

Chapter - 5

Fluorimetric Analysis : Theory, Concept of Singlet and Triplet Electronic States, Internal and External Conversions, Intersystem Crossing, Factors affecting Fluorescence, Quenching, Instrumentation - Study of Fluorimeter, Spectrofluorimeter and Applications.

Unit - II

SYLLABUS

FLUORIMETRIC ANALYSIS

2.1 INTRODUCTION

A large number of substances absorb ultraviolet and visible light energy. But, there are some substances which lose excess energy as heat through collisions with neighboring atoms or molecules. The energy emitted by these substances has a wavelength larger than that of absorbed. This process of emitting radiation with larger wavelength than that of absorbed is known as Luminiscence.

Luminiscence is mainly of two types :

1. Fluorescence, and
2. Phosphorescence

In order to understand the basics of fluorimetry, the following general considerations of electronic states should be known :

2.2 CONCEPT OF SINGLET AND TRIPLET ELECTRONIC STATES

The concept of singlet and triplet electronic states arise from the multiple considerations of atomic spectroscopy and they simply define the number of unpaired electrons in the absence of a magnetic field. If there are ' n ' number of unpaired electrons, $(n + 1)$ equal energy states are associated with electronic spin, regardless of the molecular orbital occupied. If unpaired electrons are not present ($n = 0$), there is only one spin state. Such a state is called singlet state, similarly, systems having 1, 2, 3, 4 unpaired electrons are referred as doublet, triplet, quartet, quintet states respectively.

Many molecules do not have unpaired electrons in their ground state. When, these molecules absorb ultraviolet or visible radiations, one or more electrons of the pair get raised to an excited singlet state. In this excited state, the spin of electrons do not change and the net spin is zero. Another possibility is one set of electron spins may undergo unpairing, resulting in two unpaired electrons which make an excited triplet state. The probability of direct transition from the singlet ground state to the triplet excited state is very small.

- (a) **Ground State :** It is the lowest energy state wherein the molecule is at rest (But, random motion may occur within the molecule).
- (b) **Excited State :** It is the highest energy state wherein the molecule is unstable with rapid electronic motion and tends to return to ground state.

- (c) **Singlet Ground State** : It is the ground state consisting of **paired** electrons in an orbital or suborbital with opposite spin.



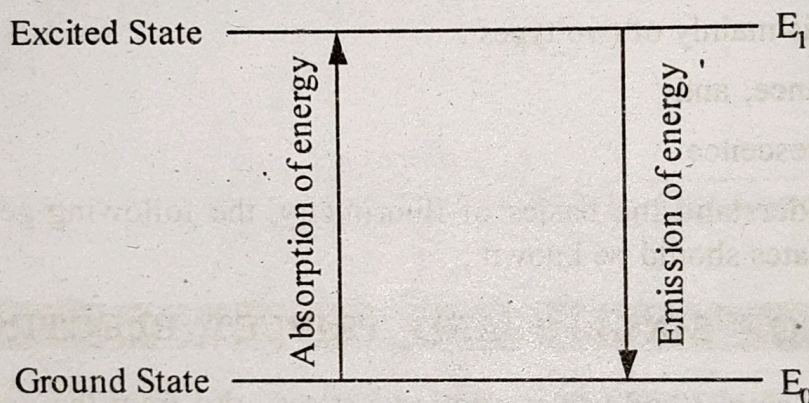
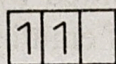
- (d) **Singlet Excited State** : It is the excited state consisting of **unpaired** electrons in orbitals or suborbital with opposite spin.



- (e) **Doublet State** : It is the state of molecule consisting of **unpaired** electrons.

Example : Free radicals.

- (f) **Triplet State** : It is the state consisting of unpaired electrons with same spin.

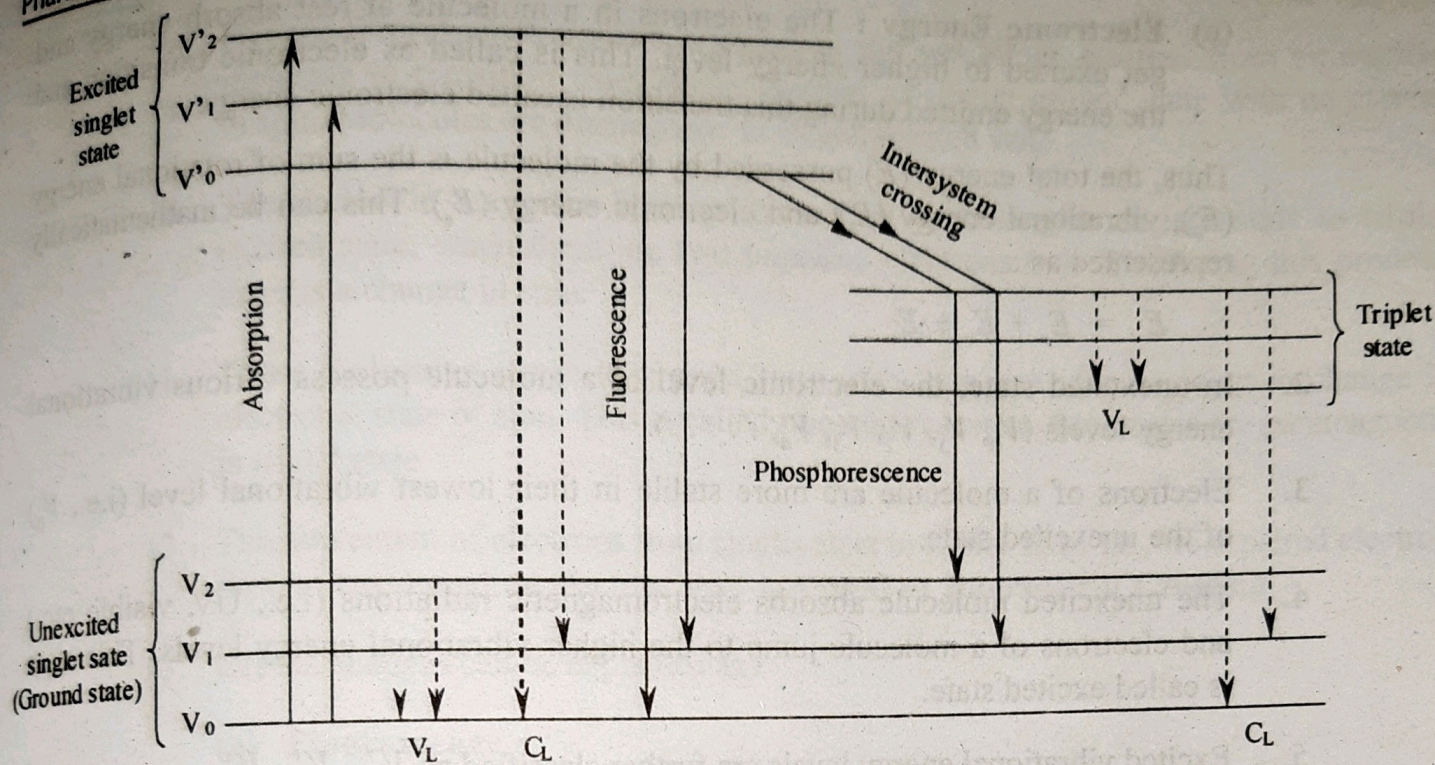


Fluorimetry is an analytical method for the measurement of fluorescence, based upon emission of absorbed radiations by the molecules. When a molecule absorbs incident electromagnetic radiations, it is excited to higher energy level, where it is unstable. Therefore, it returns to the ground state by emitting the absorbed radiations. When the emission occurs directly from singlet excited state to singlet ground state without any transitions, it is referred as **fluorescence**. And when the emission occurs within a fraction of second to few days of absorption of radiations (due to transitions from singlet excited state to triplet state and then to singlet ground state), it is referred as **phosphorescence**. Fluorescence and phosphorescence are combinedly called **photoluminescence**.

The basis of fluorimetry is the measurement of fluorescence. Drugs which are intrinsically fluorescent, are determined fluorimetrically.

Examples :

1. Quinine sulphate in 0.1N H_2SO_4 .
2. Ergometrine in 1% tartaric acid etc.



V_L – Loss of vibrational energy to solvent molecule.

C_L – Loss of energy due to collisions between excited molecule.

2.3 PRINCIPLE

A molecule absorbs incident electromagnetic radiations and gets excited. It is unstable in its excited state and tends to return to ground state by emitting radiations. Fluorescence can be referred as the radiations emitted from an excited molecule in transition from singlet excited state to singlet ground state. The molecules are diamagnetic in singlet excited state.

In fluorimetry, radiations emitted are of longer wavelength than absorbed radiations. This is because, when the radiations fall on the molecule, vibration occurs (in 10^{-13} seconds), and has an average life of 10^{-9} seconds. During the vibration period, loss of energy occurs due to intermolecular collisions and some energy is lost to solvent molecules (molecules of the solvent used for the dissolution of the sample in fluorimetry). Hence, the emitted radiations are of longer wavelength and have less

$$\text{energy} \left(E \propto \frac{c}{\lambda} \right).$$

2.4 THEORY

- Under unexcited condition (rest) a molecule possess three types of internal energies.
 - Rotational Energy** : It rotates the molecule around the 3 axes i.e., x, y and z.
 - Vibrational Energy** : It vibrates atoms present in a molecule relative to one another.

- (c) **Electronic Energy** : The electrons in a molecule at rest absorb energy and get excited to higher energy level. This is called as electronic transition and the energy emitted during this transition is called electronic energy.

