UNIT 5 FLUORIMETRY AND PHOSPHORIMETRY

Structure

5.1 INTRODUCTION

In the previous units, you have studied about UV-VIS, IR and Raman spectrometric methods. These methods were based on the absorption of radiation in UV-visible and IR regions and on the scattering of radiation. You have learnt that the absorption of electromagnetic radiation in the UV-visible region leads to an electronic excitation of the absorbing species whereas in case of IR radiations the transitions occur in the vibrational energy levels. In Raman spectrometry, on the other hand, we measure the scattered radiation arising as a consequence of elastic and inelastic collisions of radiation with matter.

In this unit, you would learn about two more molecular spectroscopic methods, namely fluorescence and phosphorescence. These are based on the phenomenon of emission of radiation by electronically excited species and are collectively termed as photoluminescence methods. We shall begin with the understanding of the origin of fluorescence and phosphorescence spectra in terms of the radiative relaxation processes associated with electronically excited species. It will be followed by the study of the relationship of fluorescence and phosphorescence with chemical structure and the dependence of the spectra on the environment of the species. A brief account of the essential components and the principles of the spectrofluorimeters is also given. Thereafter, some simple applications based on measurement of fluorescence and phosphorescence spectra have been discussed in brief. In the next unit, we shall take up the applications of fluorescence and phosphorescence measurements in detail.

Objectives

After studying this unit, you should be able to:

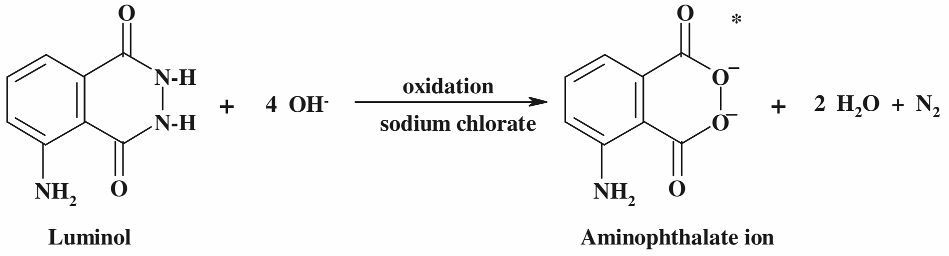
- explain the origin of fluorescence and phosphorescence spectra,
- differentiate between fluorescence and phosphorescence,
- define and differentiate between radiative and nonradiative relaxation mechanisms,
- relate fluorescence and phosphorescence emission to the chemical structure,
- compare fluorescence and phosphorescence measurements with absorption measurements,
- explain fluorescence quenching and state reasons for the same,
- describe briefly the components of fluorescence and phosphorescence instruments,
- explain the principles of different types of instruments used for the measurement of fluorescence and phosphorescence, and
- outline analytical applications of fluorescence and phosphorescence.

5.2 ORIGIN OF FLUORESCENCE AND PHOSPHORESCENCE SPECTRA

You would recall from Unit 2 that when a radiation in the UV-visible region interacts with a molecule, it causes transition among the quantised electronic energy levels. Have you ever thought what happens to the excited molecules so obtained? These relax back to the ground state by using different processes. In some of the processes the absorbed energy is given off as heat to the surroundings and in some it is emitted as radiation. When such a relaxation is accompanied by the emission of a radiation it is called **luminescence**. There are two types of luminescence phenomena namely, **fluorescence** and **phosphorescence** that arise from the excited state generated by absorption of electromagnetic radiation. Fluorescence is the emission of visible light by a substance that has absorbed light of a different wavelength. The emitted photon has a longer wavelength. Phosphorescence is related to fluorescence in emitting a photon, however, a phosphorescent material does not immediately re-emit the radiation it absorbs. As the excitation of the molecule is due to the absorption of a photon (light), these types of luminescence are called **photoluminescence.**

The term 'fluorescence' was coined by G. G. Stokes in 1852 on the name of the mineral fluorspar (CaF_2) that emits visible light on illumination with the UV light.

Chemiluminescence is another phenomenon that falls in the category of luminescence. This refers to the emission of radiation during a chemical reaction. However, in such cases the excited state is not a result of absorption of electromagnetic radiation. The oxidation of luminol (3-aminophthalhydrazide) in an alkaline solution is an example of chemiluminescence. An aqueous solution of luminol is oxidised by a solution of sodium chlorate (commercially available bleaching powder) and gives out a blue glow without increase in temperature. Luminol is oxidised to aminophthalate ion which is produced in an excited state and emits blue light on dropping to the ground state. The reaction can be written as below.



The processes involved in the excitation by radiation and the radiative emissions can be understood with the help of **Jablonski diagram**. Let us learn about Jablonski diagram and see how it explains the phenomena of fluorescence and phosphorescence.

5.2.1 Jablonski Diagram

The Jablonski diagram gives a representation of ground and different excited electronic states of a molecule and the processes associated with absorption and emission (radiative and nonradiative) of energy. A typical Jablonski diagram is given in Fig. 5.1.

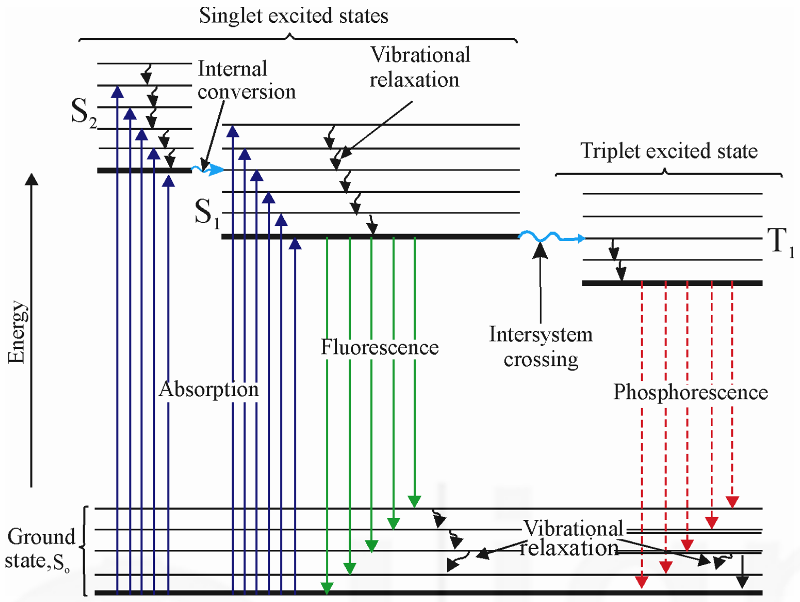


Fig. 5.1: The Jablonski diagram showing the phenomena of fluorescence and phosphorescence

The set of lines at the bottom of the figure represents the ground state and the ones in the upper portion indicate the excited electronic states. To begin with, the molecule is in the electronic ground state. In this state, the molecular orbitals are occupied by two electrons. You would recall from your earlier knowledge that according to Pauli's principle, the spins of the two electrons in the same orbital must be antiparallel. This implies that the total spin, **S,** of the molecule in the ground state is zero [½ + (– ½)]. This energy state is called "**singlet state**" and is labeled as **S_0**. The electron spins in the excited state achieved by absorption of radiation may either be parallel or antiparallel. Accordingly, this may be a **triplet** (**parallel**) or a **singlet** (**antiparallel**) **state**. These are designated as **T_1** or **S_1,** respectively as the case may be. The spin states are depicted in Fig. 5.2.

