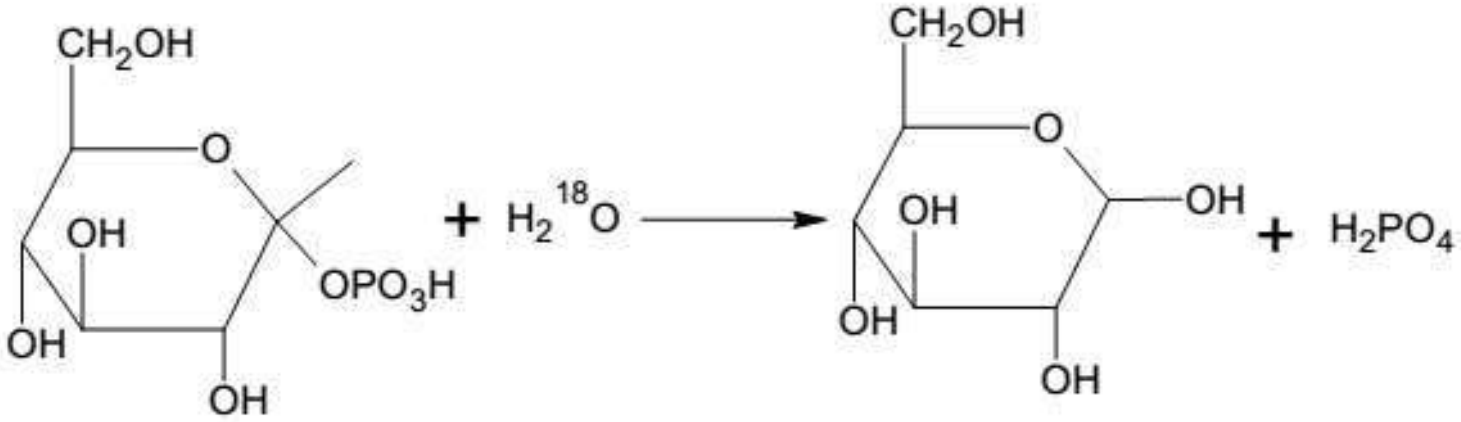
Application: -

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



4. **ISOTOPE INCORPORATION**: - This method provides information about the position of bond cleavage & their formation during reaction.

E.g. Glucose – 1- phosphatase cleavage as catalyzed by alkaline phosphatase this reaction occur with cleavage of either C – O bond or P – O bond.



5. **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.*

APPLICATION OF TRACER TECHNIQUE

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

Detection & Measurement of Radioactivity

Various methods for measuring radioactivity

1. Autoradiography

2. Gas ionization detectors
    I. Geiger counter
    II. Scintillation counter
    III. Liquid scintillators

3. Fluorescent scintillation , are the basis to detect & measure radioactivity in clinical laboratory .

AUTORADIOGRAPHY

- In autoradiography a photo graphic emulsion is used to visualize molecules labeled with a radioactive element .
- The emulsion consists of a large number of **silver halide crystals** embedded in a solid phase such as gelatin .
- As energy from radioactive material dissipated in the emulsion , the silver halide becomes negatively charged & is reduced to metallic silver.
- Photographic developers are designed to show **these silver grains as blackening of the film , & fixers remove any remaining silver halide** .
- Techniques of autoradiography have become more important in molecular biology .
- **Weak β – emitting isotopes (^{3}H ,^{14}C ,^{35}S) are most suitable for autoradiography** , particularly for cell & tissue localization experiments .
- Low energy of negatrons & short ionizing track of isotope will result in discrete image .

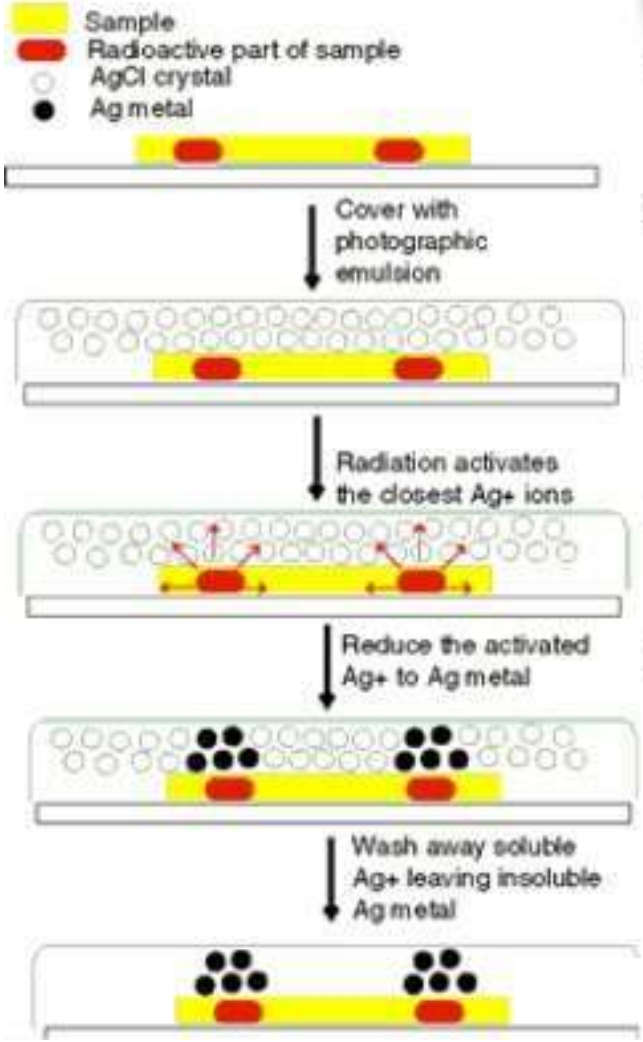
- β emitting radioisotopes are used when radioactivity associated with subcellular organelles is being located .

