which stoichiometric amounts of NADH were bound. The enzyme, in this case, consisted of four chemically identical subunits, so the bound-NADH concentration was 10 $\mu mol \cdot dm^{-3}$. Comment on the results.

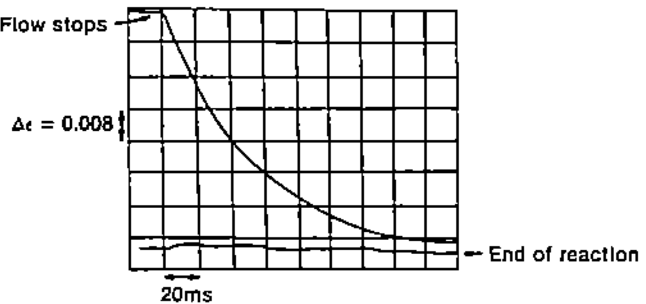


Figure 4.24

8. The interaction of chlorophyll molecules with one another is important for an understanding of photosynthesis. Studies of electronic spectra have been used to provide information relevant to the structure of the dimers. For example, bacterio-chlorophyll can form dimers in carbon tetrachloride, and a simple monomer-dimer equilibrium exists. The absorption spectra of monomers and dimers are shown in Figure 4.25. Comment on the shape of the band at the longest wavelengths.

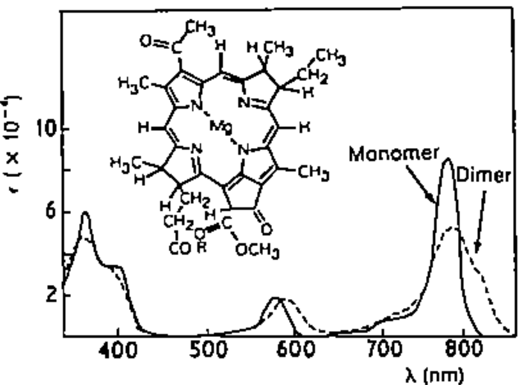


Figure 4.25

9. In principle, what information can be obtained from linear dichroism measurements on oriented samples?
10. The dichroic ratio for an oriented sample is independent of wavelength. What can you deduce about the number of overlapping bands making up the absorption?
11. The increase in intensity in the absorption spectrum of viral DNA on denaturation is much smaller than the increase on denaturation of the replicative form of this DNA (found in host bacteria). Explain this.
12. Suggest a simple reason for the intensity of charge-transfer bands in terms of the strength of the electric transition dipole.

Sct 2-8

CHAPTER 5

Fluorescence

OVERVIEW

1. Fluorescence is the emission of radiation that occurs when a molecule in an excited electronic state returns to the ground state. It involves excitation (10^{-15} s) followed by emission. There is a finite lifetime (10^{-6}–10^{-9} s) in the excited state.
2. A physical picture of fluorescence comes from considering the energy levels involved in electronic transitions using Morse curves and the Franck-Condon principle. There are various processes by which the excited state can lose energy—only one of which is fluorescence. The emission spectrum is red shifted with respect to the excitation spectrum.
3. The measurable parameters are the excitation and emission spectra and the intensity, lifetime, and polarization of the fluorescence.
4. The term **fluorophore** describes the molecular group giving rise to fluorescence. The relatively few naturally occurring fluorophores in biology include tryptophan, the Y-base of t-RNA, NADH, and chlorophyll. Synthetic fluorescent probes are widely used.
5. The fluorescence intensity and λ_{max} (the position of the maximum of the emission) are very sensitive to environment.
6. The quantum yield, Φ_F, is the fraction of molecules that becomes deexcited by fluorescence. It is defined as $\Phi_F = \tau/\tau_F$, where τ is the observed lifetime of the excited state and τ_F the lifetime that it would have if fluorescence was the only deexcitation process.
7. There are several important deexcitation processes that can be characterized by first-order rate constants

$$(1/\tau) = k = k_F + k_E + K_q[Q] + \Sigma_i ki$$

where $k_F = 1/\tau_F$, k_E represents energy transfer to a neighboring chromophore on the same molecular complex, $k_q[Q]$ represents deexcitation by an added solute $[Q]$, and $\Sigma_i k_i$ represents other processes.

8. Molecular events on the time scale of the fluorescence lifetime can be investigated by analyzing quenching processes and depolarization.
9. Distances between fluorophores can be obtained from energy-transfer processes.
10. Applications include ligand binding (including H^+) to macromolecules, environmental probes (including polarity and conformational changes), molecular dynamics (including molecular tumbling and oxygen quenching), distance r between fluorophores (energy transfer $\sim 1/r^6$), and various assays (including those using fluorescent antibodies).
11. Phosphorescence is the emission of radiation observed when a forbidden transition occurs from an excited state (usually a triplet state) to the ground state. The lifetime in these excited states is 10^{-2} to 10^2 s. Phosphorescence is rarely observed in solution.

INTRODUCTION

In discussing absorption processes between electronic energy levels, we have mainly been concerned with the excitation of a molecule from a ground state to a higher energy level. In many instances, the excitation energy is lost as heat to the surroundings as the molecules relax (return) to the ground state. However, in some cases, reradiation (emission) occurs. This process is called **fluorescence.**

Fluorescence, therefore, involves two processes: absorption and subsequent emission. Each process occurs in the time scale given by the inverse of the transition frequency (about 10^{-15} s), but there is a time lag of about 10^{-9} s when the molecule exists in the excited state. The lifetime of the molecule in the excited state depends on competition between the radiative emission and any radiationless process, such as the transfer of the excitation energy to the surrounding medium. These nonradiative processes provide an alternative mechanism for the excited molecules to relax back to the ground state, and their presence will result in a diminution or a **quenching** of the fluorescence intensity.

As we shall see, fluorescence occurs at a lower frequency than that of the incident light. Since the detection frequency is different from the incident frequency, sensitivity is high because there is no background signal from the excitation source. It is often possible to measure fluorescence at concentrations in the $10^{-8} M$ range, which is about two orders of magnitude below those generally used for absorption spectra.

Many of the uses of fluorescence are very similar to those described in the section on ultraviolet—for example, measuring binding, monitoring conformational changes, or following a reaction. In addition, fluorescence is a particularly powerful technique because there are many reactions, solvent rearrangements, and molecular-motion processes that take place on the same time scale as the lifetime of the excited state. The resulting sensitivity of fluorescence to this time scale and to environment is the basis of many of its applications to biochemistry. By contrast, we note that absorption of light in the ultraviolet occurs in about 10^{-15} s—a time scale during which the chromophore and its environment are essentially static.

Worked Example 5.1 Fluorescence in Green Plants

In photosynthesis, green plants harvest light by trapping energy from electromagnetic radiation. This energy is then utilized in a series of reactions to produce ATP. These reactions can be blocked, e.g., by a weedkiller (see Figure 5.1(a)). Explain why the fluorescence of the receptor molecule is then much increased (Figure 5.1(b)) in the presence of weedkiller.

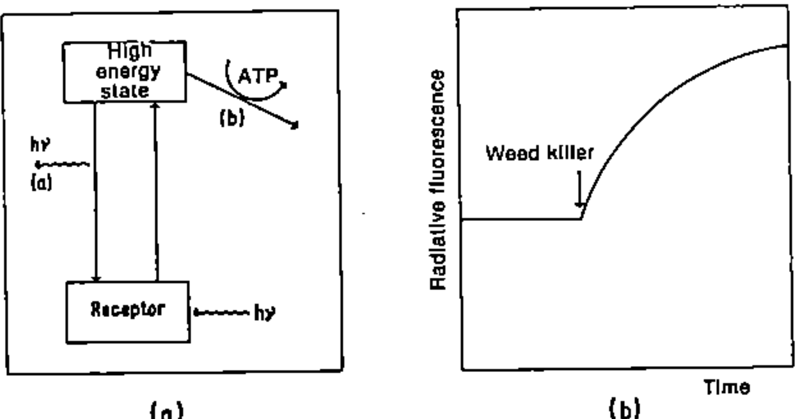


Figure 5.1

Solution

There are at least two routes by which energy can be removed from the initial reaction in photosynthesis—energy transfer and fluorescence. In the absence of weedkiller, the fluorescence is quenched as the excitation energy is used to make ATP. In the presence of weedkiller, the energy-transfer reactions leading to the formation of ATP are shut off. The molecules can then relax back to their initial state by giving up their excitation energy, using the only "open" route—fluorescence.

PHYSICAL PICTURE

To obtain a simple physical picture of fluorescence, we return once again to a discussion of the energy levels involved in electronic transitions. In general, at room temperature a molecule will be in its ground electronic state and its lowest vibrational level. Absorption of the appropriate energy results in excitation into the upper vibrational levels of the singlet excited state.

The spectral transitions between the vibrational levels of different electronic states are governed by the Franck-Condon principle. On the energy diagram (see Figure 4.3), we illustrate this by a vertical transition in which the internuclear separation is the same in both electronic states. Different vibrational levels in the excited state will receive the transition depending on the relative positions of the

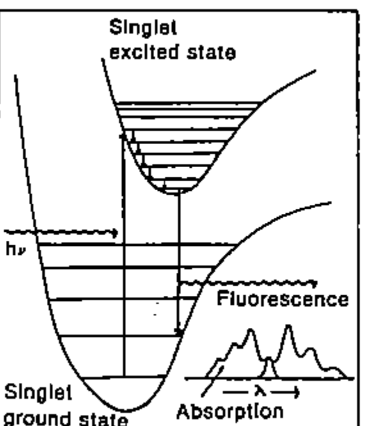
ground- and excited-state energy curves (see Figure 4.3). In solutions, when molecules have reached the Franck-Condon excited state, several events can occur as the molecules reestablish (or relax back to) their equilibrium populations (mainly in the lowest vibrational energy level in the ground electronic state). The first event is that the molecules in vibrational levels above the lowest one in the excited state lose their excess energy (usually as heat) and return to the lowest level (thus establishing a Boltzmann population distribution in the excited state). The process takes about 10^{-12} to occur. From this excited state, spontaneous emission can occur.* The emitted light is fluorescence.

EXCITATION AND EMISSION SPECTRA

The dependence of the fluorescence intensity on the wavelength of the *exciting* light is referred to as the **excitation spectrum**. Conversely, the **emission spectrum** describes the variation of the fluorescence intensity with the wavelength of the *emitted* light. The position of the maximum in the emission spectrum (λ_{max}) is sensitive to the polarity of the environment and the mobility of the fluorophore. There is often a mirror-image relationship between the excitation and emission spectra.