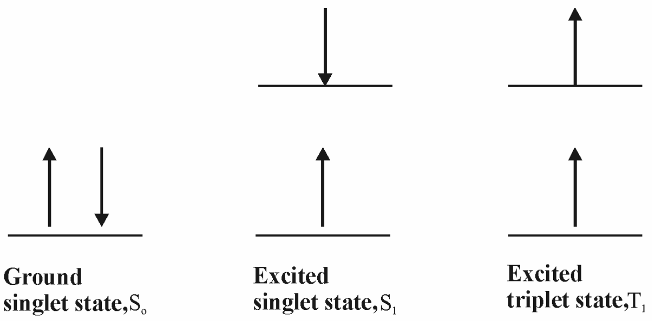


Fig. 5.2: Possible electronic spin states of the molecules

The energies of the two excited spin states are also different due to the difference in the interactions between electrons; the energy of the triplet state usually being lower than that of the singlet state. In the Jablonski diagram two excited singlet states (S_1 and S_2) and a triplet state (T_1) are shown. The absorption and emission of the radiation leads to the activation and deactivation processes. Let us learn about the way activation (by absorption of radiation) and deactivation processes take place.

5.2.2 Activation

The absorption of a photon of suitable energy causes the molecule to get excited from the ground state to one of the excited states. This process is called as **excitation** or **activation** and is governed by **Franck-Condon principle**. According to this principle, the electronic transition takes place so fast ($\sim10^{-15}$ s) that the molecule does not get an opportunity to execute a vibration, i.e., when the electrons are excited the internuclear distance does not change. The basis for the principle is that the nuclei are very massive as compared to the electrons and therefore move very slowly.

The excitation to a triplet state requires a reversal of the electron spin and such a transition is forbidden by quantum theory. Accordingly, the probability of such a transition is very low.

The implication of Frank -Condon principle is that the transition from the ground state to excited state can be represented by vertical arrows in the Jablonski diagram. The electronic transition takes place from the state of lowest vibrational energy of an electronic ground state to any of the vibrational levels of the excited electronic states as shown by the vertical upward arrows in the diagram. The excited state achieved would depend on the energy of the photon absorbed. In the diagram the two excited states as S_1 and S_2 have been shown. These are obtained by the absorption of the radiation in the region of wavelength λ_1 and λ_2 respectively. Once the molecule gets excited by the absorption of radiation, it does not stay excited indefinitely; a number of processes bring it back to the ground state. This is called **deactivation**. Let us learn about different means of deactivation of excited states.

5.2.3 Deactivation

Once excited, the molecule can undergo a number of relaxation processes during the time it spends in the excited state. The deactivation processes can be broadly categorised into two groups given below.

- Nonradiative deactivation
- Radiative deactivation

Let us learn about these.

- **Nonradiative Deactivation**

As the name suggests, these deactivation mechanisms are not accompanied by any radiative loss. There are three different nonradiative means of deactivation. These are described in the following paragraphs.

Vibrational Relaxation

In the higher vibrational levels of an excited state, the molecule rapidly loses (in < 10^{-12} s) its excess vibrational energy by collision with other molecules and falls to the lowest vibrational level of the excited state. In this nonradiative mode of relaxation, called **vibrational relaxation**, the energy lost is dissipated as heat to the surroundings. In principle, the nonradiative or radiationless deactivation may also lead to the relaxation of the S_1 state to S_0 state. However, such a relaxation is quite slow.

Internal Conversion

Once the molecules that are excited to an electronic state (say S_2) higher than the S_1 state reach the vibrational ground state in the electronic level, these can pass to a higher vibrational level of a lower excited state (S_1) which has the same energy. This process is referred to as **internal conversion**. The molecule can continue to lose energy in this state in a nonradiative way (vibrational relaxation) until it reaches the lowest vibrational level in this excited state.

Intersystem Crossing

In rare occasions, the molecule in the vibrational states of a singlet excited state may cross over to a vibrational level of a triplet state if the two have same energy. This process is called **intersystem crossing.** This **spin-exchange** mechanism may also lead, though very rarely, to the crossing over from a triplet state to a singlet state.

- **Radiative Deactivation**

When the molecule in the excited state (S_1) relaxes down to the lowest vibrational level it may emit a photon and come down to the electronic ground state (S_0). This process is called **fluorescence** and takes about 10^{-9} s. Another radiative relaxation process can arise from the excited molecule that had crossed over to the triplet excited state by intersystem crossing and has relaxed to the vibrational ground state in the triplet excited state. In such a case the molecule emits a photon and comes down to a vibrational mode of the electronic ground state, S_0. This phenomenon is called **phosphorescence**. As the transition from a triplet state to a singlet state is theoretically forbidden, it does not take place readily. Thus, while the fluorescence emission can take place within $10^{-9} - 10^{-6}$ seconds, the transition from an excited triplet state to the ground state in case of phosphorescence requires at least 10^{-4} seconds and may take as long as 10^{2} seconds.

In fluorescence, the spin multiplicities of the ground and emissive excited states are the same whereas for phosphorescence, the excited and ground states have different spin multiplicities.

Since the fluorescence emission takes place only after the excited molecule has relaxed to the vibrational ground state of the S_1 state i.e., after having lost part of its energy, the energy of the emitted radiation is lower than that of the excitation radiation. This means that the wavelength of fluorescence emission would be greater than the excitation wavelength. Further, since the energy of the triplet excited state of the molecule is lower than that of the associated singlet state; the transitions to the ground state are associated with the emission of light of still lower energy. As a consequence the phosphorescence occurs at longer wavelengths than fluorescence. Fig. 5.2 shows the excitation, fluorescence and phosphorescence spectra of phenanthrene

.

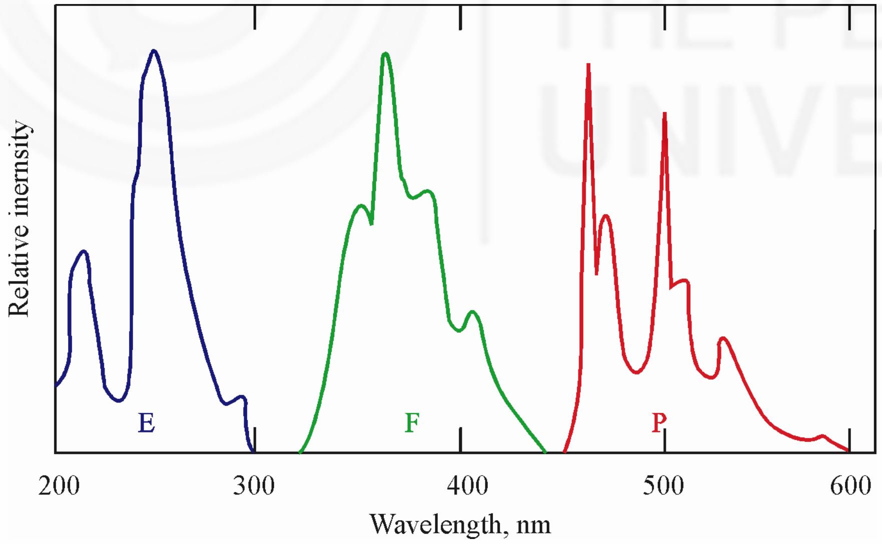


Fig. 5.3: The excitation (E), fluorescence (F) and phosphorescence (P) spectra of phenanthrene

The excited molecules when reach a triplet state, spend relatively long time in that state. Since they are at the triplet state for a long time, they can lose their energy by means other than by emission of photon i.e., they are very susceptible to collisions with the solvent molecules. Therefore, phosphorescence in solution at room temperature is a rare phenomenon. However, the phosphorescence can be observed by decreasing the temperature of the sample whereby decreasing the collisions with the solvent molecules. In addition, the quenching of the triplet excited state by oxygen is also responsible in preventing phosphorescence, and therefore the solution of the analyte needs to be thoroughly degassed before determination of its phosphorescence.