- ^{3}H is the best radioisotope , since it's all energy will get dissipated in the emulsion .

- Electron microscopy can then be used to locate the image in the developed film .
- For location of DNA bands in electrophoretic gel, ^{32}P labeled nucleic acid probes are useful .

- After hybridization ,hydrolysis & separation of DNA fragments by electrophoresis , a photographic plate is applied to to the covered gel & allowed to incubate .

Methodology



1. The radioactive sample is covered with the photographic emulsion by several described method.
2. The radioactive part of the sample activates the silver halide crystals near by.
3. This results in reduction of Ag^{+} ions to Ag atom leaving dark color bands.
4. The slide is then washed away by fixers to get insoluble Ag atom only.
5. The autoradiogram can further be viewed and observed under the microscope.

Figure 1.

Source: http://lifeofplant.blogspot.in/2011/12/autoradiography.html

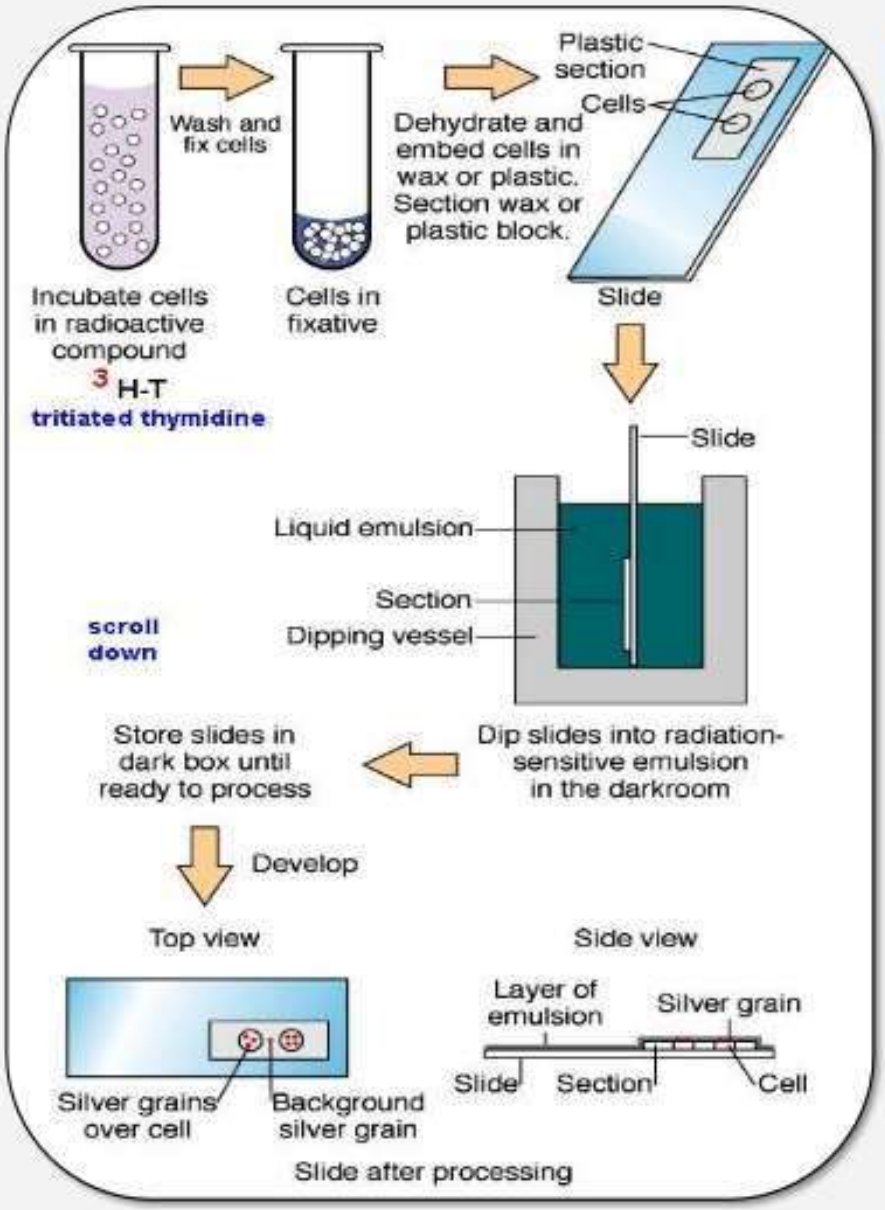
Choice of emulsion & film

X ray films are generally suitable for macroscopic samples such as whole body, electrophoretographs , chromatographs .

When light (or) electron microscopic , detection of image (cellular , subcellular localization of radioactivity) very sensitive films are necessary .

Time of exposure & film processing depends upon the isotope , sample type , level of activity , film type & purpose of the experiment.

In Direct autoradiography , the X ray film or emulsion is placed as close as possible to the sample .

