Static and Dynamic Fluorescence Quenching Experiments for the Physical Chemistry Laboratory

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Fluorescence spectroscopy is a technique that is ideally suited for the undergraduate laboratory curriculum. Several workers have published experiments designed for the undergraduate lab employing fluorescence measurements (*1–5*). In all of these cases, the measurement of the steady state fluorescence signal has been emphasized to extract information on analyte level or identity and on the efficiency of fluorescence quenching by an external quencher.

The time-dependent nature of fluorescence can also be exploited if the lifetime of fluorescence can be measured. This information is especially useful in evaluating the mechanism and efficiency of fluorescence quenching, especially when time-dependent data are combined with steady-state fluorescence measurements on the same fluorophore–quencher system. These data can often be used to determine whether the quenching mechanism is static (occurs because of a ground state complex between fluorophore and quencher), dynamic (occurs from diffusion of quencher to fluorophore while the latter is in its excited state) or if both mechanisms are occurring. Moreover, the binding constant for the ground-state quenching complex (K) and the rate constant for dynamic quenching (k_q) can often be calculated from these results. As a consequence, such measurements constitute an excellent experiment for the physical chemistry laboratory.

Theory

Intensity (Steady-State) Measurements and Fluorescence Quenching

The intensity of fluorophore fluorescence can be quenched by ground-state quencher–fluorophore reactions (static quenching) and by excited-state quencher–fluorophore reactions (dynamic quenching). In the discussion that follows, static quenching is assumed to result from the formation of a nonfluorescent quencher–fluorophore complex in the ground state. Shifts of the fluorophore absorption spectrum with added quencher provide evidence of such complex formation. Another type of static quenching is often observed at high quencher concentrations due to the existence of increasing numbers of quencher–fluorophore pairs in which the quencher is close enough to the fluorophore to instantaneously quench its excited state (*6*). Treatment of this type of quenching is less straightforward. Fortunately, it can be distinguished from quenching due to true ground-state complex formation because it does not produce changes in the fluorophore absorption spectrum.

The steady-state parameter that responds to added quencher is the fluorescence quantum yield Φ_f, which is defined as the ratio of the rate of fluorescence emission to the rate of absorption (photons out/photons in). The fluorescence quantum yield in the absence of quencher (Φ_f^o) is defined by the mechanism below, where A is the fluorophore ground state, A^* is the fluorophore emitting state (lowest excited singlet), k_A is the rate constant for photon absorption, k_f is the rate constant for fluorescence emission, and k_{nr} is the sum of first-order rate constants for nonradiative decay modes, such as internal conversion and intersystem crossing. The variables n_A and n_{A^*} are the number of fluorophores in the ground and excited state, respectively.

$$A + h\nu \rightarrow A^* \qquad \text{Rate} = k_A n_A \qquad (1)$$

$$A^* \rightarrow A + h\nu \qquad \text{Rate} = k_f n_{A^*} \qquad (2)$$

$$A^* \rightarrow A \qquad \text{Rate} = k_{nr}\, n_{A^*} \qquad (3)$$

Since steady-state conditions exist, we can assume:

$$k_A n_A = (k_f + k_{nr}) n_{A^*} \qquad \left(\frac{dn_{A^*}}{dt} = 0\right) \qquad (4)$$

Rearranging eq 4 gives:

$$n_{A^*} = \frac{k_A n_A}{(k_f + k_{nr})} \qquad (5)$$

The Φ_f is formally defined as:

$$\Phi_f = \frac{k_f n_{A^*}}{k_A n_A} \qquad (6)$$

Combining eqs 5 and 6 gives the familiar form for the fluorescence quantum yield in the absence of quencher (Φ_f^o):

$$\Phi_f^o = \frac{k_f}{k_f + k_{nr}} \qquad (7)$$

When quencher (Q) is added, another A^* decay mechanism is now possible, where k_q is the second-order rate constant for quenching and the product $k_q[Q]$ is pseudo-first-order.