Thus, the total energy (E) possessed by the molecule is the sum of rotational energy (E_r), vibrational energy (E_v) and electronic energy (E_e). This can be mathematically represented as :

$$E = E_r + E_v + E_e$$

- In unexcited state, the electronic level of a molecule possess various vibrational energy levels ($V_0, V_1, V_2, V_3, V_4, \dots$).
- Electrons of a molecule are more stable in their lowest vibrational level (i.e., V_0) of the unexcited state.
- The unexcited molecule absorbs electromagnetic radiations (i.e., UV, visible etc.) and electrons of a molecule jump to the higher vibrational energy levels. This state is called excited state.
- Excited vibrational energy levels are further classified as $V'_0, V'_1, V'_2, \dots$
- When an electron jumps to higher energy level (such as singlet, doublet, triplet, quartet or quintet excited states), unpaired electrons are formed. This occurs only in the absence of magnetic field.
- The energy states of a molecule is given by $(n + 1)$ rule, which depends on the number of electrons (n) irrespective of the orbital in which they are present.

Number of Electrons (n)	Number of Energy States ($n + 1$)
0	1 (Singlet)
1	2 (Doublet)
2	3 (Triplet)
3	4 (Quartet)
4	5 (Quintet)

- The excited molecule is highly unstable. Hence it returns to the ground state by losing energy. Loss of energy can occur by following ways :
 - The molecular collisions between excited molecules, leads to loss of energy. This results in transfer of molecule to the lowest vibrational unexcited state (C_L).
 - Vibrational energy may be taken up by the surrounding solvent molecules (V_L).
 - The excited molecule emits the radiation absorbed (fluorescence or phosphorescence).

9. Hence, fluorescence can be referred as the radiations emitted from an excited molecule in transition from singlet excited to singlet ground state with no change in spin. Molecules are diamagnetic in singlet excited state.
10. The molecule may also undergo transition from singlet excited state to triplet excited state, when there are two unpaired electrons ($n = 2$). During this process, there is a change in spin.
11. These molecules return to ground state with time gap, due to delay in change in electronic state of spin. This is called **phosphorescence**. Molecules are paramagnetic in triplet state.
12. The movement of electrons from singlet state to triplet state, i.e., from paired electron state to two unpaired electron state is termed as **Intersystem Crossing**.
13. Orbital changes can be explained as :

(a) **Fluorescence**

Singlet State $\longrightarrow$ Singlet Excited State $\longrightarrow$ Singlet Ground State
(No unpaired electrons)

$\pi \longrightarrow \pi^*$ (No change in spin)

(b) **Phosphorescence :**

Singlet State $\longrightarrow$ Singlet Excited State $\longrightarrow$ Triplet State $\longrightarrow$ Singlet Ground State
(No unpaired electrons) (2 unpaired electrons)

$\pi \longrightarrow \pi^*$ (No change in spin) $\longrightarrow$ Change in spin

14. The molecules in higher excited states can also reach the lowest vibrational state of a lower electronic level by a process known as **Internal Conversion** in which the vibrational levels from different excited states overlap and have same potential energy which is a radiationless process.

2.5 FACTORS AFFECTING FLUORESCENCE

The various factors affecting fluorescence are as follows :

Nature of Molecules :

- (a) The molecules which have the tendency to absorb electromagnetic radiations (i.e., UV, visible), can only exhibit fluorescence.
- (b) Unsaturated molecules with π bonds and good resonance stability can exhibit fluorescence.

Example : Alkenes with conjugated double bonds.

- (c) Saturated molecules with σ bonds do not exhibit fluorescence.

Example : Aliphatic and saturated cyclic organic compounds.

- (d) In general, the greater the absorbancy of a molecule, the more intense its luminiscence.

Affect of Functional Groups : Functional groups exhibit a marked effect on fluorescence oftenly.

- (a) Electron releasing groups improve fluorescence.

Example : Amine, Hydroxy and Methoxy groups.

- (b) Electron accepting groups deteriorate or completely destroy fluorescence.

Examples : Carboxylic, Nitro and Azo groups, Halides (chlorides, bromides, iodides, etc.)

High Molecular Weight Compounds :

If an electron of an atom with high molecular weight (hence, high atomic number) is excited, it exhibits decreased fluorescence.

Affect of Concentration :

- (a) There exists a linear relationship between the concentration of sample and fluorescence upto an absorbance value of 0.02. The linearity holds good to about 5% of cases with absorbances upto 0.05.

- (b) In dilute solution, the radiation distribute uniformly throughout the solution and get absorbed uniformly giving high intense fluorescence.

- (c) In highly concentrated solution, upper layers of the solution absorb more radiations and less amount of radiations are transferred to lower layers. Thus, there is no uniform radiations absorption which results in decreased fluorescence.

- (d) In highly concentrated solution, intramolecular collisions cause loss of vibrational energy and certain amount of fluorescence which is emitted is reabsorbed resulting in decrease in intensity of fluorescence. This is called as **concentration quenching**.

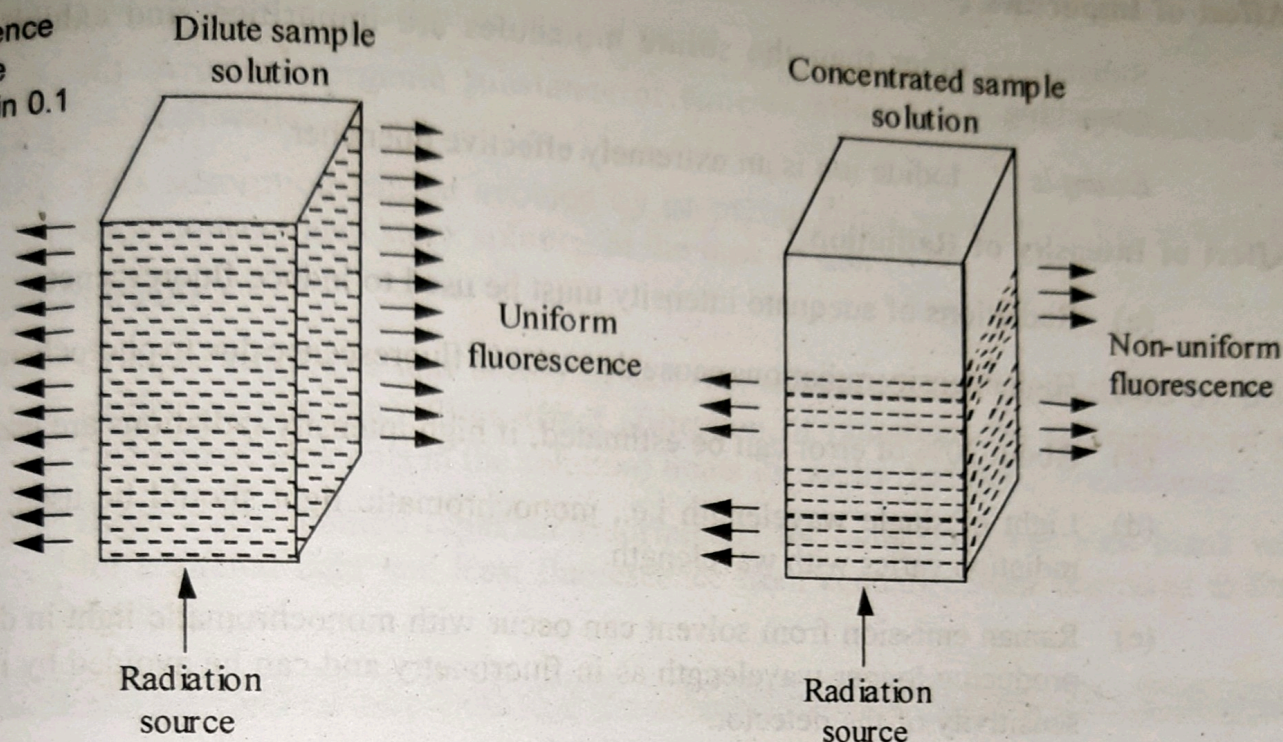
- (e) Inter - filter effect is a phenomenon which disturbs electronic excitation and fluorescence.

Inter - filter effect is due to :

- (i) Presence of non-fluorogenic material, or

- (ii) High concentration of the solution.

Figure :
Fluorescence
of Quinine
Sulphate in 0.1
 $\text{N H}_2\text{SO}_4$



Affect of Oxygen :

- (a) There is a decrease in fluorescence in the presence of oxygen due to,
 - (i) Direct conversion of fluorogenic material into non-fluorogenic material.
 - (ii) Indirectly due to quenching (decrease in sensitivity and intensity of fluorescence).

Example : Anthracene is highly prone to oxidation.

- (b) This decrease in fluorescence can be avoided by using oxygen free solutions or comparing the results obtained in the presence and absence of oxygen.

Affect of pH of the Solution :

Depending upon the acidity or alkalinity, a substance can be ionised or unionised and hence, can be fluorogenic or non-fluorogenic.

Example : Phenol in neutral and alkaline medium undergoes ionisation and gives weak fluorescence, whereas in acid medium it is unionised and gives intense fluorescence.

Affect of Temperature and Viscosity :

- (a) Alterations in temperature may affect the concentration and viscosity of the sample.
- (b) Increase in temperature may result in increase in concentration and decrease in viscosity resulting in intermolecular collisions and deactivation of excited molecule destroying fluorescence.
- (c) Some substances may exhibit fluorescence at temperature lower than room temperature or in a viscous solvent or glassy matrix.

Affect of Impurities :

Substances other than the solute molecules are impurities, and exhibit fluorescence quenching.

Example : Iodide ion is an extremely effective quencher.

Affect of Intensity of Radiation :

- (a) Radiations of adequate intensity must be used to induce fluorescence.
- (b) High intensity radiations cause decrease in fluorescence due to photochemical changes.
- (c) About 20% of error can be estimated, if high intensity radiations are used.
- (d) Light of single wavelength i.e., monochromatic light should be used, as energy of radiation varies with wavelength.
- (e) Raman emission from solvent can occur with monochromatic light in dilute solution producing longer wavelength as in fluorimetry and can be avoided by increasing the sensitivity of the detector.

