chapter**FIFTEEN**

Molecular Luminescence Spectrometry

Three related types of optical methods are considered in this chapter: **molecular fluorescence, phosphorescence**, and **chemiluminescence**. In each of these methods, molecules of the analyte are excited to produce a species whose emission spectrum provides information for qualitative or quantitative analysis. The methods are known collectively as **molecular luminescence** procedures.

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Fluorescence and phosphorescence are alike in that excitation is brought about by absorption of photons. As a consequence, the two phenomena are often referred to by the more general term *photoluminescence*. As will be shown in this chapter, fluorescence differs from phosphorescence in that the electronic energy transitions responsible for fluorescence do not involve a change in electron spin. Because of this, the excited states involved in fluorescence are short-lived ($<10^{-5}$ s). In contrast, a change in electron spin accompanies phosphorescence, and the lifetimes of the excited states are much longer, often on the order of seconds or even minutes. In most instances, photoluminescence, be it fluorescence or phosphorescence, occurs at wavelengths longer than that of the excitation radiation.

The third type of luminescence, chemiluminescence, is based on the emission of radiation by an excited species formed during a chemical reaction. In some instances, the excited species is the product of a reaction between the analyte and a suitable reagent (usually a strong oxidant such as ozone or hydrogen peroxide). The result is an emission spectrum characteristic of the oxidation product of the analyte or the reagent rather than the analyte itself. In other instances, the analyte is not directly involved in the chemiluminescence reaction. Instead, the analyte inhibits or has a catalytic effect on a chemiluminescence reaction.

Measurement of the intensity of photoluminescence or chemiluminescence permits the quantitative determination of a variety of important inorganic and organic species in trace amounts. Currently, the number of fluorometric methods is far greater than the number of applications of phosphorescence and chemiluminescence procedures.

One of the most attractive features of luminescence methods is their inherent sensitivity, with detection limits that are often one to three orders of magnitude lower than those encountered in absorption spectroscopy. In fact, for selected species under controlled conditions, single molecules have been detected by fluorescence spectroscopy. Another advantage of photoluminescence methods is their large linear concentration ranges, which also are often significantly greater than those encountered in absorption methods. Because excited states are quite susceptible to being deactivated by collisions and other processes, many molecules do not fluoresce or phosphoresce at all. Because of

such deactivation processes, quantitative luminescence methods are often subject to serious interference effects. For this reason luminescence measurements are often combined with such separation techniques as chromatography and electrophoresis. Fluorescence detectors are particularly valuable as detectors for liquid chromatography (Chapter 28) and capillary electrophoresis (Chapter 30).

Generally, luminescence methods are less widely applicable for quantitative analyses than are absorption methods because many more species absorb ultraviolet (UV) and visible radiation than exhibit photoluminescence when radiation is absorbed in this region of the spectrum.[1]

15A THEORY OF FLUORESCENCE AND PHOSPHORESCENCE

Fluorescence occurs in simple as well as in complex gaseous, liquid, and solid chemical systems. The simplest kind of fluorescence is that exhibited by dilute atomic vapors, which was described in Chapter 9. For example, the 3*s* electrons of vaporized sodium atoms can be excited to the 3*p* state by absorption of radiation of wavelengths 589.6 and 589.0 nm (see Figure 8-1a). After about 10^{-8} s, the electrons return to the ground state and in so doing emit radiation of the same two wavelengths in all directions. This type of fluorescence, in which the absorbed radiation is reemitted without a change in frequency, is known as *resonance radiation* or *resonance fluorescence*.

Many molecular species also exhibit resonance fluorescence. Much more often, however, molecular fluorescence (or phosphorescence) bands occur at wavelengths longer than the absorption band. This shift toward longer wavelengths is termed the *Stokes shift* (see Section 6C-6).

15A-1 Excited States Producing Fluorescence and Phosphorescence

The characteristics of fluorescence and phosphorescence spectra can be rationalized by means of the molecular orbital considerations described in Section 14B-1. However, an understanding of the difference between the two photoluminescence phenomena requires a review of electron spin and the differences between singlet and triplet excited states.

Electron Spin

The Pauli exclusion principle states that no two electrons in an atom can have the same set of four quantum numbers. This restriction requires that no more than two electrons occupy an orbital and furthermore the two have opposed spin states. Under this circumstance, the spins are said to be paired. Because of spin pairing, most molecules exhibit no net magnetic field and are thus said to be diamagnetic—that is, they are neither attracted nor repelled by static magnetic fields. In contrast, free radicals, which contain unpaired electrons, have a magnetic moment and consequently are attracted by a magnetic field. Free radicals are thus said to be *paramagnetic*.

Singlet and Triplet Excited States

A molecular electronic state in which all electron spins are paired is called a *singlet state*, and no splitting of electronic energy levels occurs when the molecule is exposed to a magnetic field. The ground state for a free radical, on the other hand, is a *doublet state* because there are two possible orientations for the odd electron in a magnetic field, and each imparts slightly different energies to the system.

When one of a pair of electrons of a molecule is excited to a higher energy level, a singlet or a *triplet state* is formed. In the excited singlet state, the spin of the promoted electron is still paired with the ground-state electron. In the triplet state, however, the spins of the two electrons have become unpaired and are thus parallel. These states can be represented as in Figure 15-1, where the arrows represent the direction of spin. The nomenclature of singlet, doublet, and triplet derives from spectroscopic *multiplicity* considerations, which need not concern us here. Note that the excited triplet state is less energetic than the corresponding excited singlet state.

The properties of a molecule in the excited triplet state differ significantly from those of the excited singlet state. For example, a molecule is paramagnetic in the triplet state and diamagnetic in the singlet. More important, however, is that a singlet-to-triplet transition (or the reverse), which

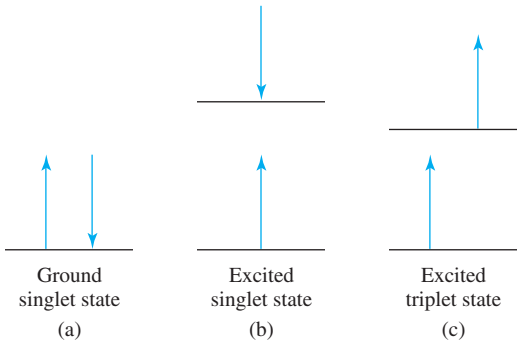


FIGURE 15-1 Electronic spin states of molecules. In (a) the ground electronic state is shown. In the lowest energy, or ground, state, the spins are always paired, and the state is said to be a singlet state. In (b) and (c), excited electronic states are shown. If the spins remain paired in the excited state, the molecule is in an excited singlet state (b). If the spins become unpaired, the molecule is in an excited triplet state (c).

[1]For further discussion of the theory and applications of fluorescence, phosphorescence, and luminescence, see D. M. Jameson, *Introduction to Fluorescence*, Boca Raton, FL: Taylor & Francis, 2014; J. R. Lakowicz, Principles of Fluorescence Spectroscopy, 3rd ed., New York: Springer, 2006; *Molecular Luminescence Spectroscopy*, S. Schulman, ed., New York: Wiley, Part 1, 1985; Part 2, 1988; Part 3, 1993; E. L. Wehry, in *Physical Methods of Chemistry*, 2nd ed., Volume VIII, Chap. 3, B. W. Rossiter and R. C. Baetzold, eds., New York: Wiley, 1993.

also involves a change in electron spin, is a significantly less probable event than the corresponding singlet-to-singlet transition. As a result, the average lifetime of an excited triplet state may range from 10^{-4} to several seconds, compared with an average lifetime of $\sim 10^{-8}$ s for an excited singlet state. Furthermore, radiation-induced excitation of a ground-state molecule to an excited triplet state has a low probability of occurring, and absorption bands due to this process are several orders of magnitude less intense than the analogous singlet-to-singlet absorption. We shall see, however, that an excited triplet state can be populated from an excited singlet state of certain molecules. Phosphorescence emission is often a result of such a process.

Energy-Level Diagrams for Photoluminescent Molecules

Figure 15-2 is a partial energy-level diagram, called a *Jablonski diagram*, for a typical photoluminescent molecule. The lowest heavy horizontal line represents the ground-state energy of the molecule, which is normally a singlet state, and is labeled S_0. At room temperature, this state represents the energies of most of the molecules in a solution.

The upper heavy lines are energy levels for the ground vibrational states of three excited electronic states. The two lines on the left represent the first (S_1) and second (S_2) electronic singlet states. The one on the right (T_1) represents the energy of the first electronic triplet state. As is normally the case, the energy of the first excited triplet state is lower than the energy of the corresponding singlet state.

Numerous vibrational energy levels are associated with each of the four electronic states, as suggested by the lighter horizontal lines. As shown in Figure 15-2, absorption transitions can occur from the ground singlet electronic state (S_0) to various vibrational levels of the excited singlet electronic states (S_1 and S_2). Note that direct excitation to the triplet state is not shown. Because this transition involves a change in multiplicity, it has a very low probability of occurrence. A low-probability transition of this type is called a *forbidden transition*.

Molecules excited to electronic states S_1 and S_2 rapidly lose any excess vibrational energy and relax to the ground vibrational level of that electronic state. This nonradiational process is termed *vibrational relaxation*.

15A-2 Rates of Absorption and Emission

Photon absorption is a very rapid process, which takes place in about 10^{-14} to 10^{-15} s. Fluorescence emission, on the other hand, occurs at a significantly slower rate. Typically, fluorescence occurs in 10^{-5} to 10^{-10} s. As we have noted, the average rate of a triplet-to-singlet transition is less than that of a corresponding singlet-to-singlet transition. Thus, phosphorescence emission requires 10^{-4} to 10 s or more to occur.

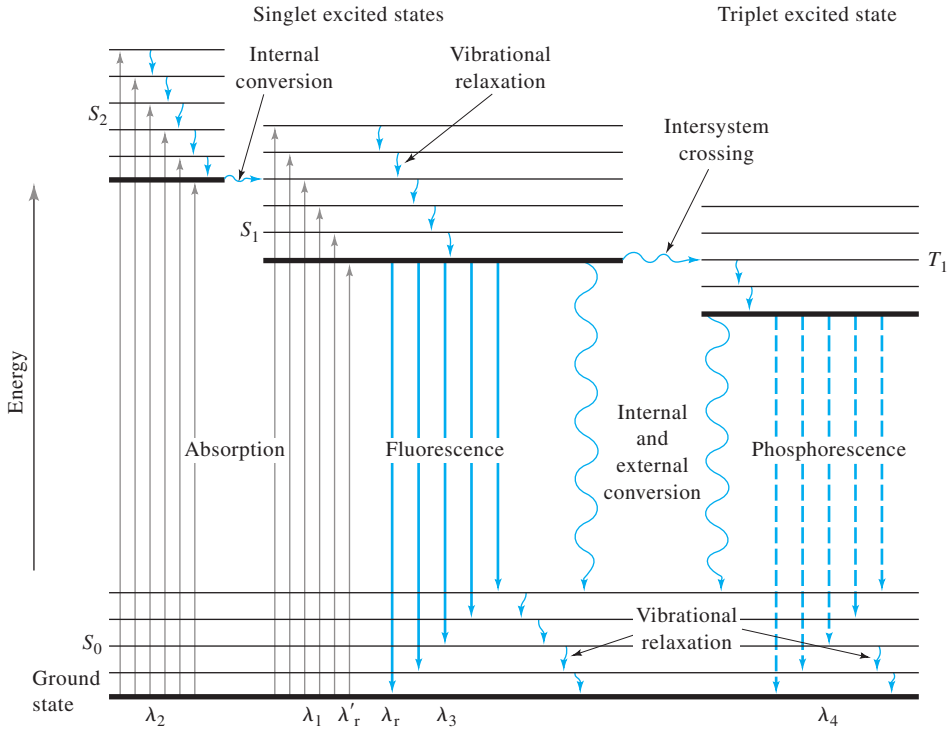


FIGURE 15-2 Partial energy-level diagram for a photoluminescent system, often called a Jablonski diagram.

15A-3 Deactivation Processes

An excited molecule can return to its ground state by a combination of several mechanistic steps. As shown by the straight, downward pointing, vertical arrows in Figure 15-2, two of these steps, fluorescence and phosphorescence, involve the emission of a photon. The other deactivation steps, indicated by wavy arrows, are radiationless processes. The favored route to the ground state is the one that minimizes the lifetime of the excited state. Thus, if deactivation by fluorescence is rapid with respect to the radiationless processes, such emission is observed. On the other hand, if a radiationless path has a more favorable rate constant, fluorescence is either absent or less intense.

Photoluminescence is limited to systems incorporating structural and environmental features that cause the rate of radiationless relaxation or deactivation processes to be slowed to a point where the emission process can compete kinetically. We have a good quantitative understanding of luminescence processes. However, we are only beginning to understand deactivation processes in any detail.

Vibrational Relaxation

As shown in Figure 15-2, a molecule may be promoted to any of several vibrational levels during the electronic excitation process. Collisions between molecules of the excited species and those of the solvent lead to rapid energy transfer with a minuscule increase in temperature of the solvent. Vibrational relaxation is so efficient that the average lifetime of a *vibrationally* excited molecule is 10^{-12} s or less, a period significantly shorter than the average lifetime of an *electronically* excited state. As a consequence, fluorescence from solution always involves a transition from the *lowest vibrational level of an excited electronic state*. Several closely spaced emission lines are produced, however, and the transition can terminate in any of the vibrational levels of the ground state (see Figure 15-2).

A consequence of the efficiency of vibrational relaxation is that the fluorescence band for a given electronic transition is displaced toward lower frequencies or longer wavelengths from the absorption band (the Stokes shift). Overlap occurs only for the resonance peak involving transitions between the lowest vibrational level of the ground state and the corresponding level of an excited state. In Figure 15-2, the wavelength of absorbed radiation that produces the resonance peak λ_r is labeled λ_r'.

Internal Conversion

The term *internal conversion* describes intermolecular processes that leave the molecule in a lower-energy electronic state without emission of radiation. These processes are neither well defined nor well understood, but they are often highly efficient.

