Quenching of Fluorescence

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching. These include excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching. In this chapter we will be concerned primarily with quenching resulting from collisional encounters between the fluorophore and quencher, which is called collisional or dynamic quenching. We will also discuss static quenching, which can be a valuable source of information about binding between the fluorescent sample and the quencher. Static quenching can also be a complicating factor in the data analysis. In addition to the processes described above, apparent quenching can occur due to the optical properties of the sample. High optical densities or turbidity can result in decreased fluorescence intensities. This trivial type of quenching contains little molecular information. Throughout this chapter we will assume that such trivial effects are not the cause of the decreases in fluorescence intensity.

Fluorescence quenching has been widely studied both as a fundamental phenomenon, and as a source of information about biochemical systems. These biochemical applications of quenching are due to the molecular interactions that result in quenching. Both static and dynamic quenching require molecular contact between the fluorophore and quencher. In the case of collisional quenching, the quencher must diffuse to the fluorophore during the lifetime of the excited state. Upon contact, the fluorophore returns to the ground state, without emission of a photon. In general, quenching occurs without any permanent change in the molecules, that is, without a photochemical reaction. In static quenching a complex is formed between the fluorophore and the quencher, and this complex is nonfluorescent. For either static or dynamic quenching to occur the fluorophore and quencher must be in contact. The requirement of molecular contact for quenching results in the numerous applications of quenching. For example, quenching measurements can reveal the accessibility of fluorophores to quenchers. Consider a fluorophore bound either to a protein or a membrane. If the protein or membrane is impermeable to the quencher, and the fluorophore is located in the interior of the macromolecule, then neither collisional nor static quenching can occur. For this reason quenching studies can be used to reveal the localization of fluorophores in proteins and membranes, and their permeabilities to quenchers. Additionally, the rate of collisional quenching can be used to determine the diffusion coefficient of the quencher.

It is important to recognize that the phenomenon of collisional quenching results in the expansion of the volume and distance within the solution which affects the fluorophore. The root-mean-square distance $\sqrt{\Delta x^2}$ that a quencher can diffuse during the lifetime of the excited state (τ) is given by $\sqrt{\Delta x^2} = \sqrt{2D\tau}$, where D is the diffusion coefficient. Consider an oxygen molecule in water at 25°C. Its diffusion coefficient is 2.5 x 10^{-5} cm^2/s. During a typical fluorescence lifetime of 4 ns the oxygen molecule can diffuse 45 Å. If the lifetime is longer, diffusion over still larger distances can be observed. For example, for lifetimes of 20 and 100 ns the average distances for oxygen diffusion are 100 and 224 Å, respectively. With the use of longer-lived probes with microsecond lifetimes (Chapter 20), diffusion over still larger distances can be observed. Hence, fluorescence quenching can reveal the diffusion of quenchers over moderately large distances comparable to the size of proteins and membranes. This situation is different from solvent relaxation. Spectral shifts resulting from reorientation of the solvent molecules are due primarily to the solvent shell immediately adjacent to the fluorophore.

8.1. QUENCHERS OF FLUORESCENCE

A wide variety of substances act as quenchers of fluorescence. One of the best-known collisional quenchers is molecular oxygen,[1] which quenches almost all known fluorophores. Depending upon the sample under investigation, it is frequently necessary to remove dissolved oxygen to obtain reliable measurements of the fluorescence yields or lifetimes. The mechanism by which oxygen quenches has been a subject of debate. The most likely mechanism is that the paramagnetic oxygen causes the fluorophore to undergo intersystem crossing to the triplet state. In fluid solutions the long-lived triplets are completely quenched, so that phosphorescence is not observed. Aromatic and aliphatic amines are also efficient quenchers of most unsubstituted aromatic hydrocarbons. For example, anthracene fluorescence is effectively quenched by diethylaniline.[2] For anthracene and diethylaniline the mechanism of quenching is the formation of an excited charge-transfer complex. The excited-state fluorophore accepts an electron from the amine. In nonpolar solvents fluorescence from the excited charge-transfer complex (exciplex) is frequently observed, and one may regard this process as an excited state reaction rather than quenching. In polar solvents the exciplex emission is often quenched, so that the fluorophore–amine interaction appears to be that of simple quenching. While it is now known that there is a modest through-space component to almost all quenching reactions, this component is short range (<2 Å), so that molecular contact is a requirement for quenching.

