CHAPTER I

FLUORESCENCE SPECTRA OF PHTHALEIN DYES

- A -

FLUORESCENCE SPECTROSCOPY

1.1 INTRODUCTION

Absorption spectroscopy can be used for detection of species down to 10^{-7} M. Although over conventional chemical methods this is an achievement by an order, the needs of both bioscientists working in enzymology and physicists working in the field of ultrapure materials and semiconductors are more stringent and hence a technique capable of going to 10^{-12} M is very important. The activities in this field may be twofold. In one direction it should lead to development of suitable instrumentation, theory and technique and search for new specific and sensitive reagents should continue in the other direction. Modern microprocessor controlled, double monochromator instruments with photomultiplier, response curve corrector electronic cam and computer programmes cater for very critical usage but simple filter fluorometers can serve the analyst to his satisfaction. Needless to say, once the instrumentation is developed to a particular level much remains to be done in the direction of study of new fluorogens and their applications. This quest is therefore an almost endless one.

Fluorescence is observed in extracts or components of natural materials and the branches of fluorescence spectroscopy and fluorescence microscopy are well developed and many biological problems are now routinely and exclusively studied by using these methods. More refinements and modifications of existing methods as well as development of new methods are welcome.

1.2 HISTORICAL NOTE

Many teletell stories of gemstones glowing in darkness are heard often in connection with Royal Jewellery or misticism. We wonder at the fascinating beauty of the glow-worms. For more than a century it is known that mineral fluorspar on heating emits radiations. These all related observations refer to luminescence phenomenon. In general the emission of long wavelength visible region radiation from substances when irradiated with short wavelength of ultra violet wavelength are referred to as fluorescence. The scientific investigations of this phenomenon start with Bequerel's design of phosphoroscope around 1850 and Stokes' early observations that the luminescence emission peak wavelength is always longer and less energetic than the absorption maximum. This marks the beginning of development of science spectroscopy. However up to first quarter of the present century there were very few significant contributions in this field. Jablonski introduced in 1930 the energy level diagrams and metastable stage and differentiated fluorescence from phosphorescence.

The rapid upsurge of the research activities in the frontier accumulated sufficient data and Pringsheim published in 1948 a comprehensive book on the subject. By this time fluorescence spectrometer became a markatable instrument and the Klett fluorometer became available to many researchers. From this very simple filter photometer slow sophisticated instruments appeared as it was necessary for more detailed investigations. Simultaneously, the technological developments demanded luminescent screens, phosphors for watch dials and hands, inner-coating fo r fluorescent tubes, television tubes and schintillators in detection of nuclear radiations which lead to development of theory and technique. In general purely from chemical stand point many biochemical measurements at low concentration levels were developed and this expanded the activity in fluorescence spectroscopy enormously. Industrial production of optical brightners, fluorescent paints and invisible marking inks have brought this subject to a very rapid R and D activity in recent years. The recent introduction of microprocessed technology had added a new dimension to photometric measurements and it is now possible to carry on complicated manipulations and processing of experimental data.

1.3 FLUORESCENCE

Molecules which contains π electron systems have two electrons in each π bond, such a system of paired electrons in π level in the ground state configuration is a singlet state and

the spins of π electrons are paired up. When π electrons are excited by retaining the electron spin the resultant excited states S_1, S_2, S_3, etc. are also singlet states. It is possible that the spin of the excited electron may be reversed while going from ground to excited state and thus an excited triplet state will result from ground singlet state. Quantum mechanical selection rules state that electronic transitions between singlet and triplet states are forbidden. The absorption of radiation generally takes place between the ground state S_0 and the singlet states S_1, S_2, S_3, etc. Excitation to still higher level leads to excitation of σ-electrons ultra-violet or visible region and thus the excited states of the σ-electron compete, with π-electron states for the excitation energy. The process of absorption of secondary radiation by organic molecules is schematically shown in Fig. 1.1. Here each triplet state (T_1, T_2, T_3) lies below the corresponding singlet state. As excepted the vibrational energy of the main molecular skeleton is superimposed on each of the electronic levels. Luminescence may be produced by absorption into any of the excited singlet states and the primary fluorescence emission occurs, from the lowest excited singlet state S_1, back to ground state S_0. The molecular vibration requires very small time of the order of 10^{-12}s, however the radiative life time of S_1 is almost more (i.e. 10^{-9}s) and therefore for most of the molecules the emission spectrum, the fluorescence life time, and the fluorescence quantum efficiency are independent of the state of initial excitation. In addition to sinking of S_2 to S_1, a non-radiative process known as intersystem crossing also takes place.

