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Quenching of Fluorescence by Oxygen. A Probe for Structural Fluctuations in Macromolecules†

Joseph R. Lakowicz and Gregorio Weber*

ABSTRACT: Quenching of the fluorescence of various fluorophores by molecular oxygen has been studied in aqueous and nonaqueous solutions equilibrated with oxygen pressures up to 100 atm. Temperature dependence of quenching, agreement with the Stern–Volmer equation, and fluorescence lifetime measurements indicate that essentially all the observed quenching is dynamic and close to the diffusion-controlled limits. Studies of charged polyamino acids containing tryptophan show that oxygen quenching, in contrast to I^-, is completely insensitive to charge effects. Ethidium bromide, when intercalated into double helical DNA, is quenched with 1/30th of the efficiency of the free dye in solution. Three dyes bound to bovine serum albumin were also found to be relatively protected from the free diffusion of oxygen. Quenching of intrinsic or bound fluorophores by molecular oxygen is therefore an appropriate method to determine the accessibility to oxygen of regions of the macromolecule surrounding the fluorophore and indirectly the structural fluctuations in the macromolecule that permit its diffusion to the fluorophore.

Molecular oxygen is known to be an efficient quencher of the fluorescence of aromatic hydrocarbons (Berlman, 1965; Ware, 1962). The studies so far published show quenching by oxygen to be a diffusion-controlled process in which virtually every collision with the excited fluorophore is effective in quenching. Although a good deal of work has been done in nonaqueous solutions, very little work has been done in aqueous solutions. This discrimination is due to the low solubility and diffusion coefficient of oxygen in water as compared to organic solvents, and the concomitant low levels of quenching that are observed in solutions equilibrated with air, or even with pure oxygen at atmospheric pressure.

Previous work in aqueous systems used pyrenebutyric acid (Vaughan and Weber, 1970).[1] Due to the long fluorescent lifetime of pyrenebutyric acid (100–200 nsec) it was possible to quench a significant fraction of its fluorescence using dissolved oxygen at 1 atm pressure. It was shown that when pyrenebutyric acid was bound to bovine serum albumin and other proteins the amount of quenching was greatly reduced. The protein structure prevented the diffusion of oxygen to the fluorophore.

Typical fluorescence probes have lifetimes of 10–20 nsec. Native tryptophan fluorescence of proteins displays lifetimes ranging from 2 to 6 nsec. Upon equilibration with oxygen at a pressure of 1 atm the fluorescence of a dye solution with a 2-nsec lifetime would decrease by only 3%; in a system where there is some steric barrier to the diffusion of oxygen to the fluorophore the decrease in signal would be even less. This problem may be overcome by increasing the oxygen concentration *via* an increase in the oxygen pressure. Equilibration of an aqueous solution with oxygen at a pressure of 100 atm results in a dissolved oxygen concentration (0.13 M) sufficient to quench more than one-half of the fluorescence of a dye with 1-nsec lifetime.

Since oxygen is an uncharged molecule of very small dimensions, and not particularly hydrophilic, as shown by its small solubility, the potential exists for yielding fundamentally different information from that obtained by the use of iodide, bromate, or mercuric ions as quenchers. Iodide quenching is known to be extremely sensitive to charge effects (Lehrer, 1971). In opposition to Hg^{2+}, oxygen at any of the concentrations used here does not form significant complexes with any of the dyes or proteins used in these studies. Hg^{2+} has a radius of action determined by the possibility of energy transfer from those fluorophores with emission spectra within the absorption band of the mercury–sulfur bond (Chen, 1971). In contradistinction, oxygen has no appreciable absorption at wavelengths longer than 250 nm, so that quenching requires actual short-range interaction with the fluorophore.

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[1] Barenboim (1963) reported the use of oxygen to quench photo- and radioluminescence of aromatic amino acids, proteins, and nucleic acids in solution and as dry powders. The use of polychromatic excitation (240–293 nm) comprising wavelengths of strong O_2 absorption, the smaller range of O_2 pressures (16 atm), the small number of proteins examined, and the absence of fluorescence lifetime measurements preclude comparison of his results with the present studies.

Because of the necessity of actual collisions with the fluorophore, and the high probability of quenching at any collision, oxygen must be considered an ideal probe to obtain information about the existence of local structural fluctuations in the nanosecond range in proteins and nucleic acids in water solutions.

Experimental Procedures

Dynamic quenching of fluorescence is described by the Stern–Volmer equation (Stern and Volmer, 1919)

$$F_0/F = 1 + k_+{}^*\tau_0[O_2]$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, τ_0 is the fluorescence lifetime in the absence of quencher, and $k_+{}^*$ is the bimolecular quenching constant. $K = k_+{}^*\tau_0$ is the Stern–Volmer quenching constant. The proportionality of K to τ_0 is easily explained by noting that a long-lived fluorophore has a higher probability of undergoing a collision with oxygen than a fluorophore which emits at an earlier time. The diffusion-controlled bimolecular rate constant $k_+{}^*$ is given by

$$k_+{}^* = 4\pi aDN'$$

D is the sum of the diffusion coefficients of oxygen and fluorophore, a is the sum of the molecular radii, and N' is the number of molecules per millimole (Weller, 1959). Using the value of 2.6×10^{-5} cm^2/sec for the diffusion coefficient of oxygen at 25° (Millington, 1955), a diffusion coefficient of tryptophan at 25° of 0.66×10^{-5} cm^2/sec (Landolt-Börnstein, 1969), and a collision radius (a) equal to 4 Å, $k_+{}^*$ is found to be 1×10^{10} M^{-1} sec^{-1}.

The static quenching of fluorescence is dependent upon formation of a nonfluorescent complex between fluorophore and quencher predating excitation. A modified form of the Stern–Volmer equation which describes quenching data when both dynamic and static quenching are operative is

$$F_0/F = (1 + k_+{}^*\tau_0[O_2])(1 + K_s[O_2])$$

K_s is the equilibrium constant for complex formation or static quenching constant (Vaughan and Weber, 1970). Dynamic quenching is a process which competes with emission for depopulation of the excited state; thus the fluorescence lifetime decreases in proportion to the yield. Static quenching reduces only the yield since the lifetime of the uncomplexed fluorophores remains constant. Perrin (1929) and Wawilov (1936) first pointed out that the dynamic nature of the quenching process may be proven by the equivalence of F_0/F and τ_0/τ. This was experimentally shown by Szymanowsky (1936) and, in the case of oxygen in water, by Vaughan and Weber (1970). A static component in the quenching is apparent by upward curvature in the Stern–Volmer plots. In this case $\tau_0/\tau < F_0/F$. Calculations show that if the emitting population is heterogeneous, either by lifetime or bimolecular quenching constant, τ_0/τ may be greater or less than F_0/F. In no case, however, is upward curvature in the Stern–Volmer plots possible unless two modes of quenching, *i.e.*, static and dynamic, act on the same population.

Using any two data points from a Stern–Volmer plot one may solve for K and K_s. A computer program was written which does this calculation for every possible pair of data points and averages the resulting K and K_s values. This program was used to separate the static and dynamic quenching constants for fluorophores in aqueous and nonaqueous solvents.

