# Oxygen Quenching of Pyrenebutyric Acid Fluorescence in Water. A Dynamic Probe of the Microenvironment*

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ABSTRACT: Solutions of pyrenebutyrate have fluorescence lifetimes of 135 nsec in deoxygenated water and 100 nsec, and a corresponding diminished yield in air-equilibrated water. The oxygen quenching of the fluorescence is shown by means of measurements of lifetime and relative yield of fluorescence to be a diffusion-controlled phenomenon in which virtually every collision is effective. Adsorbates or chemical conjugates of pyrenebutyric acid with bovine albumin are not affected by changing the concentrations of $O_2$ in the medium, while in concentrated urea the quenching is markedly increased. Observations of a similar kind have been carried out in polylysine conjugates and apohemoglobin adsorbates. The lifetime in the absence of oxygen ($\tau_0$) in the systems studied varied from 100 to 205 nsec. From observations of glycerol–water mixtures it is suggested that $\tau_0$ may depend upon the polarity of the environment. The observations reported here show that pyrenebutyric acid may be used to determine the accessibility of $O_2$ in microenvironments of biological interest. In principle, such a probe could have a resolution time of 1 $\mu$sec. A general treatment of quenching by collisions and complex formation is developed from considerations of the rate equations involved.

Free pyrenebutyric acid has a fluorescent lifetime in the order of 100 nsec in air-saturated water solutions. Substances which can perturb the excited state thus have 5 to 10 times longer to diffuse to these excited molecules than they would for the usual case of lifetimes in the order of 10–20 nsec. This extra time during which diffusion processes may occur increases the volume of the solution that influences the excited state of each pyrenebutyric acid molecule from 100 to 1000 times over the volume that can affect the shorter lived molecules.

Oxygen is known to be a powerful quencher of electronic excited states but due to the circumstances mentioned above, its influence upon short lifetime fluorescence in air-saturated water solutions is negligible. On the other hand, oxygen quenching of pyrenebutyric acid is found to be appreciable in such solutions. Therefore, changes in the fluorescent lifetime and yield of pyrenebutyric acid can be used to determine the oxygen concentration in a microenvironment (*e.g.*, the surface or interior of membranes, particles, macromolecules, etc.).

We present studies in simple systems that demonstrate the usefulness of pyrenebutyric acid in this connection.

## Experimental Procedures

*Materials.* Pyrenebutyric acid (Eastman Distilled Products) was twice recrystallized from 70% ethanol–30% water. A 0.1 M stock solution in dimethylformamide was prepared. This stock solution was diluted approximately $10^5$ in water for study. For covalent labeling pyrenebutyric acid was activated by forming the mixed anhydride with sulfuric acid as described by Rawitch *et al.* (1969). Labeling was carried out by adding activated pyrenebutyric acid to a solution of macromolecules in amounts determined by assuming a labeling efficiency of 30%. Care was taken to dilute the activated pyrenebutyric acid at least 100 times with buffer before its slow addition to the solution to be labeled, in order to avoid dimerization of the pyrenebutyric acid.

Bovine serum albumin was obtained in crystallized form from Armour Pharmaceutical Co. After bovine serum albumin was covalently labeled, it was passed through a Dowex 2 (200 mesh) column to remove free or activated pyrenebutyric acid. Poly-L-lysine I (mol wt 150,000) was obtained from Sigma Chemical Co. It was dissolved in glass-distilled water to yield a solution of pH between 6.7 and 6.8. The reaction mixture was dialyzed against distilled water for 24 hr to remove the excess activated pyrenebutyric acid. The degree of labeling achieved was one pyrenebutyric acid for about four poly-L-lysine chains. The pH of the poly-L-lysine samples was raised by the addition of two or three drops of 2 N $NH_4OH$ to 3 ml of sample to attain the pH necessary for $\alpha$-helix formation in poly-L-lysine.

Apohemoglobin was prepared by the method of Rossi-Fanelli *et al.* (1958) and stored lyophilized. It was dissolved in 0.01 M $PO_4$ buffer (pH 7.0) to give a final concentration of 0.9 mg/ml ($5.6 \times 10^{-5}$ M assuming mol wt 16,000). Human and bovine thyroglobulin were prepared by the method of Pierce *et al.* (1965) and dissolved in 0.1 M $PO_4$ buffer (pH 7.0) to give approximately 30 mg/ml. It was labeled to the extent of 0.7–0.8 mole of pyrenebutyric acid/mole of thyroglobulin (mol wt 660,000).

*Methods.* A Korad K-1QP Q-switched ruby laser was used to excite the fluorescence of pyrenebutyric acid. Its output was frequency doubled to produce a pulse at 347 nm having

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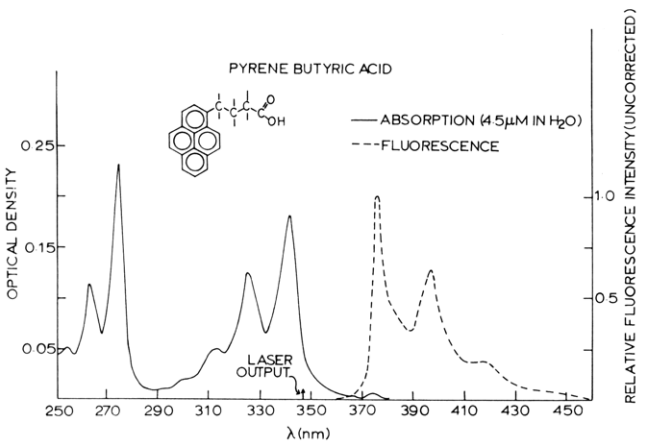



FIGURE 1: The structure of pyrenebutyric acid along with its absorption and fluorescence spectra.

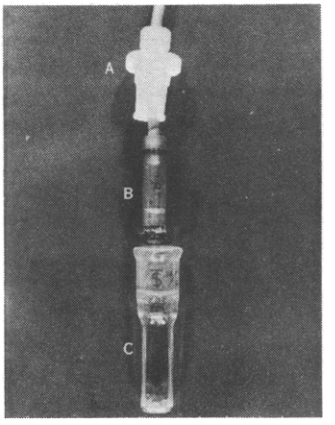



FIGURE 2: The cuvet and probe assembly. A is the 14/20 tapered thermometer holder. B is the Clark oxygen probe. C is the Pyrex cuvet fitted with a 14/20 ground glass joint.

a half-width of less than 20 nsec and a decay time of 7 nsec. The 347-nm output is ideal for studying the fluorescence decay of pyrenebutyric acid since it lies within the first absorption band of pyrenebutyric acid (Figure 1). The fluorescence signal passed through a Jarrell–Ash (82–410) monochromator set at 376 ± 0.8 nm and was monitored by an Amperex 56-UVP photomultiplier and displayed on a Dumont 766 H/T oscilloscope. The oscilloscope trace was triggered by an RCA 1P-28 phototube monitoring the air scatter from the exciting pulse. The oscilloscope traces were photographed and analyzed for least-squares fit to a straight line when the natural logarithm of the fluorescent intensity was plotted against time. No data were gathered closer than 55 nsec from the rise of the fluorescent signal so that deconvolution corrections were not required in the analysis of the emission. The rate of emission is given by the slope of the least-squares straight line. The fluorescence lifetime, $\tau$, is the reciprocal of the rate of emission.