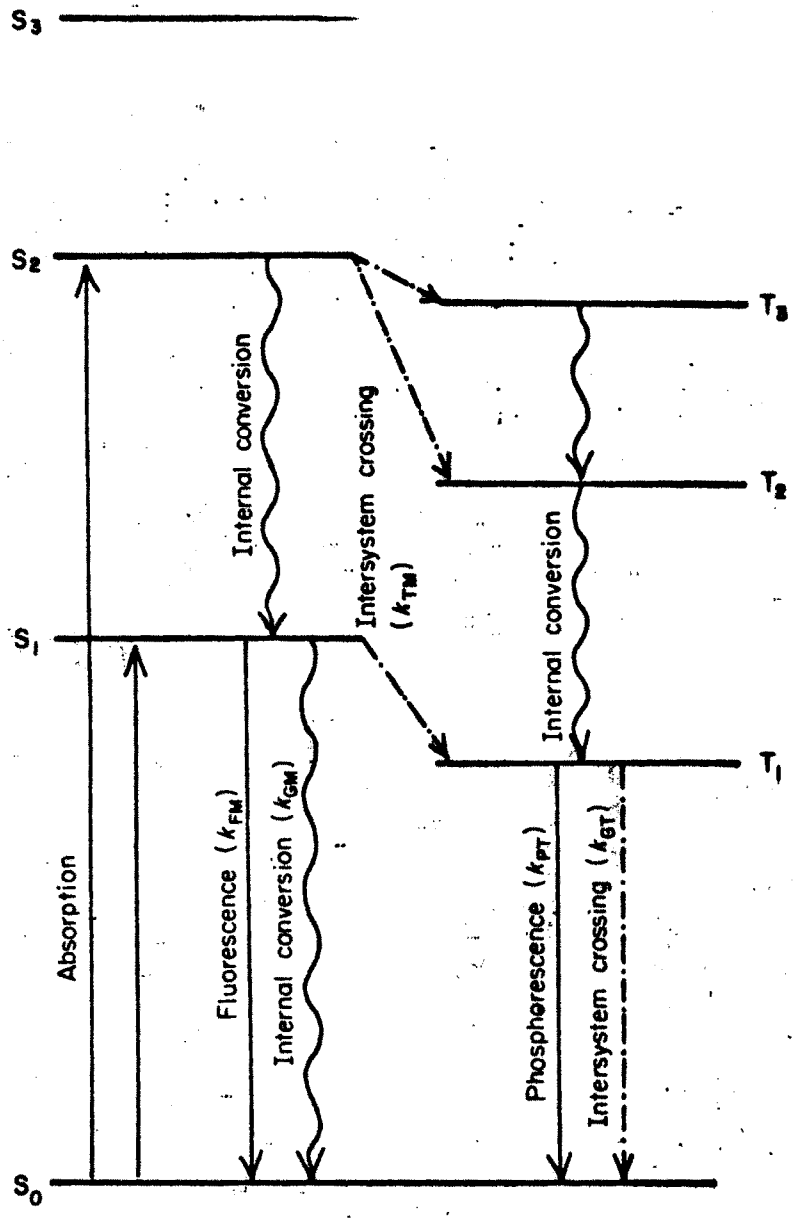


Fig. 1. π-Electron luminescence processes (rate parameters in parentheses): —— absorption; ——, emission (radiative process); ∿, internal conversion (non-radiative); — · — ·, intersystem crossing (non-radiative process).

Intersystem crossing is defined as a process of non-radiative transfer of energy from the singlet manifold to the triplet manifold. It has been said above that normally singlet-triplet transitions are forbidden. However, there is a finite rate constant $\approx 10^6$ s^{-1} for intersystem crossing from the excited singlet to lowest triplet state T_1 that is $S_1 \rightsquigarrow T_1$. It is possible that internal conversion may take place even between higher triplet to lower triplet ($T_2 \longrightarrow T_1$). The sinking of T_1 to S_o is known as phosphorescence which involves intersystem crossing, $S_1 \rightsquigarrow T_1$ and therefore the probability of radiative emission from T_1 to S_o depends on non-radiative intersystem crossing from S_1 to T_1. Fig. 1.1 shows molecular radiative and non-radiative energy transfer processes.

1.4 REQUIREMENTS OF FLUORESCENCE

1.4.1 Electronic Considerations

As stated previously, the mechanism of fluorescence is defined on the basis of excitation and de-excitation (or sinking) without intersystem crossing.

Following structural features will be necessary for exhibiting fluorescence.

(a) The compound must possess high extinction coefficient in ultraviolet or visible region corresponding $\pi \longrightarrow \pi^*$ transition, and must have absorption band in the long wavelength region.

A molecule with $n \longrightarrow \pi^*$ transition as above shows phosphorescence and not fluorescence [1].

