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Mechanism of Ethidium Bromide Fluorescence Enhancement on Binding to Nucleic Acids†

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ABSTRACT: The mechanism of the enhancement of the fluorescence of ethidium bromide on binding to double helical RNA and DNA has been investigated. From an examination of the effect of different solvents on the fluorescence lifetime, quenching of fluorescence by proton acceptors, and the substantial lengthening of lifetime observed upon deuteration of the amino protons, regardless of the medium, we conclude that proton transfer from the excited singlet state is the process primarily responsible for the low fluorescence yield in most polar solvents. Enhancement of fluorescence upon intercalation is attributed to a reduction in the rate of excited state proton transfer to solvent molecules. The proposed mechanism accounts for the ~3.5-fold increase in the lifetime of free ethidium bromide in going from H_2O to D_2O; the fact that addition of small amounts of water to nonaqueous solvents decreases the fluorescence whereas addition of small amounts of D_2O enhances the fluorescence; and the enhancement of the ethidium bromide triplet state yield on binding to DNA. Other proposed mechanisms are shown to be inconsistent with our findings.

Ethidium bromide, a cationic dye (structure I) which interacts strongly and specifically with double helical RNAs and DNAs, is widely used in spectrofluorimetric studies because of the striking fluorescence enhancement it displays upon binding (Waring, 1965; LePecq and Paoletti, 1967; Bittman, 1969; Burns, 1969, 1971; Tao et al., 1970; LePecq, 1971; Angerer et al., 1974; Bontemps and Fredericq, 1974; Genest et al., 1974; Gatti et al., 1975). It is generally agreed that strong fluorescence enhancement accompanies intercalation of the dye into the double helix conformation of the nucleic acid but there is also evidence for additional nonintercalative, less fluorescence-enhanced sites which are presumed to involve electrostatic binding (Waring, 1965; LePecq and Paoletti, 1967; Bittman, 1969). Recently, ethidium bromide has been used to probe tRNA structure (Bittman, 1969; Urbanke et al., 1973), 5S RNA (Gray and Saunders, 1971; Feunteun et al., 1975), circular DNA (Hudson et al., 1969), chromatin structure (Ide and Baserga, 1976), ribosomal RNA (Lawrence and Daune, 1976), synthetic DNA (Aktipis and Martz, 1974), tRNA protein interactions (Rigler et al., 1971), and to determine the molecular weight of DNA (Weissman et al., 1976). Ethidium bromide also elicits a wide range of biochemical effects (Lurquin and Buchet-Mahieu, 1971; Avadhani et al., 1973; Kramer et al., 1974; Criddle et al., 1976).

Despite extensive studies of ethidium bromide complexes with polynucleotides, a satisfactory mechanism explaining the high degree of fluorescence enhancement upon binding has not yet emerged. When ethidium bromide intercalates into a double helix, both its solvent environment and equilibrium conformation are modified, and either one or both of these changes might be involved in the enhancement mechanism. LePecq and Paoletti (1967) suggested the enhancement resulted from immersion of ethidium bromide in a hydrophobic region where it would no longer be quenched by aqueous solvent but made no proposal with respect to the quenching mechanism. Burns (1969) attributed the enhancement to a change in the conformation of ethidium bromide which renders a previously forbidden transition allowed. Recently, Hudson and co-workers (Hudson and Jacobs, 1975; Waleh et al., 1976) presented evidence for a triplet state nearly degenerate with the lowest excited singlet in ethidium bromide and postulated that the fluorescence enhancement is due to environment-induced shifts in the relative singlet–triplet energy separation leading to a reduction in the singlet-to-triplet intersystem crossing rate.

The experimental evidence presented to date is insufficient to decide unequivocally among these or other possible enhancement mechanisms. Without such knowledge, the usefulness of ethidium bromide fluorescence assaying of the extent of double helical structure (LePecq, 1971) remains somewhat open to question, especially in view of reports that the fluorescence parameters of ethidium bromide vary with extent of binding (Bontemps and Fredericq, 1974). A proper interpretation of ethidium bromide fluorescence studies of the interaction of nucleic acid with proteins such as the synthetases (Rigler et al., 1971), ribosomal proteins, and histones (Lawrence and Daune, 1976) also requires an understanding of the enhancement mechanism and how it may be affected by such interactions. Once the enhancement mechanism is known, it

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might be possible to identify other dyes which could serve as fluorescent probes of biomolecular structure and function.

