

Chapter 5

FLUOROMETRIC ANALYSIS

I. INTRODUCTION

Fluorescence is the emission of light of one wavelength by a molecule a very short time after it absorbs light of some other, usually shorter, wavelength. Measurements based upon the intensity of fluorescence emission are widely used in trace analysis because excellent sensitivity can be achieved — often to concentrations as low as 10^{-4} $\mu\text{g}/\text{ml}$ — and because the measurements are more selective than ultraviolet or visible absorbance measurements. The intensity of emitted light is often linear over a range of three to four orders of magnitude in concentration. However, practical fluorescence measurements are subject to a number of sources of both inaccuracy and imprecision which have limited their applications in pharmaceutical analysis to cases where high sensitivity is critical. Fluorescence measurements can nonetheless achieve a high degree of reliability when modern instruments are used with carefully controlled experimental conditions.

II. PRINCIPLES OF FLUORESCENCE

A. Excitation and Relaxation

Figure 1 is a schematic representation of several low lying energy levels of a polyatomic molecule. The electronic energy levels are divided into two groups — singlet states in which all electron spins are paired and triplet states in which the spins of two electrons are not paired. Several vibrational levels corresponding to each electronic level are also shown.

At room temperature most molecules are in their lowest electronic and vibrational energy levels (ground state). Absorption of a photon lifts the molecule from the ground state into a higher energy state which is usually excited both vibrationally and electronically, as indicated by the vertical arrows in Figure 1. The spectroscopic selection rules require that the upper and lower states connected by photon absorption have the same multiplicity (both singlets or both triplets). After photon absorption occurs, several processes may reduce the energy of the molecule and return it towards its ground state. These include:

1. Vibrational relaxation
2. Internal conversion
3. Intersystem crossing
4. Photon emission (fluorescence and phosphorescence)
5. Energy transfer

1. *Vibrational Relaxation*

A molecule in an upper vibrational level of any state quickly loses its excess vibrational energy through collisions with other molecules. This process is fast enough that most molecules in an excited electronic state will be found in the lowest vibrational level of that state.

2. *Internal Conversion and Intersystem Crossing*

It is very common for the upper vibrational levels of one state to be of the same

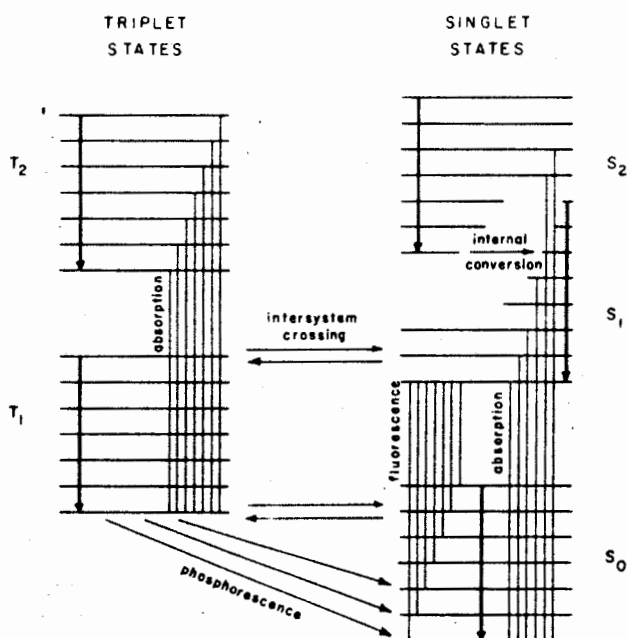


FIGURE 1. Transitions between electronic and vibrational excited states for polyatomic molecules with singlet ground states. Heavy arrows represent vibrational relaxation.

energy as the lower vibrational levels of a higher state, as shown in Figure 1. When this occurs, molecules may pass between vibrational levels of the same total energy and thus move from a higher electronic state to a lower one. When the upper and lower electronic states have the same multiplicity (both singlets or both triplets), the process is referred to as "internal conversion"; when the multiplicities are different, it is called "intersystem crossing". Once internal conversion has occurred, the molecule quickly loses excess vibrational energy and settles into the lowest vibrational levels of its new state.

3. Photon Emission: Fluorescence and Phosphorescence

A molecule in an *excited* electronic state may return to the ground electronic state by emission of a photon. The emission is called fluorescence when it originates from an excited singlet state and phosphorescence when it originates from a triplet state. The wavelength of light required to excite the molecule is shorter than the wavelength emitted. There are several important differences between fluorescence and phosphorescence. Transitions between singlet and triplet states involving emission or absorption of photons are "forbidden" transitions, i.e., they occur with very low probability. As a result of the low probability, lifetimes for phosphorescent emission range from 10^{-4} sec to greater than 1 sec. Thus phosphorescence may continue for a relatively long time after the source of excitation is removed. Fluorescence lifetimes on the other hand fall in the range of 10^{-6} to 10^{-9} sec,¹ and thus fluorescence emission decays rapidly when the source of excitation is removed.

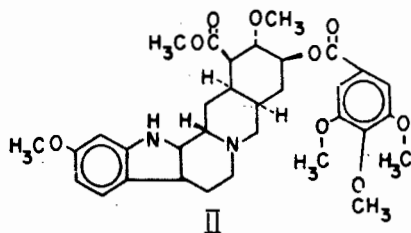
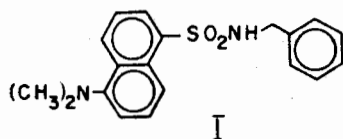
Vibrational relaxation is a much more rapid process than photon emission, so essentially all emission occurs from the lowest vibrational levels of the excited state. The lowest vibrational level of a triplet state is however of lower energy than the lowest level of the corresponding singlet state (compare Hund's Rule for the relative energy

of atomic states with paired and unpaired electron configurations). Therefore phosphorescent emission will be at longer wavelengths (lower energy) than fluorescent emission from the same molecule.

Rapid vibrational relaxation also causes the wavelength of emitted light to be longer than that of the absorbed light. As shown in Figure 1, absorption takes the molecule from the lowest vibrational level of S_0 to a higher vibrational level of S_1 , and thus involves a larger energy change than emission which takes the molecule from the lowest vibrational level of S_1 to one of the vibrational levels of S_0 . The fact that transitions may terminate in a number of different vibrational levels leads to rather broad excitation and emission spectra for most substances, as shown in Figure 2. The true excitation spectrum for a substance is essentially identical to its absorption spectrum, but experimental excitation spectra are often distorted by instrumental effects. The true (corrected) excitation and emission spectra are usually, but not always,²⁻⁶ independent of the wavelength used to record them. The excitation and emission spectra of a compound are nearly mirror images of one another in many cases because the vibrational energy level distributions are often similar in the ground and excited states.

4. Energy Transfer

A molecule in an excited electronic state can return to the ground state by a direct, nonradiative transfer of energy to an acceptor molecule.^{2,7,8} The transfer may occur over long distances (50 to 100 Å)⁹⁻¹⁰ and may be an important process if (1) there is a large overlap in the absorption spectra of the acceptor molecule and the emission band of the donor molecule and (2) if the donor molecule has a high fluorescence efficiency (*vide infra*).¹¹ Transfer may also occur between isolated chromophores within the same molecule; examples include the efficient transfer of excitation from the phenyl to the naphthyl chromophore in I,¹² and from the indole to the trimethoxybenzoate chromophore in reserpine(II).^{13,14} In I and II the acceptor chromophore dissipates its excitation energy through fluorescent emission, but in other cases the energy is dissipated without emission.



B. Fluorescence Intensity

1. Quantitative Description

The fluorescence quantum efficiency, ϕ_F , is the fraction of light absorbed by a chromophore that is re-emitted as fluorescence:

$$\phi_F = \frac{\text{Number Photons Emitted}}{\text{Number Photons Absorbed}} \quad (1)$$

ϕ_F ranges from zero to one depending upon the relative effectiveness of the various relaxation mechanisms. The exact value of ϕ_F depends upon both the structure of the molecule and its environment. The experimental determination of ϕ_F has been reviewed by Demas and Crosby.¹⁵ Efficiencies for several substances are presented in Table 1. An expression for the intensity of fluorescence, F , is easily derived in terms of ϕ_F since

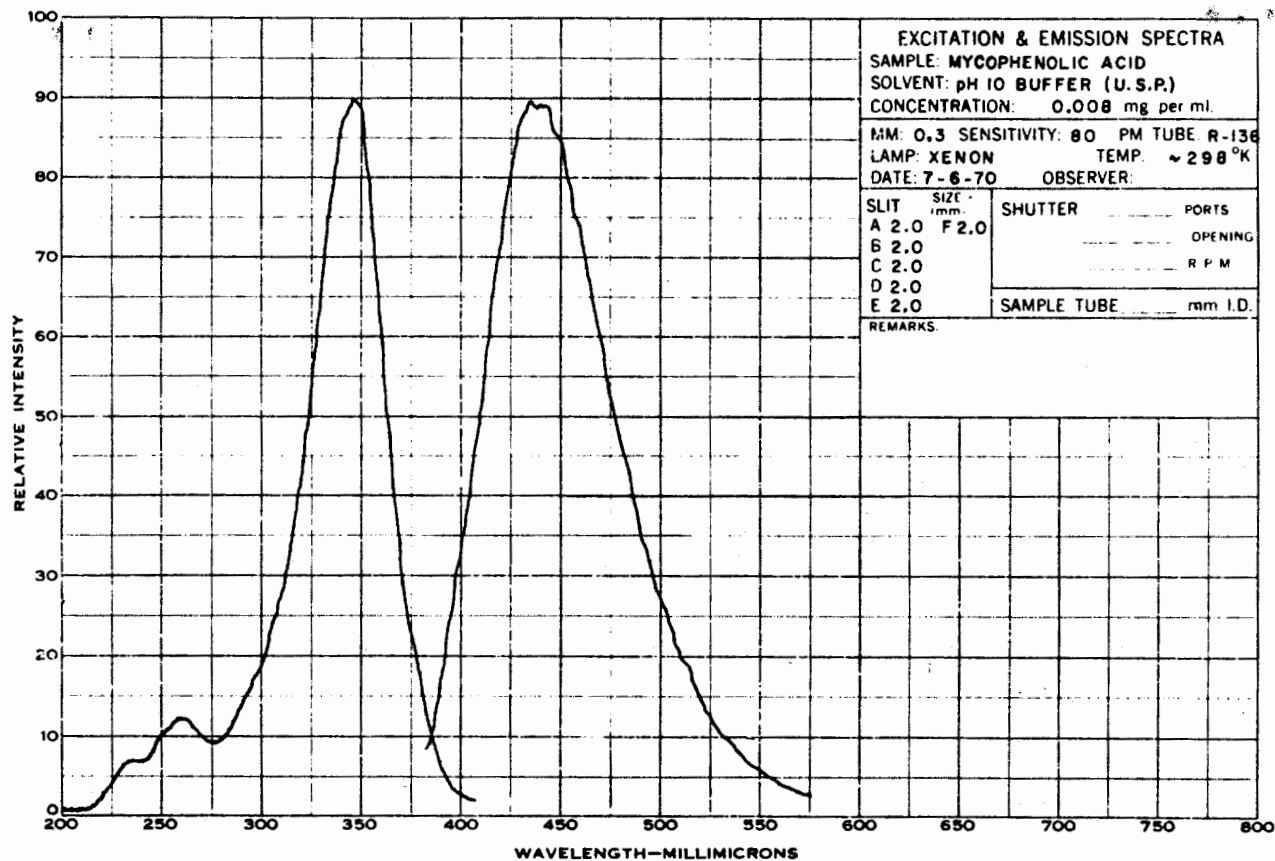
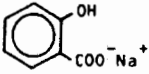
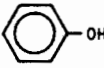
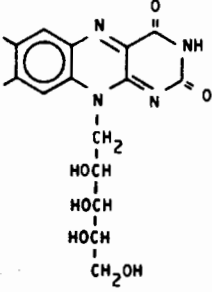
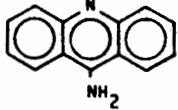
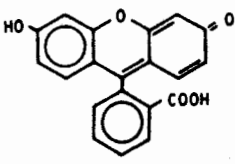
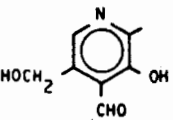


FIGURE 2. Fluorescence spectrum of mycophenolic acid in alkaline solution. The left hand curve (shorter wavelength) is the excitation spectrum and the right hand curve (longer wavelength) is the emission spectrum.

Table I
FLUORESCENCE QUANTUM EFFICIENCIES

Compound	Solvent	ϕ_F	Ref.
 Sodium salicylate (III)	Water	0.28	21
 Phenol (IV)	Water	0.22	21
 Riboflavin (V)	Water	0.26	21
 9-Aminoacridine (VI)	Water	0.98	21
 Fluorescein (VII)	0.1 N NaOH	0.92	21
 Pyridoxal (VIII)	0.05 M, pH 7.0 Phosphate buffer	0.05	22

$F = \phi_F (I_0 - I)$ where $(I_0 - I)$ is the amount of light absorbed by the analyte. By Beer's Law (Equation 2, Chapter 2, Section II.C.1) $I = I_0 \exp(-\epsilon cl)$, so

$$F = I_0 (1 - e^{-\epsilon cl}) \phi_F \quad (2)$$

where ϵ = molar absorptivity, c = molarity of solution, l = length of light path through the solution in centimeters, I_0 = intensity of light incident on sample, and ϕ_F = fluorescence quantum efficiency. In solutions with small absorbance values (0.05 or less), $\exp(-\epsilon cl)$ will be small, and Equation 2 reduces to the usual relation

$$F = I_0 \epsilon c l \phi_F \quad (3)$$

which is the basis of most quantitative fluorimetric analyses. Four assumptions underlay the quantitative use of Equation 3:

1. ϕ_F must be constant from sample to sample and measurement to measurement.
2. The absorbance of the solution at the excitation wavelength must be small.
3. Either I_0 must be constant or corrections must be made for variations in it.
4. The observed signal must be directly proportional to the total fluorescence emitted (F).

It is important that these assumptions be clearly recognized because they are easily violated in practical fluorescence measurements, as is discussed in the following section.

2. Experimental Factors Affecting Fluorescence Intensity

a. Factors affecting ϕ_F

i. Temperature and Viscosity

The rates of internal conversion, intersystem crossing, and energy transfer all increase with increasing frequency and energy of molecular encounters,^{17,18} whereas the rate of emission is insensitive to molecular collisions. Therefore lower temperature and greater viscosity increase ϕ_F by decreasing the efficiency of these alternate relaxation mechanisms. ϕ_F often changes by about 1%/1°C temperature change, but changes as large as 5%/1°C have been observed in solutions of tryptophan, Rhodamine B, and others.^{19,20} Of course increasing viscosity or lowering the temperature will only be effective means of increasing fluorescence yields when ϕ_F is significantly less than one.

ii. Solvent and pH

The excited states of polar molecules are often more polar than the ground states and therefore are more strongly solvated. The greater solvation of the excited state results in a smaller energy difference between the ground and excited states in polar solvents than in nonpolar solvents, and therefore a red shift is observed in the emission spectrum as solvent polarity increases. The red shift in the emission spectrum is usually larger than the shift observed in the corresponding absorption spectrum. Fluorescence efficiency on the other hand is affected very little by solvent polarity unless hydrogen bonding or complex formation with the solvent occurs.

Hydrogen bonding of the excited state of a fluorescence molecule often decreases ϕ_F . The decrease in ϕ_F appears to be due to an enhanced rate of internal conversion from S_1 to S_0 in the presence of hydrogen bonding.^{15,16} The decrease in ϕ_F may be quite large, as can be seen from the data on 5-hydroxyquinoline in Table 2. It is apparent that fluorometric analyses of substances in which $-\text{OH}$, $-\text{CO}_2\text{H}$, $-\text{NH}_2$, $-\text{SH}$ or other hydrogen bonding groups are part of the chromophore should be carried out in non-hydrogen bonding solvents whenever possible.

Solvents containing one or more heavy atoms may also decrease ϕ_F because heavy atoms have large spin-orbit couplings which make them very effective in inducing intersystem crossing during molecular collisions. Solvents containing halogens or other heavy atoms should therefore be avoided whenever possible.

The fluorescence spectra of weak acids and bases are also very sensitive to pH be-

Table 2
SOLVENT EFFECTS ON
FLUORESCENCE EFFICIENCY OF 5-
HYDROXYQUINOLINE¹⁶

Solvent	$-\Delta H$, kcal/mol*	ϕ_f , 25°C
Isopentane	—	0.30
Acetonitrile	3.5	0.24
Sulfolane	3.5	0.21
Dioxane	4.4	0.19
Diethyl ether	5.1	0.12
Dimethylformamide	5.3	0.09
Tetrahydrofuran	5.5	0.09
Dimethylsulfoxide	6.4	0.07

* Enthalpy of hydrogen bond formation between 5-hydroxyquinoline and solvent molecules.

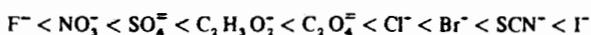
From Wehry, E. L., *Fluoresc. News*, 6, 1, 1971. With permission.