Chemical Quenching :

This can occur in two ways :

- (a) The excited molecule transfers its fluorescent intensity to surrounding molecules, ions or impurity by intercollisions, thereby destroying fluorescence.
- (b) The unexcited molecule may form a stable complex with quencher molecule inhibiting excitation and fluorescence.

Affect of Structure of the Compounds :

- (a) Closed ring aromatic compounds exhibit fluorescence

Example : Fluorescein, Eosin.

- (b) Compounds with more than two cyclic structures (polycyclic), exhibit fluorescence

Example : Vitamin K, Nucleosides, Purines

- (c) Rigid molecules like metal complexes have enhanced fluorescence as they inhibit the liberation of excitation energy.
- (d) The position of functional group (chromophore) which is responsible for absorption affects fluorescence.

Adsorption of Solute on Cell Walls :

- (a) Fluorimetry requires dilute solutions (concentration 10 - 100 times less than that in spectrophotometry) of the solute to obtain fluorescence. But this solution gets adsorbed onto the cell wall.

Examples :

- (i) Quinine is adsorbed on the cell walls.
 - (ii) Aromatic organic substance of concentration 1 – 2 ppm is adsorbed onto cell walls.
- (b) This adsorption can be avoided by preparing dilute solution of the sample from fresh concentrated stock solution at the time of use.

Illumination Methods :

- (a) Opaque solutions and solids of any concentration can be radiated by frontal method, while inter-filter effect (filtration of radiation due to presence of non-fluorogenic materials in the solution) limits its use by destroying fluorescence.
- (b) Right angle method (spekker fluorimeter) for radiation gives least blank values for scattered light and least fluorescence from container walls compared to frontal method.

2.6 QUENCHING

Quenching refers to the decrease in intensity of fluorescence as a result of decrease in the sensitivity of constituents.

Types of Quenching :

Quenching can be due to either high concentration of solution or due to the presence of specific functional groups or due to chemical reaction etc. Hence, it is classified into following types.

1. Chemical Quenching
2. Collisional Quenching
3. Self/Concentration Quenching
4. Static Quenching

1. **Chemical Quenching :** Chemical Quenching is further classified as :

(a) pH Dependent Quenching :

Example : When aniline is exposed to excitation radiations of 290 nm, it exhibits blue fluorescence if the pH of the solution is between 5 and 13. At pH below 5, it exists in the cationic form and above 13 it exists in the anionic form and does not exhibit fluorescence in these forms.

(b) Oxygen dependent Quenching : Many organic compounds in the presence of oxygen (dissolved) gets excited from excited singlet state to triplet state, exhibiting paramagnetism.

- (c) **Quenching due to Halides** : Iodide ion is an extremely effective quencher. It causes extreme decrease in the intensity of fluorescence due to decreased sensitization. Chloride, bromide ions also exhibits quenching.
- (d) **Quenching due to Electron withdrawing Groups** : Nitro ($-\text{NO}_2$) and carboxylic groups ($-\text{COOH}$) causes desensitization of the constituents by withdrawing electrons, resulting in decreased fluorescence.
- (e) **Heavy Metal Quenching** : Presence of heavy metals cause collisions and excitation of the molecules from excited state to triplet ground state, resulting in loss of energy. This results in quenching.

2. **Collisional Quenching** : High temperature of the sample and presence of very low viscous samples causes increased collisions between the molecules resulting in deactivation of excited molecule. This causes decrease in fluorescence.

3. **Self/Concentration Quenching** : There exist a linear relationship between fluorescence and concentration in dilute solution. In concentrated solution, the linearity is not seen. This is called as concentration dependent quenching. It can be due to :

- (a) Loss in vibrational energy due to collisions between atoms or molecules of a compound.
- (b) The emitted fluorescent radiations can be reabsorbed by the sample itself.

4. **Static Quenching** : The substance to be examined may form a complex with the quencher molecule, inhibiting the excitation of the substance and hence the fluorescence.

Example : The fluorescent intensity of riboflavin is reduced when it is complexed with caffeine.

Relationship between Concentration and Intensity of Fluorescence :

According to Beer-Lambert's Law, there exists a linear relationship between the concentration of the solution and emitted fluorescent intensity in dilute solutions. But, the direct application of this law to fluorescence is restricted as fluorescence is an emission phenomenon of a substance. It may be represented as :

$$F \propto I_0 - I$$

.....[1]

where,

F – Fluorescent Intensity.

I_0 – Intensity of Incident Radiation.

I – Intensity of Emitted Radiation.

$$F = K(I_0 - I)$$

.....[2]

K – Proportionality constant, it represents fraction of incident radiation absorbed.

$$I = I_0 10^{-abc}$$

.....[3]

where,

 a - Absorptivity b - Pathlength in cm c - Concentration in gm/LSubtracting both sides with I_0

$$I - I_0 = I_0 10^{-abc} - I_0 \quad \text{.....[4]}$$

$$I_0 - I = I_0 - I_0 10^{-abc} \quad \text{.....[5]}$$

Substituting $I_0 - I$ in equation - (2)

$$\Rightarrow F = K(I_0 - I_0 10^{-abc}) \quad [\text{As } F = K(I_0 - I)] \quad \text{.....[6]}$$

$$F = KI_0 - KI_0 10^{-abc} \quad \text{.....[7]}$$

(Or)

$$F - KI_0 = -KI_0 10^{-abc}$$

(Or)

$$\frac{F - KI_0}{KI_0} = -10^{-abc} \quad \text{.....[8]}$$

Inversing both the sides

$$\frac{KI_0}{F - KI_0} = -10^{-abc}$$

Taking (-) common

$$-\left(\frac{KI_0}{F - KI_0}\right) = -10^{-abc}$$

$$\frac{KI_0}{KI_0 - F} = 10^{-abc}$$

Applying logarithms on both the sides

$$\log\left(\frac{KI_0}{KI_0 - F}\right) = \log 10^{-abc} \quad \text{.....[9]}$$

$$\log\left(\frac{KI_o}{KI_o - F}\right) = abc \log 10 \quad [\because \log a^x = x \log a]$$

$$\log\left(\frac{KI_o}{KI_o - F}\right) = abc \quad [\because \log 10 = 1] \quad \dots[10]$$

where,

$$F = 2.303 KI_o \cdot abc$$

$$F = K''c \quad \dots[11]$$

where,

$$K' = 2.303 KI_o \cdot ab$$

From equation - (11), it indicates that fluorescent intensity is proportional to concentration. This law is applicable when concentration is expressed in ppms. In equation - (10), it is considered that fluorescent radiation and incident radiation takes the same path. But, practically, incident radiation and fluorescent radiation are perpendicular to each other where, pathlength (b) is replaced by the length of the cell in the direction of the detector. But, still the equation holds good and is applicable.

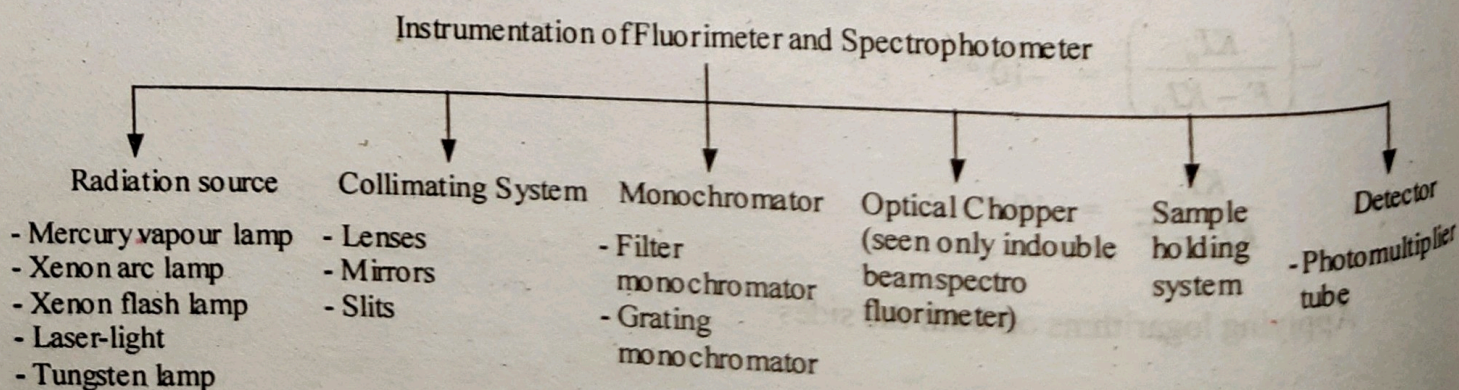
Factors affecting Beer-Lambert's Law can be applied to fluorescence as well. They are :

1. Association between molecules present in the solution.
2. Dissociation of molecules into ions.
3. Type of solvent used.

2.7 INSTRUMENTATION

General Instrumentation of Fluorimeter and Spectrofluorimeter :

The components used in fluorimeter and spectrofluorimeter are :



- (a) **Mercury Vapour Lamp** : It is widely used in filter fluorimeter. A mercury vapour lamp covered with glass or fused silica envelope, is used as a source of radiation. Both high pressure and low pressure mercury vapour lamps are available. In low pressure mercury vapour lamps, when electric discharge occurs, radiations of various wavelengths like 253.7 nm (strong intensity wavelength), 313, 365, 404.7, 407.8, 546.1, 577 and 579.1 nm are obtained. At low pressure, the discharge is less intense and diffused so the lamp is relatively stable. Radiation of desired intensity is then isolated using primary filter.

Mercury vapour lamp may be coated with phosphorous which emits abundant radiations. Interference filters select the exact wavelength of excitation from emission lines of mercury lamp. Band pass filters select the band of light for excitation.