Calculations of theoretical quenching data in a heterogeneous system were carried out as follows. Assuming two populations of fluorophores having τ_{01} and τ_{02} for fluorescent lifetimes, and assuming the fraction of the fluorescence intensity from each population, α_{01} and α_{02}, one may calculate the lifetime that would be measured by phase, $\tau_0(\phi)$, or modulation, $\tau_0(M)$ (Spencer and Weber, 1960; Spencer, 1970). Oxygen bimolecular quenching constants may be assigned to each population. A computer program was written to calculate F_0/F, $\tau_0(\phi)/\tau(\phi)$ and $\tau_0(M)/\tau(M)$ as a function of oxygen concentration when τ_{01}, τ_{02}, α_{01}, α_{02}, k_1, and k_2 are given. Due to the large number of parameters which must be assigned for even the simplest two-population model and the possibility of achieving the same results for different combinations of these parameters, no attempt was made to fit any of our data to these calculations, but they were useful in demonstrating the type of data that result from a heterogeneous population.

8-Anilinonaphthalene-1-sulfonic acid (ANS)[2] was prepared according to Weber and Young (1964) and used as the magnesium salt. 6-Diethylaminonaphthalene-1-sulfonic acid (DENS) was prepared and purified by J. Stewart and G. Weber (unpublished results) by the reaction of 6-aminonaphthalene-1-sulfonic acid with diethyl sulfate, using pyridine–water as a solvent. Indole was obtained from Aldrich (Puriss grade) and recrystallized three times from petroleum ether. L-Tryptophan, L-tyrosine, and riboflavine were purchased from Nutritional Biochemicals. Lumiflavine was prepared by irradiation of a basic solution of riboflavine with a mercury arc for 2 hr. The solution was acidified with concentrated HCl and extracted two times with chloroform. The extracts were evaporated to dryness. Chromatography according to Cairns *et al.* (1968) indicated at least 90% purity. A trace of lumichrome may be present. Ethidium bromide was a gift from Dr. James Wetmur, Department of Chemistry, University of Illinois, Urbana. Spectrophotometric grade dodecane, 9-vinylanthracene, 2-methylanthracene, and anthracene-9-carboxylic acid were obtained from Aldrich. The latter compound was recrystallized from alcohol–water. Zone-refined perylene was purchased from Aremco. Calf thymus DNA was obtained from Sigma as the sodium salt, lot 101C-9520. Bovine serum albumin was obtained from Armour, lot D71002. Poly(L-glutamate^{99}-L-tryptophan[1]) and poly(L-lysine^{97}-L-tryptophan[3]) were generous gifts from Dr. Gerald D. Fasman, Brandeis University. These high molecular weight, random sequence copolymers were identical with those used by Lehrer (1971).

Unless otherwise stated, all experiments were performed at 25.0 ± 0.2°, traceable to National Bureau of Standards (NBS) thermometers 151998 and 172631. The temperature was maintained by a circulating bath from which the temperature was monitored. For temperatures other than 25°, the temperature of the actual sample being used was monitored. Absorption spectra were recorded on a Cary Model 15 recording spectrophotometer. Optical densities were measured with a Zeiss spectrophotometer. Emission spectra were obtained on the instrument described by Weber and Young

[2] Abbreviations used are: ANS, 8-anilinonaphthalene-1-sulfonic acid; DENS, 6-diethylaminonaphthalene-1-sulfonic acid; N-Ac-Trp-NH$_2$, *N*-acetyl-L-tryptophanamide; Gdn·HCl, guanidine hydrochloride.

(1964) using right angle optics. Both the excitation and emission monochromators were recalibrated or their calibration checked several times during the course of these experiments. A low-pressure mercury lamp was used to supply the wavelength standards (Pen Ray Lamp Model 11 SC-1C, Ultra-Violet Products, Inc.). The calibration is accurate to ±1 nm. Whenever accurate intensity measurements were required, at least 50 readings were averaged using a digital ratio meter and averager. Fluorescence intensities were always recorded as the ratio of the fluorescence intensity to the intensity of the exciting light. This corrects for variations in the xenon lamp output. Fluorescence lifetimes were recorded on the phase-modulation cross-correlation fluorometer described by Spencer and Weber (1969). If the fluorophore was bound to a macromolecule the exciting beam was polarized 35° from the vertical position. This cancels the effects of Brownian rotations on the measured lifetimes (Spencer and Weber, 1970). The frequency of the exciting light was 14.2 MHz.

Special Equipment. These experiments required the capability of performing fluorescence, absorbance and enzyme activity determinations at oxygen pressures up to 100 atm. Two stainless steel cells were constructed, one provided with three windows for fluorescence measurements, and one provided with a pair of in-line windows. The latter was used for absorption measurements and for determination of enzyme activities under high pressures of oxygen. Both of these cells use quartz windows and Viton O-ring seals. Buna-N O-rings were found to release ultraviolet absorbing impurities into the solutions and thus considered unsuitable. All quartz windows were obtained from George Behm and Sons, Dayton, Ohio. The temperature of both cells was controlled by internal stainless steel tubing (0.25-in. o.d.) through which fluid from an external temperature-controlled bath was circulated.

Figure 1 is a photograph of the high-pressure fluorescence cell. The black tubing is connected to a temperature control coil which is inside the cell. The smaller tubing visible in this picture is for delivery and release of gas pressure. The windows were type 125 quartz, 1 in. diameter × 0.25 in. thick. The light path is 0.5 in. in diameter. For the excitation and emission windows special T-shaped windows were used, one of which is visible in Figure 1. When these windows are in place they essentially simulate the conditions in a 1-cm square cuvet in the center of the cell. This geometry allowed the use of solutions of normal optical densities, rather than the more dilute solutions that would be required if the excitation beam entered the solution 3 cm in front of the point of observation. In addition to the three windows on the sides, a fourth window was present on the top of the cell for observation. A mount was built which held this cell rigidly in the fluorometer previously described. An integral part of this mount is a magnetic stirrer which allowed agitation of the solution inside the cell *via* an internal Teflon-coated stirring bar. The cell was typically filled with solution to about 1 cm above the top of the windows. About 30–60 min was required to equilibrate the solution with a given gas pressure. Due to this long equilibration time experiments sometimes lasted 10 hr. It was very important for the absolute gain of the instrument to remain unchanged over this time period since all intensity readings are relative to the initial fluorescence prior to any increase in oxygen pressure. In order to monitor the gain of the instrument provisions were made to accurately position a reference solution in front of the emission monochromator and to divert the exciting beam to this solution. The required movable mirror and reference cell traveled on precision dovetail mounts. The accuracy of their movements was such as to yield better than

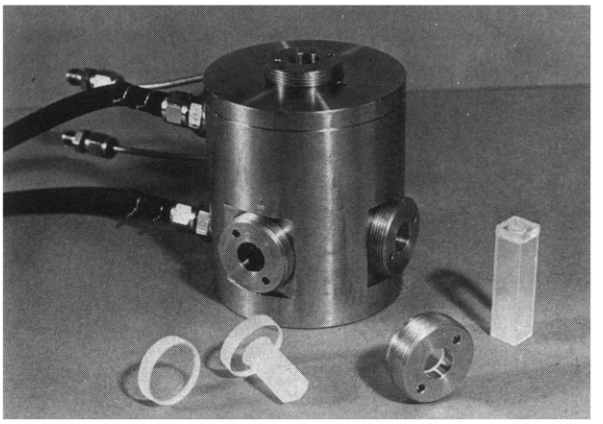


FIGURE 1: High-pressure fluorescence cell.