Internal conversion is a crossover between two states of the same multiplicity (singlet-singlet or triplet-triplet). It is particularly efficient when two electronic energy levels are sufficiently close for there to be an overlap in vibrational energy levels. This situation is illustrated in Figure 15-2 for the two excited singlet states S_2 and S_1. At the overlaps shown, the potential energies of the two excited states are essentially equal, which permits an efficient crossover from S_2 to S_1. Internal conversion can also occur between state S_1 and the ground electronic state S_0. Internal conversion through overlapping vibrational levels is usually more probable than the loss of energy by fluorescence from a higher excited state. Thus, referring again to Figure 15-2, excitation by the band of radiation labeled λ_2 usually produces a fluorescence band centered at wavelength λ_3 to the exclusion of a band that would result from a transition between S_2 and S_0. The excited molecule makes the transition from the higher electronic state to the lowest vibrational state of the lower electronic excited state via a series of vibrational relaxations, an internal conversion, and then further relaxations. Under these circumstances, the fluorescence occurs at λ_3 only, regardless of whether radiation of wavelength λ_1 or λ_2 was responsible for the excitation. Quinine provides a classical example of this type of behavior (see Problem 15-13). This naturally occurring substance possesses two analytically useful excitation bands, one centered at 250 nm and the other at 350 nm. Regardless of which wavelength is used to excite the molecule, however, the wavelength of maximum emission is 450 nm (see Figure 15-3).

The mechanisms of the internal conversion process $S_1 \rightarrow S_0$ shown in Figure 15-2 are not totally understood. For some molecules, the vibrational levels of the ground state overlap those of the first excited electronic state, and deactivation occurs rapidly by the mechanism just described. This situation prevails with aliphatic compounds, for example, and accounts for these species seldom fluorescing. With such compounds, deactivation by energy transfer through overlapping vibrational levels occurs so rapidly that fluorescence does not have time to occur.

Internal conversion may also result in the phenomenon of *predissociation*. In this process, the electron moves from a higher electronic state to an upper vibrational level of a lower electronic state in which the vibrational energy is great enough to cause rupture of a bond. Large molecules have an appreciable probability for the existence of bonds with strengths less than the electronic excitation energy of the chromophores. Rupture of these bonds can occur as a consequence of absorption by the chromophore followed by internal conversion of the electronic energy to vibrational energy associated with the weak bond.

Predissociation should be differentiated from *dissociation*, in which the absorbed radiation directly excites the electron of a chromophore to a sufficiently high vibrational level to cause rupture of the chromophoric bond. In this case, no internal conversion is involved. Dissociation processes also compete with the fluorescence process.

External Conversion

Deactivation of an excited electronic state may involve interaction and energy transfer between the excited molecule and the solvent or other solutes. This process is called *external conversion*. Evidence for external conversion includes a marked solvent effect on the fluorescence intensity of most species.

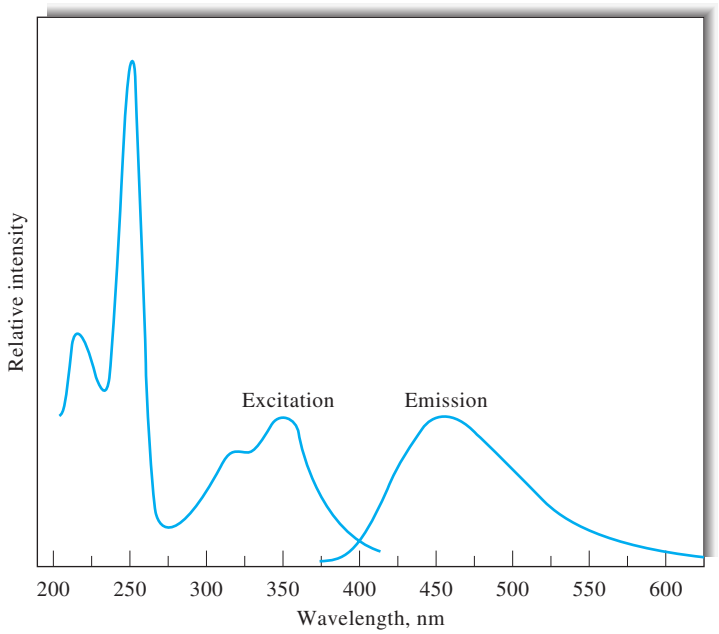


FIGURE 15-3 Fluorescence excitation and emission spectra for a solution of quinine.

Furthermore, those conditions that tend to reduce the number of collisions between particles (low temperature and high viscosity) generally lead to enhanced fluorescence. The details of external conversion processes are not well understood.

Radiationless transitions to the ground state from the lowest excited singlet and triplet states (Figure 15-2) probably involve external conversion, as well as internal conversion.

Intersystem Crossing

Intersystem crossing is a process in which there is a crossover between electronic states of different multiplicity. The most common process is from the singlet state to the triplet state ($S_1 \rightarrow T_1$) as shown in Figure 15-2. As with internal conversion, the probability of intersystem crossing is enhanced if the vibrational levels of the two states overlap. Note that in the singlet-triplet crossover shown in Figure 15-2, the lowest singlet vibrational level overlaps one of the upper triplet vibrational levels and a change in spin state is thus more probable.

Intersystem crossing is most common in molecules that contain heavy atoms, such as iodine or bromine (the *heavy-atom effect*). Spin and orbital interactions increase in the presence of such atoms, and a change in spin is thus more favorable. The presence of paramagnetic species such as molecular oxygen in solution also enhances intersystem crossing with a corresponding decrease in fluorescence.

Phosphorescence

Deactivation of electronic excited states may also involve phosphorescence. After intersystem crossing to the triplet state, further deactivation can occur either by internal or external conversion or by phosphorescence. A triplet → singlet transition is much less probable than a singlet-singlet conversion. Transition probability and excited-state lifetime are inversely related. Thus, the average lifetime of the excited triplet state with respect to emission is large and ranges from 10^{-4} to 10 s or more. Emission from such a transition may persist for some time after irradiation has ceased.

External and internal conversion compete so successfully with phosphorescence that this kind of emission is ordinarily observed only at low temperatures in highly viscous media or by using special techniques to protect the triplet state.

15A-4 Variables Affecting Fluorescence and Phosphorescence

Both molecular structure and chemical environment influence whether a substance will or will not luminesce. These factors also determine the intensity of emission when luminescence does occur. The effects of some of these variables are considered briefly in this section.

Quantum Yield

The *quantum yield*, or *quantum efficiency*, for fluorescence or phosphorescence is simply the ratio of the number of molecules that luminesce to the total number of excited molecules. For a highly fluorescent molecule such as fluorescein, the quantum efficiency approaches unity under some conditions. Chemical species that do not fluoresce appreciably have efficiencies that approach zero.

Figure 15-2 and our discussion of deactivation processes suggest that the fluorescence quantum yield ϕ for a compound is determined by the relative rate constants k_x for the processes by which the lowest excited singlet state is deactivated. These processes are fluorescence (k_f), intersystem crossing (k_i), external conversion (k_{ec}), internal conversion (k_{ic}), predissociation (k_{pd}), and dissociation (k_d). We can express these relationships by the equation

$$\phi = \frac{k_f}{k_f + k_i + k_{ec} + k_{ic} + k_{pd} + k_d} \qquad (15\text{-}1)$$

where the k terms are the respective rate constants for the various deactivation processes.

Equation 15-1 permits a qualitative interpretation of many of the structural and environmental factors that influence fluorescence intensity. Those variables that lead to high values for the fluorescence rate constant k_f and low values for the other k_x terms enhance fluorescence. The magnitude of k_f, the predissociation rate constant k_{pd}, and the dissociation rate constant k_d mainly depend on chemical structure. Environment and to a somewhat lesser extent structure strongly influence the remaining rate constants.

Transition Types in Fluorescence

It is important to note that fluorescence seldom results from absorption of UV radiation of wavelengths shorter than 250 nm because such radiation is sufficiently energetic to cause deactivation of the excited states by predissociation or dissociation. For example, 200-nm radiation corresponds to about 140 kcal/mol. Most organic molecules have at least some bonds that can be ruptured by energies of this magnitude. As a result, fluorescence due to $\sigma^* \rightarrow \sigma$ transitions is seldom observed. Instead, such emission is confined to the less energetic $\pi^* \rightarrow \pi$ and $\pi^* \rightarrow n$ processes.

As we have noted, an electronically excited molecule ordinarily returns to its *lowest excited state* by a series of rapid vibrational relaxations and internal conversions that produce no emission of radiation. Thus, fluorescence most commonly arises from a transition from the lowest vibrational level of the first excited electronic state to one of the vibrational levels of the electronic ground state. For most fluorescent compounds, then, radiation is produced by either a $\pi^* \rightarrow n$ or a $\pi^* \rightarrow \pi$ transition, depending on which of these is the less energetic.

Quantum Efficiency and Transition Type

It is observed empirically that fluorescence is more commonly found in compounds in which the lowest energy transition is of a $\pi \rightarrow \pi^*$ type (π, π^* excited singlet state) than in compounds in which the lowest energy transition is of the $n \rightarrow \pi^*$ type (n, π^* excited state); that is, the quantum efficiency is greater for $\pi^* \rightarrow \pi$ transitions.

The greater quantum efficiency associated with the π, π^* state can be rationalized in two ways. First, the molar absorptivity of a $\pi \rightarrow \pi^*$ transition is ordinarily 100- to 1000-fold greater than for an $n \rightarrow \pi^*$ process, and this quantity represents a measure of the transition probability. Thus, the inherent lifetime associated with the π, π^* state is shorter (10^{-7} to 10^{-9} s compared with 10^{-5} to 10^{-7} s for the n, π^* state) and k_f in Equation 15-1 is larger.

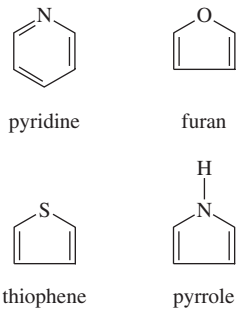
The most efficient phosphorescence often occurs from the n, π^* excited state, which tends to be shorter lived and thus less susceptible to deactivation than a π, π^* triplet state. Also, intersystem crossing is less probable for π, π^* excited states than for n, π^* states because the energy difference between the singlet and triplet states is larger and spin-orbit coupling is less likely.

In summary, then, fluorescence is more commonly associated with the π, π^* state because such excited states exhibit relatively short average lifetimes (k_f is larger) and because the deactivation processes that compete with fluorescence are less likely to occur.

Fluorescence and Structure

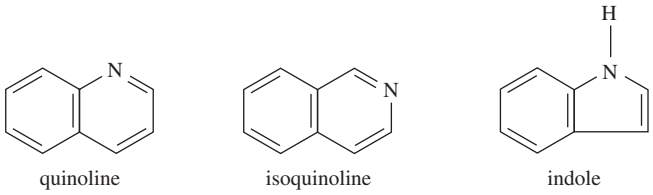
The most intense and the most useful fluorescence is found in compounds containing aromatic functional groups with low-energy $\pi \rightarrow \pi^*$ transitions. Compounds containing aliphatic and alicyclic carbonyl structures or highly conjugated double-bond structures may also exhibit fluorescence, but the number of these is smaller than the number in the aromatic systems.

Most unsubstituted aromatic hydrocarbons fluoresce in solution, the quantum efficiency usually increasing with the number of rings and their degree of condensation. The simple heterocyclics, such as pyridine, furan, thiophene, and pyrrole,



do not exhibit fluorescence. On the other hand, fused-ring structures ordinarily do fluoresce. With nitrogen heterocyclics, the lowest-energy electronic transition is believed to involve an $n \rightarrow \pi^*$ system that rapidly converts to the triplet state and prevents fluorescence. Fusion of benzene rings to a heterocyclic nucleus, however, results in an increase in the molar absorptivity

of the absorption band. The lifetime of an excited state is shorter in such structures, and fluorescence is observed for compounds such as quinoline, isoquinoline, and indole.



quinoline isoquinoline indole

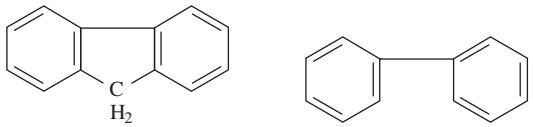
Substitution on the benzene ring causes shifts in the wavelength of absorption maxima and corresponding changes in the fluorescence emission. In addition, substitution frequently affects the quantum efficiency. Some of these effects are illustrated by the data for benzene derivatives in Table 15-1.

The influence of halogen substitution is striking; the decrease in fluorescence with increasing molar mass of the halogen is an example of the heavy-atom effect (page 365), which increases the probability for intersystem crossing to the triplet state. Predissociation is thought to play an important role in iodobenzene and in nitro derivatives as well, because these compounds have easily ruptured bonds that can absorb the excitation energy following internal conversion.

Substitution of a carboxylic acid or carbonyl group on an aromatic ring generally inhibits fluorescence. In these compounds, the energy of the $n \rightarrow \pi^*$ transition is less than in the $\pi \rightarrow \pi^*$ transition, and as discussed previously, the fluorescence yield from the $n \rightarrow \pi^*$ systems is ordinarily low.

Effect of Structural Rigidity

It is found empirically that fluorescence is particularly favored in molecules with rigid structures. For example, the quantum efficiencies for fluorene and biphenyl are nearly 1.0 and 0.2, respectively, under similar conditions of measurement. The difference in behavior is largely a result of the increased rigidity furnished by the bridging methylene group in fluorene. Many similar examples can be cited.



fluorene biphenyl

The influence of rigidity has also been invoked to account for the increase in fluorescence of certain organic chelating agents when they are complexed with a metal ion. For example, the fluorescence intensity of 8-hydroxyquinoline is much less than that of its zinc complex:

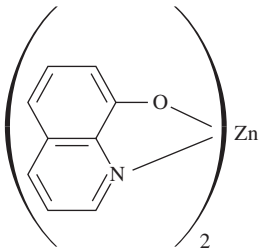


TABLE 15-1 Effect of Substitution on the Fluorescence of Benzene

Compound	Formula	Wavelength of Fluorescence, nm	Relative Intensity of Fluorescence
Benzene	C_6H_6	270–310	10
Toluene	$C_6H_5CH_3$	270–320	17
Propylbenzene	$C_6H_5C_3H_7$	270–320	17
Fluorobenzene	C_6H_5F	270–320	10
Chlorobenzene	C_6H_5Cl	275–345	7
Bromobenzene	C_6H_5Br	290–380	5
Iodobenzene	C_6H_5I	—	0
Phenol	C_6H_5OH	285–365	18
Phenolate ion	$C_6H_5O^-$	310–400	10
Anisole	$C_6H_5OCH_3$	285–345	20
Aniline	$C_6H_5NH_2$	310–405	20
Anilinium ion	$C_6H_5NH_3^+$	—	0
Benzoic acid	C_6H_5COOH	310–390	3
Benzonitrile	C_6H_5CN	280–360	20
Nitrobenzene	$C_6H_5NO_2$	—	0

Lack of rigidity in a molecule probably causes an enhanced internal conversion rate (k_{ic} in Equation 15-1) and a resulting increase in the likelihood for radiationless deactivation. One part of a nonrigid molecule can undergo low-frequency vibrations with respect to its other parts. These vibrations undoubtedly account for some energy loss.