Another type of quenching is due to heavy atoms such as iodide and bromide. Halogenated compounds such as trichloroethanol and bromobenzene also act as collisional quenchers. Quenching by the larger halogens such as bromide and iodide may be a result of intersystem crossing to an excited triplet state, promoted by spin–orbit coupling of the excited (singlet) fluorophore and the halogen.[3] Since emission from the triplet state is slow, the triplet emission is highly quenched by other processes. The quenching mechanism is probably different for chlorine-containing substances. Indole, carbazole, and their derivatives are uniquely sensitive to quenching by chlorinated hydrocarbons and by electron scavengers[4] such as protons, histidine, cysteine, NO_3^-, fumarate, Cu^{2+}, Pb^{2+}, Cd^{2+}, and Mn^{2+}. Quenching by these substances probably involves a donation of an electron from the fluorophore to the quencher. Additionally, indole, tryptophan, and its derivatives are quenched by acrylamide, succinimide, dichloroacetamide, dimethylformamide, pyridinium hydrochloride, imidazolium hydrochloride, methionine, Eu^{3+}, Ag^+, and Cs^+. Quenchers of protein fluorescence have been summarized in several insightful reviews.[5–7] Hence a variety of quenchers are available for studies of protein fluorescence, especially to determine the surface accessibility of tryptophan residues and the permeation of proteins by the quenchers.

Additional quenchers include purines, pyrimidines, N-methylnicotinamide and N-alkyl pyridinium, and picolinium salts.[8–9] For example, the fluorescence of flavin adenine dinucleotide (FAD) and reduced nicotinamide adenine dinucleotide (NADH) are both quenched by the adenine moiety. Flavin fluorescence is quenched by both static and dynamic interactions with adenine,[10] whereas the quenching of dihydronicotinamide appears to be primarily dynamic.[11] These aromatic substances appear to quench by formation of charge-transfer complexes. Depending upon the precise structure involved, the ground-state complex can be reasonably stable. As a result, both static and dynamic quenching are frequently observed. A variety of other quenchers are known. These are summarized in Table 8.1, which is intended to be an overview and not a complete list. Known collisional quenchers include hydrogen peroxide, nitric oxide (NO), nitroxides, BrO_4^-, and even some olefins.

Because of the variety of substances that act as quenchers, one can frequently identify fluorophore–quencher combinations for a desired purpose. It is important to note that not all fluorophores are quenched by all the substances listed above. This fact occasionally allows selective quenching of a given fluorophore. The occurrence of quenching depends upon the mechanism, which in turn depends upon the chemical properties of the individual molecules. Detailed analysis of the mechanism of quenching is complex. In this chapter we will be concerned primarily with the type of quenching, that is, whether quenching depends on diffusive collisions or formation of ground-state complexes. Later in this chapter we describe biochemical applications of quenching. The mechanisms of quenching will be discussed in the following chapter.

8.2. THEORY OF COLLISIONAL QUENCHING

Collisional quenching of fluorescence is described by the Stern-Volmer equation:

$$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q] \qquad (8.1)$$

Table 8.1. Quenchers of Fluorescence

Quenchers	Typical fluorophore	References
Acrylamide	Tryptophan, pyrene, and other fluorophores	5–7, 176–180
Amines	Anthracene, perylene	2, 124, 181–186
Amines	Carbazole	187
Amine anesthetics	Perylene, anthroyloxy probes	188–190
Bromate	–	191
Bromobenzene	Many fluorophores	192
Carbon disulfide	Laser dyes, perylene	193
Carboxy groups	Indole	194
Cesium (Cs^+)	Indole	195
Chlorinated compounds	Indoles and carbazoles	196–199
Chloride	Quinolinium, SPQ	200–203
Cobalt (Co^{2+})	NBD, PPO, Perylene (Energy transfer for some probes)	204–210
Dimethylformamide	Indole	211
Disulfides	Tyrosine	212
Ethers	9-Arylxanthyl cations	213
Halogens	Anthracene, naphthalene, carbazole	214–229
Halogen anesthetics	Pyrene, tryptophan	230–232
Hydrogen peroxide	Tryptophan	233
Iodide	Anthracene	234–237
Imidazole, histidine	Tryptophan	238
Indole	Anthracene, pyrene, cyanoanthracene	239–241
Methylmercuric chloride	Carbazole, pyrene	242
Nickel (Ni^{2+})	Perylene	243–244
Nitromethane and nitro compounds	Polycyclic aromatic hydrocarbon	245–256
Nitroxides	Naphthalene, PAH, Tb^{3+}, anthroyloxy probes	257–266
NO (nitric oxide)	Naphthalene, pyrene	267–270
Olefins	Cyanonaphthalene, 2,3-dimethylnaphthalene, pyrene	271–273
Oxygen	Most fluorophores	274–290
Peroxides	Dimethylnaphthalene	291
Picolinium nicotinamide	Tryptophan, PAH	292–296
Pyridine	Carbazole	297
Silver (Ag^+)	Perylene	298
Succinimide	Tryptophan	299–300
Sulfur dioxide	Rhodamine B	301
Thallium (Tl^+)	Naphthylamine sulfonic acid	302
Thiocyanate	Anthracene, 5,6-benzoquinoline	303–304
Xenon		305

In this equation F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively; k_q is the bimolecular quenching constant; τ_0 is the lifetime of the fluorophore in the absence of quencher, and Q is the concentration of quencher. The Stern-Volmer quenching constant is given by $K_D = k_q\tau_0$. If the quenching is known to be dynamic, the Stern-Volmer constant will be represented by K_D. Otherwise this constant will be described as K_{SV}.

Quenching data are usually presented as plots of F_0/F versus $[Q]$. This is because F_0/F is expected to be linearly dependent upon the concentration of quencher. A plot of F_0/F versus $[Q]$ yields an intercept of one on the y-axis and a slope equal to K_D (Figure 8.1). Intuitively, it is useful to note that K_D^{-1} is the quencher concentration at which $F_0/F = 2$ or 50% of the intensity is quenched. A linear Stern-Volmer plot is generally indicative of a single class of fluorophores, all equally accessible to quencher. If two fluorophore populations are present, and one class is not accessible to quencher, then the Stern-Volmer plots deviate from linearity toward the x-axis. This result is frequently found

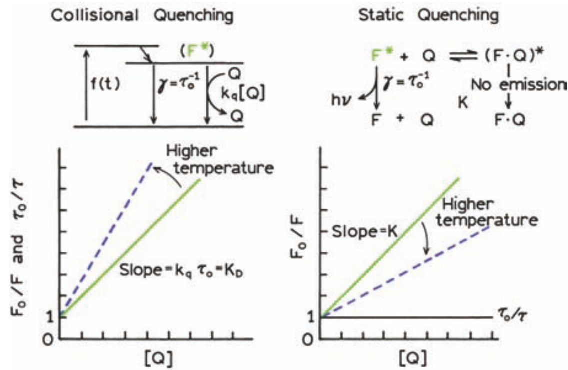

Figure 8.1. Comparison of dynamic and static quenching.

for the quenching of tryptophan fluorescence in proteins by polar or charged quenchers. These molecules do not readily penetrate the hydrophobic interior of proteins, and only those tryptophan residues on the surface of the protein are quenched.

It is important to recognize that observation of a linear Stern-Volmer plot does not prove that collisional quenching of fluorescence has occurred. In Section 8.3 we will see that static quenching also results in linear Stern-Volmer plots. Static and dynamic quenching can be distinguished by their differing dependence on temperature and viscosity, or preferably by lifetime measurements. Higher temperatures result in faster diffusion and hence larger amounts of collisional quenching (Figure 8.1). Higher temperature will typically result in the dissociation of weakly bound complexes, and hence smaller amounts of static quenching.