Fluorescence intensities were measured in a spectrophotofluorometer (Weber and Young, 1964) modified to collect the emission perpendicular to the exciting light. Intensities at 376 ± 5 nm excited by 347 ± 5 nm light were measured to determine the effect of oxygen on the quantum yield. To compensate for changes in lamp intensity, the ratio of the fluorescent signal to the exciting signal was recorded by a Dana digital voltmeter (Model 5403). The photomultiplier voltages were adjusted to give a constant reading from a standard solution prior to each reading to compensate for drifts in the power supplies. The printed output of the voltmeter was averaged to give the fluorescence, $F$, at a given oxygen concentration.

Oxygen concentrations were monitored by a Clark (1956) oxygen electrode (Yellowsprings Instrument Co., Model 5331 probe and power supply from Model 53 biological oxygen monitor). The Pyrex fluorescence cuvet used for these studies was fitted with a 14/20 taper-ground glass joint as shown in Figure 2. A nylon thermometer holder, also with a 14/20 taper, was drilled out to accommodate the Lucite body of the oxygen probe. An O-ring provided the gas-tight seal between the probe and the nylon. This design enabled the probe to be raised and lowered in the cuvet to accommodate varying amounts of sample (4 ml or less). The seal of the entire assembly was ensured by the use of stopcock grease at all points of contact with joints.

The oxygen probe was calibrated by reference to an air-saturated sample of distilled water maintained at the same temperature. The concentration of oxygen in such a solution $C_{sat}$, was taken from Linke and Seidell (1965). The oxygen concentration for a full scale deflection on the chart recorder (Honeywell Electronik 19) connected to the probe's power supply, $C_{100}$, was determined by

$$C_{100} = (C_{sat}P)/(760\,f) \quad (1)$$

where $P$ is the atmospheric pressure in millimeters of Hg and $f$ is the fraction of full scale deflection in the calibrating solution. $P$ was measured on a Fortin-type barometer (Central Scientific Co.) to compensate for the changes in oxygen solubility with pressure.

The oxygen concentration was lowered from the air-saturated level of approximately 8 ppm (0.25 mM) by bubbling nitrogen through the sample for 10 min. This operation lowered the oxygen concentration to 0.3–0.8 ppm (0.01–0.025 mM). After 6–10 min of rapid stirring in the sealed sample cuvet, the decay curve or intensity of the fluorescence was determined while the oxygen concentration was simultaneously displayed on the chart recorder.

The oxygen concentration was then raised in increments by briefly removing the probe from the solution and bubbling small volumes of air or pure oxygen from a hypodermic syringe through the solution. Approximately 30 ml of air was divided into 5 or 6 increments to raise the air-saturation level from 5% to nearly 100%. An additional 50 ml of pure oxygen was delivered in 5 or 6 additional increments to bring the saturation level to 65% of oxygen saturation (approximately 300% of air saturation). Further increments were less effective due to increased diffusion of oxygen from the concentrated solution into the atmosphere. Measurements were made at 25° unless stated otherwise.

As developed in the Appendix (eq 17) the dependence of "strong" quenching upon quencher concentration $Q$ is given by

$$\frac{F_0}{F} = (1 + K_0[Q])(1 + k_+^*\tau_0[Q]) \quad (2)$$

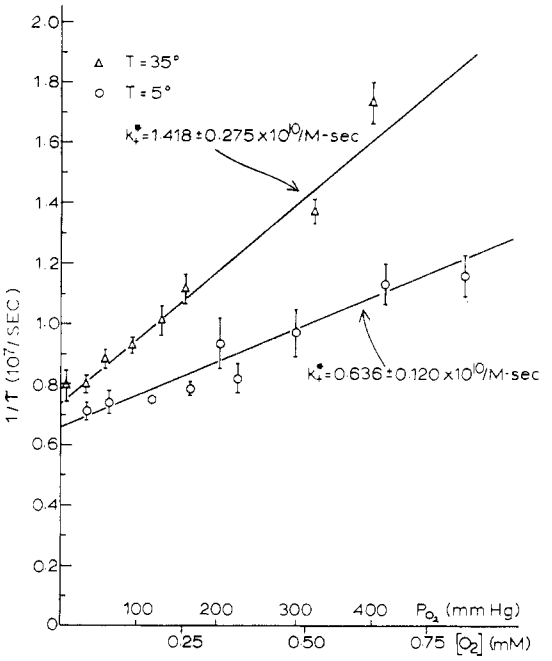



FIGURE 3: Stern–Volmer lifetime plots of free pyrenebutyric acid in $PO_4$ buffer (pH 7.0) at 5 and 35°.

where $K_0$ is the equilibrium constant of the nonfluorescent dark complexes, $k_+^*$ the rate of quenching of the excited state of the fluorescent molecules by encounters with quencher, $\tau_0$ the fluorescence lifetime in the absence of quencher, and $F_0$ and $F$ are, respectively, the fluorescence intensity in the absence and presence of quencher. At low and medium quencher concentration, and at all concentrations if either $K_0$ or $k_+^*\tau_0$ is negligible compared with the other, the process is described by

$$\frac{F_0}{F} = 1 + K[Q] \tag{3}$$

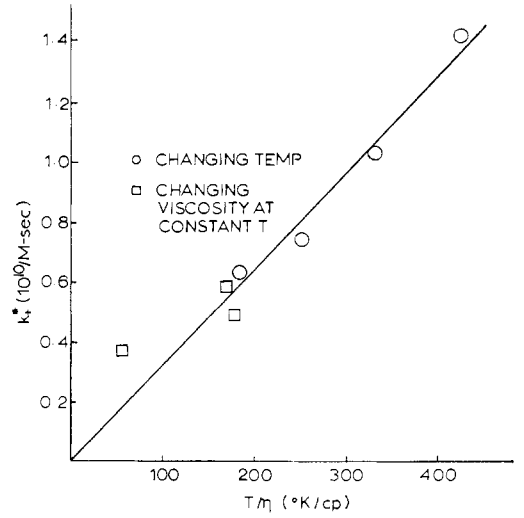



FIGURE 4: The relationship of the quenching rate constant, $k_+^*$, to changes in $T/\eta$.