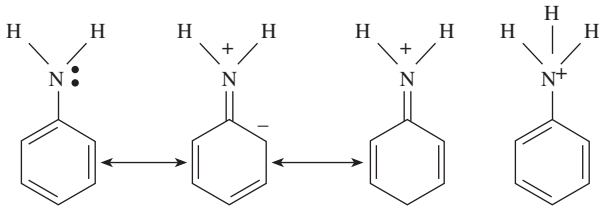
Temperature and Solvent Effects

The quantum efficiency of fluorescence in most molecules decreases with increasing temperature because the increased frequency of collisions at elevated temperatures improves the probability for deactivation by external conversion. A decrease in solvent viscosity also increases the likelihood of external conversion and leads to the same result.

The fluorescence of a molecule is decreased by solvents containing heavy atoms or other solutes with such atoms in their structure; carbon tetrabromide and ethyl iodide are examples. The effect is similar to that which occurs when heavy atoms are substituted into fluorescing compounds. Orbital spin interactions result in an increase in the rate of triplet formation and a corresponding decrease in fluorescence. Compounds containing heavy atoms are frequently incorporated into solvents when enhanced phosphorescence is desired.

Effect of pH on Fluorescence

The fluorescence of an aromatic compound with acidic or basic ring substituents is usually pH dependent. Both the wavelength and the emission intensity are likely to be different for the protonated and unprotonated forms of the compound. The data for phenol and aniline shown in Table 15-1 illustrate this effect. The changes in emission of compounds of this type arise from the differing number of resonance species that are associated with the acidic and basic forms of the molecules. For example, aniline has several resonance forms but anilinium has only one. That is,



resonance forms of aniline anilinium ion

The additional resonance forms lead to a more stable first excited state resulting in fluorescence in the UV region.

The fluorescence of certain compounds as a function of pH has been used for the detection of end points in acid-base titrations. For example, fluorescence of the phenolic form of 1-naphthol-4-sulfonic acid is not detectable by the eye because it occurs in the UV region. When the compound is converted to the phenolate ion by the addition of base, however, the emission band shifts to visible wavelengths, where it can readily be seen. It is significant that this change occurs at a different pH than would be predicted from the acid dissociation constant for the compound. The explanation of this discrepancy is that the acid dissociation constant for the excited molecule differs from that for the same species in its ground state. Changes in acid or base dissociation constants with excitation are common and are occasionally as large as four or five orders of magnitude. These observations suggest that analytical procedures based on fluorescence frequently require close control of pH.

The presence of dissolved oxygen often reduces the intensity of fluorescence in a solution. This effect may be the result of a photochemically induced oxidation of the fluorescing species. More commonly, however, the quenching takes place due to the paramagnetic properties of molecular oxygen, which promotes intersystem crossing and conversion of excited molecules to the triplet state. Other paramagnetic species also tend to quench fluorescence.

Effect of Concentration on Fluorescence Intensity

The power of fluorescence emission F is proportional to the radiant power of the excitation beam that is absorbed by the system. That is,

$$F = \phi_f K''(P_0 - P) = K'(P_0 - P) \tag{15-2}$$

where P_0 is the power of the beam incident on the solution, P is its power after traversing a length b of the medium, ϕ_f is the quantum efficiency of the fluorescence process, and K'' is a constant dependent on geometry and other factors. The quantum efficiency of fluorescence is a constant for a given system, and so the product $\phi_f K''$ is lumped into a new constant K' on the right side of Equation 15-2. To relate F to the concentration c of the fluorescing species, we write Beer's law in the form

$$\frac{P}{P_0} = 10^{-\varepsilon bc} \tag{15-3}$$

where ε is the molar absorptivity of the fluorescing molecules and εbc is the absorbance A. By substitution of Equation 15-3 into Equation 15-2, we obtain

$$F = K'P_0(1 - 10^{-\varepsilon bc}) \tag{15-4}$$

The exponential term in Equation 15-4 can be expanded as a Maclaurin series to

$$F = K'P_0\left[2.303\varepsilon bc - \frac{(2.303\varepsilon bc)^2}{2!} + \frac{(2.303\varepsilon bc)^3}{3!} + \cdots\right] \tag{15-5}$$

Provided that $2.303\varepsilon bc = A < 0.05$, all of the subsequent terms in the brackets are small with respect to the first term. Under these conditions, the maximum relative error caused by dropping all but the first term is 0.13%. Thus, we may write

$$F = 2.303\, K'\varepsilon bcP_0 \tag{15-6}$$

Simulation: Learn more about **luminescence spectroscopy** at www.tinyurl.com/skoogpia7

or, at constant P_0

$$F = Kc = 2.303K'\varepsilon bcP_0 = 2.303\phi_f K''\varepsilon bcP_0 \qquad (15\text{-}7)$$

Thus, a plot of the fluorescence radiant power of a solution versus concentration of the emitting species should be linear at low concentrations. When c becomes great enough that the absorbance is larger than about 0.05, the higher-order terms in Equation 15-5 become important and linearity is lost; F then lies below an extrapolation of the straight-line plot. This excessive absorption is known as *primary absorption*.

Another factor responsible for negative departures from linearity at high concentration is *secondary absorption*. Secondary absorption occurs when the wavelength of emission overlaps an absorption band. Fluorescence is then decreased as the emission traverses the solution and is reabsorbed by other molecules in solution. Secondary absorption can be absorption by the analyte species itself or absorption by other species in the solution. The effects of these phenomena are such that a plot of fluorescence versus concentration may exhibit a maximum. Absorption effects are often termed *inner filter effects*.

Dynamic Quenching

The term *quenching* usually refers to nonradiative energy transfer from an excited species to other molecules. *Dynamic quenching*, also called *collisional quenching*, requires contact between the excited species and the quenching agent (Q). Dynamic quenching occurs as rapidly as the collision partners can diffuse together. The rate is temperature and viscosity dependent. The quencher concentration must be high enough that there is a high probability of a collision between the excited species and the quencher during the lifetime of the excited state.

For external conversion controlled by dynamic quenching with a single quencher, the external conversion rate constant can be written as

$$k_{ec} = k_q[Q] \qquad (15\text{-}8)$$

where k_q is the rate constant for quenching and [Q] is the concentration of the quencher. In the absence of quenching ($k_{ec} = 0$) and where predissociation and dissociation are absent ($k_{pd} = k_d = 0$), the fluorescence quantum efficiency ϕ_f^0 can be written from Equation 15-1 as

$$\phi_f^0 = \frac{k_f}{k_f + k_i + k_{ic}} \qquad (15\text{-}9)$$

With quenching, we can write

$$\phi_f = \frac{k_f}{k_f + k_i + k_{ic} + k_q[Q]} \qquad (15\text{-}10)$$

By taking the ratio of Equation 15-9 to Equation 15-10, we obtain the Stern-Volmer equation

$$\frac{\phi_f^0}{\phi_f} = 1 + K_q[Q] \qquad (15\text{-}11)$$

where K_q is the Stern-Volmer quenching constant defined as $K_q = k_q/(k_f + k_i + k_{ic})$. Rearrangement of this equation yields,

$$\frac{1}{\phi_f} = \frac{1}{\phi_f^0} + \frac{K_q[Q]}{\phi_f^0}$$

A plot of $1/\phi_f$ versus [Q] should be a straight line with a slope of K_q/ϕ_f^0 and an intercept of $1/\phi_f^0$. The Stern-Volmer constant can be obtained from the ratio of the intercept and the slope. Dynamic quenching reduces both the fluorescence quantum yield and the fluorescence lifetime.

Because the fluorescence emission F is directly proportional to the quantum efficiency ϕ_f (Equation 15-2), we can also write the Stern-Volmer equation in terms of easily measured quantities

$$\frac{F_0}{F} = 1 + K_q[Q] \qquad (15\text{-}12)$$

where F_0 and F are the fluorescence signals in the absence and in the presence of quencher, respectively. The Stern-Volmer constant is then just the slope of a plot of F_0/F versus [Q], and the intercept of the plot is unity. Example 15-1 illustrates the use of Equation 15-12 in determining K_q values.

EXAMPLE 15-1

The fluorescence of quinine sulfate (see Figure 15-3) is quenched by high concentrations of chloride ion. The following fluorescence signals F were obtained as a function of the concentration of chloride ion.

Fluorescence Intensity, F	$[Cl^-]$, M
180.0	0.00
87.5	0.005
58.0	0.010
43.2	0.015
35.0	0.020
28.5	0.025
14.5	0.030
19.1	0.040
15.7	0.050

Set up a spreadsheet and determine the quenching constant K_q.

Solution

The spreadsheet and Stern-Volmer plot are shown in Figure 15-4. The data are entered into columns B and C, and F_0/F is calculated in column D. The slope and its standard deviation are $K_q = 210 \pm 0.9\ M^{-1}$, as obtained from the spreadsheet statistics. Note that the intercept is very nearly unity.

	A	B	C	D
1	**Fluorescence Quenching**			
2		*F*	[Cl⁻]	F_0/F
3		180.0	0.000	1.0000
4		87.5	0.005	2.0571
5		58.0	0.010	3.1034
6		43.2	0.015	4.1667
7		35.0	0.020	5.1429
8		28.5	0.025	6.3158
9		24.5	0.030	7.3469
10		19.1	0.040	9.4241
11		15.7	0.050	11.4650
12	**Slope**	209.9462	1.009155	**Intercept**
13	**SD slope**	0.868963	0.023127	**SD intcpt**
14	R^2	0.99988	0.040292	s_r
15	*F*	58373.19	7	**df**
16	**SSregress**	94.7664	0.011364	**SSresid**
17				
18	**Spreadsheet Documentation**			
19	Cell D3=B3/B3			
20	Cells B12:C16=LINEST(D3:D11,C3:C11,1,1)			

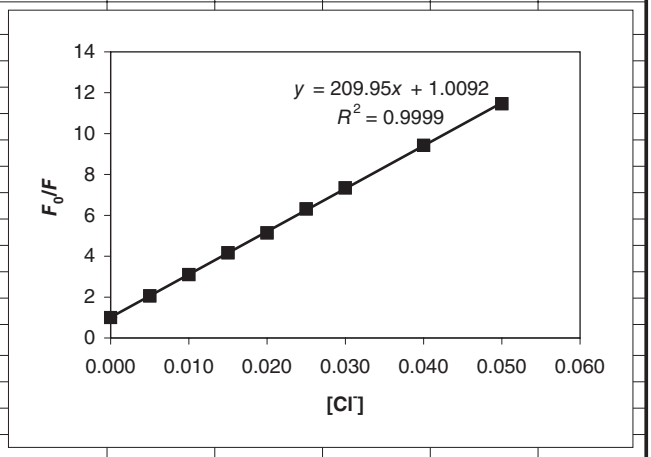


FIGURE 15-4 Spreadsheet and plot to determine quenching constant for chloride ion quenching of quinine.

The presence of dissolved oxygen often reduces the intensity of fluorescence in a solution. This effect is sometimes the result of a photochemically induced oxidation of the fluorescing species. More commonly, however, the quenching takes place because molecular oxygen is paramagnetic, which promotes intersystem crossing and conversion of excited molecules to the triplet state. Other paramagnetic species also tend to quench fluorescence. Dissolved oxygen is also an efficient quencher of the triplet state. For this reason, solutions are usually deoxygenated before phosphorescence measurements are made.

Other Types of Quenching

In *static quenching*, the quencher and the ground-state fluorophore form a complex called the *dark complex*. Fluorescence is usually observed only from the unbound fluorophore. The decrease in fluorescence intensity can also be described by the Stern-Volmer equation in the case of static quenching. However, K_q in Equation 15-11 is now the formation constant of the fluorophore-quencher complex. With static quenching, the lifetime is not affected. Hence, lifetime measurements can allow dynamic quenching to be distinguished from static quenching.

In *long-range*, or *Förster*, *quenching*, energy transfer occurs without collisions between molecules. Dipole-dipole coupling between the excited fluorophore and the quencher account for the transfer. The dependence of the degree of quenching on the quencher concentration is complicated and does not follow Stern-Volmer behavior.

15A-5 Emission and Excitation Spectra

Figure 15-5 shows three types of photoluminescence spectra for tryptophan. An excitation spectrum labeled *E* in the figure is obtained by measuring luminescence intensity at a fixed wavelength while the excitation wavelength is varied. Because the first step in generating fluorescence emission is absorption of radiation to create excited states, an excitation spectrum is essentially identical to an absorption spectrum taken under the same conditions. Fluorescence and phosphorescence spectra (*F* and *P*, respectively), on the other hand, involve excitation at a fixed wavelength while recording the emission intensity as a function of wavelength.

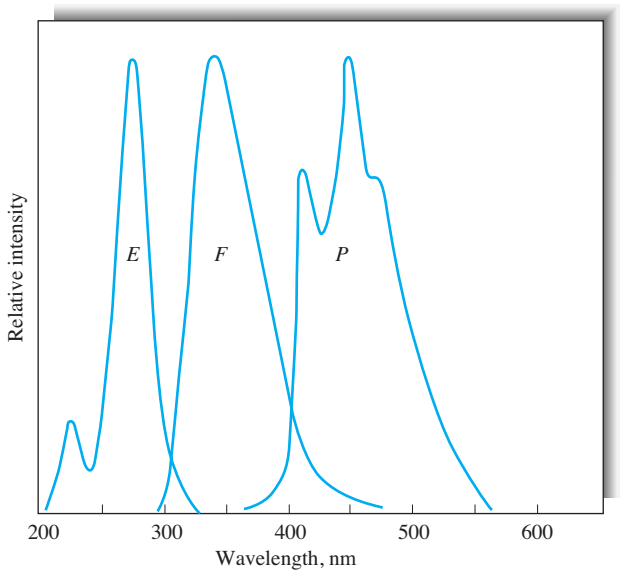


FIGURE 15-5 Spectra for tryptophan: *E*, excitation; *F*, fluorescence; *P*, phosphorescence. (Adapted from G. G. Guilbault, *Practical Fluorescence*, New York: Marcel Dekker, 1973, p. 164. Courtesy of Marcel Dekker, Inc.)