Mercury vapour lamp provides good sensitivity for the material whose wavelength of excitation is equivalent to emission lines of mercury vapour. Mercury vapour is less sensitive, if the material is highly fluorescent and emit fluorescent radiations at excitation radiations other than emission lines of mercury vapour. (i.e. 253.7, 313, 365, 404.7, 407.8, 435.8, 546.1, 577, 579.1 nm)

- (b) **Xenon-arc Lamp** : It is widely used as a radiation source in spectrofluorimeter. The lamp acts under discharges of high pressure direct currents produced by compressing the arc discharge between the narrow gap of electrodes. Xenon lamp emits intense and stable radiations in the wavelength range of 300 - 1300 nm which includes strong emission lines in the wavelength range of 800 - 1100 nm range.

The short term stability of the lamp (about 0.3%) is determined by arc flicker (i.e., sudden movement in the arc lamp leading to amplitude variation), whereas the long term stability of the lamp (about 1% drift per hour) depends upon electrode wear (i.e., deformation at the tip face of the electrodes) and arc wander (i.e., random shift of the lamp arc relative to one of the electrodes). Radiations emitted from xenon arc lamp are equivalent to black body radiator giving a colour temperature of 6000 K.

- (c) **Xenon-Flash Lamp** : It consists of a lamp filled with xenon gas and causes excitation of the sample by high energy flash produced by the discharge of a charged capacitor. Repetitive flashing and AC methods of amplification produces a high intensity flash with a capillary diameter of 0.8 mm which produces an image on the sample position of dimensions 2mm wide and 18mm high. Xenon-flash lamp is advantageous in micro cell and continuous-flow arrangements. It is compact and has low cost.

The instability of xenon source (xenon-arc lamp and xenon-flash lamp) can be corrected by ratio-mode operation. It employs two photo cells, reference photodetector for exciting radiation and sample photodetector for emission radiation. The output from reference photodetector is sent to photomultiplier dynode voltage control circuit, where it controls the dynode voltage of both photomultiplier tubes in inverse relationship.

Fluctuations in xenon source (xenon-arc lamp and xenon-flash lamp) causes a corresponding change in fluorescence. The reference photodetector reads the range and speed of fluctuation in the radiation source and signals to the excitation monochromator. Thus, it compensates for wavelength dependent fluctuations in excitation spectra.

- (d) **Laser-light** : It is used when monochromatic light or high intensity radiations are desired. Laser excited fluorescence is used in determination of ultra-trace inorganic ion.
- (e) **Tungsten Lamp** : It is used when excited radiations above 450 nm are required. Tungsten lamp gives a group of lines (broad band) unlike the sharp lines of mercury vapour lamp.

2. **Collimating System** : In filter fluorimeter, lenses, mirrors and slits are used to render the emitted radiations parallel. Lenses made up of either quartz or glass are used.

In spectrofluorimeter, mirrors and slits are used to render the emitted radiations parallel. Mirrors can reflect, focus or collimate the radiations in spectrofluorimeter. Mirrors which are aluminized on front surface is called as front surface mirrors. These mirrors reduce the light losses. Lattice mirrors are flat mirrors and are half aluminized and half transmitting. Lattice mirrors reflect the sample beam and transmit the reference beam to emission grating monochromator.

Both front surface and lattice mirror plays a major role in spectrofluorimeter. Large entrance slits are used to allow more excitation radiations to fall on the sample. This is very advantageous when sample concentration is low.

3. **Monochromator** : The various types of monochromators used are :

- (a) **Filter Monochromator** : Glass filters and interference filters are used in fluorimetry. Glass filters allows broad band of light whereas interference filters allows a narrow band of light. Photodetectors have poor sensitivity towards broad band of light.

In filter fluorimeter, two types of filters are used : primary filter and secondary filter. Primary filters are also called as excitation filters which allows a broad band of light to pass through. Off-band transmission can occur which may be due to certain unavoidable pin holes and defects in film coatings. Secondary filters are also called as emission filters, which allows a narrow band of light to pass through. Sharp-cutoff transmission occurs, allowing longer wavelengths to pass through and absorbing smaller wavelength radiation.

Primary filters allow passage of UV radiations which are responsible for wavelength of excitation, but not visible radiations. Secondary filters absorb incident ultraviolet radiation and transmit visible fluorescent radiation.

- (b) **Grating Monochromator** : Grating monochromator of Czerny-turner type is used in spectrofluorimeter with 0.25 m focal length and f/4 or f/5 aperature. Grating with 600 grooves/mm is used when excitation radiation has wavelength of 300 nm and emission radiation has wavelength of 500 nm. To isolate the wavelength of excitation, excitation monochromator is placed between radiation source and sample. Excitation monochromator absorbs visible radiations and transmits UV radiations.

Wavelength of emission can be isolated by placing emission monochromator between sample and photomultiplier tube (detector). Emission monochromator transmits visible radiations and absorbs UV radiations.

In a good monochromator, the emission-lines are resolved such that they are 1 nm apart. This provides spectral selectivity and fine spectral structure.

The filter or grating monochromators are placed at right angles to incident light to avoid scattering and reflection of light. The 90° geometry efficiently separates wavelength of excitation and wavelength of emission.

4. **Chopper** : An optical rotatory chopper is present only in double beam spectrofluorimeter, splits the excitation radiation from source. Chopper creates alternate current signals by blocking the radiations to one photocell and transmitting to another photocell and vice versa. Any signal in intermediate period is due to dark current of the detector. The signals from both photocells are separated and passed to differential amplifier, which eliminates dark current and gives the exact measurements.
5. **Sample Holding System** : These are called as cuvettes or cells which are cylindrical or rectangular in shape having an area of 1 cm^2 . These are made of good quality glass or quartz. Cuvettes are provided with lid to prevent vaporization of volatile materials. The cuvette is placed in a chamber having light absorbing surface.

The 90° sample cell geometry between the radiation source and the emission monochromator avoids scattered radiations reaching the detector. Baffles can also be used to avoid scattered radiations reaching the detector.

Sample Cell Geometry :

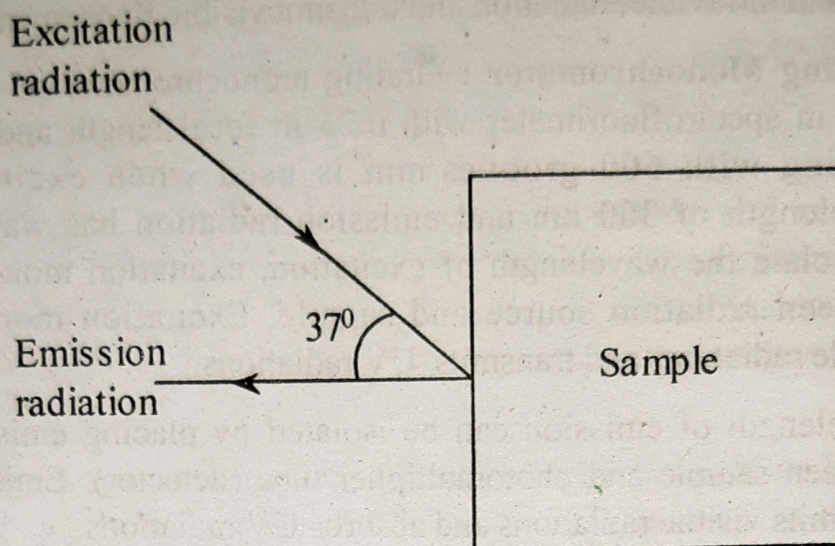
The arrangement between the excitation/radiation source and the emission from the sample refers to sample cell geometry.

Sample cell geometry can be performed in four ways :

1. Frontal (37°) Method
2. Right angle (90°) Method
3. Rotating cell method
4. Straight-through (transmission) method

1. Frontal (37°) Method :

Figure :
Schematic
Representation
of Frontal
Method

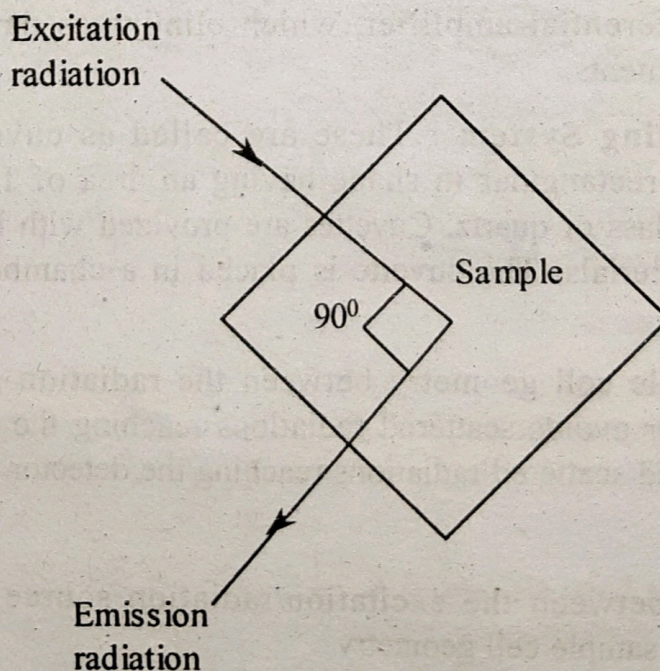


- The angle formed between the excitation radiation and the emission radiation is 37° .
- This method of illumination is used for semi-opaque (translucent) materials, solids or high absorbing solutions.

Disadvantage : Emission monochromator receives fluorescence from cell surface and also the reflected radiations, which are not desired. Reflected radiations can be reduced to some extent by choosing 37° as the take-off angle.