Worked Example 5.2 Fluorescence Occurs at Longer Wavelengths than Absorption

Figure 5.2 illustrate the processes leading to fluorescence. Explain why the fluorescence spectrum is shifted to longer wavelengths than those of the absorption spectra and why there is a mirror-image relationship between them. Does the shape of the fluorescence spectrum depend on the wavelength of the exciting light?



Processes leading to fluorescence
Figure 5.2

Solution

The fluorescence usually appears at longer wavelength (lower frequency) than the incident light because the energy of the emitted electromagnetic radiation differs from that absorbed by an amount equivalent to the vibrational energy lost to the surroundings. Only one transi-

(continued)

*Stimulated emission does not occur, since there is usually no applied electromagnetic radiation at the correct frequency.

Worked Example 5.2 (continued)

tion between the lowest vibrational levels (termed 0–0) coincides in the two processes of the absorption and emission, namely the one involving the lowest vibrational states of the ground *and* excited states. Transitions probable in absorption are also probable in emission because the transition dipole connecting the two states is the same each time, hence the mirror-image relationship. Since the fluorescence originates from molecules in the lowest vibrational level of the excited state, the shape of the spectrum is independent of the wavelength of the exciting light. (However, as we shall see later, the intensity of the spectrum does depend on the wavelength of the exciting light.)

TRANSITION PROBABILITY AND LIFETIME

In absorption, the probabilities of electronic transitions, together with the populations of the energy levels, determine the intensities of the corresponding absorption bands. The same factors apply in fluorescence. However, other radiationless processes can deplete the excited state, thus reducing its population and decreasing the observed fluorescence intensity. The intensity will therefore depend on the relative rates of the competing processes. If fluorescence is the only means of depopulating the excited state, then this process is kinetically a first-order one, in which the rate constant k_f is that for spontaneous emission.* The inverse of the rate constant in a first-order process is defined as a **relaxation time**.† In this instance, it is referred to as the **radiative lifetime** τ_F, such that $\tau_F = 1/k_F$. Since the absorption probability is related to the emission probability, τ_F can often be related to the molar extinction coefficient (ϵ_{max} in units of $cm^2\ mol^{-1} \equiv (mol \cdot dm^{-3})^{-1}\ cm^{-1}$). For example, the relationship

$$\frac{1}{\tau_F} \simeq 10^4 \epsilon_{max}$$

can be used to obtain a very approximate value of τ_F in seconds.

In discussing the various time scales mentioned so far, we must be careful not to confuse lifetime (or relaxation time) with the time for a transition. The latter is given by the reciprocal of the frequency of the transition (see Worked Example 4.5). The lifetime refers to a bulk property that is a measure of how long the molecules exist in a particular state. Figure 5.3 summarizes some of the pathways that are discussed in this chapter with typical values of their rate constants. The wavy lines indicate nonradiative pathways, which are often termed **internal conversion**. As we shall see, measurement of lifetimes can be used in a number of ways to obtain dynamic and structural information about molecules.

*Spontaneous emission, like that from radioactive decay, is a first-order process, since it does not involve collisions between molecules.

†Strictly speaking from elementary kinetics, relaxation time is the correct definition of the inverse rate constants, rather than the more commonly used *lifetime*. The relaxation time is the time for the process to fall to 1/*e* of its initial value.

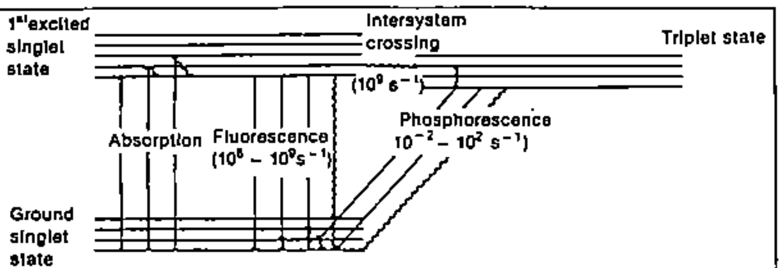


Figure 5.3 Some pathways of relaxation from the excited state.

Worked Example 5.3 The Radiative Lifetime Provides a Time Scale for Events in the Excited State

In the photosynthetic unit, hundreds of chlorophyll molecules transfer their energy to a site at the reaction center. The chlorophyll molecule at this center has unique properties because of its environment, which lowers the energy of its excited state. This makes it an energy trap (see Figure 5.4). The reaction centers mediate the change of light into chemical energy. Can you give a lower limit for the rate of energy transfer? Chlorophyll has an extinction coefficient of about 10^5 $(\text{mol}\cdot\text{dm}^{-3})^{-1}\cdot\text{cm}^{-1}$.

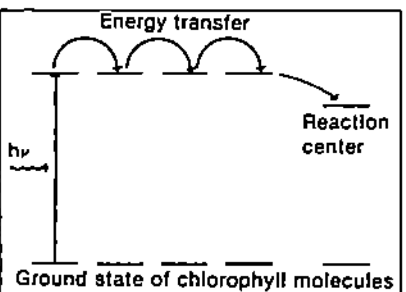


Figure 5.4

Solution

We can estimate the lifetime of the excited state from $1/\tau_F \sim 10^4\epsilon_{max}$, which gives $\tau_F \sim 10^{-9}$ s. To be effective, energy transfer must depopulate the excited state faster than does the fluorescence process. The time of energy transfer must therefore be faster than 10^{-9} s. (Values of about 10^{-10} s or less would ensure that most of the energy found its way to the reaction center.)

QUANTUM YIELD

The last worked example illustrated that other processes can compete with fluorescence to depopulate the excited state. These competing processes will cause a more rapid decay of the excited molecules than expected if fluorescence is considered to be the only process by which the molecules can return to the ground

state. The overall rate constant (k) for the depopulation of the excited state is obtained by summing the individual rate constants for all the competing processes (which are also assumed to be first-order processes). That is,

$$k = k_F + \Sigma k_i$$

where k_i represents the various competing radiationless processes. The lifetime (τ) or relaxation time, for the overall process is given by

$$\tau = \frac{1}{k} = \frac{1}{k_F + \Sigma k_i}$$

The fraction of excited molecules that becomes deexcited by fluorescence is called the **quantum yield** (Φ_F) (or the **fluorescence efficiency**) and is given by the equation

$$\Phi_F = \frac{k_F}{k_F + \Sigma_i k_i}$$

Substituting for τ and τ_F, we also obtain that

$$\Phi_F = \frac{\tau}{\tau_F}$$

Absolute values of Φ_F are difficult to measure experimentally because instrumental correction factors have to be known. In practice, they are obtained by comparison with a standard sample for which the quantum yield is known. For instance, quinine sulfate in $0.5\text{-mol}\cdot\text{dm}^{-3}$ H_2SO_4 has $\Phi_F = 0.7$.

The quantum yield is sensitive to the immediate surroundings of the fluorescent chromophore (**fluorophore**) and also to specific quenching processes. Changes in quantum yield are accompanied by changes in fluorescence intensity. Measurements of intensity are usually sufficient if only relative values of quantum yield are to be studied.

FLUORESCENCE INTENSITY

The quantum yield (fluorescence efficiency) Φ_F will reflect all the processes that compete with fluorescence for the depopulation of the excited state. The fluorescence intensity (F_λ) will therefore depend on the initial population of the excited state (I_A) multiplied by the quantum yield Φ_F.

$$F_\lambda = I_A \Phi_F$$

This expression refers to the total fluorescence intensity emitted in all directions. In practice, only a small amount is collected by the spectrometer. The equation has to be multiplied by an instrumental factor (Z) that allows for this.

Worked Example 5.4 Fluorescence Intensity Depends on the Excitation Wavelength

Using the Beer-Lambert law, deduce how the intensity of the fluorescence spectrum will depend on the excitation wavelength.

Solution

The intensity of the fluorescence is given by $F_\lambda = I_A \Phi_F Z$. The population in the excited state depends on the amount of light absorbed, I_A, which can be calculated from the Beer-Lambert law as

$$I_A = I_t - I_0 = I_0\{1 - \exp[-(2.3\epsilon(\lambda_A)cl]\}$$

where $\epsilon(\lambda_A)$ is the extinction coefficient at the exciting (absorbing) wavelength, c is the concentration of the absorbing molecules, and l is the path length. For small absorbances, $\exp[-(2.3\epsilon(\lambda_A)cl)] << 1$, so we can replace the exponential by $(1 - 2.3\epsilon(\lambda_A)cl)$. Thus

$$I_A = I_0\{1 - [1 - 2.3\epsilon(\lambda_A)cl]\} = I_0(2.3\epsilon(\lambda_A)cl)$$

The intensity of the fluorescence $F(\lambda)$ is thus given by

$$F_\lambda = I_0(2.3\epsilon(\lambda_A)cl\Phi_F Z)$$

From this equation, if the intensity I_0 of the exciting light is kept constant, and if we assume that Φ_F and Z are independent of the wavelength of the exciting light, then for a given solution

$$F_\lambda \propto \epsilon(\lambda_A)$$

That is, the intensity in the fluorescence spectrum will depend on the extinction coefficient at the wavelength of the absorbing species. Since the extinction coefficient also reflects the probability of a transition, this result is expected because transitions probable in absorption are also probable in emission. Thus, while the *shape* of the fluorescence spectrum is independent of the exciting wavelength, the *intensity* is not.

FLUORESCENCE POLARIZATION

Measurements of fluorescence polarization can give information on rotational motions in proteins.

If the chromophore is excited with plane polarized light and the fluorescence is observed through analyzing polarizers, then it is found that the degree of polarization of the fluorescence usually decreases. This phenomenon is called **fluorescence depolarization**, and the reasons for its occurrence are discussed later in the chapter. The polarization is conventionally defined as

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp}$$

where $I_\parallel$ and $I_\perp$ are, respectively, the fluorescence intensities resolved in directions parallel and perpendicular to the direction of the exciting beam. In solutions in which the molecules are randomly oriented but immobilized, the value of P is called the **intrinsic polarization** (P_0). If the molecules in that system undergo motion during the lifetime of the excited state, then P may differ from the P_0 value.

Another relationship, similar to P, is sometimes used—the **anisotropy factor***

$$A = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

Measurements of P and A are sometimes carried out under steady-state conditions, that is, using constant illumination. In such cases only the average motion of the system is observed. However, nanosecond pulses of polarized light can be used to measure $I_\parallel$ and $I_\perp$ separately and as a function of time. This technique often enables different motions to be probed or **time resolved**.

NATURAL FLUOROPHORES AND FLUORESCENT PROBES

The main fluorophores used in biochemistry can be classified into natural fluorophores and fluorescent indicators, or **probes**.