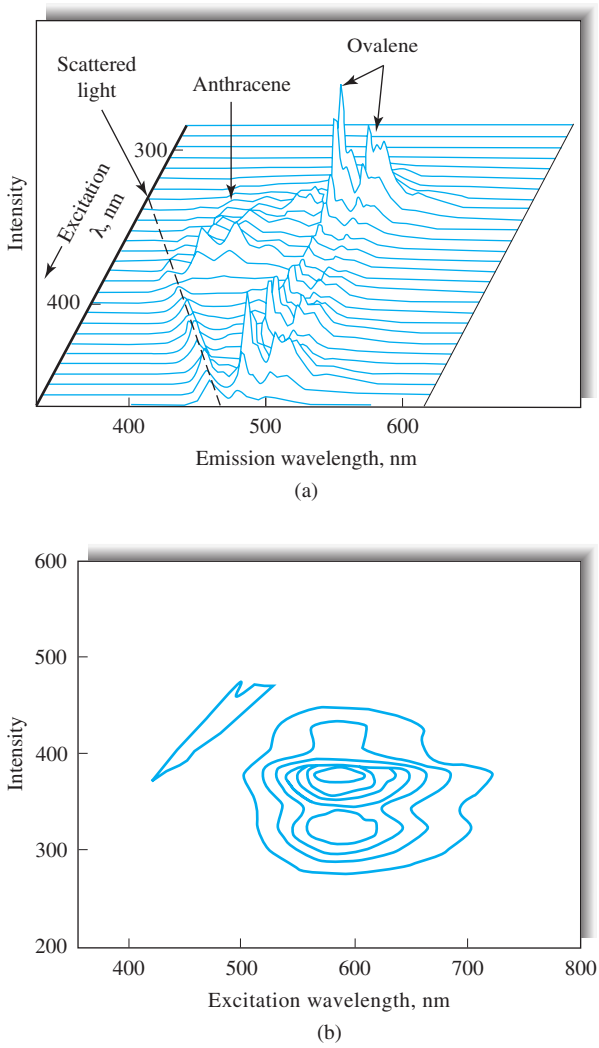


FIGURE 15-6 Total luminescence spectra. In (a), the total fluorescence spectrum of a mixture of anthracene and ovalene is shown as a three-dimensional plot. In (b), the total fluorescence spectrum of 8-hydroxybenzo[a]pyrene is shown as a contour plot. Each line represents a particular fluorescence intensity. (Part a from Y. Talmi et al., *Anal. Chem.*, **1978**, *50*, 936A, **DOI**: 10.1021/ac50033a719. Figure 11, p. 948A. Part b adapted from J. H. Rho and J. L. Stewart, *Anal. Chem.*, **1978**, *50*, 620, **DOI**: 10.1021/ac50026a020. Figure 2, p. 622. Copyright 1978 American Chemical Society.)

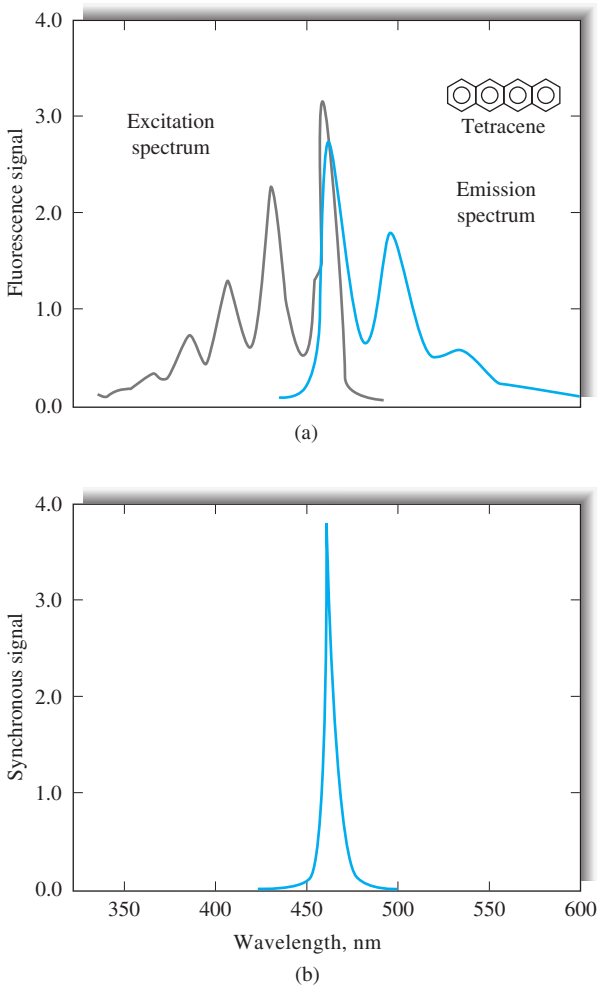


FIGURE 15-7 Synchronous fluorescence spectra. In (a), the excitation and emission spectra of tetracene are shown. In (b), the synchronous spectrum is shown for a fixed-wavelength difference of 3 nm. (From T. Vo-Dinh, *Anal. Chem.*, **1978**, *50*, 396, **DOI**: 10.1021/ac50025a010. Figure 1, p. 397. Copyright 1978 American Chemical Society.)

Another type of luminescence spectrum is shown in Figure 15-6. The *total luminescence spectrum* is either a three-dimensional representation or a contour plot. Both simultaneously show the luminescence signal as a function of excitation and emission wavelengths. Such data are often called an *excitation-emission matrix*. Although the total luminescence spectrum can be obtained on a normal computerized instrument, it can be acquired more rapidly with array-detector-based systems (see next section).

Some luminescence instruments allow simultaneously scanning both the excitation and the emission wavelengths with a small wavelength difference between them. The spectrum that results is known as a *synchronous spectrum*. A luminescence signal is obtained only at wavelengths where both excitation and emission occur for the wavelength difference chosen as shown in Figure 15-7. The synchronous spectrum can also be generated from the total luminescence spectrum by appropriate software.

As has been pointed out earlier, photoluminescence usually occurs at wavelengths that are longer than the excitation wavelength. Furthermore, phosphorescence bands are generally found at longer wavelengths than fluorescence bands because the excited triplet state is, in most instances, lower in energy than the corresponding singlet state. In fact, the wavelength difference between the two provides a convenient measure of energy difference between triplet and singlet states.

15B FLUORESCENCE AND PHOSPHORESCENE INSTRUMENTATION

The components of instruments for measuring photoluminescence are similar to those found in UV-visible photometers or spectrophotometers. Figure 15-8 shows a typical configuration for these components in *fluorometers* and *spectrofluorometers*. Nearly all fluorescence instruments use double-beam optics as shown to compensate for fluctuations in radiant power. The upper sample beam first passes through an excitation wavelength selector (filter or monochromator), which transmits radiation that excites fluorescence but excludes or limits radiation of the fluorescence emission wavelength. Fluorescence is emitted from the sample in all directions, but is most conveniently observed at right angles to the excitation beam. The right-angle geometry minimizes the contributions from scattering and from the intense source radiation. The emitted radiation then passes through an emission wavelength selector (filter or monochromator) that isolates the fluorescence emission. The isolated radiation then strikes a phototransducer, where it is converted into an electrical signal for measurement.

The lower reference beam passes through an attenuator that reduces its power to approximately that of the fluorescence radiation (the power reduction is usually by a factor of 100 or more). The attenuated reference beam then strikes a second transducer and is converted to an electrical signal. Electronics and a computer data system then process the signals to compute the ratio of the fluorescence emission intensity to the excitation source intensity and produce the resulting spectrum or single-wavelength data.

The sophistication, performance characteristics, and costs of fluorometers and spectrofluorometers differ as widely as do those of the corresponding instruments for absorption measurements. If only filters are used for wavelength selection, the instrument is called a *fluorometer*. True *spectrofluorometers* use two monochromators for wavelength isolation. Some instruments are hybrids in that they use a filter for selecting the excitation wavelength and a monochromator for choosing the emission wavelength. These are, often, still called spectrofluorometers. Several commercial spectrophotometers can be purchased with adapters that permit their use as spectrofluorometers.

True spectrofluorometers allow production of a fluorescence excitation spectrum or a fluorescence emission spectrum. Figure 15-9a shows an excitation spectrum for anthracene in which the fluorescence emission was measured at a fixed wavelength while the excitation wavelength was scanned. With suitable corrections for variations in source output intensity and detector response as a function of wavelength, an absolute excitation spectrum is obtained that closely resembles an absorption spectrum.

Figure 15-9b is the fluorescence emission spectrum for anthracene. The spectrum was obtained by holding the excitation wavelength constant while the emission wavelengths were scanned. These two spectra are approximately mirror images of one another because the vibrational energy differences for the ground and excited electronic states are roughly the same (see Figure 15-2).

The selectivity of spectrofluorometers is important in electronic and structural characterization of molecules and is valuable in both qualitative and quantitative analytical work. For concentration measurements, however, relatively inexpensive fluorometers often suffice. These are usually designed specifically to solve the measurement problems peculiar to fluorescence methods and are frequently as specific and selective as modified absorption spectrophotometers.

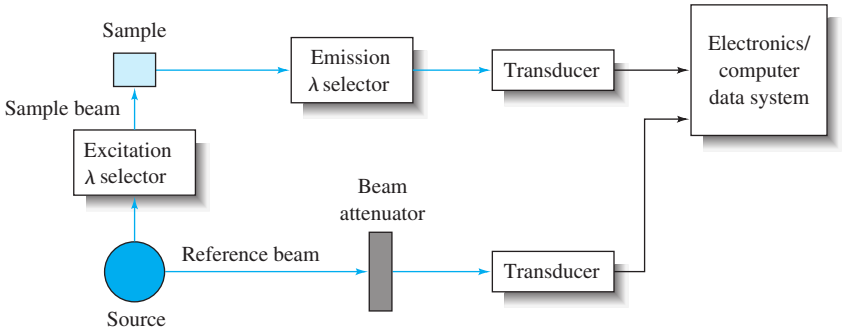


FIGURE 15-8 Components of a fluorometer or spectrofluorometer. Source radiation is split into two beams. The sample beam passes through the excitation wavelength selector to the sample. The emitted fluorescence is isolated by the emission wavelength selector before striking the transducer. The reference beam is attenuated before striking the transducer. The electronics and computer system compute the ratio of the fluorescence intensity to the reference beam intensity, which cancels the effect of source intensity fluctuations.

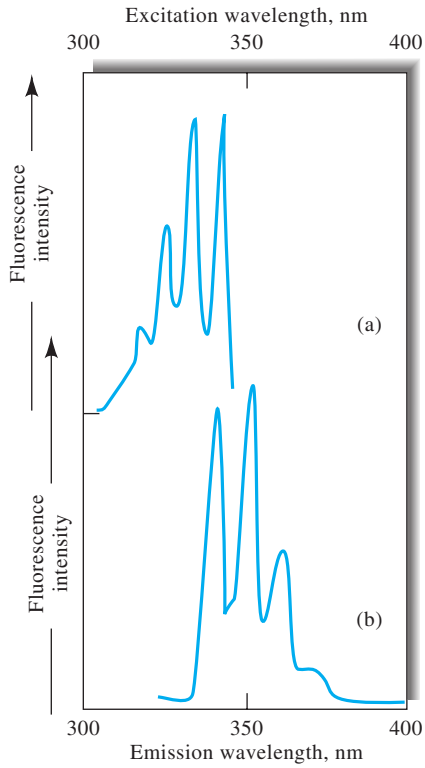


FIGURE 15-9 Fluorescence spectra for 1 ppm anthracene in alcohol: (a) excitation spectrum and (b) emission spectrum.

15B-1 Components of Fluorometers and Spectrofluorometers

The components of fluorometers and spectrofluorometers are quite similar to those we have discussed for absorption photometers and spectrophotometers. However, several differences are discussed here.[2]

Sources

As shown by Equation 15-6, the magnitude of the output signal in luminescence measurements, and thus the sensitivity, is directly proportional to the source radiant power P_0. For this reason, more intense sources are used in luminescence methods than the tungsten or deuterium lamps used in absorption measurements.

Lamps. The most common source for filter fluorometers is a low-pressure mercury vapor lamp equipped with a fused silica window. This source produces useful lines for exciting fluorescence at 254, 302, 313, 546, 578, 691, and 773 nm. Individual lines can be isolated with suitable absorption or interference filters. Because fluorescence can be induced in most fluorescing compounds by a variety of wavelengths, at least one of the mercury lines ordinarily proves suitable.

For spectrofluorometers, where a source of continuum radiation is required, a 75- to 450-W high-pressure xenon arc lamp is commonly used. Such lamps require a power supply capable of producing direct currents of 5 to 20 A at 15 to 30 V. The spectrum from a xenon arc lamp is a continuum from about 300 to 1300 nm. The spectrum approximates that of a blackbody (see Figure 6-22). In some instruments, a capacitor is discharged through the lamp at a constant frequency to provide flashes that are regularly spaced in time; higher peak intensities are obtained by pulsing. In addition, the outputs of the transducers are then ac signals that can be readily amplified and processed.

Blue light-emitting diodes (LEDs) have also been used in fluorescence instruments. These lamps emit radiation at 450–475 nm and are suitable for exciting some fluorophores. Mixtures of phosphors in some LEDs can provide wavelengths in the UV region to about 375 nm (see Section 13D-1).

Lasers. Since the 1970s, various types of lasers have also been used as excitation sources for photoluminescence measurements. Of particular interest are tunable dye lasers pumped by a pulsed nitrogen laser or a Nd-YAG laser. Fixed-wavelength lasers are also used, particularly in detectors for chromatography and electrophoresis.

Most commercial spectrofluorometers use lamp sources because they are less expensive and more applicable to determining multiple analytes with different excitation wavelengths. Laser sources, however, offer significant advantages in certain instances: for example, (1) when samples are very small, as in microbore chromatography and capillary electrophoresis where the amount of sample is a microliter or less; (2) in remote sensing, as in fluorometric detection of hydroxyl radicals in the atmosphere or of chlorophyll in bodies of water, where the collimated nature of laser beams is vital; or (3) when highly monochromatic excitation is needed to minimize the effects of fluorescing interferences.

Filters and Monochromators

Interference and absorption filters have been used in fluorometers for wavelength selection of both the excitation beam and the resulting fluorescence radiation. Spectrofluorometers are equipped with at least one and often two grating monochromators.

Transducers

Luminescence emission signals are typically quite low in intensity. Therefore, sensitive transducers are required. Photomultiplier tubes are the most common transducers in sensitive fluorescence instruments. Often, these are operated in the photon-counting mode to give improved signal-to-noise ratios (see page 184). Transducers are sometimes cooled to

[2]For a review of commercial fluorescence instruments, see J. Kling, *Anal. Chem.*, **2000**, *72*, 219A, **DOI**: 10.1021/ac002755u.