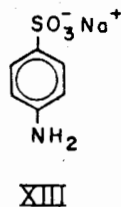
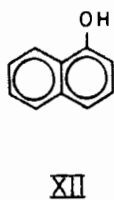
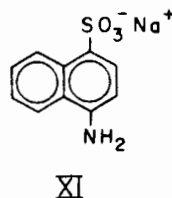
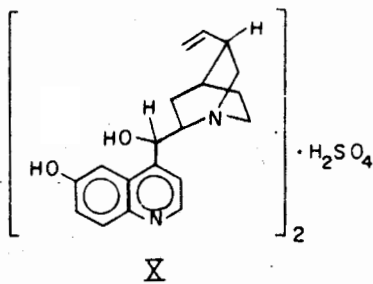
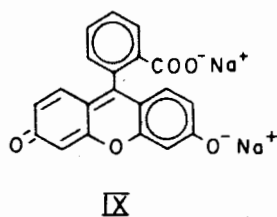
cause the principal light absorbing and emitting species will change from the protonated to the nonprotonated form of the molecule as the pH is increased. The excitation spectrum will show essentially the same acid-base shifts as the UV absorption spectrum of the compound. In particular, the shift in λ_{MAX} will occur at pH values near the pK value of the functional group. The emission spectrum will have a different pH dependence than the excitation spectrum because (1) the pK values for a molecule in the S_1 state are typically 4 to 5 units different from its pK in the S_0 state, and (2) proton transfers are much faster than emission from S_1 . Thus the changes in the emission spectrum occur when the pH is near the pK of the excited singlet state rather than the pK of the ground state. pK values for the ground and excited states of several drug substances are presented in Table 3. A more comprehensive discussion of the acid and base properties of excited molecules can be found in the review by Ireland and Wyatt.²³ The possibility of large differences between pK_s and pK_s^* should be kept in mind when attempting to find a solvent system that will optimize a particular fluorimetric analysis.

iii. Effects of Other Solutes

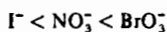
Fluorescence is not useful for accurate analysis of substances in complex mixtures because the fluorescence efficiency can be affected in an unpredictable manner by the other solutes. Solute containing halogens or other heavy atoms quench fluorescence in the same way as halogenated solvents. Paramagnetic solutes, including molecular oxygen, cause significant decreases in ϕ_f . Guilbault²¹ generalizes that oxygen is present at a concentration of about 10^{-3} M in normal aerated solutions and that this concentration is sufficient to decrease ϕ_f by 20% in many cases. Thus the sensitivity of a fluorometric assay can often be improved by purging the solutions with nitrogen before measuring the fluorescence intensity. Paramagnetic metal ions such as Cu^{2+} , Cr^{3+} , Ni^{2+} , and Fe^{2+} are also strong quenchers of fluorescence.²² Diamagnetic cations of light metals such as Na^+ , K^+ , Ca^{2+} and Mg^{2+} do not alter ϕ_f .

Inorganic anions may also reduce ϕ_f . Jette et al.²³⁻²⁵ studied the effects of several anions on the fluorescence of sodium fluorescein(uranin, IX), quinine sulfate(X), and uranyl sulfate($UO_2SO_4 \cdot 3H_2O$) and found that the ability of these ions to quench fluorescence increased in the following order:





The decrease in ϕ_f observed for the ions from F^- to $C_2O_4^{2-}$ was quite small in this study. The relative effectiveness of a series of quenchers will, however, depend strongly on the fluorescent compound itself as can be seen by comparing the results obtained by Jette et al. with those of Rollefson and Stoughton.³⁶ The latter authors used sodium naphthionate(XI), 1-naphthol(XII), and sodium sulfanilate(XIII) to study anion quenching and found the following order of quenching strength



which reverses the order of I^- and NO_3^- found by Jette et al.

Organic substances may affect ϕ_f through energy transfer, reaction or complexing with the analyte in the ground state, or reaction with the analyte in the excited state. For example, charge transfer reactions between the excited states of nitrogen heterocycles and traces of peroxides or aliphatic alcohols are known to decrease the fluorescence yield of these compounds.^{37,38} Solvents marketed specifically for fluorescence or gas chromatographic use are generally pure enough to be used as supplied. Other grades of solvents should be purified by careful washing and/or distillation before use. Note that energy transfer can lead to either positive or negative changes in ϕ_f depending upon whether the extraneous solute is the energy recipient or the energy donor.

b. Factors Affecting I_0 and F Directly

i. Inner Filter Effects

Equation 3 was derived assuming that ϵ was small and therefore the light intensity available across the sample was essentially a constant equal to I_0 . If ϵ is not small, the intensity of light will decrease sharply across the sample and the fluorescence intensity will not be directly proportional to concentration, c , but will follow equation 2 instead.

The same effect results from other solutes which absorb at the excitation wavelength: the observed fluorescence becomes dependent on the total optical density of the solution as well as on the concentration of analyte. This is known as the "inner filter effect". Parker and Barnes³⁹ have given the following expression for correcting measured fluorescence intensities, F , for the inner filter effect in experiments where the emission is observed at right angles to the excitation, and where the absorbance of the

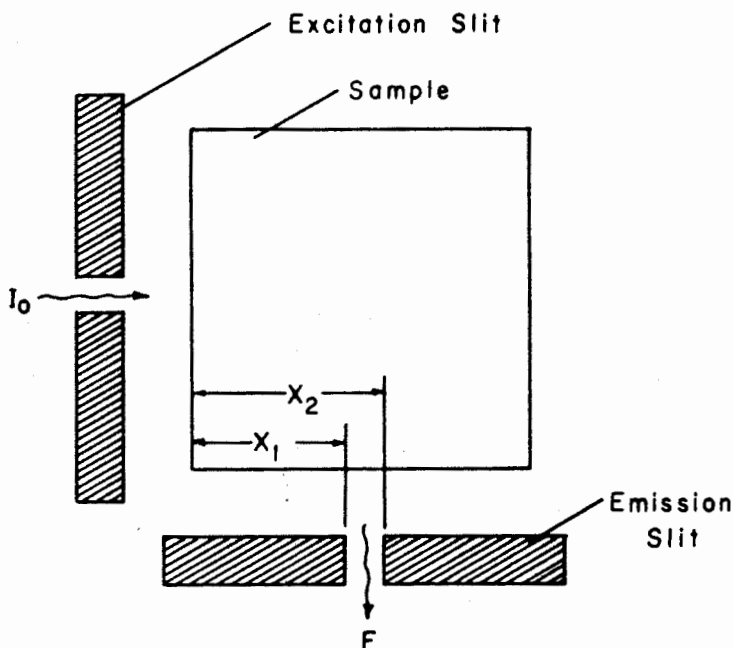


FIGURE 3. Cell and slit arrangement for observing fluorescence perpendicular to the incident beam.

analyte is small:

$$F_{\text{CORRECTED}} = \frac{2.303 A (x_2 - x_1)}{10^{-Ax_1} - 10^{-Ax_2}} F_{\text{OBSERVED}} \quad (4)$$

where A = total optical density of the solution at the excitation wavelength and x_1 , x_2 = position of the slit edges as defined in Figure 3.

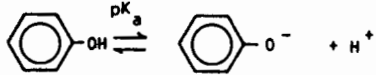
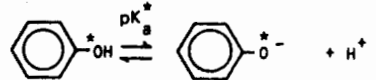
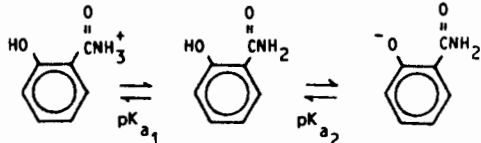
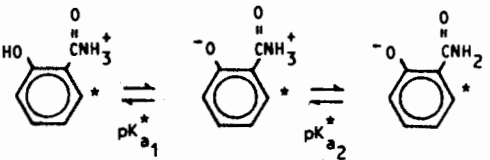
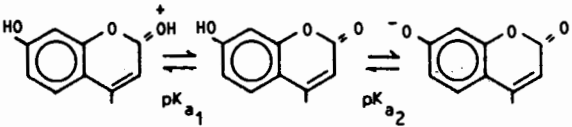
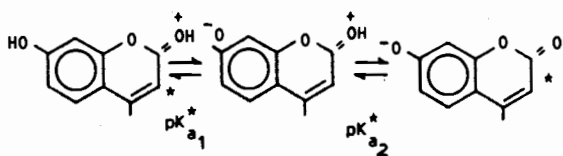
The correction is on the order of 1% for an optical absorbance of 0.01 for all slit widths in the experimental geometry shown in Figure 3 and increases at higher absorbance values as shown in Table 4. Equation 4 is not accurate when $F_{\text{CORRECTED}}/F_{\text{OBSERVED}}$ exceeds 3.0. The inner filter effect in strongly absorbing solutions may be a little smaller with frontal observation of the sample as shown in Figure 4, B: corrections for this geometry are given in Table 5.⁴⁰ The inner filter effect in other experimental geometries is discussed in Reference 1. A good description of correction of right angle fluorescence measurements has also been given by Holland et al.⁴¹

The observed fluorescence intensity, F , will also be decreased by solutes that absorb at the emission wavelength.⁴² An error on the order of 1% will result if the solution absorbance is 0.01 at the emission wavelength and 1-cm cells are used in the configuration shown in Figure 3.

ii. Scattered Light and Emission from Impurities

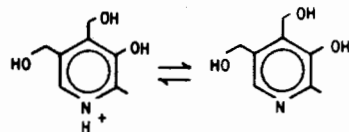
The observed intensity, F , may also contain contributions from scattered light and from fluorescent emission of other solutes. Light scattering may arise from the cuvette walls, Tyndall scattering by suspended particles, and Rayleigh and Raman scattering from the solvent. Light arising from scattering by the cuvette and from Tyndall and Rayleigh scattering will be of the same wavelength as the excitation source. Light arising from the fluorescence of other solutes or the cuvette glass, second order Rayleigh

Table 3
COMPARISON OF GROUND (S_0) AND FIRST EXCITED SINGLET (S_1) pK VALUES

Substance	State and pK	Reaction	Ref.
Phenol	S_0 $pK_a = 10.0$		25
	S_1 $pK_a^* = 3.7$		.
Salicylamide	S_0 $pK_{a1} = -2.6$ $pK_{a2} = +8.3$		24
	S_1 $pK_{a1}^* = -5.3$ $pK_{a2}^* = 2.1$		
4-Methyl-7-hydroxy coumarin	S_0 $pK_{a1} = -5.0$ $pK_{a2} = 7.8$		27
	S_1 $pK_{a1}^* = -4.1$ $pK_{a2}^* = 2.2$		

Pyridoxal

S_0 $pK_a = 5.0$



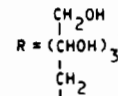
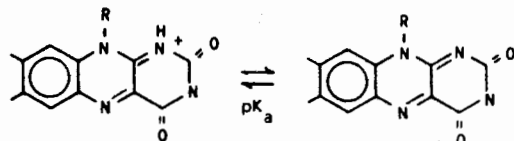
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S_1 $pK_a^* = -2.2$

Same as S_0

Riboflavin

S_0 $pK_a = -0.1$



28

S_1 $pK_a^* = 1.7$

Same as S_0

Lumiflavin

S_0 $pK_A = 0.0$

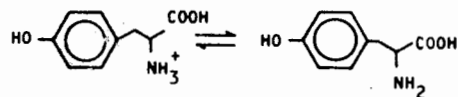
Same as riboflavin, $R = -\text{CH}_3$

28

S_1 $pK_a^* = 1.7$

Tyrosine

S_0 $pK_A = 2.1$



6

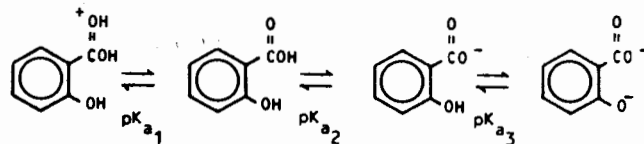
S_1 $pK_a^* = 1.8$

Salicylic acid

S_0 $pK_{a_1} = -8.0$

$pK_{a_2} = 3.0$

$pK_{a_3} = 14.0$



29.30

S_1 $pK_{a_1}^* = -7.0$

$pK_{a_2}^*$

$pK_{a_3}^* = 16.0$

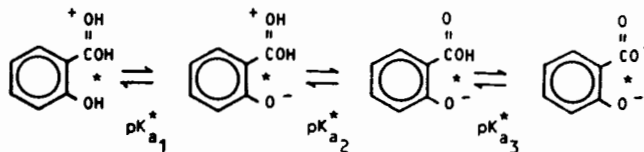


Table 4
CORRECTION FACTORS, ζ , FOR THE INNER
FILTER EFFECT.* FLUORESCENCE OBSERVED AT
RIGHT ANGLES TO THE DIRECTION OF
ILLUMINATION

Absorbance	Slit width, mm					
	.5	1.0	2.0	3.0	4.0	5.0
0.005	1.0060	1.0060	1.0060	1.0060	1.0060	1.0060
0.01	1.0118	1.0118	1.0118	1.0118	1.0118	1.0118
0.05	1.0594	1.0594	1.0594	1.0594	1.0594	1.0593
0.10	1.1222	1.1222	1.1221	1.1220	1.1218	1.1216
0.15	1.1887	1.1887	1.1885	1.1882	1.1878	1.1872
0.20	1.2591	1.2590	1.2587	1.2582	1.2574	1.2564
0.25	1.3337	1.3336	1.3330	1.3321	1.3308	1.3292
0.30	1.4127	1.4125	1.4117	1.4103	1.4083	1.4058
0.35	1.4964	1.4961	1.4949	1.4929	1.4900	1.4864
0.40	1.5850	1.5846	1.5829	1.5802	1.5763	1.5713
0.45	1.6789	1.6784	1.6761	1.6724	1.6672	1.6605
0.50	1.7783	1.7776	1.7747	1.7698	1.7630	1.7543
0.60	1.9952	1.9940	1.9893	1.9814	1.9705	1.9565
0.70	2.2385	2.2367	2.2295	2.2175	2.2008	2.1797
0.80	2.5115	2.5088	2.4982	2.4807	2.4564	2.4257
0.90	2.8176	2.8139	2.7988	2.7740	2.7398	2.6966
1.00	3.1611	3.1559	3.1351	3.1008	3.0538	2.9947

$$* F_{\text{corrected}} = \zeta \times F_{\text{observed}}$$

scattering, or Raman scattering will be of longer wavelength than the excitation source.

The intensity of scattered light entering the detector optics will be smaller if the detector views the sample at right angles to the excitation beam, than it will be in other configurations. Background from scattering and fluorescence of the cuvette walls can be minimized by arranging the slits so that the portion of the wall that is directly illuminated by the excitation source is not viewed by the detector. The cuvette must be kept meticulously clean, but use of dichromate cleaning solutions or fluorescent detergents must be avoided. Tyndall scattering is best controlled by careful preparation of the sample so that suspended particles or droplets of immiscible solvents are not present. Since most scattered light will be at the excitation wavelength, placing a filter between the sample and detector to block light of shorter wavelength than the emission will greatly reduce the background from scattered light. Use of a monochromator with relatively narrow band pass is also effective in reducing the background from scattered light. Second order Rayleigh scattering and Raman scattering may be observed at longer wavelengths than the excitation, but are of low intensity and generally will not interfere in any but the most sensitive of assays. The second order Rayleigh scatter occurs at twice the wavelength of the incident light, e.g., excitation at 300 nm will give rise to strong Rayleigh scattering at 300 nm and weak second order scattering at 600 nm. Raman scattering will also be observed at longer wavelengths, but the wavelength is a characteristic of the solvent. In terms of frequency,^{41,42}

$$\nu_{\text{RF}} = \nu_{\text{R}} + \nu_{\text{F}} \quad (5)$$

where ν_{R} = frequency of the Raman scattered light, ν_{R} = frequency of the Raman band of the solvent, and ν_{F} = frequency of the excitation beam. Equation 5 can be

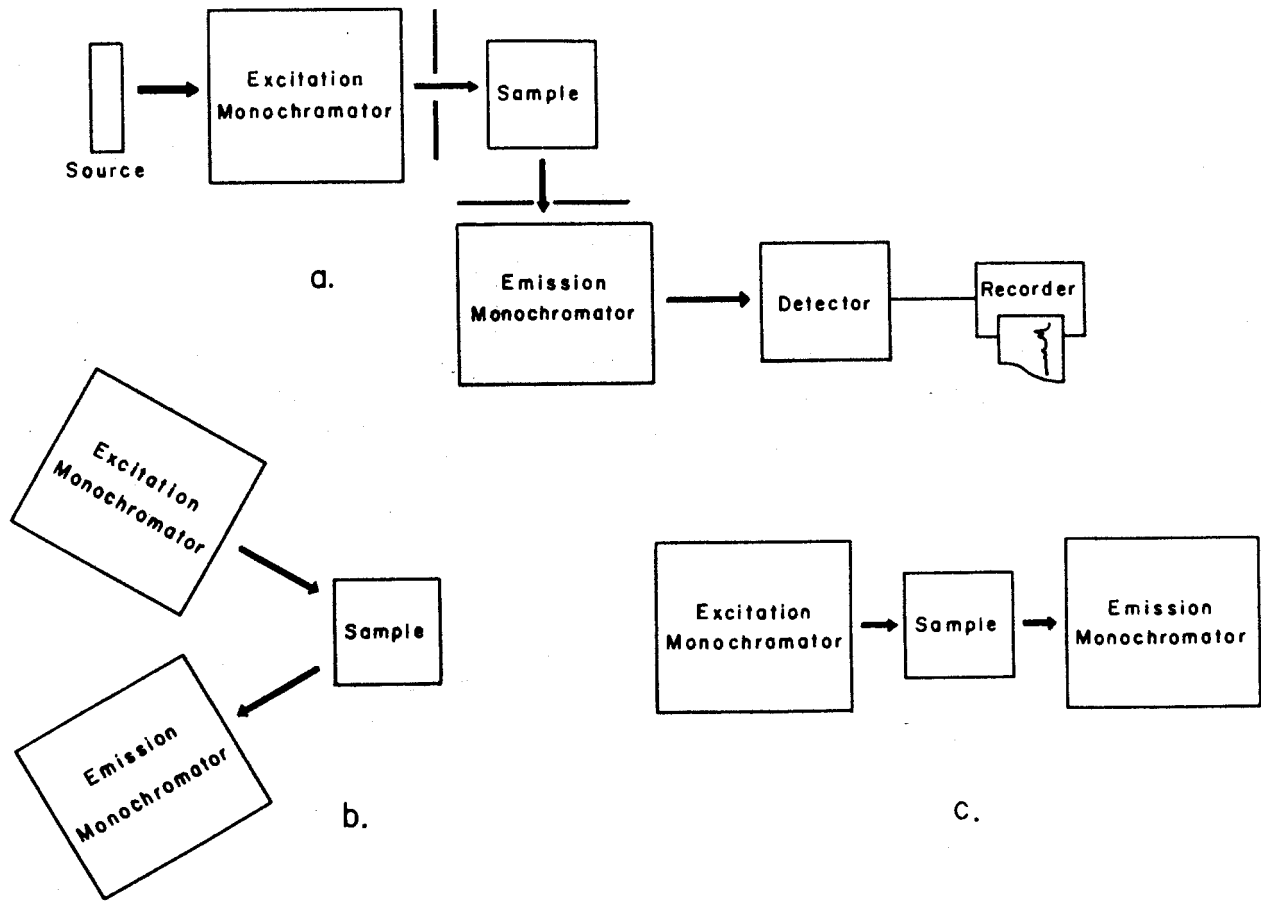


FIGURE 4. A. Block diagram of a fluorometer employing the 90° geometry; B. frontal geometry; C. in-line geometry.