1% reproducibility of the signal from the reference cell. The gain drift of this instrument over a 10-hr period was typically less than 5%. By the use of the external standard all fluorescence intensities reported here are corrected for any significant drifts in gain. The reference solution was identical with the solution being quenched and was contained in a 1-cm square stoppered fluorescence cuvet.

Filling the cell to a point above the windows requires about 60 ml of solution. For some experiments with proteins this volume is excessive. Mounts were constructed which allowed placement of 1- or 2-cm square section fluorescence cuvets in the center of the pressure cell, thus reducing the required volume. Equilibration with the increased gas pressure proved to be too slow using 1-cm cuvets. Using the 2-cm square cuvet 30–60 min was needed for equilibration. A 2-cm cuvet was used in all the studies involving 6 M guanidine hydrochloride (Gdn·HCl). Contact of stainless steel with 6 M Gdn·HCl resulted in formation of a brown precipitate.

The measurement of fluorescence lifetimes under oxygen pressure required extensive modification of the phase fluorometer described by Spencer and Weber (1969). In the measurement of lifetimes with this instrument the phase angle of the exciting light and fluorescence is measured, and the former is provided by the scattered light from a glycogen solution. Because of the impossibility of rapid replacement of the high-pressure fluorescence cell with the glycogen solution, two movable mirrors were used to reflect the exciting beam to the glycogen solution. If a scattering solution is placed in both the reference position and the pressure cell a phase delay corresponding to a nonzero lifetime is observed. This is due to the different geometry of the two light paths. The phase and modulation of the exciting beam are not constant across the beam itself. Depending on the exact positioning of the lenses in each light path, varying portions of this beam may be blocked at the entrance to the scattering cuvet or the pressure cell. Thus the overall phase of the beam is altered. The amount of blockage varies with wavelength since the focal lengths of the lenses vary. In all the measurements reported here the phase difference due to the beam splitting was measured before and after each high-pressure run and the measured lifetimes were corrected by addition or subtraction of the spurious "lifetime" measured for the two scattering solutions.

The high-pressure absorption cell is shown in Figure 2. This cell was designed to fit into the sample compartment of a Cary 15 spectrophotometer. The windows are 1.5 in. in diameter × 0.5 in. thick. The light path is 0.75 in. in diameter.

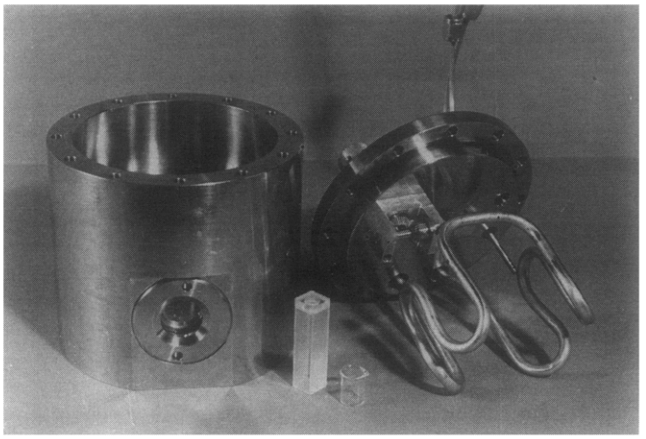


FIGURE 2: High-pressure absorbance and enzyme assay cell.

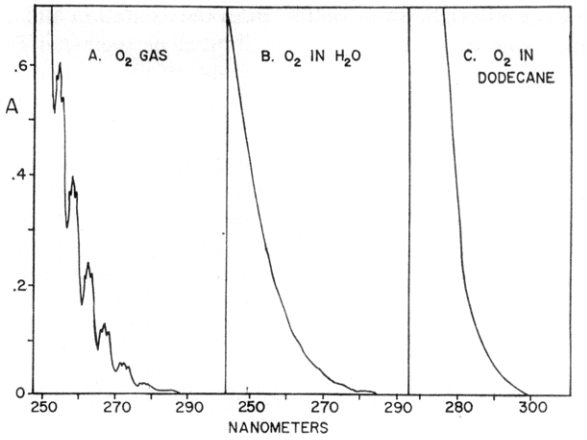


FIGURE 3: Absorption spectra of oxygen, 1500 psi, 10.7-cm light path.

All the quartz components of this cell are type 151 laser quality quartz. This type of quartz has higher transmission in the ultraviolet. This attribute is valuable because use of the internal accessories of this cell can increase the light path through quartz to 10 cm.

The tubing visible on the right of Figure 2 is for temperature control. The two bevelled gears visible in this picture are part of the mechanism which allows the measurement of enzymatic activity. For enzyme assays an internal cell is filled with 20 ml of substrate solution and enzyme is placed into the 1-ml beaker. Both substrate and enzyme are preequilibrated with the increased oxygen pressure prior to initiation of the assay. Agitation of the solution is accomplished by an external magnetic stirrer. By rotation of the knob present on top of the cell it is possible to drop the 1-ml beaker visible in Figure 2 into the internal cell to initiate the reaction. This beaker remains outside the light path.

The light path of the internal stainless cell may be varied by movement of the 0.75 in. diameter quartz rods which form its windows. Using light paths of 0.5, 1, or 2 cm for this cell it is possible to fill the remaining space between the internal and external windows with quartz rods. This is useful in the 250-nm region and at shorter wavelengths to cancel to absorbance due to dissolved or gaseous oxygen in the space between the internal and external windows; 1-, 2-, or 5-cm cuvets can also be mounted inside the cell.

The pressure of the gas phase above the solutions was determined with a Roylyn Precision Gauge; 0–1500-psi scale, 5-psi divisions, 0.25% accuracy.

Oxygen concentrations were calculated using Henry's law and the following oxygen solubilities at 1 atm oxygen pressure: 5°, 0.001933 M; 15°, 0.001541 M; 25°, 0.001275 M; 35°, 0.00103 M (Richardson, 1928; Winkler, 1891). No corrections were made for the effect of ionic strength on oxygen solubility. This effect depends on the particular ionic species (MacArthur, 1916). Since data are not available for all the buffers used, no corrections were attempted. NaCl (0.5 M) can decrease the oxygen solubility by 15% compared to pure water. Since controls using free fluorophores were performed in all these experiments the apparent bimolecular rate constants may be compared without knowing the precise effect of the salts on the oxygen solubility.

In absolute ethanol and dodecane the following solubilities were used: ethanol, 0.00987 M/atm; dodecane, 0.00826 M/atm (Landolt-Börnstein, 1962).