8.2.1. Derivation of the Stern-Volmer Equation

The Stern-Volmer equation can be derived by consideration of the fluorescence intensities observed in the absence and presence of quencher. The fluorescence intensity observed for a fluorophore is proportional to its concentration in the excited state, $[F^*]$. Under continuous illumination a constant population of excited fluorophores is established, and therefore $d[F^*]/dt = 0$. In the absence and presence of quencher the differential equations describing $[F^*]$ are

$$\frac{d[F^*]}{dt} = f(t) - \gamma[F^*]_0 = 0 \tag{8.2}$$

$$\frac{d[F^*]}{dt} = f(t) - (\gamma + k_q[Q])[F^*] = 0 \tag{8.3}$$

where $f(t)$ is the constant excitation function, and $\tilde{a} = \tau_0^{-1}$ is the decay rate of the fluorophore in the absence of quencher. In the absence of quenching the excited-state population decays with a rate $\tilde{a} = (\Gamma + k_{nr})$, where Γ is the radiative rate and k_{nr} is the non-radiative decay rate. In the presence of quencher there is an additional decay rate $k_q[Q]$. With continuous excitation the excited-state population is constant, so the derivatives in these equations can be set to zero. Division of eq. 8.3 by 8.2 yields

$$\frac{F_0}{F} = \frac{\gamma + k_q[Q]}{\gamma} = 1 + k_q\tau_0[Q] \tag{8.4}$$

which is the Stern-Volmer equation.

The Stern-Volmer equation may also be obtained by considering the fraction of excited fluorophores, relative to the total, which decay by emission. This fraction (F/F_0) is given by the ratio of the decay rate in the absence of quencher (γ) to the total decay rate in the presence of quencher $(\gamma + k_q[Q])$:

$$\frac{F}{F_0} = \frac{\gamma}{\gamma + k_q[Q]} = \frac{1}{1 + K_D[Q]} \tag{8.5}$$

which is again the Stern-Volmer equation. Since collisional quenching is a rate process that depopulates the excited state, the lifetimes in the absence (τ_0) and presence (τ) of quencher are given by

$$\tau_0 = \gamma^{-1} \tag{8.6}$$

$$\tau = (\gamma + k_q[Q])^{-1} \tag{8.7}$$

and therefore

$$\frac{\tau_0}{\tau} = 1 + k_q\tau_0[Q] \tag{8.8}$$

This equation illustrates an important characteristic of collisional quenching, which is an equivalent decrease in fluorescence intensity and lifetime (Figure 8.1, left). For collisional quenching

$$\frac{F_0}{F} = \frac{\tau_0}{\tau} \tag{8.9}$$

The decrease in lifetime occurs because quenching is an additional rate process that depopulates the excited state.

The decrease in yield occurs because quenching depopulates the excited state without fluorescence emission. Static quenching does not decrease the lifetime because only the fluorescent molecules are observed, and the uncomplex fluorophores have the unquenched lifetime τ_0.

8.2.2. Interpretation of the Bimolecular Quenching Constant

In papers on quenching one frequently encounters the bimolecular quenching constant (k_q), which reflects the efficiency of quenching or the accessibility of the fluorophores to the quencher. As shown below, diffusion-controlled quenching typically results in values of k_q near 1 x 10^{10} M^{-1} s^{-1}. Values of k_q smaller than the diffusion-controlled value can result from steric shielding of the fluorophore or a low quenching efficiency. Apparent values of k_q larger than the diffusion-controlled limit usually indicate some type of binding interaction.

The meaning of the bimolecular quenching constant can be understood in terms of the collisional frequency between freely diffusing molecules. The collisional frequency (Z) of a fluorophore with a quencher is given by

$$Z = k_0[Q] \tag{8.10}$$

where k_0 is the diffusion-controlled bimolecular rate constant. This constant may be calculated using the Smoluchowski equation:

$$k_0 = 4\pi RDN/1000 = \frac{4\pi N}{1000}(R_f + R_q)(D_f + D_q) \tag{8.11}$$

where R is the collision radius, D is the sum of the diffusion coefficients of the fluorophore (D_f) and quencher (D_q), and N is Avogadro's number. The collision radius is generally assumed to be the sum of the molecular radii of the fluorophore (R_f) and quencher (R_q). This equation describes the diffusive flux of a molecule with a diffusion coefficient D through the surface of a sphere of radius R. The factor of 1000 is necessary to keep the units correct when the concentration is expressed in terms of molarity. The term N/1000 converts molarity to molecules/cm^3.