$$A^* + Q \rightarrow A + Q \qquad \text{Rate} = k_q[Q] n_{A^*} \qquad (8)$$

Now eq 5 becomes:

$$n_{A^*} = \frac{k_A n_A}{\left(k_f + k_{nr} + k_q[Q]\right)} \qquad (9)$$

Combining eq 9 with eq 6 gives an expression for the fluorescence quantum yield in the presence of quencher, providing dynamic quenching (eq 8) is the only quenching mechanism.

$$\Phi_f = \frac{k_f}{(k_f + k_{nr} + k_q[Q])} \qquad (10)$$

Dividing eq 7 by eq 10 produces the familiar Stern–Volmer equation(*7*):

$$\frac{\Phi_f^o}{\Phi_f} = 1 + \frac{k_q[Q]}{(k_f + k_{nr})} \qquad (11)$$

In the more familiar form of this equation Φ_f^o and Φ_f are replaced by F^o and F, the intensities of fluorescence at a given wavelength, in the absence and presence of Q, respectively, and the term $[k_q/(k_f + k_{nr})]$ is set equal to K_{SV}, the Stern–Volmer quenching constant (*6*).

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$$\frac{F^o}{F} = 1 + K_{SV}[Q] \tag{12}$$

If this quenching mechanism alone obtains, a plot of F^o/F versus [Q] is linear with an intercept of 1 and a slope equal to K_{SV}.

When quenching can also occur by ground-state complex formation (static quenching) another reaction must be considered in the overall quenching mechanism(*8*):

$$A + Q \overset{K}{\rightleftarrows} AQ \xrightarrow{h\nu} AQ^* \longrightarrow AQ + \text{heat} \tag{13}$$

K is the equilibrium constant for the formation of the "dark" complex, AQ. If both static and dynamic quenching are occurring, the intensity ratio (F/F^o) can be expressed as the fractional reduction due to quenching of A^* (dynamic) times the fractional reduction due to complexation of A (f, static) (*6*).

$$\frac{F}{F^o} = \left(\frac{1}{1 + K_{SV}[Q]}\right) f \tag{14}$$

The fraction f can be expressed in the following way, given the definition of K in eq 13:

$$f = \frac{[A]}{[A] + [AQ]} = \frac{1}{1 + K[Q]} \tag{15}$$

Thus, it follows that:

$$\frac{F}{F^o} = \left(\frac{1}{1 + K_{SV}[Q]}\right)\left(\frac{1}{1 + K[Q]}\right) \tag{16}$$

This, in turn, can be rearranged to give a modified form of the Stern–Volmer equation:

$$\frac{F^o}{F} = (1 + K_{SV}[Q])\,(1 + K[Q]) \tag{17}$$

Equation 17 predicts upward curvature of the plot of F^o/F versus [Q] in the event that both static and dynamic quenching are occurring.

If quenching *only* occurs by the static mechanism (k_q, $K_{SV} = 0$), eq 17 simplifies to:

$$\frac{F^o}{F} = 1 + K[Q] \tag{18}$$

Note that eq 18 also predicts a linear relation between F^o/F and [Q] with an intercept of 1, as does eq 12 derived for the case where dynamic quenching alone is occurring. In the former case the slope equals K, while in the latter it equals K_{SV}. Thus, one cannot determine whether quenching is static or dynamic on the basis of a single linear Stern–Volmer plot. If such plots are obtained as a function of temperature, a determination might be made from the change in slope. Increased temperature often causes the slope (K_{SV}) to increase if quenching is dynamic (k_q increases with temperature), while the slope (K) should decrease with increasing temperature if the quenching is static (*6*). Even so, there is no way to extract a value for k_q using steady-state measurements only.

Fluorescence Lifetimes as a Function of Quencher Concentrations for Three Systems[a]

N-MEAI		PSA		2-AN	
[GMP]/10^{-3}M	τ/ns	[I^-]/10^{-3}M	τ/ns	[β-CD]/10^{-3}M	τ/ns
0.0	34.8	0.0	58.6	0.0	2.0
1.0	30.9	3.0	37.2	1.0	1.9
2.0	27.8	6.0	26.4		
3.0	25.0	9.0	20.7		
4.0	22.5	12.0	17.1		
6.0	18.8				
8.0	15.7				

[a]The systems are (1) *N*-MEAI/GMP, (2) PSA/Iodide, and (3) 2-AN/β-CD.