Results and Discussion

Absorption Spectrum of Oxygen. Figure 3 shows the absorption spectrum of oxygen as a gas and dissolved in water and dodecane, respectively. For these spectra the entire 10.7-cm light path of the high-pressure absorption cell was used. Knowledge of the absorption of oxygen in aqueous solutions at pressures up to 1500 psi of O_2 is necessary to determine if there is significant absorption at 280 nm, the shortest excitation wavelength used in these studies. Such absorption could lead to artificially higher Stern–Volmer quenching constants due to absorption of the exciting light with the resultant decrease in the emission intensity. The shape of the emission spectra could also be affected by such absorption. Previous data concerning the absorption of oxygen in aqueous solution were obtained at 1 atm pressure and do not permit any prediction of the absorption at 100 atm. Figure 3 demonstrates that there is no significant increase in the absorption at 280 nm in aqueous solution due to the increased oxygen concentration. This facts provides a small experimental advantage over iodide quenching, in the study of which excitation must be at 290 nm or longer wavelengths to reduce the absorbance. The concentration of gaseous oxygen at 100 atm is about 4.5 M and the concentration of dissolved oxygen is about 0.13 M. The larger extinction coefficient of dissolved oxygen is thought to be due to formation of water–oxygen adducts (Heidt and Johnson, 1957). Heidt's work was performed at 1 atm, using a 10-cm light path over the wavelength range from 200 to 215 nm. In order to compare results an absorption spectrum of oxygen in water at 1500 psi was recorded using a 0.5-cm light path. The remainder of the light path was filled with quartz rods. An optical density of 0.51 at 215 nm was observed. This value allows us to predict an optical density at 1 atm using a 10-cm light path of 0.102. Heidt and Johnson found 0.098. This lends strong support to the use of Henry's law over our entire pressure range.

Absorption Spectra of Fluorophores; Effect of Oxygen Concentration. Oxygen is known to enhance singlet to triplet transitions which are shown by increased absorption at the red edge of the longest wavelength absorption band (Evans, 1953, 1957). We were concerned about the effect of oxygen on the absorbance from the ground state to the first excited singlet state since this is where fluorophores are excited in these experiments. The absorption spectra of indole, tryptophan, ANS, and DENS in 0.1 M sodium phosphate buffer, pH 7.0, are completely unchanged when the solutions are equilibrated with 1500 psi of O_2. The absorption spectrum of perylene in

TABLE I: Stern–Volmer Constants and Fluorescence Lifetimes of Fluorophores in Aqueous Solutions.

Fluorophore	K (M^{-1})	τ_0 (nsec)	$K/\tau_0 \times 10^{-10}$ (M^{-1} sec^{-1})
Indole[a]	50.4	4.1	1.23
Tryptophan[a]	32.5	2.7	1.2
Tryptophan[b]	38.8	2.7	1.44
Tryptophan[c]	36.8	2.9	1.27
Tryptophan[d]	34.2	2.9	1.18
N-Ac-Trp-NH_2[a]	33.8	2.8	1.16
Tyrosine[e]	39.0	3.2	1.2
DENS[a]	230	30	0.77
Anthracene-9-carboxylic acid[a]	15.3	2.0	0.77
Riboflavine[a]	16.9	4.2	0.40
Lumiflavine[a]	22.6	4.9	0.46

[a] 0.1 M sodium phosphate, pH 7.0. [b] 0.025 M Tris-HCl, pH 7.5. [c] 0.01 M Tris-HCl, pH 7.5. [d] 0.01 M Tris-HCl + 0.5 M KCl, pH 7.5. [e] 0.005 M sodium phosphate, pH 7.1.

dodecane equilibrated with 1500 psi of O_2, in which the oxygen concentration is about 1 M, is also unperturbed. Enhanced singlet–triplet transitions were not seen in any of these spectra. Such observations require very concentrated solutions of the chromophores which only allow observation of the red edge of the absorption band. Lack of perturbation of the absorption spectrum is evidence against complex formation between chromophore and oxygen, and thus supports the dynamic nature of the quenching observed by us.

Oxygen Quenching of Free Fluorophores in Aqueous Solutions. Figure 4 shows typical Stern–Volmer plots of *N*-acetyl-L-tryptophanamide and riboflavine in aqueous solutions. Note the lack of deviation from linearity. Appreciable complex formation between oxygen and the fluorophore would yield positive deviations from the straight line. Thus these data are evidence against any significant amount of static quenching. Calculations of oxygen solubility using generalized gas fugacities and the Krichevsky–Karsarnovsky equation (Hougen *et al.*, 1959) predict negative deviations from Henry's law and thus negative deviations in the Stern–Volmer plots. As an example, using a partial molar volume for oxygen of 31 ml (Lauder, 1959) such a calculation indicates that at 90 atm of oxygen pressure the oxygen solubility would be 21% less than that predicted by Henry's law. If such a deviation from Henry's law actually occurred it would be apparent in our data. It seems unlikely that precisely the correct amount of static quenching occurs to cancel this effect in virtually all the cases studied. Evans (1957) has shown that the deviation from Henry's law for the solubility of oxygen in chloroform is less than 10% at 1500 psi. Since the solubility of oxygen in water is less than in chloroform, we would expect the deviation from Henry's law to be smaller than 10%. Perhaps formation of an oxygen hydrate (Heidt and Johnson, 1957) is the reason for lack of agreement with the Krichevsky–Karsonovsky equation. In any case it appears that within the accuracy of these experiments, Henry's law is a quite valid approximation.

Table I summarizes Stern–Volmer quenching constants and fluorescence lifetimes for a number of fluorophores. Table II lists experimental conditions which were used for the studies on free fluorophores described here. In determining the Stern–Volmer quenching constants, both excitation and emission monochromators were used, but no filters. For the measurement of fluorescent lifetimes the exciting light was isolated by using both a monochromator and a bandpass filter, whereas only a low pass filter was used for the emission. We note that most of the bimolecular rate constants are in close agreement with the diffusion-controlled value of 1.0×10^{10} M^{-1} sec^{-1}. Thus we conclude that every collision of oxygen with an excited fluorophore is effective in quenching its potential fluorescence. This fact is known to be true for fluorophores in

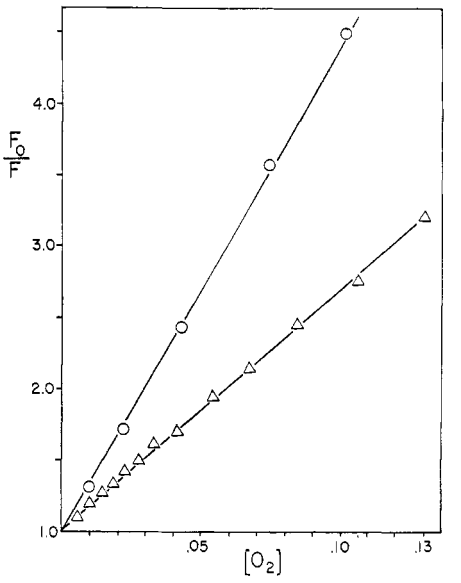


FIGURE 4: Oxygen quenching of *N*-acetyl-L-tryptophanamide (○) and riboflavine (△) in 0.1 M sodium phosphate, pH 7.0.