The collisional frequency is related to the bimolecular quenching constant by the quenching efficiency f_Q:

$$k_q = f_Q k_0 \tag{8.12}$$

For example, if f_Q = 0.5 then 50% of the collisional encounters are effective in quenching and k_q will be half the diffusion-controlled value k_0. Since k_0 can be estimated with moderate precision, the observed value of k_q can be used to judge the efficiency of quenching. Quenchers like oxygen, acrylamide, and I^- generally have efficiencies near unity, but the quenching efficiency of weak quenchers like succinimide depends on the solvent and/or viscosity. The efficiency is generally less with the lighter halogens. The quenching efficiency depends upon the reduction potentials of the fluorophore and amine quencher, as expected for a charge-transfer reaction (Chapter 9).

The efficiency of quenching can be calculated from the observed value of k_q, if the diffusion coefficients and molecular radii are known. The radii can be obtained from molecular models, or from the molecular weights and densities of the quencher in question. Diffusion coefficients may be obtained from the Stokes-Einstein equation:

$$D = kT/6\pi\eta R \tag{8.13}$$

where k is Boltzmann's constant, η is the solvent viscosity, and R is the molecular radius. Frequently, the Stokes-Einstein equation underestimates the diffusion coefficients of small molecules. For example, quenching efficiencies of 2–3 were calculated for oxygen quenching of fluorophores dissolved in various alcohols.[12] These impossibly large efficiencies were obtained because the diffusion coefficient of oxygen in organic solvents is several fold larger than predicted by eq. 8.13. This equation describes the diffusion of molecules that are larger than the solvent molecules, which is not the case for oxygen in ethanol. As an alternative method, diffusion coefficients can be obtained from nomograms based upon the physical properties of the system.[13] Once the diffusion coefficients are known, the bimolecular quenching constant for f_Q = 1 can be predicted using Smoluchowski eq. 8.11.

It is instructive to consider typical values for k_q and the concentrations of quencher required for significant quenching. For example, consider the quenching of tryptophan by oxygen.[14] At 25°C the diffusion coefficient of oxygen in water is 2.5 x 10^{-5} cm^2/s and that of tryptophan is 0.66 x 10^{-5} cm^2/s. Assuming a collision radius of 5 Å, substitution into eq. 8.11 yields k_0 = 1.2 x 10^{10} M^{-1} s^{-1}. The observed value of the oxygen Stern-Volmer quenching constant was 32.5 M^{-1}. The unquenched lifetime of tryptophan is 2.7 ns, so that k_q = 1.2 x 10^{10} M^{-1} s^{-1}, which is in excellent agreement with the predicted value. This indicates that essential-

ly every collision of oxygen with tryptophan is effective in quenching, that is $f_Q = 1.0$. A bimolecular quenching constant near 1×10^{10} M^{-1} s^{-1} may be considered as the largest possible value in aqueous solution. Many quenchers are larger than oxygen. Smaller diffusion-limited quenching constants are expected because the larger molecules have smaller diffusion coefficients. For example, the acrylamide quenching efficiency of tryptophan fluorescence is also near unity,[15] but $k_q = 5.9 \times 10^9$ M^{-1} s^{-1}. This somewhat smaller value of k_q is a result of the smaller diffusion coefficient of acrylamide relative to oxygen. Frequently data are obtained for fluorophores that are bound to macromolecules. In this case the fluorophore is not diffusing as rapidly. Also, the quenchers can probably only approach the fluorophores from a particular direction. In such cases the maximum bimolecular quenching constant is expected to be about 50% of the diffusion-controlled value.[16]

8.3. THEORY OF STATIC QUENCHING

In the previous section we described quenching that resulted from diffusive encounters between the fluorophore and quencher during the lifetime of the excited state. This is a time-dependent process. Quenching can also occur as a result of the formation of a nonfluorescent ground-state complex between the fluorophore and quencher. When this complex absorbs light it immediately returns to the ground state without emission of a photon (Figure 8.1).