Dynamic Measurements (Fluorescence Lifetime) and Fluorescence Quenching

When fluorescence is excited by a pulsed rather than a continuous source, the decrease of fluorophore fluorescence after the pulse is extinguished normally follows a single exponential decay in solution. The lifetime of fluorescence, defined as the time for the fluorescence signal to decay to 1/e of its original value, is given by eq 19 in the absence of quencher and eq 20 in the presence of quencher.

$$\tau^o = \frac{1}{(k_f + k_{nr})} \tag{19}$$

$$\tau = \frac{1}{(k_f + k_{nr} + k_q[Q])} \tag{20}$$

Dividing eq 19 by eq 20 gives another form of the Stern–Volmer equation, *which applies only if quenching is dynamic.*

$$\frac{\tau^o}{\tau} = 1 + \frac{k_q[Q]}{k_f + k_{nr}} = 1 + k_q\tau^o[Q] \tag{21}$$

We now recognize that the Stern–Volmer constant (K_{SV}) obtained from steady state measurements is equal to $k_q\tau^o$. Thus, if lifetime measurements are possible, the value of k_q can be extracted from plots of eq 21 or eq 12 and the value of the lifetime in the absence of quencher (τ^o).

It is important to note that lifetime measurements are not affected by the formation of a ground-state "dark" complex (AQ). Consequently, lifetime measurements can be used to separate dynamic quenching from static. Moreover, when both dynamic and steady-state measurements are possible, it is often possible to extract k_q if dynamic quenching only is occurring; K, if static quenching only is occurring; or k_q and K, if both mechanisms are occurring, from these measurements.

We report here three separate experiments that illustrate the usefulness of both types of quenching measurements for this end.

Experimental

Fluorescence spectra and intensity data were obtained on a Perkin–Elmer Lambda 5B Spectrofluorometer attached to a Perkin–Elmer R100 recorder. Excitation and emission slits were both set at either 5 or 10 nm for all measurements. The fluorescence source is a 8.3-W xenon discharge lamp. Fluorescence lifetime data were obtained on a Photon Technology Incorporated Fluorescence Lifetime System, which employs a pulsed nitrogen discharge lamp for excitation and an optical boxcar detector. Lifetime values were extracted from the data by convolution of the lamp decay with exponential decays until the fit to the observed fluorescence decay was deemed acceptable on the basis of the χ^2 statistic. The convolution was done on an IBM PC compatible computer. We include tabulated lifetime data in the table for all of the fluorophore/quencher systems we studied so that others can perform these experiments without having access to lifetime measuring instrumentation. We are also willing to send copies of the lamp and fluorescence intensity profiles and fitted decay curves to anyone who would like to show these to students.

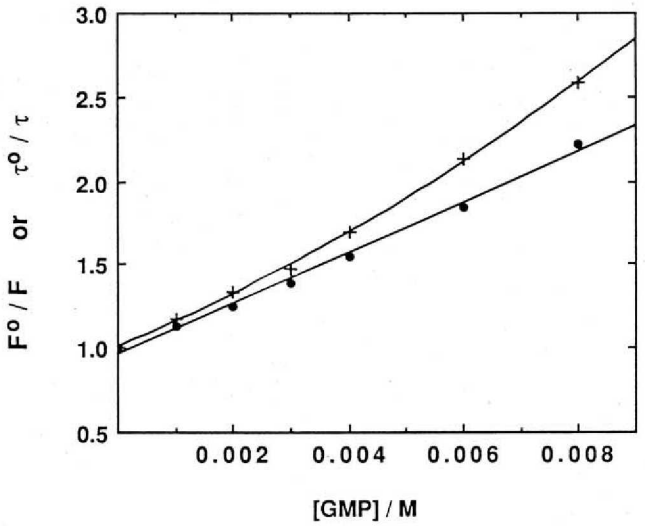

Figure 1. Fluorescence intensity and lifetime of *N*-MEAI plotted against the concentration of GMP quencher. The excitation wavelengths for the fluorescence intensity and lifetime determinations were 380 and 358 nm, respectively. In both experiments the fluorescence emission was monitored at 485 nm. The solid circles correspond to τ^0/τ and the crosses to F^0/F.