TABLE II: Instrumental Conditions Used for These Quenching Experiments.

Fluorophore	Excitation Wavelength ±2 nm	Emission Wavelength ±3 nm	Excitation Filter[a]	Emission Filter
Indole (H_2O)	280	350	7-54	0-54
Indole (EtOH)	280	328	7-54	0-54
Indole (dodecane)	280	302	7-54	0-54
Tryptophan	280	355	7-54	0-54
N-Ac-Trp-NH_2	280	355	7-54	0-54
Tyrosine	280	304	7-54	0-54
DENS (H_2O)	370	460	7-59	3-73
Anthracene-9-carboxylic acid	364	408	7-54	3-74
Riboflavine	445	515	0-51	3-71
Lumiflavine	440	510	0-51	3-71
DENS (EtOH)	370	440	7-51	3-73
ANS (EtOH)	380	465	7-51	3-73
Perylene	410	438	7-59	3-72
9-Vinylanthracene	370	425	7-59	3-74
2-Methylanthracene	360	405	7-59	3-75

[a] Corning filters were used in the measurement of fluorescence lifetimes. When using filters in the 3 series, a sodium nitrite filter was also used to prevent scattered ultraviolet light from reaching these filters.

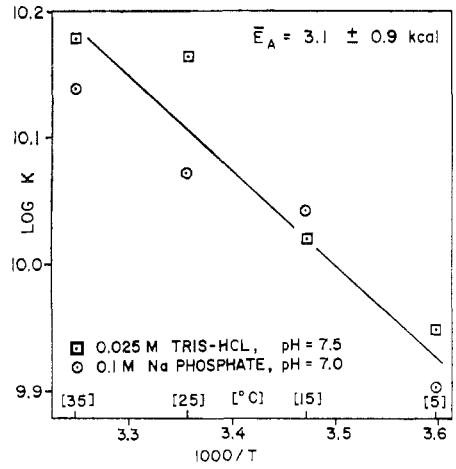


FIGURE 5: Arrhenius plot of the oxygen bimolecular quenching constant of tryptophan.

organic solvents (Berlman, 1965) and pyrenebutyric acid in aqueous solution (Vaughan and Weber, 1970). However, this fact has not been demonstrated for these short-lifetime fluorophores presented in the table in aqueous solution over a reasonable range of oxygen concentration.

It is not surprising that molecules like DENS, riboflavine, and lumiflavine have bimolecular quenching constants somewhat below the diffusion-controlled value. Collisions of oxygen with the ethyl groups of DENS or the ribose or methyl groups of riboflavine would not be expected to deactivate the excited state. Lumiflavine, which lacks the ribose moiety, is quenched more efficiently.

When the pressure is released from all these solutions the original fluorescence intensity returns. If the absorption spectrum is taken after the quenching experiment no change is observed in comparison with a sample which was not subjected to oxygen pressure and illumination. We conclude that no appreciable photochemical degradation occurs in the course of these experiments for any of the fluorophores listed in Table 1. Samples were only subjected to illumination during times of actual measurements, not during the lengthy equilibration times.

With one exception, the shape of the fluorescence emission spectra and the emission maxima of all the compounds studied

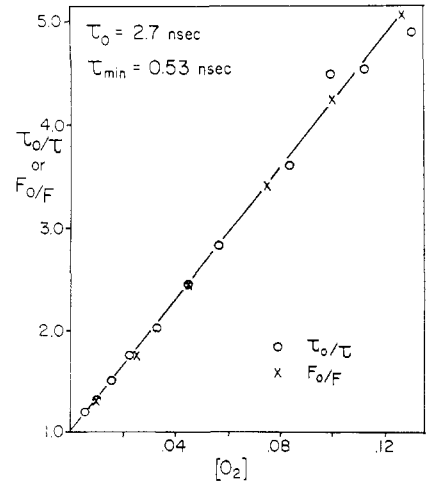


FIGURE 6: Oxygen quenching of tryptophan as observed by fluorescence yield and lifetime. Solvent is 0.1 M sodium phosphate, pH 7.0.

TABLE III: Dynamic and Static Quenching Constants in Aqueous and Nonaqueous Solvents.

Fluorophore	Solvent	K (M^{-1})	K_s (M^{-1})	% Static Quenching
Tryptophan	0.1 M sodium phosphate, pH 7	32	0.2	0.6
Indole	0.1 M sodium phosphate, pH 7	51	−0.8	−1.6
Riboflavine	0.1 M sodium phosphate, pH 7	20	−1.4	−7.0
Lumiflavine	0.1 M sodium phosphate, pH 7	22	0.3	1.4
Anthracene-9-carboxylic acid	0.1 M sodium phosphate, pH 7	15	−0.1	−0.67
Tryptophan	0.025 M Tris, pH 7.5	36	0.7	1.9
Indole	100% ethanol	138	6.9	5.0
Perylene	Dodecane	112	2.6	2.3
9-Vinylanthracene	Dodecane	234	4.6	2.0
2-Methyl-anthracene	Dodecane	130	3.4	2.6

in aqueous solutions are unchanged under 1500 psi of O_2. Tryptophan displays a small blue shift at 1441 psi of approximately 2 nm. Under these conditions about 80% of the fluorescence intensity is quenched. This shift was noted in several experiments and proved to be completely reversible. This solution, when equilibrated with 1500 psi of O_2, displays a fluorescence lifetime of 0.5 nsec. Possibly solvent relaxation around the increased dipole moment of the excited fluorophore is not quite complete within 0.5 nsec (Bakshiev, 1964; Brand and Gohlke, 1971). This reorientation of the solvent dipoles after excitation is known to be responsible for the red shift of fluorescence emission spectra in polar solvents. Oxygen quenching, by decreasing the fluorescence lifetime to rates comparable with the solvent relaxation time, allows in fact one to investigate this latter process (Weber and Lakowicz, 1973). This possibility is more fully discussed at the end of this section.

Table III lists some dynamic and "static quenching constants" for fluorophores in aqueous solutions. These values were calculated using the computer program described under Experimental Procedures. These calculations rule out any significant static quenching in aqueous solutions.

The Stern–Volmer quenching constants and fluorescence lifetimes of tryptophan were measured at four different temperatures (5, 15, 25, and 35°) and in two different buffers. Plots of the bimolecular quenching constant *vs.* T/η (T, absolute temperature; η, viscosity) are linear within experimental error. This supports the dynamic nature of the observed quenching. The slope of a plot of the bimolecular quenching constant *vs.* $1/T$ allows one to calculate the activation energy for the quenching process (Figure 5). A least-squares fit of these eight data points yields an activation energy for oxygen quenching of 3.1 ± 0.9 kcal, which is within experimental error of the activation energy for oxygen diffusion calculated from available diffusion data, 3.6 ± 0.14 kcal (Landolt-Börnstein, 1969). Thus, the temperature dependence of quenching supports dynamic quenching.

TABLE IV: Oxygen Quenching Constants in Nonaqueous Solvents.