For static quenching the dependence of the fluorescence intensity upon quencher concentration is easily derived by consideration of the association constant for complex formation. This constant is given by

$$K_S = \frac{[F-Q]}{[F][Q]} \tag{8.14}$$

where $[F-Q]$ is the concentration of the complex, $[F]$ is the concentration of uncomplexed fluorophore, and $[Q]$ is the concentration of quencher. If the complexed species is nonfluorescent then the fraction of the fluorescence that remains (F/F_0) is given by the fraction of the total fluorophores that are not complexed: $f = F/F_0$. Recalling that the total concentration of fluorophore $[F]_0$ is given by

$$[F]_0 = [F] + [F-Q] \tag{8.15}$$

substitution into eq. 8.14 yields

$$K_S = \frac{[F]_0 - [F]}{[F][Q]} = \frac{[F_0]}{[F][Q]} - \frac{1}{[Q]} \tag{8.16}$$

We can substitute the fluorophore concentration for fluorescence intensities, and rearrangement of eq. 8.16 yields

$$\frac{F_0}{F} = 1 + K_S[Q] \tag{8.17}$$

Note that the dependence of F_0/F on $[Q]$ is linear, which is identical to that observed for dynamic quenching, except that the quenching constant is now the association constant. Unless additional information is provided, fluorescence quenching data obtained by intensity measurements alone can be explained by either dynamic or static processes. As will be shown below, the magnitude of K_S can sometimes be used to demonstrate that dynamic quenching cannot account for the decrease in intensity. The measurement of fluorescence lifetimes is the most definitive method to distinguish static and dynamic quenching. Static quenching removes a fraction of the fluorophores from observation. The complexed fluorophores are nonfluorescent, and the only observed fluorescence is from the uncomplexed fluorophores. The uncomplexed fraction is unperturbed, and hence the lifetime is τ_0. Therefore, for static quenching $\tau_0/\tau = 1$ (Figure 8.1, right). In contrast, for dynamic quenching $F_0/F = \tau_0/\tau$.

One additional method to distinguish static and dynamic quenching is by careful examination of the absorption spectra of the fluorophore. Collisional quenching only affects the excited states of the fluorophores, and thus no changes in the absorption spectra are expected. In contrast, ground-state complex formation will frequently result in perturbation of the absorption spectrum of the fluorophore. In fact, a more complete form of eq. 8.17 would include the possibility of different extinction coefficients for the free and complexed forms of the fluorophore.

8.4. COMBINED DYNAMIC AND STATIC QUENCHING

In many instances the fluorophore can be quenched both by collisions and by complex formation with the same quencher. The characteristic feature of the Stern-Volmer

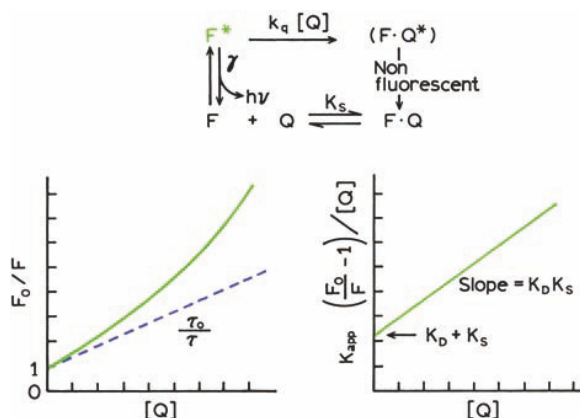

Figure 8.2. Dynamic and static quenching of the same population of fluorophores.

plots in such circumstances is an upward curvature, concave towards the y-axis (Figure 8.2). Then the fractional fluorescence remaining (F/F_0) is given by the product of the fraction not complexed (f) and the fraction not quenched by collisional encounters. Hence

$$\frac{F}{F_0} = f\frac{\gamma}{\gamma + k_q[Q]} \quad (8.18)$$

In the previous section we found that $f^{-1} = 1 + K_S[Q]$. Inversion of eq. 8.18 and rearrangement of the last term on the right yields

$$\frac{F_0}{F} = (1 + K_D[Q])(1 + K_S[Q]) \quad (8.19)$$

This modified form of the Stern-Volmer equation is second order in $[Q]$, which accounts for the upward curvature observed when both static and dynamic quenching occur for the same fluorophore.