A clear understanding of the factors affecting the lifetime of ethidium bromide might help resolve the apparent disagreement between NMR studies of ethidium bromide binding to tRNA in solution, which indicate a unique intercalating binding site (Jones and Kearns, 1975) and x-ray diffraction studies ofeethidium bromide binding in yeast $tRNA^{Phe}$ crystals which indicate a nonintercalating binding site (Warrant et al., 1976).

In this paper we have carried out experiments designed to elucidate the fluorescence quenching processes in ethidium bromide. These include measurements of solvent and deuterium isotope effects on fluorescence lifetimes and yields, quenching experiments and sensitized photooxidation experiments utilizing bound and free ethidium bromide. The results are not readily reconciled with intersystem crossing to the triplet state as the primary mechanism by which excitation energy is dissipated in ethidium bromide but are easily explained by quenching of the excited singlet by solvent molecules. To account for the experimental results, we propose that the major pathway for deactivation of free ethidium bromide in aqueous solution involves proton transfer from the excited singlet state to water. The enhancement of the ethidium bromide fluorescence observed on binding to DNA, or on going to other solvents, is attributed to a reduction in the excited state proton transfer rate.

Materials and Methods

Ethidium bromide (Calbiochem) showed only a single spot upon TLC[1] (4:1:1 butanol–acetic acid–water) and was used without further purification as was 1,3-diphenylisobenzofuran (Aldrich). Rose Bengal was a gift from Dr. Juan Yguerabide and was used as received. Solutions of unfractionated *E. coli* tRNA and calf thymus DNA (Calbiochem) were of the order of 10^{-4} M in phosphate in 0.01 N cacodylate buffer, pH 7.0 (instrument reading), 0.1 N NaCl, and 0.01 M $MgCl_2$. All solvents used were reagent or spectral grade. Acetone was dried over calcium sulfate, fractionally distilled, and then stored over calcium sulfate to reduce water content to a minimum (ca. 0.03% by NMR analysis). D_2O (Mallincrodt Deuter AR grade) and methanol-d_1 (Norell Chemical) were both 99% deuterated, according to manufacturer's specifications.

Sensitized photooxidation experiments were carried out on air-saturated solutions in methanol (free ethidium bromide) or 60% methanol–40% water (ethidium bromide bound to DNA) containing either Rose Bengal or ethidium bromide as sensitizer at optical densities between 0.1 and 0.2. Solutions were excited at 546 nm by a 200-W Hg lamp filtered by Corning CS 3-69 and 4-96 filters. The substrate was 10^{-6} M 1,3-diphenylisobenzofuran, and its disappearance by sensitizer–triplet-generated singlet oxygen photooxidation (Olmsted and Akashah, 1973) was determined by fluorescence intensity measurements using 404-nm excitation and viewing emission through a Corning 3-72 filter. Control solutions lacking sensitizer displayed no loss of fluorescence when irradiated under these conditions. Some solutions were oxygen saturated by bubbling tank oxygen through them for 2 min immediately prior to stoppering and irradiation.

Steady-state fluorescence intensity measurements were carried out using a 200-W xenon lamp and Bausch and Lomb high intensity monochromator for excitation and Corning CS 2-58 filter and RCA 7265 photomultiplier to view emission at 90° to excitation. Ethidium bromide solutions in different solvents were matched in absorbance at one or more wavelengths using a Beckman Acta CIII recording spectrophotometer.

TABLE I: Solvent Effects on Ethidium Bromide Fluorescence.

Solvent	τ_f	λ_{max}(absorb)	ϵ	Viscosity (cP)
H_2O	1.8	480	78.5	1.0
Me_2SO	5.0	535	46.7	2.0
Pyridine	5.8	540	12.3	0.97
Glycerol	5.9	515	42.5	954
Methanol	6.0	520	32.6	0.55
Ethanol	6.9	532	24.3	1.20
Acetone	9.3	520	20.7	0.32