Natural fluorophores include the aromatic amino acids, flavins, vitamin A, chlorophyll, and NADH. Nucleic acids do not have appreciable fluorescence, with the exception of the Y-base in t-RNA. Typical fluorescence characteristics of some of these are listed in Table 5.1. The relative experimental sensitivity will be governed by the product of ϵ_{max} and Φ_F.

Worked Example 5.5 Protein Fluorescence Arises from Tryptophan Residues

Use Table 5.1 to explain the statement in the title of the problem.

Solution

From Table 5.1, the relative sensitivity of Trp suggests that the fluorescence from this residue will dominate the fluorescence spectrum of a protein. (Additionally, in proteins the fluorescence of Tyr residues is usually quenched in the presence of Trp residues because of the transfer of excitation energy from Tyr to Trp residues by the Förster resonance-transfer mechanism; see later).

Because there are so few natural fluorophores, many of the applications of the fluorescence technique in biochemistry involve adding a fluorescent probe or labeling reagent to the system. These probes may be bound either covalently or

*The value of $I_\parallel + 2I_\perp$ expresses the total incident light—parallel to the incident axis and the *two* directions at right angles to it.

Table 5.1

Fluorophore	Conditions	Absorption λ_{max} (nm)	Absorption ϵ_{max} ($\times 10^{-3}$)	Fluorescence λ_{max} (nm)	ϕ_F	τ_F (ns)	Sensitivity $\epsilon_{max}\phi_F$ ($\times 10^{-2}$)
Trp	H_2O pH 7	280	5.6	348	0.20	2.6	11.
Tyr	H_2O pH 7	274	1.4	303	0.1	3.6	1.4
Phe	H_2O pH 7	257	0.2	282	0.04	6.4	0.08
Y-base	Yeast t-RNA^{Phe}	320	1.3	460	0.07	6.3	0.91

Table 5.2 Typical fluorescent probes[a]

Probe	Uses	Absorption λ_{max} (nm)	Absorption ϵ_{max} ($\times 10^{-3}$)	Emission[b] λ_{max} (nm)	Emission[b] ϕ_F	τ_F (ns)	Sensitivity $\epsilon_{max}\phi_F$ ($\times 10^{-2}$)
Dansyl chloride	Covalent attachment to protein: Lys, Cys	330	3.4	510	0.1	13	3.4
1,5-I-AEDANS		360	6.8	480	0.5	15	34
7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD)	Lys, Tyr	345	9.5	—	~1		
Fluorescein isothiocyanate (FITC)	Covalent attachment to protein: Lys	495	42	516	0.3	4	116
8-Anilino-1-naphthalene sulfonate (ANS)	Noncovalent binding to proteins	374	6.8	454	0.98	16	67
Pyrene and various derivatives	Polarization studies in membranes	342	40	383	0.25	100	100
Ethenoadenosine and various derivatives	Analogues of nucleotides bind to proteins. Incorporate into nucleic acids	300	2.6	410	0.40	26	10
Ethidium bromide	Noncovalent binding to nucleic acids	515	3.8	600	~1	26.5	38
Proflavine monosemicarbazide	Covalent attachment to RNA 3'-ends	445	15	516	0.02	—	30

[a] After C. R. Cantor and P. R. Schimmel, *Biophysical Chemistry*, (New York: W. H. Freeman and Company, Publishers, 1980).

[b] Values shown for ϕ_F and τ_F are near maximum typically observed in biological samples at ambient temperature. Other (considerably smaller) values are often found.

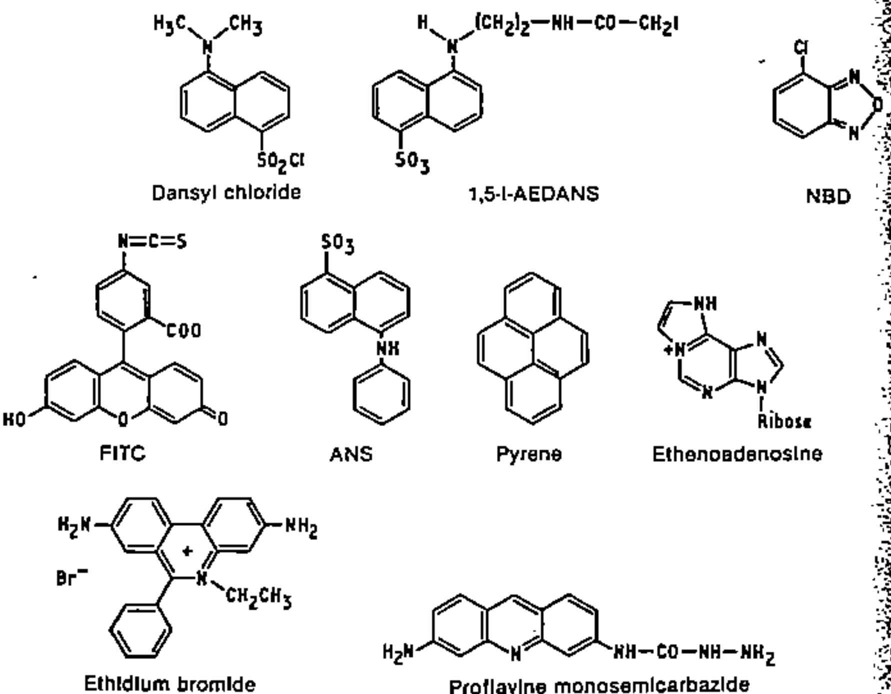


Figure 5.5 Structures of some fluorescent probes.

noncovalently, and much ingenuity has gone into designing site-specific probes. Some of the more common probes are listed in Table 5.2 and their structures are shown in Figure 5.5.

Transition metal ions are not usually fluorescent, however, a number of the lanthanide ions do have observable fluorescence. These can often be used as probes for nonfluorescent ions, notably calcium and, sometimes, magnesium.

EFFECT OF ENVIRONMENT ON FLUORESCENCE PARAMETERS

Fluorescence is very sensitive to environment and the various parameters (e.g., λ_{max}, ϕ_F, τ_F) can be affected in a variety of ways. Some examples of these are given next.

Environmental Effects on λ_{max}

In general, a molecule in the first excited electronic state will have a charge distribution different from that in the ground state. Interactions of the chromophore with the surrounding solvent molecules may occur prior to emission. These interactions will alter the energy of the excited state and also the frequency of the fluorescence emission. This can result in the nonequivalence of the absorption and emission transitions between the lowest vibrational levels in each state (the 0–0) and the breakdown of the mirror-image relationship.

This sensitivity of the fluorescence to environment can be used in a number of ways, of which one of the best known is to estimate polarity. In general, the excited state will be more polar than the ground state; therefore, the excited molecules will tend to interact with a polar solvent (or environment) so as to align the solvent dipoles. This alignment decreases the energy of the excited state and causes the emission spectrum to shift toward the red.

The position of λ_{max} as a measure of polarity must, however, be used with caution. A blue shift (with respect to the probe's fluorescence) can arise if the molecules do not have time to undergo rearrangement (and hence lower the energy of the excited state) during the lifetime of the excited state. This phenomenon is known as **orientation constraint** and emphasizes the ambiguities that may arise in using λ_{max} as a measure of the polarity of an environment.

Worked Example 5.6 Fluorescent Probes of Environmental Polarity

The fluorescent spectra of 1-anilino-8-naphthalene sulfonate (ANS) shifts toward the blue, and the quantum yield increases as the solvent polarity decreases in the order: ethylene glycol, methanol, *n*-propanol, and *n*-octanol (see Figure 5.6). ANS binds to apomyoglobin with a 1:1 stoichiometry and is displaced by the heme. When ANS binds, λ_{max} shifts from 515 nm (in water) to 454 nm and is accompanied by an increase in quantum yield from 0.004 to 0.98. What can you deduce about the ANS binding site?

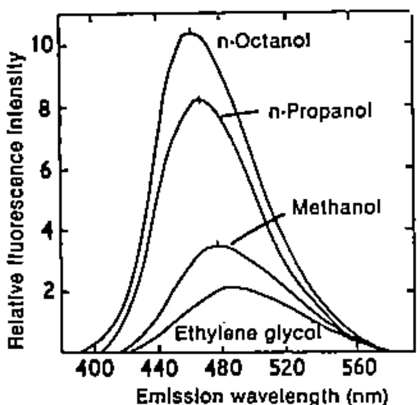


Figure 5.6

Solution

The shift toward the blue suggests that ANS binds in a (nonpolar) hydrophobic environment. The increase in quantum yield also supports this conclusion. The displacement of ANS by the heme suggests that ANS binds in or near the heme pocket (which one might expect to be hydrophobic).

Environmental Effects on Quantum Yield

The immediate surroundings can modify the fluorescence. A large number of applications of fluorescence in biochemistry simply involve monitoring the intensity of the fluorescence of an intrinsic or extrinsic probe.

In general, the quantum yield (fluorescence intensity) increases as the polarity of the solvent (or environment) decreases. One postulated mechanism for this is that the rate of intersystem crossing (transferring to different excited states) is reduced in nonpolar solvents.

Any constraint in the excited state increases the probability of fluorescence and hence the quantum yield. Measurement of the lifetime can be used to help to distinguish between a polarity effect and an environmental constraint. The consequence of constraint will make the observed value of τ_F closer to the expected value.

Worked Example 5.7 Changes in the Fluorescence Intensity of a Probe Can Be Used to Monitor Binding

Glutamate dehydrogenase consists of six identical subunits, and its activity is allosterically regulated. The enzyme forms ternary complexes with the substrate analogue glutarate and NAD^+ in which the oxidized coenzymes are more firmly bound than in their binary complexes with the enzyme alone.

The fluorescence quantum yield of NADH bound to the enzyme is markedly *enhanced* compared with that of the free coenzyme and λ_{max} (460 nm) is blue shifted by about 10 nm compared with free NADH. With a trace amount of NADH present as a probe, the binding of NAD^+ in the presence of a saturating concentration of glutarate was monitored by measuring the fluorescence enhancement. The results are shown in Figure 5.7, where the percent saturation of NAD^+ is also shown (obtained from binding studies under the same conditions). At half-saturation of the enzyme sites, the NADH probe signals a sudden change. Suggest a biochemical explanation for this.

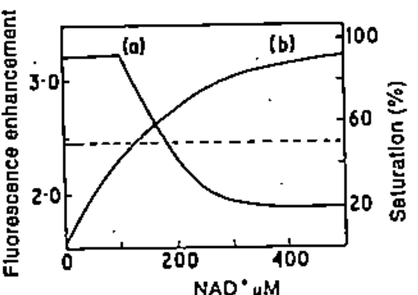


Figure 5.7

(*Clue:* NAD^+ and NADH compete for the same site.)