The dynamic portion of the observed quenching can be determined by lifetime measurements. That is, $\tau_0/\tau = 1 + K_D[Q]$—the dashed line in Figure 8.2. If lifetime measurements are not available, then eq. 8.19 can be modified to allow a graphical separation of K_S and K_D. Multiplication of the terms in parentheses yields

$$\frac{F_0}{F} = 1 + (K_D + K_S)[Q] + K_DK_S[Q]^2 \quad (8.20)$$

$$\frac{F_0}{F} = 1 + K_{app}[Q] \quad (8.21)$$

where

$$K_{app} = \left[\frac{F_0}{F} - 1\right]\frac{1}{[Q]} = (K_D + K_S) + K_DK_S[Q] \quad (8.22)$$

The apparent quenching constant is calculated at each quencher concentration. A plot of K_{app} versus $[Q]$ yields a straight line with an intercept of $K_D + K_S$ and a slope of K_SK_D (Figure 8.2). The individual values can be obtained from the two solutions of the quadratic equation (see eq. 8.23 below). The dynamic component can generally be selected to be the solution comparable in magnitude to the expected diffusion-controlled value, by the temperature or viscosity dependence of the values, or from other available information about the sample.

8.5. EXAMPLES OF STATIC AND DYNAMIC QUENCHING

Before proceeding with additional theories and examples of quenching it seems valuable to present some examples which illustrate both static and dynamic quenching. Data for oxygen quenching of tryptophan are shown in Figure 8.3.[14] The Stern-Volmer plot is linear, which indicates that only one type of quenching occurs. The proportional decrease in the fluorescence lifetime and yields proves that the observed quenching is due to a diffusive process. From the

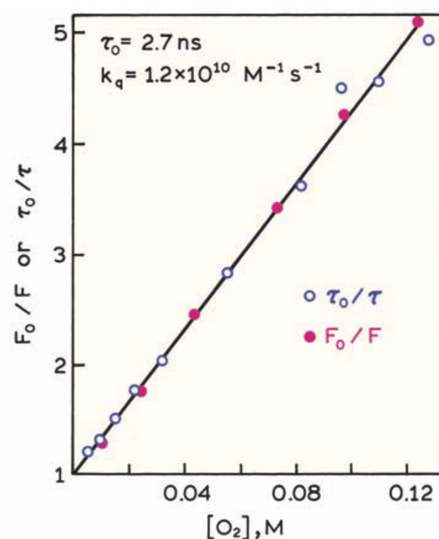

Figure 8.3. Oxygen quenching of tryptophan as observed by fluorescence lifetimes and yields. Revised and reprinted with permission from [14]. Copyright © 1973, American Chemical Society.

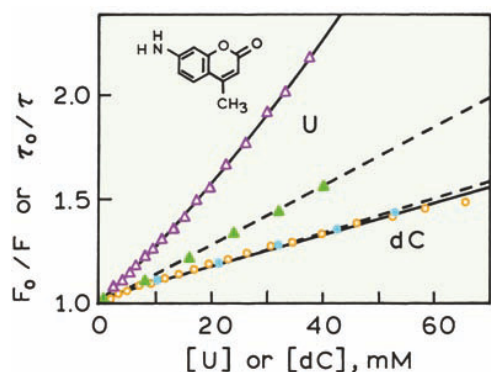

Figure 8.4. Quenching of coumarin C-120 by the nucleotides uridine (F_0/F, open triangle; τ_0/τ, solid triangle) or deoxycytosine (F_0/F, ○; τ_0/τ, ●). The sample was excited at the isoelectric point at 360 nm. Revised and reprinted with permission from [19]. Copyright © 1996, American Chemical Society.