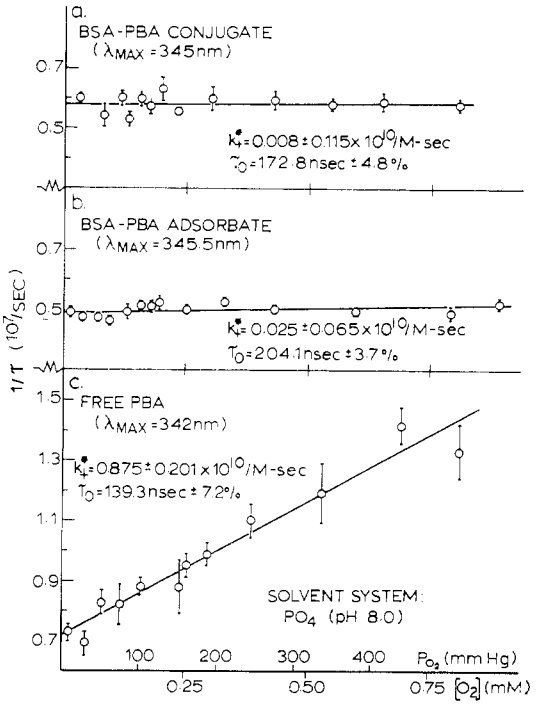



FIGURE 5: Comparison of the oxygen quenching of pyrenebutyric acid lifetimes at pH 8.0 when (a) covalently bound to bovine serum albumin, (b) adsorbed to bovine serum albumin, and (c) free in solution at 25°.

where $K$ is the observable or overall quenching constant. By comparison of eq 2 and 3 it is seen that the equality

$$K = k_+^*\tau_0 \tag{4}$$

obtains only if $K_0 \ll k_+^*\tau_0$ that is when dark association processes have negligible effect in comparison with encounters with quencher during the lifetime of the excited state.

A less direct, though equally convincing test of the dominance of excited-state encounters in the quenching process depends upon the diffusion-limited character of such processes (Wawilov, 1929). This reflects itself in the linear dependence of $k_+^*$ or $K$ upon $T/\eta$, where $T$ is the absolute temperature and $\eta$ the viscosity of the solvent.

The efficiency of a collisional quenching process, $\gamma$, may be calculated from the expression

$$k_+^* = \gamma(4\pi aDN') \tag{5}$$

with the assumption that either the fluorophore or $Q$ is uncharged (Weller, 1959). In eq 5 $a$ is the encounter distance (assumed to be the sum of the molecular radii), $D$ is the sum of the respective diffusion coefficients, and $N'$ is the number of molecules per millimole (to correct the dimensions of eq 5). The term in parenthesis in eq 5 is the number of collisions of A and Q per mole per second.

For the pyrenebutyric acid–$O_2$ system studied here, $D$ and $a$ were determined in the following manner. The diffusion coefficient for oxygen in water at 25° is given by Millington (1955) as $2.6 \times 10^{-5}$ cm²/sec. The diffusion coefficient for pyrenebutyric acid was estimated to be $0.5 \times 10^{-5}$ cm²/sec

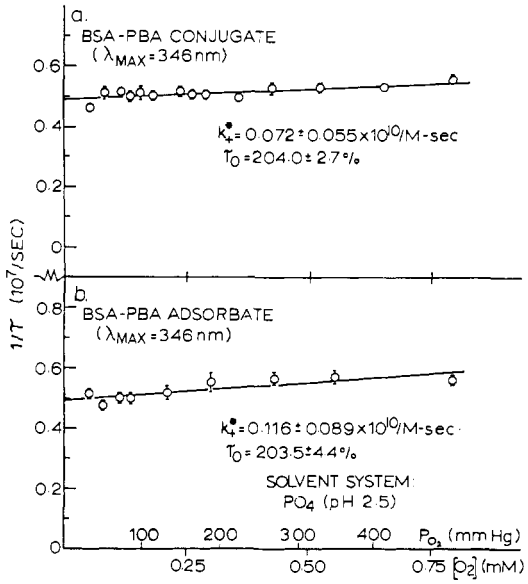



FIGURE 6: Oxygen quenching of pyrenebutyric acid lifetimes at pH 2.5 when (a) covalently bound to bovine serum albumin and (b) adsorbed to bovine serum albumin.

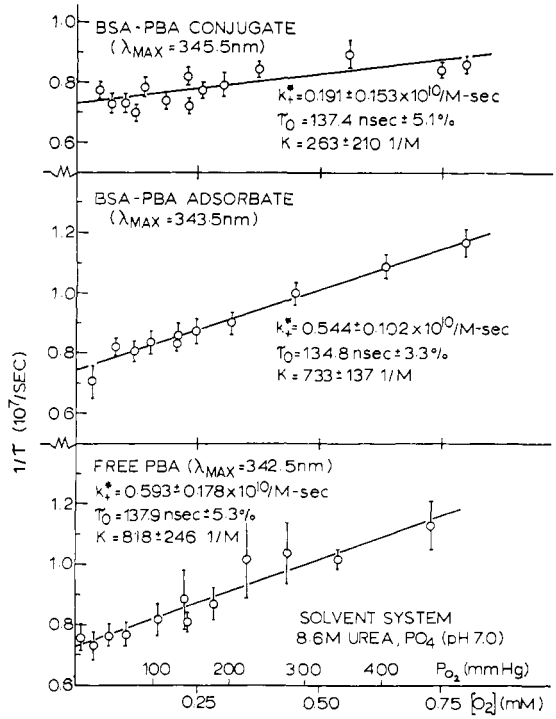



FIGURE 7: Comparison of the oxygen quenching of pyrenebutyric acid lifetimes in 8.6 M urea solutions when (a) covalently bound to bovine serum albumin, (b) adsorbed to bovine serum albumin, and (c) free in solution. The Stern–Volmer constants, $K$, are given for comparison with Figure 8.

by the nomogram of Othmer and Thakar (1953). Thus, we obtain $D = 3.1 \times 10^{-5}$ cm$^2$/sec. The molecular diameter of oxygen was taken to be $2.95 \times 10^{-8}$ cm from the average of two determinations given by Weast (1968). In calculating the molecular diameter of pyrenebutyric acid, it was assumed that collisions of oxygen with the butyric acid side chain would have no quenching effect. From the X-ray results of Camerman and Trotter (1965) for pyrene, the long axis of pyrenebutyric acid was determined to be $7.02 \times 10^{-8}$ cm and the short axis $4.95 \times 10^{-8}$ cm. Taking these values as possible diameters for the equivalent collisional sphere for pyrenebutyric acid, the sum of the radii of pyrenebutyric acid and $O_2$ is somewhere between $4.99 \times 10^{-8}$ and $3.94 \times 10^{-8}$ cm. The average of these two extremes, $4.48 \times 10^{-8}$ cm, was taken as the value of $a$ used for the computation of $\gamma$.