(b) The molecules having excited singlet state with a half-life of above 10^{-8}s may show fluorescence. If the half-life is more than 10^{-8}s intersystem crossing will be possible. But if the half-life is less than 10^{-8}s rapid deactivation will result.

(c) The gap between the excited singlet state S_1 and triplet state T_1 must be sufficiently wide, otherwise intersystem crossing will be favoured.

1.4.2 Structural Requirements

(a) It was formerly thought that a fluorescent molecule has two specific functions allocated to two specific structural features. The molecules were supposed to have a chromophoric part responsible for colour and absorption spectra, and a fluorophoric part responsible for fluorescence and emission spectrum. We know now that in most of the cases absorption and fluorescence are the characters of the molecule as a whole. It does not mean that structural alteration will not be possible and that any change will lead to a non-fluorescent molecule. This statement does not imply a guaranty of retention of fluorescence on moderate structural alteration.

(b) Nature of carbon skeleton

(i) Saturated substances are not fluorescent.

(ii) Compounds which only absorb at high energy frequency than the strength of their weakest bonds, do not usually show fluorescence. Nitrobenzene with C-NO_2, bond cleavage energy of 60 Kcal mol^{-1}, bond cleavage energy of least stable bond with absorption band at 260 nm corresponding to 110 Kcal mol^{-1} or bromobenzene with C-Br bond cleavage energy of 66 Kcal mol^{-1} as against absorption band at 261 nm corresponding to 100 Kcal mol^{-1} energy do not show fluorescence. On the contrary eosin with weakest C-Br bond of 66 Kcal mol^{-1} with absorption band at 520 nm having energy 55 Kcal mol^{-1} or 1,4-di-pyrenebutadiene with C-C bond energy of 125 Kcal mol^{-1} with absorption maximum at 350 nm corresponding to 80 Kcal mol^{-1} show fluorescence [2].

All fluorescent compounds possess a conjugated system of double bonds but the converse is not true.

(iii) Linear chain of fused benzene rings favours fluorescence [3].

(iv) The conjugated system must have a planar structure, then only fluorescence will be exhibited. Sometimes steric hindrance destroys planarity as in o-diphenylbenzene.

(v) Molecular frame work must be rigid otherwise the absorbed energy is dissipitated as heat and then there will be no fluorescence. This is why cyclic compounds are prone to exhibit fluorescence compared to chain structure. It is obvious therefore that biphenyl with rigid, non-planar configuration does

not fluoresce and fluorene in which $-CH_2$ group ties. The two benzene rings in 2,2-positions fix them in a plane shows fluorescence. Likewise the same, although phenolphthalein or fluorescein have comparable structures, since the prior has non-rigid structure, needless to say, the later has strong fluorescence.

(vi) Substituents which increase or induce conjugation may enhance fluorescence [4, 5].

(vii) Intramolecular hydrogen bonding changes the fluorescence unpredictable [6].

(viii) Organic compounds which are fluorescent are said to possess native fluorescence but in many inorganic systems chemically induced fluorescence is common.

1.5 FACTORS WHICH AFFECT FLUORESCENCE

(i) Presence of other substances which absorb exciting short wavelength radiations or which absorb long wavelength radiation emission will cause hindrance in excitation or emission and fluorescence does not be observed.

(ii) By interaction with fluorescent substance the ground state becomes the light absorbing species and prevents fluorescence.

(iii) By interaction with fluorescent substance in the excited state similar effect is expected.

(iv) There may be chemical reactions and products may not be fluorescent.

(v) Fluorescence may be statically on dynamically quenched by solvents or contaminents or impurities. Similar effects are expected on formation of dimers/excimers [7-10].

(vii) Fluorescence is quenched by certain trace impurities, fluorescence of anthracene disappears if a trace of phenazine is added [11].

(viii) Oxygen in the ground state exists as a triplet. When it comes in contact its molecules in the excited singlet state there may be exchange of singlet and triplet state leading to quenching of fluorescence [12].

(ix) Sometimes excited state spacies form aggregates. Such dimerization gives an excimer. The excimer gives the bathochromic shift in the fluorescence [13-15].

(x) Fluorescence of a compound may vary with pH. The effect of pH is of great importance in the study of solution chemistry. Sulphapyridine exhibits fluorescence over the range of H_o value of -1 to 4. pH dependance of fluorescence may be utilized for finding the pK values. It is possible to determine pK by making use of the relation :

$$pK_a - pK_a^* = 21187 \left[\frac{\lambda_f - \lambda_a}{\lambda_f \lambda_a} \right] \qquad \text{----- 1}$$

where λ_a and λ_f are absorbance and fluorescence maximum [16-19].