SAQ 1

Which of the statements that follow the following incomplete statements can be used to correctly complete it?

a) Fluorescence occurs when ………………..

  i) a molecule returns to the electronic ground state from an excited triplet state by losing its excess energy as a photon.

  ii) a molecule returns to the electronic ground state from an excited singlet state by losing its excess energy as a photon.

  iii) a molecule lowers its vibrational energy by losing its excess energy as a photon.

b) Intersystem crossing refers to…………………..

  i) the reversal of the spin of an excited electron, changing the state of the molecule (from singlet state to triplet state or vice – versa).

  ii) the loss of excess energy by the molecule by emitting a photon.

  iii) the conversion of the excess electronic energy by the molecule to vibrational energy.

5.3 FLUORESCENCE SPECTRUM

You have learnt that the fluorescence arises when the electronically excited molecule relaxes back from S_1 state to S_0 state accompanied by an emission of radiation. You would have noticed from Fig. 5.1 that when this radiative relaxation takes place, the molecule can come to any of the vibration levels of S_0 state. This implies that the emitted radiation is constituted of different wavelengths. A plot of the emitted radiation as a function of wavelength for any given excitation wavelength is known as the **emission fluorescence spectrum**. In Fig. 5.1, the excitation radiation is shown to have caused the transition from S_0 to S_2 state. What do you think would happen to the fluorescence emission if we change the wavelength of the excitation radiation?

Yes, you thought it right; the emission spectrum would still remain the same because the fluorescence emission generally occurs only from the first excited singlet state irrespective of the excited singlet state produced initially. You would observe in Fig. 5.1 that the fluorescence emission remains the same for both the excitations viz., $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$. However, the intensity of the emission would be expected to change because the excitation radiation is absorbed to different extents (recall the UV-VIS spectra). Now if we change the wavelength of the exciting light and plot the emission from the sample at a given emission wavelength against the wavelength of

If the intensity of exciting light is kept constant as its wavelength is changed, the plot of emission against exciting wavelength is known as the corrected excitation spectrum.

exciting radiation, the result is known as the **excitation spectrum**. The emission fluorescence and excitation spectra of 9-methylanthracene is given in Fig. 5.4.

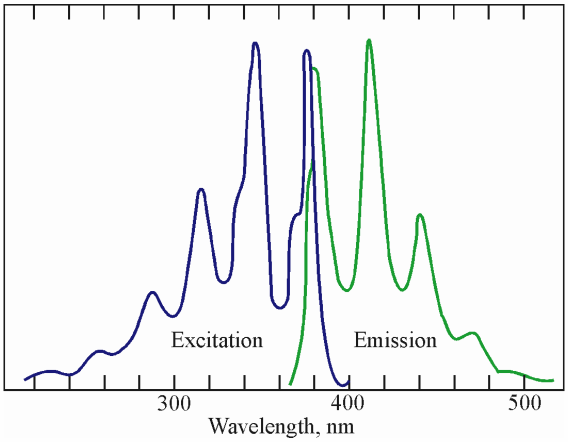


Fig. 5.4: The excitation and emission fluorescence spectra of 9-methylanthracene

Since a given analyte can fluoresce only after it has absorbed radiation, an excitation spectrum consists of the wavelengths of light that the analyte is able to absorb. In other words generally the excitation spectrum of a molecule is the same as its UV-VIS absorption spectrum. Therefore, the excitation spectrum for a compound should not change if we change the emission wavelength that is being measured. Thus, if the excitation spectrum varies with choice of emission wavelength, we can say that the observed fluorescence is arising from two or more different substances. This fact may be exploited to check for the purity of the fluorescent analyte. Generally, the maximum in the fluorescence spectrum of a compound occurs at longer wavelength than the maximum in the absorption spectrum. The wavelength difference between the absorption and fluorescence maxima is called the **Stokes shift**.

You have so far learnt about the phenomenon of fluorescence and phosphorescence and their origin in terms of the excitation and emission processes. A question arises, do all the molecules show these phenomena or not? Is there any correlation between the molecular structure and the fluorescence or phosphorescence behaviour? Let us find answers to these questions in the next section. You can test your understanding of the origin of fluorescence by answering the following SAQ.

SAQ 2

Why does the fluorescence occur at longer wavelengths than the absorption?

……………………………………………………………………………………………...

……………………………………………………………………………………………...

……………………………………………………………………………………………...

……………………………………………………………………………………………...

……………………………………………………………………………………………...

5.4 FLUORESCENT AND PHOSPHORESCENT SPECIES

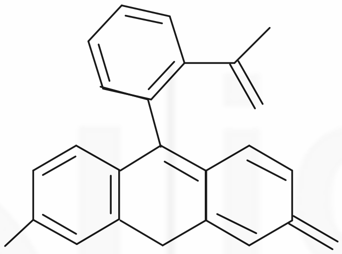
Molecular structure is one of the important parameters in determining the possibility and extent of fluorescence in a chemical species. In principle, every molecule has an inherent capability to show fluorescence, however, in reality only a few molecules actually do so. Besides structure, the fluorescent behaviour of a molecule is affected by the chemical environment in which it exists. Similarly the phosphorescence is also shown by a few species and there is a kind of correlation with the structure and environment. You know the phenomena of fluorescence and phosphorescence together are termed photoluminescence.

Let us learn what type of molecules are photoluminiscent and how does the photoluminescence depend on the environment of the photoluminiscent species.

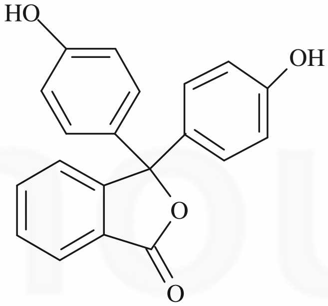
5.4.1 Photoluminescence and Structure

We will first explain the relationship between the structure and the characteristic of fluorescence of a molecule. There is no specific requirement in a molecule that makes it fluorescent. However, there are certain common observations. For example, the presence of the benzene ring and the nature of substituents on it seem to favour the fluorescent behaviour of the molecule. The halogen substituents tend to decrease the fluorescence and shift the fluorescence bands to longer wavelengths; the effects increase with increase in the atomic mass of the substituted halogen.

Compounds with fused ring are found to be especially fluorescent, and the extent of fluorescence is found to be directly proportional to the number of rings in the molecule. The structural rigidity in a molecule favours fluorescence. For example, fluorescein is a highly fluorescent molecule, while the structurally similar phenolphthalein is essentially nonfluorescent.

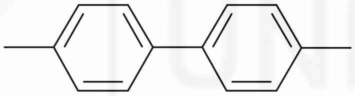


Fluorescein

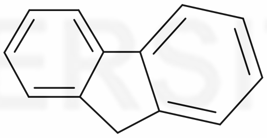


Phenolphthalein

The increase in fluorescence is due to the lack of collisions in the rigid molecules. Another example is biphenyl which has a low fluorescence quantum efficiency i.e. ~ 0.2 while fluorene with a rigid structure has a quantum efficiency almost equal to 1.

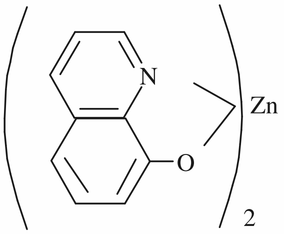


Biphenyl



Fluorene

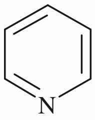
Complex formation also leads to the rigidity in molecules and shows higher fluorescence. For example, 8-hydroxyquinoline when complexed with zinc ions increases its fluorescence to a good extent.



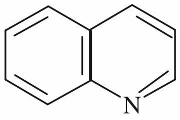
8-Hydroxyquinoline complexed with zinc ions

The fluorescence observed with rigid cyclic molecules with π-bonds is found to be enhanced by electron donating groups e.g., $-NH_2$, OR, – OH and $-OCH_3$, while the electron withdrawing groups such as COOH, NO_2, N=N and Br, I and CH_2COOH tend to reduce it. On the other hand the nonrigid molecules do not fluoresce much, as these rapidly lose the absorbed energy through nonradiative means like, vibrational relaxation or even degradation. The substituents on the aromatic ring may affect both, the intensity and the position of the fluorescence band.