## Results

*Free Pyrenebutyric Acid.* It was observed that bubbling oxygen through a dilute solution of pyrenebutyric acid in water lowered the fluorescent lifetime from about 100 to 65 nsec. Figure 3 shows the Stern–Volmer plots for free pyrenebutyric acid at 5 and 35°. The effect of reduced viscosity at 35° can be seen by the increased slope indicating an increased value of $k_+^*$.

If this quenching were primarily collisional, as previously assumed, the collisional rate constant, $k_+^*$, would be proportional to $T/\eta$, where $T$ is the absolute temperature and $\eta$ is the viscosity of the solution. The viscosity of several samples was changed both by changing the temperature and adding sucrose to a sample at constant temperature. The relationship of $k_+^*$ to changes in $T/\eta$ is seen in Figure 4. The fact that the plot was linear confirmed the idea that oxygen was indeed a collisional quencher of pyrenebutyric acid.

The efficiency of the oxygen quenching was calculated from eq 5 using the average of three determinations of $k_+^*$ at 25°, $k_+^* = 1.03 \times 10^{10}$ (moles/sec)$^{-1}$. This value of $k_+^*$ combined with the parameters mentioned above indicated that the efficiency for the pyrenebutyric acid–$O_2$ system at 25° was approximately 99.5 ± 10%.

*Cases Where Pyrenebutyric Acid Accessibility to $O_2$ May Be Restricted.* When pyrenebutyric acid was covalently conjugated with bovine serum albumin (such that the average number of pyrenebutyric acid's bound per bovine serum albumin was 0.5) or adsorbed to bovine serum albumin (*i.e.*, pyrenebutyric acid free in solution with a tenfold excess of bovine serum albumin) in 0.1 M $PO_4$ buffer (pH 8.0) two marked changes occurred in the Stern–Volmer plots. As seen in Figure 5, the slopes, hence the values of $k_+^*$, were greatly reduced compared with that of free pyrenebutyric acid. In addition, the intercept was lowered when the protein was present, thus raising $\tau_0$. The values of $k_+^*$ and $\tau_0$ suggested that not only was the pyrenebutyric acid less accessible to oxygen ($k_+^*$ lowered) but also it was in an environment which stabilized its excited state ($\tau_0$ increased). It was noted that in the two samples with high values of $\tau_0$, the first absorption maximum for pyrenebutyric acid, $\lambda_{max}$, was shifted to ~345 nm compared with 342 nm in the free state.

When the pH of a bovine serum albumin solution is lowered below pH 3.0, it is known that this protein undergoes expansion (Foster, 1960; Weber and Young, 1964). Lowering the pH of the bovine serum albumin solutions to 2.5 or less gave the results shown in Figure 6. The pyrenebutyric acid appeared to be more accessible to oxygen than at the higher pH (the $k_+^*$ values were 5–10 times larger) but its environment was not appreciably changed ($\tau_0$ was still in the 200-nsec range).

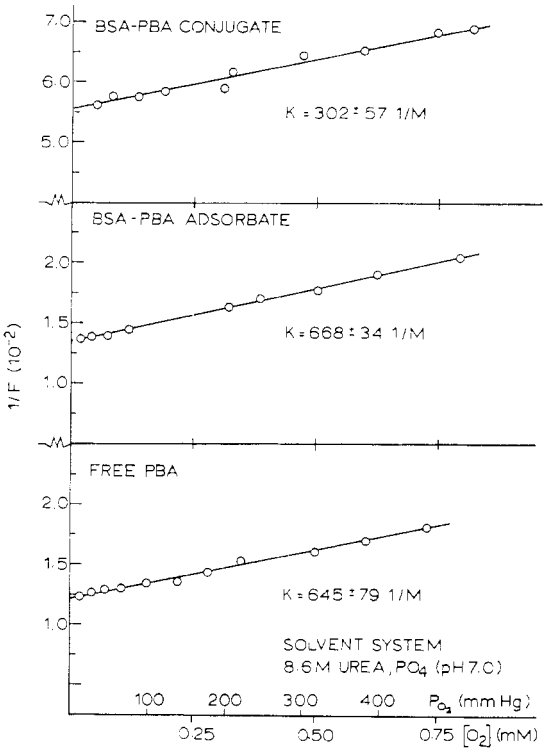



FIGURE 8: Oxygen quenching of pyrenebutyric acid fluorescence for the same solutions as shown in Figure 7.

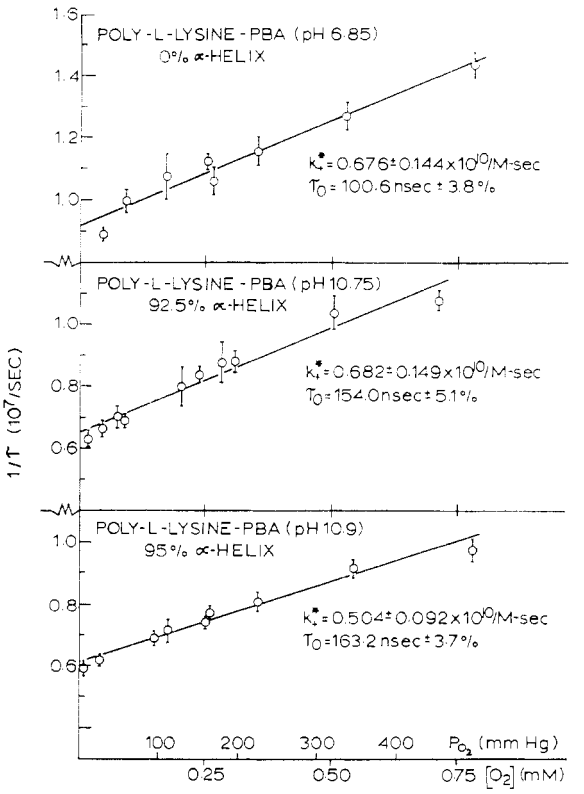



FIGURE 9: The effect of pH on the oxygen accessibility of pyrenebutyric acid covalently bound to poly-L-lysine. The change of $k_+^*$ and $\tau_0$ with pH (or $\alpha$-helix content) can be seen.