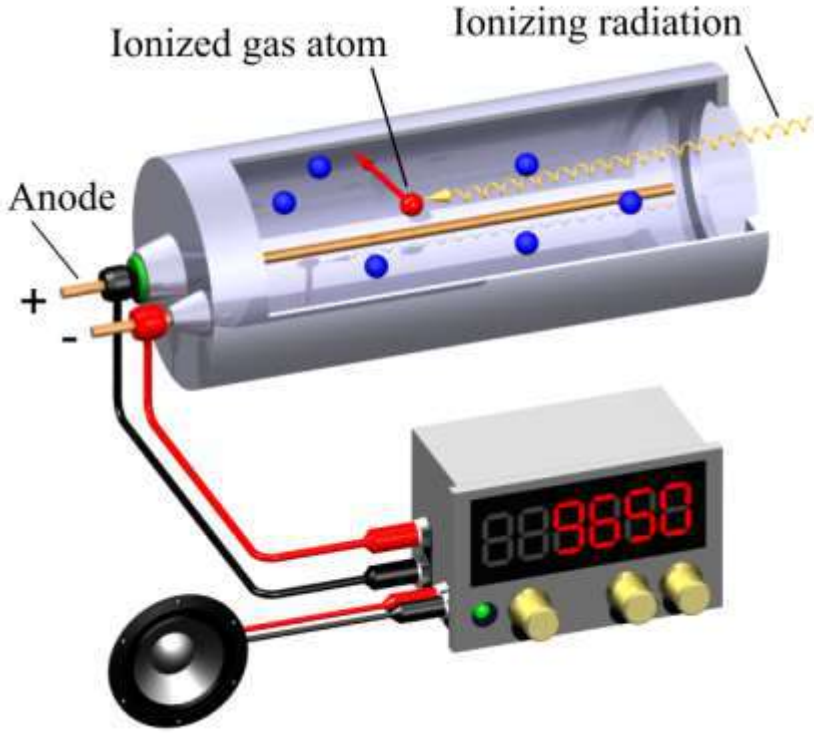


AUTORADIOGRAPHY

- Radioisotopes are taken up selectively by cells to be studied
- Exposure of photographic film to their emitted radiation reveal presence of such isotopes in the vicinity of these target cells
- Silver bromide crystals in emulsion detect radiation, that reduce them to visible black granules.

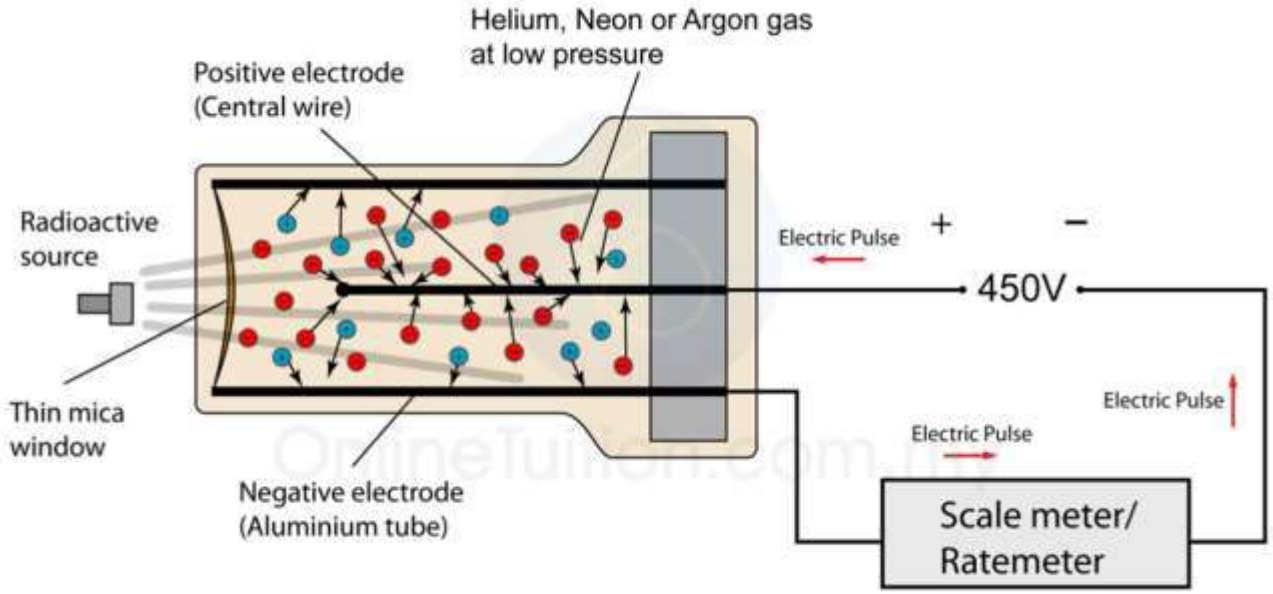
Geiger Counter

The **Geiger counter** is an instrument used for measuring ionizing radiation used widely in such applications as radiation dosimetry,radiological protection, experimental physics and the nuclear industry.



Inert Gas (Principle gas) Bromine Dead time Gaseous Ionization Detector Hance Geiger and W. Muller- 1928

Principle: Avalanche : Free electron cause electrical conduction by causing more ionization + ions massive _ Dead time (10-4 s), Secondary electron emitted: further count wrong: quenching gas = bromine gas



1.The tube contains argon gas at low pressure.
2.The end of the tube is sealed by a mica 'window' thin enough to allow alpha particles to pass into the tube as well as beta and gamma radiation.
3.When a charged particle or gamma-radiation enters the tube, the argon gas becomes ionized. This triggers a whole avalanche of ions between the electrodes.
4.For a brief moment, the gas conducts and a pulse of current flows in the circuit.
5.The circuit includes either a scaler or a ratemeter. A scaler counts the pulses and shows the total on a display.
6.A ratemeter indicates the number of pulses or counts per second. The complete apparatus is often called a Geiger counter.