Table 5
CORRECTION FACTORS, ζ , FOR INNER FILTER
EFFECT: FLUORESCENCE OBSERVED WITH
FRONTAL GEOMETRY (FIGURE 4)^a

Optical density	L = 0.25 ^a	L = 0.50 ^a	L = 1.00 ^a
1.0×10^{-4}	9.6236×10^{-2}	1.6194×10^{-1}	2.4998×10^{-1}
2.0	9.6233	1.6193	2.4995
3.0	9.6231	1.6192	2.4993
4.0	9.6228	1.6191	2.4991
5.0	9.6226	1.6191	2.4989
6.0	9.6223	1.6190	2.4986
7.0	9.6221	1.6189	2.4984
8.0	9.6218	1.6188	2.4982
9.0	9.6215	1.6187	2.4979
1.0×10^{-3}	9.6213	1.6187	2.4977
2.0	9.6187	1.6178	2.4954
3.0	9.6162	1.6170	2.4931
4.0	9.6136	1.6162	2.4909
5.0	9.6111	1.6154	2.4886
6.0	9.6085	1.6146	2.4863
7.0	9.6060	1.6138	2.4840
8.0	9.6034	1.6130	2.4818
9.0	9.6009	1.6122	2.4795
1.0×10^{-2}	9.5983	1.6114	2.4772
2.0	9.5729	1.6033	2.4548
3.0	9.5476	1.5953	2.4326
4.0	9.5223	1.5874	2.4107
5.0	9.4972	1.5795	2.3892
6.0	9.4721	1.5717	2.3679
7.0	9.4471	1.5640	2.3469
8.0	9.4223	1.5563	2.3262
9.0	9.3975	1.5486	2.3058
1.0×10^{-1}	9.3728	1.5410	2.2857
2.0	9.1308	1.4680	2.0984
3.0	8.8975	1.3999	1.9342
4.0	8.6725	1.3363	1.7897
5.0	8.4555	1.2770	1.6620
6.0	8.2462	1.2216	1.5490
7.0	8.0442	1.1698	1.4484
8.0	7.8493	1.1213	1.3586
9.0	7.6611	1.0758	1.2783
1.0×10	7.4795	1.0332	1.2060
2.0	5.9684	7.2400×10^{-2}	7.6139×10^{-2}
3.0	4.8809	5.4620	5.5495
4.0	4.0813	4.3530	4.3747
5.0	3.4805	3.6102	3.6158
6.0	3.0197	3.0826	3.0841
7.0	2.6589	2.6899	2.6903
8.0	2.3710	2.3865	2.3866
9.0	2.1373	2.1451	2.1451
1.0×10^1	1.9444	1.9484	1.9484
2.0	1.0196	1.0196	1.0196
3.0	6.9172×10^{-1}	6.9172×10^{-1}	6.9172×10^{-1}
4.0	5.2373	5.2373	5.2373
5.0	4.2151	4.2151	4.2151
6.0	3.5273	3.5273	3.5273
7.0	3.0328	3.0328	3.0328
8.0	2.6600	2.6600	2.6600
9.0	2.3689	2.3689	2.3689

Table 5 (continued)
CORRECTION FACTORS, ξ , FOR INNER FILTER
EFFECT: FLUORESCENCE OBSERVED WITH
FRONTAL GEOMETRY (FIGURE 4)^a

Optical density	L = 0.25 ^a	L = 0.50 ^a	L = 1.00 ^a
1.0 × 10 ⁴	2.1354	2.1354	2.1354
2.0	1.0757	1.0757	1.0757
3.0	7.1908 × 10 ⁻⁴	7.1908 × 10 ⁻⁴	7.1908 × 10 ⁻⁴
4.0	5.4009	5.4009	5.4009
5.0	4.3246	4.3246	4.3246
6.0	3.6061	3.6061	3.6061
7.0	3.0924	3.0924	3.0924
8.0	2.7067	2.7067	2.7067
9.0	2.4067	2.4067	2.4067

^a L = cell depth in cm., Cell width is 1.0 cm. (see Figure 4) $F_{CORR} = F_{OBS} \times \xi$

^b From Mode, V. A. and Sisson, D. H., *Anal. Chem.*, 200, 1831, 1974. With permission.

rewritten in terms of the wavelengths λ_{RE} of the scattered light and λ_E of the excitation light as:

$$\lambda_{RE} = \frac{\lambda_e}{1 - \lambda_e/\Delta\lambda_R} \quad (6)$$

where $\Delta\lambda_R$ is the wavelength corresponding to the characteristic frequency shift of the Raman band. Values of $\Delta\lambda_R$ in nanometers for some prominent Raman bands of common solvents are given in Table 6. Positions of bands for other solvents may be calculated from Raman band positions given in Reference 46. The dependence of the emission wavelength on the excitation wavelength can be used experimentally to distinguish Raman from other types of scattering and emission.

The presence of emission from solutes other than the analyte is more difficult to identify, but it can sometimes be detected through the differences that exist between the excitation spectra of the two compounds. In particular, if the fluorescence efficiencies of the substances differ, then the efficiency calculated from the corrected fluorescence spectra of the sample will depend upon the excitation wavelength. This test must be used with caution because a natural dependence of ϕ_f on excitation wavelength is found in some cases,³⁻⁶ and because an apparent dependence of ϕ_f on excitation wavelength can be an artifact resulting from inner filter effects in optically dense solutions.

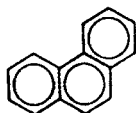
The effect of emission from extraneous fluorescent solutes on an analysis can be minimized by careful selection of the excitation and emission wavelengths. However, even when the excitation wavelength selected is well to the long wavelength side of the normal absorption band of an impurity, the impurity can still contribute to the observed fluorescence intensity. This situation arises because a small percentage of the impurity molecules will be in a vibrationally excited state and may therefore be excited to a level in the S₁ state by absorption of a photon at longer wavelength than is characteristic of the substance. The fluorescence emission spectrum will be the same as the normal emission spectrum of the molecule, but a portion of the emission may actually be of shorter wavelength than the exciting light.⁴⁷ Fluorescent emission at wavelengths shorter than the excitation wavelength is known as anti-Stokes fluorescence and has been studied in detail for a number of compounds including phenanthrene(XIV)⁴⁷ and 1,12-benzoperylene(XV).⁴⁸ Anti-Stokes emission is low intensity and can be reduced

Table 6
RAMAN BANDS FOR COMMON SOLVENTS⁴⁵

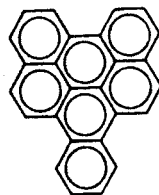
Solvent	Frequency shift, cm^{-1}	$\Delta\lambda_s$, nm	Source
Water	3380	2959	Hydrogen bonded O-H stretch
Ethanol	2920	3425	C-H Stretch
	1400	7143	CH_2 Rocking/twisting
Cyclohexane	2880	3472	C-H Stretch
	1360	7353	CH_2 Wag
Carbon tetrachloride	700	14,290	C-Cl Stretch (?)
Chloroform	3020	3311	C-H Stretch
	700	14,290	C-Cl Stretch

From Parker, C. A., *Analyst (Amsterdam)*, 84, 446, 1959. With permission.

further by operating at lower temperatures since the intensity of emission is proportional to the population of the upper vibrational levels of S_0 .



XIV



XV

Complete removal of the interference can only be accomplished by chemical means. The procedure for preparing samples must be designed to remove likely fluorescent impurities from the sample. Solvents and reagents should be checked for fluorescent impurities before use. As impurities in solvents may quench fluorescence as well as add to the luminescence background, it is advisable to use solvents marketed specifically for fluorescence work, or that have been redistilled or otherwise purified before use.

III. FLUOROMETERS

A. Instrument Geometry

A fluorometer consists of a sample compartment situated between a light source and a light detector as shown in the block diagram in Figure 4, A. The most common arrangement is for the detector to view the sample at right angles to the incident light beam because this configuration minimizes the background signal from scattered light. A frontal arrangement (Figure 4, B) is necessary with opaque samples and is often used for optically dense solutions. The frontal arrangement has the disadvantage that scattering and fluorescence from the cuvette wall are viewed directly by the detector slit. Inner filter effects are smaller but far from negligible in frontal geometries (see Tables 4 and 5).

The in-line geometry of Figure 4, C can also be used. This geometry can be used for strongly absorbing and strongly fluorescent solutions, but has the serious disadvan-

tage that the detector directly views the source. In-line instruments therefore require much better wavelength selectivity in both the excitation and emission monochromators than are necessary with other instrument geometries. All three configurations should give comparable spectra for weakly absorbing sample solutions.'

B. Cells

The excitation wavelength in many fluorescence applications is in the UV region of the spectrum, so silica or quartz cells must be used. Cells intended for fluorescence work must be selected for low fluorescence under UV irradiation: properly annealed cells of synthetic silica are generally the most satisfactory.

Cells should be handled very carefully to avoid scratching or chipping, since defects in the surface will increase the amount of scattered light reaching the detector. They should be cleaned by rinsing with solvent and then washing with a nonfluorescent inorganic detergent, such as Calgonite[®], immediately after use. A solution of hydrochloric acid in ethanol may also be used, but concentrated acids and alkalis should be avoided.

C. Light Source

Mercury vapor and xenon lamps are the usual light sources for fluorometers. The mercury lamps offer intense light output at select wavelengths, good stability, and the economies of a simple power supply and low lamp cost. The wavelengths of the mercury emission lines and their relative intensities are given in Table 7. In many cases, the high intensity and narrow frequency range of the mercury lines more than compensate for the loss in signal that results from exciting at a wavelength different than the optimum for the substance being studied. The frequency coverage of a mercury lamp can be increased by increasing the vapor pressure, which broadens the emission lines, or by coating the inside of the lamp with phosphors that emit light over a broader band.

The high pressure xenon lamp provides a continuum with good intensity from below 250 nm up into the visible. The intensity of the xenon lamp is only about one half that of the mercury vapor lamp at the emission maxima of the mercury lamp. In spite of somewhat lower intensity, the xenon lamp is widely used because it provides complete coverage of the UV spectrum and because a continuous source is essential for a scanning spectrophotofluorometer. A disadvantage of the xenon lamp is instability of the output intensity and arc wander. As the arc shifts back and forth, slightly different wavelengths are focused on the exit slit and the fluorescence intensity fluctuates accordingly. A significant contribution to assay imprecision can result from these fluctuations. A good discussion of the xenon, mercury vapor, and other lamps can be found in Reference 1.

D. Wavelength Selection

Wavelength selection for both the excitation and emission sides of the instrument may be accomplished by either filters or monochromators, or both. Filter fluorometers require a filter between the sample and the source (excitation filter) and another between the sample and the detector (emission filter). The excitation filter is chosen to block all wavelengths of light greater than the desired excitation wavelength: this prevents the longer wavelengths from the source from being scattered into the detector and causing an unacceptably high background signal. The emission filter is chosen to block all wavelengths shorter than the desired emission wavelength: this prevents scattered light at the excitation wavelengths from entering the detector and again causing a high background signal. When placed in series, a properly chosen pair of excitation and emission filters will block light at essentially all wavelengths.

Table 7
MERCURY EMISSION
LINES

λ , nm	Relative intensity
253.7	10,000
296.5	60
302.2	110
312.2	71
313.2	11
365.0	89
365.5	21
366.3	14
404.7	89
435.8	170
546.1	120
577.0	17
579.0	18

Filter fluorometers offer the advantage of relatively low cost, easy operation, and good sensitivity. They have the disadvantages that (1) the excitation and emission wavelengths must be quite different in order for them to be properly isolated by the filters, (2) they are not useful when extremely high sensitivity is required because the Raman emission of the solvent will occur at wavelengths not blocked by the emission filter, and (3) filter instruments cannot be scanned to record the entire fluorescence spectrum.

Monochromator instruments may use either prisms or gratings to disperse the light. Very narrow excitation and emission bands can be selected which reduce the magnitude of interference from scattering, other fluorescent species in the solution, etc. Grating instruments have the additional advantage that resolution is constant across the entire spectrum, which makes them especially well suited for use in scanning fluorometers. Filters may be used in conjunction with a grating monochromator in high sensitivity work in order to eliminate the second order spectrum passed by the monochromator.

E. Detectors

Two types of photodetectors are commonly used in fluorescence work: barrier layer cells and photomultiplier tubes. A barrier layer cell consists of a thin layer of a semiconductor on a metal base. Light incident on the semiconductor surface generates an emf, and hence a current, which can be amplified and recorded. These cells generally are most sensitive to light in the 500- to 600-nm region. Barrier cells have the disadvantages that they fatigue rather rapidly and are not very sensitive.

The photomultiplier tube is a very sensitive light detector which provides both detection and amplification of the incident light signal. Radiation incident on the photomultiplier photocathode causes electrons to be ejected from it. The ejected electrons are accelerated by high voltage toward a second photosensitive electrode (called a dynode), where each incident electron displaces several more electrons. This process is repeated several times and results in signal amplification as high as 10^4 or 10^6 .

The maximum sensitivity of a particular photomultiplier tube occurs in a narrow wavelength band, but a variety of photomultiplier tubes are available which cover the UV and visible spectrum quite well. For high sensitivity work it is important that the photomultiplier tube be selected for maximum sensitivity at the emission wavelength of the analyte.

F. Correction of Spectra

The true excitation spectrum of a substance is a plot of $\epsilon \phi_f$ vs. wavelength, where ϵ

is the molar absorptivity and ϕ_f is the fluorescence quantum efficiency. The emission spectrum is a plot of the relative number of quanta emitted as a function of wavelength under constant excitation conditions. Experimentally the excitation spectrum is obtained by setting the emission monochromator at the wavelength of the fluorescence maximum and then scanning the excitation monochromator. The emission spectrum is obtained by setting the excitation monochromator at the wavelength of the excitation maximum and scanning the emission monochromator. The intensities recorded in this way do not give the true fluorescence spectrum because they are affected by several instrumental factors.

The excitation spectrum obtained in this way is actually proportional to $I_o\phi_f$ as a function of wavelength and must be corrected for variations in I_o with wavelength in order to obtain the true spectrum. I_o varies across the spectrum because both the lamp output and the efficiency of the monochromator are wavelength dependent. The excitation spectrum may be corrected easily by splitting the exciting light into two beams. One of the beams irradiates the sample. The other is monitored by an auxiliary detector with flat spectral response in order to obtain a measure of I_o . The corrected excitation spectrum is obtained by recording the ratio, F/I_o , of the output of the fluorescence detector to that of the auxiliary detector. Using the ratio F/I_o has the additional advantage in analytical work of improving precision by correcting for fluctuations and drift in the lamp output.

The emission spectrum is affected by the wavelength dependence of the emission monochromator efficiency and the detector sensitivity. Correction of the emission spectrum is more difficult than correction of the excitation spectrum and requires measurement of the response curve of the detector-monochromator combination. The experimental emission spectra are then divided by the response curve to obtain the corrected emission spectrum. Correction of the emission spectrum offers no advantage in quantitative analysis, although knowledge of the corrected spectrum can be very helpful in optimizing assay conditions when maximum sensitivity is necessary. For maximum sensitivity, a lamp should be chosen with the highest possible output near the maximum in the corrected excitation spectrum, and a detector should be chosen with maximum sensitivity near the maximum in the corrected emission spectrum. A more detailed discussion of correcting fluorescence spectra can be found in References 1, 40, 41, and 49.

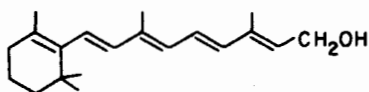
IV. RELATION OF FLUORESCENCE TO MOLECULAR STRUCTURE

A. General Considerations

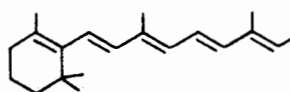
A necessary condition for a compound to fluoresce is that it absorb light in the UV or visible region of the spectrum. While a number of chromophores absorb above 200 nm, only those based upon a conjugated π electron system will generally give efficient re-emission of the absorbed energy. In particular, the most intense fluorescence is associated with molecules whose lowest energy electronic transitions are $\pi \rightarrow \pi^*$, as these molecules have the greatest fluorescence efficiency and the highest ϵ values. Simple aliphatic compounds that absorb above 200 nm (RSH, $\lambda_{MAX} = 225$ to 230 nm; R_2S , $\lambda_{MAX} = 210$ to 215 nm and 235 to 240 nm; RBr, $\lambda_{MAX} = 200$ to 210 nm; RI, $\lambda_{MAX} = 255$ to 260 nm) generally dissipate absorbed light energy through photo-dissociation, reaction, or other nonradiative processes and are not fluorescent. The lowest energy transition for compounds whose principal chromophore is a simple group with a π -bonded heteroatom (ketones, aldehydes, carboxylic acids, amides, esters, azo and nitro compounds, etc.) is $n \rightarrow \pi^*$. These substances generally undergo efficient internal conversion and show very low fluorescence efficiencies.