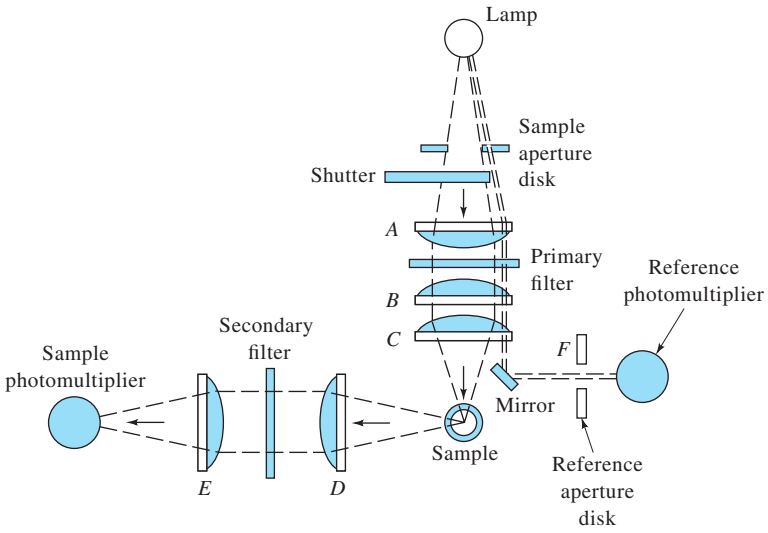


FIGURE 15-10 A typical fluorometer. (Courtesy of Farrand Optical Components and Instruments Division of Ruhle Companies, Inc.)

improve signal-to-noise ratios. Charge-transfer devices, such as charge-coupled devices (CCDs), are also used for spectrofluorometry.[3] This type of transducer permits the rapid recording of both excitation and emission spectra and is particularly useful in chromatography and electrophoresis.

Cells and Cell Compartments

Both cylindrical and rectangular cells fabricated of glass or silica are used for fluorescence measurements. Care must be taken in the design of the cell compartment to reduce the amount of scattered radiation reaching the detector. Baffles are often introduced into the compartment for this purpose. Even more than in absorbance measurements, it is important to avoid fingerprints on cells because skin oils often fluoresce.

Low-volume micro cells are available for situations in which sample volumes are limited. Several companies make flow cells for fluorescence detection in chromatography and in continuous flow analysis. Sample-handling accessories include micro-plate readers, microscope attachments, and fiber-optic probes. Low-volume cells are often used for room-temperature phosphorescence and for chemiluminescence. Special cells and sample handling are needed for low-temperature phosphorescence measurements.

Although the right-angle geometry shown in Figure 15-8 is most common, two other cell configurations are used. Front-surface geometry allows measurements on solutions with high absorbances or on opaque solids. The 180°, or in-line, geometry is rarely used because the weak luminescence signal must be isolated from the intense excitation beam by the emission wavelength selector.

Data Manipulation

Modern computer-based luminescence instruments have many different data-manipulation schemes available in software. Common data-manipulation and display options include blank signal subtraction, production of corrected excitation and emission spectra, calculation and display of difference and derivative spectra, peak detection and processing, deconvolution, production of three-dimensional total luminescence plots, fitting of calibration data, calculation of statistical parameters, and smoothing of spectra by various methods. Specialized software is available for kinetics, for high-performance liquid chromatography (HPLC) detection, for analysis of mixtures, and for time-resolved measurements.

15B-2 Instrument Designs

Fluorometers

Filter fluorometers provide a relatively simple, low-cost way of performing quantitative fluorescence analyses. As noted earlier, either absorption or interference filters are used to limit the wavelengths of the excitation and emitted radiation. Generally, fluorometers are compact, rugged, and easy to use.

Figure 15-10 is a schematic of a typical filter fluorometer that uses a mercury lamp for fluorescence excitation and a pair of photomultiplier tubes as transducers. The source beam is

Tutorial: Learn more about **fluorescence instrumentation** at www.tinyurl.com/skoogpia7

[3]R. S. Pomeroy in *Charge Transfer Devices in Spectroscopy*, J. V. Sweedler, K. L. Ratzlaff, and M. B. Denton, eds., New York: VCH, 1994, pp. 281–314; P. M. Epperson, R. D. Jalkian, and M. B. Denton, *Anal. Chem.*, **1989**, *61*, 282, **DOI**: 10.1021/ac00178a020.

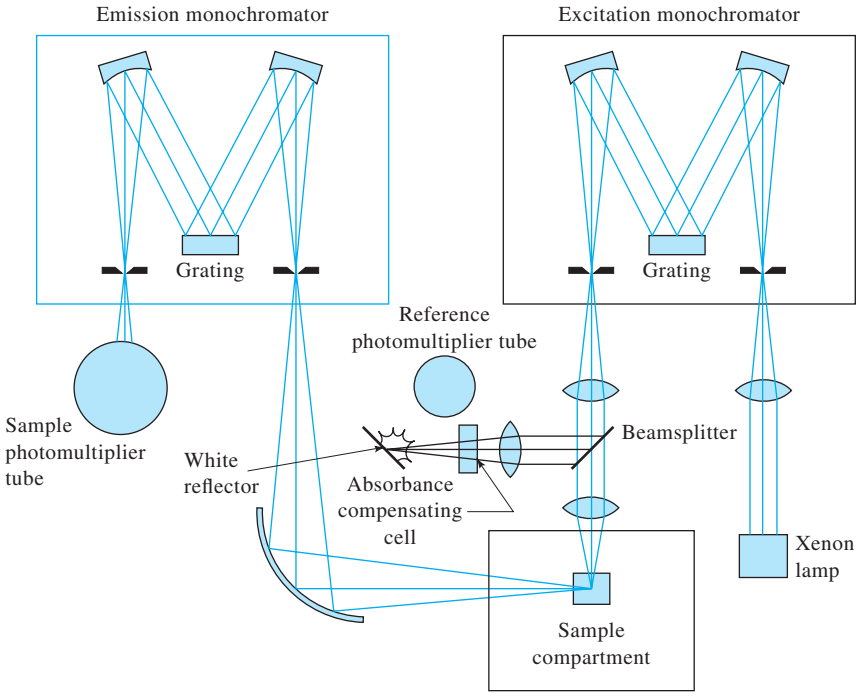


FIGURE 15-11 A spectrofluorometer. (Courtesy of Horiba Jobin Yvon, Edison, NJ.)

split near the source into a reference beam and a sample beam. The reference beam is attenuated by the aperture disk so that its intensity is roughly the same as the fluorescence intensity. Both beams pass through the primary filter, with the reference beam then being reflected to the reference photomultiplier tube. The sample beam is focused on the sample by a pair of lenses and causes fluorescence emission. The emitted radiation passes through a second filter and then is focused on the second photomultiplier tube. The electrical outputs from the two transducers are then processed to compute the ratio of the sample to reference intensities, which serves as the analytical variable.

The instrument just described is representative of the dozen or more fluorometers available commercially. Some of these are simpler single-beam instruments. Some are portable instruments for fieldwork. The cost of such fluorometers ranges from several hundred dollars to more than $10,000.

Spectrofluorometers

Several instrument manufacturers offer spectrofluorometers capable of providing both excitation and emission spectra. The optical design of one of these, which uses two grating monochromators, is shown in Figure 15-11. Radiation from the excitation monochromator is split, part passing to a reference photomultiplier and part to the sample. The resulting fluorescence radiation, after dispersion by the emission monochromator, is detected by a second photomultiplier.

An instrument such as that shown in Figure 15-11 provides perfectly satisfactory spectra for quantitative analysis. The emission spectra obtained will not, however, necessarily compare well with spectra from other instruments because the output depends not only on the intensity of fluorescence but also on the characteristics of the lamp, transducer, and monochromators. All of these instrument characteristics vary with wavelength and differ from instrument to instrument. A number of methods have been developed for obtaining a corrected spectrum, which is the true fluorescence spectrum freed from instrumental effects (see next section).

Spectrofluorometers Based on Array Detectors

Several spectrofluorometers based on diode-array and charge-transfer devices have been described that permit fluorescence spectra to be obtained in fractions of a second.[4] Commercial instruments are now available with CCD detectors.[5]

One of the unique uses of an array-detector spectrofluorometer is the production of total luminescence spectra. Such a spectrum is a plot of the emission spectrum at every excitation wavelength usually presented as a three-dimensional plot. Total luminescence spectra can be obtained in a conventional manner with a standard photomultiplier transducer, although collection of the spectra involved is quite time-consuming. The emission spectrum is obtained at one excitation wavelength, and then,

[4]See, for example, R. S. Pomeroy in *Charge Transfer Devices in Spectroscopy*, J. V. Sweedler, K. L. Ratzlaff, and M. B. Denton, eds., New York: VCH, 1994, pp. 281–314; P. M. Epperson, R. D. Jalkian, and M. B. Denton, *Anal. Chem.*, **1989**, *61*, 282, **DOI**: 10.1021/ac00178a020.

[5]For example, see instruments made by Ocean Optics, Inc., Dunedin, Florida, and Horiba Jobin Yvon, Edison, New Jersey.

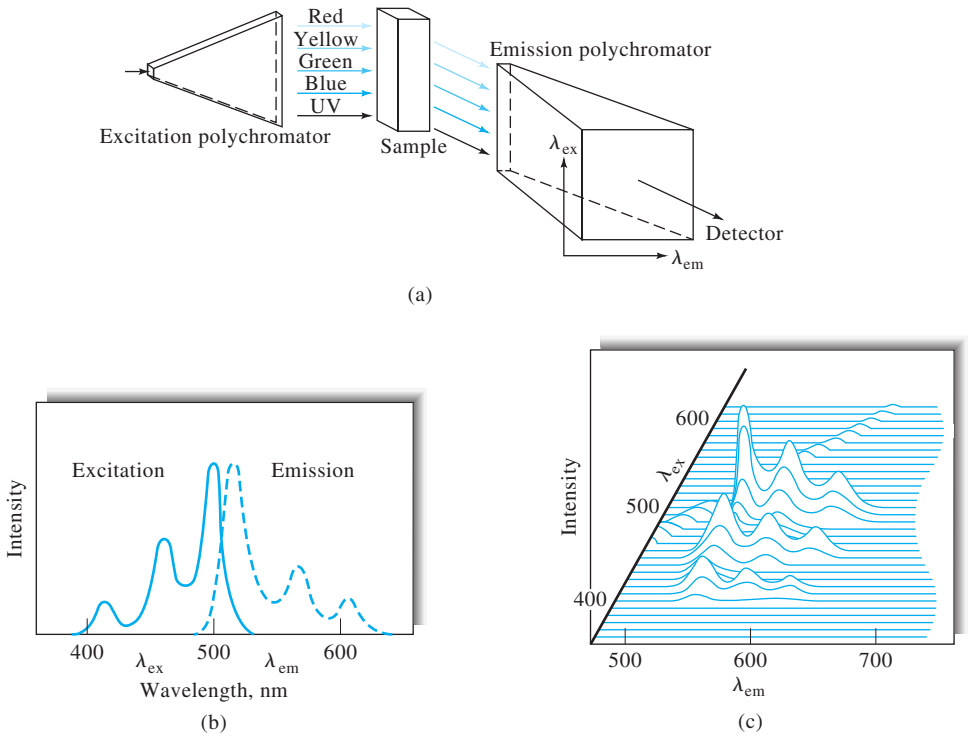


FIGURE 15-12 Three-dimensional spectrofluorometer. (a) Schematic of an optical system for obtaining total luminescence spectra with a CCD detector. (With permission from G. W. Suter, A. J. Kallir, and U. P. Wild, *Chimia*, **1983**, *37*, 413.) (b) Excitation and emission spectra of a hypothetical compound. (c) Total luminescence spectrum of compound in (b). (With permission of D. W. Johnson, J. P. Callis, and G. C. Christian, *Anal. Chem.*, **1977**, *49*, 747A, **DOI**: 10.1021/ac50016a769. Figure 3, p. 749A. Copyright 1977 American Chemical Society.)

the excitation monochromator is moved to another wavelength and the emission spectrum scanned again. The process is then repeated until sufficient range is achieved in the excitation. A computer stores the various spectra and presents the total three-dimensional luminescence display.

The principle of a sophisticated array-detector-based instrument to produce total luminescence spectra is illustrated in Figure 15-12a. Here, the length of a sample cell is irradiated with an excitation beam that has been dispersed along the *xy* plane by a monochromator that has been rotated 90° with respect to its exit slit. The transducer is a two-dimensional CCD that sees the dispersed excitation radiation in the *xy* plane and the dispersed radiation from the emission monochromator in the *yz* plane. Figure 15-12b shows traditional excitation and emission spectra for a hypothetical molecular species. Figure 15-12c shows the total luminescence spectrum for this compound, which is an isometric projection, sometimes called a *stack plot*, of the complete excitation and emission spectra of the compound obtained with the arrangement shown in Figure 15-12a. Total spectra of this type can be obtained in a few seconds or less and are of particular use for analyzing mixtures of fluorescing species. A commercial instrument of this type is available.[6]

[6]Horiba Jobin Yvon, Edison, New Jersey.

Fiber-Optic Fluorescence Sensors

With fiber-optic probes, fluorescence determinations can be carried out at various locations well away from a source and a detector. With such probes, radiation from a laser source travels through an optical fiber and excites fluorescence in sample solutions. Fluorescence emission then travels back through the same fiber to a detector for measurement. The applicability of this type of device has been extended to nonfluorescing analytes by fixing a fluorescing indicator material to the end of the fiber.[7] A discussion of the properties of fiber optics and applications to chemical instrumentation is found in Section 7G.

Many fluorescence sensors are based, not on direct fluorescence, but on the quenching of fluorescence.[8] Molecular oxygen, for example, is one of the best collisional quenchers. Oxygen can quench the fluorescence from polycyclic aromatic hydrocarbons; complexes of ruthenium, osmium, iridium, and platinum; and a number of surface-adsorbed heterocyclic molecules. An oxygen sensor can be made by immobilizing the fluorophore in a thin

[7]For a discussion of fiber-optic fluorescence sensors, see O. S. Wolfbeis, in *Molecular Luminescence Spectroscopy*, S. G. Schulman, ed., Part 2, Chap. 3, New York: Wiley, 1988.

[8]See W. Trettnak, in *Fluorescence Spectroscopy: New Methods and Applications*, O. S. Wolfbeis, ed., New York: Springer-Verlag, 1993, Chap. 7.

layer of silicone on the end of a fiber-optic bundle.[9] Sensors for SO_2, halides, H_2O_2, and several other molecules have been based on fluorescence quenching.

Phosphorimeters

Instruments used for studying phosphorescence are similar in design to the fluorometers and spectrofluorometers just considered except that two additional components are required.[10] The first is a device that alternately irradiates the sample and, after a suitable time delay, measures the intensity of phosphorescence. The time delay is required to differentiate between long-lived phosphorescence emission and short-lived fluorescence emission, both of which would originate from the same sample. Both mechanical and electronic devices are used, and many commercial fluorescence instruments have accessories for phosphorescence measurements. Many of the current instruments use a gated scheme for the delay. A pulsed xenon arc lamp is often used to excite the sample. After a delay time, specified by the user, the data-acquisition system is activated to obtain the phosphorescence signal. Often, the signal is integrated during this period when the lamp is off and fluorescence has decayed to a very small value.