1.6 INSTRUMENTATION OF FLUOROMETRY

Essentially fluorometer is designed on the principle of excitation by homogeneous solution which does not scatter light.. Molecular which possess fluorescence are filled in silica cuvette in the form of dilute solution. The emission traverses in the direction at right angles to the incident beam and is then analysed with respect to wavelength as well as intensity. In the simplest instrument like a Klett fluorometer on L.P. fluorometer, a radiation from mercury lamp passes through "UV pass-visible cutt off" black filter which cuts off all the visible component and allows only the UV radiation to pass on from the top of a glass cuvette, thus avoiding UV cut off property of the cell wall. The secondary radiation may contain some scattered component or reflected component of ultraviolet radiation which is cut off by "UV cut off visible pass" filter. The polychromatic visible radiation may be as such measured as total signal or it can be passed through various optical transmission filters to give the emission spectrum. This spectrum may not be of much use since transmission characteristics of the filter and the photocell response at the nominal wavelength are ignored which introduce a very distorted picture. Such instruments are comparable with simple colorimeters and are mainly used in biochemical and medical routine analysis.

A modification incorporating use of mercury lamp and "UV pass visible cut off" filter as excitation optics and using a prism or grating monochromator for recording of emission spectrum has an advantage that use of selected emission wavelength is possible for the quantitative measurement. An electronic cam

which applies "photocell response curve correction" can give a fairly good emission spectrum. This instrument cannot be used when the dependence of emission characteristics with excitation wavelength is to be studied. Instruments of this type have xenon lamp in place of mercury lamp.

A highly sophisticated instrument incorporate prism or grating monochromators both in excitation and emission processes. Aminco Bowman SPF spectrofluorometer, Shimadzu R.F. 540 spectrophotofluorometer, Perkin-Elmer L.S.-5 fluorescence spectrofluorometer, Baird Atomics Fluorispec, Farrand spectrofluorometer and Turner spectrofluorometer belong to this category. Some of these instruments can be connected to a mini-computer or a thermal printer giving a wealth of information and data of corrected emission and excitation spectra. Such instruments are well suited for advanced research and very accurate quantitative work.

1.7 EXPERIMENTAL MEASUREMENTS

The experimental measurements of (i) absorption spectra, (ii) emission spectra, (iii) excitation spectra, (iv) life times and rate parameters and (v) quantum efficiency and yield are important.

1.7.1 Absorption spectra

Einstein relation $\Delta E = h\nu = hc/\lambda$ describes the absorption of a photon by a chromophoric absorbing center,

h is Planck's constant, ν , λ and c are the frequency, wavelength and velocity of the incident photon. This relation also states the quantization condition for absorption or emission of light. For any particular light absorption system chosen for study, the energies of electronic, vibrational and rotational changes are involved and therefore Einstein relation becomes

$$h\nu = \Delta E_{el} + \Delta E_{vib} + \Delta E_{rot} \quad ---- 1.2$$

where ΔE_{el}, ΔE_{vib} and ΔE_{rot} are the changes in electronic, vibrational and rotational energies. The electronic absorption involves absorption of energy for migration of an electron from one orbital to other of the absorbing species and occurs in long wavelength ultraviolet or visible or NIR region. Electronic spectrum has broad bands and occassionally there is moderate to severe overlap giving variation from well separated symmetric bands standing side by side, to moderate overlapping giving side bands. The overlap of weak band with a strong one gives a shoulder on one limb. An extreme type of overlap is such that there is no visible sign of a bulge, but the weak band causes distortion of the symmetry. The complete analysis of such curves can be done by laborious manual curve-fitting analysis or by using appropriate computer programme [20-21]. The vibrational spectrum usually contains fine structure and hence there are several narrow bands in the spectrum. It generally covers the range of 5000 to 200 cm^{-1}. In polyatomic molecules the rotational fine structure cannot be observed at room temperature as the thermal excitations at room temperature are not much different with respect to energy

than the rotational absorption. Therefore rotational excitations average out and broaden vibrational bands [22].

In practice the absorption spectrum is plotted in a variety of ways. It is known for long time that absorption spectrum lies between excitation and emission spectrum and it is a mirror image of fluorescence emission as shown in Fig. 4.1 (chapter 4).

1.7.2 Emission spectra

The absorption centre in a fluorescent molecule absorbs radiation depending exclusively on the basis of its absorption characteristics faithfully expells the energy almost in a duplicating pattern of the absorption phenomenon. Only difference in that the loss of energy in the process results in emission at longer wavelength. It is therefore expected that absorption and emission curves have comparable profile. Also it is necessary to remember that the profile of the emission line is dependent on exciting wavelength. Just as in absorption phenomenon the intensity of emission is a function of population of absorbing centers and pertinent selection rules restricting the number of absorbing centers are valid. The nature of excitation of the absorbing centers (i.e. absorbing atoms) is explained by drawing Morse potential energy diagrams for the ground and the excited states. According to Franck-Condon principle the life-time of excitation from ground state to excited state is of the order of 10^{-15}s. The change in bond length resulting from vibration is not seen in the diagram since the bond length of an excited

molecule is usually greater than the ground state molecule. The excitation molecule finds itself in a highly compressed state. This excess vibration energy is rapidly lost and gets transferred to the neighbouring molecules. If the excited molecule initially under compression mentioned above were at higher vibrational level of the excited state, on dissipation of energy it will comedown to lowest vibration level in the excited state. At this stage the molecule has an option to revert to ground state by emission of light. Since due to loss of vibrational energy, the emitted photon has less energy than the absorbed photon and therefore the emission spectrum is shifted to longer wavelengths with respect to the absorption spectrum as per Stokes' law. It is also obvious that intensity of absorption or emission depends on the amount of overlap of the vibrational wavelength function of ground and excited states. It is also obvious that the mirror image of emission and absorption spectrum indicates the similarity of the respective vibrational wave functions in the excited and ground states. If such symmetry is lacking it must be a result of some hindrance to the relaxation processes in the excited and ground state [23].

1.7.3 Excitation spectra

Excitation spectrum of an organic molecule is obtained by observing the variation in the luminescence quantum intensity with change in excitation wavelength. The internal conversion from higher excited state to the lower excited state is very

straight forward, easy and efficient process in most of the organic molecules and therefore the quantum yield is independent of excitation wavelength even fof such wavelength independent processes. Excitation spectra are very much useful for measuring absorption spectra of extremely dilute solutions. This is possible because for dilute solution the quantum intensity I is proportional to the extinction coefficient,

$$I = I_o (1 - 10^{-\epsilon cd}) q \qquad \text{----- 1.3}$$

$$I = I_o \epsilon cdq \qquad \text{--- 1.4}$$

when $\epsilon cd \ll 1$

where I_o is the incident excitation intensity, q is the quantum efficiency and $(1-10^{-\epsilon cd})$ is the fraction of photons absorbed for concentration c and sample thickness d. This means that the excitation spectra and absorption spectra are identical for extremely dilute solution. Parker has shown that an excitation spectra can be run quite accurately down to 10^{-12} M and this is an advantage, since absorption spectra are hardly reliable below 10^{-8} M.

1.7.4 Life Time and Rate Parameters

The decay time of fluorescence of organic molecules is generally 10^{-9}s and hence experimental procedures require skill and sophisticated instruments based on either stroboscopic or phase-shift techniques.

The molecules absorb radiation and then may re-emit this excitation energy as fluorescence. The emission probability for any excited state is directly proportional to the related absorption probability, therefore, the radiative transition probability or probability per unit time, K_{FM}, that an electron in a particular excited state will sink to the ground state, can be worked out from the absorption spectrum and the mean wavelength of emission. This radiation

$$K_{FM} = 2.88 \times 10^{9}\, n^{2} \langle \bar{\nu}^{-3} \rangle_{av}^{-1} \int \frac{\epsilon(\bar{\nu})\, d\bar{\nu}}{\bar{\nu}} \qquad \text{---- 1.6}$$

where n is the refractive index and the medium $\epsilon(\bar{\nu})$ is the extinction coefficient. $(\bar{\nu}^{-3})_{av}^{-1}$ is associated with mean wave-number of the fluorescence spectrum. $F_M(\bar{\nu})$ is given by

$$\{\bar{\nu}^{-3}\}_{av}^{-1} = \frac{F_M(\bar{\nu})\, d\bar{V}}{F_M(\bar{\nu})\, d\bar{\nu}/\bar{\nu}^{3}} \qquad \text{-------- 1.7}$$

This was based on assumption that the absorption and fluorescence spectra are sharp and fully overlapping. Further work of Strickler and Berg considers broad band molecular spectra [24] Birks and Dyson modified the equation as follows :

$$K_{FM} = 2.88 \times 10^{-9}\, n_f^3/n_a \langle \bar{\nu}^{-3} \rangle_{av}^{-1} \int \frac{\epsilon(\bar{V})\, d\bar{\nu}}{\bar{\nu}} \qquad \text{... 1.8}$$

where n_f and n_a are refractive indices at the emission and absorption wavelengths respectively.

Since K_{FM} is known which is the probability per unit time that the electron will fall to a lower energy state, the average

length of time that the electron remains in the excited state will be $1/K_{FM}$ which is radiative life time τ_o, of the excited state. The fluorescence life time, τ depends on the competition between the radiative and non-radiative processes. If K_{GM} is non-radiative rate parameter for internal conversion and K_{IM} is the non-radiative rate parameter for intersystem crossing from the lowest excited state S_1 the sum of non-radiative parameters is $K_{GM} + K_{IM}$. The total probability per unit time for the transition S_1 and S_o will be $K_{FM} + K_{IM}$ therefore observed life time will be

$$\tau = \frac{1}{K_{FM} + K_{IM}} \qquad \ldots 1.9$$

If M^* mol L^{-1} is the concentration of excited singlet state, molecules at dM^*/dt is the rate of change of M^* after excitation by a δ-pulse for a transition and

$$\frac{dM^*}{dt} = -(K_{FM} + K_{IM})\ [M^*] \qquad \ldots 1.10$$

The fluorescence quantum intensity I at any instance is

$$I = K_{FM}\ [M^*] \qquad \ldots 1.11$$

On integration of equation 1.10 we get expression for exponential decay as

$$I = I_o \exp -(K_{FM} + K_{IM})t = I_o \exp\left(-\frac{t}{\tau}\right) \qquad \ldots 1.12$$

1.7.5 Quantum Efficiency and Quantum Yield

The ratio of number of fluorescence photon to the number of fluorescence photon absorbed is known as quantum efficiency yield q_{FM}

$$q_{FM} = \frac{K_{FM}}{K_{FM} + K_{IM}} = \frac{\tau}{\tau_0} \qquad \ldots 1.13$$

q_{FM} and τ are measured whereby it becomes possible to determine and K_{FM} and K_{IM} quantum efficiency and quantum yield are distinctly different. The fraction of excited molecules which may take up a given radiative or non-radiative path way is defined as quantum yield. Triplet quantum yield Φ_{TM} is given by

$$\Phi_{TM} = \frac{K_{TM}}{K_{TM} + K_{GM} + K_{FM}} = K_{TM}\tau \qquad \ldots 1.14$$

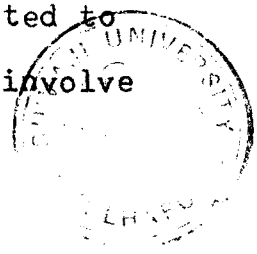
This gives the fraction of molecules in the excited state S_1 which will cross to T_1 by intersystem crossing. From this phosphorescence quantum efficiency q_{PT} can be calculated

$$q_{PT} = \frac{K_{PT}}{K_{PT} + K_{GT}} \qquad \ldots 1.15$$

and therefore Φ_{PT} the phosphorescence quantum yield is

$$\Phi_{PT} = \Phi_{TM} \cdot q_{PT} \qquad \text{--- --- 1.16}$$

It is clear now that the term quantum efficiency is related to the fundamental related processes but quantum yield may involve any other processes.



1.8 APPLICATIONS OF FLUORESCENCE SPECTROSCOPY

A complete listing of applications of fluorescence will run in large bulk hence a brief listing is only presented here.

Qualitative and quantitative determination of about 250 species is given in Meites' Hand Book of Analytical Chemistry [25-32].

The applications in analytical chemistry are the study of fluorescent metal chelates and use of luminescence indicators in titrimetry. The technique finds the use in analysis of agricultural produce and in bioscience. There are many applications in proteins, amino acids, vitamins and steriods. There are a host of clinical micromethods. In clinical examination fluorometer is used to estimate Catacholamine, Serotonin, Histamine, Estrogen, Estradiol, Estriol, Equiline, Equilenin, Corticosterone, Cortisol, Pregnandiol, Androsterone, Progesterone and vitamins.

Pharmacological analysis of crude drugs, quality control of pharmceuticals and study of metabolism of medicine related to Barituric acid, tetracycline, Aspirin, Morphine, Amphetamine, Phenothiazins, LSD, Tetrahydrocannabinol, Adenine, Adenosine - ATP and ADP, Guthionpurine and Thyopyridine. In biological work it is mainly applied in enzyme, co-enzyme assay. Canning industry makes use of fluorometry to analyse packed foods to measure amino acid and vitamin contents. In polution chemistry it is mainly used in analysis of carcinogen and pesticide residues.

- B -

PHTHALEIN DYES

1.9 SYNTHESIS OF PHTHALEIN DYES

A phenol is mixed with phthalic anhydride with a few drops of sulphuric acid or fused zinc chloride and heated. The reaction product on pouring in water gives an impure mass, which when treated with sodium hydroxide solution dissolves out and intensely coloured form of the phthalein dye is formed [33]. If resorcinol is used in the preparation we get fluorescein. In order to enhance the solubility a sulphonic group must be introduced, for this purpose, o-sulphobenzoic anhydride is used in place of phthalic anhydride. The dyes prepared from sulphobenzoic anhydride are known as sulphonephthalein dyes [34]. These compounds find use in titrimetric analysis as acid-base, precipitation, fluorescence or redox titrations. The esters of phthalein dyes find use in kinetic methods of analysis of some enzymes [35].

1.10 IONIC EQUILIBRIA IN PROTONATION AND DEPROTONATION OF PHTHALEIN DYES

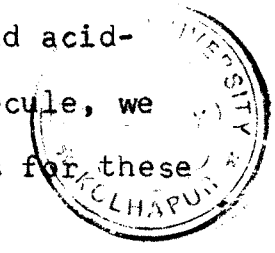
The structural framework of phenolphthalein when in solid state are prepared by acid precipitation has a lactone structure. The lactone form is insoluble in water and more soluble in alcohol. On addition of alkali a deep purple or blue colour is obtained and therefore they are useful as chemical indicators. On addition of dilute alkali deeply coloured quinonoid structure is obtained

which on addition of very strong alkali changes to colourless carbinol base.

Phenolphthalein forms esters which are useful in biochemical work. Cresolphthaleins were coupled with iminodiacetic acid or amino acetic acid to give metallochromic cresolphthalein complexones, calmagite , Naphthol violet [36], Phthalein violet, Glycine cresol red [37], Glycine thymol blue [38], Methyl thymol blue [39], Xylenol orange [40-48].

1.11 USE OF PYROMELLITIC ACID IN THE SYNTHESIS OF PHTHALEIN DYES (MELLITEIN DYES)

It is possible to create exactly analogous work by replacing pyromellitic acid anhydride in place of phthalic anhydride [49]. Pyromellitic acid dianhydride allows coupling with two molecules of phenol which may be considered as a diphenolphthalein. Likewise same coupling with two molecules and resorcinol may give what we may call as difluoromellitein. However it is possible to couple different phenols to give a series of mixed indicator type molecules. In case one of the phenols is fixed as resorcinol and the other is varied, a series of fluorescein-like compounds is obtained. As has been said earlier, one should not expect that there will be an existance of fluorescein-like part and phenolphthalein-like part, operating independent of each other. On the contrary we can expect that since fluorescein and acid-base character are both attributable to the entire molecule, we can confine the expectation to having graded properties for these



molecules so that there will be slightly varying optical character combined with feeble substituent effect and different degree of reactivity. We have at our disposal a pliable system which can be adopted to specific uses. In our laboratory we intend to explore and exploit these interesting molecules.

1.12 FLUORESCEIN AND FLUORESCENCE

Fluorescein was basically introduced as an acid-base indicator to substitute litmus. Its analytical uses as acid-base indicator became shadowed when the use of eosin in precipitation titration was known (Table 1.1). Several other derivatives of fluorescein were reported. The use of pyromellitic acid dianhydride made it possible to have several analogues of fluorescein in which half the part of molecule will have sufficiently strong cromogenic function. This system is worth exploiting in a manner parallel to that of fluorescein.

1.13 RECENT WORK ON FLUORESCEIN

Recent work on fluorescein is related to exploration of many fine details of the solution chemistry studied by fluorometry. Koichi studied the pH transition intervals of uranin and dichlorofluorescein in mixed aqueous solvents [50]. Doshkeivich and Kisarev worked out the full details of synthesis and purification of fluorescein [51]. Histochemical properties of the dye were studied by Csuri [52]. The red shift in alkaline solution of fluorescein was studied by Tripathi *et al.* [53]. The same

Table No. 1.1

No.	Mechanism of action	Colour change	Titratable ions (the titrant in paranthesis)	Trivial name	Chemical name
1	Surface precip.	greenish-yellow pink	Cl, Br, I, SCN (Ag)	Fluorescein	Resorcinol phthalein
2	"	greenish-yellow pink	Cl, Br, I, (Ag)	-	Dimethyl (R) fluorescein
3	"	greenish-yellow pink	Cl, Br, I, (Ag)	-	Dichloro (P) fluorescein
4	"	greenish-yellow red	Cl, Br, I, (Ag)	-	Dichloro (R) fluorescein
5	"	yellowish-red reddish-violet	Br, I, (Ag)	phloxin BA extra	Tetrachloro (P) tetrabromo (R) fluorescein
6	"	yellowish-red reddish-violet	Br, I, (Ag)	-	Dibromo (R) fluorescein
7	"	yellowish-red reddish-violet	Br, I, (Ag)	phloxin	Dichloro (P) tetrabromo (R) fluorescein
8	"	yellowish-red reddish-violet	Br, I, (Ag)	Eosin	Tetrabromo (R) fluorescein
9	"	yellowish-red reddish-violet	I (Ag)	Erythrosin	Diiodo (R) fluorescein
10	"	yellowish-red reddish-violet	I (Ag)	-	Dimethyl (R) diiodo (R) fluorescein
11	"	red bluish-red	I (Ag)	Rose bengal	Dichloro (P) tetraiodo (R) fluorescein
12	"	red reddish-violet	I (Ag)	Erythrosin B	Tetraiodo (R) fluorescein

authors further continued the study of the tetrabromoderivative [54]. Guyot et al. studied the cationic, neutral, mono and dianionic forms of fluorescein and calculated the quantum yield [55]. Pandya and Machwe studied the polarization of fluorescein spectrum and analysed the overlapping band of the dimeric form of the dye [56]. An exhaustive and interesting large paper has been written by Martin and Lindquist over a wide pH range covering 10 M H_2SO_4 to 0.01 M NaOH in aqueous solution. Kurucsev used the five parameter equations by using non linear least squares programme [57] and carried out vibronic analysis of the visible absorption and fluorescence spectra of fluorescein dianion [58]. The effect of halogen substitution on fluorescein was studied spectrophotometrically by Matveets, Scherbov and Akhmetove [59]. The red edge effect in excited states for protolytic reactions in fluorescein cation was studied by Shah, Joshi and Pant [60]. Radiobromination of fluorescein was reported by Gobuty, Spicer and Dressler [61]. Lopez studied the fluorescence quantum yield and corrections for reabsorption and reemission [62]. Paucescu and Ionescu showed that fluorescein is brominated to a degree of not reported so far [63]. Use of H_3PO_3 and reaction condition of use of 200-205° C temperature using 4:1:5 butanol acetic acid and water mixture are worked out for the synthesis of fluorescein when by product purpuroxanthene is not formed [64]. Abromochkin et al. gave procedure for improved yield of nitrofluorescein by using H_3PO_4 at 135-140° C [65]. Absorption and fluorescence spectra of bromo or iodo substituted fluorescence in water propionol and DMF at different concentrations and temperatures

in presence of various amounts of HCl were analysed by Zakhidow, Nizamov and Atakhodzhaev [66]. The effect of concentration on the emission spectra and decay of phosphorescence of fluorescence in boric acid glass was studied by Burak [67]. Idriss et al. studied the medium effect on the absorption spectra of fluorescence [68]. Fluorescein hydrazide reacts with H_2O_2 and hemin or ferricyanide in aqueous alkaline media [69]. Khanna applied for a patent for uses of alkyl substituted fluorescein derivatives [70]. The lactone and amphotenic forms of fluorescein and eosin and the stability of lactone ring in organic solvents showed that lactone ring in fluorescein is more stable than eosin while in the solid state or aqueous media the reverse is true [71]. Fluorescein S isothiocyanate on treatment with H-Asp. Arg. Val. Tyr-Ile-His-Pro-Phe-OH gave a new derivative which was purified and characterised by Barnes et al. Its infusion in the renal artary of anesthetised rat reduced the blood flow by 12-27 %. This compound may find use in characterisation of angiotestin receptors [72]. A patent has been filed by a Japanese firm that claims the four new alkoky substituted fluorescein derivatives are useful as fluorescent indicators for antigens and antibodies [73]. The cationic and mono and dianionic forms of fluorescein and its derivatives by IR, UV, H and ^{13}C NMR were studied and its was found that the positive charge of the cationic form is largely delocalised over the xanthene ring [74]. An amine derivative of fluorescein was prepared by Abramochikin et al. [75]. 3,5-Dichlorotriazinyl amino fluorescein confers fluorescence

to a large variety of biologically active compounds [76]. Photooxidation study of fluorescein showed that a neutral radical is generated by a photoreaction and was characterised [77]. The ionic equilibria involved in deprotonation of mono-protonated (OH^+-), neutral (COOH group) and anionic (OH group) forms of fluorescein in the normal S_o and S_1 states were studied and band assignments were made [78]. The spectral properties of fluorescein and carboxy fluorescein were measured by Babcock and Kramp [79]. The change of free energy, enthalpy and entropy products by dimerisation of trimerization of fluorescein, eosin and erythrosin were reported by Lopez [80]. Estradiol takes up fluorescein and forms a conjugation which is reported to be useful for study of vaginal epithelium of women of child bearing age [81]. The formation constants of dimers and trimers and their structures were studied by Lopez [82]. The recent work clearly shows the current trends in research and it can be said with certainty that more interest is being shown in biologically significant reactions and determinations.

1.14 CONCLUSION

From the above review it is obvious that interesting chemistry may possibly emerge from the study of mellitein dyes. In particular the set which exhibits fluorescence has great potential as a flexible system that may generate a series of useful compounds with small successive differences covering quite a range in several steps.