SCINTILLATION COUNTING

Scintillator are material that produces light when ionizing radiation passes through it. Can be solid liquid and gas. Eg. NaI, CsI etc.

Operation :

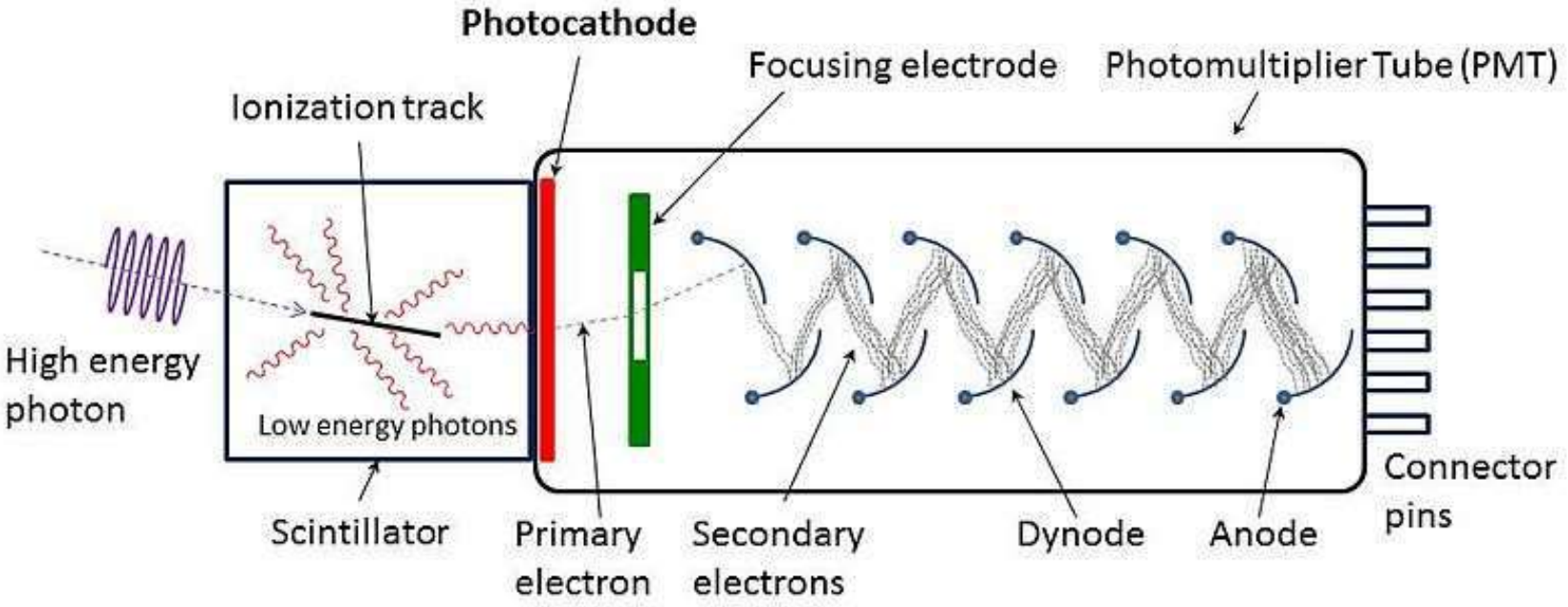
1. Absorption of incident radiation raising electron to excited state
2. After subsequent de excitation the scintillator emits a photons in the visible light range
3. The light emitted from the scintillator interacts with the photot cathods of a photomultiplier tube releasing the electrons
4. Electron are guided with the help of an electric field toward the first dynode: dynode is coated with the a substance which emits secondary electrons
5. Secondary electrons from the first dynode to another dynode and so on
6. Final amplification of about 10^6 of higher.