A second new component is needed because phosphorescence measurements are usually performed at liquid nitrogen temperature in a rigid medium to minimize collisional deactivation of the long-lived triplet state. Usually, a Dewar flask with quartz windows, as shown in Figure 15-13, is a part of a phosphorimeter. At the temperature used, the analyte exists as a solute in a glass or solid solvent. A common solvent for this purpose is a mixture of diethylether, pentane, and ethanol.

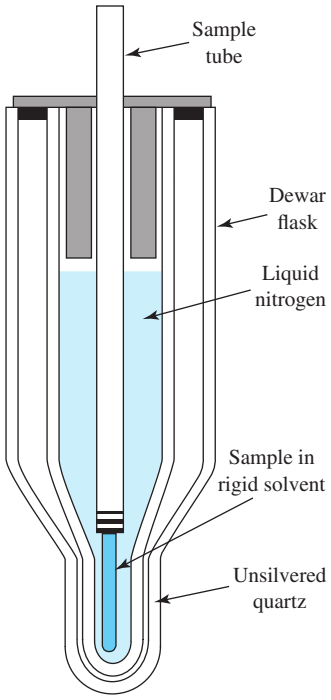


FIGURE 15-13 Dewar flask and cell for low-temperature phosphorescence measurements. The optical path traverses the unsilvered part of the flask.

15B-3 Correction and Compensation Schemes

Several different schemes are used to correct luminescence spectra for some of the many variables that influence them. Source stability, source spectral distribution, inner filter effects, efficiencies of optical components, and spectral responses of instrument components are among the variables that can influence luminescence intensities and spectra. Many instruments have provision to compensate or correct for some of these effects.

Source Compensation

The luminescence instruments shown in Figures 15-10 and 15-11 both monitor the source intensity via a reference photomultiplier. Most commonly, the ratio of the sample luminescence signal to the signal from the reference detector is continuously obtained. This can compensate for source intensity fluctuations and drift. Both double-beam-in-space and double-beam-in-time designs are used.

Corrected Excitation Spectra

Source compensation does not correct for the wavelength dependence of the source or the efficiencies of the optical components and excitation wavelength selector. Correction for these effects can be done in several ways. In some commercial instruments, a *quantum counter* has been used. The quantum counter is a reference cell filled with a concentrated fluorophore of high quantum efficiency, such as Rhodamine B. A 3- to 8-g/L solution in glycerol essentially absorbs all incident light from 220 to 600 nm. The quantum efficiency is virtually constant over this excitation range. The excitation beam is split after the wavelength selector, and a fraction excites the reference material. The same emission spectral distribution results from all excitation wavelengths so that the photomultiplier spectral response is not of concern. The resulting reference signal is directly proportional to the source radiant power striking the sample.

Modern computerized spectrofluorometers often achieve a similar correction by storing the reference spectrum of the source in computer memory. After the sample spectrum is scanned, the correction is made by calculating the ratio of the sample spectrum to the reference spectrum.

An example of the differences between corrected and uncorrected spectra is given in Figure 15-14. An appropriately

[9] J. N. Demas, B. A. DeGraff, and P. B. Coleman, *Anal. Chem.*, **1999**, *71*, 793A, **DOI**: 10.1021/ac9908546.

[10] See R. J. Hurtubise, *Anal. Chem.*, **1983**, *55*, 669A, **DOI**: 10.1021/ac00257a783; R. J. Hurtubise, *Phosphorimetry: Theory, Instrumentation, and Applications*, Chap. 3, New York: VCH, 1990.

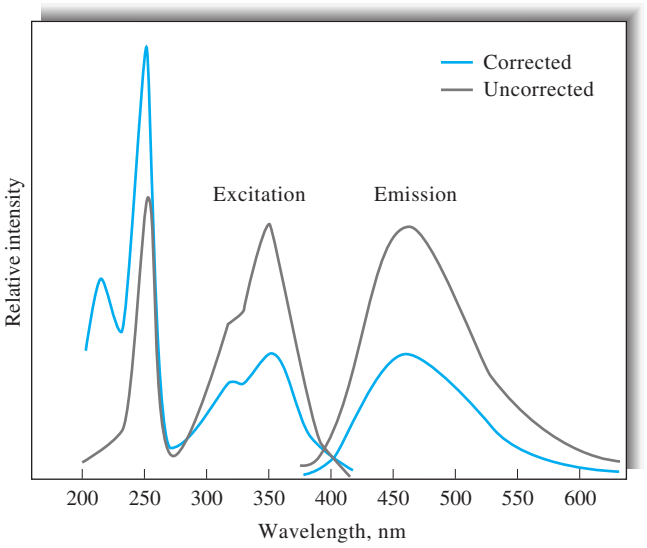


FIGURE 15-14 Corrected and uncorrected spectra for quinine sulfate in 0.2 M H_2SO_4. Note that the corrected excitation spectrum shows a higher intensity in the short-wavelength (~250 nm) region than in the longer-wavelength region (~350 nm) because the molar absorptivity of the 250-nm band is larger. The uncorrected excitation spectrum shows a much lower intensity in the 250-nm region because the source radiant power is lower. The 350-nm band is of higher intensity in the uncorrected spectrum because of a high source radiant power. (From *NIST Tech. Note* 584, p. 55, December 1971.)

corrected excitation spectrum should be identical to the absorption spectrum of the same compound.

Corrected Emission Spectra

Emission spectra collected on different instruments will vary because of different wavelength dependencies of the wavelength selectors and transducers. The shape of a properly corrected emission spectrum reflects only how the luminescence efficiency varies with excitation wavelength, as shown in Figure 15-14. To correct the emission spectrum, a calibrated light source is used, and calibration factors for the emission monochromator and transducer are determined. These are stored in computer memory. The uncorrected emission results are then multiplied by appropriate correction factors to give the corrected spectrum.

15B-4 Instrument Standardization

Because of variations in source intensity, transducer sensitivity, and other instrumental variables, it is nearly impossible to obtain with a given fluorometer or spectrophotometer exactly the same reading for a solution or a set of solutions from day to day. For this reason it is common practice to standardize an instrument and set it to a reproducible sensitivity level. Standardization is often carried out with a standard solution of a stable fluorophore. The most common reagent for this purpose is a standard solution of quinine sulfate having a concentration of about 10^{-5} M. It is generally excited by radiation at 350 nm and emits radiation of 450 nm (see Figure 15-14). Other compounds have been described for other wavelength regions.

Several instrument manufacturers offer a set of fluorescence standards. In some cases the standards are dissolved in a plastic matrix to give stable solid blocks that can be used indefinitely without special storage. With these, the instrument is easily standardized for the wavelength region to be used for the analysis.

Comparing fluorescence spectra among different instruments should be done only with corrected spectra, as discussed in Section 15B-3.

15C APPLICATIONS OF PHOTOLUMINESCENCE METHODS

Fluorescence and phosphorescence methods have inherently lower limits of detection (LODs) than absorption-based spectrophotometric measurements. They are among the most sensitive analytical techniques available to the scientist. The enhanced sensitivity arises from the concentration-related parameter for fluorometry and phosphorimetry F being directly proportional to the source radiant power P_0. The luminescence intensity can be measured independently of P_0. In contrast, an absorbance measurement requires evaluation of both P_0 and P, because absorbance, which is proportional to concentration, depends on the ratio of these two quantities.

The sensitivity of a fluorometric method can be improved by increasing P_0 or by further amplifying the fluorescence signal. In actuality, the luminescence signal is not zero, however, when the analyte concentration is zero. Background luminescence and signals from scattering and other sources determine the ultimate LODs that can be achieved. In spectrophotometry, in contrast, an increase in P_0 results in a proportionate change in P and therefore fails to affect A. Thus, fluorometric methods generally have detection limits one to three orders of magnitude better than the corresponding spectrophotometric procedures. On the other hand, the precision and accuracy of photoluminescence methods is usually poorer than spectrophotometric procedures by a factor of perhaps 2 to 5. The precision of photoluminescence methods is often limited by source flicker noise and drift. The accuracy is often limited by concomitants, or particles, in the sample that cause additional fluorescence and scattering or that quench the analyte fluorescence. Generally, phosphorescence methods are less precise than their fluorescence counterparts.

Luminescence methods usually have wider linear dynamic ranges than the corresponding absorption methods. Calibration curves are often linear from slightly above the detection limit to the point where absorption, and thus the primary inner-filter effect, becomes significant. These methods also show increased selectivity over absorption methods because not as many

molecules show significant luminescence as absorb radiation, and both the excitation and the emission wavelengths can be varied. On the other hand, contamination from reagents, laboratory glassware, and other interferents is more significant for luminescence methods.

15C-1 Fluorometric Determination of Inorganic Species

Inorganic fluorometric methods are of two types.[11] Direct methods involve the formation of a fluorescing chelate and the measurement of its emission. A second group of methods is based on the decrease in fluorescence emission because of the quenching action of the substance being determined. The latter technique has been most widely used for anion analysis.

Cations Forming Fluorescing Chelates

Two factors greatly limit the number of transition-metal ions that form fluorescing chelates. First, many of these ions are paramagnetic, which increases the rate of intersystem crossing to the triplet state. Deactivation by fluorescence is thus unlikely, although phosphorescence may be observed. A second factor is that transition-metal complexes are characterized by many closely spaced energy levels, which enhance the likelihood of deactivation by internal conversion. The principal inorganic applications of fluorimetry are thus to nontransition-metal ions, which are less susceptible to these deactivation processes. Note that such cations are generally colorless and tend to form colorless chelates. Thus, fluorometry often complements spectrophotometry.

Fluorometric Reagents

The most successful fluorometric reagents for cation analyses have aromatic structures with two or more donor functional groups that permit chelate formation with the metal ion.[12] The structures of four common reagents are shown in Figure 15-15.

Selected fluorometric reagents and their applications are presented in Table 15-2. As can be seen, LODs are quite low by most of these methods.

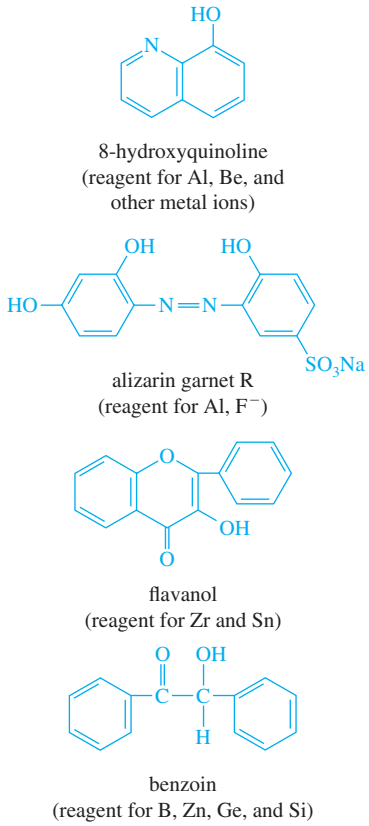


FIGURE 15-15 Some fluorometric chelating agents for metal cations. Alizarin garnet R can detect Al^{3+} at levels as low as 0.007 μg/mL. Detection of F^- with alizarin garnet R is based on fluorescence quenching of the Al^{3+} complex. Flavanol can detect Sn^{4+} at the 0.1-μg/mL level.

15C-2 Methods for Organic and Biochemical Species

The number of applications of fluorometric methods to organic chemistry is impressive. Dean has summarized the most important of these in a table.[13] The heading *Fluorescence Spectroscopy of Some Organic Compounds* has more than 200 entries, including such diverse compounds as adenine, anthranilic acid, aromatic polycyclic hydrocarbons, cysteine, guanine, isoniazid, naphthols, nerve gases sarin and tabun, proteins, salicylic acid, skatole, tryptophan, uric acid, and warfarin (coumadin). Many medicinal agents that can be determined fluorometrically are listed, including adrenaline, morphine, penicillin, phenobarbital, procaine, reserpine, and lysergic acid diethylamide (LSD). Without question, the most important application of fluorometry is in the analysis of food products, pharmaceuticals, clinical samples, and natural products. The sensitivity and selectivity of the method make it a particularly valuable tool in these fields. Numerous physiologically important compounds fluoresce.

15C-3 Phosphorimetric Methods

Phosphorescence and fluorescence methods tend to be complementary because strongly fluorescing compounds exhibit weak phosphorescence and vice versa.[14] For example, among

[11]For a review of fluorometric determination of inorganic species, see A. Fernandez-Gutierrez and A. M. De La Pena, in *Molecular Luminescence Spectroscopy*, S. G. Schulman, ed., Part 1, Chap. 4, New York: Wiley, 1985.

[12]For a more detailed discussion of fluorometric reagents, see pp. 384–426 of *Molecular Luminescence Spectroscopy* (note 11), and G. Guilbault, in *Comprehensive Analytical Chemistry*, G. Svehla, ed., Vol. VIII, Chap. 2, pp. 167–178, New York: Elsevier, 1977.

[13]J. A. Dean, *Analytical Chemistry Handbook*, pp. 5.63–5.69, New York: McGraw-Hill, 1995.

[14]See R. J. Hurtubise, *Phosphorimetry: Theory, Instrumentation, and Applications*, Chap. 3, New York: VCH, 1990.

TABLE 15-2 Selected Fluorometric Methods for Inorganic Species

Ion	Reagent	Wavelength, nm		LOD, μg/mL	Interferences
		Absorption	Fluorescence		
Al^{3+}	Alizarin garnet R	470	500	0.007	Be, Co, Cr, Cu, F^-, NO_3^-, Ni, PO_4^{3-}, Th, Zr
F^-	Quenching of Al^{3+} complex of alizarin garnet R	470	500	0.001	Be, Co, Cr, Cu, Fe, Ni, PO_4^{3-}, Th, Zr
$B_4O_7^{2-}$	Benzoin	370	450	0.04	Be, Sb
Cd^{2+}	2-(*o*-Hydroxyphenyl) benzoxazole	365	Blue	2	NH_3
Li^+	8-Hydroxyquinoline	370	580	0.2	Mg
Sn^{4+}	Flavanol	400	470	0.1	F^-, PO_4^{3-}, Zr
Zn^{2+}	Benzoin	—	Green	10	B, Be, Sb, colored ions

From J. A. Dean, *Analytical Chemistry Handbook*, New York: McGraw-Hill, 1995, pp. 5.60–5.62.

condensed-ring aromatic hydrocarbons, those containing heavier atoms such as halogens or sulfur often phosphoresce strongly. However, the same compounds in the absence of the heavy atom tend to exhibit fluorescence rather than phosphorescence.