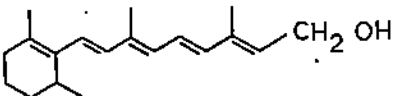
Solution

At half-saturation, three of the six subunits will have NAD^+ tightly bound; the NADH probe cannot be on these subunits. The most likely explanation is that the binding of NAD^+ as a ternary complex with glutarate to three of the six subunits causes a conformational change in the other three subunits. The reduction in fluorescence quantum yield of NADH must also result from the change in environment caused by the conformational change.

Worked Example 5.8 Probing Rigidity of Environment

The radiative lifetime of Vitamin A_1 calculated from the absorption and emission spectra is about 2.4 ns. A solution of retinol has a quantum yield of 0.004 and a measured lifetime of 2.4 ns in water, but when bound to BSA has a quantum yield of 0.04 and a measured lifetime of 8.0 ns. What can you conclude about the rigidity of the retinol environment in BSA?

Vitamin A_1 (all *trans*-retinol)



Solution

From $\Phi_F = \tau/\tau_F$, we obtain that in water, $\tau_F = 2.4 \times 10^{-9}/0.004 = 600$ ns, while when bound to BSA, $\tau_F = 8 \times 10^{-9}/0.04 = 200$ ns. These values are much longer than the calculated value of τ_F of 2.4 ns because Vitamin A_1 is undergoing rearrangement in the excited state. The decrease in τ_F when retinol is bound to BSA suggests that the probe is so constrained that it does not have sufficient freedom to undergo rearrangement on excitation.

Specific Quenching Processes. In favorable cases, an analysis of specific quenching processes can lead to useful biochemical information. A low quantum yield may also reflect the ground-state environment of the fluorophore. For instance, the low quantum yield found for some tyrosines in proteins has been attributed to hydrogen bonding with peptide carbonyl groups.

There are two types of quenching frequently studied in biological systems. The first is a result of a long-range (up to a distance of about ~5 nm) nonradiative process called **resonance energy transfer** from one chromophore to another. Analysis of this quenching allows the distance between the chromophores (which need not be the same molecule) to be measured. The second type of quenching arises from collisional processes. Here the addition of oxygen or other paramagnetic species or of heavy ions, such as I^- or Cs^+ all lead to an enhanced rate of intersystem crossing. The fluorescence quenching requires a bimolecular collision between quencher and fluorophore. Analysis of this quenching allows information on the dynamics of the collision process to be obtained. These two quenching processes are dealt with separately later. Note that collisional processes have also been used qualitatively to test the "availability" of a fluorophore in a macromolecule to the quencher.

Worked Example 5.9 Fluorescence Quenching by I^- Ions Gives Information on Tryptophan Accessibility in Lysozyme

Iodide acts as a collisional quencher of tryptophan fluorescence. 80% of the fluorescence from the six tryptophans in lysozyme arises from Trp-62 and Trp-108. The tryptophan fluorescence is significantly quenched (about 60%) in the presence of I^- ions (about 0.2*M*). When Trp-62 is oxidized with N-bromosuccinimide, which makes it nonfluorescent, the residual fluorescence is hardly quenched at all by the same concentration of I^- ions. Explain what this means in terms of accessibility to solvent of the two tryptophan residues.

Solution

In the modified enzyme, the fluorescence of Trp-108 is not quenched by I^-, which indicates that this residue may not be accessible to solvent. The quenching of the fluorescence of the native enzyme suggests that Trp-62 is the more exposed to the solvent. Note, however, that the accessibility of I^- to the tryptophans may be influenced by interaction of its charge with any charges on the protein, thus creating a possible ambiguity in the results.

Environmental Effects on Lifetimes

If a molecule contains two or more fluorophores, their environments may be different, but their individual emissions will probably not be resolved in the fluorescence spectrum in solution. If they have different fluorescence lifetimes, however, then following the decay of the fluorescence gives another chance to resolve them. The simplest way of doing this is to excite the sample with a short pulse of light (~1 ns) and monitor the emission $S(t)$ as a function of time. $S(t)$ is related to the initial emission intensity $S(0)$ following the pulse and the lifetime by

$$S(t) = S(0)e^{-t/\tau}$$

where $1/\tau$ is the sum of the rates of the transitions from the excited state. When there are two or more fluorophores, $S(t)$ will be expressed as a sum of exponential terms. Analysis of these may resolve the individual fluorophore lifetimes, which can then be used as probes of the different environments.

Worked Example 5.10 Resolving Lifetimes of Tryptophan Residues in Lysozyme

Lysozyme has six tryptophan residues located at positions 28, 62, 63, 108, 111, and 123. Trp-62, -63, and -108 are located in the active site. As mentioned before, however, most of the fluorescent emission arises from Trp-62 and Trp-108. The excitation pulse and fluorescence decay are shown in Figure 5.8. To analyze the fluorescence decay, a correction had to be made for the shape of the excitation pulse. The fluorescence decay was then analyzed in terms of two exponentials. Measurements were also made in samples where Trp-62 and -108 were oxidized either individually or together (to make them nonfluorescent).

(continued)

Worked Example 5.10 (continued)

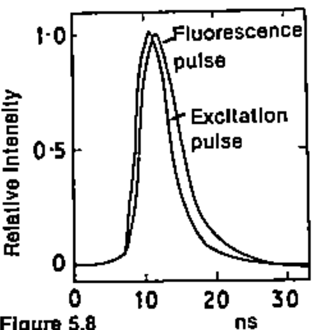


Figure 5.8

The following results were obtained:

Sample	*Lifetimes (ns)*	
Lysozyme	2.7	0.6
Oxidized Trp-62 lysozyme	1.5	0.3
Oxidized Trp-108 lysozyme	2.7	0.3
Oxidized Trp-108 and -62 lysozyme	—	0.3

What can you deduce about the lifetimes for the individual tryptophans?

Solution

When Trp-108 and -62 are oxidized, the single lifetime of 0.3 ns must represent the weighted mean of the lifetimes of all the other tryptophans. From the preceding results, Trp-62 must have a lifetime of 2.7 ns and Trp-108 of 1.5 ns. This latter value is contributing to the value of 0.6 ns in native lysozyme (for more details, see C. Formoso and L. S. Förster, *J. Biol. Chem.*, 250 (1975):5738–5745). (One of the limitations is the difficulty of quantitatively analyzing contributions from two or three similar exponentials. This is also a problem in the analysis of several similar rotational motions. Note also that quite a large "correction" for the pulse may have to be made, which presents a further difficulty in analysis of lifetimes of the same order as the pulse response.)

MEASUREMENTS OF MOLECULAR DYNAMICS

The lifetime of the molecule in the excited state before fluorescence occurs is finite—about 10^{-9} s. Any molecular events, such as tumbling or collisions, on this sort of time scale can lead to changes in the observed fluorescence. These changes can, in turn, be analyzed to yield information on molecular dynamics.

Dynamic Quenching: The Stern-Volmer Relationship

The effect of collisions with other molecules can shorten the fluorescence lifetime by providing additional processes that depopulate the excited state. There can also be a dynamic quenching of the fluorescence as a result of the encounters between an added quencher (such as I^- or O_2) and the fluorophore. The rate of quenching encounters is given by the product of the bimolecular quenching rate constant, k, and the quencher concentrations $[Q]$. Since the concentration of Q is often much greater than that of the excited molecule, the rate process may be regarded as essentially pseudo first order with a rate constant $k[Q]$. The quantum yield in the absence of quencher is (see the discussion of quantum yield earlier in the chapter)

$$\Phi_F = \frac{k_F}{k_F + \Sigma_i k_i}$$

In the presence of the quencher, the quantum yield will be

$$(\Phi_F)_Q = \frac{k_F}{k_F + \Sigma_i k_i + k[Q]}$$

Thus

$$\frac{\Phi_F}{(\Phi_F)_Q} = \frac{k_F + \Sigma_i k_i + k[Q]}{k_F + \Sigma_i k_i}$$

$$= 1 + \frac{k[Q]}{\Sigma_i k_i + k_F}$$

$$= 1 + k[Q]\tau$$

where τ is the lifetime in absence of quencher. This equation is often written in terms of the fluorescence intensities in the presence (I) and absence (I_0) of quencher, that is,

$$\frac{I_0}{I} = 1 + K[Q]$$

This expression is called the **Stern-Volmer** equation, which is characterized by a quenching constant K. Thus a plot of $[(I_0/I) - 1]$ versus $[Q]$ will give a straight line of slope K. Note that $K = k\tau$, where τ is the lifetime in the absence of the quencher. This emphasizes that the larger the lifetime of a fluorophore, the higher the probability of its undergoing a collision with the quencher.

The rate constant for quenching (k) depends on the probability of a collision between fluorophore and quencher. This probability depends on their rate of diffusion (D), their size, and concentration. It can be shown that

$$k = 4\pi a D \mathfrak{N}/10^3$$

where D is the sum of the diffusion coefficients of quencher and fluorophore, a is the sum of the molecular radii, and $\mathfrak{N}$ is Avogadro's number. (For example, for

oxygen quenching of tryptophan fluorescence in solution, $a = 0.4$ nm, $D_{O_2} = 2.6 \times 10^{-9}\text{cm}^2\cdot\text{s}^{-1}$, and $D_{Trp} = 0.66 \times 10^{-9}\text{cm}^2\cdot\text{s}^{-1}$, which gives $k = 1 \times 10^{10}$ $(\text{mol}\cdot\text{dm}^{-3})^{-1}\cdot\text{s}^{-1}$.

The determination of the dynamic quenching constant K can thus lead to information on diffusion coefficients. Measurements of K are better obtained from lifetimes rather than intensities because there may be other processes that affect the intensity. One example is **static quenching**, in which formation of a complex between quencher and fluorophore (characterized by an equilibrium constant K_{eq}) predates the excitation. Excitation of the fluorophores will then result in instantaneous quenching of those that are complexed. Static quenching thus reduces the concentration of the excited molecules, which alters the intensity of the fluorescence but not the lifetime.

A modified form of the Stern-Volmer equation that describes quenching data when both dynamic and static quenching are operative is

$$\frac{I_0}{I} = \frac{(1 + K_{eq}[Q])(1 + K[Q])}{K[Q]}$$

The first term describes the static quenching, which predates the excitation, and the second the dynamic quenching resulting from encounters between quencher and fluorophore during the fluorescent lifetime. A static component in the quenching results in an upward curvature in the Stern-Volmer plots. (Note that in this case, $I_0/I > \tau/\tau_Q$).