2. Right Angle (90°) Method :

Figure :
Schematic
Representation
of Right Angle
Method

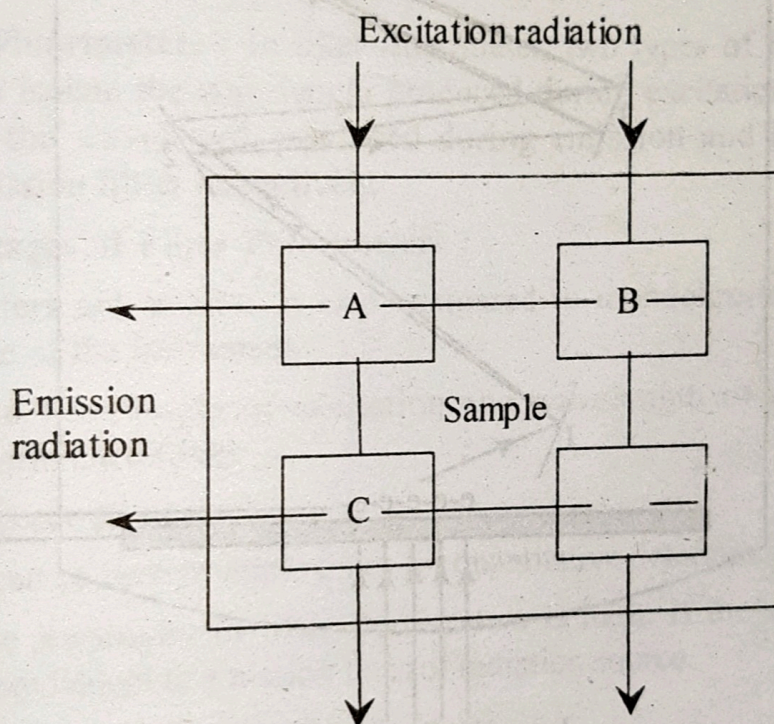


- The angle between the excitation radiation and the emission radiation is 90° .
- The 90° arrangement provides efficient separation of excitation radiations and emission radiations.

- (c) Emission monochromator cannot receive the fluorescent radiation from sample cell surface, as the illumination from sample cell surface cannot be viewed by the emission monochromator. Hence, it avoids fluorescent radiations from the cell and also the reflected radiations.
- (d) Scattered radiations are produced from the bulk of the solution.
- (e) The arrangement of entrance slits and exit slits determines the effective pathlength, where the exciting radiations maintains the linearity between the intensity of fluorescence and solute concentration.
- (f) This method is used for clear or nearly clear solutions having low absorption.

3. Rotating Cell Method :

Figure :
Schematic
Representation
of Rotating Cell
Method



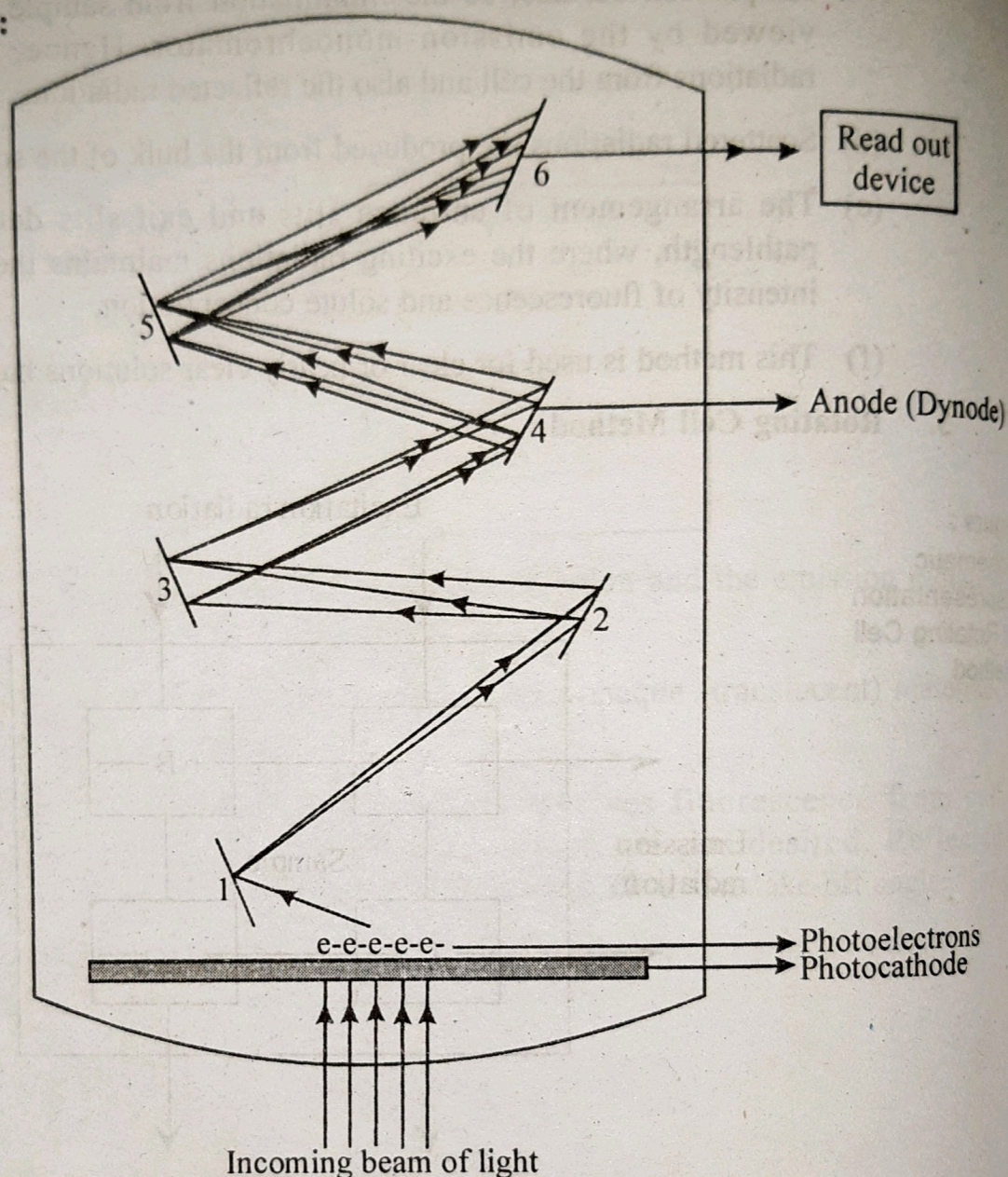
- (a) It is a novel method, in which the cell is rotated in 3 positions. A, B and C to calculate the exact fluorescence.
- (b) The difference of measurement readings of position A and B gives the amount of fluorescent radiations absorbed by the sample.
- (c) The difference of measurement readings of position A and C gives the amount of excitation radiation absorbed by the sample.
- (d) The corrections of the fluorescent radiations give a linear graph between the fluorescence concentration versus absorbances upto 2.7.

4. Straight Through Transmission Method :

- (a) Uranium is fused with $\text{LiF} - \text{Na}_2\text{CO}_3$ to form fusion fluxes.
- (b) Fusion fluxes are cooled to fluorescent pellets which helps in determination of uranium.
- (c) This method is rarely used now.

Detector :**Photomultiplier Tube :**

Figure :
Photomultiplier
Tube



Photomultiplier tube is quite sensitive and can detect extremely weak signals. Therefore, it is used in sophisticated instruments. It should be protected from stray light in order to obtain accurate results.

Construction : It consists of a light sensitive cathode (photocathode) and about 10 anodes (dynodes) which are maintained at a potential of 75 – 100 volts.

Working : It works on the principle of multiplication of the photoelectrons by secondary emission of electrons. When a beam of light falls on the photocathode, photoelectrons are generated which are accelerated towards the anodes. At each stage of acceleration from one anode to another anode, the emission of electrons is increased by a factor of 4 or 5 due to secondary emission of electrons. Thus, several electrons are liberated by each original electron, as a result of secondary emission. Due to this a net factor of 10^6 is achieved from the last electrode.

The detector such as Photomultiplier tube should be covered up to avoid unnecessary radiation and it can detect weak signals.

Types of Fluorimeters :

The instruments used for measurement of fluorescence utilize either filter or grating monochromator. Depending upon the monochromator utilized, the two types of instruments are :

1. Filter Fluorimeters :
 - (a) Single beam filter fluorimeter
 - (b) Double beam filter fluorimeter
2. Spectrofluorimeters :
 - (a) Single beam spectrofluorimeter
 - (b) Double beam spectrofluorimeter

1. **Filter Fluorimeters :** In filter fluorimeter, two types of filters are used : Primary filter to isolate the wavelength produced during excitation and secondary filter to isolate the wavelength produced during emission and hence, called excitation and emission filters respectively.

Advantages of Filter Fluorimeters :

- (i) Filters are smaller in size compared to monochromators, resulting in small size of the instrument.
- (ii) The wavelength of excitation and wavelength of emission are chosen by interference filters.
- (iii) It is economical.
- (iv) It can be used multiple times for quantitative determinations.
- (v) The sensitivity of filter fluorimeters is high, if the wavelength of excitation is equivalent to emission lines of radiation source.