Most fluorescent organic compounds are therefore either polyenes, aromatics, or

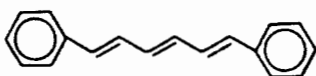
derivatives of these two classes. Among these compounds, fluorescence efficiencies are greatest when the π system is rigid and planar in both the ground and excited state.⁵⁰ Acyclic polyenes tend to have lower fluorescence efficiencies than their cyclic analogues because they are more flexible and their chromophores are often nonplanar due to steric interactions. Retinol (Vitamin A, XVI) is a simple polyene which exhibits fairly strong fluorescence emission at 470 nm when excited at 325 nm.⁵¹ The retinals (XVI, XVII) with $n \rightarrow \pi^*$ being the lowest energy transition⁵² are not fluorescent at room temperature. Other examples include 1,8-diphenyloctatetraene (XVIII),⁵⁰ with principal excitation bands at 355 nm, 374 nm, and 398 nm, emission bands at 453 nm, 487 nm, 520 nm, and 560 nm, and a quantum efficiency of 0.09; and *trans*-stilbene (XIX)^{53,54} with principal excitation bands at 278 nm, 294 nm, and 308 nm, emission bands at 334 nm, 350 nm, and 368 nm, and a quantum efficiency of 0.01. *cis*-Stilbene (XX) is not fluorescent at all.⁵⁴ The low fluorescence efficiency in the stilbenes appears to be the result of significant deviations from planarity in the excited states rather than in the ground state.^{55,56} The stilbenes also undergo photo-isomerization and photochemical reactions.^{53,59}



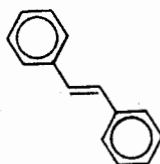
XVI



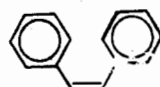
XVII



XVIII

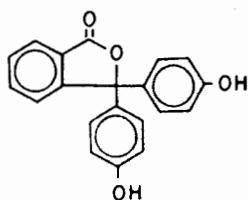


XIX

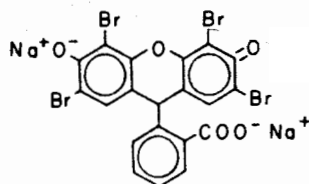


XX


Phenolphthalein (XXI), fluorescein (VII), and eosin yellow (XXII) provide other examples of the importance of rigidity in achieving high fluorescence efficiencies. Phenolphthalein does not fluoresce at any wavelength. Fluorescein, which differs from phenolphthalein only in the oxygen bridge restraining the rings being coplanar, emits with an efficiency of 0.92 when excited at 366 nm in 0.1 N NaOH.¹² Eosin yellow emits with an efficiency of 0.19 when excited at 366 nm.¹² Eosin yellow has a lower quantum efficiency than fluorescein because the substitution with bromine increases intersystem crossing — a general phenomenon observed with heavy atom substitutions.



XXI



XXII

Table 8
 FLUORESCENCE CHARACTERISTICS OF SEVERAL
 SUBSTITUTED BENZENES⁶² R_1 -- R_2

R_1	R_2	Excitation, nm	Emission, nm	Quantum efficiency
H	OH	270	330	0.032
	OCH ₃	270	303	0.034
	NH ₂	280	350	0.025
	N(CH ₃) ₂	286	365	0.097
	F	257	289	0.007
NH ₂	Cl, Br, I, NO ₂	NHCCH_3 , COOH	No fluorescence	
	CH ₃	289	357	0.028
	OCH ₃	297	375	0.042
	SO ₂	254	352	0.050
	F	289	362	0.123
	Cl _o	290	362	0.017
		NHCCH_3 , Br, NO ₂	290	352
OH	CH ₃	278	313	0.088
	OCH ₃	289	328	0.059
	F	276	315	0.030
	Cl	280	317	0.089
	Br, NO ₂		No fluorescence	

From Bridges, J. W. and Williams, R. T., *Nature (London)*, 196, 4849, 1962. With permission.

The strong fluorescence of many metal chelates^{58,59} is also attributable in part to the formation of rigid, planar ring structure in the complex. However, shifts in the electronic energy levels of the ligands do occur upon formation of the complex as evidenced by shifts of their absorption bands to longer wavelengths, and these shifts may also contribute to the efficiency of the fluorescence.

B. Substituent Effects on the Fluorescence of Benzene Derivatives

Benzene itself is only weakly fluorescent. In solution in ethanol it emits at 270 nm with an efficiency of 0.04 when excited at 248 nm.^{60,61} Substitutions on the ring often have a strong effect on the fluorescence of benzene,⁶²⁻⁶⁵ and these effects provide insights into the way substituents affect the fluorescence of aromatic systems in general. The fluorescence characteristics of a number of substituted benzenes are given in Table 8. The following generalizations can be made:

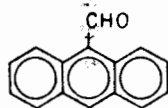
- Most groups that are *ortho/para* directors in electrophilic aromatic substitution shift the excitation and emission to longer wavelengths.⁶³ They may enhance fluorescence efficiency or have no effect upon it, but generally will not decrease it. This group includes -OH, -NH₂, -NHR, -NRR', -OR, and R, where R and R' are alkyl groups. -NHC-R and -O⁻ (phenolate anion) are exceptions as they are *ortho/para* directing but strongly inhibit fluorescence in substituted benzenes: however, in polycyclic phenols such as α -naphthol and 4-hydroxybiphenyl, only the phenolate anion and not the neutral species are fluorescent.
- Groups with direct *meta* in electrophilic aromatic substitution reduce fluorescence efficiency and often eliminate it altogether.⁶⁴ This group includes -NR₃⁺, -COOH, -NO₂, $\text{RC}(=\text{O})-$, -CHO, -N=N-, -I, -Br, -Cl, and -F. The nitrile group, -C \equiv N, is an exception as it is *meta* directing, but does not inhibit fluorescence.

Table 9
SUBSTITUENT EFFECTS ON
THE FLUORESCENCE OF
BENZENE

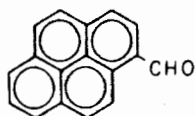
Positive or no effect	Negative effect
-OH	-NR ₂ ⁺ -O ⁻
-NH ₂	-NO ₂ -COOH
-NHR	$\begin{array}{c} \text{O} \\ \parallel \\ \text{RC}- \end{array}$
-NHRR'	-CHO
-OR	-N=N- $\begin{array}{c} \text{O} \\ \parallel \\ \text{NH}-\text{C}-\text{R} \end{array}$
-R	
-CN	-I -Br -Cl -F

The effect of halogen substitution decreases regularly from iodine to fluorine, and is usually very small for fluorine. Rules 1 and 2 are summarized in Table 9.

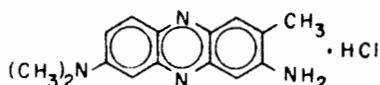
3. When both *ortho/para* and *meta* directing groups are present, the compound is usually fluorescent. The transitions giving rise to the fluorescence in this case may be of the charge transfer type rather than $\pi \rightarrow \pi^*$. Simple aromatic aldehydes are an exception to this rule in that their lowest energy transition is almost always $n \rightarrow \pi^*$, and so they are very rarely fluorescent, although they may become fluorescent in acidic alcohols due to acetal formation.⁶⁶ Introduction of a heteroatom into the conjugated system (see Section C below), or increasing the size of the system lowers the energy of the $\pi \rightarrow \pi^*$ transition more rapidly than that of the $n \rightarrow \pi^*$ transition with the result that complex aromatic aldehydes and heteroaromatic aldehydes are often fluorescent.⁶⁷ Thus 9-anthraldehyde (XXIII) is very weakly fluorescent, pyrene-3-aldehyde (XXIV) fluoresces fairly strongly in polar hydrogen-bonding solvents, while pyridoxal (VIII)⁵¹ and Nuclear Fast Red (XXV)⁶⁸ fluoresce strongly in neutral aqueous solution.



XXIII



XXIV



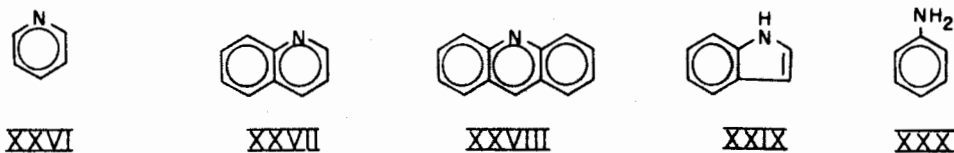
XXV

4. Substitution of a heavy atom for a lighter one, e.g., sulfur for oxygen or bromine for chlorine, tends to decrease the fluorescence efficiency.

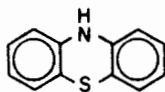
C. Fluorescence of Anilines and Nitrogen Heterocycles

The fluorescence of anilines,⁶⁹ sulfonamides,⁷⁰ and indoles⁶⁹ have been systematically studied. Fluorescence in these compounds and other small nitrogen heterocycles is dependent upon the nature of the nitrogen lone pair electrons. In compounds such as pyridine(XXVI) and quinoline(XXVII), the unshared pair occupies an sp^2 orbital which is orthogonal to the π -orbitals as shown in Figure 5. The lowest energy transition is $n \rightarrow \pi^*$ for small heterocycles with this electron configuration, and they do not fluoresce. However, increasing solvent polarity and increasing the size of the conjugated system both lower the energy of $\pi \rightarrow \pi^*$ transitions more rapidly than $n \rightarrow \pi^*$ transitions. As a result, quinoline does show some fluorescence in highly polar solvents.⁷¹ Acridine(XXVIII) fluoresces⁷² blue in its uncharged form, green in its protonated form, and the fluorescence yield increases with the polarity of the solvent.⁷¹

The unshared pair of electrons in indole(XXIX) and aniline(XXX) participate in the π -system as shown in Figure 5. The lowest energy transition for compounds with this electronic configuration is a $\pi \rightarrow \pi^*$ transition, and the free bases of these compounds can be expected to fluoresce in the absence of fluorescence inhibiting substituents. The protonated forms of anilines and indoles are not fluorescent; the unshared pair is no longer available to the π system, and they behave like aromatic compounds substituted with a strong fluorescence inhibiting group ($-NH_2^+$, see Table 9).



The effects of ring substituents on the fluorescence of anilines are similar to their effects on phenols and other benzene derivatives,⁶⁵ as discussed in the preceding section. Substituent effects on the fluorescence of heterocyclic ring systems have been studied in only a few special cases, including the barbiturates(XXXI),⁷³⁻⁷⁵ thiobarbiturates(XXXII),⁷⁶ and phenothiazines(XXXIII).^{77,78A,B} The fluorescence of the barbiturates arises from their dianions and is therefore only observed in strongly basic solutions.⁷³⁻⁷⁵ Barbiturates with at least one hydrogen on carbon 5 form the dianion shown in Figure 6, A, and are only weakly fluorescent with an emission maximum at 400 nm. 5,5-Disubstituted barbiturates form the dianion shown in Figure 6, B, and this dianion fluoresces strongly at 420 nm when excited at 277 nm. N-alkylation destroys the fluorescence entirely as dianion formation is no longer possible. The fluorescence intensity of 5,5-disubstituted barbiturates is reduced if one of the substituents is a phenyl or other unsaturated group.



XXXIII

Thiobarbiturates exist as zwitterions up to pH 11 and as anions at higher pH values⁷⁹ as shown in Figure 7. Both the zwitterion and anion are fluorescent, but the anion fluoresces more strongly.⁷⁶ The thiobarbiturates studied have excitation maxima at 312 nm and emission maxima at 510 nm. The excitation and emission wavelengths are independent of pH. Substitutions on the ring nitrogens decrease the fluorescence, while

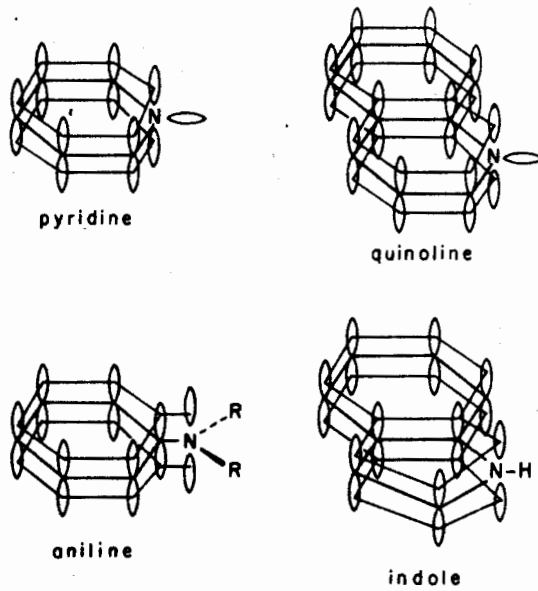


FIGURE 5. Electron orbitals in nitrogen heterocycles.

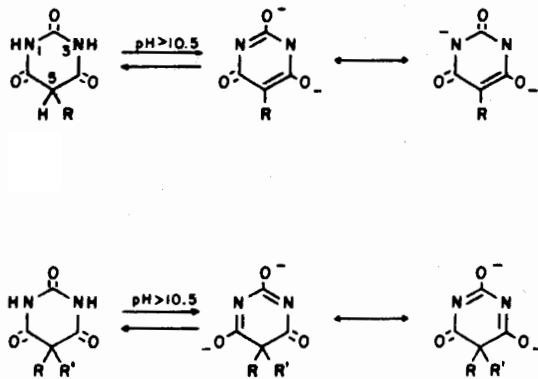


FIGURE 6. Dianions of 5-substituted (upper) and 5,5-disubstituted (lower) barbiturates.

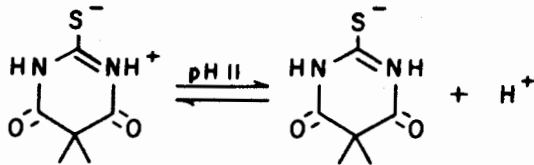


FIGURE 7. Ionization of thiobarbituric acid.

substitution on carbon 5 with a phenyl or other unsaturated group enhances the fluorescence intensity — just opposite the effect of unsaturated substituents on the fluorescence of oxo-barbiturates.

Phenothiazines^{77,78} have excitation maxima at 250 to 275 nm and 305 to 325 nm,

and a single emission maximum at 450 to 475 nm (uncorrected). The exact wavelengths for both excitation and emission depend upon the substituents on the ring. The effect of substituents at carbon 2 on the wavelengths of the excitation and emission follows the order:

shorter wavelength: $-\text{OCH}_3$, $-\text{H}$, $-\text{Cl}$, $-\text{CF}_3$, $-\text{SCH}_3$; longer wavelength

The wavelengths are, as expected, independent on the alkyl chain at N-10.

D. Fluorescence of Miscellaneous Compounds

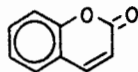
Systematic studies of the fluorescence of classes of compounds other than those already discussed have not been made, although several groups are typically fluorescent. The fluorescence of oxazoles(XXXIV) and oxadiazoles(XXXV) has been reported.⁶⁰ Many coumarins(XXXVI) are fluorescent. The hydroxycoumarins fluoresce strongly when the hydroxyl is at C-7, much weaker when at C-3 or C-4, and nonfluorescent when at C-6 or C-8.⁶¹ Tetracyclines(XXXVII) fluoresce in alkaline solutions, with the excitation maximum near 390 nm and an emission maximum near 520 nm.



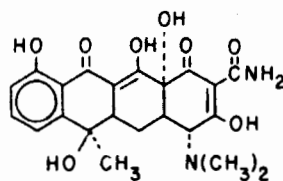
XXXIV



XXXV



XXXVI



XXXVII

V. PHARMACEUTICAL APPLICATIONS OF FLUORESCENCE ANALYSIS

A. Scope

A substance must absorb light in order to fluoresce and as a result, any substance that can be measured fluorimetrically can also be measured spectrophotometrically. The choice of method is made according to the requirements of the problem at hand. Fluorimetric methods offer greater sensitivity than spectrophotometric methods, but are less precise and more susceptible to systematic errors. As a result, spectrophotometric methods are widely used in the analysis of drug substances and formulations, while fluorimetric methods are generally reserved for analysis of drugs in biological samples where great sensitivity is needed and greater variability can be tolerated. However, fluorometric methods should be considered when devising methods for small amounts of material, such as encountered in the analysis of trace impurities in a drug substance or in unit dose assays of certain steroids, alkaloids, and other drugs which are administered in very low doses.

The pharmaceutical applications of fluorescence reviewed in the following sections will be limited to methods using the native fluorescence of the drug substance or the development of fluorescence through a reaction unique to a particular drug or group of drugs. Additional references to the fluorescence of certain classes of drugs or to drugs containing specific chromophores can be found in Section IV of this chapter. Many other applications of fluorescence can be found in books by Parker,¹ Guilbault,¹⁹ Pringsheim,²⁷ White and Argauer,⁶⁶ Pesez and Bartos,⁶⁴ Udenfriend,^{65,66} Hercules,⁶⁷ and Passwater.⁶⁸⁻⁷⁰ The fluorescence spectra of a large number of aromatic compounds can be found in Berلمان's book.⁷¹ In addition, recent developments in

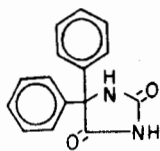
fluorescence analysis are reviewed every other year in the annual review issue of *Analytical Chemistry*.

B. Diphenylmethane Derivatives

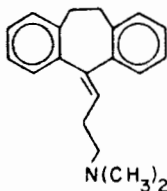
A number of drugs contain the diphenylmethane group and can be analyzed fluorimetrically using either the native fluorescence of this group or the fluorescence of its oxidation products. Wirz et al.⁹² have studied the fluorescence and phosphorescence of a number of antihistamines containing the diphenylmethane group. The characteristic wavelengths and quantum efficiencies found for these compounds are given in Table 10. The fluorescence spectra of the compounds in Table 10 are quite similar to one another, are the same in dilute aqueous acid and in ethanol, and are relatively insensitive to pH. The fluorescence arises from the entire diphenylmethane group, since the spectra are quite different from those of benzene, toluene, and other substances containing only isolated phenyl groups.⁹² The limit of detection for the antihistamines ranged from 10^{-7} to 10^{-9} M. Wirz et al.⁹² also reported a specific fluorometric procedure for the analysis of phenhydramine hydrochloride injection in this paper.

Diphenylmethane derivatives can also be oxidized to benzophenone derivatives which fluoresce in strongly acidic solutions. Cyclizine(XXXXIV), chlorcyclizine(XXXX), meclizine(XXXXIII), and diphenhydramine(XXXIX) can be analyzed by oxidation with 3% hydrogen peroxide at 90°C for 30 min and measured at the wavelengths given in Table 10.⁹³

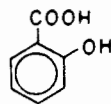
Diphenylhydantoin(XXXXV) has been analyzed in plasma by oxidation with alkaline potassium permanganate, extraction of the resulting benzophenone into heptane, and back extraction into concentrated sulfuric acid. The fluorescence emission is then measured at 485 nm with excitation at 355 nm.⁹⁴ Amitriptyline(XXXXVI) has been quantitated in biological samples by measuring the emission at 555 nm with excitation at 305 nm after extraction and heating with aqueous perchloric acid⁹⁵ to develop the fluorescence.