Worked Example 5.11 Quenching of Protein Fluorescence by Oxygen

The quenching of tryptophan fluorescence of lysozyme by oxygen was studied by using oxygen concentration up to 0.13*M* (corresponding to equilibration with oxygen at a pressure of 100 atm). This pressure has no effect on the activity of the protein. A plot of the ratio of the fluorescence intensity in the absence (I_0) and presence (I) of oxygen versus $[O_2]$ is shown in Figure 5.9. What can you deduce from this plot?

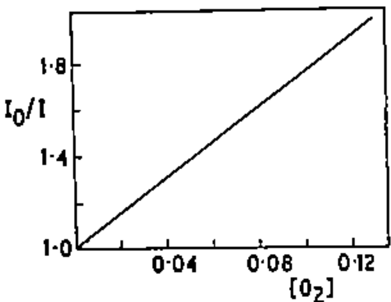


Figure 5.9

Solution

The figure shows a Stern-Volmer plot of the quenching of fluorescence by O_2. It is linear, showing no upward curvature, so the quenching is dynamic. This can be checked from lifetime measurements. A plot of τ/τ_Q versus $[O_2]$ should have the same shape. From the slop of the curve we can calculate that K is about 7 $(\text{mol}\cdot\text{dm}^{-3})^{-1}$. The linearity of the plot also suggests that the tryptophans contributing to the fluorescence do not have significantly different Stern-Volmer quenching constants and therefore are accessible to oxygen.

> ***Worked Example 5.12 Nanosecond Fluctuations in Proteins Detected by Oxygen Quenching of Fluorescence***
>
> In the previous example, the fluorescence lifetime was found to be 1.5 ns. The rate constant for quenching of tryptophan fluorescence by oxygen in water can be calculated to be 10^{10} (mol·dm^{-3})$^{-1}$s^{-1}. What is the value of the rate constant in lysozyme? What are the implications of this result?
>
> *Solution*
>
> From the Stern-Volmer equation for dynamic quenching, $k = K/\tau$ = $(7/1.5 \times 10^{-9}) = 0.5 \times 10^{-10}$ (mol·dm^{-3})$^{-1}$s^{-1}. From our previous calculations, this would imply that oxygen is diffusing through the protein at almost the same rate as in free solution. The protein must be undergoing rapid structural fluctuations on the nanosecond time scale, which permits diffusion of oxygen.

Fluorescence Depolarization

Steady-State Fluorescence Depolarization: Photoselection. The excitation of molecules to their excited state depends on the angle θ between the plane of polarization of the incident light and the transition dipole moment of the transition. The probability of absorption is proportional to $\cos^2\theta$, and the preferential absorption that takes place with polarized excitation is called **photoselection.** The measured value of the polarization P will depend not only on (1) the orientation of the absorption transition dipole moment but also on (2) the orientation of the emission transition dipole with respect to that of the absorption. A third factor, obviously, is the ordering in the sample. In a randomly oriented sample, the $\cos^2\theta$ dependence results in the excitation of a significant number of molecules that do not have transition dipoles aligned exactly along the plane of polarization. (This number is actually proportional to $\sin\theta$.) When these nonaligned molecules emit, they will have components of both parallel and perpendicular polarized light. Therefore, P will always have a value of less than one. In fact, if the averaging over all θ is considered, it turns out that P has the characteristic value $P_0 = 0.5$ if the transition dipole moments of excitation and emission are parallel. In general, however, the absorption and emission transition dipoles are not parallel but at some fixed angle θ to each other, and this reduces P_0 further.

The term P_0 is called the **intrinsic polarization** and is given by

$$P_0 = \frac{3\cos^2\theta - 1}{\cos^2\theta + 3}$$

Thus if $\theta = 90°$, $P_0 = -\frac{1}{3}$. P_0 can take values between $\frac{1}{2}$ and $-\frac{1}{3}$.

Motional Depolarization. Photoselection "selects," at an instant, those molecules whose chromophores have components of their transition dipole moments oriented along the direction of polarization. At that *instant*, we can regard those selected chromophores as rigid, and the system will have a characteristic value of

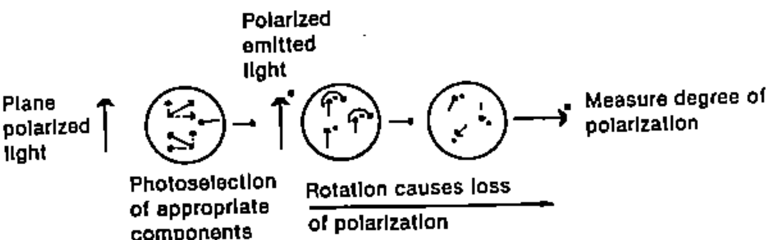


Figure 5.10 Depolarization resulting from rotation.

$P = P_0$. If in the time between absorption and emission the molecules rotate, the effects of photoselection will be lost and P will be less than P_0. Rotation thus causes depolarization (see Figure 5.10 for a simple physical picture). The relationship between P and P_0 is

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{\tau_F}{\tau_R}\right)$$

where τ_F is the radiative lifetime and τ_R is the rotational correlation time of the molecule (see Chapter 6 for a definition of correlation time). It is important to stress that this equation, known as the **Perrin equation**, assumes that the rotational motion is isotropic.

The anisotropy of polarization (A) is also affected by motion, as shown by the equation

$$\frac{1}{A} = \frac{1}{A_0}\left(1 + \frac{\tau_F}{\tau_R}\right)$$

where A_0 is the intrinsic anisotropy (in the absence of all motion).

Measures of A and P are carried out under steady state conditions, that is, using constant illumination. The values of P_0 and A_0 can be extracted from the foregoing equations by varying τ_R or by altering either the viscosity η (by addition of sucrose and glycerol) or the temperature. Since $\tau_R \propto \eta/T$, extrapolation of plots of $1/A$ or $(1/P_0 - \frac{1}{3})$ versus T/η to infinite viscosity ($T/\eta \rightarrow 0$) will give the values of $1/A_0$ and $(1/P_0 - \frac{1}{3})$.

Disadvantages of this technique are that the addition of sucrose and glycerol may alter the conformation of the molecule and that P_0 may depend on the solvent. In addition, τ_F has to be known to calculate τ_R. Because steady-state conditions are used, the values of τ_R obtained will be average ones. If the molecule has several types of rotation (e.g., a rodlike molecule can rotate about its long axis and rotate end over end), then it is not possible to separate the different motions. This method of obtaining τ_R is now rarely used, because it is easier to obtain molecular dynamic information *directly* from pulsed fluorescence techniques. Nevertheless, measurements of P are fairly straightforward and can still give useful empirical information.

Worked Example 5.13 Fluorescence Polarization as a Binding Assay

When an antigen labeled with a fluorescent group binds to its antibody, the polarization of the fluorescence increases from 0 to 0.5. Why?

Solution

When the fluorophore is free in solution, its rotational motions must result in small values of the polarization. On binding to the antibody, the fluorophore is immobilized and the polarization increases.

Worked Example 5.14 Motion of Retinol in a Membrane

The observed fluorescence lifetime of retinol in an erythrocyte membrane is 10 ns. When polarized light was used for excitation, the polarization of fluorescence was 0.35. What can you conclude about the motion of retinol in the membrane? If you assume $P_0 = 0.5$, what other assumptions do you have to make?

Solution

From the Perrin equation, we obtain that $(1/0.35 - \frac{1}{3}) = (1/0.5 - \frac{1}{3})(\times 1 + 10/\tau_R)$, from which $\tau_R \simeq 20$ ns. Tumbling of the membrane as a whole is expected to be much slower than this, which suggests that retinol has some local mobility, resulting from the "fluidity" of the membrane.

It is necessary to assume that the Perrin equation is valid here. In fact it really only applies for isotropic motion—which is unlikely in a membrane.

Time-Resolved Depolarization of Fluorescence Using Nanosecond Pulses. In time-resolved depolarization, the sample is excited with nanosecond pulses of polarized light. (The intensity of the fluorescence emitted in directions parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the direction of excitation are measured as a function of time. There is an *initial* difference in these intensities (because of photoselection, and hence in the anisotropy A_0, which usually decays in a few nanoseconds if the molecule rotates in solution. The results are expressed as plots of the anisotropy (A) as a function of time because $A(t)$ can be *directly* related to the correlation time.* In the simplest case, for isotropic motion in which $A(t)$ decays exponentially,

$$A(t) = A_0 e^{-t/\tau_R}$$

A plot of log A versus time is linear, with slope of $-1/\tau_R$. Note the simplicity of this method compared with the steady-state method and the fact that τ_F does not need to be known to calculate τ_R.

If there is *more* than one motion that can contribute to the decay curve, the plots of log $A(t)$ versus t will be nonlinear. The curves are then analyzed in terms

*The equation in terms of $P(t)$ is of the form $P(t) = C/(\exp(t/\tau_R) + D)$, where C and D are constants, so it is not so easy to use experimentally.

of two or more exponentials to give the rate constants and amplitudes of each motion. (The relative amplitudes give the weighting factors for the different types of motion to the decay). Actually, the analysis is not as straightforward as indicated because the various motions will occur on similar time scales and it is not always easy to separate two exponentials that have similar rates. Further, the response of the illumination (switching-on and -off time) is frequently of the order of 10^{-9} s, so the values of $I_\parallel$ and $I_\perp$ have to be corrected for this process before any further analysis.

These difficulties have limited the use of the technique somewhat; many applications involve using it empirically to detect changes rather than to obtain structural details.

Worked Example 5.15 Flexibility in Antibody Molecules

A schematic picture of an antibody molecule is shown in Figure 5.11. The fluorescent probe dimethylnaphthalenesulfonyl–lysine (DNS–lysine) binds to anti-DNA antibodies and is rigidly held in the combining site. The correlation time obtained from nanosecond-pulse fluorescence measurements of DNS–lysine was 100 ns. When a protein of molecular weight 50,000 is attached to the Fc part of the molecule, the value of the correlation time is unchanged. Can you suggest a reason for this?

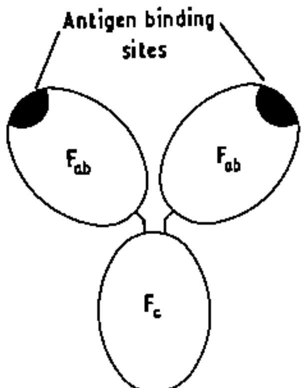


Figure 5.11

Solution

The constant value of the correlation time suggests that it is not associated with the motion of the whole molecule but rather with the Fab arms of the molecule. This indicates flexibility between the Fab and Fc parts of the molecule.