Fluorophore	Solvent	K (M^{-1})	τ_0 (nsec)	$K/\tau_0 \times 10^{-10}$ (M^{-1} sec^{-1})
Perylene	Dodecane	112	5.4	2.08
9-Vinylanthracene	Dodecane	234	11.1	2.11
2-Methylanthracene	Dodecane	130	5.2	2.51
Indole	Dodecane	236	8.5	2.78
Indole	Ethanol	138	5.0	2.76
DENS[a]	Ethanol	485	20	2.43
ANS	Ethanol	250	13.4	1.86

[a] A tenfold excess of NaOH was added to dissolve DENS in ethanol.

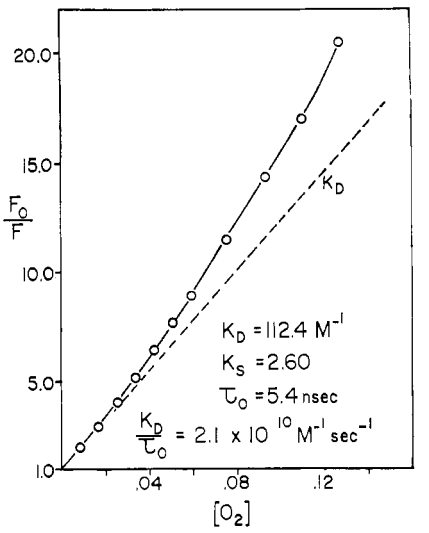


FIGURE 7: Oxygen quenching of perylene in dodecane.

A definitive proof of the dynamic nature of the quenching observed in our experiments is the equivalence of F_0/F and τ_0/τ. Figure 6 contains F_0/F and τ_0/τ data for tryptophan at oxygen pressures up to 100 atm. Within experimental error these two values are equal. We conclude that no significant static quenching occurs under our experimental conditions. When this tryptophan solution is equilibrated with 100 atm of oxygen pressure the lifetime is reduced to almost 0.5 nsec. We must point out that measurement of this short lifetime, although compounded with the usual sensitivity problems associated with excitation in the short ultraviolet region, was quite easily accomplished using the cross-correlation lifetime instrument of Spencer and Weber (1969).

Oxygen Quenching of Free Fluorophores in Nonaqueous Solutions. Positive deviations in the Stern–Volmer plots are generally seen in nonaqueous solutions at oxygen concentrations where greater than 80% of the initial fluorescence is quenched. Oxygen quenching of perylene in dodecane is an example (Figure 7). Such positive deviations may be described in terms of a static quenching constant (K_s) which is the equilibrium constant for the formation of a nonfluorescent dark complex between oxygen and the fluorophore. Judged from these data (Table III) the static component is in the range of 3% of the dynamic component in nonaqueous systems. A small static component of this magnitude is sufficient to explain the apparently large positive deviation visible on Figure 7. Note that perylene was quenched to 4% of the original intensity in this experiment ($F_0/F_{min} = 25$). At F_0/F values less than 5 no static component was detectable.

Table IV summarizes some bimolecular rate constants in nonaqueous solvents. A value of 2–3 $\times$ 10^{10} M^{-1} sec^{-1} is in agreement with the larger diffusion coefficient of oxygen in these solvents (Ware, 1962). Thus, we conclude that the quenching radii or effective encounter distance for oxygen and fluorophore is independent of solvent.

Observation of a static quenching component in quenching data does not prove that the formation of a nonfluorescent complex is the reason for the observed positive deviations. One might expect complex formation to be detectable in the absorption spectra taken under the same conditions as the quenching data. It was already noted that perylene in dodecane displays no change in its absorption spectrum at 1500 psi of O_2, where the oxygen concentration is about seven times the highest concentration in Figure 7. If the positive deviation on Figure 7 is interpreted as complex formation, then about 1.5% of the perylene is "complexed" with oxygen at 0.127 M O_2. Under the experimental conditions this corresponds to $K_s^{-1} = 8.3$ M or a free energy of complex formation (ΔG_0) of +1 kcal/mol. Thus, the hypothetical complex is very weak, perhaps too weak to be formally designated as such. Since 8 M O_2 would be needed for 50% "complex" formation, it seems we are dealing not with a real complex, but rather with the amount of oxygen next to a fluorophore being determined by the mole fraction of oxygen in the solvent.

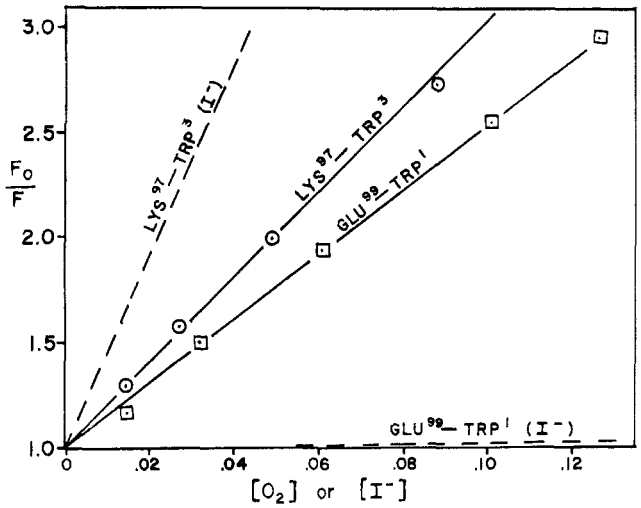
This phenomenon has been observed by others (Boaz and Rollefson, 1950; Rollefson and Boaz, 1948). Frank and Vavilov (1931) suggested the apparent "static" quenching may be due to the presence of a quencher molecule within a certain distance of the fluorophore at the time of excitation. This distance defines the "sphere of action" within which the probability of quenching is unity. They modified the Stern–Volmer equation by introducing an exponential factor that accounts for the probability of a quencher being within the sphere of action

$$F_0/F = (1 + K[O_2]) \exp([O_2]vN/1000)$$

N is Avogadro's number and v is the volume of the sphere of action. The quenching data for perylene in dodecane are consistent with a 9-Å sphere of action. This distance is evidently the same as the sum of the radii of fluorophore and quencher and therefore our "complex" is equivalent in its effect to oxygen being adjacent to the fluorophore.

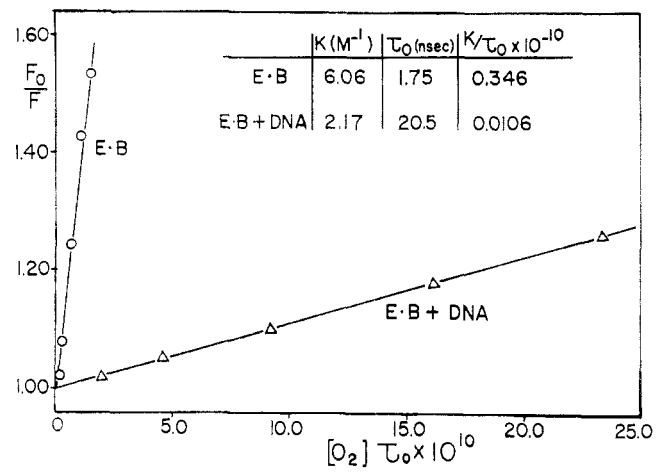
It is not the purpose of this paper to make a definitive statement on reasons for deviations from the Stern–Volmer equation. Such discussion would involve us in the definition of a molecular complex. Does the presence of oxygen within the "sphere of action" constitute a complex, or is some more intimate association necessary? We note that these deviations are only apparent at large values of F_0/F (>5) in organic solvents. We shall be concerned primarily with aqueous solution and F_0/F values less than 5 where no deviations are seen.