XXXXV



XXXXVI



XXXXVII

C. Salicylates

Salicylic acid(XXXXVII) and many of its derivatives fluoresce strongly in solution. The corrected emission and excitation wavelengths of XXXXVII, *o*-anisic acid(XXXXVIII) and methyl salicylate(XXXXXIX) are given in Table 11 as a function of acidity in water and chloroform. The unusually long emission wavelength for XXXXVII is the result of transfer of the proton from the hydroxyl to the carboxyl group during the lifetime of the excited state.^{10, 96, 97} The fluorescence of free salicylic acid has been widely used for the analysis of salicylic acid, acetylsalicylic acid, and related substances in plasma and urine samples. Total salicylates in plasma can be determined by precipitating proteins with tungstic acid, diluting the protein-free sample with 10 N sodium hydroxide, and measuring the fluorescence emission at about 389 nm while exciting at 322 nm.⁹⁸ The optimum wavelengths for the measurement will vary somewhat from instrument to instrument. Samples are stable in the sodium

Table 10
 FLUORESCENCE OF SEVERAL ANTIHISTAMINES CONTAINING
 THE DIPHENYLMETHANE GROUP

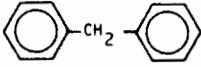
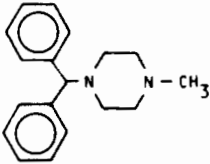
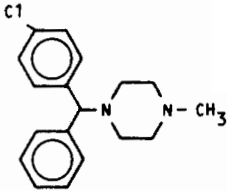
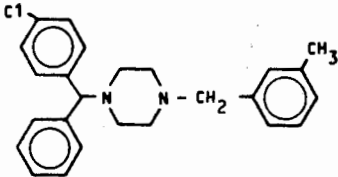
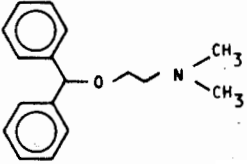
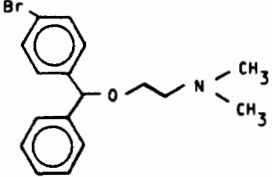
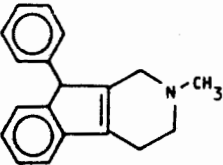
	Natural fluorescence ^a			After H ₂ O ₂ ^b	
	Excitation	Emission	Quantum efficiency	Excitation	Emission
 XXXVIII Diphenylmethane	260	282	0.16	—	—
 XXXXIV Cyclizine	—	—	—	305 & 335	417 & 449
 XXXX Chlorcyclizine	265	292	—	345	451
 XXXXIII Meclizine	265	291	—	310 & 345	420 & 444
 XXXIX Diphenhydramine	258	285	0.01	305 & 345	412 & 454

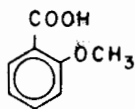
Table 10 (continued)
 FLUORESCENCE OF SEVERAL ANTIHISTAMINES CONTAINING
 THE DIPHENYLMETHANE GROUP

	Natural fluorescence ^a			After H ₂ O ₂ ^b	
	Excitation	Emission	Quantum efficiency	Excitation	Emission
 XXXXI Bromodiphenhydramine					
				Photolyzes to a second, unidentified fluorescent specie	
 XXXXII Phenindamine	260	313	0.04	—	—

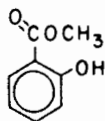
^a Data from Wirz, D. R., Wilson, D. L., and Schenk, G. H., *Anal. Chem.*, **47**, 896, 1974.

^b Data from Jensen, R. E. and Pflaum, R. T., *J. Pharm. Sci.*, **53**, 835, 1964.

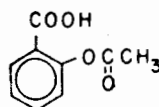
hydroxide solution for several hours if carefully protected from air. The limit of detection is on the order of 1 $\mu\text{g}/\text{ml}$. This procedure measures total plasma salicylates since many salicylate derivatives fluoresce at the same wavelengths and because salicylate esters, such as aspirin(L), hydrolyze rapidly to salicylate in alkaline solution. Salicylic acid(LI), the principal substance appearing in the urine after ingestion of XXXXVII or L, can be quantitated using the same procedure as for plasma except that excitation and emission shifted to slightly longer wavelengths. Salicylic acid standards should be used in place of salicylate standards for urine analysis. Both free salicylate and aspirin can be determined in biological samples by separating them on a dextran gel column,⁹⁹ by selective extraction,¹⁰⁰ or by paper chromatography¹⁰¹ prior to the fluorimetric measurement. Rowland and Riegelman¹⁰² used gas chromatography to determine L and fluorimetry for XXXXVII in plasma.



XXXXVIII



XXXXIX



L

Miles and Schenk¹⁰³ reported a simple fluorimetric procedure for L in tablets. In 1% acetic acid in chloroform, the lowest energy excitation and emission wavelengths

Table 11
FLUORESCENCE OF SALICYLATES AS A FUNCTION OF ACIDITY IN WATER AND CHLOROFORM

	Aqueous solutions:					Chloroform solutions:		
	18 M H ₂ SO ₄	0.1 M H ₂ SO ₄	pH 7	0.01 M NaOH	7 M NaOH	2% TFA*	Neutral	2% Morpholine
	Salicylic Acid							
λ_{EXC} , nm	329	302	296	296	322	—	—	—
λ_{EMIS} , nm	418	442	408	408	389	391 & 446	444	413
	o-Anisic Acid							
λ_{EXC} , nm	321	296	279	279	—	—	—	—
λ_{EMIS} , nm	420	364	345	345	—	402	340	—
	Methyl Salicylate							
λ_{EXC} , nm	330	302	302	331	—	—	—	—
λ_{EMIS} , nm	418	366 & 448	366 & 448	408	—	397 & 446	450	450

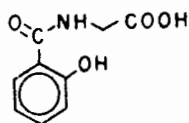
* TFA = Trifluoroacetic acid.

for L are 280 nm and 335 nm, respectively, whereas the wavelengths for XXXXVII are 308 nm and 450 nm, respectively. Thus L can be measured without interference from XXXXVII in this solvent. Measurable hydrolysis of L to XXXXVII did not occur over a period of several hours provided a spectroscopic quality chloroform was used. Tablets were analyzed by dispersing them in chloroform, filtering, and measuring. The calibration curve for the analysis was linear from 0 to 2.8×10^{-4} M, with a limit of detection of about 10^{-6} M. This procedure is not as sensitive as the methods for XXXXVII because L has a quantum efficiency of only 0.015 to 0.020 and a molar absorptivity of 1446 in the chloroform solvent employed.

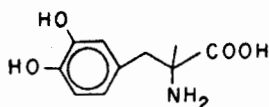
D. Catecholamines

Derivatives of 4-(2-aminoethyl)catechol can be analyzed fluorimetrically using two different procedures: oxidation and rearrangement to a fluorescent lutin, or oxidation and subsequent condensation with 1,2-diamino-ethane(LII). The ring closure reaction is an intramolecular oxidative coupling (see Chapter 2, Section V.C.2.a.i and Section V.C.2.c). Both reactions are shown for epinephrine(LIII) in Figure 8.

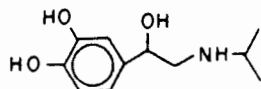
Conversion of a catecholamine to a lutin, often called the trihydroxyindole reaction, is usually carried out between pH 6.0 and 7.0 using 10^{-3} to 10^{-4} M potassium ferricyanide as the oxidant. Iodine can be used as oxidant in place of ferricyanide. The reaction must be timed and reaction conditions carefully controlled. When the oxidation is complete, sodium hydroxide is added to make the solution alkaline and an antioxidant is added to prevent further oxidation of the lutin. Ascorbic acid is the most widely used antioxidant,¹⁰⁴⁻¹⁰⁷ although thioglycolic acid,¹⁰⁸ cysteine hydrochloride,¹⁰⁹ 2-mercaptoethanol,¹¹⁰ and 2,3-dimercaptopropanol¹¹¹ have all been used and offer the advantage of lower background fluorescence than ascorbic acid.



LII



LIV



LV

The *U.S.P. XIX*¹¹² specifies the trihydroxyindole reaction with ferricyanide and ascorbic acid for the determination of epinephrine in Lidocaine Hydrochloride Injection. An automated system for carrying out the *U.S.P. XIX* procedure has also been reported.¹¹³ The measurements are made with excitation at 420 nm and emission at 520 nm. Similar procedures have been used for the analysis of levarterenol (excitation 400 nm, emission 500 nm),¹¹⁰ and for methyl dopa(LIV) in biological fluids (excitation 400 nm, emission 510 nm).¹¹⁴ The use of iodine in place of ferricyanide or oxidant, but retaining ascorbic acid as the stabilizer has been used in the analysis of levarterenol¹¹⁵ and isoproterenol(LV).¹¹⁶ Levarterenol has also been measured spectrophotometrically by maintaining a pH of 6 after the oxidation step in order to avoid conversion of the noradrenochrome to noradrenolutin: the noradrenochrome was quantitated directly by measuring its absorbance at 529 nm.¹¹⁵

Weil-Malherbe and Bone¹¹⁷ originally reported the condensation of LIII and LVI with LII to form fluorescent products as shown in Figure 8. This is usually referred to as the ethylenediamine procedure. The reaction is carried out in acidic aqueous solution at 50°C.¹¹⁵⁻¹¹⁷ LIII emits at 510 and 600 nm when excited at 420 nm, and as little as 2 ng of LIII can be measured. The ethylenediamine procedure has been criticized

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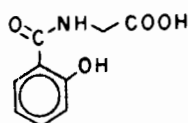
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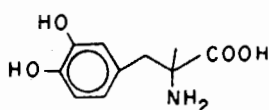
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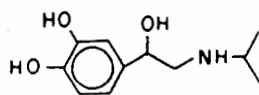
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II



LIV



LV

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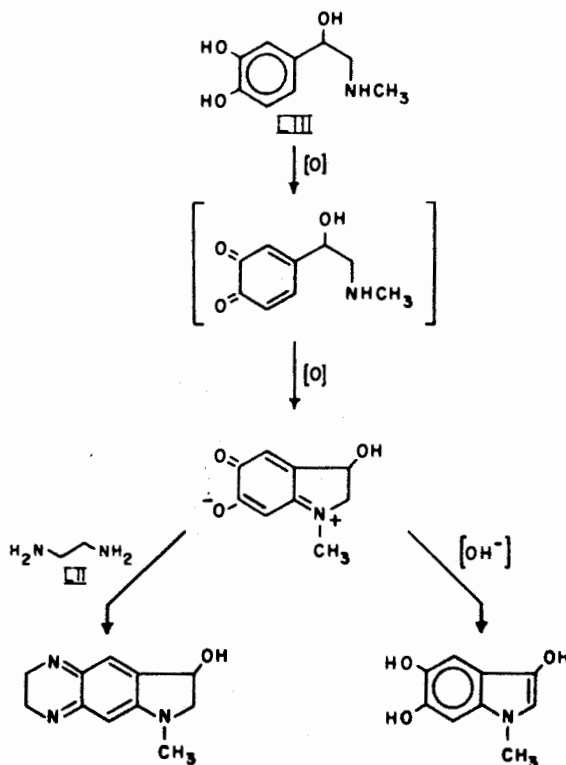


FIGURE 8. Reactions for preparation of fluorescent derivatives of catecholamines. The catecholamine (e.g., epinephrine, LIII) is oxidized through an intermediate orthoquinone to an adrenochrome. The adrenochrome is then converted to a fluorescent product by reaction with 1,2-diaminoethane or by rearrangement in alkali to adrenolutin.

for lack of specificity, but a careful comparison with the trihydroxyindole method showed no significant differences when used to measure catecholamines in human plasma samples.



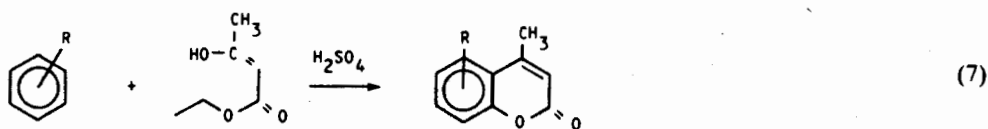
Other fluorimetric procedures also find occasional use in the analysis of catecholamines in pharmaceutical preparations. For example, the native fluorescence of LIII in aqueous 0.1 M HCl has been used to quantitate LIII in preparations containing lidocaine hydrochloride. LIII was first separated from other components of the formulation by ion-pair column chromatography and then measured with an excitation wavelength of 281 nm and an emission wavelength of 334 nm. The procedure has the advantage of separating LIII from the physiologically inactive sulfonic acid derivatives, which form by reaction of LIII with the bisulfite, which is added to many formulations to prevent adrenochrome formation.^{120,121} Some catecholamines will also

condense with formaldehyde to form 6,7-dihydroxy-3,4-dihydroisoquinolines(LVII) which can be quantitated fluorimetrically.¹²²

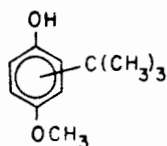
E. Miscellaneous Phenols and Oxygen Heterocycles

Many phenolic compounds, including the catechols discussed in the preceding section, exhibit fluorescence which is strong enough to be useful for their analysis. Butylated hydroxyanisol (BHA, LVIII) is used as an antioxidant in many pharmaceutical preparations and in food products. Low levels of LVIII can be determined in these products by first extracting with diethyl ether, then removing potential interferences by thin layer chromatography, and finally quantitating by measuring the intensity of emission at 327 nm while exciting at 290 nm.¹²³ Propyl gallate(LIX), another antioxidant, has also been determined fluorimetrically in foodstuffs.^{124,125} LIX is measured at 352 nm with excitation at 272 nm.

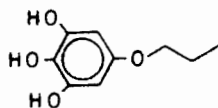
Phenols which do not fluoresce with sufficient natural intensity can be converted to fluorescent derivatives by condensation with ethyl acetoacetate(LX) to form a coumarin derivative as shown in Equation 7.^{126,127}



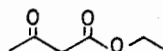
The procedure consists of dissolving the sample in ethanol, adding an excess of LX, and then diluting the solution with an equal volume of concentrated sulfuric acid. The reaction is usually complete within 15 min at room temperature although phenol(LXI) itself requires heating at 80°C for 15 min to force completion. The emission wavelengths of the products from several phenols tested in this reaction by Pesez and Bartos are given in Table 12.



LVIII

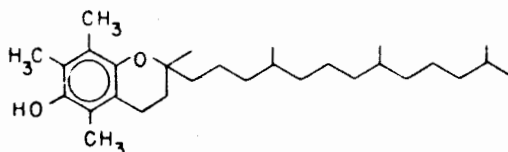


LIX



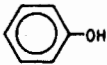
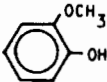
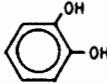
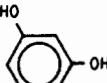
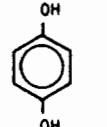
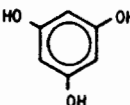
LX

α -Tocopherol (Vitamin E, LXVII) can be measured in plasma and other biological materials by extraction and fluorimetry.^{128,129} The measurements are made in 3:1, hexane:ethanol with excitation at 295 nm and emission at 340 nm. The limit of detection is approximately 0.01 μg of LXVII. Esters of LXVII do not fluoresce and must be hydrolyzed to the free phenol if they are to be measured. LXVII has also been determined in plant oils using high pressure liquid chromatography with a fluorescence detector,¹³⁰ a procedure that should be easily adapted for the analysis of multi-vitamins and other pharmaceutical preparations containing this vitamin.



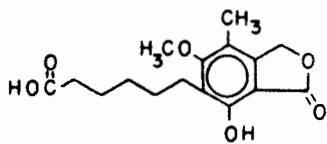
LXVII

Table 12
 PHENOLIC COMPOUNDS FORMING FLUORESCENT
 PRODUCTS WITH ETHYL ACETOACETATE^a

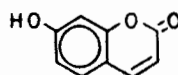
Compound	Structure	Emission wavelength, nm
Phenol (LXI)		460
Guaiacol (LXII)		495
Pyrocatechol (LXIII)		495
Resorcinol (LXIV)		430
Hydroquinone (LXV)		475
Phloroglucinol (LXVI)		490

^a From Pesez, M. and Bartos, J., *Colorimetric and Fluorimetric Methods of Analysis*, Marcel Dekker, New York, 1974, 109. With permission.

A large number of simple phenols and oxygen heterocycles have been determined fluorimetrically in biological samples. For example, mycophenolic acid (LXVIII) has been determined in plasma and tissue samples by extraction with dichloroethane, back extraction into a pH 10 borate buffer, and measuring its emission at 438 nm with excitation at 350 nm.¹³¹ Coumarin (LXIX) and umbelliferone (LXIX) have been determined in blood by extraction and measurement of their fluorescence in alkaline media.¹³² Coumarin emits at 491 nm when excited at 361 nm and umbelliferone emits at 450 nm when excited at 370 nm. Bufuralol (LXXa)¹³³ has also been determined fluorimetrically in blood and urine. LXX emits at 305 nm when excited at 250 nm in a 0.1 N hydrochloric acid solution. Other examples may be found in References 85 to 90.



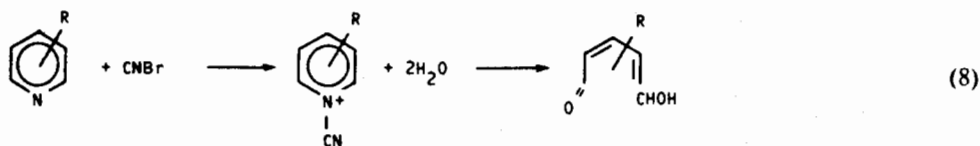
LXVIII



LXIX

F. Pyridines

Many pyridine compounds form fluorescent products when treated with cyanogen bromide.^{135, 136} Jensen and Pflaum¹³⁵ studied the use of this reaction for development of fluorescence in a number of antihistamines containing the pyridyl group. The excitation and emission wavelengths they found are presented in Table 13. The reaction of cyanogen bromide with pyridine derivatives bearing at least one α -hydrogen produces a derivative of glutamic aldehyde (LXXb)¹³⁷ as shown in Equation 8. LXXb



may be responsible for the fluorescence, but the intermediate pyridinium cation is also a possible source of the emission. The reaction may be carried out by warming a neutral aqueous solution of the analyte with an equal volume of saturated aqueous cyanogen bromide.^{136, 137} An analyte concentration between 5×10^{-6} and 5×10^{-4} M is usually sufficient. The product LXXb may also be reacted further with an aromatic amine to convert it to the more stable, fluorescent Schiff base. *p*-Aminobenzoic acid is frequently used in this modification of the cyanogen bromide reaction.^{138, 139}



Many pyridine derivatives also develop fluorescence when treated with hydrogen peroxide. Jensen and Pflaum¹³⁵ also tested this reaction on a number of pyridine containing antihistamines and obtained the results summarized in Table 13. The reaction gives the strongest fluorescence with 2-aminopyridine, moderate fluorescence with other pyridines, and weak fluorescence with nonpyridine antihistamines.¹³⁵ The procedure of Jensen and Pflaum consisted of heating an aqueous solution of the antihistamine containing 1.5% hydrogen peroxide at 90°C for 30 min. Several nonpyridine antihistamines also develop fluorescence under these reaction conditions.¹³⁵

Many 2-aminopyridine compounds have a natural fluorescence that can be used for analysis. Wilson et al.¹⁴⁰ investigated the fluorescence and phosphorescence of a number of antihistamines containing the 2-aminopyridine group. The fluorescence emission and excitation wavelengths of these compounds are also listed in Table 13. Wilson et al.¹⁴⁰ showed that the principal emitting specie is the pyridinium (+1) ion. They also presented a specific procedure for fluorimetric analysis of methapyrilene (LXXI) in tablets. The fluorimetric method would be suitable for use in content uniformity studies or other studies requiring single dose assays.