DETERMINATION OF DISTANCES BETWEEN CHROMOPHORES BY RESONANCE-ENERGY TRANSFER

The quenching of fluorescence by collisions is a very short range effect. It depends on the rate of diffusion of the molecule, which is governed by factors such as molecular size and the viscosity of the solution.

Fluorescence quenching can also occur by the transfer of excitation energy between chromophores, over much greater distances, without emission and reabsorption of radiation. This process is independent of the solution viscosity. The most common type of energy transfer is from the excited singlet state of a donor to the excited singlet state of an acceptor. In this transfer of energy, the energy donor returns from the excited state to the ground state and the energy

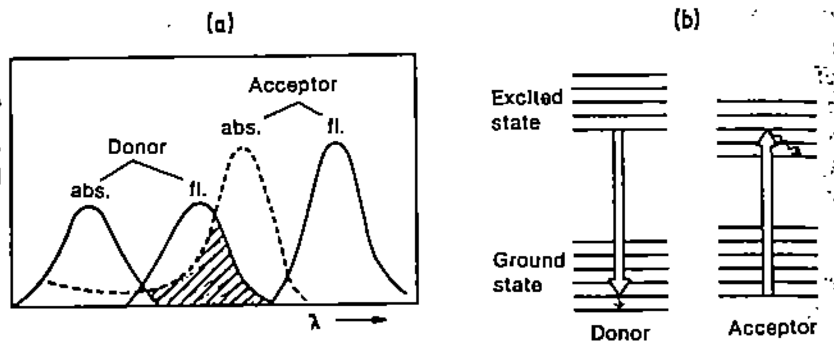


Figure 5.12 Resonance-energy transfer between donor and acceptor.

(a) The shaded area represents the region of overlap between donor fluorescence and acceptor absorption.

(b) Nonradiative relaxation to the lowest vibrational state of the ground state (by the donor) and of the first excited state (by the acceptor) leads to a loss of energy matching. Hence, continuous energy transfer back and forth between donor and acceptor cannot occur (from Cantor and Schimmel, *Biophysical Chemistry*, vol. 2 (San Francisco: W. H. Freeman and Company, Publishers, 1980)).

acceptor is simultaneously excited from its ground to its excited state. The energy separations in each case must match—that is, be in resonance.

A measure of the amount of resonance that will occur is given by the overlap of the fluorescence spectrum of the donor and the absorption spectrum of the acceptor. Figure 5.12 illustrates this resonance-energy transfer. Note that the donor and acceptor end up in excited vibrational states. Vibrational relaxation rapidly converts these to the lowest vibrational energy level in each state. The energies no longer match, so the transfer of energy back again to the original donor is unlikely. If the acceptor fluoresces, the original excitation energy reappears as acceptor emission. This is termed **sensitized fluorescence**. When the acceptor molecule is nonfluorescent, the transferred energy is dissipated by nonradiative processes.

If k_T is the rate constant for depopulation of the excited state by resonance-energy transfer, then the measured lifetime τ_T is given by

$$\frac{1}{\tau_T} = \frac{1}{\tau} + k_T$$

where $1/\tau$ is the rate of depopulation, in the absence of resonance-energy transfer.

The efficiency (E_T) of depopulation by resonance-energy transfer is then

$$E_T = \frac{k_T}{1/\tau + k_T} = \frac{1/\tau_T - 1/\tau}{1/\tau_T} \tag{5.1}$$

$$\therefore \frac{\tau_T}{\tau} = 1 - E_T$$

If Φ_T and Φ_D represent the quantum yields of the donor in the presence and absence of resonance-energy transfer, then

$$\frac{\Phi_T}{\Phi_D} = 1 - E_T$$

Förster has explained this resonance-energy transfer in terms of a dipole-dipole interaction between the donor and acceptor pair. The energy of a dipole-dipole interaction depends on $1/R^3$, where R is the intermolecular distance. The rate of energy transfer (k_T) is proportional to the square of this interaction and hence to $1/R^6$. If we define R_0 as the distance at which the energy transfer is 50% efficient, i.e., when $1/\tau = k_T$, then equation 5.1 can be rewritten as

$$E_T = \frac{R^{-6}}{R_0^{-6} + R^{-6}} = \frac{R_0^6}{R^6 + R_0^6} \tag{5.2}$$

or

$$R = R_0\left(\frac{1 - E_T}{E_T}\right)^{1/6}$$

where R_0 is a constant for each donor-acceptor pair. From a measurement of the quenching of the fluorescence (ϕ_T/ϕ_D) (or better, the lifetime τ_T/T) in the presence and absence of the acceptor, R can be calculated if R_0 is known.

Worked Example 5.16 Energy Transfer Depends on the Sixth Power of the Distance Between the Donor and Acceptor Pair

A model system for the study of the dependence of energy transfer on the separation of donor (napthyl) is dansyl-(L-propyl)n-α-naphthyl semicarbazide (see L. Stryer and R. P. Haughland, *Proc. Nat. Acad. Sci. U.S.A.*, 58 (1967): 719–726). The proline residues form a helix, the dimensions of which are known, so the distances between the napthyl and dansyl groups can be worked out. The measured efficiencies of transfer for these compounds fit well with those from (5.2) (confirming the R^6 dependence). (a) From the data in Figure 5.13, estimate R_0. (b) Very often the efficiency of transfer is calculated by measuring the quenching of the fluorescence intensity. Calculate what value of this you would expect at a distance of 6.0 nm between donor and acceptor in the system here. Comment on the use of the method in light of this.

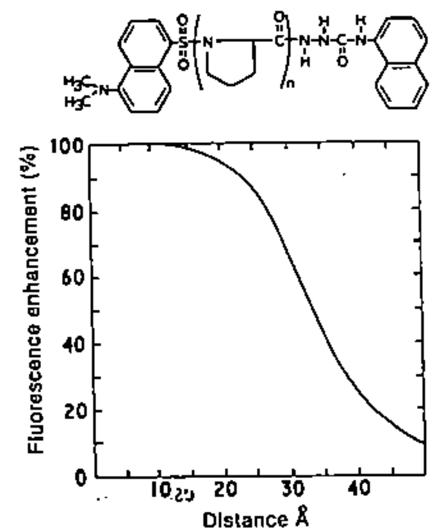


Figure 5.13

(continued)

Worked Example 5.16 (continued)

Solution

a. From the graph, R_0 (the distance at which the energy transfer is 50% efficient) is about 3.4 nm.

b. When $R = 6.0$ nm, then from equation 5.2,

$$E = \frac{6^6}{6^6 + 3.4^6} = 0.97$$

Now

$$\frac{I_T}{I_D} = \frac{\phi_T}{\phi_D} = 1 - E$$

when I_T and I_D are the fluorescence intensities in the presence and absence of energy transfer, respectively.

$$\therefore \frac{I_T}{I_D} = 1 - 0.97 = 0.03$$

The fluorescence signal is quenched 3%.

This quenching is very small and suggests that distances as large as this cannot be reliably measured in this system by energy transfer. The range of distances that can be measured will depend on the value of R_0 for the system. The quantity R_0 can be evaluated from the parameters of the absorption and fluorescence spectra as shown in the next section.

Calculation of R_0. The value of R_0 (in nanometers) can be obtained from the expression

$$R_0 = 9.79 \times 10^2 (J\bar{\nu}n^{-4}k^2\Phi_D)^{1/6}$$

where n is the refractive index of the medium, Φ_D is the quantum yield of the energy donor (in the absence of the acceptor), k^2 is the orientation factor between the donor and acceptor electric transition dipole moments (see Appendix IX), and J is the integral of the spectral overlap of the absorption spectrum of the acceptor and the emission spectrum of the donor. In Figure 5.14 J is given by

$$J = \int F_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda$$

The term $\epsilon_A(\lambda)$ represents the extinction coefficient of the acceptor at each value λ and therefore the absorption spectrum of the acceptor. The term $F_D(\lambda)$ is the fraction of total donor fluorescence occurring at λ.* The value J is usually calculated graphically.

*The emission spectrum cannot be quantified in the same way as the absorption spectrum, since the observed fluorescence intensity depends on the intensity of the light absorbed multiplied by a factor that comprises both the quantum yield and the response of the instrument to fluorescence. This means that the observed fluorescence intensity is in arbitrary units.

From the expression for R_0, two conditions favor singlet-singlet resonance-energy transfer: (1) a large spectral overlap, J, between the absorption bands of the donor and acceptor molecules and (2) a high quantum yield ϕ of the donor molecule in the absence of the acceptor. However, the major uncertainty in calculating R_0 arises from the lack of knowledge of the orientation factor (k^2). This term is equal to $(3\cos^2\theta - 1)^2$, where θ is the angle between the electric transition dipole moments of the donor-acceptor pair. In principle, k^2 can vary between 0 (the two transition dipoles perpendicular) and 4 (parallel) (see Appendix IX). The theory will apply in macromolecules, provided the transition dipoles are rigidly fixed, but the orientation factor will not generally be known. Most authors assume a value of k^2 of $\frac{2}{3}$, which is the factor for an isotropic rapidly rotating system. If this is not the case, then some uncertainty may be introduced into the calculated distance. (For a good discussion on this problem see the article "Fluorescence Energy Transfer as a Spectroscopic Rule" by L. Stryer *Ann. Rev. Biochem.*, 47(1978):819–46.) Stryer concludes that this uncertainty is likely to be small—usually less than 20%.

Worked Example 5.17 Graphical Evaluation of J

Figure 5.14 shows hypothetical fluorescence and absorption spectra for a hypothetical donor-acceptor pair. Evaluate J.

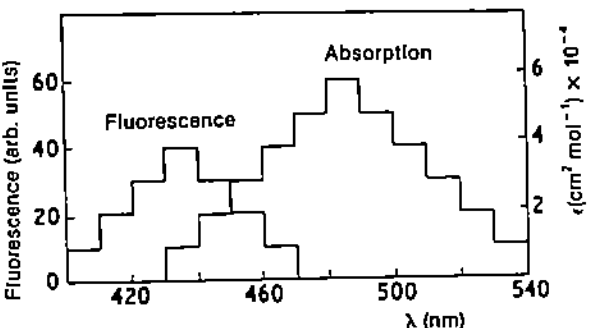


Figure 5.14

Solution

Since it is not possible to write exact expressions for $F_D(\lambda)$ or $\epsilon_A(\lambda)$ as functions of λ, we must replace the integral with a sum

$$J = \sum_{i=1}^{i=4} J_i = \sum_{i=1}^{i=4} F_D(\bar{\lambda}_i)\epsilon_A(\bar{\lambda}_i)\bar{\lambda}^4 \Delta\lambda$$

Here we have divided the shaded overlap area into four numbered rectangles, each of width $\Delta\lambda = 10$ nm. We can then determine the values of F_D and ϵ_A at the midpoint

(continued)

Worked Example 5.17 (continued)

wavelength ($\bar{\lambda}_i$) of each rectangle. Similarly the fluorescence spectrum can be divided into seven rectangles.