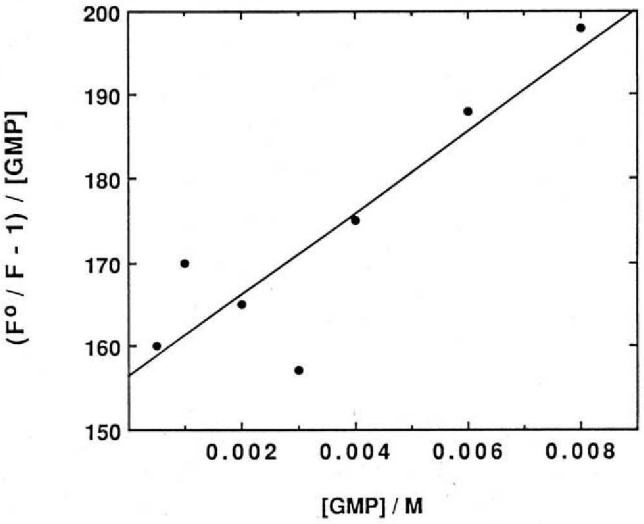

Figure 2. A plot of the *N*-MEAI fluorescence intensity data of Figure 1 in a way suggested by eq 22.

N-methylacridinium iodide (*N*-MEAI) and 1-pyrenesulfonic acid, sodium salt (PSA) were obtained from Molecular Probes (Eugene, OR). The GMP (guanosine 5′-monophosphate, disodium salt trihydrate), 2-acetylnaphthalene (2-AN), and β-cyclodextrin (β-CD) were obtained from Aldrich Chemical; 2-AN and β-CD were recrystallized from water before use. All other chemicals were reagent grade or better. Solutions were prepared using distilled water.

- **Note:** *N*-methylacridinum iodide is a potential **skin and eye irritant.**

Solutions of GMP should be prepared within 1 day of usage. *N*-MEAI/GMP solutions were prepared in 0.1 M phosphate buffer at pH 7.0 . The PSA/iodide solutions where prepared at a constant ionic strength of 0.0135 M with KCl. No attempt to control either pH or ionic strength was made in the 2-AN/β-CD system. Fluorophore concentrations were used that corresponded to absorbances of less than 0.11 measured at the excitation wavelength used in the fluorescence experiments.

Results and Discussion

Quenching of N-methylacridinium Iodide Fluorescence by GMP

Figure 1 shows typical results for the effect of quencher concentration on the fluorescence intensity and lifetime of *N*-MEAI. Lifetime data for this system are given in the table. The linear dependence of τ^0/τ and quadratic dependence of F^0/F on GMP concentration indicates the presence of both dynamic and static quenching in this system. From eq 21, the slope of the τ^0/τ plot and the independently measured value for τ^0 of 35 ns, one calculates a value for k_q of 4.4×10^9 M^{-1} s^{-1} in good agreement with 4.3×10^9 M^{-1} s^{-1} obtained by Kubota et al. (*9*). Using eq 17 as a guide, one can calculate the *N*-MEAI/GMP association constant K from the coefficients obtained by fitting a second-order polynomial to the intensity data shown in Figure 1. Alternatively, rearranging eq 17 to give eq 22 shows that K can be obtained by fitting a first-order polynomial to a plot of $(F^0/F - 1)/[Q]$ vs. $[Q]$.

$$\frac{\frac{F^0}{F} - 1}{[Q]} = (k_q\tau^0 + K) + k_q\tau^0 K[Q] \qquad (22)$$

This is the usual method employed to analyze these data (*6, 9*) and is the procedure we have used. Figure 2 is a plot of $(F^0/F - 1)/[Q]$ versus [GMP]. From the slope and intercept one calculates K to be 48 M^{-1} in good agreement with Kubota et al.'s value of 44 M^{-1} (*9*). Alternately, using just the slope and the previously determined k_q and τ^0, one calculates K to be 34 M^{-1}. That GMP is both a static and dynamic quencher is not surprising. Energy transfer between the conjugated π-systems of the rings in *N*-MEAI and GMP should be efficient and the opposite charges on the fluorophore and quencher are certainly consistent with formation of a complex involving the ground state species.