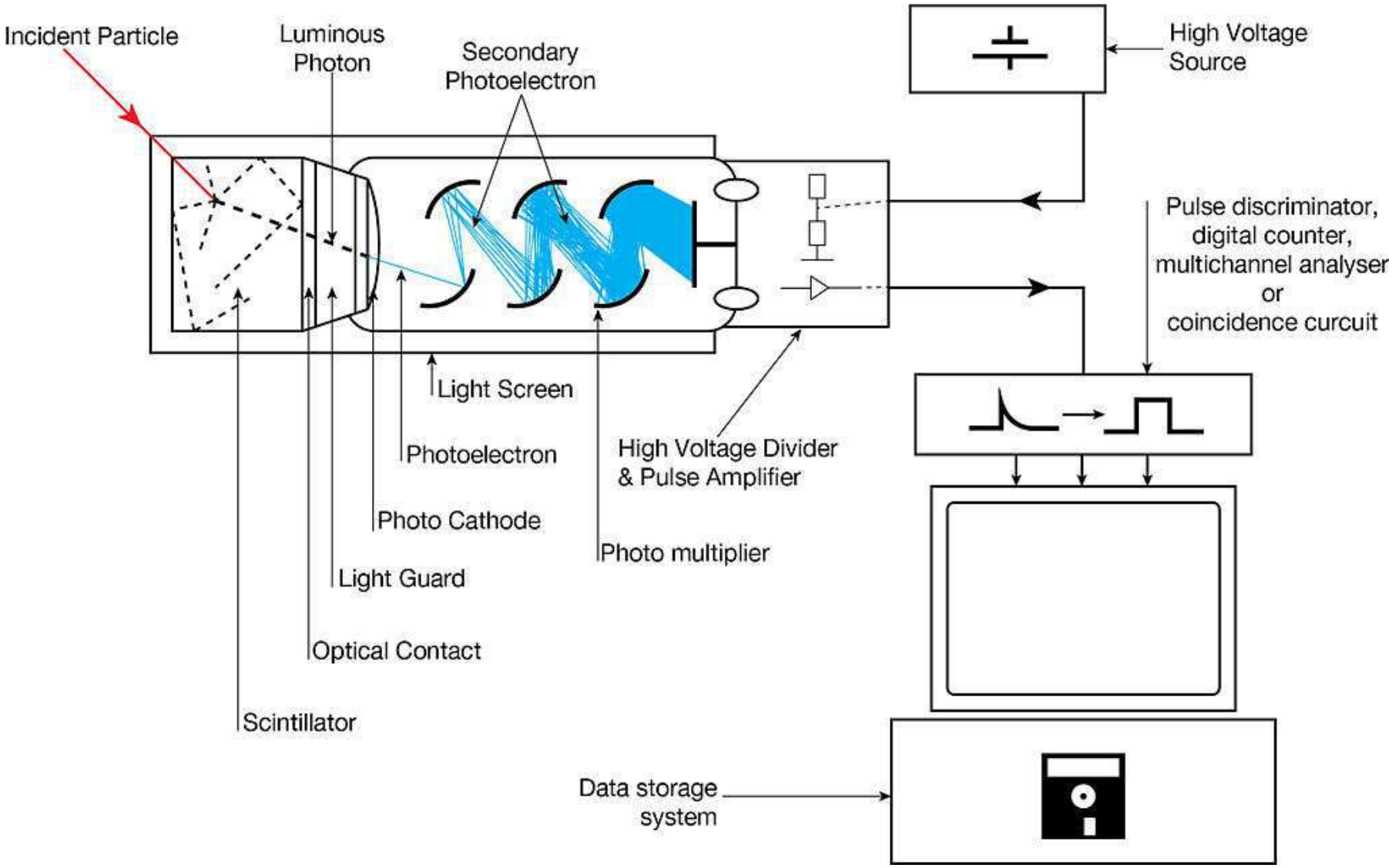
Scintillation energy Resolution :

1- large amplification needed because primary signal is low

2- large amplification leads to poor energy resolution

SCINTILLATION COUNTING



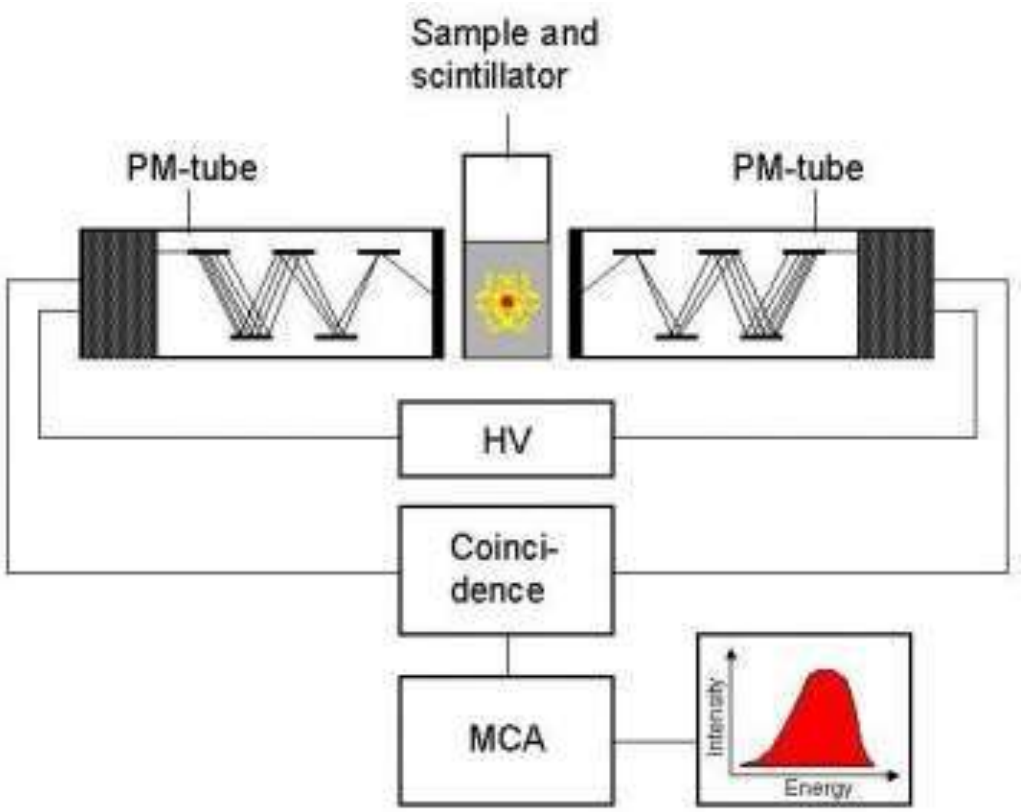

Incident Particle
Luminous Photon
Secondary Photoelectron
High Voltage Source
Pulse discriminator, digital counter, multichannel analyser or coincidence curcuit
Light Screen
Photoelectron
High Voltage Divider & Pulse Amplifier
Photo Cathode
Photo multiplier
Light Guard
Optical Contact
Scintillator
Data storage system

LIQUID SCINTILLATION COUNTING

Liquid scintillation counting is the measurement of activity of a sample of radioactive material which uses the technique of mixing the active material with a liquid scintillator, and counting the resultant photon emissions. The purpose is to allow more efficient counting due to the intimate contact of the activity with the scintillator. It is generally used for alpha and beta particle detection.