First, we evaluate $F_D(\bar{\lambda}_i)$. This is given by

$$F_D(\bar{\lambda}_i) = F_i \Big/ \sum_{i=1}^{i=7} F_j \Delta\lambda$$

where F_i is the fluorescence intensity at $\bar{\lambda}_i$. Note that the sum in the denominator is taken over the entire fluorescence spectrum.

Hence

$$\Sigma F_j \Delta\lambda = (10 + 20 + 30 + 40 + 30 + 20 + 10) \cdot 10 = 1600 \text{ nm} = 1.6 \times 10^{-4} \text{ cm}$$

This value can be used in conjunction with the fluorescence intensities and extinction coefficients at the four midpoint wavelengths to construct the following table:

i	$\bar{\lambda}_i$ (cm)	$\bar{\lambda}_i^4$ (cm^4)	$F_D(\bar{\lambda}_i)$ (cm^{-1})	$\epsilon_A(\bar{\lambda}_i)$ (mol·dm^{-3})$^{-1}$·cm^{-1}	J_i cm^3·M^{-1}
1	4.35×10^{-5}	3.58×10^{-18}	25.0×10^4	1×10^4	8.95×10^{-15}
2	4.45×10^{-5}	3.92×10^{-18}	18.8×10^4	2×10^4	1.47×10^{-14}
3	4.55×10^{-5}	4.29×10^{-18}	12.5×10^4	3×10^4	1.61×10^{-14}
4	4.65×10^{-5}	4.68×10^{-18}	6.25×10^4	4×10^4	1.17×10^{-14}

Hence $J = 5.14 \times 10^{-14}$ cm^3·(mol·dm^{-3})$^{-1}$

Note: Sometimes the unnormalized form of $F_D(\lambda)$ is used directly in J, in which case J becomes

$$J = \frac{F_D(\lambda)\epsilon_A(\lambda)\lambda^4 \, d\lambda}{F_D(\lambda)\, d\lambda}$$

Worked Example 5.18 Energy Transfer as a Spectroscopic Ruler: Can Rhodopsin Traverse the Disc Membrane?

Rhodopsin is a photoreceptor protein that is in integral part of the disc membranes of vertebrate retinal rod cells. Rhodopsin consists of opsin, a protein, and 11-*cis* retinal (see Figure 5.15(a)), a prosthetic group. The

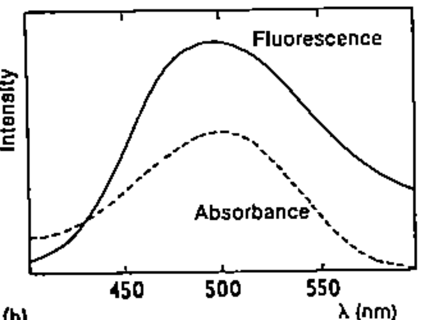
(a)

(continued)

Worked Example 5.18 (continued)

color of rhodopsin and its responsiveness to light depend on the presence of the 11-*cis* retinal, which is a very effective chromophore giving a broad absorption band at 500 nm.

Rhodopsin was covalently labelled with 1.5-I AEDANS, (formula is shown in Figure 5.5). The labeled rhodopsin retained the 500-nm absorption band and was regenerable after bleaching (removal of 11-*cis* retinal), which suggests that the introduction of the probe had not altered the overall conformation significantly. The overlap between the fluorescence emission spectrum of the 500-nm absorption band of 11-*cis* retinal is shown in Figure 5.15(b). Use the data to show whether rhodopsin is sufficiently long to traverse the membrane, which is estimated from electron microscopy to be 7.5 nm wide.



(b)

Figure 5.15

The quantum yields in the presence and absence of 11-*cis* retinal are 0.68 and 0.75, respectively. The refractive index n of the medium is 1.33; the orientation factor $k^2 = \frac{2}{3}$. The overlap integral $J = 1.84 \times 10^{13}$ cm^3·(mol·dm^{-3})$^{-1}$.

Solution

We first calculate R_0 from

$$\begin{aligned} R_0 &= 9.79 \times 10^2 (Jn^{-4}k^2\phi_D)^{1/6} \\ &= 9.79 \times 10^2 (1.84 \times 10^{13} \times 1.33)^{-4} \times 0.67 \times 0.75)^{1/6} \\ &= 5.4 \text{ nm} \end{aligned}$$

The ratio of the quantum yields in the presence and absence of 11-*cis* retinal is $0.68/0.75 = 0.91$. The transfer efficiency is therefore $1 - 0.91 = 0.09$. We use this value to obtain

$$\begin{aligned} R &= 5.4\left(\frac{0.91}{0.98}\right)^{1/6} \\ &= 7.9 \text{ nm} \end{aligned}$$

Rhodopsin is thus long enough to traverse the membrane.

FLUORESCENT ANTIBODIES

The specific binding of antibodies, coupled with the sensitivity of fluorescence, forms the basis of the use of fluorescent antibodies in various processes such as cell sorting. After a cell population is stained with fluoresceinated antibody, the mixed cell suspension can be examined under a fluorescence microscope (see Chapter 11) and the number of stained cells counted.

PHOSPHORESCENCE

The presence of a second exicted state is responsible for phosphorescence. In most biological chromophores, this other excited state is a triplet state. We recall that the triplet state has the spins of two of its electrons arranged in a parallel, rather than in an opposed, manner. Transitions between states of different multiplicities are theoretically forbidden, so the population and depopulation of the triplet state proceeds as follows (see Figure 5.16):

Excitation of electrons from the ground singlet state results in transitions to the upper singlet state. Here any excess vibrational energy in the excited state is rapidly transferred to the surroundings as heat. As the molecule "steps down" the vibrational energy levels, there is a finite possibility that if a vibrational energy level of the triplet state coincides with one from the excited singlet state,

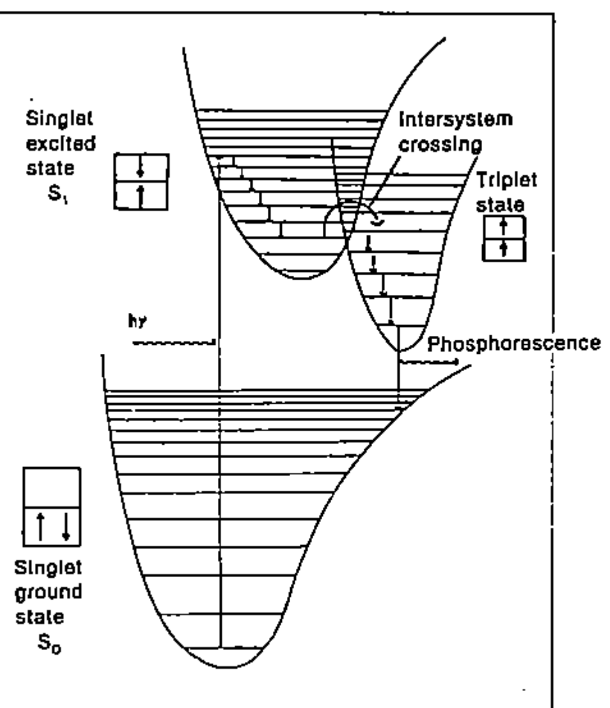


Figure 5.16 Processes leading to phosphorescence.

then the molecule will transfer to the triplet state, reversing the orientation of the spin of one of its electrons.* This switch is known as **intersystem crossing**. The reversal of the spin is characterized by a first-order rate constant, which is about $10^9\ s^{-1}$. Once in the triplet state, the molecule continues losing energy until it reaches the lowest triplet vibrational level. From here it can return (relax) to the ground singlet state by the spontaneous emission of radiation known as **phosphorescence**. Note that the emission wavelength is red shifted with respect to fluorescence.

The rate of depopulation of the triplet level by spontaneous emission will be very slow because the transition dipole connecting the two states is very small (forbidden transition). The lifetime of this state is therefore very long (typically 10^{-2} s to 10^2 s). The long lifetime of the triplet state makes it highly vulnerable to any quenching process that will remove energy—such as collisions with the solvent. For this reason phosphorescence is rarely observed in solution.

Worked Example 5.19 Triplet Probes of Molecular Motion

Eosin derivatives (Figure 5.17) are useful as triplet probes. In these, the nature of the group X is such that it can be covalently attached to proteins. When the triplet state is populated with molecules that have been photoselected following flash excitations of the S_0-S_1 transition with plane-polarized light, the resulting polarization of the phosphorescence is anisotropic. The time dependence of this anisotropy can be measured.

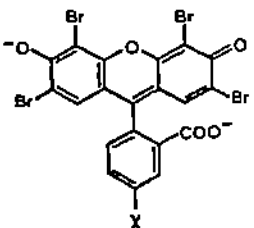


Figure 5.17

What is the main difference in the information on molecular motion available from such measurements in phosphorescence compared with fluorescence?

Solution

The main difference results from the lifetimes of the triplet and S_1 states. The lifetimes give the time scale over which the emission anisotropy can detect molecular motion. The lifetime of the triplet state (milliseconds) means that relatively slow molecular motions can be detected. An example would be the rotation of a protein molecule in a membrane. The fluorescence method detects motions in the nanosecond range (for an introduction to triplet probes, see R. J. Cherry, *Biochim. Biophys. Acta,* 559 (1979):289–327.)

*The triplet state is lower in energy than the first excited singlet state.

PROBLEMS

1. What is the difference between the time for a transition and the relaxation time (or lifetime) in fluorescence?
2. Several competing events depopulate the excited state. Write down an expression for the lifetime τ in terms of the rate constants of the following processes: (a) Intersystem crossing k_{IC}, (b) energy transfer k_T, (c) internal conversion k_C, (d) collisional quenching k_Q in the presence of a large amount of quencher Q, (e) fluorescence k_F.
3. In solution of chlorophyll, the measured lifetime is about 7 ns. However, in a photosynthetic unit, the lifetime is estimated to be about 0.1 ns. Can you suggest a reason for this difference? What are the relative fluorescence yields of chlorophyll in solution and in the photosynthetic unit? The radiative lifetime of chlorophyll is 25 ns.
4. *N*-bromosuccinimide (NBr) oxidizes tryptophan, and the product is non-fluorescent. Modification of papain by NBr showed that 2 molar equivalents modified Trp-69 and 4 molar equivalents modified Trp-69 and -177. The variation of the fluorescence spectra (excitation at 288 nm) with pH of the native, Trp-69 modified, and Trp-69 and -177 modified enzymes are shown in Figure 5.18. What conclusions can you draw from the data?