Quenching of 1-Pyrenesulfonic Acid Fluorescence by Potassium Iodide

As shown by the linearity and nearly identical slopes of the plots in Figure 3, quenching of PSA fluorescence by po-

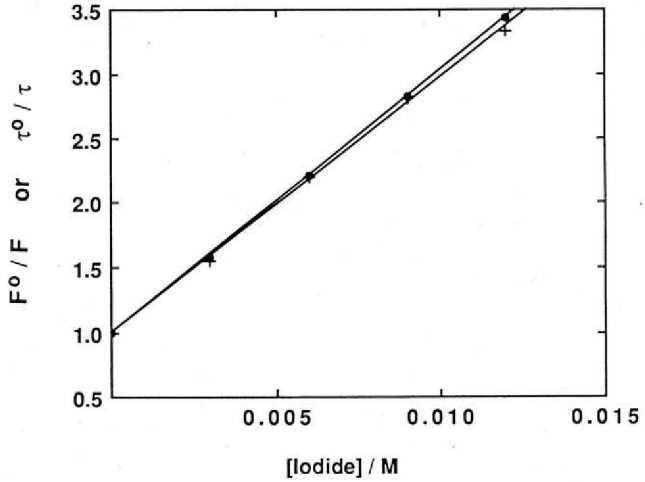

Figure 3. Fluorescence intensity and lifetime of PSA plotted against the concentration of iodide ion. The excitation wavelengths for the fluorescence intensity and lifetime measurements were 335 and 337 nm, respectively. In both experiments the fluorescence emission was monitored at 395 nm. The solid circles correspond to τ^0/τ and the crosses to F^0/F.

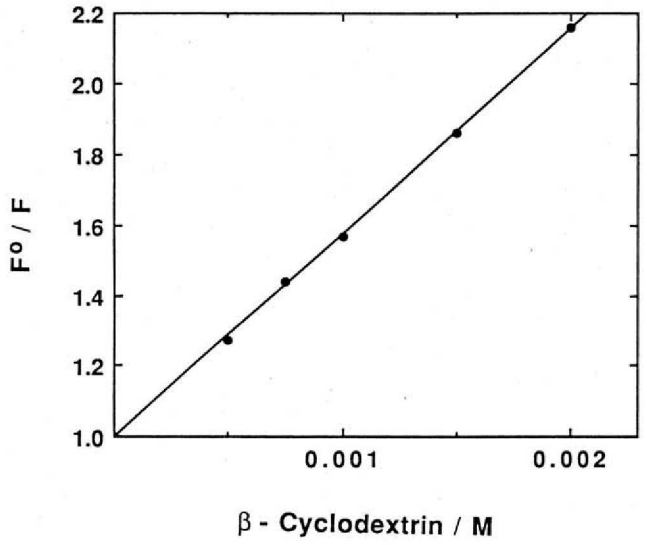

Figure 4. Fluorescence intensity of 2-AN plotted against the concentration of β-cyclodextrin quencher. The fluorescence excitation and emission wavelengths were 340 and 435 nm, respectively.

tassium iodide only occurs dynamically. Lifetime data for this system are given in the table. From eq 21, the slope of these plots, and τ° one calculates a value for the dynamic quenching rate constant of 3.4×10^9 M^{-1} s^{-1}. The absence of static quenching is expected since both fluorophore and quencher exist as anions in aqueous solution.

Quenching of 2-Acetylnaphthalene Fluorescence by β-Cyclodextrin

During the course of an independent investigation by two of the authors, it was observed that the fluorescence of 2-acetylnaphthalene (2-AN) is quenched when this molecule binds in the internal cavity of β-cyclodextrin (β-CD) (*10*). As the concentration of β-CD increases from 0 to 0.001 M, the intensity of 2-AN fluorescence decreases by 50%, while the lifetime of 2-AN fluorescence remains unchanged (2.0 ± 0.1 ns). This small difference is within the error of the lifetime measurement. Consequently, the 2-AN/β-CD system is an example of the third possibility mentioned above, where only static quenching is occurring. The slope of the fluorescence intensity plot, shown in Figure 4, is equal to the equilibrium constant for formation of the ground-state 2-AN/β-CD complex. The value we obtain for this equilibrium constant is 581 M^{-1} at 21 °C.