Quenching of Charged Copolymers; Comparison of O_2 and I^-. Iodide (I^-) is also known to be an efficient quencher of fluorescence. The dynamic nature of the iodide quenching of indole was proven by a comparison of fluorescence lifetime and yield measurements. F_0/F and τ_0/τ values were equivalent within experimental error and yielded $K = 34$ M^{-1} (for 0.025 M Tris-HCl, [KI] + [KCl] = 0.5 M, *ca.* 10^{-4} M $Na_2S_2O_3$).



	OXYGEN			IODIDE $^-$	
COPOLYMER	K(M^{-1})	τ_0 (nsec)	K/τ_0·10^{-10}	K(M^{-1})$^+$	K/τ_0·10^{-10}
LYS^{97}TRP^3	20.3	2.0	1.02	46	2.3
GLU^{99}TRP^1	15.4	1.2±.3	1.2±.2	0	0

FIGURE 8: Comparison of quenching by oxygen (this paper) and iodide (Lehrer, 1971) of charge copolymers. The same conditions as described for tryptophan (Table II) were used, except 360-nm emission for the glutamate–tryptophan copolymer. The solvent was 0.06 M Tris-HCl, pH 7.54.



	K(M^{-1})	τ_0 (nsec)	K/τ_0 × 10^{-10}
E·B	6.06	1.75	0.346
E·B + DNA	2.17	20.5	0.0106

FIGURE 9: Oxygen quenching of ethidium bromide when free in solution (○) and when intercalated into double helical DNA (△). The solvent was 0.01 M Tris-HCl–0.001 M EDTA–1 M NaCl, pH 7.8; free dye, 480 ± 2 nm excitation, 585 ± 3 nm emission; ethidium bromide–DNA complex, 505 ± 2 nm excitation, 582 ± 3 nm emission, [DNA] = 3.6 × 10^{-4} M, [ethidium bromide] = 0.24 × 10^{-4} M.

By the use of charged polymers of lysine or glutamic acid, each containing a small percentage of tryptophan, Lehrer (1971) has shown quenching by iodide to be extremely sensitive to local charge and ionic strength. Using these same copolymers we demonstrate in Figure 8 the complete insensitivity of oxygen quenching to local charge. Quenching by oxygen, due to its insensitivity to charge effects, yields a more accurate picture of the steric impediments to quenching in a macromolecule in solution. If inefficient quenching by I^- is observed, either steric or charge and solvation effects may be the cause. If similar observations are made in quenching by O_2, we can exclude the effect of solvation or charge as responsible for this effect, and must conclude that accessibility of the O_2 to the fluorophore is hindered by an interposed molecule.

Oxygen Quenching of an Ethidium Bromide–DNA Complex. It was shown by LePecq and Paoletti (1967) that ethidium bromide binds to base-paired regions of deoxyribonucleic acid according to two modes. The first mode of binding (complex I) appears to be intercalation of this dye into base-paired regions of the DNA. The second mode of binding (complex II) appears to be simple electrostatic binding of the positively charged ethidium group to the negatively charged phosphates. Complex II only appears in low ionic strength solutions. Complex I is characterized by a large increase in quantum yield and a dissociation constant that is almost independent of ionic strength. Experiments with synthetic polynucleotides indicate that complex I can only form in the presence of a double-stranded structure. These experiments led to speculation about the protection that DNA would provide to the intercalated dye from the free diffusion of oxygen. Indeed, good protection may be taken as further evidence for actual intercalation. Under the conditions of the experiment the association constant was 8 × 10^4 (LePecq and Paoletti, 1967). This allows us to calculate that 97% of the ethidium bromide was complexed with DNA. The contribution of bound ethidium bromide to the total fluorescence was 99.7%. Different excitation and emission wavelengths were used for free and bound dye because of the changed optical properties of ethidium bromide when complexed with DNA. In Figure 9 the data from this experiment are shown. The measured lifetimes were incorporated in the x axis to demonstrate the large difference in the bimolecular quenching constant for free and bound ethidium bromide. This experiment demonstrates the necessity of an independent measurement of the unquenched lifetime for meaningful interpretation of the data. The amount of protection of the dye from the free diffusion of oxygen is striking. The K/τ_0 ratio of 0.01 for the ethidium bromide–DNA complex is the lowest found for any system studied. It can only be concluded that the double helical structure does not allow diffusion of oxygen on the time scale of the fluorescence lifetime. This experiment also demonstrates the necessity of a collision for quenching to occur. Even though oxygen can certainly diffuse close to the ethidium bromide molecule, little quenching is observed, indicating that quenching by oxygen is not possible without actual collision. These experiments, taken together with the quenching data of the fluorophores in aqueous and nonaqueous solvents, are only consistent with a quenching mechanism in which the quenching radius is equivalent to the collision radius, regardless of the medium that surrounds fluorophore and quencher.

The direct interpretation possible for O_2 quenching of ethidium bromide may be contrasted with the uncertainty involved in the case of other quenchers, such as bromate (BrO_3^-) or iodide. For the later cases the interpretation would be greatly complicated by charge effects. The positive charge of ethidium bromide would increase the local quencher concentration for the free dye, and the negative charges of the phosphates on the DNA would be expected to have the opposite effect. The efficiency of quenching would result from a weighted balance of these opposing effects. The advantage of an uncharged quencher is thus apparent. On the other hand, if O_2 and I^- quenching are compared on the same system, it becomes possible to calculate the separate effects of charge and steric factors.

Quenching of Dye–Bovine Serum Albumin Systems. The previous work of Vaughan and Weber (1970) demonstrated

TABLE V: Bimolecular Quenching Constants for Dyes Bound to Bovine Serum Albumin.[a]

Fluorophore	K (M^{-1})	τ_0 (nsec)	$K/\tau_0 \times 10^{-10}$ (M^{-1} sec^{-1})
DENS ($\bar{n}$ = 2)	22	30	0.073
ANS ($\bar{n}$ = 0.5)	9.45	17[b]	0.055
ANS ($\bar{n}$ = 9)	11.7	12[b]	0.098
9-Vinylanthracene	13.3	9.9	0.134

[a] 0.1 M sodium phosphate, pH 7.0, was used for all solutions. ANS–bovine serum albumin, $\bar{n}$ = 0.5: 375 ± 2 nm excitation, 465 ± 4 nm emission, [ANS] = 2.4×10^{-5}, [bovine serum albumin] = 4×10^{-5}; ANS–bovine serum albumin, $\bar{n}$ = 9: 430 ± 2 nm excitation, 475 ± 4 nm emission, [ANS] = 3.9×10^{-4}, [bovine serum albumin] = 3.9×10^{-5}; 9-vinylanthracene–bovine serum albumin, 371 ± 2 nm excitation, 430 ± 4 nm emission, OD (371) = 0.11, [bovine serum albumin] = 4×10^{-5}. [b] Average of phase and modulation lifetimes: $\bar{n}$ = 0.5, $\tau(\phi)$ = 16.5, τ(M) = 17.7; $\bar{n}$ = 9, $\tau(\phi)$ = 9.9, τ(M) = 13.6.

that pyrenebutyric acid was protected from collisions with oxygen when bound to bovine serum albumin. We decided to try similar experiments using shorter lifetime fluorophores. Table V lists the quenching constant of DENS when bound to bovine serum albumin. The bound dye is only 10% as available to the free diffusion of oxygen as the free dye (Table I). This indicates the protein matrix provides a barrier to the diffusion of oxygen in the region of the fluorophore.