Fluorescence methods are frequently used in the analysis of compounds in the vitamin B₆ group, including pyridoxine (LXXXII), pyridoxal (VIII), pyridoxamine (LXXX), and pyridoxic acid (LXXXI). The fluorescence of these compounds as a function of pH and structure has been studied by Bridges et al.²⁶ The characteristic wavelengths of their natural fluorescence are given in Table 14. Natural fluorescence has been used to determine VIII, LXXX, and LXXXII in blood samples,¹⁴¹ but the sensi-

Table 13
EXCITATION AND EMISSION WAVELENGTHS FOR SEVERAL PYRIDINE
COMPOUNDS AND THEIR REACTION PRODUCTS

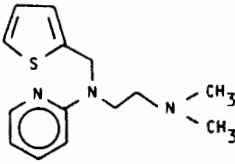
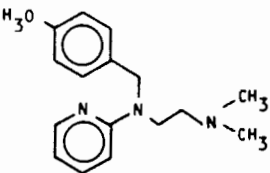
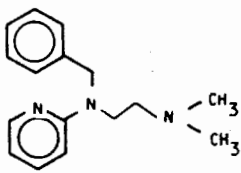
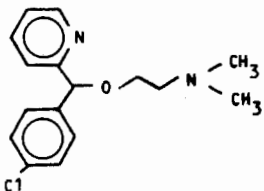
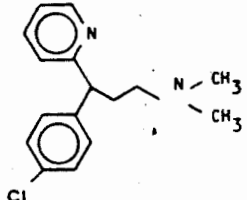
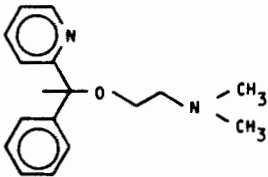
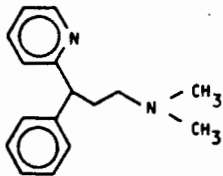
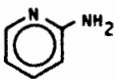
Compound	Natural fluorescence		After H ₂ O ₂		After CNBr	
	Excitation ^a	Emission ^b	Excitation	Emission ^c	Excitation	Emission ^c
 <p>Methapyrilene (LXXI)</p>	238, 303	363	345	409	350	412
 <p>Pyrilimine (LXXII)</p>	242, 306	366	340	408	350	419
 <p>Tripelennamine (LXXIV)</p>	248, 306	363	345	408	355	419
 <p>Carbinoxamine (LXXV)</p>	-	-	335	394	275	467
 <p>Chlorpheniramine (LXXVI)</p>	-	-	350	436	280	447

Table 13 (continued)
 EXCITATION AND EMISSION WAVELENGTHS FOR SEVERAL PYRIDINE
 COMPOUNDS AND THEIR REACTION PRODUCTS

Compound	Natural fluorescence		After H ₂ O ₂		After CNBr	
	Excitation ^a	Emission ^b	Excitation	Emission ^c	Excitation	Emission ^c
 Doxylamine (LXXVII)	—	—	370	449	280	388
 Pheniramine (LXXVIII)	—	—	330	352	275	434
 2-Aminopyridine (LXXIX)	230, 295	363	—	—	—	—

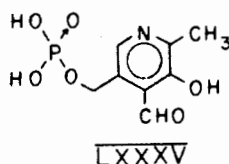
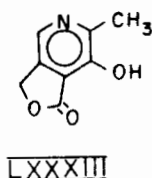
^a The excitation band giving the highest quantum efficiency is listed first.

^b Data from Pesez, M. and Bartos, J., *Colorimetric and Fluorimetric Methods of Analysis*, Marcel Dekker, New York, 1974, 392.

^c Data from Perlman, E., *J. Pharmacol. Exp. Ther.*, 95, 465, 1949.

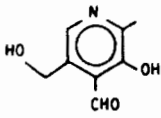
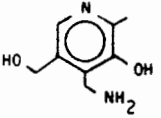
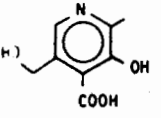
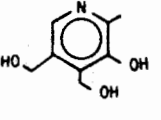
tivity and selectivity are not as good as in methods using derivatization prior to measurement.

Pyridoxic acid lactone(LXXXIII) exhibits strong fluorescent emission at 450 nm when excited at 350 nm.¹⁴² Reddy et al.¹⁴² measured LXXXI in urine using an anion exchange column to isolate it from other urine components and then converting it to the lactone by heating at 100°C for 15 min in 5 N hydrochloric acid. Fujita et al.¹⁴³ used a similar procedure to determine VIII, LXXX, LXXXII, as well as LXXXI in biological samples after converting them to the lactone.



VIII can also be determined by converting it to the cyanohydrin(LXXXIV) with

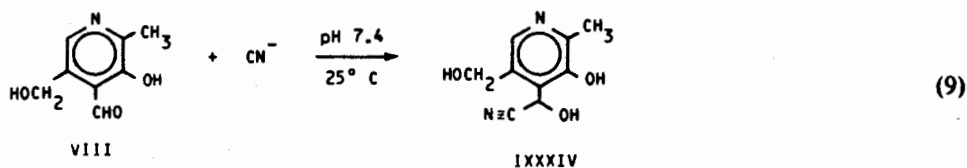
Table 14
FLUORESCENCE OF THE B₆ VITAMINS

Compound	Natural fluorescence		
	Excitation ^a	Emission ^a	Quantum efficiency ^b
 VIII Pyridoxal	330 nm	385 nm	0.048
 LXXX Pyridoxamine	335 nm	400 nm	0.110
 LXXXI Pyridoxic acid	—	—	—
 LXXXII Pyridoxine	340 nm	400 nm	—

^a Data from Coursin, D. B. and Brown, V. C., *Proc. Soc. Exp. Biol. Med.*, 98, 315, 1958.

^b Data from Chen, R. F., *Science*, 150, 1593, 1965.

potassium cyanide:

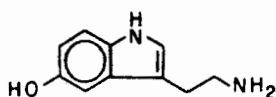


This reaction was first studied by Bonavita and Scardi¹⁴⁴ as the basis for a spectrophotometric assay for pyridoxal-5-phosphate (LXXXV) and was later developed into a fluorimetric procedure by the same investigators.^{145,146} The reaction is run in a pH 7.4 phosphate buffer which is 0.01 M in KCN and the reaction is complete within 30 min at 25°C.¹⁴⁶ VIII and LXXXV can be determined in the presence of one another using this procedure.¹⁴⁶ LXXX can also be determined using this procedure¹⁴⁷ after convert-

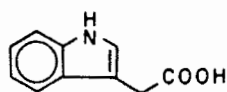
ing it to VIII by transamination with glyoxylate as described by Metzler et al.¹⁴⁶ The limit of detection for these substances is approximately 10^{-9} M.¹⁴⁷

G. Indoles

Most of the work on fluorimetric methods of analysis for indoles has been directed to the biologically important 3-alkylindoles such as tryptamine(LXXXVI), 5-hydroxytryptamine (serotonin, LXXXVII), and indole-3-acetic acid(LXXXVIII). Methods based upon the natural fluorescence of these compounds and upon the fluorescence of derivatives have been reported. LXXXVII¹⁴⁹ and LXXXVIII¹⁵⁰ have been determined in biological samples using their natural fluorescence in solution in hydrochloric acid which affords a limit of detection of about $0.03 \mu\text{g}/\text{ml}$. Maximum sensitivity may not have been achieved in these early analyses since the fluorescence of indoles is reduced in acidic solutions (see Section IV.C. and Reference 69).

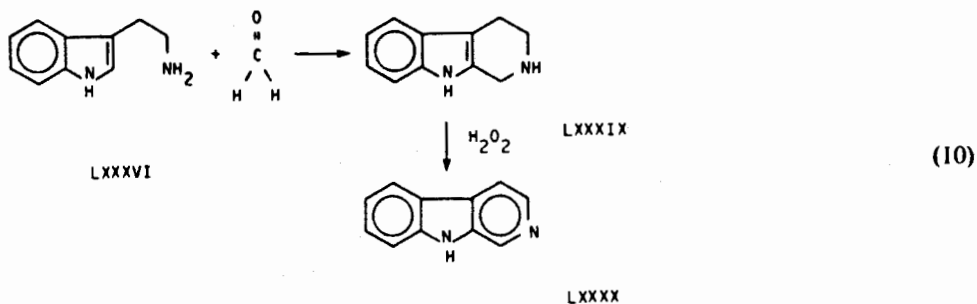


LXXXVII



LXXXVIII

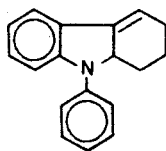
Both the sensitivity and selectivity of the fluorimetric procedures can be improved by converting the indole into strongly fluorescent derivatives. Hess and Udenfriend¹⁵¹ developed a procedure for analysis of LXXXVI which improved the specificity but not the sensitivity of the assay. In their procedure LXXXVI is converted to tetrahydronorharman(LXXXIX) by condensation with formaldehyde and subsequently oxidized to norharman(LXXXX) with peroxide as shown in Equation 10. The analyte



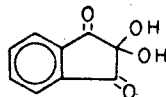
is extracted into 0.1 N sulfuric acid, enough concentrated formaldehyde solution is added to make the mixture 0.5% in formaldehyde, and the reaction is allowed to proceed at 100°C for 20 min. Hydrogen peroxide is added to make the solution approximately 0.15% in this reagent and heating is continued for another 20 min. The fluorescence is measured at 440 nm with excitation at 365 nm. This procedure has also been used for the determination of N-terminal tryptophan dipeptides¹⁵² and polypeptides.¹⁵³

A significant improvement in sensitivity can be achieved by reacting the indole amine with *o*-phthalaldehyde(LXXXXI) instead of formaldehyde.¹⁵⁴ The reaction is carried out by heating the indole derivative at 100°C for 1 hr in 10 N hydrochloric acid containing 0.005% LXXXXI. Maickel and Miller¹⁵⁴ found the presence of a hydroxy or methoxy group at position 5 of the indole ring and an alkyl substituent at 3 were both required to produce a strongly fluorescent product in this reaction. Indole itself was not converted to a fluorescent product, and substances having only a single substituent

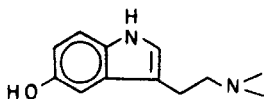
is carried out in a 1:1 mixture of acetic anhydride:trifluoroacetic acid at 0°C and is complete in 15 min. Approximately 5×10^{-6} M LXXXVIII can be measured using the emission at 500 nm with excitation at 440 nm.



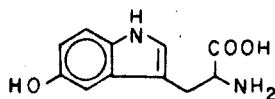
LXXXIV



LXXXV



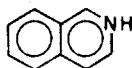
LXXXVI



LXXXVII

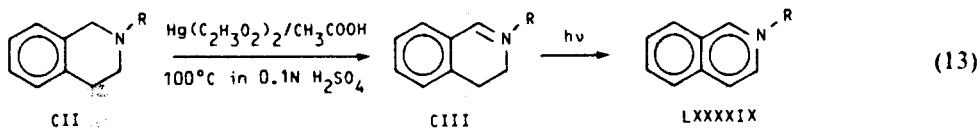
H. Quinolines and Isoquinolines

Quinoline(XXVII) and isoquinoline(IC) derivatives are often strongly fluorescent. The fluorescence of this group of compounds is usually greatest in polar solvents. Quinine(X), which contains the quinoline nucleus, is often used as a fluorescence standard. Both X and its isomer, quinidine, can be measured in biological samples by extracting from an alkaline solution into benzene, back extracting into 0.1 N sulfuric acid, and measuring the emission at 450 nm with excitation at 350 nm.¹⁶³ A number of other Cinchona alkaloids containing a quinoline group can also be analyzed fluorimetrically,¹⁶⁴ as can most quinoline antimalarial drugs.



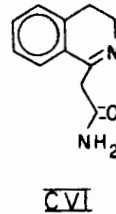
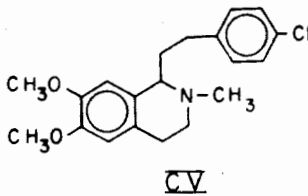
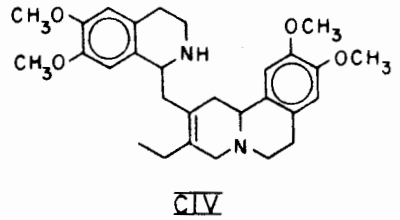
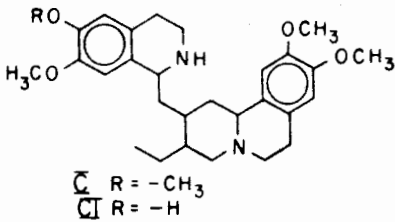
IC

Emetine(C), cephaeline(CI), and a number of other alkaloids contain a tertiary 1,2,3,4-tetrahydroisoquinoline group, (CII) which is not fluorescent. However, tertiary tetrahydroisoquinoline groups are readily oxidized to 3,4-dihydroisoquinolines (CIII)^{165, 166} or isoquinolines(IC)¹⁶⁷⁻¹⁶⁹ which fluoresce strongly in acid solution.



(13)

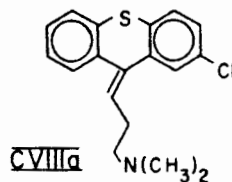
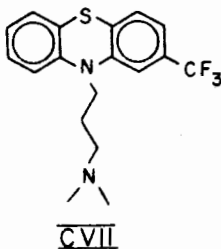
Knabe¹⁶⁵ originally reported using mercuric acetate in ethylenediaminetetraacetic acid to carry out the conversion CII to CIII, but Schwartz and Rieder¹⁶⁶ found that mercuric acetate in acetic acid gave a higher and more reproducible yield. Mercuric acetate oxidation to the dihydroisoquinoline derivative has been used to determine emetine(C),¹⁶⁶ dehydroemetine(CIV),¹⁶⁶ and methopholine(CV)¹⁷⁰ in biological samples.



deSilva et al.¹⁶⁹ used the photochemical conversion of CIII to IC to provide a very sensitive fluorimetric analysis for 3,4-dihydro-1-isoquinoline acetamide(CVI) in blood and urine. The photochemical oxidation was accomplished by irradiating a solution of the sample in 30% trichloroacetic acid for 1 hr under an intense UV lamp. The emission was measured at 380 nm with excitation at 335 nm. Methods for metabolites were also given in this paper.¹⁶⁹

I. Phenothiazines and Thioxanthenes

Most phenothiazine drugs are fluorescent and have similar excitation and emission spectra.^{77,78A,78B} The excitation and emission maxima for a number of phenothiazines and thioxanthenes are listed in Table 15. The natural fluorescence of these compounds can be used to analyze for them in biological samples. Martin,¹⁷¹ for example, determined triflupromazine(CVII) by measuring its emission at 500 nm using excitation at 325 nm in concentrated sulfuric acid. The limit of detection for CVII was approximately 0.05 $\mu\text{g}/\text{ml}$. deSilva and D'Arconte¹⁷² determined chlorprothixene(CVIIIa) in blood after extracting into concentrated sulfuric acid. The limit of detection for CVIIIa was approximately 0.01 $\mu\text{g}/\text{ml}$.



The sensitivity of the fluorimetric procedures can be improved by oxidation of the phenothiazine to a sulfoxide prior to measurement.

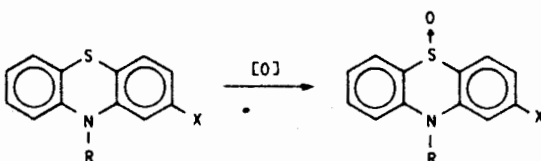


Table 15
FLUORESCENCE EXCITATION AND EMISSION WAVELENGTHS FOR SEVERAL
PHENOTHIAZINES AND THEIR OXIDATION PRODUCTS^a

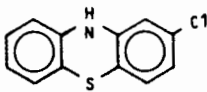
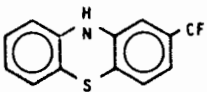
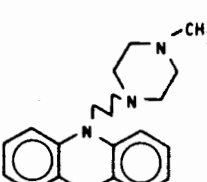
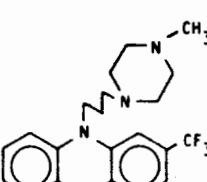
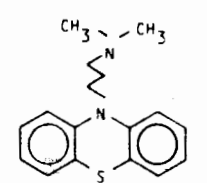
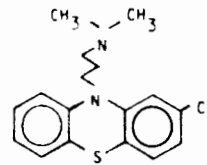
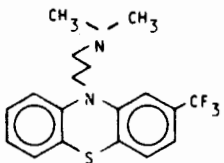
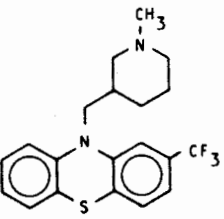
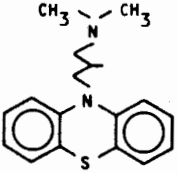
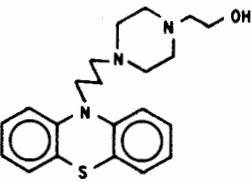
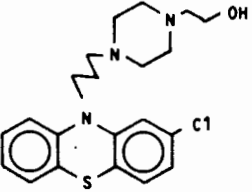
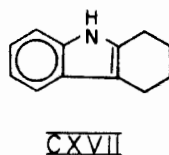
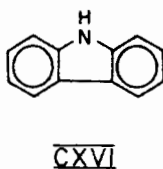
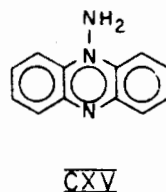
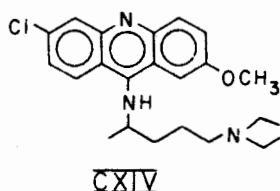
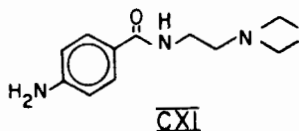
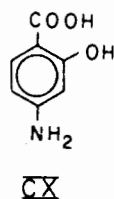
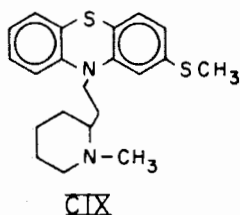
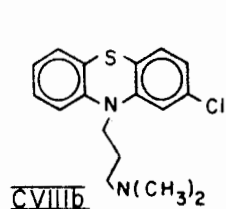
Compound	Natural		Oxidized	
	Excitation	Emission	Excitation	Emission
 Chlorphenothiazine	340	470	360	440
 Trifluorophenothiazine	300	350	350	410
 Prochlorperazine	325	450	340	380
 Trifluoroperazine	320	470	350	405
 Promazine	320	450	340	375
 Chlorpromazine	325	455	340	380

Table 15 (continued)
 FLUORESCENCE EXCITATION AND EMISSION WAVELENGTHS FOR SEVERAL
 PHENOTHIAZINES AND THEIR OXIDATION PRODUCTS^a

Compound	Natural		Oxidized	
	Excitation	Emission	Excitation	Emission
 Trifluopromazine	330	475	350	405
 Trifluomepazine	325	480	350	405
 Methiomepazine	330	470	360	440
 Fluphenazine	325	475	350	405
 Perphenazine	330	460	345	380

^a From Ragland, J. B. and Kinross-Wright, V. J., *Anal. Chem.*, 36, 1356, 1964. With permission.