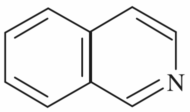
Another observation is that the simple heterocyclic compounds like pyridine, furan, thiophene and pyrrole do not fluoresce significantly, but when fused to a benzene ring, they may become highly fluorescent. For example, the heterocyclic pyridine is nonfluorescent, but quinoline and isoquinoline in which the pyridine ring is fused to a benzene ring are highly fluorescent.



Pyridine



Quinoline



Isoquinoline

Aliphatic and alicyclic carbonyl compounds or highly conjugated double bond structures also show fluorescence.

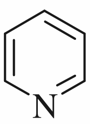
Despite the above mentioned categories of molecules, the number of molecules having intrinsic fluorescence capabilities is very small. However this does not limit the applications of the fluorescence measurements. The applicability of fluorimetry can be extended by derivatising the compounds that do not exhibit intrinsic fluorescence to make fluorescent derivatives. Another possibility is that the nonfluorescent molecule may be chemically attached with a **fluorescent tag** or **label**.

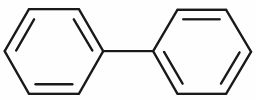
Let us now understand how structure of a molecule affects its characteristic of phosphorescence. As regards phosphorescence, it has been observed that the introduction of certain paramagnetic metal ions such as copper and nickel give rise to phosphorescence. These ions do not induce fluorescence, on the contrary Mg and Zn compounds show strong fluorescence. Phosphorescence is affected by the molecular structure such as unsubstituted cyclic and polycyclic hydrocarbons and those containing $-CH_3$, $-NH_2$, $-OH_2$, –COOH, $-OCH_3$ substituents which have lifetimes in the range of 5–10 seconds for benzene derivatives and 1– 4 seconds for naphthalene derivatives. The introduction of a nitro group (NO_2) in a structure diminishes the intensity of phosphorescence, as does the introduction of aldehyde and ketonic carbonyl groups. The emission life time (τ) is in seconds in rigid media and is 10^{-2} –100 seconds in fluid media.

Metal ions having unpaired electrons show paramagnetic behaviour.

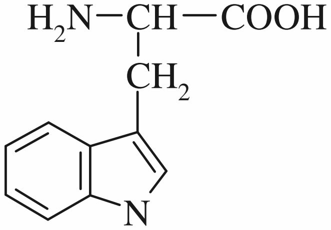
SAQ 3

Which of the following molecules are expected to be fluorescent?

i) 

ii) 

iii)



5.5 FACTORS AFFECTING FLUORESCENCE AND PHOSPHORESCENCE

The fluorescence spectrum and intensity of a molecule often depend strongly on the molecule's environment. The common factors affecting the fluorescence are as follows.

- Temperature
- pH
- Dissolved oxygen
- Solvent

Let us learn about these in brief.

Fluorophore: A component of the molecule that makes it fluorescent.

Temperature: A rise in temperature is almost always accompanied by a decrease in fluorescence. The change in temperature causes the viscosity of the medium to change which in turn changes the number of collisions of the molecules of the **fluorophore** with solvent molecules. The increase in the number of collisions between molecules in turn increases the probability for deactivation by internal conversion and vibrational relaxation. In such cases, it is recommended to use thermostated cell holders.

pH: Relatively small changes in pH can sometimes cause substantial changes in the fluorescence intensity and spectral characteristics of fluorescence. For example, serotonin shows a shift in fluorescence emission maximum from 330 nm at neutral pH to 550 nm in strong acid without any change in the absorption spectrum. In the molecules containing acidic or basic functional groups, the changes in pH of the medium change the degree of ionisation of the functional groups. This in turn may affect the extent of conjugation or the aromaticity of the molecule which affects its fluorescence. For example, aniline shows fluorescence while in acid solution it does not show fluorescence due to the formation of anilinium ion. Therefore, pH control is essential while working with such molecules and suitable buffers should be employed for the purpose.

Dissolved oxygen: The paramagnetic substances like dissolved oxygen and many transition metals with unpaired electrons dramatically decrease fluorescence and cause interference in fluorimetric determinations. The paramagnetic nature of molecular oxygen promotes intersystem crossing from singlet to triplet states in other molecules. The longer lifetimes of the triplet states increases the opportunity for radiationless deactivation to occur.

Presence of dissolved oxygen influences phosphorescence too and causes a large decrease in the phosphorescence intensity. It is due to the fact that oxygen which is in triplet state at the ground state gets the energy from an electron in the triplet state and gets excited. This is actually the oxygen emission and not the phosphorescence. Therefore, it is advisable to make phosphorescence measurement in the absence of dissolved oxygen.

Solvent: The changes in the “polarity” or hydrogen bonding ability of the solvent may also significantly affect the fluorescent behaviour of the analyte. The difference in the effect of solvent on the fluorescence is attributed to the difference in their ability to stabilise the ground and excited states of the fluorescent molecule. This alters the probability and the energy of both absorption and emission bands.

Besides solvent polarity, solvent viscosity and solvents with heavy atoms also affect fluorescence and phosphorescence. Increased viscosity increases fluorescence as the deactivation due to collisions is lowered. A higher fluorescence is observed when the solvents do not contain heavy atoms while phosphorescence increases due to the presence of heavy atoms in the solvent.

Thus, the fluorescence characteristics of probe molecules may be used to make inferences about their immediate microenvironments.

5.6 FLUORESCENCE QUENCHING

The fluorescence emission is quite sensitive to the presence of impurities and other species in the sample. These cause a decrease in the intensity of fluorescence emission. This decrease in the fluorescence intensity arising out of the interaction of the excited state of the fluorophore with its surroundings is called **quenching**. For example, the quinine fluorescence is quenched by the presence of halide ions. One of the mechanisms of quenching involves collisions between excited and ground state molecules leading to an increase in the amount of radiationless relaxation. It is called **self-quenching** and it alters the ratio of excited molecules that relax via the fluorescence pathway. Since self-quenching depends on the rate at which collisions occur, it increases with an increase in the concentration of the analyte. Due to self-quenching, the photoluminescence efficiency varies with the concentration.

The effect of self-quenching and self-absorption mechanisms can be so large that the observed fluorescence intensity may actually decrease with the increase in concentration.

Another mechanism that leads to the decrease in fluorescence intensity is called **self-absorption** or the **inner-cell effect**. It is observed in the molecules in which the absorption band overlaps with the wavelength of the emitted (fluoresced) photon. In such a situation some of the emitted photons are reabsorbed before they can escape the solution. This is called **self-absorption** and similar to the self-quenching, this is also significant at high analyte concentrations. A significant portion of the excitation radiation is absorbed by the sample before it reaches the centre of the cuvette. As self-absorption is a physical artifact, in principle it can be eliminated by the proper cell design and measurement conditions.

The intermolecular electronic energy transfer from the excited molecule to a quencher molecule is one of the common ways by which the fluorescence quenching occurs. The process can be represented as follows.

$$M^* + Q \rightarrow Q^* + M$$

where, the excited analyte molecule (M^*) transfers its excitation energy to a quencher molecule Q, whereby it gets de-excited to M forming an excited quencher molecule, Q^*. In case Q^* happens to be a fluorescent species, then its fluorescence is called **sensitised fluorescence**. The sensitisation of fluorescence can be one of the ways to observe fluorescence from a molecule (Q) that may otherwise be difficult to excite directly. However, the quenchers are generally not desirable for the fluorimetric determinations. Mathematically, the quenching generally follows the **Stern–Volmer equation** given below.

$$\frac{\varphi_F^0}{\varphi_F} = 1 + K_{SV}(Q)$$

where φ_F^0 and φ_F are the fluorescence quantum yields for the analyte in the absence and presence of quencher respectively, and K_{SV} is the **quenching constant**.