Phosphorimetry has been used for determination of a variety of organic and biochemical species, including such substances as nucleic acids, amino acids, pyrine and pyrimidine, enzymes, petroleum hydrocarbons, and pesticides. The method has not, however, found as widespread use as fluorometry, perhaps because of the need for low temperatures and the generally poorer precision of phosphorescence measurements. On the other hand, the potentially greater selectivity of phosphorescence procedures is attractive. The reason for this difference in behavior is that efficient phosphorescence requires rapid intersystem crossing to populate the excited triplet state, which in turn reduces the excited singlet concentration and thus the fluorescence intensity.

During the past two decades, considerable effort has been expended in the development of phosphorimetric methods that can be carried out at room temperature.[15] The first observations of room-temperature phosphorescence were made with the analyte bound to a solid support, such as filter paper or silica gel. In these applications, a solution of the analyte is dispersed on the solid, and the solvent is evaporated. The phosphorescence of the surface is then measured. The rigid matrix minimizes deactivation of the triplet state by collisional quenching. Collisional quenching has much more of an effect on phosphorescence than on fluorescence because of the much longer lifetime of the triplet state.

[15]T. Vo-Dinh, *Room Temperature Phosphorimetry for Chemical Analysis*, New York: Wiley, 1984.

Room-temperature phosphorescence in solution has been observed in organized media containing micelles. With micelles, the analyte is incorporated into the core of the micelle, which serves to protect the triplet state. Cyclodextrin molecules, which are doughnut-shape polymers, have also been used. In most room-temperature experiments, heavy atoms, such as Tl(I), Pb(II), Ag(I), and halide ions, are used to promote intersystem crossing.

15C-4 Fluorescence Detection in Liquid Chromatography

Photoluminescence measurements provide an important method for detecting and determining components of a sample as they elute from a chromatographic or capillary electrophoresis column. Laser-excited fluorescence is particularly important for these applications because the beam can be readily focused to a size on the order of the column diameter. Applications in liquid chromatography and capillary electrophoresis are discussed in more detail in Chapters 28 and 30.

15C-5 Lifetime Measurements

Fluorescence lifetime measurements can give information about collisional deactivation processes, about energy transfer rates, and about excited-state reactions. Lifetime measurements can also be used analytically to provide additional selectivity in the analysis of mixtures containing luminescent species. The measurement of luminescence lifetimes was initially restricted to phosphorescent systems, where decay times were long enough to permit the easy measurement of emitted intensity as a function of time. In recent years, however, it has become relatively routine to measure rates of luminescence decay on the fluorescence time scale (10^{-5} to $<10^{-9}$ s).

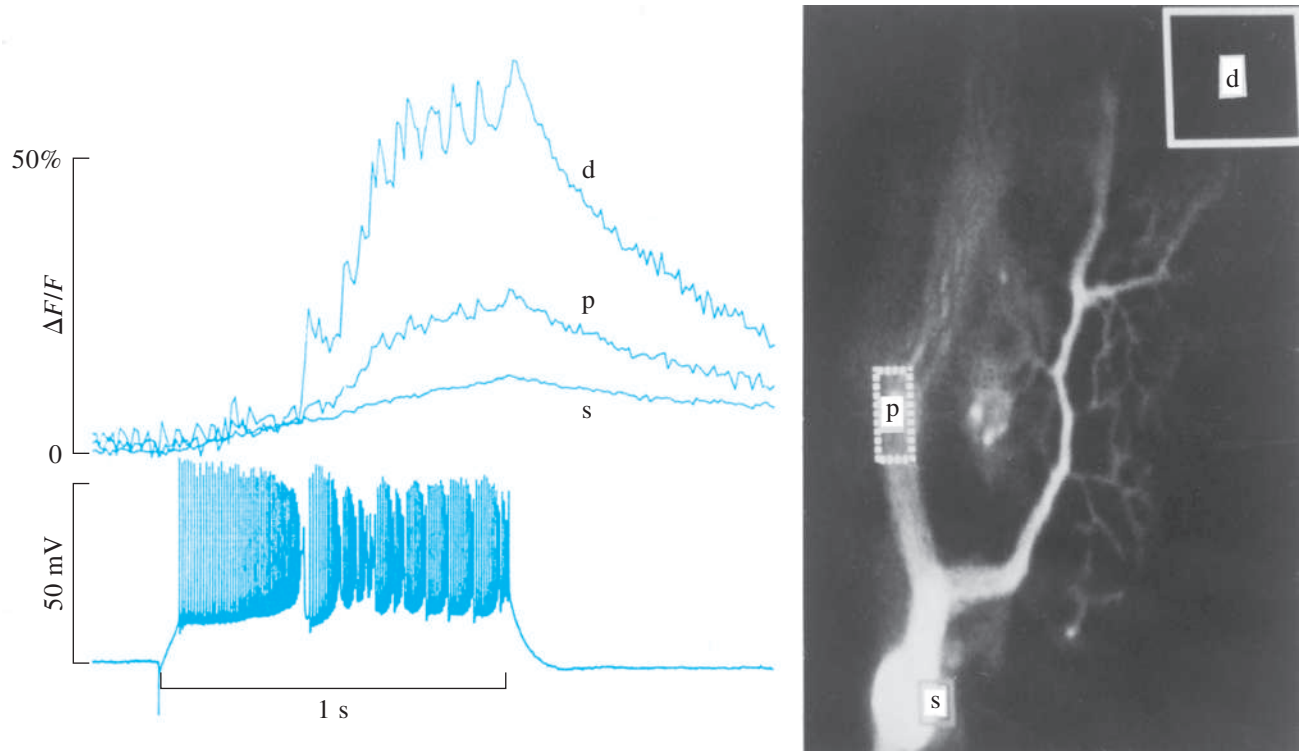


FIGURE 15-16 Calcium transients in a cerebellar Purkinje cell. The image on the right is of the cell filled with a fluorescent dye that responds to the calcium concentration. Fluorescent transients are shown on the top left recorded at areas d, p, and s in the cell. The transients in region d correspond to the dendrite region of the cell. Specific calcium signals can be correlated to the action potentials shown on the bottom left. (From V. Lev-Ram, H. Mikayawa, N. Lasser-Ross, W. N. Ross. Calcium Transients in Cerebellar Purkinje Neurons Evoked by Intracellular Stimulation, *J. Neurophysiol*, **1992**, *68*, 1170–1177, figure 2A.)

Two widely used approaches are used for lifetime measurements, the *time-domain* approach and the *frequency-domain* approach. In time-domain measurements, a pulsed source is used and the time-dependent decay of fluorescence is measured. In the frequency-domain method, a sinusoidally modulated source is used to excite the sample. The phase shift and demodulation of the fluorescence emission relative to the excitation waveform provide the lifetime information. Commercial instrumentation is available to implement both techniques.[16]

15C-6 Fluorescence Imaging Methods

In recent years, it has become possible to combine fluorescence spectroscopy with optical microscopy to produce localized images of fluorophores in complex matrices such as single cells.[17] In some cases the intrinsic (native) fluorescence of biomolecules can be used in conjunction with microscopy to monitor dynamics in cells.[18] In the absence of a native fluorophore, fluorescent indicators can be used to probe biological events. A particularly interesting probe is the so-called ion probe that changes its excitation or emission spectrum on binding to specific ions such as Ca^{2+} or Na^{+}. These indicators can be used to record events that take place in different parts of individual neurons or to monitor simultaneously the activity of a collection of neurons.

In neurobiology, for example, the dye Fura-2 has been used to monitor the free intracellular calcium concentration following pharmacological or electrical stimulation. By following the fluorescence changes as a function of time at specific sites in the neuron, researchers can determine when and where a calcium-dependent electrical event took place. One cell that has been studied is the Purkinje neuron in the cerebellum, which is one of the largest in the central nervous system. When this cell is loaded with the Fura-2 fluorescent indicator, sharp changes in fluorescence can be measured that correspond to individual calcium action potentials. The changes are correlated to specific sites in the cell by means of fluorescence imaging techniques. Figure 15-16 shows the fluorescence image on the right along

[16]For references dealing with lifetime measurements, see F. V. Bright and C. A. Munson, *Anal. Chim. Acta*, **2003**, *500*, 71, **DOI**: 10.1016/S0003-2670(03)00723-2; J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 2nd ed., Chaps. 4 and 5, New York: Kluwer Academic Publishers/Plenum Press, 1999.

[17]X. F. Wang, B. Herman, eds., *Fluorescence Imaging Spectroscopy and Microscopy*, New York: Wiley, 1996.

[18]See, for example, E. S. Yeung, *Anal. Chem*., **1999**, *71*, 522A, **DOI**: 10.1021/ac9906025.

with fluorescence transients, recorded as the change in fluorescence relative to the steady fluorescence $\Delta F/F$, correlated with sodium action potential spikes. The interpretation of these kinds of patterns can have important implications in understanding the details of synaptic activity. Commercial fluorescence microscopes and microscopy attachments are available from several sources.

Fluorescence microscopy and fluorescence lifetime methods have been combined in the technique known as *fluorescence lifetime imaging*. Here, molecular lifetimes can be used to create contrast in two-dimensional fluorescence images.[19]

15D CHEMILUMINESCENCE

The application of chemiluminescence to analytical chemistry is a relatively recent development. The number of chemical reactions that produce chemiluminescence is small, which limits the procedure to a relatively small number of species. Nevertheless, some of the compounds that do react to give chemiluminescence are important components of the environment. For these, the high selectivity, the simplicity, and the extreme sensitivity of the method account for its growth in usage.[20]

15D-1 The Chemiluminescence Phenomenon

Chemiluminescence is produced when a chemical reaction yields an electronically excited species that emits light as it returns to its ground state. Chemiluminescence reactions are encountered in a number of biological systems, where the process is often termed *bioluminescence*. Examples of species that exhibit bioluminescence include the firefly, the sea pansy, and certain jellyfish, bacteria, protozoa, and crustacea. The chemistry of the various natural bioluminescence processes is incompletely understood.

Over a century ago, it was discovered that several relatively simple organic compounds also are capable of exhibiting chemiluminescence. The simplest type of reaction of such compounds to produce chemiluminescence can be formulated as

$$A + B \rightarrow C^* + D$$

$$C^* \rightarrow C + h\nu$$

where C* represents the excited state of the species C. The luminescence spectrum is that of the reaction product C. Most chemiluminescence processes are considerably more complicated than is suggested by these simple reactions.

For chemiluminescence, the radiant intensity I_{CL} (photons emitted per second) depends on the rate of the chemical reaction ($d[C]/dt$) and the chemiluminescence quantum yield ϕ_{CL} (photons per molecule reacted). The latter term is equal to the product of the excitation quantum yield ϕ_{EX} (excited states per molecule reacted) and the emission quantum yield ϕ_{EM} (photons per excited state). These relationships are described by the equation

$$I_{CL} = \phi_{CL}\frac{d[C]}{dt} = \phi_{EX}\phi_{EM}\frac{d[C]}{dt} \qquad (15\text{-}13)$$

Chemiluminescence systems useful in analytical chemistry generally have values of ϕ_{CL} of 0.01 to 0.2.

15D-2 Measurement of Chemiluminescence

The instrumentation for chemiluminescence measurements is remarkably simple and may consist of only a suitable reaction vessel and a photomultiplier tube. Generally, no wavelength selection device is necessary because the only source of radiation is the chemical reaction between the analyte and reagent. Several instrument manufacturers offer chemiluminescence photometers.

The typical signal from a chemiluminescence experiment is a time-dependent signal that rises rapidly to a maximum as mixing of reagent and analyte completes. Then, a more or less exponential decay of signal follows (see Figure 15-17). Usually for quantitative analysis, the signal is integrated for a fixed time and compared with standards treated in an identical way. Alternatively, peak heights are used for quantitation. Often a linear relationship between signal and concentration is observed over a concentration range of several orders of magnitude.

15D-3 Analytical Applications of Chemiluminescence

Chemiluminescence methods[21] are generally highly sensitive because low light levels are readily monitored in the absence of noise. Furthermore, radiation attenuation by a filter or a monochromator is usually unnecessary. In fact, detection limits are usually determined not by transducer sensitivity but rather by reagent purity. Typical detection limits lie in the parts-per-billion (or sometimes less) to parts-per-million range. The

[19]See, for example, J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 2nd ed., New York: Kluwer Academic Publishing/Plenum Press, 1999; B. Herman, *Fluorescence Microscopy*, 2nd ed., New York: Springer-Verlag, 1998.

[20]For some reviews of chemiluminescence and its analytical applications, see S. Das et al., *Anal. Chem.*, **2012**, *84*, 597, **DOI**: 10.1021/ac202904n and previous reviews in *Analytical Chemistry*; T. A. Nieman, *in Handbook of Instrumental Techniques for Analytical Chemistry*, F. A. Settle, ed., Chap. 27, Upper Saddle River, NJ: Prentice-Hall, 1997.

[21]For some recent applications, see F. J. Lara, A. M. Garcia-Campaña, and J. J. Aaron, *Anal. Chim. Acta*, **2010**, *679*, 17, **DOI**: 10.1016/j.aca.2010.09.001.

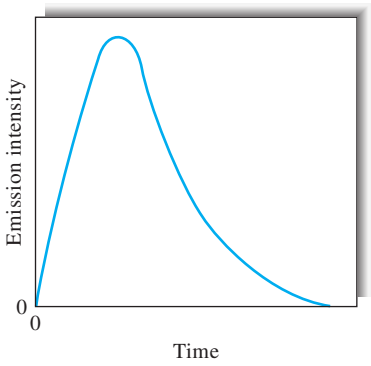


FIGURE 15-17 Chemiluminescence emission intensity as a function of time after mixing reagents.

precision of determinations varies depending on the instrumentation and care that is used.

Analysis of Gases

Chemiluminescence methods for determining components of gases originated with the need for highly sensitive methods for determining atmospheric pollutants such as ozone, oxides of nitrogen, and sulfur compounds. One of the most widely used of these methods is for the determination of nitrogen monoxide with the reactions

$$NO + O_3 \rightarrow NO_2^* + O_2$$

$$NO_2^* \rightarrow NO_2 + h\nu \ (\lambda = 600 \text{ to } 2800 \text{ nm})$$

Ozone from an electrogenerator and the atmospheric sample are drawn continuously into a reaction vessel, where the luminescence radiation is monitored by a photomultiplier tube. A linear response is reported for nitrogen monoxide concentrations of 1 ppb to 10,000 ppm. Chemiluminescence has become the predominant method for monitoring the concentration of this important atmospheric constituent from ground level to altitudes as high as 20 km.