(a) Single Beam Filter Fluorimeter :

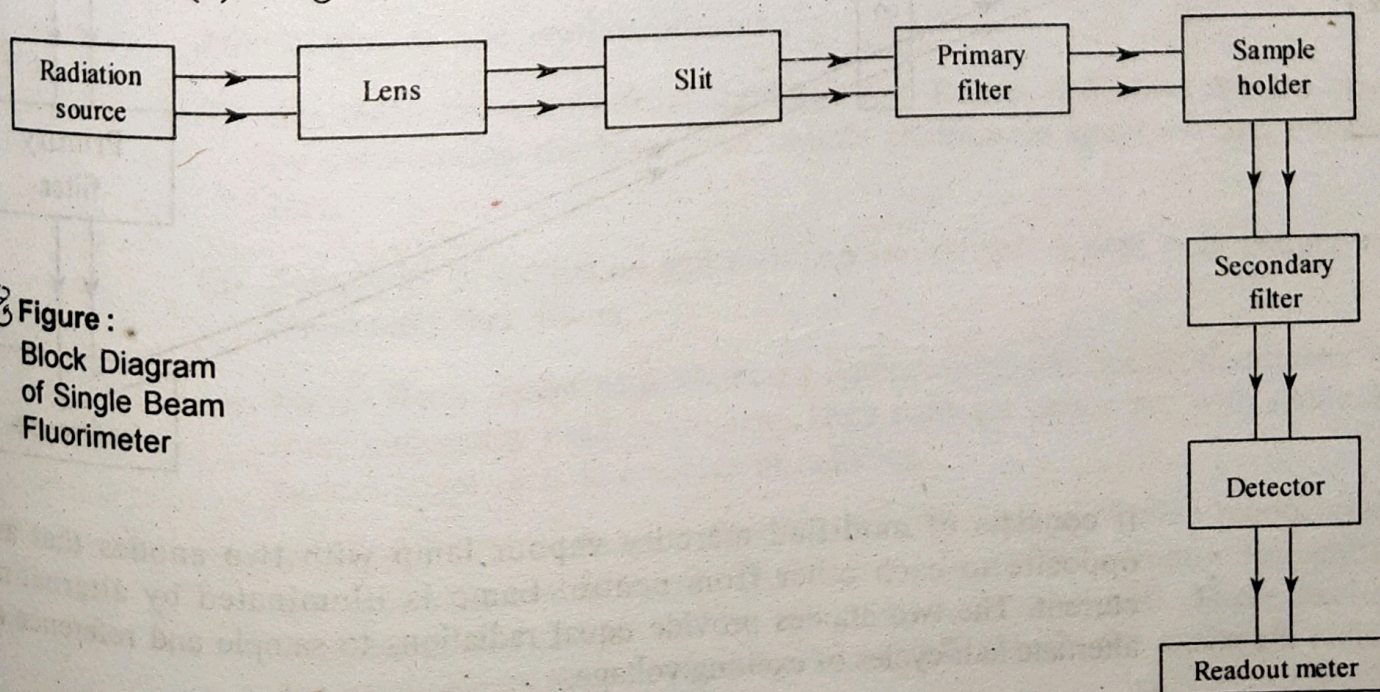


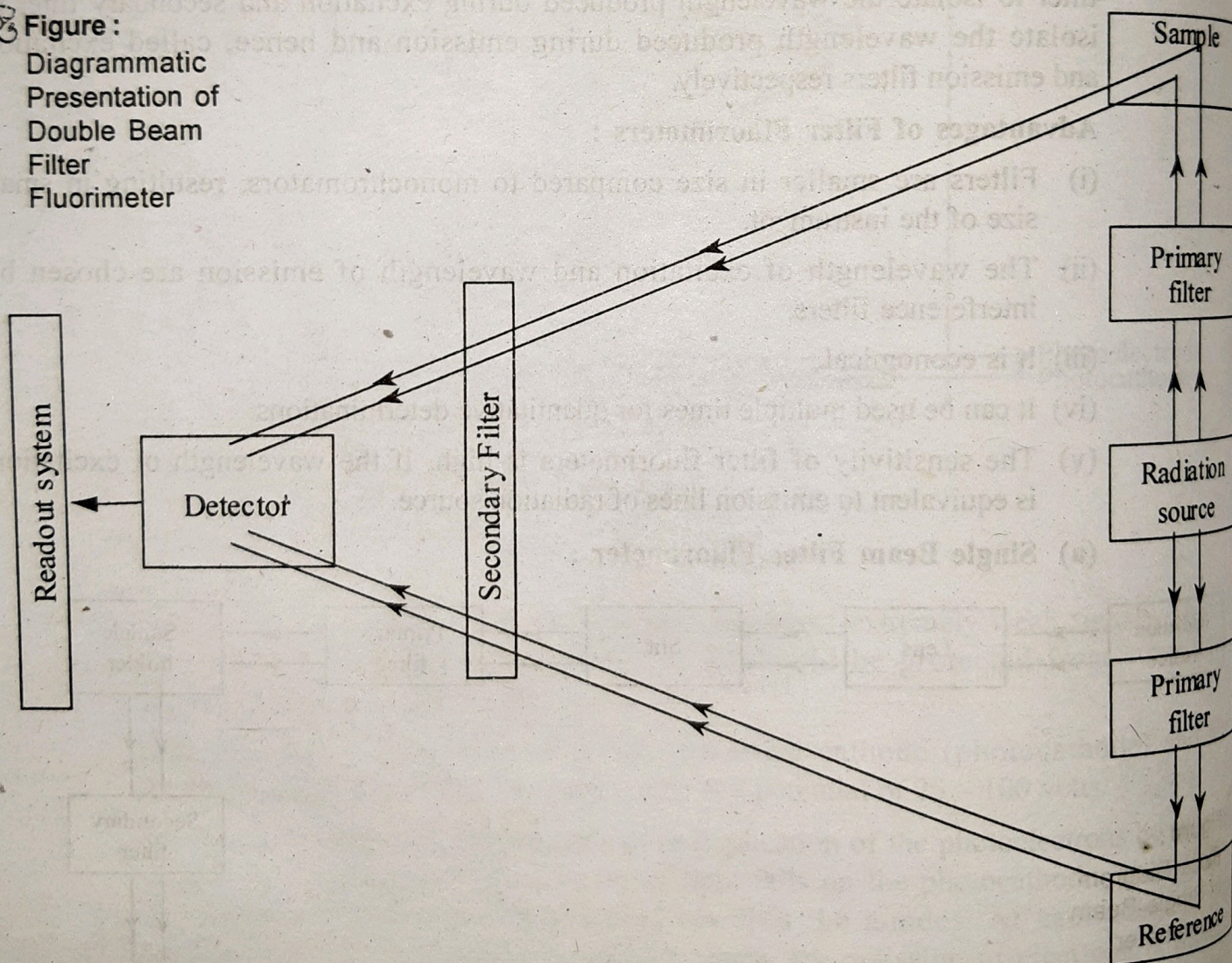
Figure :
Block Diagram
of Single Beam
Fluorimeter

Working : The radiations from radiation source are allowed to pass through the lens and slits to make them parallel. The radiations received by primary filter, transmits UV-radiation and absorbs visible radiations. UV-radiations are excitation radiations which fall on the sample cell.

A secondary filter placed at right angles to sample cell receives emitted fluorescent radiations. It absorbs UV radiations and transmits visible radiations. The 90° geometry of fluorimeter separates wavelength of excitation and wavelength of emission. The fluorescent radiations are then received by a suitable detector (photomultiplier tube). The detector is connected to a suitable readout meter like galvanometer, which reads the fluorescence.

(b) Double Beam Filter Fluorimeter :

Figure :
Diagrammatic
Presentation of
Double Beam
Filter
Fluorimeter



It consists of modified mercury vapour lamp with two anodes that are opposite to each other from centre. Lamp is illuminated by alternating current. The two anodes provide equal radiations to sample and reference alternate half cycles of exciting voltage.

Working : The radiations are passed through primary filters of reference and sample cell. Primary filter absorbs visible radiations and transmit UV radiations to reference cell and sample cell. The radiations from reference cell and sample cell are focussed on a single secondary filter, which absorbs UV radiations and transmit visible radiations.

The transmitted radiations are sent to the detector, connected to suitable readout system. The fluorescent signals obtained from sample and reference cells are compared and analysed. In double beam system, variations in temperature and fluctuations in source and detector can be neglected to some extent.

Calibration of Filter Fluorimeter : Fluorescence of sample is measured with respect to standard.

Fluorimeter reading for standard is first obtained. The standard is then removed and the sample of known concentration is analysed. The fluorimeter readings due to solvent and cuvette are also recorded. The readings are taken without any adjustment of the instrument for each measurement. This helps in determining the fluorimeter's true-zero reading.

A graph of fluorimeter reading on Y-axis and concentration on X-axis is called as calibration curve.

Some known fluorescent standards are rhodamine B in ethylene glycol, tryptophan in water.

2. **Spectrofluorimeters :** In spectrofluorimeters, 2 grating monochromators are present, that have replaced the filters of fluorimeter. The grating monochromators measures the fluorescence in the region of 200 - 800 nm, which is the useful zone of fluorescence technique.

Advantages of Spectrofluorimeters :