In an alternative mechanism of quenching the quencher molecule forms a complex with the analyte molecule, which then deactivates using some internal conversion mechanisms. This mechanism can be represented as follows.

$$A^* + Q \rightleftharpoons AQ^* \longrightarrow AQ$$

In this mechanism, the effect of the concentration of the quencher molecule or the analyte can be understood in terms of simple law of mass action. A higher concentration of any of the two shifts the equilibrium to the right and the analyte fluorescence is quenched to a greater extent.

Generally in the compounds containing heavy atoms like, halogens are expected to cause fluorescence quenching. In addition, the paramagnetic species like oxygen, containing unpaired electrons are prominent quenchers.

5.6.1 Quantum Yield

As discussed in section 5.2.3, a number of processes contribute towards the deactivation of the excited state. Only a certain fraction of the excited molecules relax through the fluorescence pathway i.e., by emission of radiation. This fraction is quantified in terms of a parameter called **quantum efficiency** or **quantum yield**. Mathematically, the quantum yield is defined as the quotient of the number of photons that are emitted and the number of photons that are absorbed. It can be written as the following equation.

$$\text{Quantum yield} = \varphi = \frac{\text{Number of emitted photons}}{\text{Number of absorbed photons}} \leq 1$$

Thus, the quantum yield refers to the contribution of fluorescence to the various deactivation processes of the excite a state. The quantum yield is related to the absorption properties of the molecule as well as the fluorescence life times (τ). The relationship with the life times is as follows.

$$\varphi = {}^{\tau}\!/_{\tau_0}$$

where, τ and τ_0 are the fluorescence lifetimes in the presence and in the absence of nonradiative processes, respectively. With the transition rates k_f for the fluorescence, k_{ic} and k_{isc} for the internal conversion and for the intersystem crossing, and k_Q with the fluorescence quenching by quencher molecules, the quantum yield can be expressed as follows,

$$\varphi = \frac{k_f}{k_f + k_{ic} + k_{isc} + k_Q}$$

SAQ 4

Which of the following statements are true about self-absorption mechanism of fluorescence quenching?

i) It is associated with the formation of complex of the analyte molecule.

…………………………………………………………………………………

ii) It involves collision between excited and ground state molecule.

…………………………………………………………………………………...

iii) It occurs in molecules having overlapping of absorption band with the wave length of the emitted photon.

……………………………………………………………………………….................

iv) It increases with decrease in concentration of the analyte.

……………………………………………………………………………………

5.7 INSTRUMENTATION FOR FLUORESCENCE MEASUREMENT

All fluorescence instruments use essentially the same components as are used in absorption spectrophotometers. However, the geometric arrangement of the components is somewhat different. This is due to the reason that any transmitted radiation is not measured along with the fluorescence. You know that the absorption and transmission of radiant energy occur only along the direction of the incident light whereas the fluorescence radiation emanates in all directions. The detection of transmitted radiation is avoided by placing the detector at a right angle to the transmitted beam, as shown in Fig. 5.5.

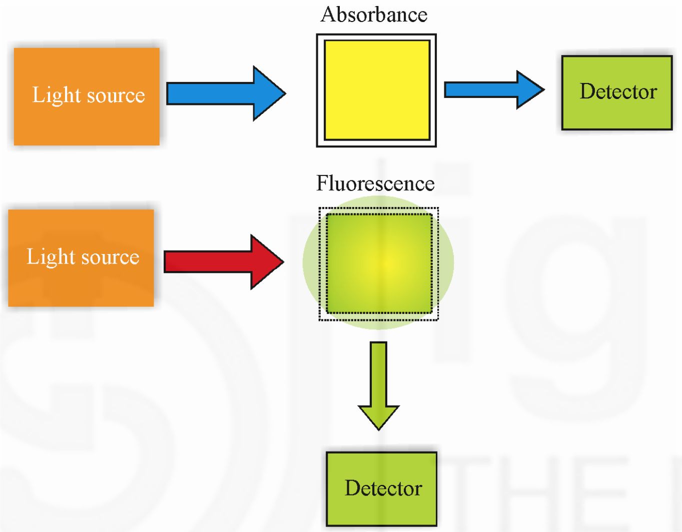


Fig. 5.5: The relative location of the light source and the detector in a fluorescence measurement set up

The essential components of an instrument used to measure fluorescence of the sample are:

- Excitation light sources
- Filters or monochromators
- Sample holder
- Detector
- Readout device

In addition to the optical components, most fluorimeters and spectrofluorometers have dedicated computers. These are required to control the instrumental operating parameters like, excitation and emission wavelengths, scan rates, monochromator slit widths, detector parameters, etc. These help in the acquisition and post processing of the spectral data.

Currently, two broad categories of commercial instruments are being manufactured. The low cost instruments needed for routine measurements are based on filters as wavelength selectors whereas the more sophisticated spectrofluorometers use monochromators. A typical configuration of different components of the fluorimeter is given in Fig. 5.6.

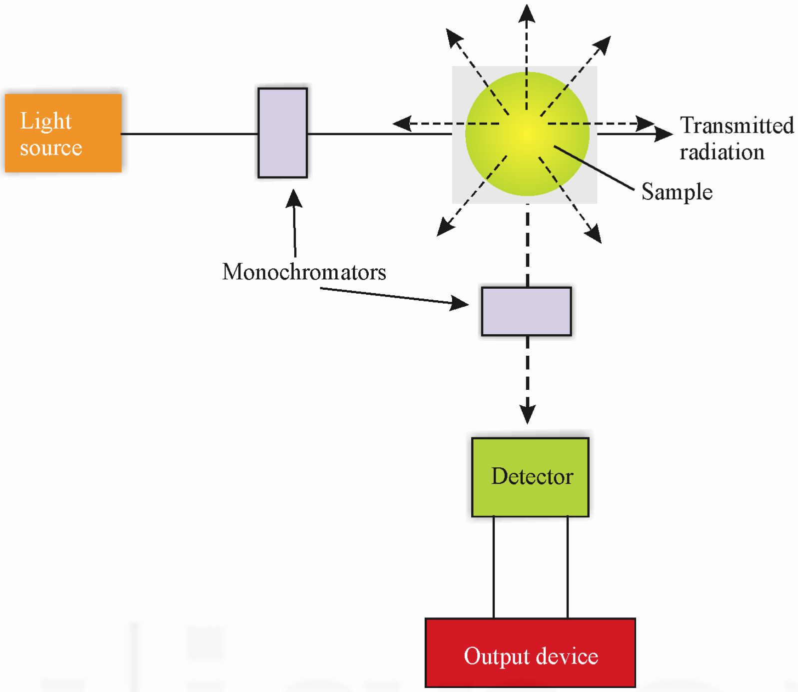


Fig. 5.6: A schematic layout of a fluorimeter

Let us learn about the essential components of a fluorimeter in the following subsections.

5.7.1 Sources

As mentioned earlier, the fluorescence power or intensity, I_F, is directly proportional to the source power, P_0. An increase in P_0 will produce a larger signal for a given concentration and thereby improve sensitivity. Therefore, the source must be more intense than that required for UV-VIS absorption spectroscopy. The tungsten filament and deuterium lamps used in absorption spectrophotometers are generally not suitable for fluorescence instruments as they lack the desired intensity. As molecular absorption spectra usually are broad, a highly monochromatic source is generally not required; an intense continuum source that emits throughout the UV, visible, and near infrared regions is adequate. However, in fluorescence measurements a source with intense emission lines at certain frequencies are desirable.