Oxidation shifts the excitation maximum to a longer wavelength and the emission maximum to a shorter wavelength as indicated in Table 15. The oxidation can be accomplished with hydrogen peroxide,^{78B,173} potassium permanganate,^{77,78A} ceric sulfate,^{174,175} cupric perchlorate,¹⁷⁵ and others. Hydrogen peroxide and potassium permanganate are most frequently used in the fluorimetric analysis of phenothiazines.

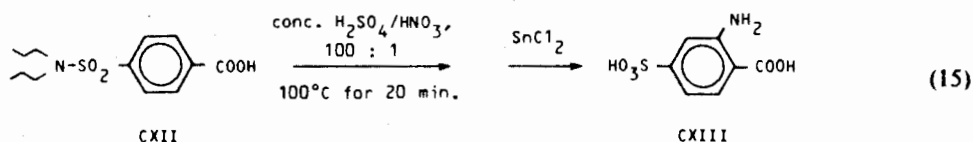


The usual procedure when potassium permanganate is used is to treat a solution of the analyte in ethanol with twice its volume of 6.3×10^{-5} M KMnO_4 (0.001%) in 0.1 M sulfuric acid and then dilute 1:2 with 0.1 M sulfuric acid. The reaction is complete within $\frac{1}{2}$ hr at room temperature. Oxidation with hydrogen peroxide can be accomplished in 10 min at 100°C in 3% hydrogen peroxide in 50% acetic acid (dilute 30% H_2O_2 1:2 with 50% acetic acid). Tompsett employed the hydrogen peroxide procedure to determine CVII, and Ragland et al.¹⁷³ used it for the analysis of CVII, chlorpromazine(CVIIIb), and thioridazine(CIX) in blood, urine, and tissue samples. Mellinger and Keeler⁷⁷ analyzed CIX in urine, saliva, spinal fluid, stomach juice, and bile using the potassium permanganate oxidation procedure.

J. Miscellaneous Anilines and Nitrogen Heterocycles

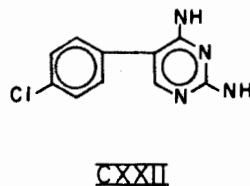
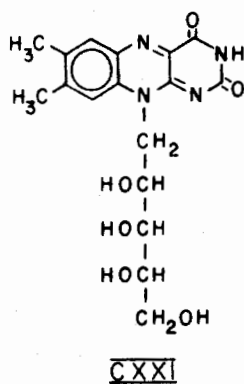
Aniline derivatives including aminobenzoic acids are often fluorescent when present as a neutral amine. The anilinium ions and amides are usually not fluorescent. The fluorescence of *p*-aminosalicylic acid(CX) and procaine amide(CXI) have been reported,⁸² but have not been used for analysis of these materials since UV and colorimetric methods give adequate sensitivity for these materials in biological samples. Cunningham et al.¹¹⁷ have reported a fluorescence method for probenecid(CXII) in which the drug is first converted to *p*-sulfoanthranilic acid(CXIII). CXIII is quantitated by measuring its emission at 415 nm in 50% aqueous ethanol at pH 3.3 to 3.9 with exci-

tation at 340 nm.



Quinacrine(CXIV)¹⁷⁸⁻¹⁸⁰ and other acridine derivatives^{181,182} can be quantitated fluorimetrically. The closely related phenazines also fluoresce, and 5-aminophenazine(CXV) has been analyzed in a variety of pharmaceutical preparations using its emission at 415 nm with excitation at 365 nm.¹⁸³ deSilva et al.¹⁸⁴ have reported the fluorescence and phosphorescence characteristics of a large number of drug substances containing the carbazole(CXVI), tetrahydrocarbazole(CXVII), and 1,4-benzodiazepin(CXVIII) groups. The fluorescence characteristics of several of these compounds are presented in Table 16. The 1,4-benzodiazepines can be quantitated using either their native fluorescence or the fluorescence or phosphorescence developed by converting them to quinazolinones(CXIX) or 9-acridinones(CXX) as shown in Figure 9.¹⁸⁴

Riboflavin(vitamin B₂, CXXI), an isoalloxazine derivative, is usually analyzed by fluorescence in biological samples and pharmaceutical preparations. The U.S.P. procedure for CXXI and CXXI Injection specifies measuring the fluorescence emission at approximately 530 nm in a water solution with excitation at about 444 nm.¹⁸⁵ After measurement, the sample is treated with hydrosulfite to reduce CXXI to a nonfluorescent specie so that the fluorescence background in the sample can be determined. CXXI in tablets is determined fluorimetrically after a more extensive sample work-up.¹⁸⁵



Pyrimethamine(CXXII), a 2,4-diaminopyrimidine, has been measured in urine using the compound's natural fluorescence.¹⁸⁶ Simmons and DeAngelis¹⁸⁷ have quantitated several diaminopyrimidines *in situ* on thin layer plates after spraying the plates with 2.0 M aqueous ammonium hydrogen sulfate to enhance their natural fluorescence. Thiamine(vitamin B₁, CXXIII), a 4-aminopyrimidine, is nonfluorescent but can be quantitatively oxidized to the highly fluorescent compound, thiochrome(CXXIV), as shown in Equation 16.¹⁸⁸⁻¹⁹¹ The oxidation is

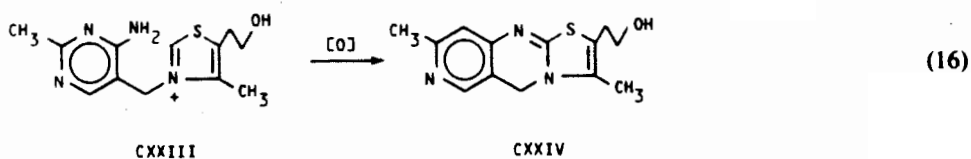


Table 16

FLUORESCENCE EXCITATION AND EMISSION WAVELENGTHS FOR SEVERAL TETRAHYDROCARBAZOLES, CARBAZOLES, AND BENZODIAZEPINES¹⁸⁴ AT 77 K

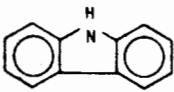
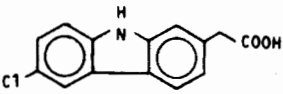
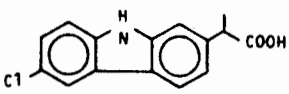
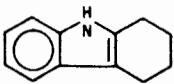
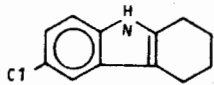
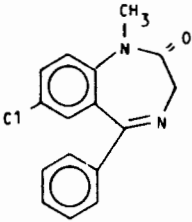
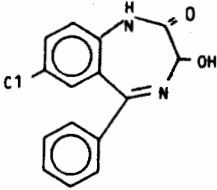
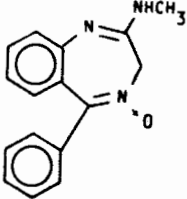
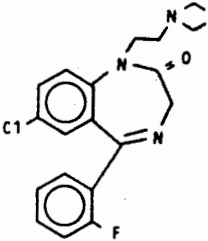
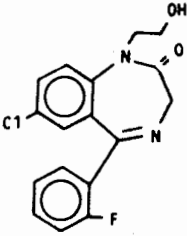
Compound	Solvent	Excitation Maximum, nm	Emission Maximum, nm
Carbazoles			
	a	265, 290, 340	355, 370
Carbazole			
	a	270, 312, 370	395
6-Chloro-2-carbazole acetic acid			
	a	270, 300, 340	370
(d,1)-6-Chloro-α-methylcarbazole-2-acetic acid			
Tetrahydrocarbazoles			
	a	290	360
1,2,3,4-Tetrahydrocarbazole			
	a	297	340
6-Chloro-1,2,3,4-tetrahydrocarbazole			
1,4-Benzodiazepines			
	b	290, 380	480
Diazepam			

Table 16 (continued)
 FLUORESCENCE EXCITATION AND EMISSION WAVELENGTHS FOR SEVERAL
 TETRAHYDROCARBAZOLES, CARBAZOLES, AND BENZODIAZEPINES¹⁸⁴ AT 77 K

Compound	Solvent	Excitation Maximum, nm	Emission Maximum, nm
1,4-Benzodiazepines (continued)			
 Oxazepam	b	285, 325, 365	490, 510
 Chlordiazepoxide	b	Not fluorescent	
 Flurazepam	b	280, 375	475
 N-1-Hydroxyethylflurazepam	b	260, 280, 375	475, 500

Note: a = ethanol; b = 1% sulfuric acid in ethanol.

usually accomplished with alkaline ferricyanide and the fluorescence emission is measured at 435 nm with excitation at 365 nm.¹⁹² This procedure has been automated for applications requiring large numbers of samples of CXXIII to be analyzed.¹⁹³

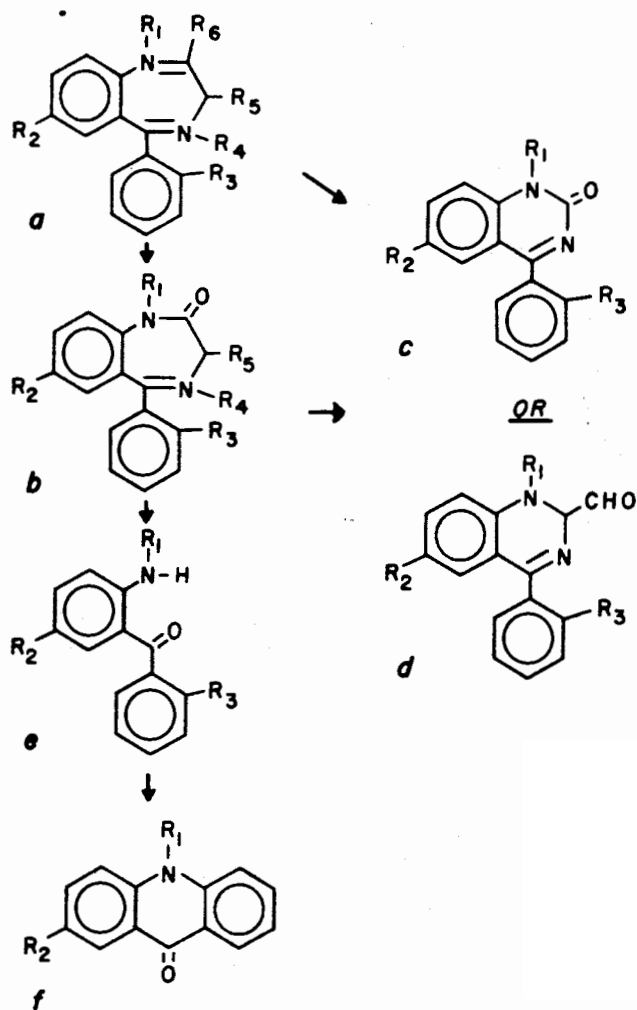


FIGURE 9. Chemical reactions of 1,4-benzodiazepines and their fluorescent derivatives. Species *a* and *b* rearrange in concentrated acids to form quinazolinones, *c*, and quinazoline-carboxaldehydes, *d*. Oxidation of *a* at C2 yields the amide, *b*, which can be hydrolyzed to the *o*-aminobenzophenone, *e*, by treatment with 6 *M* hydrochloric acid at 100°C. *e* can be catalyzed to a 9-acridinone, *f*, by treatment at 100°C with potassium carbonate in dimethylformamide. Substituent groups: R_1 = H or alkyl; R_2 = halogen or $-\text{NO}_2$; R_3 = H or halogen; R_4 = \rightarrow O; R_5 = H or $-\text{OH}$; and R_6 = H, $-\text{NH}_2$, or $-\text{NHCH}_3$.

Many indole alkaloids are fluorescent or can easily be converted to a fluorescent specie. Ergotamine(CXXXV) has been determined in tablets by extraction and measurement of the fluorescence emission at 402 nm in ethanol with excitation at 318 nm.¹⁹⁴ CXXXV has also been analyzed by high pressure liquid chromatography using fluorimetric detection.^{195,196} Lysergic acid diethylamide(CXXXVI) emits at 445 nm when excited at 325 nm in dilute acid.¹⁹⁷ Fluorimetric procedures have been widely used to determine trace levels of CXXXVI in blood.^{198,199} Reserpine(II) has relatively weak natural fluorescence, but is easily oxidized to the strongly fluorescent 3,4-didehydroreserpine(CXXVII) as shown in Figure 10.²⁰⁰ Oxidation with nitrite in dilute acid has been

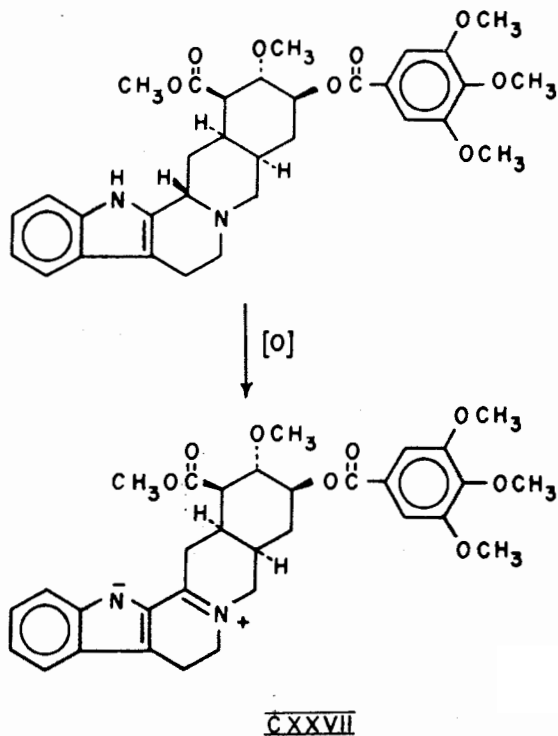
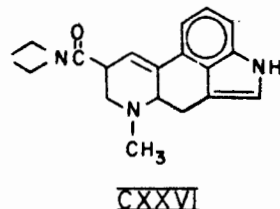
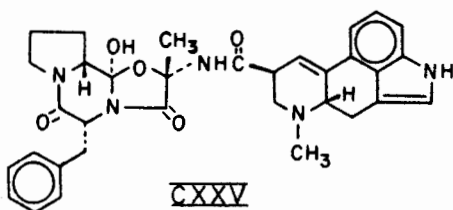


FIGURE 10. Oxidation of reserpine to 3,4-dihydroreserpine.

used for analysis of II in tablets,²⁰¹ including single tablet assays.^{202,203} Hydrogen peroxide,²⁰³⁻²⁰⁶ selenious acid,²⁰⁷ and vanadium pentoxide²⁰⁸⁻²¹¹ have also been used for the oxidation. CXXVII emits at 510 nm when excited at 390 nm. The oxidation of II to CXXVII and measurement of the absorbance rather than the fluorescence of CXXVII is also used in the analysis of reserpine.²¹²



K. Steroids

Most steroids are not naturally fluorescent, but many of them will develop intense fluorescence if treated with concentrated sulfuric acid or sulfuric acid/alcohol mixtures.²¹³ Sadee et al.²¹⁴ have shown that 17 α -alkyl-17 β -hydroxy-4,6-dien-3-ones (CXXVIII) are converted to highly fluorescent 17 β -methyl-17 α -alkyl-4,6,8(14)trien-3-ones upon treatment with sulfuric acid, as shown in Figure 11. The products formed upon treatment of other steroids with sulfuric acid have not been identified, but the reactions are probably similar to the one in Figure 11.