To study the change in accessibility following a more complete unfolding of the structure, bovine serum albumin samples were dialyzed against 8.6 M urea for 48 hr and then analyzed for oxygen quenching. Since the initially absorbed pyrenebutyric acid was dialyzed away in the change of solvent, free pyrenebutyric acid was added to restore the initial concentration ratio of bovine serum albumin to pyrenebutyric acid.

Figure 7 shows the effect of oxygen on pyrenebutyric acid in 8.6 M urea in the presence and absence of bovine serum albumin. It should be noted that the bovine serum albumin conjugate had a $k_+^*$ value almost 20% of the free pyrenebutyric acid in solution. Previously the pyrenebutyric acid conjugate had a $k_+^*$ value that was only 1–8% of the free pyrenebutyric acid. The accessibility of pyrenebutyric acid to oxygen was thus further increased by urea denaturation. The bovine serum albumin–pyrenebutyric acid adsorbate showed an even more marked increase in quenching by oxygen to the extent that it closely approximated the free pyrenebutyric acid in urea.

The values for $\tau_0$ free, "absorbed," or covalently bound in 8 M urea were now practically identical. This change indicated that in all three systems pyrene butyric acid was in the same environment.

Table I and a comparison of Figures 7 and 8 show that $K$ and $k_+^*\tau_0$ obtained by the two studies on the same solution are equal within experimental uncertainty. It should be noticed that the three quantities $K$, $\tau_0$, and $k_+^*$ are all independently determined. Less information is obtained from plots of $1/F$ *vs.* $[O_2]$ than when the lifetimes are analyzed since one can see independent changes of the dynamic parameters $k_+^*$ and $\tau_0$ in the lifetime studies but cannot do so in the $1/F$ plots.

The study of oxygen quenching of polylysine–pyrenebutyric acid conjugates was undertaken as complementary to that of the protein conjugates. Polylysine exists as a loosely open or "random coil" structure at pH lower than 8.5 and as a compact $\alpha$ helix at pH higher than 11 (Applequist and Doty, 1962).

Figure 9 shows that the structure of poly-L-lysine influenced both $k_+^*$ and $\tau_0$. The initial low $\tau_0$ (100 nsec) at pH 6.85 can be attributed to the charged atmosphere due to the $NH_3^+$ groups on the poly-L-lysine at this pH or to a relatively nonrigid environment. Once the charged atmosphere was elim-

TABLE I: Comparison of Overall Quenching Constant, $K$, and Dynamic Quenching Constant $k_+^*\tau_0$.[a]

| System | $K$ (l./mole) | $k_+^*\tau_0$ |
|---|---|---|
| Free pyrenebutyric acid | 645 ∓ 79 | 818 ∓ 246 |
| Pyrenebutyric acid–bovine serum albumin absorbate | 668 ∓ 34 | 733 ∓ 137 |
| Pyrenebutyric acid–bovine serum albumin conjugate | 302 ∓ 57 | 263 ∓ 210 |

[a] Solvent: 8.6 M urea, pH 7.0, 25°.

TABLE II: Rate of Quenching ($k_+^*$), Lifetime in the Absence of Oxygen ($\tau_0$), Quenching Efficiency ($\gamma$), and Wavelength of Maximum Absorption ($\lambda_{max}$).[a]

| Conditions (pH) | $k_+^*$ (l. mole$^{-1}$ sec$^{-1}$) $\times 10^{-10}$ | $\gamma$ | $\tau_0$ (nsec) | $\lambda_{max}$ (nm) |
|---|---|---|---|---|
| Polylysine | | | | |
| (6.85) | 0.68 ± 0.1 | 0.78 | 101 | |
| (10.75) | 0.68 ± 0.2 | 0.78 | 154 | |
| (10.90) | 0.50 × 0.1 | 0.57 | 163 | |
| Bovine serum albumin–pyrenebutyric acid | | | | |
| (7) conj[b] | 0.01 ± 0.1 | 0.01 | 173 | 345 |
| 8 M urea (7), conj | 0.19 ± 0.1 | 0.32 | 137 | 343.5 |
| (2) conj | 0.07 ± 0.05 | 0.08 | 204 | 346 |
| (7) ads | 0.02 ± 0.06 | 0.02 | 204 | 345.5 |
| 8 M urea, ads | 0.54 ± 0.1 | 0.91 | 135 | 343.5 |
| (2) ads | 0.12 ± 0.09 | 0.14 | 203 | 346 |
| Apohemoglobin | | | | |
| (7) ads | 0.112 ± 0.04 | 0.12 | 196 | |
| Human thyroglobulin[c] | | | | |
| (7) conj | 0.12 ± 0.07 | 0.13 | 181 | |
| Bovine thyroglobulin[d] | | | | |
| (7) conj | 0.24 ± 0.09 | 0.28 | 150 | |
| Free pyrenebutyric acid | | | | |
| (7) | 0.87 ± 0.20 | 1.0 | 139 | 342 |
| 8.6 M urea | 0.59 ± 0.2 | 1.0 | 138 | 342.5 |

[a] All observations at 25°. [b] conj = covalent conjugates; ads = adsorbate; [c] Iodine Content 0.32% (Pierce *et al.*, 1965). [d] Iodine content 1% (A. Rawitch, personal communication).

inated and the helix content became significant, the change of environment was reflected in an increase in $\tau_0$ to 154 nsec. As the helix content approached 95%, the relative exclusion of oxygen was seen in the reduction of $k_+^*$.

The results of all the observations are summarized in Table II where the values of $k_+^*$, $\tau_0$, and $\gamma$ have been collected. $\gamma$ is calculated as the ratio of $k_+^*$ in the system to $k_+^*$ observed for free pyrenebutyric acid in the same solvent and temperature. The accessibility of the pyrenebutyric acid residue in the system may be characterized by the value of $\gamma$ recorded. The lowest values (0.01–0.02) were observed in the conjugates and adsorbates of bovine serum albumin in agreement with other observations which indicate that hydrophobic ligands may be virtually buried within the structure of bovine serum albumin (Weber and Young; 1964; Anderson and Weber, 1969). Conjugates of thyroglobulin ($\gamma$ = 0.12, 0.24) were intermediate between the values for bovine serum albumin and a virtually open structure like polylysine where $\gamma$ = 0.8. The value of $\gamma$ = 0.32 observed for albumin conjugates in 8.6 M urea indicates accessibility still far from complete in this medium. The lower value of the bovine thyroglobulin's $\tau_0$ compared with the human thyroglobulin may be due to the greater iodine content of the bovine protein and a resulting perturbation of the excited state.