Filter fluorimeters often employ a low-pressure mercury vapour lamp. This source produces intense lines at certain wavelengths. One of these lines will usually be suitable for excitation of a fluorescent sample. Spectrofluorometers, on the other hand, need a continuous radiation source, are often equipped with a 75-450 W high-pressure **xenon arc lamp**. These produce an intense continuum between about 250 and 600 nm. As the xenon arc lamp produces lot of heat, the lamp assembly needs to be cooled therefore, these instruments cannot be used for routine work.

For certain applications, it is preferable to use a laser excitation source. A tuneable dye laser, using a pulsed nitrogen laser as the primary source can produce monochromatic radiation between 360 and 650 nm. Since the radiation produced is monochromatic, there is no need for an excitation monochromator. A few fluorescence spectrometers using laser sources are commercially available; most such instruments are intended for highly specific applications such as analysis of uranium in the nuclear industry.

5.7.2 Wavelength Selectors

The low-cost instruments designed for routine determinations are simple filter fluorimeters. Such instruments are used when it is sufficient to measure fluorescence intensity at a single excitation and emission wavelength. These employ fixed filters to isolate both the excitation and emission wavelengths. In order to isolate one particular wavelength from a source what we need is just a pair of cut-off filters. Absorption filters are comprised of a suitably absorbing substance or substances dispersed in gelatin, glass or plastic. The filter fluorimeters are used primarily in environmental field screening, hospital or clinical settings and other applications in which low cost and small size are crucial.

Nowadays, interference filters having high transmission (≈ 40%) over a narrow range (10 – 15 nm) of wavelengths have become available. Today it is possible to purchase filters giving the maximum transmission at any desired wavelength. Interference filters consist of a thin transparent layer of CaF_2 or MgF_2 sandwiched between parallel, partially reflecting metal films. A portion of the radiation entering the filter is reflected back and forth between the two metallic films constituting the filter (Fig. 5.7).

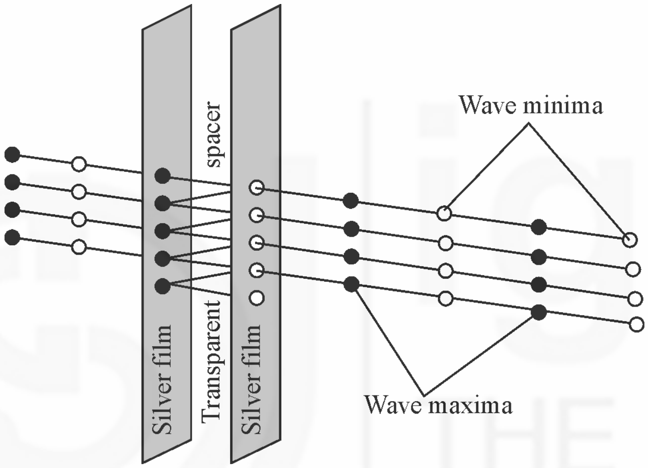


Fig. 5.7: Schematic diagram of an interference filter used in fluorescence measurements

The escaping radiation then undergoes interference with the transmitted portion of the radiation. The radiation for which the filter spacing is a multiple of a half wavelength ($n\lambda/2$) undergoes **constructive interference** whereas the radiation of other wavelengths undergoes **destructive interference**. The destructive interference of the radiation is not complete in the cases where the half wavelength multiples are close to, but not exactly equal to the spacing between the metal films. These also appear in the transmitted radiation and as a consequence the transmitted radiation is a band of radiant energy and not a single wavelength.

Most modern fluorimeters used in analytical laboratories generally use diffraction grating monochromators about which you have learnt in Unit 2. Such a fluorescence spectrometer is capable of recording both excitation and emission spectra and therefore makes full use of the analytical potential of the technique. When monochromators are employed, it should be possible to change the slit width of both the excitation and emission monochromators independently.

5.7.3 Sampling

The majority of fluorescence measurements of the analyte are carried out in solution. For this the sample is taken in a **cuvette** or in a **flow cell**. The cuvettes generally are circular, or square shaped. These are constructed of a material that will transmit both the incident as well as the emitted light. Glass and quartz both qualify this criterion. However, the quartz cuvettes are generally used when the radiation belongs to the UV region. The square cuvettes are most common and are found to be quite precise as the parameters of a square cuvette are easier to maintain during manufacture. On the other hand the circular cuvettes though not quite accurate are suitable for routine applications and have the advantage of being less expensive.

The cuvette or cell is placed normal to the incident beam as shown in Fig. 5.5. Though resulting fluorescence is given off equally in all directions, it is collected from the right angles to the incident beam. As fluorimetry is a very sensitive technique, the following precautions should be followed without exception while handling the cuvettes.

- The optical surfaces of the cell should not be touched with hand as it invariably leaves an invisible film that may change the light transmission and reflection characteristics of the cell, especially in the ultraviolet region.
- The cuvettes should be handled only at the top portions of the side plates that do not face the optical axis.
- The samples should be transferred to the cuvettes with the help of a dropper or a pipette.
- The cuvettes should be rinsed with the analyte solution before filling and the overfilling should be avoided.

5.7.4 Detectors

The fluorescence signals for an analyte present at low concentration is weak. It is partly due to low photoluminescence efficiencies and partly because only a small fraction of the fluorescence radiation reaches the detector. Thus the basic requirement for the detector is that it should be able to detect weak optical signals. Therefore, **photomultiplier tubes** with their high sensitivity and low noise are preferred over simple phototubes. You have learnt about these detectors in Unit 2 of Block 1. However, the shortcoming of photomultiplier tubes is that these are single channel detectors. One needs to scan the appropriate monochromator across the whole wavelength range to obtain a spectrum. This does not allow taking the spectra of transient species or short lived analytes. To circumvent this problem we need a multi channel instrument using an array of detectors. In recent past some instruments using **charge coupled device** (CCD) as detectors have been developed and are likely to become common in future.

5.7.5 Read out Devices

The output from the detector is suitably amplified and displayed on a read out device like a meter or digital display. The sensitivity of the amplifier can be changed so as to be able to analyse samples of varying concentrations. In some instruments the display can be adjusted to directly give the output in terms of the concentration. Nowadays the instruments have microprocessor controlled electronics that provides outputs compatible with the printers and computers whereby minimising the possibility of operator error in transferring data.

SAQ 5

Complete the following sentences with appropriate words.

a) The fluorescence power or intensity is directly proportional to the …………. .

b) In order to measure fluorescence intensity at a single excitation and emission wavelength, ………… instruments are used.

c) Photomultiplier tubes with low noise and high sensitivity are preferred over simple photo tubes so as to detect …………... signals.

5.8 INSTRUMENTATION FOR PHOSPHORESCENCE MEASUREMENT

You know that the basic difference between fluorescence and phosphorescence is that the phosphorescence emission occurs at a different time frame and can be measured only if the sample is solid or is at liquid nitrogen temperatures. Therefore, the basic instrumentation for phosphorescence is similar to that of fluorescence; however, two aspects of the measurement need to be modified. The first is the sampling technique and second being the recording procedure. Let us study about the modifications in the fluorimeter done regarding these two aspects.

5.8.1 Sampling

Since most of the measurements in phosphorescence are carried out in rigid media at cryogenic temperatures of liquid nitrogen we need to use solvents that have certain special characteristics. It is the most important requirement besides good solubility of the analyte. The solvent must form a clear rigid glass at 77 K i.e., the temperature of measurement. In addition, it should be highly pure so that there is practically nil background phosphorescence. Not many solvents qualify to be used for the phosphorescence measurement. Ethanol is an excellent solvent for polar molecules though it may require addition of small quantities of acid or base to produce a clear solid. On the other hand a mixture of diethyl ether, isopentane and ethanol in the ratio of 5: 5: 2 respectively, commonly called **EPA** is an excellent choice for non-polar compounds.