Samples are dissolved or suspended in a "cocktail" containing a solvent (historically aromatic organics such as benzene or toluene, but more recently less hazardous solvents are used), typically some form of a surfactant, and small amounts of other additives known as "fluors" orscintillators. Scintillators can be divided into primary and secondary phosphors, differing in their luminescence properties

Liquid scintillation process is the conversion of the energy of a radioactive decay event into photons of light in a liquid. Photomultipliers (PM-tubes) detect the emission of light and convert the light pulse into an electrical signal. The intensity of the light pulse (number of photons emitted) is proportional to the energy of the radioactive decay event. Further, the size (height) of the electrical pulse is proportional to the intensity of the light and, accordingly, also proportional to the energy of the decay event.



The electrical pulse can be handled in an electronic system, which measures its height and stores the events in an intensity-energy array, a so-called multichannel analyzer (MCA) system. Thus, an energy spectrum can be recorded of the decaying radionuclide

In this way liquid scintillation counting (LSC) is a detection technique for radioactivity. Normally, the radioactive substance is intimately mixed with the detector which is the liquid scintillator cocktail. The radioactive substance should then, preferably, be in liquid form.

The emission of light in the liquid scintillator is an isotropic process. By applying two PM-tubes instead of only one the noise in the detection process (background counts not due to the decay process) may then be reduced. Only those events that are recorded in both PM-tubes simultaneously (in coincidence within the required time window, often 10-30 ns width) are recorded as “true” counts. A principle sketch of the detection system is given in the figure above

CHERENKOV RADIATION

Cherenkov radiation, also known as **Vavilov–Cherenkov radiation**, is electromagnetic radiation emitted when a charged particle (such as an electron) passes through a dielectric medium at a speed greater than the phase velocity of light in that medium.

The characteristic blue glow of an underwater nuclear reactor is due to Cherenkov radiation.

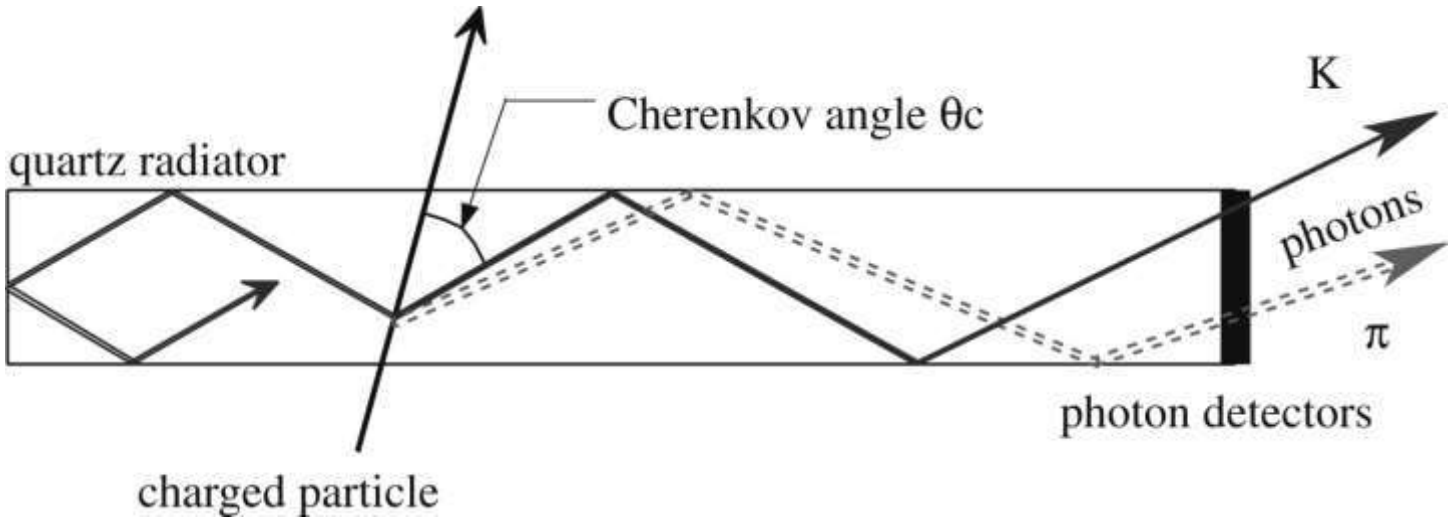
High-energy beta emitters, such as phosphorus-32, can also be counted in a scintillation counter without the cocktail, instead using an aqueous solution. This technique, known as **Cherenkov counting**, relies on the Cherenkov radiation being detected directly by the photomultiplier tubes. Cherenkov counting in this experimental context is normally used for quick, rough measurements, since the geometry of the sample can create variations in the output.