Throughout these studies the observation was made that a $\tau_0$ in the region of 175–200 nsec could be expected when the location of $\lambda_{max}$ was closer to 346 nm than 342 nm. Since it was felt that the pyrene ring structure of pyrenebutyric acid would be stabilized by a hydrophobic environment, a study of pyrenebutyric acid lifetimes as a function of the water content of a system was carried out.

The results of increasing the water content of a glycerol–water mixture after bubbling with $N_2$ for 10 min are shown

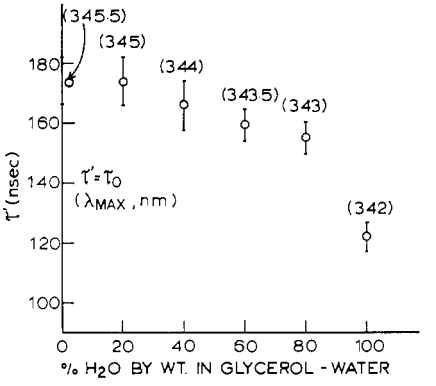



FIGURE 10: The lifetimes of oxygen-depleted solutions as a function of water concentration. Above each point is indicated the absorption maximum, $\lambda_{max}$, of each solution.

in Figure 10. Since the $N_2$ bubbling lowered the oxygen level to less than 0.015 mM, the lifetimes were a close approximation to $\tau_0$ values for each solution. The values of $\lambda_{max}$ are also indicated for the various solutions. One can observe that the absorption maximum shifted to 342 nm and the $\tau_0$ value decreased as the water content of the solutions increased. The lower $\tau_0$ values seem to correlate well with the presence of an aqueous environment.

During all of the above studies, the fluorescence peaks showed no detectable shifts in position. The only observed changes in the emission involved appearance of additional fine structure in the region of 380–390 nm for pyrenebutyric acid in a nonaqueous, well-protected environment such as bovine serum albumin. These spectral changes were slight compared with the change of 70 nsec in $\tau_0$ and the 20-fold increase in $K$ as the bovine serum albumin–pyrenebutyric acid conjugate was taken from the native state to the urea-denatured one.

## Discussion

We have presented a perturbation method that appears to be a more sensitive indication of chromophore accessibility to solvent than similar methods previously used. The solvent perturbation method of Herskovits and Laskowski (1960, 1962) involves the addition of nonaqueous perturbants in a concentration range of 10–20%. The observed difference spectra result from contact of the residue examined with the nonaqueous component in the system and are potentially able to reveal the exposure of chromophores, for example, tyrosyl residues in bovine serum albumin under various conditions.

Absorption perturbation gives a measure of the equilibrium distribution of chromophores as regards contact with the perturbant. It does not depend upon a kinetic property of the solution and gives no information as to the kinetic steps by which the perturbation is achieved.

Fluorescence lifetime studies in pure solvents show that oxygen quenching of pyrenebutyric acid fluorescence is an excited-state phenomenon essentially dependent upon the respective rates of emission of the fluorophore and of contact with free-diffusing oxygen. By following quantum yields alone, the relative rate of these two processes may be gauged, while observations of the dependence of lifetime upon the partial pressure of oxygen permit the determination of the absolute rate of oxygen–pyrene butyric acid quenching encounters.

Studies of the perturbation of the fluorescence efficiency have been carried out in several protein systems (Steiner, *et al.*, 1964; Lehrer, 1967; Winkler, 1969). The perturbants were various solvents, $I^-$ and $BrO_3^-$, all relatively large perturbants compared with oxygen. Solvent perturbation studies of fluorescence lifetimes do not appear to have been done.

In recent studies Winkler (1969) used the $BrO_3^-$ ion in concentrations up to 0.3 M as the quenching probe for the accessibility of the adsorbed aminonaphthalenesulfonic acid to the quenching ion.

The approach presented here has several important features that lend it further application. (1) The probe for accessibility is the free-diffusing, neutral oxygen molecule whose unsolvated diameter is approximately 3 Å. "Holes" and "clefts" in the structure of a protein can thus be examined with one of the smallest probes possible. (2) The concentration of the probe can be directly monitored by its chemical activity at a platinum electrode and can be reversibly and easily changed so that the quenching in a given solution can be studied under various conditions. (3) The observable changes are large (a 35% decrease in $\tau_0$ going from air-saturated solutions to oxygen free and a 20-fold change in $k_+^*$ for different states of bovine serum albumin) and the concentration range of the probe over which changes are most evident is in the region of 0–0.75 mM, thus eliminating the need for large changes in the solvent system in the course of an investigation.

Future applications of the above approach are possible in the correlation of other structural changes of labeled macromolecules with the parameters used here. One could conceivably monitor changes in the oxygen concentration in the microenvironment in mitochondria under various conditions of inhibition and activity. Oxygen bursts in photosynthetic systems could be monitored by the resulting changes in the yield or lifetime of pyrenebutyric acid added to the environment in submillimolar concentrations (the concentrations used in the studies presented here were seldom greater than 5 μM). Conceivably, pyrenebutyric acid and other long-lived fluorophores can serve as indicators of oxygen changes with resolution times of less than a microsecond.

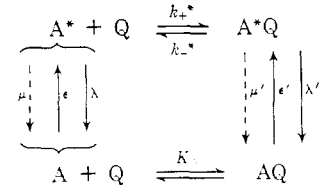
## Acknowledgments

We thank Dr. A. B. Rawitch and Miss D. Davenport for providing samples for examination in this work and Mr. E. N. Hudson for assistance in labeling samples.

## Appendix

*The Effect of Molecular Interaction upon the Fluorescence Efficiency.* We consider here the general problem of the effect of the interaction of a "fluorescent" molecule A and another molecule Q upon the fluorescence efficiency of the former. Various treatments of particular aspects of this problem are found in the literature since the original contribution of Stern and Volmer (1919). Wawilow (1928), Perrin (1929), and many others afterward have treated the problem of collisional quenching. Weber (1948, 1950), Weber and Lokar (1948), Epple and Förster (1954), and others have dealt with the description of quenching by complex formation. Weller (1959) has treated the problem of quenching in a similar though less general fashion than the one we present below.