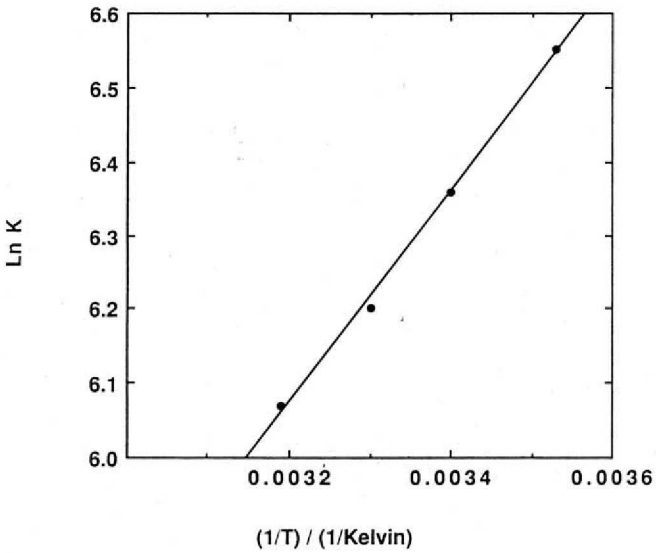

Figure 5. A van't Hoff plot of the equilibrium constant for formation of the 2-AN/β-CD complex versus the inverse Kelvin temperature. ΔH° and ΔS° for complex formation are obtained from the slope and y-intercept, respectively.

If students have sufficient time, they can repeat the fluorescence intensity measurements on this system at other temperatures and prepare a plot of ln K versus $1/T$ such as is shown in Figure 5. From the van't Hoff equation, the slope can be related to ΔH° of complex formation between 2-AN and β-CD and the intercept to ΔS°. We obtain values of -11.9 ± 0.5 kJ/mol for ΔH° and 12.5 ± 1.6 J/K mol for ΔS°. The negative sign for ΔH° is accounted for by the hydrophobic interactions between the naphthalene ring and the walls of the β–CD cavity (*11*). The overall ΔS° of complex formation is the sum of several entropy changes with differing signs. Entropy loss accompanies restriction of fluorophore motion upon binding, while entropy gain occurs due to expulsion of water from the β-CD cavity and from disruption of the solvent shell around the fluorophore when it binds to β-CD (*12, 13*). Apparently, the latter contribution dominates in this case.

This quenching of 2-AN fluorescence by β-CD is in contrast to the normally observed fluorescence enhancement when fluorophores bind to β-CD (*13–15*). The 2-AN molecule only shows significant fluorescence in strongly hydrogen-bonding solvents, such as water and fluorinated alcohols (*16*), presumably due to the ability of these solvents to blue shift the low-lying n,π^* state to an energy where it can not interfere with fluorescence (*17*). Such a strong hydrogen bond with 2-AN is not possible in the β-CD cavity and quenching therefore results.

Our students normally investigate two of the fluorophore/quencher systems during the course of two 4-hour laboratory periods. Toward the end of this same course, students also undertake a more extensive 4-week (16 laboratory hours) kinetics project. The students assigned to do the project described here are able to investigate all three fluorophore/quencher systems as well as do the thermodynamic study of 2-AN/β-CD binding.

Conclusions

Fluorescence quenching mechanisms cannot be determined from fluorescence intensity measurements alone. However, if these are combined with measurements of fluorescence lifetimes then it becomes possible to distinguish between dynamic and static quenching mechanisms. Furthermore, the rate constant for dynamic quenching and the equilibrium constant associated with static quenching can be determined. We have described here a series of three experiments suitable for the undergraduate physical chemistry laboratory which introduce students to fluorophore/quencher systems which exhibit dynamic, static, and both dynamic and static quenching. We also show how measurements of the temperature dependence of static quenching can be used to determine the ΔH°, ΔS°, and ΔG° of complex formation between a fluorescent molecule and one which can act as a quencher of this fluorescence. This experiment allows students to perform both kinetic and thermodynamic measurements on a system using experimental techniques for studying very fast molecular events. It also introduces them to the important concept of energy transfer in molecular collisions.

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