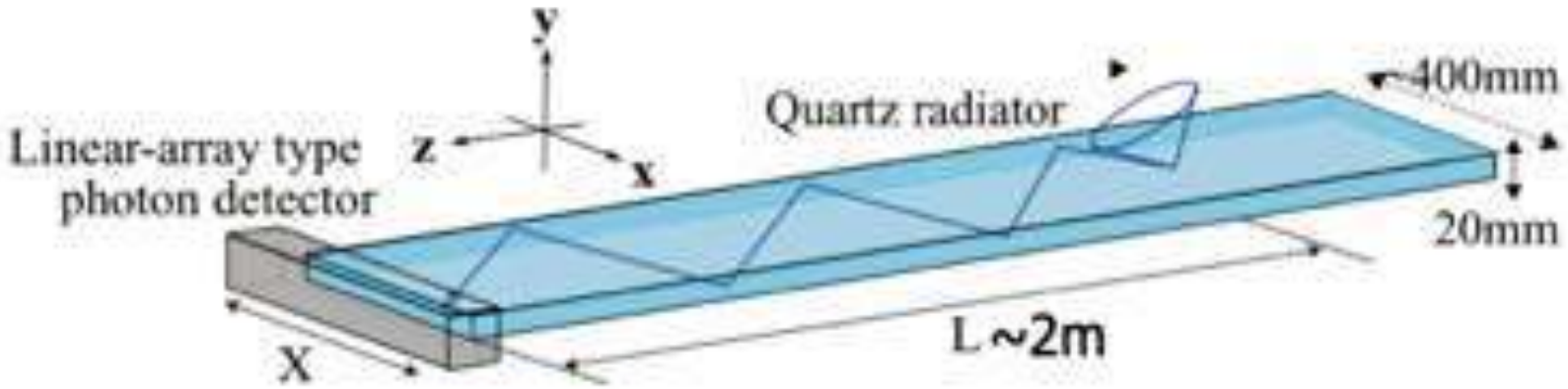
If a charged particle moves in a medium at a velocity v that is greater than the phase velocity of light waves in the medium the particle emits Cherenkov radiation.

The phase velocity of light waves in a given medium = c/n

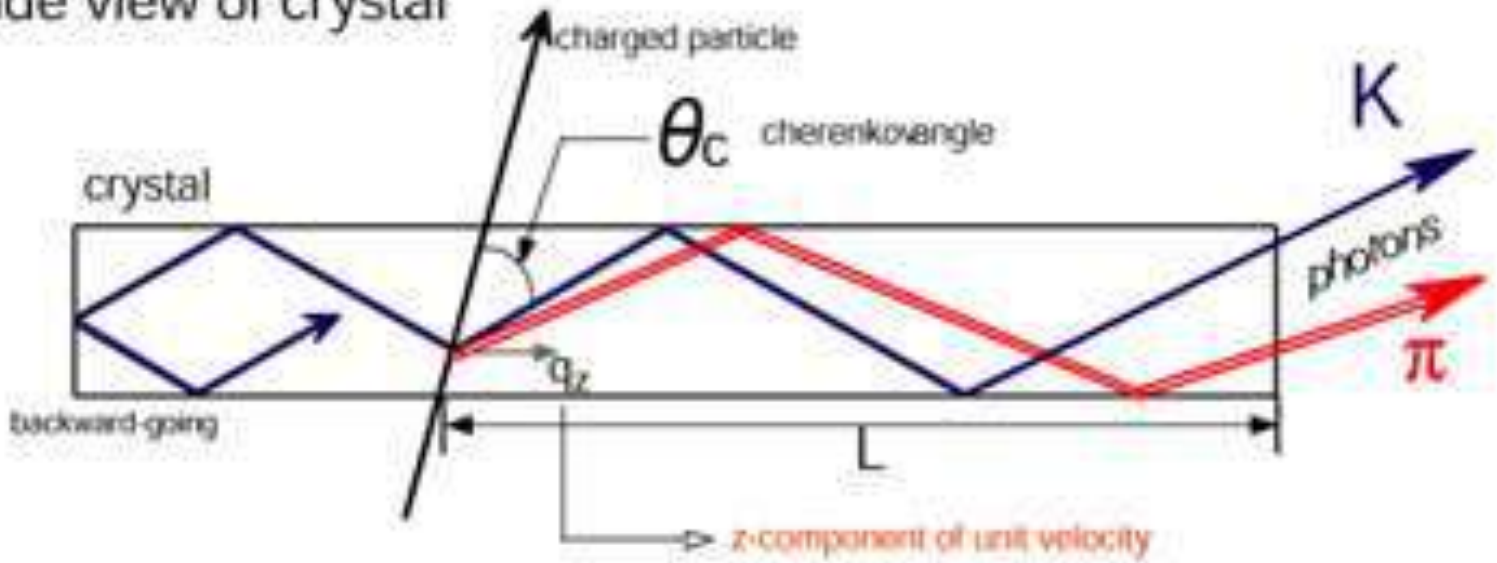
where n is therefractive index of the medium and c is the speed of light in vacuum.