The scheme below takes into account the possible existence of a dark complex AQ predating the excitation as well as an encounter between A* (excited A) and Q leading to formation of excited complexes A*Q. The scheme applies not only to quenching but to activation of fluorescence by molecular interaction. Finally this treatment describes output both under



steady illumination and when the exciting light is an arbitrary function of the time. In this scheme the fluorescent molecule A and the interacting partner Q are in equilibrium with the "dark" complex AQ. The equilibrium constant $K_0$ being defined by

$$K_0 = \frac{[AQ]}{[A][Q]} = \left(\frac{1}{\alpha} - 1\right)[Q]^{-1} \tag{1}$$

where $\alpha$ is the degree of dissociation. The dimensions of $K_0$ are therefore in liters per mole. If a light source is used which does not appreciably depopulate the ground state, the ground state concentrations [A], [Q], and [AQ] under illumination are virtually identical with those in the dark so that eq 1 is valid in both conditions. $\epsilon$ is the number of absorption transitions between A and A* per mole of A per second under the conditions of excitation prevailing. $\lambda$ is the rate of emission of excited A molecules and $\mu$ their rate of radiationless deactivation. The primed quantities $\epsilon'$, $\lambda'$, $\mu'$ refer to similar values of the complex QA. $k^*_+$ and $k^*_-$ are the rates of association of A* with Q and dissociation of AQ*, respectively.

The rate equations for the excited species A* and A*Q are, respectively:

$$\frac{d[A^*]}{dt} = \epsilon[A] - (\lambda + \mu + k^*_+[Q])[A] + k^*_-[A^*Q] \tag{2}$$

$$\frac{d[A^*Q]}{dt} = \epsilon'[AQ] - (\lambda' + \mu' + k^*_-)[A^*Q] + k^*_+[A][Q]$$

These may be put under the convenient matrix form

$$\begin{bmatrix} D + \lambda + \mu + k^*_+[Q] & -k^*_- \\ -k^*_+[Q] & D + \lambda' + \mu' + k^*_- \end{bmatrix} \begin{bmatrix} [A^*] \\ [A^*Q] \end{bmatrix} = \begin{bmatrix} \epsilon[A] \\ \epsilon'[AQ \end{bmatrix} \tag{3}$$

where $D = d/dt$.

In eq 3 we have written $\epsilon(t)$ and $\epsilon'(t)$ to stress the fact that the rates of excitation may not be constants but arbitrary functions of the time.

Solving eq 3 gives

$$\Delta[A^*Q] = \begin{bmatrix} D + \lambda + \mu + k^*_+[Q] & \epsilon(t)[A] \\ -k^*_+[Q] & \epsilon'(t)[AQ] \end{bmatrix} \tag{4}$$

$$\Delta[A^*] = \begin{bmatrix} \epsilon(t)[A] & -k^*_- \\ \epsilon(t)[AQ] & D + \lambda' + \mu' + k^*_- \end{bmatrix} \tag{5}$$

where $\Delta$ is the linear operator.

$$\frac{d^2}{dt} + (a + b)\frac{d}{dt} + (ab - c) \tag{6}$$

$$a = \lambda + \mu + k^*_+[Q]$$

$$b = \lambda' + \mu' + k^*_-$$

$$c = k^*_- k^*_+[Q]$$

*Steady-State Solution.* The steady-state solution is obtained by setting $D = 0$, and replacing $\epsilon(t)$ and $\epsilon'(t)$ by constant values $\epsilon$ and $\epsilon'$, respectively, in eq 3. Solving the simultaneous equations gives:

$$[A^*] = \frac{(\lambda' + \mu' + k^*_-)\epsilon[A] + k^*_-\epsilon'[AQ]}{(\lambda' + \mu')(\lambda + \mu + k^*_+[Q]) + k^*_-(\lambda + \mu)} \tag{7a}$$

$$[A^*Q] = \frac{(\lambda + \mu + k^*_+[Q])\epsilon'[AQ] + k^*_+[Q]\epsilon[A]}{(\lambda' + \mu')(\lambda + \mu + k^*_+[Q]) + k^*_-(\lambda + \mu)} \tag{7b}$$

Equations 7a,b may be simplified and put in a form that makes use of observable, or potentially observable experimental quantities, namely, the fluorescence yield and lifetime ($q_0$ and $\tau_0$) of the free fluorophore and the corresponding quantities ($q_0'$ and $\tau_0'$) of the AQ complex. From the definitions of these quantities

$$\begin{aligned} \tau_0 &= 1/(\lambda + \mu); \quad q_0 = \lambda\tau_0 \\ \tau_0' &= 1/(\lambda' + \mu'); \quad q_0' = \lambda'\tau_0' \end{aligned} \tag{8}$$

Furthermore if the presence of Q produces only small changes in the absorption spectrum of A it is possible to choose for the excitation wavelength an isosbestic point of A and AQ thus making $\epsilon = \epsilon'$. We can therefore assume this equivalence without loss in generality. Besides from eq 1

$$\begin{aligned} [A] &= \alpha A_0 \\ [AQ] &= (1 + \alpha)A_0 \end{aligned} \tag{9}$$

where $[A] + [AQ] = A_0$ the total amount of A present, since the fraction in the excited state is negligible. With these substitutions

$$[A^*] = \epsilon A_0\tau_0 \frac{\alpha + k^*_-\tau_0'}{1 + k^*_+\tau_0[Q] + k^*_-\tau_0'} \tag{10a}$$

$$[A^*Q] = \epsilon A_0\tau_0' \frac{1 - \alpha + k^*_+\tau_0[Q]}{1 + k^*_+\tau_0[Q] + k^*_-\tau_0'} \tag{10b}$$

The fluorescence efficiency, $F$, calculated from eq 10 and the rate relation

is $$F = \lambda[A^*] + \lambda'[A^*Q] \tag{11}$$

$$F = \epsilon A_0 \frac{q_0(\alpha + k^*_-\tau_0') + q_0(1 - \alpha + k^*_+\tau_0[Q])}{1 + k^*_+\tau_0[Q] + k^*_-\tau_0'} \tag{12}$$

The fluorescence, $F_0$, recorded in the absence of quencher, obtained by setting in the last equation $[Q] = 0$ and $\alpha = 1$, is

$$F_0 = \epsilon A_0 q_0 \tag{13}$$

Therefore

$$\frac{F}{F_0 - F} = \frac{q_0'}{q_0 - q_0'} + \frac{q_0}{q_0 - q_0'} \frac{\alpha + k_{\pm}^{*}\tau_0'}{(1 - \alpha + k_{\pm}^{*}\tau_0'[Q])} \quad (14)$$

The last equation shows that in favorable cases ($\alpha \rightarrow 0$, or $\alpha \rightarrow 1$) the fluorescence yield $q_0'$ of the complex may be estimated by extrapolation if $F/(F_0 - F)$ is plotted against $1/[Q]$.