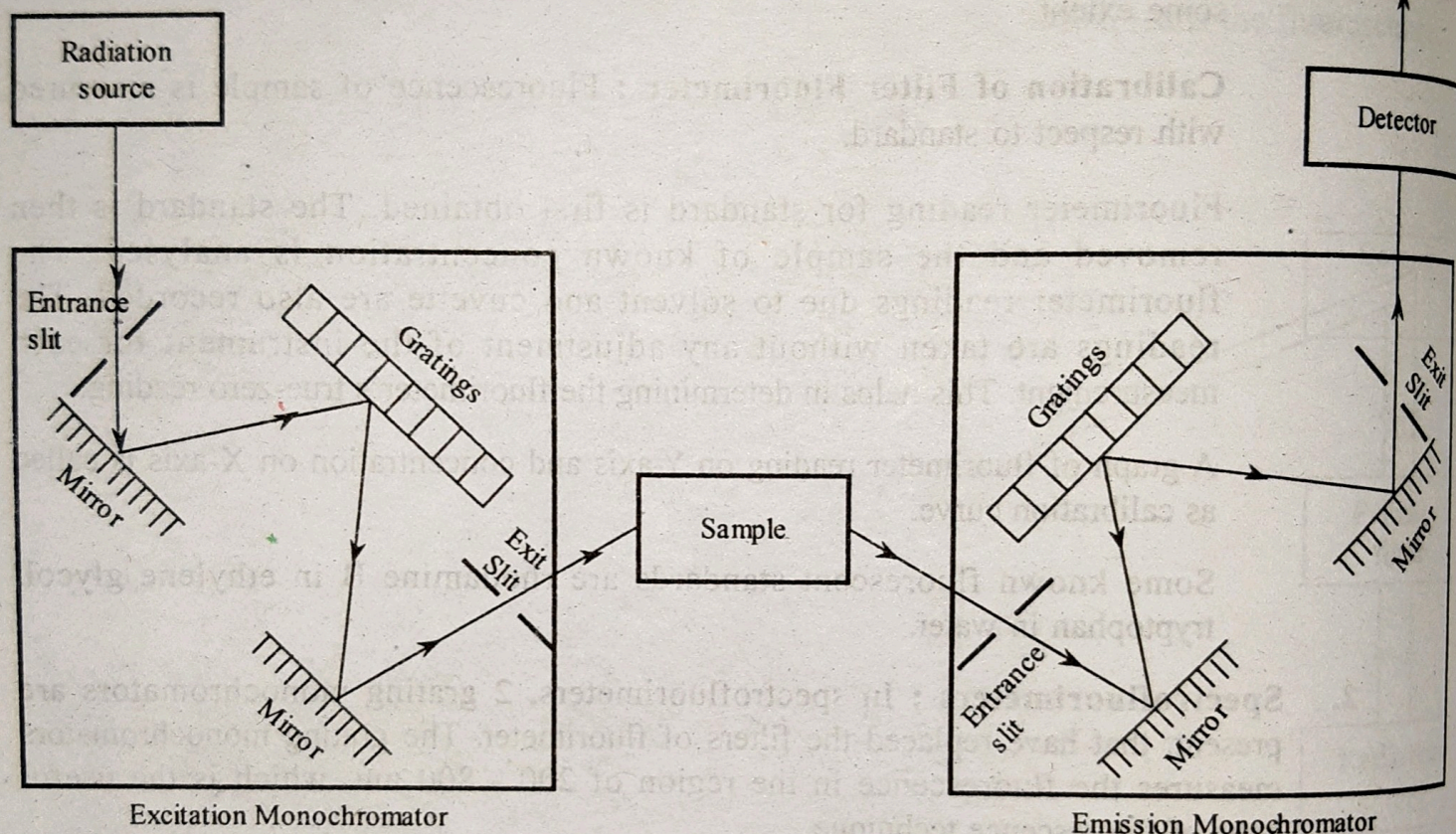
- (i) Scattered radiations from Rayleigh and Raman emission can be avoided by determining the blank and sample fluorescent spectrum and comparing both.
- (ii) Selectivity of excitation and emission wavelength is high with grating monochromators than filters.

- (a) **Single Beam Spectrofluorimeter :** Aminco-Bowman spectrofluorimeter is the most commonly used instrument. High pressure xenon arc with stable direct current supply acts as a source of radiation.

Working : The radiations from source enter into excitation monochromator through slits and mirrors. The excitation monochromator transmits the excitation wavelength of the molecule to be analysed. These excitation radiations enters the sample cell. The sample absorbs the excitation radiations

and emits fluorescent radiations. The radiations emitted from sample are passed to emission monochromator through slits and mirrors, which transmits wavelength of emission, characteristic to that compound. The emitted radiations are passed to the detector through mirror and exit slits. The signal from the detector are then amplified and sent to suitable readout system.

Figure : Diagrammatic Representation of Single Beam Spectrofluorimeter (Aminco-Bowman Spectrofluorimeter)

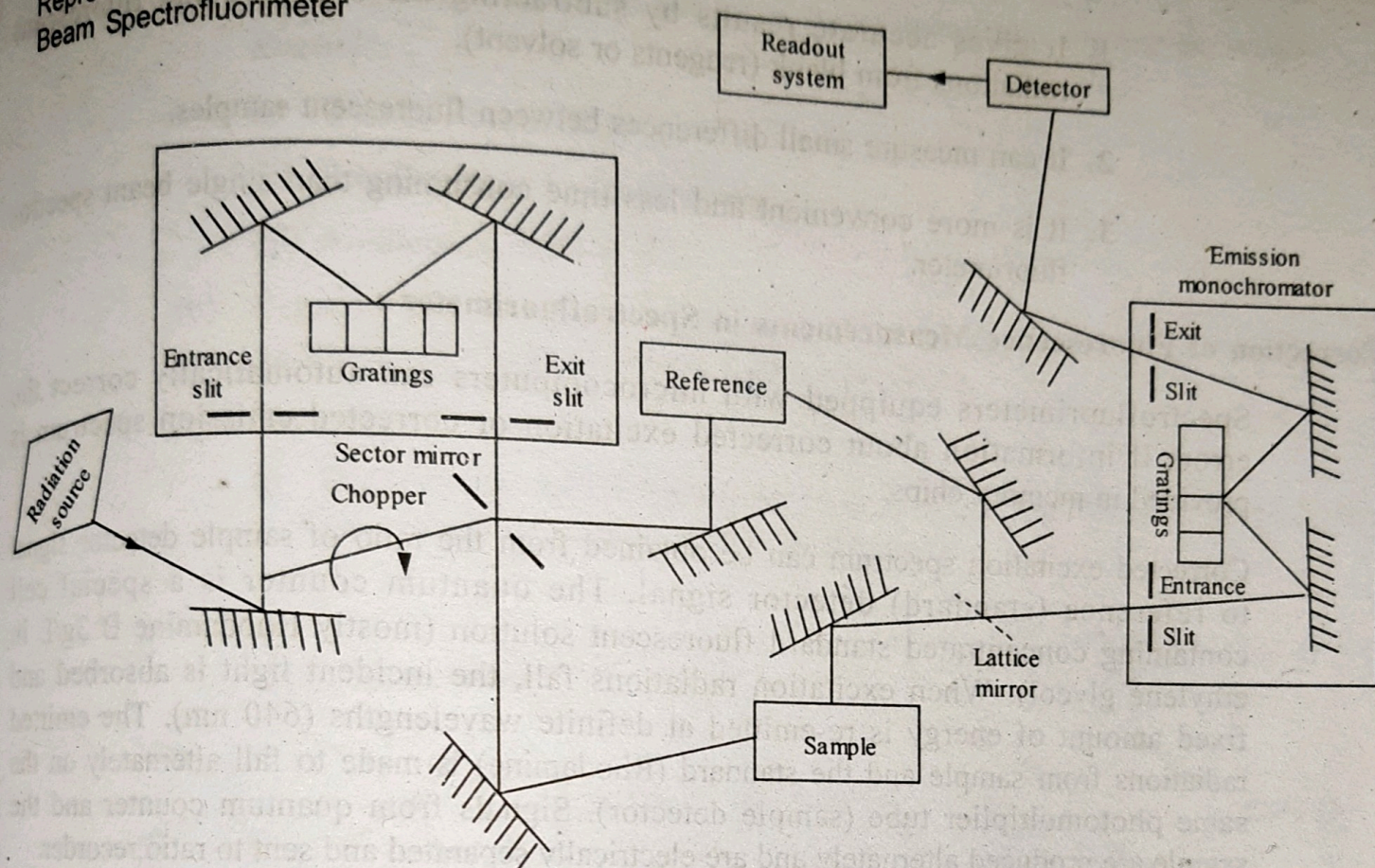


(b) Double Beam Spectrofluorimeter :

Construction : Double beam spectrofluorimeter is an electro optical system that splits radiations from source into reference and sample beams both travelling equivalent optical path. The arc of 150 W xenon lamp acts as a source of excitation radiation. Excitation monochromator (isolates the wavelength of excitation) and emission monochromator (isolates the wavelength of emission) are present. An optical chopper and sector mirror are present which transmits light to reference and sample position alternatively.

In double beam spectrofluorimeter, the chopper splits the excitation radiation from source. It creates alternate current signals by blocking the radiations to reference cell and transmitting to sample cell and vice versa. The radiation from emission monochromator is then sent to the detector. The signal from detector is read in a suitable readout system. It also consists of slits and mirrors which are used to transmit and reflect the radiations.

Figure : Diagrammatic Representation of Double Beam Spectrofluorimeter



Working : The excitation radiations which are produced from the arc of 150 W xenon lamp are chopped by bow-tie shaped disc i.e., chopper. Through the entrance slit, these radiations fall on the excitation monochromator.

A sector mirror is present after the exit slit of excitation monochromator. Sector mirror and chopper are rotated by a single common shaft. During first rotation of chopper, sector mirror reflects the beam to reference cell. During second rotation of the chopper, sector mirror opens and radiations fall on sample cell. In each revolution of chopper, the opaque portion of the disc is measured as zero. Lattice mirror reflects the sample beam (emission from the sample cell) or transmits the reference beam (emission from the reference cell) to emission monochromator by lattice mirror.

The radiations emitted from sample and reference cells are focused one after the other on the entrance slit of emission monochromator from front-surface mirrors. In fluorimetry, emission radiations and excitation radiations are obtained perpendicularly to avoid scattering of radiations. Reference and sample beams are focussed on photomultiplier tubes (i.e., detector) by passing through exit slits of emission monochromator. The signal from photomultiplier tube is measured in a photometer and recorded using suitable readout system.

Advantages :

1. It gives accurate results by subtracting the scattered and fluorescence radiations from blank (reagents or solvent).
2. It can measure small differences between fluorescent samples.
3. It is more convenient and less time consuming than single beam spectrofluorimeter.

Correction of Fluorescence Measurements in Spectrofluorimeter :

Spectrofluorimeters equipped with microcomputers can automatically correct the errors if information about corrected excitation or corrected emission spectrum is provided in memory chips.

Corrected excitation spectrum can be obtained from the ratio of sample detector signal to reference (standard) detector signal. The quantum counter is a special cell containing concentrated standard fluorescent solution (mostly Rhodamine B 3g/L in ethylene glycol). When excitation radiations fall, the incident light is absorbed and fixed amount of energy is re-emitted at definite wavelengths (640 nm). The emitted radiations from sample and the standard (Rhodamine) is made to fall alternately on the same photomultiplier tube (sample detector). Signals from quantum counter and the sample are produced alternately and are electrically separated and sent to ratio recorder.

The emission radiations from quantum counter are proportional to the amount of light falling on the sample. This gives the corrected excitation spectrum.