Uete et al.²¹⁵ have made a systematic study of the relation between steroid structure and the fluorescence induced by sulfuric acid. Over 50 steroids were examined in their

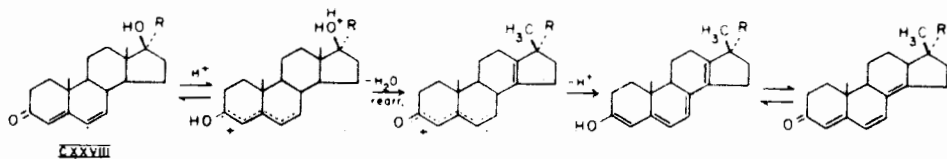
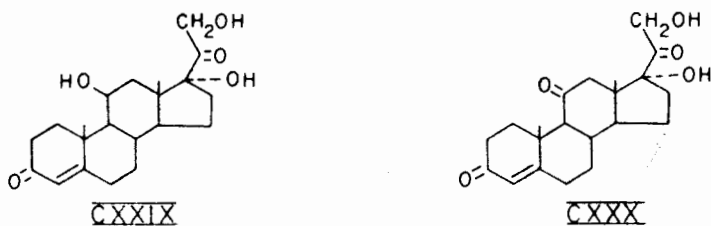
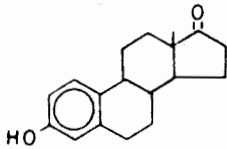
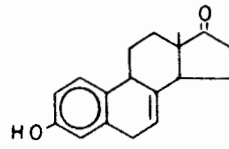
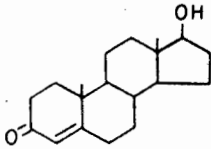
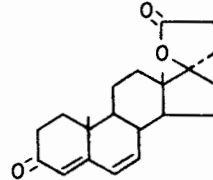
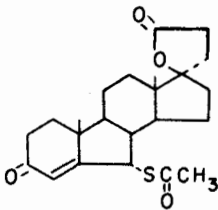
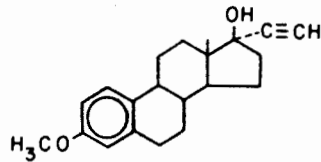
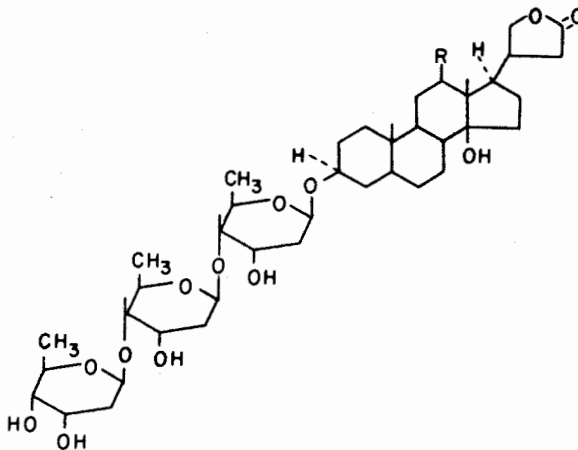


FIGURE 11. Reaction of 17- α -alkyl-17 β -hydroxy-4,6-dien-3-ones with concentrated sulfuric acid.

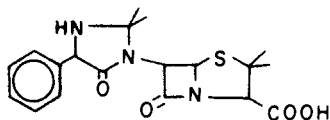
study. They found that the intensity of the fluorescence of Δ^4 -3-ketosteroids including the corticosteroids, progesterone derivatives, and androstene derivatives was increased by substituting hydroxyl groups at C-11, C-17 and C-21, but was decreased by the presence of a keto function on C-11, a double bond between C-1 and C-2, a hydroxyl on C-16, or reduction of the double bond between C-4 and C-5. The fluorescence intensity of estrogens was increased by the presence of a hydroxyl group on C-17 and decreased by a hydroxyl on C-16. The fluorescence of Δ^5 -androstenes and Δ^5 -pregnes was enhanced by a hydroxyl on either C-17 or C-21. The intensity of fluorescence from pregnenolone and dehydroepiandrosterone was also increased by the presence of a hydroxyl group on C-16. The fluorescence in these studies was developed by dissolving 3×10^{-6} mol of steroid in 2.5 ml of concentrated sulfuric acid at room temperature and measuring the intensity of the fluorescence at intervals after mixing. Most of the steroids except the estrogens yielded maximum emission near 520 nm when excited near 470 nm. Maximum fluorescence for the estrogens was observed near 480 nm with excitation at 430 nm. The emission wavelengths depend upon the concentration of sulfuric acid/alcohol used for fluorescence development.²¹⁶ The wavelength of emission can also be shifted to the red by diluting the solution with alcohol after the acid treatment.²¹⁷

The standard methods of analysis for cortisol(CXXIX) and corticosterone(CXXX) in blood employ sulfuric acid to develop fluorescence after a preliminary extraction and purification step.²¹⁸⁻²²¹ From 70 to 90% sulfuric acid in ethanol may be used with reaction times ranging from 15 min to 1 hr at room temperature. Emission is measured near 525 nm with excitation near 465 nm. In some cases the steroid is converted to an oxime or other derivative before sulfuric acid treatment.²²⁰ Estrone(CXXXI) and equilin(CXXXII) have been determined fluorimetrically after separation by liquid chromatography on a polydextran column.²²² The steroids were heated at 80°C for 15 min in a sulfuric acid:methanol:water mixture, 8:1:1 by volume, to develop the fluorescence. The fluorimetric procedure has also been automated for use in content uniformity testing of estrogen tablets.²²³ Other steroids that have been measured using the sulfuric acid reaction include testosterone(CXXXIII),^{224,225} canrenone(CXXXIV),²²⁶ spironolactone(CXXXV),²²⁶ and mestranol(CXXXVI). A number of additional references to the analysis of steroids in blood and urine using this procedure have been listed by White and Argauer.²²⁷

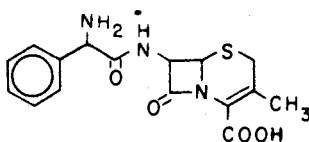


CXXXICXXXIICXXXIIICXXXIVCXXXVCXXXVICXXXVII R = -HCXXXVIII R = -OH

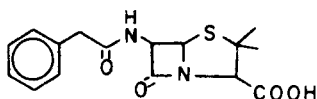
Several other reagents that are used to develop fluorescence in steroids may lead to products similar to those of the sulfuric acid reaction. Jakovljevic²²⁸ has prepared fluorescent derivatives of the cardiac glycosides digitoxin(CXXXVII) and digoxin (CXXXVIII) by warming them with a mixture of acetic anhydride, acetyl chloride, and trifluoroacetic acid. CXXXVII emits at 500 nm when excited at 470 nm after treatment, and CXXXVIII emits at 470 nm and 500 nm when excited at 345 nm and 435 nm, respectively. The difference in excitation and emission wavelengths was used to analyze mixtures of these substances. Bondjers and Björkerud²²⁹ used a zinc chloride/acetyl chloride reagent to develop fluorescence in cholesterol. Emission was at 565 nm with excitation at 528 nm.



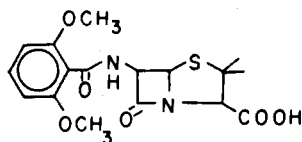
CXXXX



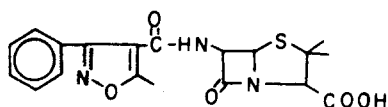
CXXXXI



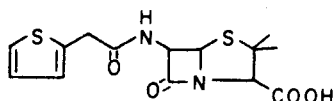
CXXXXII



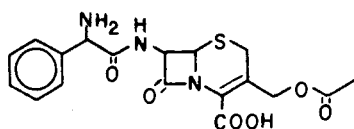
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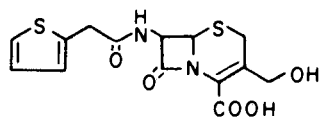
CXXXXIV



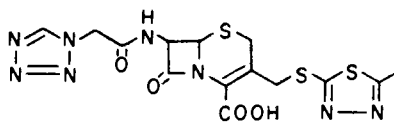
CXXXXV



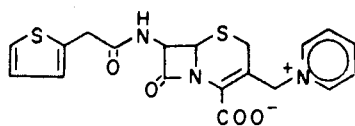
CXXXXVII



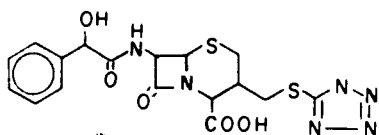
CXXXXVIII



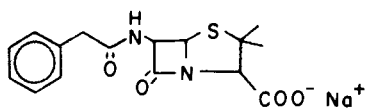
CII



CI



CII

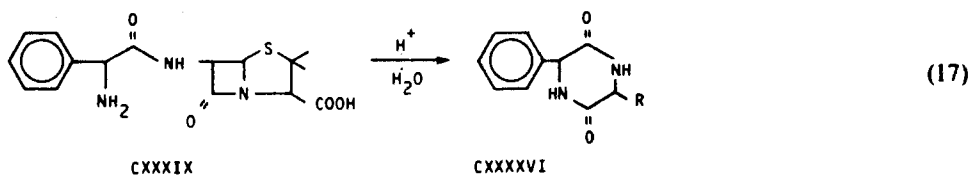


CLII

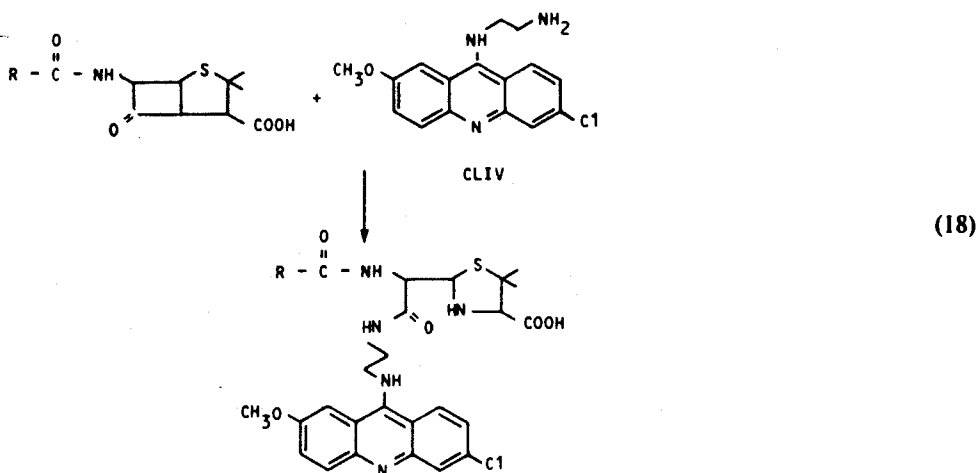
L. Penicillins, Cephalosporins, and Other Antibiotics

Smith et al.²³⁰ noted that a strongly fluorescent product was formed when ampicillin(CXXXXIX) was heated in strongly acidic solutions. Jusko²³¹ later optimized this

reaction and applied it to the analysis of CXXXIX in blood and urine. A protein-free solution containing 0.2 to 10 $\mu\text{g/ml}$ ampicillin is diluted with an equal volume of 7% formaldehyde in 0.4 M pH 2 citrate buffer and heated for 2 hr at 90°C. After cooling, the sample is taken into 2 N sodium hydroxide either by direct dilution or by extracting into acetonechloroform, 1:1 and back extracting into the sodium hydroxide solution. The fluorescence emission is measured at 422 nm with excitation at 346 nm. Hetacillin(CXXXX), which hydrolyses rapidly to CXXXIX, gave the same fluorescence intensity as CXXXIX, and cephalixin(CXXXXI) gave approximately 9% as intense a response. Benzylpenicillin(CXXXXII), methicillin(CXXXXIII), oxacillin(CXXXXIV), and cephalothin(CXXXXV) gave no response. Jusko²³¹ proposed the formation of 3,4-diketopiperazines(CXXXXVI) as shown in Equation (17) to account for the observed fluorescence.

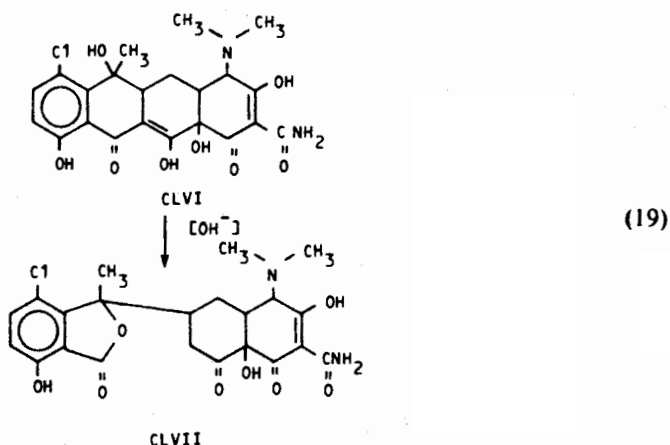


Yu et al.²³³ have reported a similar method for developing fluorescence in cephalosporin antibiotics. The cephalosporin is prepared in 0.1 N sodium hydroxide and heated at 100°C for periods ranging from 30 min to several hours. In order of decreasing fluorescence intensity, the cephalosporins tested were CXXXXV, CXXXXI, cephaloglycin(CXXXXVII), desacetyl cephalothin(CXXXXVIII), cefazolin(CL), cephaloridine(CL), and cefamandole(CLI). Emission maxima ranged from 390 nm to 435 nm with excitation between 310 and 360 nm in this series of compounds. This reaction also produces fluorescence with CXXXIX, penicillin G(CLII), and amoxicillin(CLIII). Penicillins have also been determined in biological samples by coupling with the fluorescent 2-methoxy-6-chloro-9-(2-aminoethyl)aminoacridine(CLIV) as shown in Equation 18.²³⁴

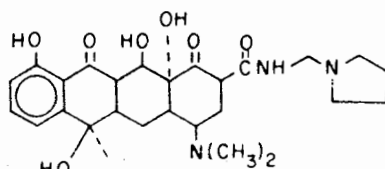
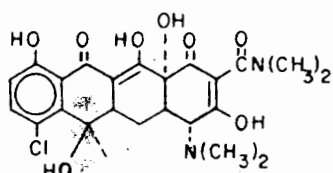
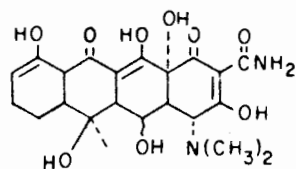
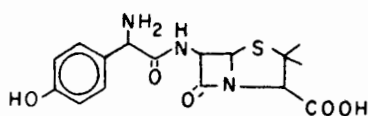


A number of the tetracycline antibiotics exhibit native fluorescence in basic aqueous solutions which can be used for analysis. Tetracycline(XXXVII) and

oxytetracycline (CLV) emit between 515 nm and 520 nm when excited at 390 nm in solution at pH 11, while chlortetracycline (CLVI) emits at 445 nm when excited at 355 nm under the same conditions.²¹ The fluorescence observed for CLVI is due to isochlortetracycline (CLVII) which is formed rapidly in neutral or alkaline solution as shown in Equation 19.²¹⁵⁻²¹⁷ CLVI has been analyzed in pharmaceutical preparations by fluorescence,²¹⁵⁻²¹⁷



and tetracyclines in general are frequently measured fluorimetrically in biological samples. Tetracyclines have also been analyzed fluorimetrically in tissues after converting them to anhydrotetracyclines by treating with 0.6 N HCl at 100°C for a few minutes.²¹⁸ The fluorescence is measured in sodium hydroxide solution with emission and excitation at 510 and 390 nm, respectively, for CLV and dimethylchlortetracycline (CLVIII), and at 560 nm and 425 nm for CLIV, CLVI, and N-pyrrolidinomethyl-tetracycline (CLIX).



Tserng and Wagner²¹⁹ have reported a fluorimetric method for erythromycin (CLX) and erythromycin propionate (CLXI) in blood after extraction as an ion pair with the fluorescent dye, Tinopal GS (CLXII). Ion-pair extraction procedures are discussed in more detail in Chapter II.

Amphotericin B (CLXIII) has also been analyzed fluorimetrically.²⁴⁰ Emission max-

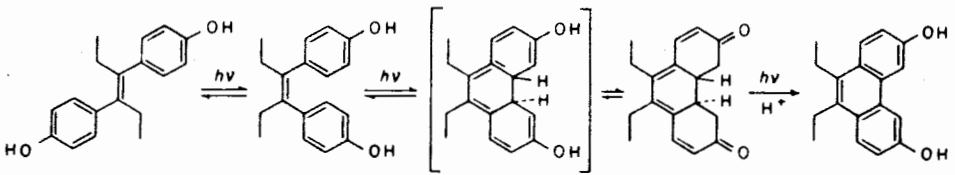
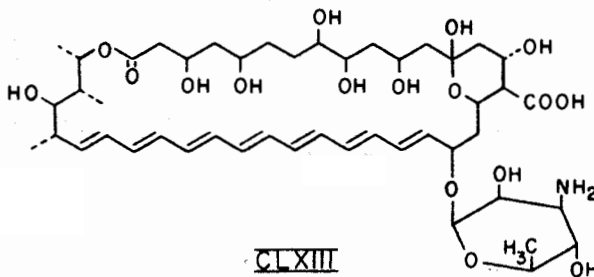
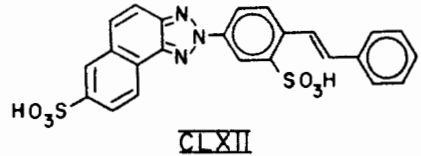
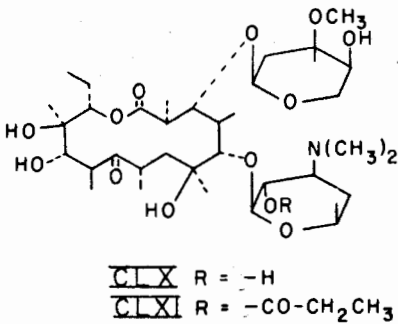


FIGURE 12. Photochemical reactions of diethylstilbestrol.

ima occur near 427 nm, 451 nm, and 472 nm with excitation at 340 nm in aqueous solution.^{241,242} However, the fluorescence spectra of CLXIII depend strongly upon the solvent used.



M. Diethylstilbestrol

Goodyear et al.²⁴³ observed that diethylstilbestrol (CLXIV) develops a yellow color ($\lambda_{\text{MAX}} = 420 \text{ nm}$) when irradiated with UV light, and used this method of color development as the basis of a spectrophotometric analysis of the drug in pharmaceutical preparations. The product of irradiation is strongly fluorescent, and Goodyear and Jenkinson²⁴⁴ reported a sensitive fluorimetric analysis of CLXIV based upon this reaction. The sample is dissolved in ethanol and irradiated with an intense source of short wavelength UV light for several minutes. The emission intensity is then measured at 435 nm with excitation at 360 nm, and compared with the intensity of standards prepared in the same manner. In alkaline ethanol the emission and excitation maxima shift to 410 nm and 525 nm, respectively. The limit of detection is on the order of 100 ng of CLXIV. The photochemical reaction converts CLXIV to the fluorescent phenanthrene derivative (CLXV) shown in Figure 12.^{245,246} Automated versions of this analysis have also been reported.²⁴⁷

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