The reaction of nitric oxide with ozone has also been applied to the determination of the higher oxides of nitrogen. For example, the nitrogen dioxide content of automobile exhaust gas has been determined by thermal decomposition of the gas at 700°C in a steel tube. The reaction is

$$NO_2 \rightleftharpoons NO + O$$

At least two manufacturers now offer an instrument for determination of nitrogen in solid or liquid materials containing 0.1% to 30% nitrogen. The samples are pyrolyzed in an oxygen atmosphere under conditions whereby the nitrogen is converted quantitatively to nitrogen monoxide. The NO concentration is then measured by the method just described.

Another important chemiluminescence method is used for monitoring atmospheric ozone. In this instance, the determination is based on the luminescence produced when the analyte reacts with the dye Rhodamine B adsorbed on an activated silica gel surface. This procedure is sensitive to less than 1 ppb ozone. The response is linear up to 400 ppb ozone. Ozone can also be determined in the gas phase based on the chemiluminescence produced when the analyte reacts with ethylene. Both reagents are reported to be specific for ozone.

Still another important gas-phase chemiluminescence method is used for the determination of atmospheric sulfur compounds such as hydrogen sulfide, sulfur dioxide, and mercaptans. In this technique, the sample is combusted in a hydrogen flame to produce a sulfur dimer, which then decomposes with the emission of light. For example, with sulfur dioxide the reactions are

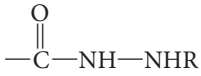
$$4H_2 + 2SO_2 \rightleftharpoons S_2^* + 4H_2O$$

$$S_2^* \longrightarrow S_2 + h\nu$$

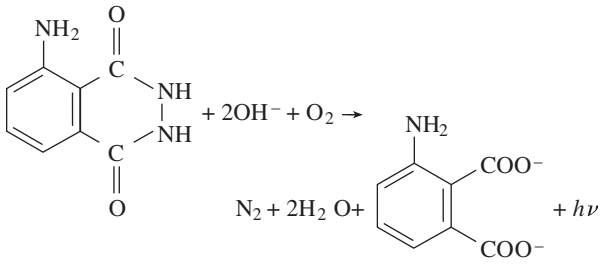
The emission occurs in the blue with maxima at 384 and 394 nm. The chemiluminescent intensity is proportional to the concentration of the excited sulfur dimer. Similarly, combustion of phosphorus compounds in a hydrogen flame gives emission due to HPO* at 526 nm. Linear working curves over four decades of concentration are reported. Both of these flame chemiluminescence techniques have been used for detection of sulfur and phosphorus species in the effluent from gas chromatographic columns.

Analysis for Inorganic Species in the Liquid Phase

Many of the analyses carried out in the liquid phase make use of organic chemiluminescing substances containing the functional group



These reagents react with oxygen, hydrogen peroxide, and many other strong oxidizing agents to produce a chemiluminescent oxidation product. Luminol is the most common example of these compounds. Its reaction with strong oxidants, such as oxygen, hydrogen peroxide, hypochlorite ion, and permanganate ion, in the presence of strong base is



Often a catalyst is required for this reaction to proceed at a useful rate. The emission produced matches the fluorescence spectrum of the product, 3-aminophthalate anion. The chemiluminescence appears blue and is centered around 425 nm.

Within certain limits, the chemiluminescence intensity of luminol is directly proportional to the concentration of either the oxidant, the catalyst, or the luminol. As a result, the reaction provides a sensitive method for determining any one of these species. Luminol is widely used in forensic science to detect small amounts of blood. The iron in hemoglobin catalyzes the reaction. As another example, using hydrogen peroxide as the oxidant, the catalyst Co^{2+} can be estimated at concentrations down to 0.01 nmol/L, Cr^{3+} down to 0.5 nmol/L, and Cu^{2+} down to 1 nmol/L. With a few cations, inhibition of luminescence occurs. For these, the decrease in intensity permits determination of concentrations.

Determinations of Organic Species

To increase the selectivity of chemiluminescence reactions and to extend chemiluminescence to analytes not directly involved in such reactions, it is common practice to precede a chemiluminescence step by an enzyme reaction for which the desired analyte is the substrate and one of the products is detected by chemiluminescence. This is most commonly done in flow systems with reactors containing immobilized enzyme. Recently, however, attention has been directed toward biosensor designs using enzymes attached to optical fibers.

Oxidase enzymes that generate H_2O_2 are commonly used in the predetection step. Not only can H_2O_2 be determined with several different chemiluminescence systems, but the necessary oxidant (O_2) is already present in most samples. Assuming quantitative conversion by the enzyme, substrates can be determined down to 10 to 100 nM, just as can H_2O_2. Substrates detected this way include glucose, cholesterol, choline, uric acid, amino acids, aldehydes, and lactate. For example:

$$\text{uric acid} + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + H_2O_2$$

The approach can be extended by using sequential enzyme steps to ultimately convert the analyte to an equivalent amount of chemiluminescent reactant. In this way, sugars other than glucose, glucosides, cholesterol esters, creatinine, and acetylcholine have been determined.[22] For example,

$$\text{sucrose} + H_2O \xrightarrow{\text{invertase}} \alpha\text{-D-glucose} + \text{fructose}$$

$$\alpha\text{-D-glucose} \xrightleftharpoons{\text{mutatrotase}} \beta\text{-D-glucose}$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

Luminol plus a peroxidase catalyst appears to be an excellent reaction medium for determining H_2O_2. Peak chemiluminescence intensity is reached in about 100 ms. The solvent is water and is compatible with some organic components. The detection limit is about 0.1 pM, with linearity for three to four decades of concentration.

[22]C. A. K. Swindlehurst and T. A. Nieman, *Anal. Chim. Acta*, **1988**, *205*, 195, **DOI**: 10.1016/S0003-2670(00)82328-4.

QUESTIONS AND PROBLEMS

*Answers are provided at the end of the book for problems marked with an asterisk.

Problems with this icon are best solved using spreadsheets.

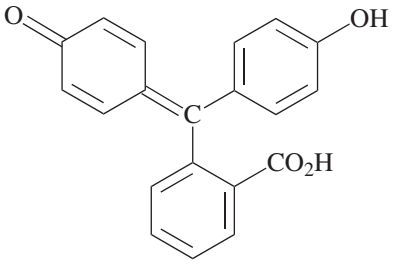
15-1 Explain the difference between a fluorescence emission spectrum and a fluorescence excitation spectrum. Which more closely resembles an absorption spectrum?

15-2 Define the following terms: (a) fluorescence, (b) phosphorescence, (c) resonance fluorescence, (d) singlet state, (e) triplet state, (f) vibrational relaxation, (g) internal conversion, (h) external conversion, (i) intersystem crossing, (j) predissociation, (k) dissociation, (l) quantum yield, (m) chemiluminescence.

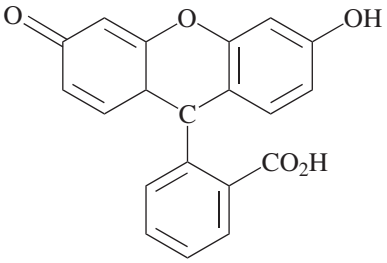
15-3 Why is spectrofluorometry potentially more sensitive than spectrophotometry?

***15-4** Which compound in each of the following pairs would you expect to have a greater fluorescence quantum yield? Explain.

(a)

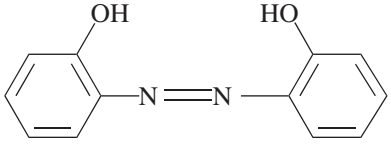


phenolphthalein

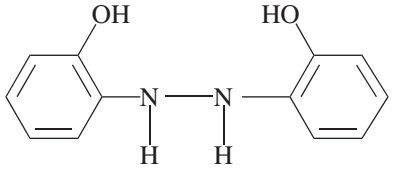


fluorescein

(b)



o,*o*′-dihydroxyazobenzene



bis(*o*-hydroxyphenyl) hydrazine

15-5 Why do some absorbing compounds fluoresce but others do not?

15-6 Discuss the major reasons why molecular phosphorescence spectrometry has not been as widely used as molecular fluorescence spectrometry.

15-7 The reduced form of nicotinamide adenine dinucleotide (NADH) is an important and highly fluorescent coenzyme. It has an absorption maximum of 340 nm and an emission maximum at 465 nm. Standard solutions of NADH gave the following fluorescence intensities:

Conc. NADH, μmol/L	Relative Intensity
0.100	2.24
0.200	4.52
0.300	6.63
0.400	9.01
0.500	10.94
0.600	13.71
0.700	15.49
0.800	17.91

(a) Construct a spreadsheet and use it to draw a calibration curve for NADH.

***(b)** Find the least-squares slope and intercept for the plot in (a).

(c) Calculate the standard deviation of the slope and the standard deviation about regression for the curve.

***(d)** An unknown exhibits a relative fluorescence intensity of 12.16. Use the spreadsheet to calculate the concentration of NADH.

***(e)** Calculate the relative standard deviation for the result in part (d).

***(f)** Calculate the relative standard deviation for the result in part (d) if a result of 7.95 was the mean of three measurements.

QUESTIONS AND PROBLEMS (continued)

15-8 The volumes of a standard solution containing 1.10 ppm of Zn^{2+} shown in the table were pipetted into separatory funnels, each containing 5.00 mL of an unknown zinc solution. Each was extracted with three 5-mL aliquots of CCl_4 containing an excess of 8-hydroxyquinoline. The extracts were then diluted to 25.0 mL and their fluorescence measured with a fluorometer. The results were the following:

Volume Std. Zn^{2+}, mL	Fluorometer Reading
0.000	6.12
4.00	11.16
8.00	15.68
12.00	20.64

(a) Construct a working curve from the data.

(b) Calculate a linear least-squares equation for the data.

(c) Calculate the standard deviation of the slope and intercept and the standard deviation about regression.

(d) Calculate the concentration of zinc in the sample.

(e) Calculate a standard deviation for the result in part (d).

***15-9** Quinine in a 1.553-g antimalarial tablet was dissolved in sufficient 0.10 M HCl to give 250 mL of solution. A 10.00-mL aliquot was then diluted to 50.00 mL with the acid. The fluorescence intensity for the diluted sample at 347.5 nm provided a reading of 196 on an arbitrary scale. A standard 100-ppm quinine solution registered 125 when measured under conditions identical to those for the diluted sample. Calculate the mass in milligrams of quinine in the tablet.

***15-10** The determination in Problem 15-9 was modified to use the standard-addition method. In this case, a 3.925-g tablet was dissolved in sufficient 0.10 M HCl to give 1.000 L. Dilution of a 20.00-mL aliquot to 100 mL yielded a solution that gave a reading of 415 at 347.5 nm. A second 20.00-mL aliquot was mixed with 10.0 mL of 50-ppm quinine solution before dilution to 100 mL. The fluorescence intensity of this solution was 503. Calculate the percentage of quinine in the tablet.

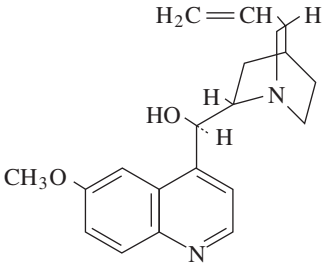
***15-11** Iron(II) ions catalyze the oxidation of luminol by H_2O_2. The intensity of the resulting chemiluminescence has been shown to increase linearly with iron(II) concentration from 10^{-10} to 10^{-8} M.

Exactly 1.00 mL of water was added to a 2.00-mL aliquot of an unknown Fe(II) solution, followed by 2.00 mL of a dilute H_2O_2 solution and 1.00 mL of an alkaline solution of luminol. The chemiluminescence from the mixture was integrated over a 10.0-s period and found to be 12.7.

To a second 2.00-mL aliquot of the sample was added 1.00 mL of a 3.27×10^{-5} M Fe(II) solution followed by the same volume of H_2O_2 and luminol. The integrated intensity was 27.9. Find the concentration of Fe(II) in the sample.

15-12 Equations for the chemiluminescence determination of SO_2 are given on page 383. Derive an expression for the relationship between the concentration of SO_2 in a sample, the luminescence intensity, and the equilibrium constant for the first reaction.

15-13 Quinine is one of the best-known fluorescent molecules, and the sensitivities of fluorometers are often specified in terms of the detection limit for this molecule. The structure of quinine is given next. Predict the part of the molecule that is most likely to behave as the chromophore and fluorescent center.



15-14 The quantum efficiency of fluorescence ϕ_f can be written as

$$\phi_f = \frac{\tau}{\tau_0}$$

where τ is the observed lifetime of the excited state in the presence of a quenching agent and τ_0 is the natural lifetime in the absence of a quencher. The fluorescence radiant power F is given by Equation 15-7. This quantity is affected by collisional quenching because the lifetime τ is influenced by collisional quenching. Derive an equation to show that the F-τ ratio is independent of collisional quenching and directly related to concentration. (From G. M. Hieftje and G. R. Haugen, *Anal. Chim. Acta*, 1981, *123*, 255, **DOI**: 10.1016/S0003-2670(01)83178-0.)

15-15 The following lifetimes were measured for the chloride quenching of quinine sulfate given in Example 15-1. The fluorescence intensities are given in the example.

Fluorescence Lifetime τ, ns	$[Cl^-]$, M
18.1	0.000
8.9	0.005
5.7	0.010
4.5	0.015
3.6	0.020
2.8	0.025
2.5	0.030
1.9	0.040
1.6	0.050

(a) Plot fluorescence intensity versus $[Cl^-]$.

(b) Plot the ratio of intensity to lifetime, F-τ versus $[Cl^-]$.

(c) Develop a normalization factor to correct the measured fluorescence intensity to that of the solution without quencher.

(d) Plot on the same graph F versus $[Cl^-]$ and F_{corr} versus $[Cl^-]$.

Challenge Problem

15-16 The following volumes of a standard 10.0 ppb F^- solution were added to four 10.00-mL aliquots of a water sample: 0.00, 1.00, 2.00, and 3.00 mL. Precisely 5.00 mL of a solution containing an excess of the strongly

» QUESTIONS AND PROBLEMS (continued)

absorbing Al-acid alizarin garnet R complex was added to each of the four solutions, and they were each diluted to 50.0 mL. The fluorescence intensities of the four solutions were as follows:

V_s, mL	Meter Reading
0.00	68.2
1.00	55.3
2.00	41.3
3.00	28.8

(a) Explain the chemistry of the analytical method.

(b) Construct a plot of the data.

(c) Use the fact that the fluorescence decreases with increasing amounts of the F^- standard to derive a relationship like Equation 1-3 for multiple standard additions. Use that relationship further to obtain an equation for the unknown concentration c_x in terms of the slope and intercept of the standard-additions plot, similar to Equation 1-4.

(d) Use linear least squares to find the equation for the line representing the decrease in fluorescence relative to the volume of standard fluoride V_s.

(e) Calculate the standard deviation of the slope and intercept.

(f) Calculate the concentration of F^- in the sample in parts per billion.

(g) Calculate the standard deviation of the result in (e).