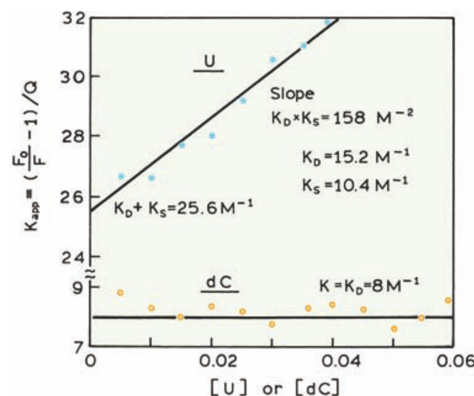

Figure 8.5. Separation of the dynamic and static quenching constants for quenching of C-120 by U or dC. Data from [19].

slope of the Stern-Volmer plot one can calculate that K_D = 32.5 M^{-1}, or that 50% of the fluorescence is quenched at an oxygen concentration of 0.031 M. The value of K_D and the fluorescence lifetime are adequate to calculate the bimolecular quenching constant, $k_q = 1.2 \times 10^{10}\ M^{-1}\ s^{-1}$. This is the value expected for the diffusion-controlled bimolecular rate constant between oxygen and tryptophan (eq. 8.11), which indicates efficient quenching by molecular oxygen.

Static quenching is often observed if the fluorophore and quencher can have a stacking interaction. Such interactions often occur between purine and pyrimidine nucleotides and a number of fluorophores.[17–19] One example is quenching of the coumarin derivative C-120 by the nucleotides uridine (U) and deoxycytosine (dC). The intensity Stern-Volmer plot for quenching by U (open triangles) shows clear upward curvature (Figure 8.4). The lifetime Stern-Volmer plot (solid triangles) is linear and shows less quenching than the intensity data. The larger amount of quenching seen from the intensity as compared to the lifetime indicates that C-120 is being decreased by both complex formation with uridine as well as collisional quenching by uridine. Contrasting data were obtained for quenching of C-120 by cytosine (dC). In this case the Stern-Volmer plots are linear for both intensities and lifetimes, and $F_0/F = \tau_0/\tau$. Hence quenching of C-120 by dC is purely dynamic.

For quenching of C-120 by uridine, the static and dynamic quenching constants can be determined by a plot of K_{app} versus [nucleotide] (see Figure 8.5). The slope (S) and intercept (I) were found to be 158 M^{-2} and 25.6 M^{-1}, respectively. Recalling $I = K_D + K_S$ and $S = K_D K_S$, rearrangements yields

$$K_S^2 - K_S I + S = 0 \tag{8.23}$$

The solutions for this quadratic equation are K_S = 15.2 or 10.4 M^{-1}. From the lifetime data we know K_D is near 13.5 M^{-1}. The lower value of 10.4 M^{-1} was assigned as the static quenching constant. At a uridine concentration of 96 mM, 50% of the ground-state C-120 is complexed and thus nonfluorescent.

It is interesting to mention why the interactions of nucleotides and C-120 were studied. The goal was to develop a method for DNA sequencing using a single electrophoretic lane for all four nucleotides.[19] This would be possible if coumarin derivatives could be identified that display different lifetimes when adjacent to each nucleotide. In this case the DNA sequence would be determined from the lifetimes observed for each band on the sequencing gel. For this fluorophore–quencher pair the quenching mechanism is a charge-transfer interaction. This mechanism is well understood, which could facilitate a rational approach to selection of the fluorophore for lifetime-based sequencing. The use of lifetime measurements in fluorescence sensing is Chapter 19 and DNA sequencing is described in Chapter 21.

8.6. DEVIATIONS FROM THE STERN-VOLMER EQUATION: QUENCHING SPHERE OF ACTION

Positive deviations from the Stern-Volmer equation are frequently observed when the extent of quenching is large. Two examples of upward-curving Stern-Volmer plots are shown for acrylamide quenching of NATA (Figure 8.6) and of the fluorescent steroid dihydroequilenin (DHE) (Figure