Advantages of Corrected Fluorescence Measurement in Spectrofluorimeter :

1. It is helpful in inter and intramolecular energy transfer studies.
2. Energy of Stoke's shift (variation of energy with wavelength of excitation and wavelength of emission) can be known.
3. Study of macromolecules.
4. It is useful in analysis of trace elements where corrected fluorescence excitaton spectrum's sensitivity is 1000 times greater than absorption spectrum.

2.8 APPLICATIONS

The important applications of Fluorimetry are found in the analysis of food products, pharmaceuticals, clinical samples and natural products due to its sensitivity and selectivity.

Applications of fluorimetry can be seen in the following fields :

Analysis of Medicinal Compounds :

A number of drugs can be estimated using fluorimetry.

Fluorescence is made to produce from non-fluorogenic drugs by following ways :

- (a) Some drugs are capable of exhibiting fluorescence in an appropriate solvent.

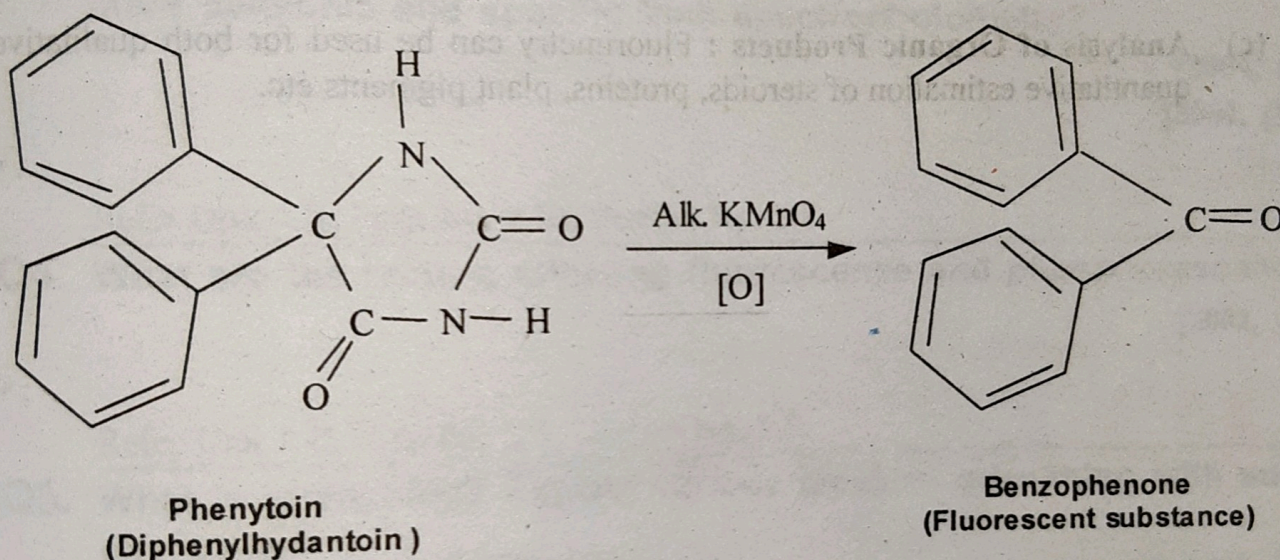
Examples :

- (i) Quinine in 0.1 N H_2SO_4
- (ii) Riboflavine in 1% tartaric acid
- (iii) Aminocrine in 0.1 N HCl.

- (b) Organic and inorganic compounds can be made fluorogenic (fluorescent substance) by chemical change such as oxidation.

Examples :

- (i) Diphenylhydantoin (phenytoin) is oxidised by alkaline KMnO_4 to form benzophenone, which exhibits fluorescence.



- (c) Organic and Inorganic compounds are complexed with suitable reagents to make them fluorogenic.
- (d) When two or more drugs are present, each drug can be estimated individually by adopting suitable methods like :
 - (i) Conversion of acidic to alkaline solution or vice versa.
 - (ii) Conversion of ionic to non-ionic compound or vice versa.
 - (iii) Selection of wavelength of excitation for each drug.
 - (iv) Extraction of any one drug from the mixture and analysing it.
- (e) Preparation of fluorogenic derivative from non-fluorogenic drug.

Examples :

- (i) Complex of atropine with eosin is soluble in chloroform and exhibit fluorescence.
- (ii) Other non-fluorogenic drugs which can be analysed are morphine and codeine.

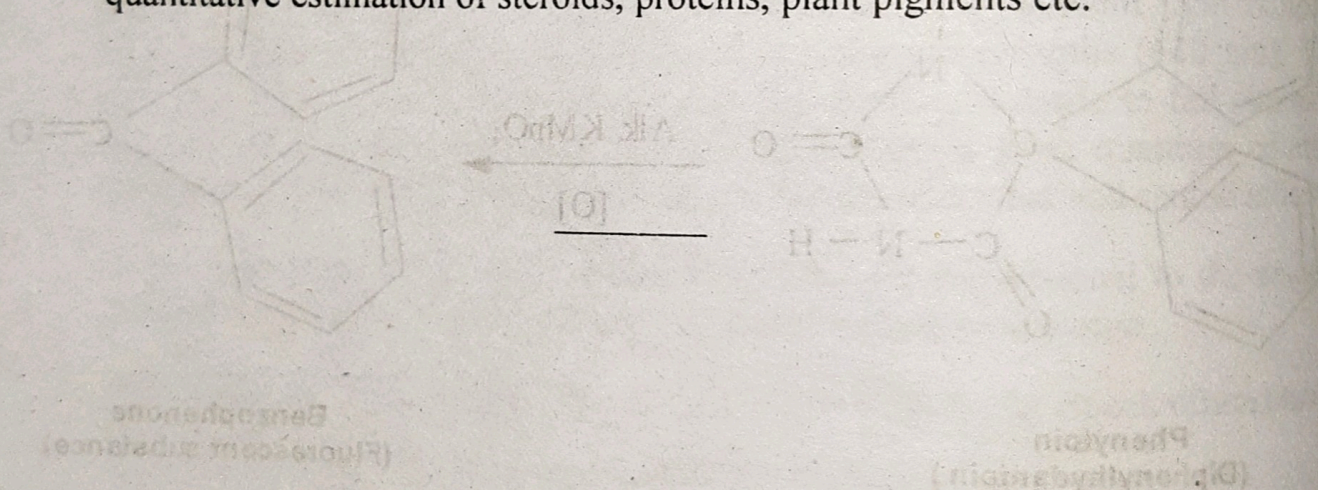
2.26 [Unit - II]

Analysis of Inorganic Compounds :

- (a) Estimation of Uranium in Salts.
- (b) Some non-fluorescent inorganic ions can be made fluorescent by complexing with non-fluorescent organic reagents. Hence, these elements can be fluorimetrically analysed.

Miscellaneous Applications :

- (a) **Measurement of Fluorescence by Fluorescent Indicators :** Fluorescent indicators are useful in fluorimetric determinations as the colour and intensity of fluorescence depends upon pH of the solution.
- (b) **Determination of Chemical Structure :** Hydrogen bonding, geometrical isomerism, polymerisation, tautomerism and reaction rates can be studied fluorimetrically.
- (c) **Analysis of Organic Products :** Fluorimetry can be used for both qualitative and quantitative estimation of steroids, proteins, plant pigments etc.



FREQUENTLY ASKED QUESTIONS

Q1. What is fluorescence and phosphorescence? Explain the concept of fluorescence through energy level diagrams?

[April, 2006, (5 M)]

[2001, (5 M)]

Answer :

Refer Unit – II, Page No. 2.2, Section No. 2.2.

Q2. What is quenching? List any two types of quenching?

[April, 2006, (2 M)]

Answer :

Refer Unit – II, Page No. 2.9, Section No. 2.6.

Q3. What are pharmaceutical applications of fluorimetry? How is fluorimetry more sensitive and specific than spectrophotometry?

[2004, (5 M)]

[2001, (10 M)]

Answer :

Refer Unit – II, Page No. 2.24, Section No. 2.8.

Q4. What are the factors affecting fluorescence and phosphorescence?

[2001, (5 M)]

Answer :

Refer Unit – II, Page No. 2.5, Section No. 2.5.

Q5. What is quenching? Explain various types of quenching with suitable examples?

[April, 2006, (5 M)]

[October, 2005, (5 M)]

[November, 2004, (5 M)]

[November, 2003, (5 M)]

Answer :

Refer Unit – II, Page No. 2.9, Section No. 2.6.

Q6. What is fluorescence? What are the factors affecting fluorescence intensity?

[October, 2005, (5 M)]

[2004, (5 M)]

[2005, (5 M)]

[November, 2003, (5 M)]

Answer :

Refer Unit – II, Page Nos. 2.2 and 2.5, Section Nos. 2.2 and 2.5.

2.28 [Unit – II]

Q7. Explain the theory, Instrumentation and Applications of spectro-fluorimetry?

[November, 2003, (10 M)]

Answer :

Refer Unit – II, Page No. 2.12, Section Nos. 2.7 and 2.8.

Q8. How are the primary and secondary filters selected in fluorimetry assay?

[2004, (2 M)]

Answer :

Refer Unit – II, Page No. 2.19, Section No. 2.7.

Q9. Show the relationship between fluorescence intensity and concentration. Describe any four factors that influence fluorescence intensity?

[November, 2002, (5 M)]

Answer :

Refer Unit – II, Page No. 2.5, Section No. 2.5.

Q10. What are “self quenching” and “true quenching”?

[November, 2002, (2 M)]

Answer :

Refer Unit – II, Page No. 2.9, Section No. 2.6.

Q11. Discuss the phenomenon of fluorescence. Explain the working of fluorimeter with suitable diagram?

[October, 2007, (10 M)]

Answer :

Refer Unit – II, Page Nos. 2.2 and 2.21, Section Nos. 2.2, 2.7.