Table V shows how the quenching of ANS bound to bovine serum albumin is affected by the number of ANS molecules bound. Bovine serum albumin is known to have four binding sites of high affinity and a larger number of sites of lesser affinity for ANS (Pasby, 1969). Heterogeneity in the measured lifetimes indicates an emitting population with more than a single lifetime (Spencer, 1970). However, the results clearly indicate that the ANS molecules which are more loosely bound to bovine serum albumin are also more accessible to oxygen. The lifetime of the $\bar{n}$ = 9 sample is shorter than the $\bar{n}$ = 0.5 sample by either the phase or modulation method. Energy-transfer mechanisms for the faster quenching of the sample with $\bar{n}$ = 9 were excluded by exciting this solution at 430 mm, where as shown by Pasby (1969) and Anderson and Weber (1969) energy transfer fails to take place. The quenching of ANS–bovine serum albumin was observed by both fluorescence intensity and lifetime measurements. Data for $\bar{n}$ = 0.5 and 9 are shown in Figures 10a and b. The deviations in τ_0/τ from F_0/F may be explained by heterogeneity in either the lifetimes or in the apparent bimolecular quenching constant. As described in the following paper (Lakowicz and Weber, 1973) effects of this type are easily possible. These data are not consistent with static quenching. If both dynamic and static quenching are operative upon the same fluorophore, an upward deviation in the Stern–Volmer plots would result. The negative deviations observed are consistent with different populations of varying quenching constant.

From these data (Table V) one could speculate that 9-vinylanthracene is less tightly bound than ANS because of its neutrality. The presence of the negative charge on the sulfonic acid group may cause the binding site to be more rigid, that is the peptide chains may be held in a more rigid configuration

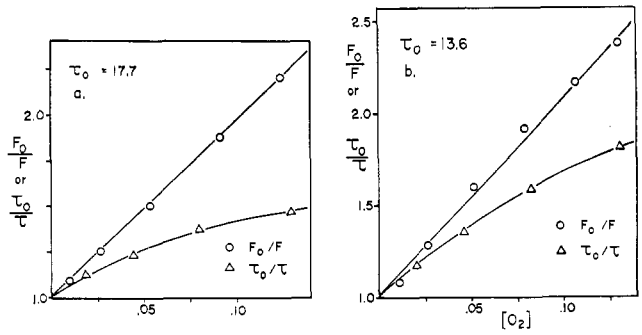


FIGURE 10: Oxygen quenching of ANS–bovine serum albumin, $\bar{n}$ = 0.5 (a) and $\bar{n}$ = 9 (b) as observed by fluorescence yield (○) and lifetime (△). See legend to Table V for experimental details. Lifetimes were measured by the modulation method, 14.2 MHz.

due to this charge. Jonas and Weber (1971) have shown that arginine residues are involved in binding ANS and that electrostatic interactions contribute some 2 kcal to the free energy of binding.

Time-Dependent Effects. The usefulness of any physical technique is determined to a large extent by the time scale of the processes that can affect the observation. Fluorophores typically display lifetimes of 1–100 nsec. Thus any process which is complete within 1 nsec, or slow relative to 100 nsec, will not affect the fluorescence experiment. The dynamic quenching of fluorescence by oxygen allows one to decrease the lifetime of the fluorophore to any desired value consistent with the gas solubility and the photometric accuracy that may be obtained in light of the decreasing quantum yield. Since the probability of quenching is proportional to the time spent in the excited state, under quenching conditions we selectively observe those fluorophores which emit sooner after excitation. Upon equilibration with 1500 psi of O_2 the lifetime of an aqueous tryptophan solution is reduced to 0.5 nsec, and the lifetime of indole in ethanol can be reduced to 36 psec. This shorter lifetime, which is presently not measurable, may be calculated with reasonable confidence from the dynamic Stern–Volmer quenching constant. Thus fluorescence may be used to investigate processes which were previously too rapid to appreciably affect the emission, such as solvent relaxation around excited fluorophores in nonviscous solvents (Bakshiev *et al.*, 1969; Ware *et al.*, 1968; Weber and Lakowicz, 1973). The time dependence of fluorescence depolarization may be obtained by steady-state polarization measurements as a function of fluorescence lifetime. In practice, oxygen quenching appears to be capable of increasing the time resolution of fluorescence by two orders of magnitude.

Conclusions

Through development of equipment and techniques for performing fluorescence and absorbance measurements on solutions equilibrated with pressures of oxygen up to 100 atm we have extended the studies of oxygen quenching to commonly used fluorescence probes and to short lifetime probes (2–10 nsec), including tryptophan. Without the use of pressure significant oxygen quenching is limited to fluorophores in organic solvents, or to aqueous solutions of fluorophores with lifetimes greater than 100 nsec.

For most fluorophores free in solution the bimolecular quenching constant observed agrees with the diffusion-controlled value. We may thus state with confidence that a bimolecular rate constant conspicuously less than the diffusion-controlled value is due to a decrease in the number of colli-

sions between oxygen and fluorophore, and not due to a change in the overall efficiency of the collisions. We have presented several examples of quenching of fluorescent probes bound to macromolecules. The observed decrease in the bimolecular quenching constants is evidently due to the reduction in the number of collisions between fluorophore and quencher due to the interposition of parts of the macromolecule which offer a steric barrier to the oxygen diffusion.

Because these are the first accurate quenching experiments using high concentrations of oxygen we felt it necessary to investigate all possible artifacts that could arise from this cause. By absorption measurements at 1500 psi we found the increase in optical density due to oxygen dissolved in water to be less than 0.01 OD unit/cm at 265 nm. Throughout these experiments no photooxidation was observed. Oxygen quenching is nondestructive and completely reversible under the experimental conditions employed. These include illumination with a monochromatic beam only for the short periods required to make measurements. After the pressure is released the oxygen diffuses from the solution and the fluorescence intensity returns to the original value. Precious samples may be reused under various experimental conditions. By the use of lifetime measurements we demonstrate that essentially all the observed quenching is dynamic in character. In contrast to I^- quenching, oxygen quenching is completely insensitive to charge effects. We conclude that oxygen quenching yields a valid representation of the probability of interaction of oxygen and fluorophore, and therefore of the ability of oxygen to diffuse to those parts of the macromolecule where the fluorophore is placed.

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