In a typical phosphorescence measurement, the sample is placed in a narrow quartz tube of an internal diameter of 1 to 3 mm. The sample tubes are placed in liquid nitrogen held in a quartz Dewar flask which is then placed in the sample holder called **phosphoroscope** (Fig. 5.8).

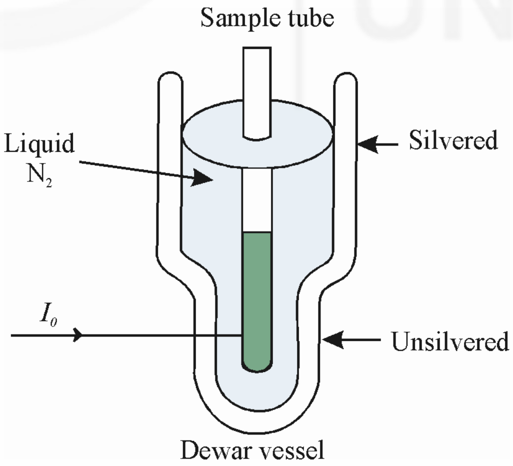


Fig. 5.8: Sample assembly for low temperature phosphorescence measurement

For room temperature measurements the sampling procedure depends on the nature of the analyte. For inorganic salts and oxides of rare earths viz., europium and uranium, which have natural phosphorescence, are used without any pretreatment. The sample is held in a solid sample accessory that fits into the instrument.

On the other hand, some compounds show phosphorescence only when adsorbed onto a substrate like, paper, cellulose, silica, etc. For the measurements of polar molecules of this category a solution of the analyte is prepared in a suitable solvent and is placed on the filter paper and thoroughly dried. The analyte adsorbed on the substrate is then used for measurement of phosphorescence. Sometimes, the addition of heavy atoms like iodine, silver or lead is found to enhance the phosphorescence.

5.8.2 Recording Procedure

Once suitable sampling procedure has been achieved the sample in principle can be used for the simultaneous measurement of fluorescence and phosphorescence as the two emissions occur at different wavelengths. However, the fluorescence emission may cause interference in the measurement of phosphorescence. This interference can be eliminated by taking advantage of the fact that once the incident radiation is cut off the fluorescence ceases immediately whereas the phosphorescence emission continues. A mechanical shutter can be used to execute recording of phosphorescence under these conditions.

One commonly used mechanical shutter system consists of a revolving can that surrounds the sample and has a small window. The can is so rotated that when it is opposite the excitation monochromator it allows a short burst of radiation to fall on the sample and immediately cut off. After a certain time delay it allows the radiation emitted during phosphorescence to pass through to the emission monochromator. A schematic diagram of the rotating can mechanical shutter system is shown in Fig.5.9.

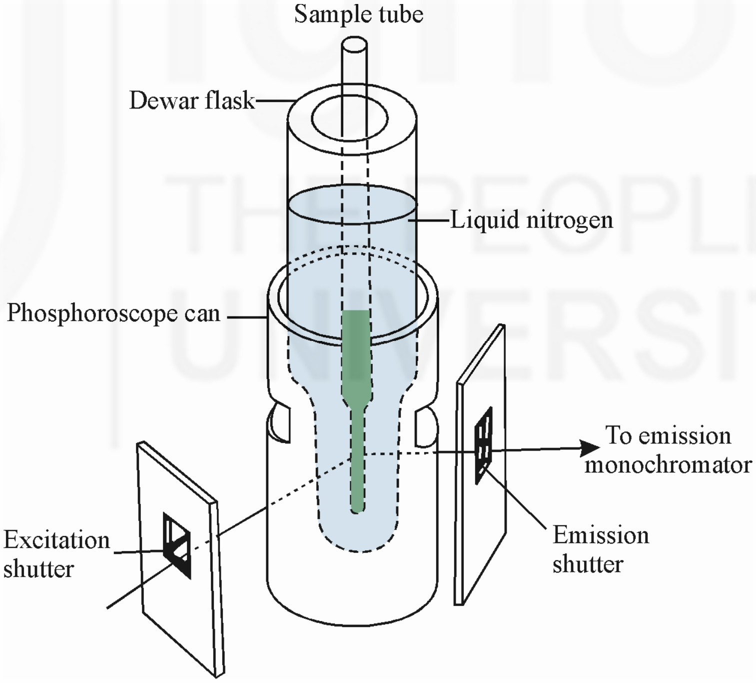


Fig. 5.9: Revolving can shutter system for recording phosphorescence and fluorescence from the same sample

Having learnt about the theory of fluorescence and phosphorescence, the species showing these phenomena, the factors affecting them and the instrumentation required for their measurement you are equipped to learn about the applications of these phenomena in the next section. However, before that why don't you assess your understanding by solving the following self assessment question.

SAQ 6

How does the revolving can shutter system help in simultaneous measurement of fluorescence and phosphorescence?

…………………………………………………………………………………………..

…………………………………………………………………………………………..

5.9 APPLICATIONS OF FLUORESCENCE AND PHOSPHORESCENCE

Fluorescence spectroscopy and its applications to the physical and life sciences have evolved rapidly during the past decade. It is widely used in the qualitative and quantitative analysis of inorganic compounds and has become extensively used spectroscopic technique in the fields of Biochemistry and Molecular Biophysics also. Despite the fact that the fluorescence measurements do not provide detailed structural information, the technique has become quite popular in these areas. This is due to its sensitivity to changes in the structural and dynamic properties of biomolecules and biomolecular complexes. The increased interest in fluorescence appears to be due to advances in time resolution, methods of data analysis, and improved instrumentation. Advances in laser and detector technology have also resulted in renewed interest in fluorescence for clinical and analytical chemistry.

In the case of inorganic analytes, though many of these are fluorescent or phosphorescent in the solid state, the majority of analysis work is undertaken in solution. For the purpose of fluorimetric analysis of inorganic analytes either direct or indirect methods are used. You know that in the direct method, the native fluorescence of the analyte is used, whereas in case of the indirect method, the analyte is converted to a fluorophore by making a complex with a suitable organic compound. For example, the fluorescent uranyl ion can be directly determined whereas in the determination of selenium, the complexing agent diaminonaphthalene is used. In general, making a complex of the metal ion with a molecule containing fused ring systems causes the development of fluorescence by making the complex rigid. Table 5.1 gives some common applications of fluorescence measurements.

Table 5.1: Some common applications of fluorescence

Element or compound	Fluorescent emission maximum (nm)	Example of application
Vitamin A	500 nm	Foodstuffs, vitamin tablets
LSD	>430 nm	Body fluids
Amphetamine Codeine, Morphine	282-300 nm 345 nm	Drug preparations and body fluids
Polyaromatic hydrocarbons (PAH)	320-550 nm	Environmental samples
Al as alizarin (garnet red complex)	580 nm	Water samples and soils
B as benzoin complex	450 nm	Water samples and soils

The phosphorescence emission has been observed in a wide variety of compounds and as you know it differs from fluorescence in terms of persistence of emission of light even after removal of the excitation source. The first analytical applications of phosphorescence were published as far back as 1957 by Kiers et al; yet the technique has not acquired the status of a routine analytical tool. This is so because we need to work at extremely low temperatures of 77 K, acquired by using liquid nitrogen. However, some recent developments leading to room temperature phosphorescence (RTP) measurements have given hope for newer practical applications and fundamental advances that may lead to the development of phosphorimetry. Currently, phosphorimetry is widely accepted in some areas like pharmaceutical analysis and forensic science. A number of the drugs belonging to sulphonamides, phenobarbital, cocaine, procaine, chlorpromazine and salicylic acid, etc. have been found to exhibit phosphorescence. Phosphorescence has also been used in the determination of the drugs present in urine and blood samples. The detection of air and water borne pollutants and analysis of pesticides are other areas of application of phosphorescence.