Lifetime measurements were performed on an Ortec Model 9200 nanosecond fluorescence spectrometer equipped with an RCA 8850 photomultiplier tube using single photon counting techniques (Yguerabide, 1972). Excitation pulses were generated by a free-running hydrogen discharge lamp (pulse half-width = 5 ns) whose output was filtered through a 540-nm interference filter. The emission was viewed at 90° to excitation through a Corning 3-66 filter and an emission polarizer oriented at 55° to eliminate anisotropic contributions to the observed decay (Yguerabide, 1972, p 524). When the fluorescence sample was replaced with a scattering solution, negligible count rates were observed. Slit widths were controlled to keep count rates below 10%, thus minimizing twin counts. The resulting decay data were deconvoluted with the lamp pulse, which was generated utilizing a scattering solution and the same 3-66 filter (but without the interference filter in the excitation beam) in order to hold to a minimum errors due to energy-dependent photomultiplier tube response. The deconvolution was accomplished by computer analysis to give single or double exponential fits by the method of moments (Yguerabide, 1972). The accuracy of the system's time scale was checked by observing signal shifts upon inserting calibrated delays (Ortec), by measuring the lifetime of quinine bisulfate in sulfuric acid and by comparing our lifetimes for ethidium bromide with literature values for aqueous (Lakowicz and Weber, 1973) and DNA-bound (Burns, 1969) media. All these criteria indicated time scale accuracy of 0.2 ns or better.

Results

Solvent Effects on Lifetime. Fluorescence lifetime variations of ethidium bromide in various solvent systems at room temperature are collected in Table I. It is readily apparent that there is no particular correlation between fluorescence lifetime and singlet energy level, λ_{max} of absorbance (as might be expected if solvent-induced reordering of excited states were occurring) nor viscosity (as would be expected if conformational rearrangements were playing a role); and there is only a partial correlation with dielectric constant, pyridine constituting a striking exception.

We also measured the fluorescence intensity ratios for ethidium bromide in different solvents, exciting the emission in each case at a wavelength where solution absorbances were matched and measuring essentially total (albeit uncorrected) fluorescence emission using a Corning CS 2-58 color filter. Results for several solvents, for ethidium bromide bound to nucleic acids at low dye/nucleotide ratios and for selected deuterated ethidium bromide systems are summarized in Table II. From these data, it is evident that the measured intensity ratios closely match the lifetime ratios, indicating there are no substantial changes in the radiative rate constant in going from low to high fluorescence efficiency environments.

[1] Abbreviations used: TLC, thin-layer chromatography; NMR, nuclear magnetic resonance.

TABLE II: Comparison of the Ethidium Bromide Fluorescence Lifetimes, τ_f, and Intensity Ratios in Various Media Relative to H_2O.[a]

Medium	$\tau_f/\tau_f(H_2O)$	$I_f/I_f(H_2O)$
Me_2SO	2.8	2.7
D_2O	3.5	3.1
Ethanol	3.8	4.3
Acetone	5.1	5.0
10^{-4} M DNA/H_2O	12.5	11.2
10^{-5} M tRNA/H_2O	13.7	13.7[b]
10^{-4} M DNA/D_2O	20.8	20.0

[a] Estimated uncertainties, ±10%. [b] Corrected for contribution due to free ethidium bromide.

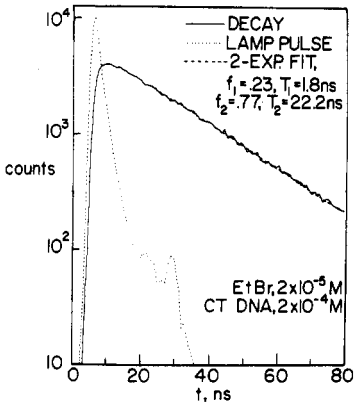

FIGURE 1: Fluorescence decay behavior of ethidium bromide bound to calf thymus DNA. The solid curve is the observed decay, displayed semilogarithmically. The dotted curve shows the lamp pulse measured under comparable conditions (see text), and the dashed curve is a computer-calculated decay curve obtained from deconvolution of the experimental decay with the lamp pulse by the method of moments.

Lifetime of Ethidium Bromide Bound to Nucleic Acid. Typical data for the fluorescence lifetime behavior of ethidium bromide bound to calf thymus DNA (pH 7.0, 0.1 M NaCl, 1 mM Mg^{2+}) are displayed in Figure 1 which shows the quality of two exponential fit obtained at moderate dye-to-nucleotide ratios. The two exponential fit shown in this figure gave a slightly better fit at moderate-to-high dye levels than did single exponentials. It consistently yielded a short lifetime component that was clearly associated with free ethidium bromide (1.8 ns). Preexponential factors gave the fractional population of each component (in these cases, free and bound dye) which could also be assayed independently by absorption spectroscopy since bound dye is significantly red-shifted in its absorption spectrum (Waring, 1965). This assay gave the same bound fraction as did the two-component analysis of the decay curves thus providing an independent check of the reliability of the fitting calculation. At lower dye-to-nucleotide ratios where negligible amounts of free dye existed, attempts at two exponential fits yielded a zero coefficient for the short-lived component (i.e., pure, single exponential decay). A further indication that bound dye exhibits only a single lifetime under these salt conditions was obtained by varying the number of channels (length of time) over which deconvolution analysis was carried out; extending the analysis out to 120 ns gave identical lifetimes as did truncation at 80 ns. If the decay curve were composed of two different lifetime contributions, in addition to that of free dye, different apparent lifetimes would result from using different analysis times.