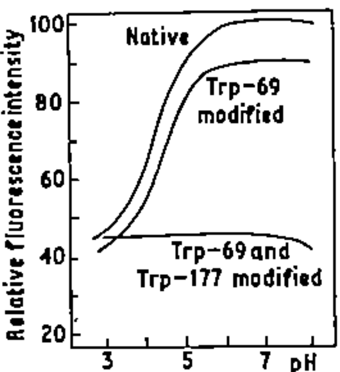


Figure 5.18

5. 1-Anilinonaphthalene-8-sulfonate (ANS) can be used as a probe for ligand binding to glutamate dehydrogenase (GDH). The ANS fluorescence alters in the presence of GDH (see Figure 5.19(a)). What conclusions might you draw from this? The addition of NADH or an inhibitor, guanosine-5′-triphosphate (GTP), alone does not alter the fluorescence of the ANS. However, in the presence of GTP, titration of the solution with NADH alters the fluorescence in a sigmoidal manner (see Figure 5.19(b)). Is ANS a useful probe for this system?

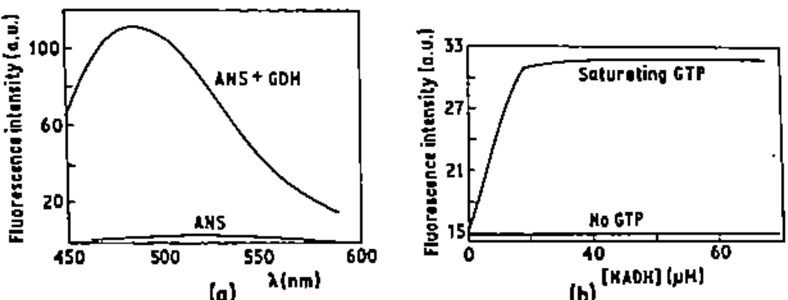


Figure 5.19

6. The intensity of ANS fluorescence increases with concentration until it reaches a maximum value, when it starts to decrease. Why?
7. Tryptophan fluoresence quenching is widely used to monitor the binding between 2,4-dinitrophenyl (Dnp) haptens and specific antibodies. Figure 5.20(a) shows the absorption and emission spectra of a purified antibody specific for Dnp ligands and the absorption spectra of ϵ-Dnp lysine. The large overlap of the absorption spectrum of ϵ-Dnp lysine with the emission spectra of the antibody results in highly efficient energy transfer. Figure 5.20(b) shows two typical titration curves for a strongly binding and a weakly binding hapten with antibody. The fluorescence quenching of a blank solution containing tryptophan (at the same concentration as the protein) and hapten is also shown. Why is it necessary to correct for the data of the blank quenching in determining the binding constant? Does this limit the utility of the method?

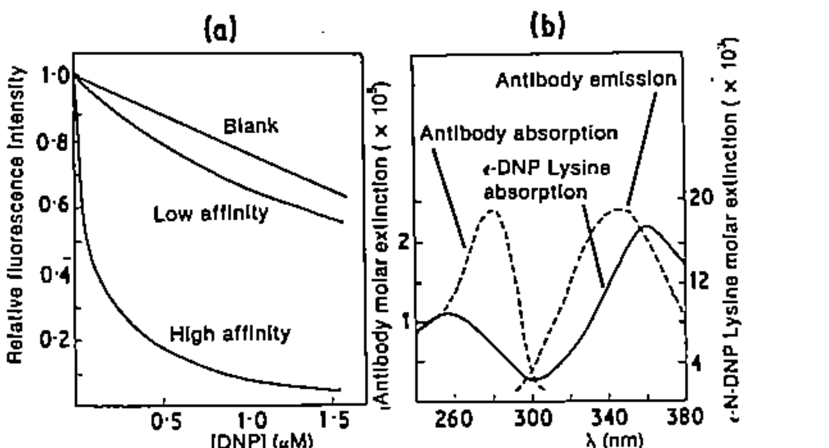


Figure 5.20

8. Thermolysin is a proteolytic enzyme (MW 37,500) that binds four calcium ions, which stabilize it against denaturation and autolysis. Two of these calcium ions are close together and can be substituted by a single terbium ion (Tb^{3+}). The active site of the enzyme also contains a zinc atom, which is essential for activity and which can be replaced by Co^{2+}. The fluorescence of terbium is partially quenched when Co^{2+} replaces zinc at the active site because of energy transfer (see Figure 5.21). Given that $R_0 = 1.63$ nm for the Tb^{3+} donor and Co^{2+} acceptor, calculate the distance between the two metal sites.

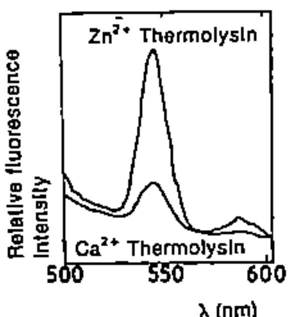


Figure 5.21

9. The kinetics of carboxypeptidase cleavage of the peptide dansyl-Gly-L-Phe were followed by monitoring the tryptophan and dansyl (DNS) fluorescence as a function of time after rapid mixing using stopped-flow methods.
   (a) Explain why the fluorescence of tryptophan increases while that of dansyl decreases.
   (b) When the zinc ion is replaced by Co^{2+}, the enzyme is active, but no dansyl fluorescence is observed. Suggest a reason for this and what information might be obtained from this observation. (The fluorescence spectra of tryptophan and DNS, together with the absorption spectra of DNS and Co^{2+} in the enzyme are shown in Figure 5.22.)

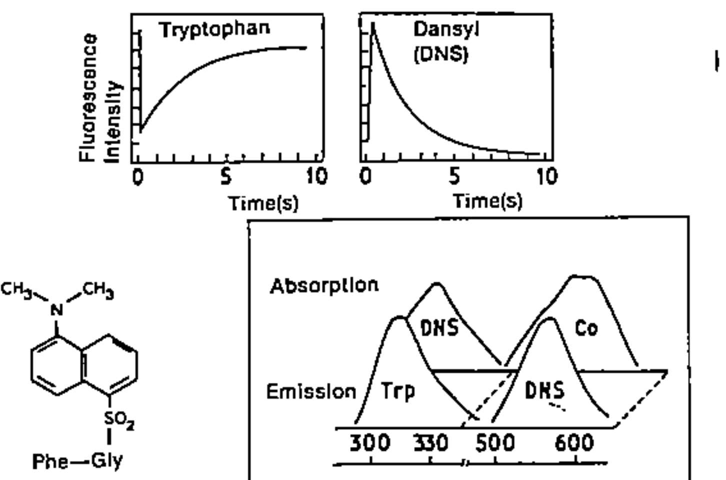


Figure 5.22

10. Figure 5.23 shows the time dependence of the anisotropy of the polarization of the fluorescence emission of dansyl-lysine in the combining sites of the Fab fragment and the $(Fab)_2$ fragment (see Figure 5.11). For Fab alone, a value of the rotational relaxation time of 26 ns was obtained from the plot. This value is about that expected if the Fab fragment behaved as a rigid unit. For the $(Fab)_2$ fragment, the curved plot of the results was analyzed in terms of *two* rotational times of 26 ns and 100 ns. What does this suggest about the site of flexibility?

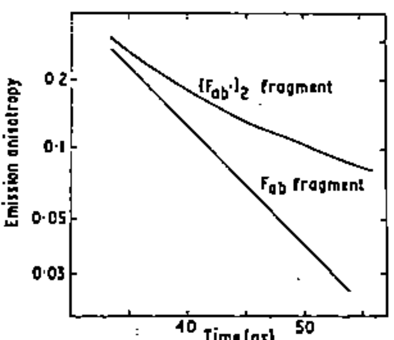


Figure 5.23

11. A protein undergoes a reversible transition between a rigid native and flexible unfolded form. A schematic plot of the predicted dependence of log A (anisotropy of the fluorescence polarization) versus time is shown in Figure 5.24(b) and (c) for two different models for the protein transition. What can you deduce about the relative rates of interconversion in each model? (Plots of log A versus time for the fully native and unfolded forms are given in Figure 5.24(a).

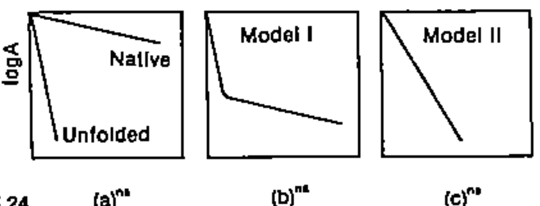


Figure 5.24

12. Energy-transfer experiments between a covalent probe and 11-*cis* retinal have suggested that rhodopsin is at least 7.5 nm long. The calculation requires the value of the overlap integral between the emission spectrum of the energy donor and the absorption spectrum of 11-*cis* retinal. These are shown in Figure 5.25. The shaded area can be divided into approximately eight rectangular strips each 25 nm wide (2.5×10^{-6} cm). From the following data, which gives the values of the absorbance and fluorescence intensity at the midpoints of these strips, evaluate the overlap integral J.

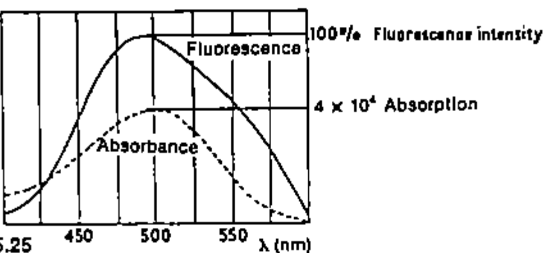


Figure 5.25

λ_i ($\times 10^5$) (cm)	$\epsilon_A(\bar{\lambda}_i)$ ($\times 10^{-4}$) $(\text{mol}\cdot\text{dm}^{-3})^{-1}\cdot\text{cm}^{-1}$	$F_D(\lambda_i)$ (%)
4.125	0.76	8
4.375	1.9	36
4.625	2.9	74
4.875	3.7	97
5.125	3.7	91
5.375	2.3	77
5.625	0.95	51
5.875	0.38	37