The general equation 12 describing the fluorescence emission as function of $Q$ reduces to well-known forms in particular cases. Thus, if $\lambda = \lambda'$ and $\mu = \mu'$ we have $q_0 = q_0'$, $\tau_0 = \tau_0'$, and $F = F_0$ for all [Q]. This is the case of a molecular interaction that does not affect the fluorescence emission.

We shall define "strong" quenching as one in which the contribution of the complexes to the fluorescence emission is negligible in all circumstances and "weak" quenching as one in which there is an appreciable contribution by the complexes to the fluorescence emission.

The conditions for "strong" quenching are:

$$\begin{aligned} q_0' &\ll q_0 \\ k_{\pm}^{*}\tau_0' &\ll 1 \end{aligned} \quad (15)$$

For "strong" quenching eq 12 may be written as

$$F = \epsilon A_0 q_0 \frac{\alpha}{(1 + k_{+}^{*}\tau_0[Q])} \quad (16)$$

From eq 1

$$\alpha = (1 + K_0[Q])^{-1}$$

$$\frac{F_0}{F} = (1 + K_0[Q])(1 + k_{+}^{*}\tau_0[Q]) \quad (17)$$

The first factor in the last equation arises from nonfluorescent complexes predating the excitation and the second from the dynamic quenching of the free fluorophore during the excited state. It will be noticed that if the quenching effect of either of these two kinds is much greater than the other the quenching follows the Stern–Volmer linear dependence of $F_0/F$ upon [Q]. From such dependence it is not possible to infer the character (static or dynamic) of the process. If there is a departure from linearity, due to the term in $[Q]^2$ being appreciable, it is certain that both ground state complexes and excited-state interactions contribute to the quenching but it is not possible to determine their relative importance except in the case in which both are equal, when $F_0/F = (1 + \text{Constant } [Q])$.[2] In all other instances the determination of the character of the process can only be done by a study of the effect of quenching upon the fluorescence lifetime (Weber, 1948; Epple and Förster, 1954). For this purpose it is necessary to examine the system under the conditions given by eq 3 with $D \neq 0$. We can envision two different cases: (1) decay of the emission after illumination has ceased (free decay); (2) fluorescence output following modulated excitation (forced decay).

We shall examine the first case only since we have used it exclusively in the experimental analysis of the system.

*Free Decay.* Free decay is the decay of the emission after excitation has ceased. Equations 4 and 5 become

$$\begin{aligned} \Delta[A^*] &= 0 \\ \Delta[A^*Q] &= 0 \end{aligned} \quad (18)$$

while $\epsilon' = \epsilon = I$ at $t = 0$. The solution involves the characteristic quadratic equation

$$y^2 + (a + b)y + (ab - c) = 0 \quad (19)$$

where $a$, $b$, and $c$ are defined in eq 6. The solutions of eq 18 are

$$[A^*] = A_1 e^{y_1 t} + B_1 e^{y_2 t} \quad (20a)$$

$$[A^*Q] = A_2 e^{y_1 t} + B_2 e^{y_2 t} \quad (20b)$$

$y_1$, $y_2$, the roots of the characteristic equation are

$$\begin{aligned} y_1 &= -\frac{a + b}{2} + \frac{(a - b)}{2}\sqrt{1 + \frac{4c}{(a - b)^2}} \\ y_2 &= -\frac{a + b}{2} - \frac{(a - b)}{2}\sqrt{1 + \frac{4c}{(a - b)^2}} \end{aligned} \quad (21)$$

Under conditions of strong quenching (eq 15) $4c \ll (a - b)^2$.

$$y_1 = -b; \quad y_2 \simeq -a \quad (22)$$

The constants $A_1$, $B_1$, $A_2$, $B_2$ are not independent

$$\begin{aligned} [A^*]_{t \rightarrow 0} &= A_1 + B_1 = I \cdot [A] \\ [A^*Q]_{t \rightarrow 0} &= A_2 + B_2 = I \cdot [AQ] \end{aligned} \quad (23)$$

so that

$$\begin{aligned} [A^*] &= A_1(e^{y_1 t} - e^{y_2 t}) + I[A]e^{y_2 t} \\ [A^*Q] &= B_2(e^{y_2 t} - e^{y_1 t}) + I[AQ]e^{y_1 t} \end{aligned} \quad (24)$$

Moreover eq 2 and 24 for $d[A^*]/dt$ and $d[A^*Q]/dt$ at $t \rightarrow 0$ together with the conditions in eq 22 give

$$A_1 = I\frac{[A] + k_{\pm}^{*}[AQ]}{y_1 - y_2} \quad (25)$$

$$B_2 = I\frac{[AQ] + k_{+}^{*}[A][Q]}{y_2 - y_1} \quad (26)$$

The fluorescence emission at time $t$, $F(t)$, is given by

$$F(t) = \lambda[A^*] + \lambda'[A^*Q] = I\frac{[A](\lambda + \lambda' k_{+}^{*}[Q]) + [AQ](\lambda' + \lambda k_{\pm}^{*})}{y_1 - y_2}(e^{y_1 t} - e^{y_2 t}) + I\lambda[A]e^{y_2 t} + I\lambda'[AQ]e^{y_1 t} \quad (27)$$

Under conditions of strong quenching the first and third terms are negligible in comparison with the second and

$$F(t) \simeq I\lambda[A]e^{-(\lambda+\mu+k_+^*[Q])} \quad (28)$$

Therefore, in such cases of strong quenching the decay after a light pulse is simple exponential with decay constant $1/\tau = \lambda + \mu + k_+^*[Q]$. The ratio of $\tau_0:\tau$ is given by

$$\frac{\tau_0}{\tau} = 1 + k_+^*\tau_0[Q] \quad (29)$$

Comparing eq 29 and 17 we see that for strong quenching

$$\frac{\tau_0}{\tau} = \frac{F_0}{F} \quad (30)$$

only if $K_0 \ll k_+^* \tau_0$, that is if dark complex formation is negligible. If such is not the case

$$1 < \frac{\tau_0}{\tau} < \frac{F_0}{F} \quad (31)$$

$\tau_0/\tau$ approaching 1 if dark complex formation is predominant, or $F_0/F$ if dynamic excited interactions are the more important. Such a rule was formulated by Weber (1948).

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