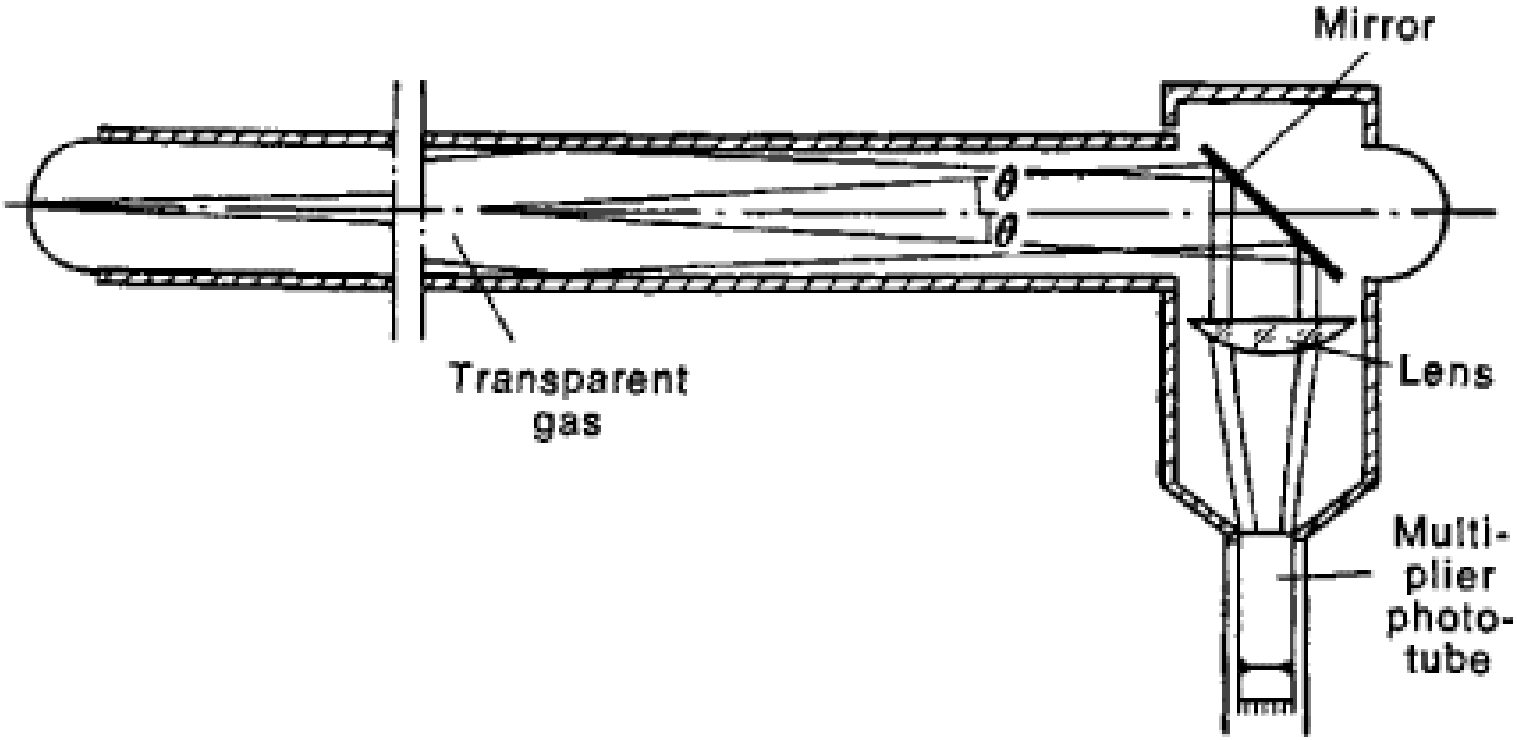
Cherenkov radiation is emitted in a specific direction and the angle θ between the direction of the radiation and the trajectory of the particle is related to v and n





Side view of crystal





Schematic of a threshold gas Cherenkov counter used with the 70-GeV particle accelerator of the Institute of High Energy Physcis (USSR).

The Cherenkov light iscollected at the cathode of the multiplier phototube by m eans of an optical system consisting of a plane mirror and a quartz lens.

Three types of Cherenkov counters are distinguished:

1. THRESHOLD,
2. DIFFERENTIAL
3. TOTAL- ABSORPTION.

The main characteristic
of threshold and differential Cherenkov counters are the detection effici ency and the velocity resolution; the latter is ameasure of a counter's a bility to distinguish two particles moving with nearly the same velocity.

A threshold Cherenkovcounter should detect all particles with velocities greater than some threshold value. Therefore, a threshold counter's op ticalsystem, which is a combination of lenses and mirrors, should— if possible—
collect all the emitted light at the cathode of themultiplier phototube.

Differential Cherenkov counters detect particles whose velocity lies between certai n limits v_1 and v_2. In conventionaldifferential Cherenkov counters, the desired parti cles are detected by using the optical system to select the light that isemitted at an angle lying between the corresponding limits θ_1, and θ_2. A lens or a spherical mirr or placed in the path of theCherenkov light focuses the light that is emitted at an a ngle θ into a ring of radius $R = f\theta$ where f is the focal length of thelens or mirror. If an annular slit diaphragm is placed at the focus of the optical system and one or m ore multiplier phototubesare placed behind the diaphragm, light will be detected on ly for particles that radiate at an angle lying within certain limits. Adifferential Chere nkov counter with a precision optical system makes it possible to single out particle s whose velocity differsby just 10^{-6} from the velocity of other particles. Such Cherenkov counters require special cont rol over the gas pressure andthe formation of a parallel particle beam.

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