TABLE III: Variation in Ethidium Bromide Fluorescence Lifetimes with Temperature.[a]

Medium	Lifetime 293 K (±0.2 ns)	195 K	77 K (±2 ns)
H_2O (10 M LiCl)	1.8	5.9[b] (g)	22[b] (g)
D_2O (10 M LiCl)	6.3	18[b] (g)	22[b] (g)
Methanol	6.0	8.9, 10[c]	23.7 (p)
Glycerol	5.9	21.8 (g)	23.5 (p)
Ethanol	6.9	9[c]	23 (p)
Methanol-d_1	12.0	21[c]	28 (p)
DNA/H_2O/glucose[d]	22.5		32.4 (p)
Duco cement	19.8 (g)		18.5 (g)
tRNA/D_2O/glucose[d]	43.0		41 (p)

[a] Values marked g were glasses; marked p were polycrystalline; all others were liquid. [b] T. Kajiwara and D. R. Kearns, unpublished results. [c] Computed from relative intensity measurements; estimated uncertainty ±1 ns. [d] 0.25% glucose and 10 mM sodium acetate added to aqueous solutions to prevent aggregation of samples upon freezing (Kleinwächter et al. (1968).

TABLE IV: Effect of Deuteration on Ethidium Bromide Fluorescence Lifetimes, τ_f.

System	τ_f(protonated)	τ_f(deuterated)
H_2O	1.8	6.3
Methanol	6.0	12.0
Acetone	9.3	15–16[a]
DNA[b]	22.5	38
tRNA[c]	25, 9[d]	43, 20[d]

[a] Obtained by extrapolating lifetimes of D_2O containing solutions to zero mole fraction D_2O (cf. Figure 2). [b] 10^{-4} M base concentration in 0.1 M NaCl, 0.01 M cacodylate buffer, pH 7.0, base/dye = 10. [c] Unfractionated *E. coli* tRNA, 10^{-5} M in 0.1 M NaCl–0.01 M Mg^{2+}–0.01 M cacodylate buffer, pH 7.0, 2 EB/tRNA. [d] Decay curves are best fitted by double exponential functions; longer decay times are attributed to intercalated ethidium bromide and short decay times to electrostatically bound ethidium bromide.

It should be emphasized that these observations of single lifetimes for ethidium bromide bound to DNA were obtained under conditions of pH and salt concentration deliberately chosen to minimize external binding. Indeed, if the pH is varied outside the 5–9 range, or the ionic strength is reduced below 0.01, a lifetime component intermediate between the free and intercalated values is consistently obtained. Emitters with intermediate lifetimes are attributed to dye molecules that are not intercalated but are bound on the outside of the DNA helix.

The lifetimes of ethidium bromide bound to tRNA were also examined. At low dye levels (less than 0.5 dye per tRNA molecule), a single exponential decay with a lifetime of 26 ns is observed (about 20% longer than for dye bound to DNA). As the dye per tRNA level is raised above 0.5, a second component of intermediate lifetime is readily observed. Again, this can be attributed to outside binding of the dye which appears to be more important for tRNA than for DNA under similar conditions. In D_2O solvent we also observe both long-lived intercalative binding and intermediate lifetime outside binding for tRNA; see Table IV.

Deuteration Effects. Included in Tables II and III are several pieces of data pertaining to media in which the amino protons of ethidium bromide have been exchanged for deuterium (NMR spectra of ethidium bromide in acetone show that

TABLE V: Fluorescence and Photochemical Parameters for Ethidium Bromide in Methanol-Water Solvent.[a]

System	Rel Photoox[b]	τ_f	Rel ϕ_T
free EtBr	1.0	4.2	1.0
EB/DNA[c]	2.3	5.7 (41%)[d] 24.5 (59%)[e]	3.9[e,f]

[a] 60% methanol by volume. [b] Obtained as slope of first-order disappearance of diphenylisobenzofuran. [c] DNA concentration $\cong 10^{-3}$ M in phosphate. [d] Free component. Percentages of free and bound EB are obtained from coefficients of two component exponential fluorescence decays. [e] