[illegible]

Relationship betn intensity of fluorescence & [illegible]

fluorescent $F = k(I_0 - I)$ ....(i)

I_0 = intensity of incident radiation

I = " " transmitted "

F = " " fluorescent

k = a proportionality constant

[illegible] that [illegible] cannot be applied directly. The above relationship [illegible]

F is assumed proportional to the intensity of [illegible] energy absorbed $(I_0 - I)$

Applying the Beer-Lambert law:

$$I = I_0 \cdot 10^{-\epsilon bc}$$

$$\Rightarrow I_0 - I = I_0 (1 - 10^{-\epsilon bc})$$

$$\Rightarrow k(I_0 - I) = k I_0 (1 - 10^{-\epsilon bc})$$

$$\Rightarrow F = k I_0 (1 - 10^{-\epsilon bc}) \quad [\text{from (i)}] \ldots$$

Writing $kI_0 = F_0$, we have

$$F = F_0 - F_0 \cdot 10^{-\epsilon bc}$$

$$\Rightarrow F_0 \cdot 10^{-\epsilon bc} = F_0 - F$$

$$\Rightarrow 10^{-\epsilon bc} = \frac{F_0 - F}{F_0}$$

$$\Rightarrow -\epsilon bc = \log \frac{F_0 - F}{F_0}$$

$$\Rightarrow \epsilon bc = \log \frac{F_0}{F_0 - F}$$

When ϵbc becomes small & approaches a [illegible] of [illegible] eq (ii) reduces to:

$$F = 2.303\, k I_0 \cdot \epsilon bc$$

$$\Rightarrow F = k' c$$

* $10^{-\epsilon bc} = e^{-2.3\epsilon bc}$

$e^{-x} = 1 - x + x^2 \ldots$

$1 - 10^{-\epsilon bc} \simeq \epsilon bc$ [illegible]

i.e. the fluorescent intensity is practically proportional to the conc of the fluorescent substance provided [illegible]

CHAPTER 4

Fluorometry

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4.1 INTRODUCTION

The mechanisms by which molecules absorb electromagnetic radiation in the visible and ultraviolet regions of the spectrum and are, as a result, raised

to excited electronic states were discussed in Chapter 1. The reverse process, loss of energy and concomitant transition of molecules from excited states to ground states of energy, can occur with reemission of radiation. Such emission is known as *luminescence*. The intensity and composition of the emitted radiation can be measured and such measurements form the basis of a sensitive method of analysis called *fluorometry*. Fluorometric methods of analysis have found application in many situations of pharmaceutical interest such as in the analysis of riboflavin, thiamine, and reserpine in drug dosage forms. More significant and widespread, however, has been the application of fluorometric techniques in the analysis of trace amounts of drugs and metabolites in biological tissues and fluids.

4.2 THEORY

Absorption of ultraviolet and visible light by molecules of an irradiated sample generates a population of molecules in excited electronic states. Each excited electronic state has many different vibrational energy levels, and excited molecules will be distributed in the various vibrational energy levels of an excited state. Most usually this state is a *singlet state*, i.e., one in which all of the electrons are paired and in each pair the two electrons spin about their own axes in opposite directions. It is intuitively apparent that since the amount of radiation absorbed by a sample does not decrease if radiation is continued, efficient rapid processes must be operant which result in the loss of energy by excited molecules and their return to the ground state. It has been calculated, in fact, that the average lifetime of a molecule in the singlet excited state is of the order of 10^{-8} sec. Molecules at each vibrational level of the excited state could, for example, lose energy by emitting photons and as a result fall to the original condition of the ground state. The energy and, therefore, the wavelength of emitted light would then be exactly the same as that absorbed. Such a process is termed *resonance fluorescence*. It is an improbable process and is rarely encountered in solution chemistry. Rather, molecules initially undergo a more rapid process, a radiationless loss of vibrational energy, and so quickly fall to the lowest vibrational energy level of the excited state. The vibrational energy is thought to be lost to solvent molecules. The process is known as *vibrational relaxation*. From the lowest vibrational level of the excited state, a molecule can either return to the ground state by photoemission or by radiationless processes. If indeed the former occurs, the emission is a type of luminescence referred to as *fluorescence*. Fluorescence is defined as the radiation emitted in the transition of a molecule from a singlet excited state to a singlet ground state. Because of vibrational relaxation in the excited state and because a molecule may return to a vibrational level in the ground state which is higher than that initially occupied prior to excitation, the radiation emitted as fluorescence is of lower energy and, therefore, of longer wavelength than that originally absorbed.

Other processes involving the excited state can occur to compete with fluorescence emission, and not all of the absorbed energy will be emitted as fluorescence. The extent to which such other processes occur is characterized by a parameter known as the *quantum efficiency of fluorescence* which is symbolized by ϕ and defined as the ratio of the number of light quanta emitted to the number absorbed. Quantum efficiency approaches 1 for highly fluorescent compounds and 0 for those which fluoresce weakly. It is interesting and pertinent to consider the processes which occur to decrease the efficiency of fluorescence. An excited molecule could, for example, undergo a radiationless loss of energy sufficient to drop to the ground state. This process is termed *internal conversion*. With some compounds a process known as *intersystem crossing* can also occur. Here a molecule in the lowest vibrational level of the excited state converts to a triplet state, a state lying at an energy level intermediate between ground and excited states and characterized by an unpairing of two electrons. Thus, in contrast to the singlet state, there is a spin reversal involving one electron of a pair and the two electrons spin about their axes in the same direction. Once intersystem crossing has occurred, a molecule quickly drops to the lowest vibrational level of the triplet state by vibrational relaxation. The triplet state is much longer lived than the corresponding singlet state with lifetimes of 10^{-4} to 10 sec. From the triplet state a molecule can drop to the ground state by emission of radiation. This type of luminescence is termed *phosphorescence* and is formally defined as emission of radiation resulting from the transition of a molecule from a triplet excited state to a singlet ground state. Phosphorescence is often characterized by an afterglow, i.e., because of the long life of the triplet state, luminescence can be observed after the source of exciting radiation has been removed. In contrast, no afterglow is observed in fluorescing systems because of the short life of the singlet excited state. A molecule in the triplet state can also undergo radiationless conversion to the ground state. Such a conversion is enhanced by the relatively long life of the triplet state so that collisions fruitful in dissipating energy can occur and by the fact that the energy difference between triplet state and ground state is not inordinately large. These processes are diagrammatically illustrated by Fig. 4.1.

4.3 FLUORESCENCE AND CHEMICAL STRUCTURE

Quantitative aspects of the processes which can involve the excited electronic state are not sufficiently well understood to permit predictions as to whether or not a particular compound will fluoresce to a degree necessary for analytical purposes. Fluorometric methods are, of course, limited to those compounds which possess a system of conjugated double bonds. A compound must absorb radiation in order to fluoresce, and it is the presence within a molecule of the mobile π electrons which is responsible for absorption

characteristics in the visible and ultraviolet regions of the spectrum. The presence of a "chromophore" does not necessarily endow a compound with the ability to fluoresce since radiationless processes and intersystem crossing can occur to decrease the quantum efficiency of fluorescence to zero or to a degree that makes practical measurement impossible. It would be expected, however, that structural features which influence the degree of conjugation of a molecule and the delocalization of π electrons might influence the likelihood

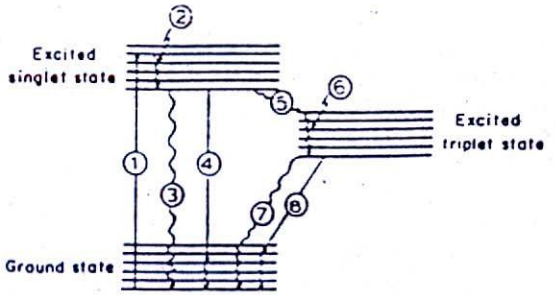
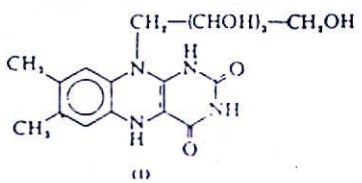
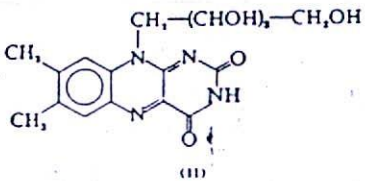


FIGURE 4.1: A diagrammatic representation of the changes in energy levels of a molecule that can occur as a result of the absorption of electromagnetic radiation: (1) absorption of radiant energy boosting molecules to various vibrational energy levels of the excited singlet state; (2) radiationless vibrational relaxation to the lowest vibrational level of the excited singlet state; (3) radiationless internal conversion from excited singlet state to ground state followed by vibrational relaxation; (4) fluorescence followed by vibrational relaxation; (5) intersystem crossing from excited singlet state to excited triplet state; (6) vibrational relaxation to the lowest vibrational level of the excited triplet state; (7) radiationless internal conversion from excited triplet state to ground state followed by vibrational relaxation; (8) phosphorescence followed by vibrational relaxation.

of measurable fluorescence. Thus, for example, saturated compounds such as cyclohexane are nonfluorescent, while benzene is weakly fluorescent, and highly unsaturated polycyclic aromatic compounds such as anthracene are strongly fluorescent. Similarly, the reduced form of riboflavin (I) does not have the degree of conjugation of the parent compound (II) and does not possess the fluorescence characteristics of riboflavin.



(I)



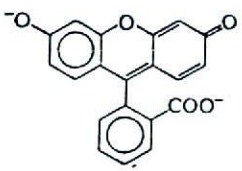
(II)

Definite correlations between chemical structure and fluorescence cannot be made. However, the work and review of Williams and Bridges[1] does provide some insight to the influence of structural features on the fluorescence of

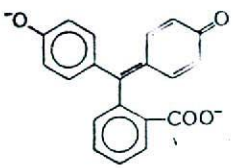
organic compounds. Monosubstitution of benzene with alkyl groupings, for example, was found to have little influence on the intensity of fluorescence of the substituted benzene relative to that of benzene. However, monosubstitution with groups known to increase electron delocalization (ortho-para-directing groups), such as fluoro, amino, alkylaminodialkylamino, hydroxy, and methoxy, yielded compounds that fluoresced more intensely than did the parent compound. Substitution with iodine, chlorine, and bromine resulted in benzene derivatives which either did not fluoresce or fluoresced to a lesser degree than benzene in spite of the fact that these substituents are also ortho-para directing. This influence of halogen substitution was also reported by McClure[3] and is apparently due to an enhanced intersystem crossing process since bromine- and iodine-substituted aromatic compounds exhibit intense phosphorescence but only weak fluorescence. Most meta-directing substituents (which tend to localize π electrons) were found to markedly decrease fluorescent intensity. Thus, benzoic acid, nitrobenzene, benzenesulfonic acid, benzenesulfonamide, and benzaldehyde were found to be nonfluorescent: Benzonitrile, in contrast, fluoresced more intensely than benzene, even though the C≡N group is meta-directing. It was postulated that electrons of the C≡N group interacted with the π electrons of the benzene ring to result in a distribution that favored fluorescence.

The fluorescence characteristics of disubstituted benzenes were also studied, but few generalities could be generated from the results. Observed effects were not predictable and apparently were the result of a combined influence on the mobility of π electrons. For example, it might be expected that substitution of the fluorescent compound, aniline, with a meta-directing group such as $-SO_2NH_2$ would result in a compound which would fluoresce to a lesser degree than aniline. Sulfanilamide, however, was found to be five times as fluorescent as aniline. Similarly, guides to predicting the behavior of heterocyclic compounds could not be made because of the uncertainty of the substituent effect. In general, it was found that a doubly bonded nitrogen (=N—) in a ring tended to decrease the likelihood of fluorescence, while the presence of —NH—, —O—, and —S— appeared to contribute to the likelihood of fluorescence.

Molecular geometry must also be considered in attempting to relate chemical structure and fluorescence. That geometric considerations are important is well illustrated by examples cited by Wehry and Rogers.[3] Fluorescein (III), for example, is highly fluorescent, while phenolphthalein (IV) is nonfluorescent. The oxygen bridge in fluorescein imparts to the molecule a rigidity and



(III)

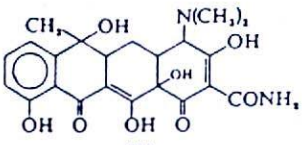


(IV)

planarity that is not present in phenolphthalein. The absence of a planar, rigid structure permits vibrations and rotations of the aromatic rings to occur which result in radiationless dissipation of excitation energy. Similarly, with a number of compounds that exhibit *cis-trans* isomerism, the *cis* isomer fluoresces much less intensely than the trans. Stilbene (V) is such a compound. This can also be ascribed to a planarity effect with the *cis* isomer being non-planar due to the bulkiness of the aromatic rings.

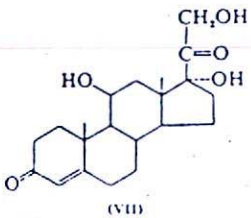
(V)

That a compound does not fluoresce or has a low intensity of fluorescence does not necessarily dismiss fluorometry as a potential tool for the analytical determination of that compound. Many well-accepted and widely used fluorometric procedures are based on chemical conversions of weakly fluorescing compounds to derivatives which fluoresce intensely. For example, tetracycline (VI) has a weak native fluorescence, but complexes of the antibiotic with Ca^{2+} and a barbiturate fluoresce quite intensely.[4] Corticosteroids



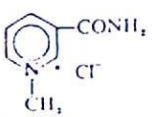
(VI)

such as hydrocortisone (VII) do not fluoresce. However, they form, in concentrated sulfuric acid and in the presence of ethanol, strongly fluorescing compounds.[5] *N*-Methylnicotinamide (VIII) is determined in biological fluids



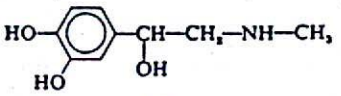
(VII)

by a fluorometric method even though it has little native fluorescence. Here the amide is condensed with acetone and treated with base to yield fluorescent



(VIII)

products.[6] Similarly, epinephrine (IX) is assayed fluorometrically by measuring the fluorescence of products resulting from oxidation and hydroxylation.[7]



(IX)

4.4 INSTRUMENTATION FOR FLUOROMETRY

In contrast to spectrophotometry, the intensity of light transmitted by a sample is not of direct concern in fluorometry. Rather, it is the intensity of radiation that is emitted as fluorescence that is measured and related to the

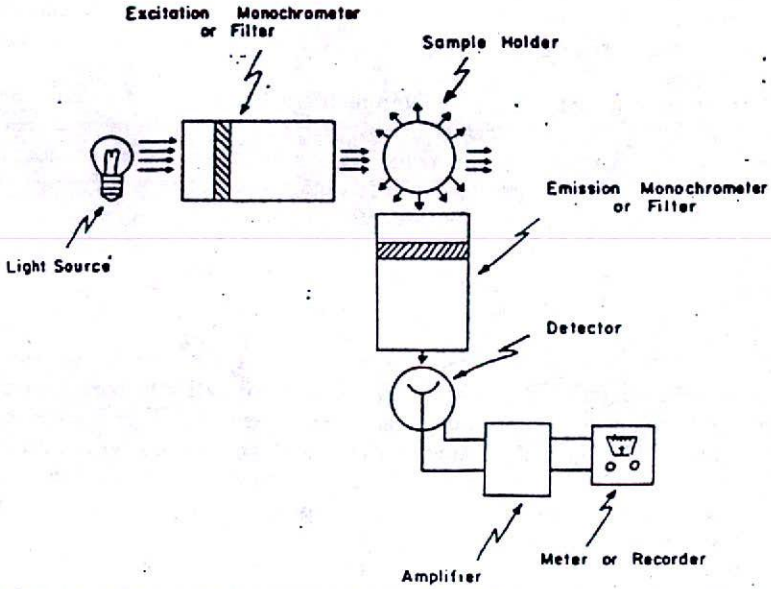


FIGURE 4.2: A diagrammatic representation of an instrument used to measure intensity of fluorescence.

concentration of fluorescing species. The components of instruments which are used in fluorometry are, however, quite similar in design and function to those employed in spectrophotometers and colorimeters. A diagrammatic representation of such a device is shown in Fig. 4.2.

The chief components are: a source of exciting radiation, an excitation filter or monochromator by which a band of exciting light can be isolated to be passed on to the sample, a sample holder, an emission filter or monochromator by which a band of fluorescence can be selected for detection, a

detector, and some means for amplifying and indicating the detector response. In most commercially available instruments, the detector is placed at a right angle to the direction of travel of the beam of exciting light. This arrangement has been found to be the most advantageous for measuring the fluorescence of dilute solutions. Other arrangements are possible, however, since fluorescence is emitted in all directions. The light source is usually a mercury or xenon arc. Those instruments which use filters as coarse monochrometers are referred to as *fluorometers*. Those employing more sophisticated and exact grating or prism monochromators are termed *spectrofluorometers*, *fluorescence spectrometers*, or *spectrophotofluorometers*. The first term appears to be the one most commonly used to designate this type of instrument. The other components of an instrument such as sample holders, detectors, amplifying and indicating devices are much the same as those discussed under spectrophotometry.

Spectrofluorometers are used in fluorometric work in a manner analogous to the use of spectrophotometers in absorption spectrophotometry. They enable an investigator to generate two types of spectra which are pertinent to fluorometry. The excitation spectrum is obtained by setting the emission monochromator at a suitable wavelength and measuring the intensity of fluorescence as a function of the wavelength of the exciting radiation. In theory, the maxima and minima exhibited by the excitation spectrum should be at wavelengths which are identical to those found in the absorption spectrum of the compound. In practice, exact coincidence may not be found due to instrumental artifacts. The *emission spectrum* of a compound is obtained by setting the excitation monochrometer at an appropriate wavelength corresponding to strong excitation and measuring the intensity of fluorescence as a function of the wavelength of emitted light. An example of excitation and emission spectra is shown in Fig. 4.3 for grisoefulvin in 1% ethanol. As would be expected from the considerations discussed in the theory section, the emission spectrum is found at longer wavelengths than the excitation spectrum. Some overlap of the two spectra is frequently observed.

Fluorometers are used in a manner somewhat analogous to colorimeters in absorption work. The excitation and emission spectra of a compound dictate the transmittance characteristics of filters that should be employed for a particular analytical determination with a fluorometer. The filters should be as much as possible mutually exclusive. That is, the emission filter should not pass wavelengths which are transmitted by the excitation filter. This precaution is necessary to preclude interferences from light which may be reflected by the sample holder and other parts of the instrument and from light scattered by the solvent used. Fluorometers are somewhat more sensitive than spectrofluorometers since filters pass a more intense radiation than prism or grating monochromators. Fluorometers are recommended for routine quantitative work, while spectrofluorometers are necessary research tools in the development of fluorometric assay methods.

A variety of fluorometers and spectrofluorometers are available from manufacturers of scientific equipment. Figure 4.4 is a schematic diagram of the optical system of a widely used filter fluorometer (model 110, G. K. Turner Associates). Figures 4.5 and 4.6 illustrate the appearance and optical characteristics of a spectrofluorometer (Aminco-Bowman spectrophotofluorometer,

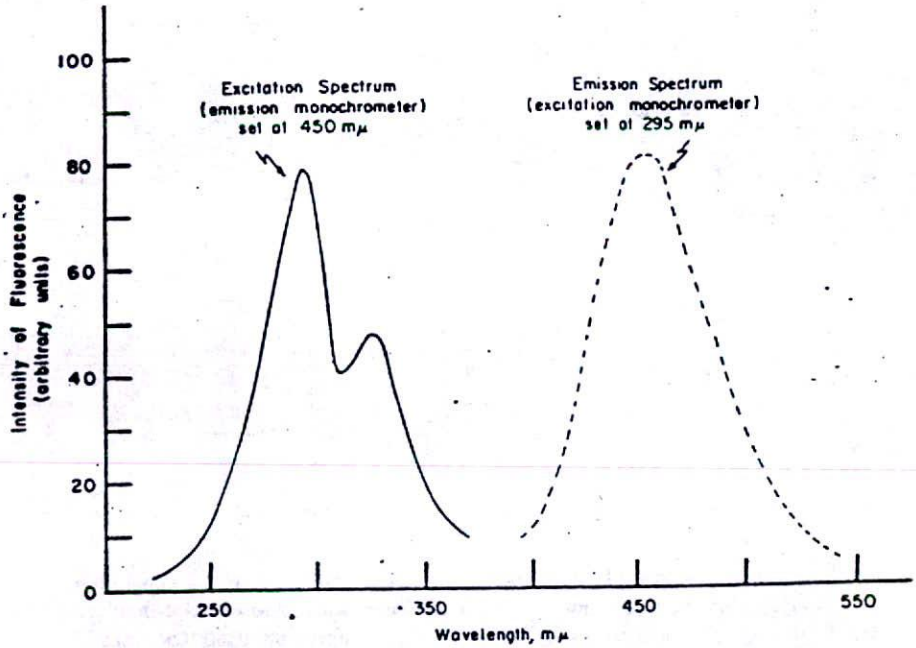


FIGURE 4.3: Excitation and emission spectra of griseofulvin in water containing 1% ethanol. Reprinted from Ref. 41, p. 365, by courtesy of *Nature*.

American Instruments Company, Inc.). The characteristics and features of many available instruments have been the subject of recent excellent reviews.[8,9] It is recommended that the reader consult such reviews and the literature available from manufacturers for specific information on commercially available instruments.

4.5 FACTORS INFLUENCING INTENSITY OF FLUORESCENCE

A. CONCENTRATION OF FLUORESCING SPECIES

The relationship between observed intensity of fluorescence and the concentration of fluorescing species is considerably more complex than that

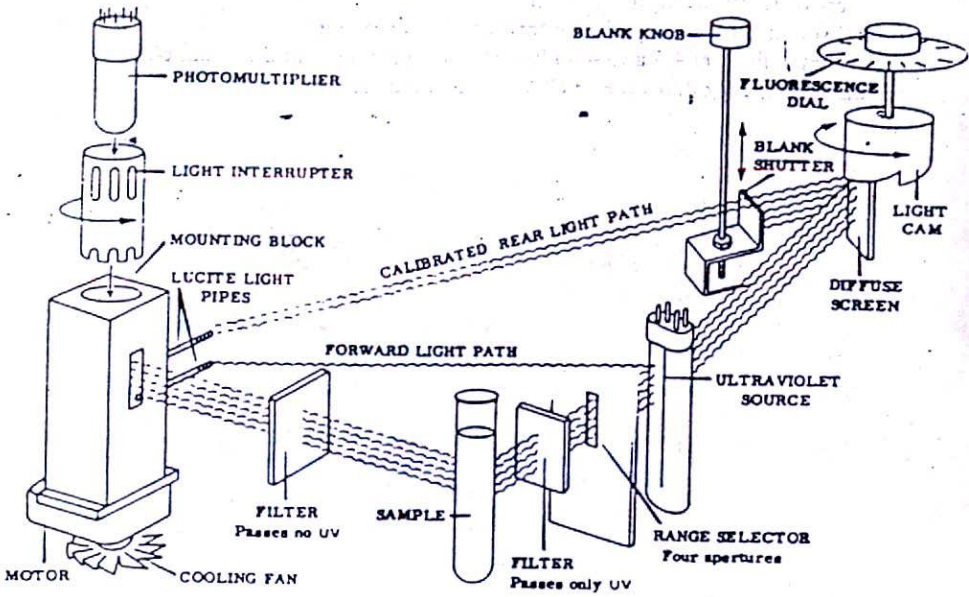


FIGURE 4.4: A schematic diagram of the optics of the Turner, model 110 fluorometer. Courtesy of G. K. Turner Associates.

between absorbance and the concentration of absorbing species which is dictated by Beer's law. Complexities arise from both theoretical considerations and from practical aspects of the instrumentation used to measure fluorescence. It might be intuitively anticipated that a linear relationship might not exist since fluorescence intensity would be expected to be proportional to the concentration of molecules in the excited state and, therefore, proportional to the intensity of radiation responsible for excitation. However,

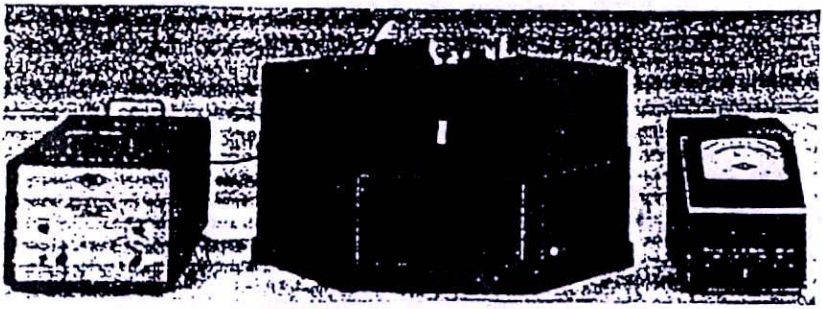


FIGURE 4.5: The Aminco-Bowman spectrophotofluorometer. Courtesy of the American Instrument Company, Inc.

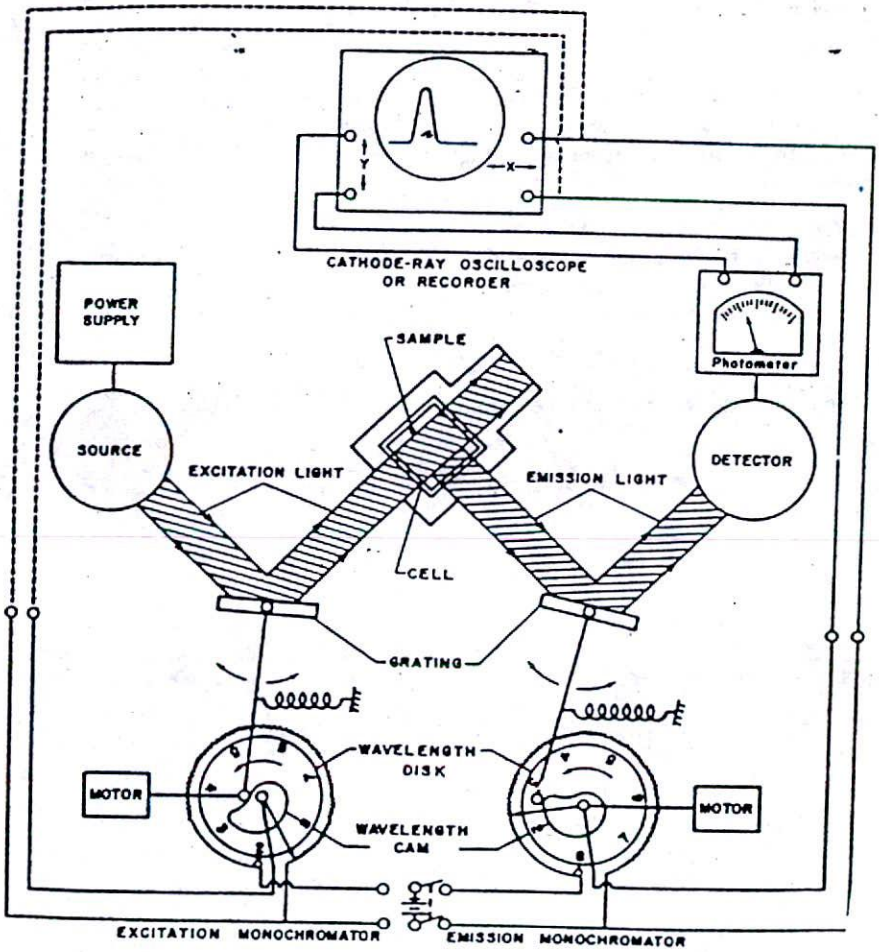


FIGURE 4.6: A schematic diagram of the optics of the Aminco-Bowman spectrophotofluorometer. Courtesy of the American Instrument Company, Inc.

light is absorbed by the sample and the intensity of exciting light does not remain constant, but diminishes as the light beam traverses a sample. If the solution is dilute, significant absorption will not occur and the decrease in intensity of exciting light will not be significant. In a more concentrated solution, the intensity of exciting light might well be different in different regions of the sample being irradiated. This consideration was formalized by

Kavanagh[10] and in more detail by Braunsberg and Osborn,[11] who reasoned that fluorescence intensity should be proportional to the amount of light absorbed by the sample, i.e.,

$$F = k\phi(I_0 - I) \tag{4.1}$$

where F is the intensity of fluorescence, k is the proportionality constant, ϕ is the quantum efficiency of fluorescence, I_0 is the intensity of light incident on a sample, and I is the intensity of light transmitted by a sample. Since, by Beer's law,

$$I = I_0 e^{-\epsilon bc} \tag{4.2}$$

where ϵ is the molar absorptivity of the compound at the wavelength of the exciting light, b is the path length along the axis of irradiation, and c is the concentration in moles per liter, Eq. (4.1) can be rewritten:

$$F = k\phi I_0(1 - e^{-\epsilon bc}) \tag{4.3}$$

The term in parentheses can be represented by a series expansion

$$e^x = 1 + x + \frac{x^2}{2!} + \cdots + \frac{x^n}{n!}$$

to yield:

$$F = k\phi I_0 \epsilon bc\left[1 - \frac{\epsilon bc}{2!} + \frac{(\epsilon bc)^2}{3!} - \cdots + \frac{(\epsilon bc)^n}{(n+1)!}\right] \tag{4.4}$$

The detector of a fluorometer does not measure total fluorescence intensity, but rather the intensity from only a segment of the sample. Equation (4.4) must then be modified:

$$S_f = k\phi g\theta I_0 \epsilon bc\left[1 - \frac{\epsilon bc}{2!} + \frac{(\epsilon bc)^2}{3!} - \cdots + \frac{(\epsilon bc)^n}{(n+1)!}\right] \tag{4.5}$$

where S_f is the electric signal generated by the detector, g is the constant reflecting the sensitivity of the detector and the amplification of the detected signal, and θ is the constant reflecting the geometry of the system, particularly the solid angle of light viewed by the detector. As recently discussed by Hercules,[12] there are two concentration regions where Eq. (4.5) can be conveniently simplified. When the concentration c is very small, the equation can be approximated by:

$$S_f = k\phi g\theta I_0 \epsilon bc \tag{4.6}$$

Such an approximation is reasonably valid when $\epsilon bc < 0.05$. Under such conditions, a linear relationship between measured intensity of fluorescence and concentration exists. When the concentration is large, $e^{-\epsilon bc}$ approaches zero and Eq. (4.5) can be approximated by:

$$S_f = k\phi g\theta I_0 \tag{4.7}$$

Under this concentration condition, measured intensity is independent of

concentration. At concentrations intermediate to the two extremes, a non-linear relationship would be theoretically expected. It is interesting and relevent to note that the concentration range over which linearity is theoretically expected is dependent on the molar absorptivity of the compound. This is illustrated in Fig. 4.7, which shows the types of intensity-concentration profiles which can be theoretically expected for compounds having different absorptivities. It can be seen that over the concentration range of the graph,

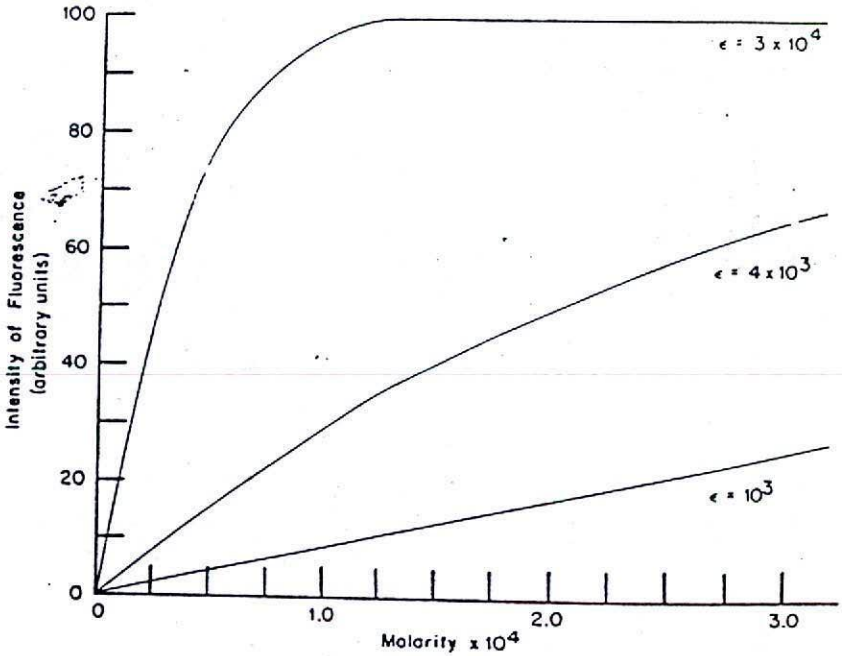


FIGURE 4.7: Intensity-concentration profiles for three different solutes having different molar absorptivities. Reprinted from Ref. 12, p. 31 A, by courtesy of *Analytical Chemistry*.

a linear relationship holds for the compound with the lowest absorptivity, while the compound with a high absorptivity exhibits a linear relationship over a rather small range of concentrations.

A further complication arises if the excitation and emission spectra of the compound overlap. In such a case, photons emitted as fluorescence can be absorbed in exciting other molecules and will not be measured as fluorescence. In dilute solution, such an occurrence will probably not be significant. However, in concentrated solutions, self-absorption of fluorescence radiation can occur and will result in a measured intensity which is less than that predictable on the basis of Eq. (4.5).

A more serious problem which is concentration-related can result due to the geometry of the measuring system. A detector set to view fluorescence at a 90° angle "sees" only a small band in the center of the sample. With dilute solutions, this band emits fluorescence which is representative of the whole cell. However, if the sample is concentrated, sufficient light absorption might occur so that the portion sensed by the detector is only weakly irradiated. This results in the phenomenon of *concentration reversal*, i.e., an

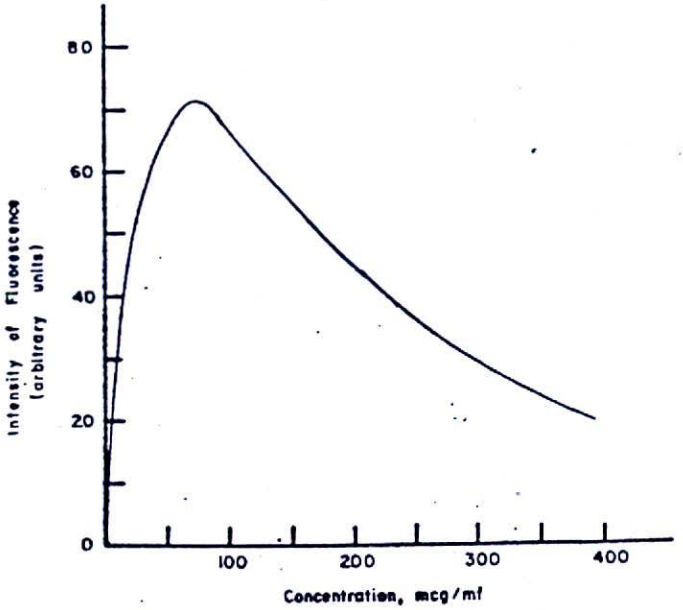


FIGURE 4.8: A plot showing the influence of concentration on the intensity of fluorescence of solutions of phenol. The excitation wavelength was 295 mμ and the emission wavelength was 330 mμ. Reprinted from Ref. 1, p. 377, by courtesy of the *Journal of Clinical Pathology* by permission of the authors, the editor, and the publishers, B.M.A. House, Tavistock Square, London W.C.1.

increase in concentration results in a decrease in measured fluorescence intensity. Such behavior is illustrated in Fig. 4.8 for phenol.

For these reasons, fluorometric measurements made for assay purposes are restricted to dilute solutions where a linear standard curve can be obtained. In general, a linear response can be expected for solutions which absorb less than 5% of the exciting radiation. It is apparent from Eq. (4.6) that a decrease in measured fluorescence resulting from a reduction in concentration can be compensated for by an increase in the intensity of exciting radiation and/or an increase in the sensitivity of the detector. Because of this, accurate measurements can be made on relatively dilute solutions and a wide range of

concentrations can be covered in the linear portion of the intensity-concentration profile. This is in marked contrast to absorption spectrophotometry, where a limited range of concentration is necessary for accurate measurements.

6. PRESENCE OF OTHER SOLUTES

The presence in a sample of solutes other than the solute being analytically determined can influence the intensity of fluorescence by one or more of a number of different effects.

1. Fluorescent Impurities

An obvious possibility is that another component of the solution might also fluoresce and thus interfere with the determination. Impurities introduced into the sample from solvents, buffers, detergents which are residual on glassware, and from the source of the sample can fluoresce and can introduce error. This possibility should always be anticipated in fluorometric work, particularly if measurements are made on very dilute solutions, and especially if the excitation radiation is in the ultraviolet region of the spectrum. Appropriate precautions must be taken which include the use of pure solvents and chemical reagents and cleanliness in all operations.

2. Inner-Filter Effect

The presence in solution of other solutes which are nonfluorescent can effect fluorescence intensity by the so-called inner-filter effect. The influence here is due to the absorption of light and is similar to the situation discussed previously where excitation and emission spectra overlap. Thus, if the nonfluorescent components absorb either excitation or emission radiation, a reduction in measured intensity of fluorescence will result. Hercules[12] has considered some theoretical aspects of the inner-filter effect and has concluded that if absorption due to other species is constant and if absorption due to the fluorescing species is small, then a linear relationship between measured fluorescence and concentration should still be observed. In such a case, Eq. (4.6) assumes the following form:

$$S_f = k\phi g\theta I_0 \epsilon bc(K) \tag{4.8}$$

where K is the constant resulting from the presence of other absorbers in the system. It is apparent that if this effect is encountered the concentration of the nonfluorescent absorber must be eliminated or be maintained constant from sample to sample and a standard curve must be used which was determined at that concentration of absorber. An alternative might be to change the wavelength of excitation or emission radiation to minimize this effect.

3. Chemical Quenching

In addition to the effects just discussed, dissolved solutes can result in decreased fluorescence by at least two types of chemical quenching processes.

One is known as *collisional quenching* and results from a diffusion-controlled process in which a molecule of "quencher" interacts with an excited molecule of the potentially fluorescing substance. Interaction results in the dissipation of excitation energy not by fluorescence but by transfer of energy to the quenching molecule. A simplified mechanism can be written to describe this situation:

$$F + h\nu_e \xrightarrow{k_1} F^*$$

$$F^* \xrightarrow{k_2} F + h\nu_f$$

$$F^* + Q \xrightarrow{k_3} F + Q$$

Here, excitation radiation $h\nu_e$ converts the "fluorophor" F to the excited state. The excited molecule F^* can dissipate excitation energy by fluorescence $h\nu_f$ or by interaction with the quencher molecule Q. The rate constants k_1, k_2, and k_3 characterize the rates of the various processes. The intensity of fluorescence will be proportional to the steady-state concentration of molecules in the excited state. In the absence of quenching agent, this concentration is given by:

$$(F^*)_0 = \frac{k_1}{k_2}(F) \tag{4.9}$$

In the presence of quencher, the concentration of excited molecules will be reduced and is given by:

$$(F^*)_e = \frac{k_1(F)}{k_2 + k_3(Q)} \tag{4.10}$$

The ratio of intensities in the absence (f_0) of quencher to that in the presence (f_e) is, therefore,

$$f_0/f_e = 1 + k_3/k_2(Q) \tag{4.11}$$

Equation (4.11) is known as the Stern-Volmer law and predicts that the ratio of the two intensities will be linearly dependent on the concentration of quenching agent.

The molecular basis for dissipation of energy by collisional quenching is poorly understood. A recent hypothesis was discussed by Hercules[13] and involves electron transfer between excited state and quenching agent. It may be represented as follows:

$$F^* + Q \rightarrow F^-{:}Q^+$$

$$\swarrow \qquad \downarrow$$

$$^3F + Q \qquad F + Q$$

The excited molecule F^* interacts with quencher Q, abstracting an electron to form an ion pair $F^-{:}Q^+$. The ion pair can dissociate to give either a triplet state ^3F and Q or a ground state F and Q. Both processes result in thermal dissipation of energy.

Another type of quenching is called *static quenching*. Here, complex formation occurs between a potentially fluorescing molecule in the ground state and a quencher molecule. If the complexed form of the potentially fluorescing molecule has different spectral characteristics than the free form, it may not undergo excitation or may be excited to a lesser degree that the noncomplexed species. Suppose, for example, that complex formation occurred and resulted in the formation of a complex, with a stability constant of K_s, that was not fluorescent, i.e.,

$$F + Q \overset{K_s}{=} F{:}Q$$

$$F + h\nu_f \xrightarrow{k_1} F^*$$

$$F^* \xrightarrow{k_2} F + h\nu_f$$

The steady-state concentration of F^* in the absence of Q is given by

$$(F^*)_0 = k_1/k_2(F)_t \tag{4.12}$$

where $(F)_t$ is the total concentration of potentially fluorescing compound. In the presence of quencher,

$$(F^*)_q = \frac{(F)_t}{1 + K_s(Q)} \frac{k_1}{k_2} \tag{4.13}$$

The ratio of fluorescence in the absence to that in the presence of static quencher is then given by:

$$f_0/f_q = 1 + K_s(Q) \tag{4.14}$$

The reduction in fluorescence caused by this type of quenching is thus dictated by the stability constant of the complex and by the concentration of quenching agent.

Many examples of chemical quenching are found in the literature. Halide ions such as iodide and chloride are well known examples of collisional quenchers. Caffeine, related xanthines, and purines[14] have been shown to influence the fluorescence of riboflavin by static mechanisms. In the usual case, quenching is an undesirable effect and the possibility of encountering this type of interference should always be evaluated in developing a fluorometric assay. It is possible to utilize this phenomenon, however, as an analytical means for determining the concentrations of compounds known to quench fluorescence. In addition, it is apparent from Eq. (4.14) that it is possible to employ fluorometric methods for the investigation of complex-forming equilibria.

C. HYDROGEN-ION CONCENTRATION

The intensity of fluorescence emitted by solutions of weak acids and weak bases can exhibit a dependency on the pH of the solution. The effect here

may be due simply to a change in the degree of ionization of the weak electrolyte. For example, suppose that the fluorescence of a weak acid HA was investigated in dilute solution using an emission filter or monochromator

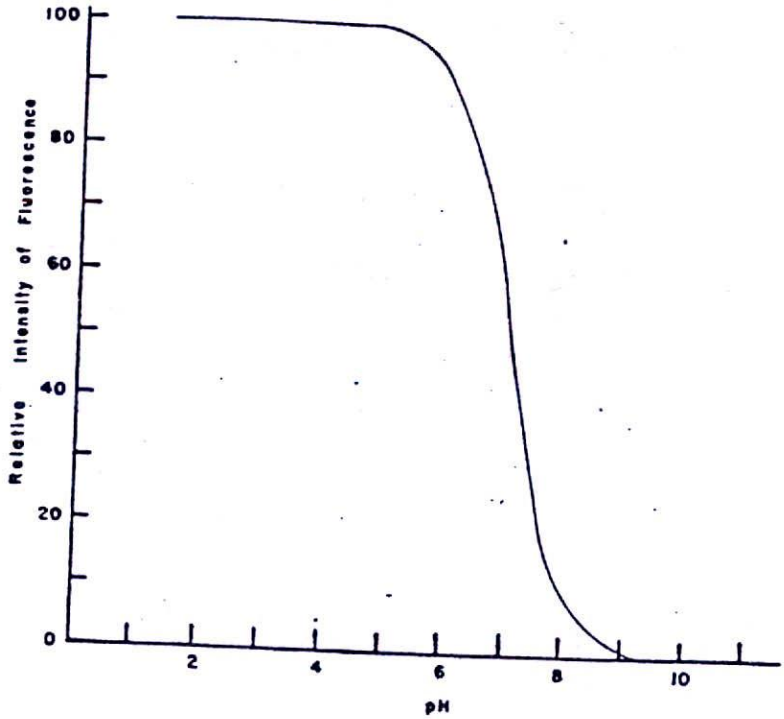


FIGURE 4.9: A plot showing the influence of pH on the fluorescence of a solution of a weak acid with a pK_a of 7. The measured fluorescence is assumed to be due to the conjugate acid. The fluorescence intensity of a strongly acid solution was assigned a value of 100 and the intensities at other values of pH were calculated relative to this.

setting that transmitted fluorescence that was specifically due to the unionized form of the acid. Then

$$S_f = k^*(HA) \tag{4.15}$$

where k^* is a combination of all the constants of Eq. (4.6). However, since

$$(HA) = \frac{C_{HA}(H^+)}{K_a + (H^+)} \tag{4.16}$$

where C_{HA} is the stoichiometric concentration of weak acid, K_a is the dissociation constant of the weak acid, and (H^+) is the concentration of solvated

protons, Eq. (4.15) becomes

$$S_f = \frac{k''C_{HA}(H^+)}{K_a + (H^+)} \tag{4.17}$$

An intensity-pH profile such as that shown in Fig. 4.9 would be expected. Similarly, if fluorescence which is specific for the conjugate base is detected,

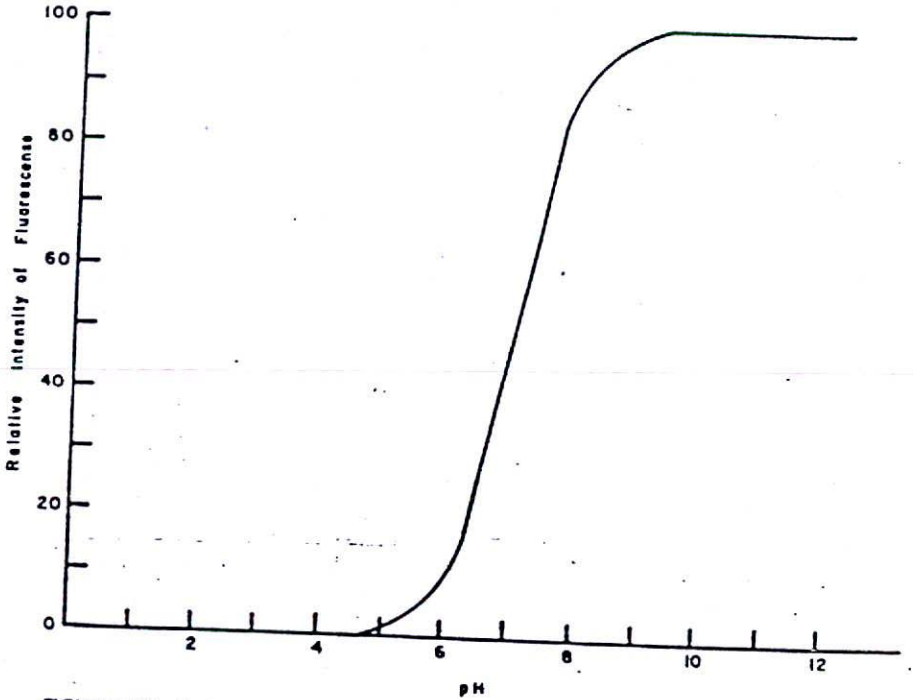


FIGURE 4.10: A plot showing the influence of pH on the fluorescence of a solution of a weak acid with a pK_a of 7. The measured fluorescence is assumed to be due to the conjugate base. The fluorescence intensity of a strongly alkaline solution was assigned a value of 100 and the intensities at other values of pH were calculated relative to this.

then Eq. (4.6) becomes

$$S_f = \frac{k''C_{HA}K_a}{K_a + (H^+)} \tag{4.18}$$

and the pH profile illustrated in Fig. 4.10 would be expected.

A more complex pH effect can be observed with some compounds and is due to the acid strength of a molecule in the excited state being different from the acid strength in the ground state. This difference has been shown to

be quite marked for a number of compounds. The phenomenon is known as *excited-state dissociation*. It was first studied by Forster[15] and was recently discussed by Ellis.[16] The occurrence is best illustrated by example, and Fig. 4.11 illustrates such a case. Here, the relative intensity of fluorescence for solutions of 2-naphthol was plotted as a function of pH. This phenolic

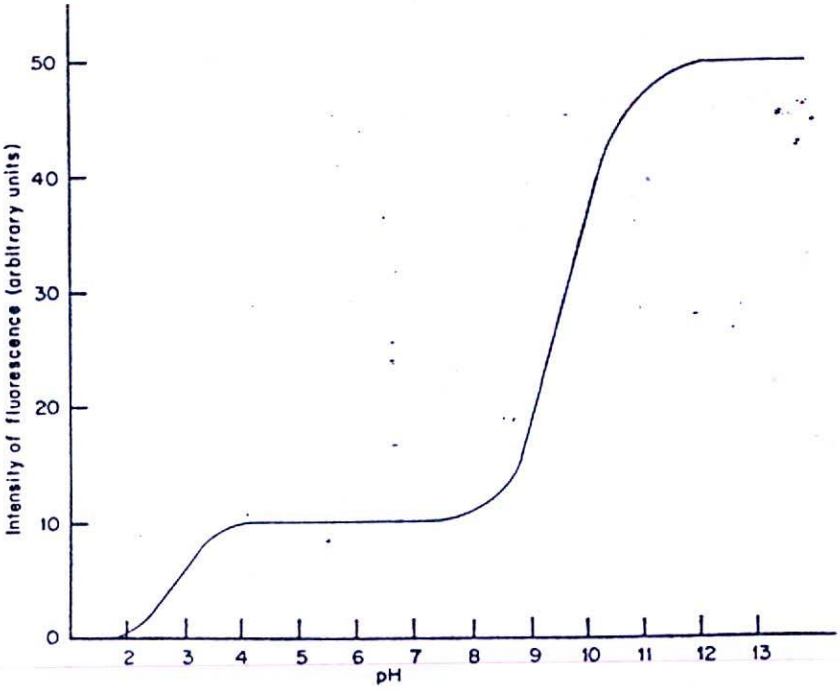
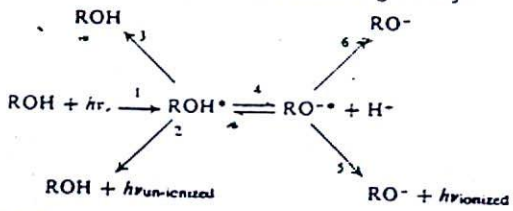


FIGURE 4.11: A plot showing the influence of pH on the intensity of fluorescence of solutions of 2-naphthol. An emission filter which passed wavelengths longer than 415 mμ was employed. The measured fluorescence was, therefore, due to excited-state anions. Reprinted from Ref. 16, p. 261, by courtesy of the *Journal of Chemical Education*.

compound has a pK_a of 9.5. Both ionized and un-ionized species fluoresce, but the fluorescence peak for the ionized form is at 429 mμ, while that for the un-ionized species is at 359 mμ. The fluorescence measurement plotted in Fig. 4.8 was, therefore, due to the 2-naphthoate anion. If the degree of ionization of the acid in the ground state was the only property influenced by a change in the concentration of hydrogen ion, then no observable fluorescence would be expected until a pH was achieved where a detectable degree of ionization occurred, i.e., a pH of 8.5 ($pK_a - 1$). That fluorescence was

observed in the region of 2 to 8.5 is indicative of excited-state dissociation. A possible mechanism, consistent with the data, is given by:



The processes are

1. Absorption of radiation by the un-ionized form
2. Fluorescence of the un-ionized form
3. Radiationless dissipation of energy
4. Excited-state dissociation to produce a proton and an anion in the excited state
5. Fluorescence of the anion
6. Radiationless dissipation of energy

The fluorescence at 429 mμ exhibited by 2-naphthol in the pH range of 2 to 8.5 means that excited-state anions did exist in this range and must have resulted from excited-state dissociation of the molecular species. The first inflection point in the pH profile corresponds to the excited-state dissociation constant, which is approximately 2.8 for 2-naphthol. The second inflection point, of course, corresponds to the ground-state dissociation constant.

Practical consequences of the pH effects on fluorescence are the same as those encountered in spectrophotometry. Thus, with certain compounds, pH must be considered as an experimental variable that is important to control. In some instances, it may be possible to utilize the influence of pH to minimize interferences and to conduct differential fluorometric determinations by appropriate pH adjustment. Additionally, fluorometry might offer a convenient approach to the determination of acidity constants of some compounds.

D. TEMPERATURE

The quantum efficiency of fluorescence is found to decrease with an increase in temperature. Thus, the higher the temperature, the more efficient and effective are radiationless processes in dissipating excitation energy. It is felt that the process of internal conversion is the one whose rate is most significantly influenced by temperature changes. The effect is most probably due to an increase in the thermal motion of molecules at higher temperatures. The increased motion favors the probability of intermolecular collision and subsequent energy loss. In general, a rise in temperature of 1°C results in a decrease in the intensity of fluorescence of about 1%. For some compounds, the sensitivity of fluorescence to temperature is even more pronounced.

Because of the temperature effect, a reasonable degree of temperature control is necessary in fluorometric methods. It is recommended, for example, that analytical samples be equilibrated to the same temperature before measurements are made. Similarly, readings should be taken within a reasonable period of time to preclude the heating of a sample contained in the sample holder of an instrument.

E. OTHER FACTORS

1. Degradation of Sample

The stability of a compound in an analytical sample is of concern in fluorometric methods, as it is in other methods of analysis. Here, in addition to autoxidative and solvolytic degradative routes, the possibility of photolytic degradation should always be anticipated. Many compounds are subject to light-catalyzed degradations and rearrangements. In fluorometric measurements the intensity of light used and the amount of sample irradiated can be sufficiently large to cause measurable loss of material during the time required for measurement. The error introduced by photodecomposition can be reduced by decreasing the intensity of exciting light and by making the measurement in as short a time period as possible.

2. Solvent Effects

The medium in which a potentially fluorescing material is dissolved can influence the intensity and characteristics of fluoresced light. As previously mentioned, impurities in solvents can contribute artifactual fluorescence. In addition, Raman scattering of the exciting light by the solvent might be erroneously measured as being due to the fluorescence of a sample. This effect is usually not of practical significance except when measurements are made on very dilute solutions. Other effects can be encountered such as the quenching of fluorescence by the solvent or by substances such as oxygen dissolved in the solvent. The spectral characteristics of fluoresced light can vary from solvent to solvent due to polarization effects and hydrogen bonding. Since these effects cannot be quantitatively predicted, the solvent cannot be changed at random in fluorometric methods of analysis.

4.6 COMPARISONS OF FLUOROMETRY WITH SPECTROPHOTOMETRY

A. SENSITIVITY

Fluorometry is significantly more sensitive as an analytical tool than is spectrophotometry. In spectrophotometry the intensity of light transmitted

by a sample is measured and compared to that transmitted by a blank. The lower limit of detectability is determined by the smallest concentration that will yield a detectable intensity *difference* between sample and blank. As the transmittance of a sample approaches 1, small errors made in measuring the difference between the two intensities result in large errors in calculated concentration. The lowest limit of concentration that can be detected with accuracy is, for all practical purposes, established by the molar absorptivity of the compound under investigation. A fluorometer measures directly the intensity of fluoresced light. Moreover, a decrease in the concentration of fluorescing species can be compensated for by an increase in the intensity of exciting light and/or an increase in the sensitivity of the detector. It is the latter variable that most significantly contributes to the sensitivity of the method. The directly measured intensity can be amplified more readily and accurately than the intensity difference measured in spectrophotometry. The lower limit of concentration here is, therefore, established by characteristics of the instrument and not usually by characteristics of the fluorescing species. In practice, it is the level of inherent "noise" of the instrument relative to the signal caused by sample fluorescence that dictates the lower limit of detectability. It has been calculated[17] that fluorescence measurements can offer sensitivity increases of as high as 10^3–10^4 over absorbance measurements.

B. SPECIFICITY

A fluorometric assay can offer a degree of specificity that might not be attainable with a corresponding spectrophotometric technique. Not all compounds which absorb ultraviolet and visible light fluoresce, and so a potentially interfering compound which absorbs light will not necessarily be a source of interference in a fluorometric method. In addition, the analyst has the ability to vary the wavelengths of both exciting and fluorescing light and to choose a combination of wavelengths which will maximize the measured fluorescence from the compound in question and minimize contributions from interfering substances. It should be noted in this respect that measurements need not be made at wavelengths corresponding to maxima or minima of the spectra. The equations relating fluorescence intensity to concentration hold for any region of the spectrum.

C. EXPERIMENTAL VARIABLES

It is obvious from the previous discussions that there are a larger number of experimental variables that must be controlled in fluorometric methods of analysis than in corresponding spectrophotometric methods. For example, temperature and the intensity of incident light must be maintained reasonably constant in a fluorometric method, but need not be rigidly controlled in a spectrophotometric procedure. Extraneous solutes can markedly affect the

intensity of fluorescence by quenching effects, whereas in spectrophotometry it is unusual to encounter a system in which the absorbance of a compound is significantly altered by the presence of other solutes. In addition, the influence of pH on fluorescence can be much more complex than on absorbance and might necessitate closer control of pH in fluorometric procedures than in spectrophotometric assays.

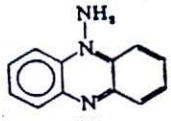
4.7 APPLICATION OF FLUOROMETRY TO PHARMACEUTICAL ANALYSIS

An examination of official compendia might lead to the impression that fluorometry has limited application in the analysis of drugs. There are, for example, only thirteen monographs in USP XVII, BP (1963), and NF XII that specify the utilization of fluorometry as an assay tool and these are concerned with the determination of only two compounds, riboflavin and thiamine. However, a survey of the literature reveals that fluorometry has enjoyed widespread use in the analysis of drugs in systems other than dosage forms. The sensitivity of the method has resulted in its application in a host of pharmacological, biochemical, toxicological, pharmacokinetic, and biopharmaceutical studies for the analysis of small amounts of drugs in biological fluids and tissues. It is not practical nor necessary to review all such applications in this chapter. These have been discussed in some detail by Udenfriend,[9] Phillips and Elevitch,[17] and Williams and Bridges.[1] More recent reviews are found in the annual *Analytical Review* editions of the journal *Analytical Chemistry*. In 1965, Wimer et al.[18] reviewed the literature appearing in the period 1962–1964 which dealt with the analysis of pharmaceuticals. In 1966, White and Weissler[19] reviewed publications pertinent to the subject of fluorometric analysis, including those relating to pharmaceutical analysis and which appeared in the period 1963–1965. It is illuminating and illustrative of the wide applicability of fluorometry to drug analysis to note from these reviews that in the period 1962–1964, publications appeared which described fluorometric procedures for the analytical determination of the following drugs or classes of drugs: adrenaline, aldosterone, androsterone, antihistamines, atropine, barbiturates, chlorpromazine, chlorprothine, codeine, dipyridamole, emetine, ergot alkaloids, estradiol, estriol, esterone, ethinyl estradiol, fursemide, imipramine, isoniazid, mephenesin, mescaline, morphine, narcotine, panthenol, papaverine, quinidine, quinine, reserpine, riboflavin, salicylates, streptomycin, sulfonamides, testosterone, tetracyclines, thebaine, tubocurarine, yohimbine.

A limited number of examples are discussed in the following section to more specifically illustrate the applicability and utility of fluorometry as an analytical tool. These examples will also demonstrate that in most instances the actual determination of the intensity of fluorescence of a sample preparation is the terminal step in a series of operations which demand that the

analyst be cognizant of a variety of separatory and chromatographic techniques, and the chemical and physical properties of the constituents of a sample.

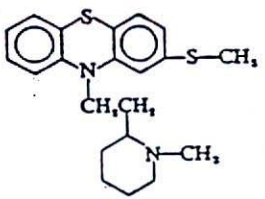
Aminocrine. A simple procedure for the determination of aminocrine (X) in drug preparations was recently described by Roberts[20] and illustrates a relatively direct fluorometric assay method. Here, the aminocrine was extracted with chloroform from a basic solution; the chloroform was evaporated and the residue was dissolved in acidic ethanol. The fluorescence of the resulting solution was determined using an excitation filter having maximum transmittance at 365 mμ and an emission filter which transmitted light of wavelengths greater than 415 mμ. The concentration of aminocrine was determined by comparing the fluorescence of the sample preparation to that of a standard preparation. The method was applied to a variety of aminocrine-containing dosage forms, including suppositories, creams, ointments, jellies, and tablets. Other constituents of the dosage forms did not interfere



(X)

with the determination. The method appears to be sensitive, as evidenced by the recommended use of a standard preparation having a concentration of 1 μg/ml.

Phenothiazines. Mellinger and Keeler[21] published an interesting study on the fluorescence characteristics of phenothiazine drugs. Thioridazine (XI) is representative of the compounds studied. All of the phenothia-

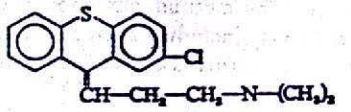


(XI)

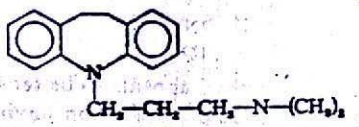
zines exhibited similar excitation and emission spectra. The emission spectrum of a solution prepared in 0.2 *N* sulfuric acid was characterized by a single peak in the range of 450 to 475 mμ. The excitation spectrum was found to have two peaks, one at approximately 250 mμ and the other in the range of 300 to 325 mμ. Addition of potassium permanganate to such solutions resulted in marked changes in spectral characteristics. The wavelength of maximum emission shifted to much lower wavelengths and the intensity of fluorescence at this maximum was much greater (15–20 times) than that

exhibited by untreated drug at its maximum. In addition, the excitation spectrum, after permanganate treatment, exhibited four peaks. Evidence was obtained to indicate that oxidation of the phenothiazine to the corresponding sulfoxide was responsible for this behavior. The spectra of phenothiazine-like compounds such as prothipendyl (XII), chlorprothixene (XIII), and imipramine (XIV) were affected by permanganate treatment, but the

(XII)



(XIII)



(XIV)

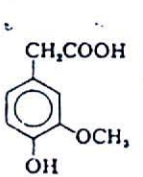
effects were qualitatively much different than those observed with phenothiazines.

Pretreatment of phenothiazines with potassium permanganate permitted quantitative fluorometric analysis of simple solutions in concentrations as low as 0.01 to 0.05 μg/ml. The sensitivity was somewhat less for samples obtained from biological sources but was significantly greater than that afforded by other techniques. For example, thioridazine in urine could be detected fluorometrically at a level as low as 0.8 μg/ml. In contrast, a concentration of 4 μg/ml was required for spectrophotometric detection, while 6 μg/ml was necessary for detection by a colorimetric method which was based on the treatment of a phenothiazine with concentrated sulfuric acid.

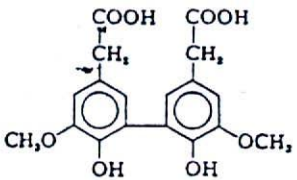
Salicylates. In 1948, Saltzman[22] described a fluorometric method for the estimation of salicylates in blood. It was based on the observation that salicylates, in alkaline medium, exhibit a blue fluorescence. The procedure involved precipitation of proteins from a sample with dilute tungstic acid and treatment with strong alkali. Alternatively, a sample of plasma was acidified and extracted with ethylene dichloride. The salicylate was then back-extracted into strong alkali. The fluorescence of the resulting solution was measured using a 370-mμ excitation filter and a 460-mμ emission filter. Chirigos and Udenfriend[23] studied the fluorescence characteristics of salicylate in more detail and determined from spectrofluorometric studies that

the excitation maximum was at 310 mμ and the emission maximum was at 400 mμ. Salicylate content of biological tissue was determined by extracting a sample with ether, back-extracting into a borate buffer, and fluorometric examination of the borate solution. More recently, Lange and Bell[24] used fluorometry coupled with paper chromatography as the basis for a micro-method for the determination of acetylsalicylic acid and salicylic acid in blood sample. Here, a small sample of blood (100 μl) was extracted with ethylene dichloride. Aliquots of the ethylene dichloride extract were spotted on paper strips and the strips were developed by the ascending technique using 0.75% nitric acid as the solvent system. Segments containing the aspirin and the salicylic acid were cut from the strip and eluted with 5 *N* sodium hydroxide. The fluorescence of solutions prepared in this manner were determined using as a blank a solution prepared by treating another portion of the chromatograph strip with alkali. Standard curves were prepared for the two compounds by subjecting blood samples containing known amounts of the drugs to the procedure and relating observed fluorescence to concentration. The method was applied to an investigation of aspirin and salicylic acid blood levels after the oral administration of aspirin tablets.

Homovanillic Acid. Homovanillic acid (3-methoxy-4-hydroxylphenylacetic acid, XV) is derived from the metabolism of 3,4-dihydroxyphenylalanine and 3,4-dihydroxyphenylethylamine. The urinary excretion of homovanillic acid (HVA) is elevated in patients with neuroblastoma and pheochromocytoma and interest has been expressed in utilizing urinary levels of HVA as a diagnostic tool. Anden et al.[25] and Sharman[26] discovered independently that HVA reacted with oxidizing agents such as potassium ferricyanide and ferric chloride, in alkaline medium, to yield a highly fluorescent solution. Although the product responsible for the fluorescence was not identified, both groups developed fluorometric procedures for the determination of HVA in biological tissues. The procedure of Anden et al. was modified by Sato,[27] who incorporated an ion-exchange treatment and solvent extraction into the procedure to isolate the HVA, which was then treated with ammonia and potassium ferricyanide. The fluorescence of the resulting solution was then determined. Corrodi and Werdinius[28] more recently studied the procedure in more detail and determined that the compound which was responsible for the fluorescence was 2,2′-dihydroxy-3,3′-dimethoxy-biphenyl-5,5′-diacetic acid (XVI). This compound exhibited an excitation maximum



(XV)

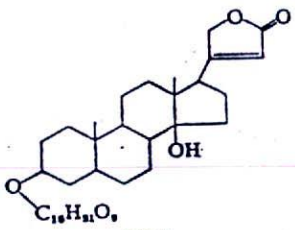


(XVI)

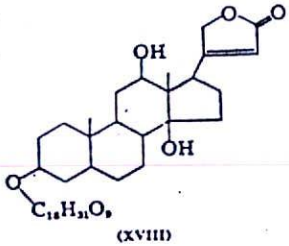
at 315 mμ and an emission maximum at 425 mμ.

An interesting application of this oxidative transformation of HVA was suggested by Guilbault et al.[29] They found that the conversion of (XV) to (XVI) could be accomplished enzymatically in a hydrogen peroxide-peroxidase system. They formulated solutions containing HVA, hydrogen peroxide, and peroxidase and measured the rate of change of fluorescence. At a constant concentration of HVA, this rate was found to be directly proportional to the peroxidase concentration and to the concentration of hydrogen peroxide. They recommended this approach for the analysis of oxidative enzymes and of hydrogen peroxide. They reported that as little as 10^{-11} mole/liter of peroxide and 10^{-3} unit/ml of peroxidase are determinable by this method.

Cardiac Glycosides. Jakovljevic[30] reviewed the many methods which have been proposed for the determination of cardiac glycosides. He noted that a number of the reported methods are fluorometric and are based on the generation of fluorophors by the action of dehydrating agents on the steroid moiety of the glycoside. He proposed a new reagent for this purpose, a mixture of acetic anhydride, acetyl chloride, and trifluoroacetic acid. He described the use of this reagent and a procedure for the simultaneous determination of digitoxin (XVII) and digoxin (XVIII) in mixtures by fluorometry. The method is an interesting illustration of how assay specificity can be attained by utilizing a knowledge of both excitation and emission characteristics of fluorescing compounds.



(XVII)

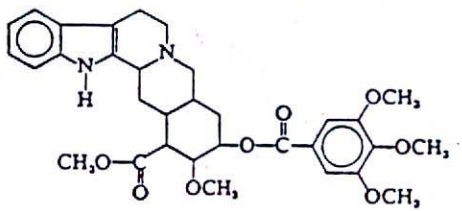


(XVIII)

Digoxin differs from digitoxin by one hydroxyl group at position 12. However, when the two compounds were treated under anhydrous conditions with the dehydrating agent, they yielded products which had significantly different fluorescence characteristics. The fluorophor generated from digitoxin exhibited a single excitation peak at 470 mμ and a single emission peak at 500 mμ. The digoxin fluorophor exhibited two excitation peaks at 345 and 470 mμ. Excitation at 345 mμ resulted in an emission peak at 435 mμ, while excitation at 470 mμ gave an emission peak at 500 mμ. Under the latter conditions, the fluorescence was approximately 30% of that obtained with digitoxin. When the digitoxin preparation was examined at an excitation wavelength of 345 mμ and an emission wavelength of 435 mμ, no fluorescence was observed. The author suggested that the treatment of digitoxin resulted

in the formation of a substituted 3,4-benzpyrene, while digoxin yielded a mixture containing compounds related to 3,4-benzpyrene and chrysine. The differences in spectral characteristics permitted the simultaneous determination of digitoxin and digoxin in samples prepared from digitalis leaf and digitalis tincture. Here, the glycosides were isolated by extraction from a sample, treated with the dehydrating agent for 30 min at 45°C, and diluted with dichloromethane. The fluorescence of the resulting solution was determined with a fluorometer using two different filter combinations. One combination was equivalent to an excitation wavelength of 470 mμ and an emission wavelength of 500 mμ. The measured fluorescence under these conditions resulted from both digitoxin and digoxin. The other combination was equivalent to an excitation wavelength of 345 mμ and an emission wavelength of 435 mμ. Fluorescence here was due to digoxin. The later reading could be used to calculate, by comparison to a standard, the concentration of digoxin. A knowledge of this concentration was then used to calculate the fluorescence due to digoxin under the excitation and emission conditions of the first filter combination and to obtain a corrected fluorescence which reflected the digitoxin concentration. The corrected fluorescence was then used to calculate the digitoxin concentration by comparison to that exhibited by a standard. The method was also applied to the determination of digitoxin in tablets and ampoules.

Reserpine. The reaction of nitrous acid with reserpine (XIX) to yield a yellow fluorescent pigment was described by Szalkowski and Mader[31] and



(XIX)

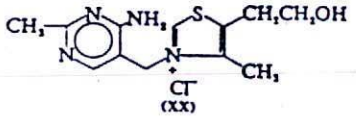
forms the basis for a widely used colorimetric method for the determination of reserpine in pharmaceutical preparations. Reserpine has been used as an additive to poultry feeds, where it is found in concentrations as low as 0.0001%. The low levels encountered in such systems could not be satisfactorily determined by the colorimetric method of analysis. Mader et al.[32,33] utilized the fluorescence characteristics of nitrous acid-treated reserpine to obtain the desired sensitivity. Treatment of reserpine with nitrous acid was found to result in the formation of a product which possessed an excitation maximum at 390 mμ and an emission maximum at 510 mμ. The intensity of fluorescence was linearly related to concentration over a wide range. The reserpine in a feed sample was isolated by a series of extractions and was eventually obtained as an assay preparation in chloroform-methanol. An aliquot of this

solution was treated with sodium nitrite and the mixture was acidified with hydrochloric acid. After an appropriate reaction time, sulfamic acid was added to consume the excess nitric acid and the fluorescence of the solution was determined. A blank was prepared by treating an aliquot of the assay preparation in a similar manner, but with the omission of the sodium nitrite. The concentration of reserpine in the assay preparation was calculated by comparison of the measured fluorescence to that found with a standard preparation which was obtained by carrying a known amount of reserpine through the extraction and reaction procedures.

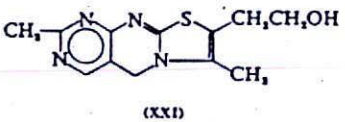
Haycock et al.[34] recently reported the results of a study in which the kinetics and mechanisms of the nitrous acid-induced fluorescence of reserpine were investigated. They presented evidence to show that the fluorescence was due to the formation of 3-dehydroreserpine. The kinetics of the reaction indicated that protonated reserpine initially reacted with nitrous acid to form an intermediate complex, which then underwent an acid-catalyzed reaction to form 3-dehydroreserpine.

Vitamins. Udenfriend[35] reviewed the many fluorometric methods that have been used for the determination of vitamins. He discussed, in some detail, procedures for vitamin A, thiamine, riboflavin and related flavins, nicotinamide, pyridoxine and related compounds, ascorbic acid, vitamin D, folic acid, *p*-aminobenzoic acid, cyanocobalamin, tocopherols, and vitamin K. The fluorometric determinations of thiamine and riboflavin are of special interest in that they serve as rather classic examples of the application of fluorometry to pharmaceutical analysis.

Thiamine (XX) possesses little native fluorescence, but is readily oxidized to thiochrome (XXI), which is highly fluorescent. Thiochrome has been



(XX)



(XXI)

reported to have an excitation maxima at 365 mμ and an emission maximum of 450 mμ.[36] The procedure described in the seventeenth revision of the USP serves as an example of the fluorometric assay. An aliquot of a sample solution of the vitamin is treated with an oxidizing reagent (an alkaline solution of potassium ferricyanide). The thiochrome which is formed is extracted into isobutanol and the fluorescence of the resulting solution is determined. The fluorescence is corrected by use of a blank and is compared to that of a standard preparation.

Riboflavin has a characteristic pronounced native fluorescence and can be assayed by direct fluorometric examination of a sample solution. As will be discussed in the practical section, fluorometric assays for riboflavin usually employ an internal standard and an internal blank.

4.8 PRACTICAL SECTION

A. GENERAL

The procedures used in fluorometric assays are, in most instances, quite similar to those encountered in colorimetry and spectrophotometry. Treatment of a sample frequently involves dilution, extraction and/or chromatographic separation, chemical reaction, and finally the determination of the intensity of fluorescence of the final assay preparation using a suitable fluorometer fitted with appropriate filters. The intensity is "read out" in arbitrary units and, after correction for the fluorescence contributions for the blank, can be used to estimate the concentration of fluorescing substances. For a nonlinear intensity-concentration profile, a standard curve must be used for this purpose. If the dilution is such that intensity is directly proportional to concentration, the concentration of the assay preparation can be obtained by comparing the intensity reading to that of a single, standard preparation. In such a case, concentration is calculated by a formula, familiar from spectrophotometric assays:

$$C_u = C_s F_u / F_s \tag{4.19}$$

where C_u is the concentration of the assay preparation, C_s is the concentration of the standard preparation, F_u is the fluorometer reading, corrected for blank, obtained with the assay preparation, and F_s is the fluorometer reading, corrected for blank, obtained with the standard preparation. The *International Pharmacopoea*[37] cautions that the ratio F_u/F_s should not be less than 0.04 and not more than 2.50 because of the limited concentration range within which fluorescence is proportional to concentration.

Frequently, internal standards are prescribed in fluorometric procedures. Here, a known quantity of pure material is added to the assay preparation to compensate for quenching effects which might be introduced during the work-up of a sample. The USP XVII assay for riboflavin[38] serves as an example. This assay specifies the treatment of 10 ml of an assay preparation with 1 ml of water and 2 ml of reagents. The fluorescence of the resulting solution is measured and designated I_u. Another 10 ml of assay preparation is treated with 1 ml of a standard preparation containing 0.001 mg of riboflavin per ml and 2 ml of reagents. The fluorescence of this solution is measured and designated I_s. The concentration of vitamin in the assay preparation in milligrams per milliliter is calculated by the formula:

$$C_u = \frac{I_u - I_b}{I_s - I_u} \times 1/13 \times 0.001 \times 13/10 = \frac{I_u - I_b}{I_s - I_u} \times 0.0001 \tag{4.20}$$

where I_b is the fluorescence reading obtained with a blank.

Various procedures are used to obtain blank readings in fluorometry. Conventional blanks are sometimes specified and are prepared by substituting

in the final step of an assay a volume of water or buffer for the required volume of assay preparation. When the possibility exists of fluorescent materials being introduced to the assay preparation by the system containing the compound of analytical interest, a more realistic blank is usually recommended. For example, plasma and urine blanks are prepared by carrying a volume of drug-free plasma or urine through the complete procedure. Internal blanks are frequently employed. Here the assay preparation is used as a blank after specifically eliminating, through chemical reaction, the fluorescence due to the drug. In the riboflavin assay, for example, a few crystals of sodium hydrosulfite are added to the cuvette immediately after the fluorescence intensity of a sample is measured. The hydrosulfite rapidly and specifically converts riboflavin to the nonfluorescent, reduced form. The fluorescence of the resulting solution is measured and is used as a blank reading to correct for fluorescence arising from sources other than riboflavin. In other instances, blanks are prepared by omitting a reagent necessary for the generation of a fluorescing species. For example, the official assay for thiamine[39] is based on the oxidation of nonfluorescent thiamine to strongly fluorescent thiochrome. The oxidizing agent employed is alkaline potassium ferricyanide solution. Blanks for both assay and standard preparations are prepared by submitting samples to the full procedure, but with the substitution of a volume of sodium hydroxide solution for the volume of oxidizing reagent which is normally used.

Numerous instrumental variables such as the intensity of exciting light, detector response, signal amplification, etc., influence the measured intensity of fluorescence. Aging of a light source and fatigue of a detector could, for example, result in nonreproducibility of results and assay error. It is important, therefore, to periodically check the sensitivity of a fluorometer and to adjust it to constant sensitivity during the course of assay measurements. A solution of a stable, strongly fluorescing substance is used for this purpose and is known as a comparison standard. The standard chosen should have fluorescence characteristics similar to those of the compound being assayed and, in fact, if that compound is sufficiently stable, no other comparison standard is needed. A solution of quinine sulfate is frequently recommended as a comparison standard. Its use is illustrated by the USP assay for thiamine. A solution of quinine sulfate in 0.1 *N* sulfuric acid at a concentration of 0.25 μg/ml is recommended since "this solution fluoresces to approximately the same degree as the thiochrome obtained from 1 μg of thiamine hydrochloride and is used to correct the fluorometer at frequent intervals for variations in sensitivity from reading to reading within an assay."

B. LABORATORY PROJECTS IN FLUOROMETRY

The following projects are offered as guides for possible laboratory exercises illustrating some principles and applications of fluorometry. Since different

makes of fluorometers differ in sensitivity, ranges of sensitivity, and the manner by which ranges of sensitivity are selected and adjusted, exact experimental details cannot be presented. The student should initially become familiar with the operational characteristics of the fluorometer available for his use by studying the instructional and descriptive literature supplied with the instrument and by appropriate laboratory demonstration.

1. Intensity of Fluorescence of Riboflavin as a Function of Concentration

Prepare a stock solution of riboflavin at a concentration of about 1 μg/ml (USP XVII or NF XII may be consulted for directions for preparing this solution). Prepare dilutions of the stock solution to obtain the following concentrations: 0.02, 0.04, 0.06, 0.08, and 0.1 μg/ml. Determine the relative intensity of fluorescence of each solution with a suitable fluorometer. An appropriate primary (excitation) filter is one that peaks at 360 mμ, while the secondary filter (emission) should pass wavelengths greater than 510 mμ. Present the results in the form of a graph in which fluorometer reading is plotted as a function of concentration. Repeat with solutions ranging in concentration from 0.002 to 0.01 μg/ml.

2. The Influence of pH on the Fluorescence Intensity of Riboflavin

Prepare buffered solutions of riboflavin ranging in pH from 2 to 11. All solutions should contain the same concentration of the vitamin, which should be such that the solution buffered to approximately pH 7 gives a reading of from 50 to 80% of full scale of the fluorometer. Determine the fluorescence of each solution and plot the fluorometer reading as a function of pH.

3. The Influence of Quenching Agents on the Fluorescence Intensity of Riboflavin

Design and conduct an experiment to demonstrate the influence of potassium iodide concentration on the intensity of fluorescence of riboflavin. Plot the results in a manner suggested by Eq. (4.11). Repeat using caffeine as a quenching agent.

4. The Intensity of Fluorescence of Salicylic Acid as a Function of Concentration

Prepare solutions of salicylic acid in 0.1 *N* sodium hydroxide to cover a range of concentrations of from 5 to 100 μg/ml. Determine the relative intensity of fluorescence of each solution and plot fluorometer reading as a function of concentration. The 360-mμ primary filter may also be used in this instance, but a secondary filter transmitting wavelengths greater than 455 mμ should be selected.

5. Assay of Riboflavin Injection

Determine the potency of a sample of riboflavin injection by employing the riboflavin-assay procedure described in USP XVII or NF XII.

6. Assay of Thiamine Hydrochloride Tablets

Employ the thiamine assay procedure described in USP XVII or NF XII to determine whether or not a sample of thiamine hydrochloride tablets meet the label claim.

7. The Determination of 9-Aminoacridine (Aminocrine) In Pharmaceutical Products

Obtain a sample of a pharmaceutical preparation containing aminocrine. Fluorometrically determine the aminocrine content by the method proposed by Roberts.[20]

8. Excited-state Dissociation of 2-Naphthol

Conduct the laboratory experiment described by Ellis[16] to demonstrate the excited-state dissociation of 2-naphthol.

9. Fluorometry In Biopharmaceutical Studies

Determine the physiological availability of riboflavin from a coated tablet using the method of Chapman et al.[40] In this procedure, the amount of riboflavin excreted in the urine following oral ingestion of a coated vitamin tablet is determined fluorometrically and compared with the amount excreted after the ingestion of a rapidly dissolving uncoated tablet.

PROBLEMS

P4.1. The molar absorptivity of riboflavin (molecular weight = 376.36) in aqueous solution at 360 mμ is approximately 7500. Fluorometric examination, using an excitation wavelength of 360 mμ, of a solution of the vitamin containing 0.010 μg/ml yielded a fluorescence intensity of 1.0 unit. Calculate the theoretically expected intensity of fluorescence for a solution containing 1.0 μg/ml. Assume that the equivalent of a 1-cm cell was used.

P4.2. The influence of pH on the intensity of fluorescence of a dilute solution of a weak acid was investigated. Solutions were prepared which varied only in the concentration of hydrogen ion. The fluorescence of each solution was determined with a fluorometer and the following results were obtained:

pH	Fluorescence (arbitrary units)
4.0	80.0
5.0	80.0
6.0	79.5
7.0	74.5
7.5	65.6
8.5	34.4
9.0	25.4
10.0	20.6
11.0	20.0
12.0	20.0

Show that the ratio of the concentration of ionized acid to that of un-ionized acid at any pH is given by $(80 - F)/(F - 20)$, where F is the observed fluorescence at that pH. Plot the logarithm of the ratio as a function of pH and determine the pK_a of the acid from the plot.

P4.3. A standard preparation of riboflavin was prepared by the following procedure. Exactly 48.5 mg of USP riboflavin reference standard was dissolved in sufficient water to make 500 ml. One ml of the resulting solution was diluted to 100 ml with water.

An assay preparation was prepared in the following manner: The riboflavin from 10 riboflavin tablets was dissolved in sufficient water to make 1 liter. One ml of this solution was diluted to 1 liter with water.

A mixture of 10 ml of assay preparation and 1 ml of standard preparation yielded a fluorescence reading of 74.0. Ten ml of the assay preparation and 1 ml of water gave a fluorescence reading of 40.0. The latter mixture was treated with 20 mg of sodium hydrosulfite and the fluorescence was again determined. The reading was found to be 2.0. Calculate the quantity in mg of riboflavin in each tablet.

P4.4. A nonfluorescent complexing agent A is known to form a nonfluorescent complex with a fluorescent compound B. A 1×10^{-6} M solution of B was placed in a fluorometer and the meter was adjusted to read 100.0. The fluorescence of a solution which was 1×10^{-6} M with respect to B and 1×10^{-2} M with respect to A was then determined and was found to be 20.0. Calculate the stability constant of the complex.

P4.5. Exactly 20 mg of USP thiamine hydrochloride reference standard was dissolved in sufficient water to make 1 liter. One ml of the resulting solution was diluted to 100 ml with water. Five ml of this solution was treated with 3 ml of oxidizing agent and the thiochrome which was formed was extracted into 20 ml of isobutanol. The fluorescence of the isobutanol phase was determined to be 67.0. A corresponding blank has a fluorescence of 4.0.

Exactly 1 ml of a thiamine hydrochloride injection was diluted with sufficient water to make 1 liter. Two ml of this solution was diluted to 1 liter with water. Five ml of the final dilution was oxidized and extracted, as previously described, and the fluorescence of the isobutanol extract was 58.0. The fluorescence of a corresponding blank was 8.0. Calculate the quantity, in milligrams, of thiamine hydrochloride in each milliter of injection.

P4.6. A preparation contains riboflavin, thiamine hydrochloride, and a fluorescent coloring agent. Describe how you would approach the problem of developing a fluorometric method for the determination of all three of these components.

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