In terms of the sensitivity, phosphorescence is comparable to that of fluorescence. Phosphorescence as a technique complements fluorescence by extending the applications of photoluminescence to a wider range of molecules. Further, uniqueness of phosphorescence lies in the time aspect as this can be used to eliminate the background interference from fluorescence. The ratios of the fluorescence to phosphorescence intensities can be used for the identification of organic compounds and the decay time measurements can be exploited for checking the purity of organic compounds.

Let us summarise what all you have learnt in this unit. In the next unit, you would learn about a number of applications of fluorimetry and phosphorimetry in detail.

5.10 SUMMARY

In this unit, you have learnt about two important spectroscopic methods, namely fluorescence and phosphorescence. These are based on the phenomenon of emission of radiation by electronically excited species and are collectively termed as photoluminescence methods. The origin of these luminescence spectra are explained in terms of Jablonski diagram which gives a representation of ground and different excited electron states of a molecule and the processes associated with absorption and emission (radiative and nonradiative) of energy.

The absorption of a photon of suitable energy causes the molecule to get excited from the ground state to one of the excited states. These excited or activated states get deactivated by a number of processes which are generally grouped under nonradiative and radiative processes. The nonradiative relaxation is through three different processes namely, vibrational relaxation, internal conversion and intersystem crossing whereas the radiative deactivation is through fluorescence and phosphorescence. The wavelength of fluorescence emission is generally greater than the excitation wavelength and that of phosphorescence emission is at still longer wavelengths.

There are two types of fluorescence spectra. These are fluorescence emission and fluorescence excitation spectrum. In the former the molecule is excited with a given wavelength and the fluorescence emission at different wavelengths is measured whereas in the later the excitation wavelength is varied and the intensity of fluorescence emission at a predetermined wavelength is monitored. Generally the excitation spectrum of a molecule is the same as its UV-VIS absorption spectrum. The wavelength difference between the absorption and fluorescence maxima is called the Stokes shift.

The fluorescent behaviour of a molecule is determined by the chemical structure of the molecule and is affected by the chemical environment in which it exists. The structural features whose presence in a molecule may make it fluorescent are the presence of benzene ring, fused ring system and rigidity in the molecule. The external factors affecting the fluorescent behaviour of a molecule are temperature, pH, paramagnetic species like dissolved oxygen and the solvent.

The fluorescence emission intensity is decreased by a number of processes like self quenching, self absorption or inner filter effects and by the presence of external species. This is called fluorescence quenching. The fraction of the excited molecules that relax through the fluorescence pathway i.e., by emission of radiation is quantified in terms of a parameter called quantum efficiency or quantum yield.

All fluorescence instruments use essentially the same components as are used in absorption spectrophotometers; however, the geometric arrangement of the components is somewhat different. In luminescence measurement the emitted radiation is measured by the detector at a right angle to the transmitted beam whereas in case of absorption spectrometers the transmitted radiation is measured along the direction of the incident light. The basic set up of the instrumentation for phosphorescence is similar to that of fluorescence. However, the sampling technique and the recording procedure need to be modified.

The applications of fluorescence spectroscopy are spread across the disciplines of physical and life sciences and it is becoming an extensively used spectroscopic technique in the fields of Biochemistry, Clinical Chemistry and Molecular Biophysics. The phosphorescence measurements on the other hand are not as common due to the requirements of cryogenic temperatures yet it finds a number of applications in the areas like pharmaceutical analysis, forensic science, environmental science and analysis of pesticides.

5.11 TERMINAL QUESTIONS

1. Differentiate between internal conversion and inter system crossing.

2. What do you understand by the spin multiplicity of an electronic energy level? What are the multiplicities of the singlet and triplet state?

3. What is the difference between chemiluminescence and photoluminescence?

4. State Franck Condon principle. What is its significance?

5. Describe briefly different mechanisms of nonradiative relaxation of an excited electronic state.

6. What is an excitation fluorescence spectrum? How is it obtained and in what way is it related to the absorption spectrum?

7. Write any three structural requirements whose presence in a molecule may make it fluorescent?

8. List different factors that may affect the fluorescence characteristics of a molecule.

9. Define fluorescence quenching. What is self-quenching?

10. What precautions should be observed while handling the sample cuvettes during fluorescence measurement?

5.12 ANSWERS

Self Assessment Questions

1. a) ii)
   b) i)

2. The fluorescence emission takes place only after the excited molecule has relaxed to the vibrational ground state of the S_1 state i.e., after having lost part of its energy. As the energy of the emitted radiation is lower than that of the excitation radiation it is observed at a longer wavelength than that of the excitation radiation

3. ii) and iii)

4. ii) and iii) are true

5. a) source power
   b) filter
   c) weak optical

6. The can is rotated in such a manner that when it is opposite the excitation monochromator it allows a short burst of radiation to fall on the sample and immediately cut off. At this point the fluorescence ceases whereas phosphorescence continues.

Terminal Questions

1. The internal conversion refers to the transfer of the molecule from an excited singlet state to another whereas in case of intersystem crossing the molecule in the vibrational states of a singlet excited state crosses over to a vibrational level of a triplet state (rarely from a triplet to singlet). However, in both the cases the energies of the initial and final level must be the same.

2. Spin multiplicity of an electronic state refers to the number of possible quantum states of the system. It is computed as (2S+1) where S is the total spin quantum number for the electronic state.

   The spin multipliticities of the singlet and triplet states are 1 and 3, respectively.

3. The difference between chemiluminescence and photoluminescence lies in the way the excited states are achieved. In chemiluminescence, the energy required for the excitation comes from the chemical reaction whereas in photoluminescence the energy is provided by a photon (light).The principle explains the phenomenon of activation process during the fluorescence and phosphorescence of a molecule.

4. According to Franck Condon principle the electronic transition takes place so fast that the molecule does not get an opportunity to execute a vibration. Therefore, in the Jablonski diagram, the transition from the ground state to excited state can be represented by vertical arrows.

5. The nonradiative relaxation refers to the deactivation of the molecule in the excited state without emission of radiation. This may occur by any of the three mechanisms viz., vibrational relaxation, internal conversion or inter-system crossing.

- In vibrational relaxation, the molecules rapidly lose their excess vibrational energy by collision with other molecules and the energy is dissipated as heat to the surroundings.
- In internal conversion the molecule is transferred from an excited singlet state to another singlet state of the same energy.
- In intersystem crossing the molecule is transferred from an excited singlet state to a vibrational level of a triplet state (or vice-versa).

6. The excitation spectrum refers to the plot of the emission intensity from the sample at a given wavelength against the wavelength of exciting radiation. This is obtained by observing the fluorescence intensity at a predetermined wavelength and the wavelength of the excitation radiation is varied continuously. Generally, the excitation spectrum of a molecule is similar to its UV-VIS absorption spectrum.

7. The following structural features in a molecule may make it fluorescent.
   - The presence of benzene ring
   - Fused ring system
   - Rigidity in the molecule

8. The following factors may affect the fluorescence characteristics of a molecule:
   - temperature
   - pH
   - dissolved oxygen
   - solvent

9. The fluorescence quenching may be defined as the decrease in fluorescence intensity of a molecule as a consequence of the interaction of the excited state of the molecule with its surroundings.

   Self quenching involves the collisions between excited and ground state molecules leading to an increase in the amount of radiation less relaxation.

10. The following precautions should be observed while handling the cuvettes during fluorescence measurement.
    - The optical surfaces of the cell should not be touched with hand.
    - The cuvettes should be handled only at the top portions of the side plates that do not face the optical axis.
    - The samples should be transferred to the cuvettes with the help of a dropper or a pipette.
    - The cuvettes should be rinsed with the analyte solution before filling and these should not be overfilled.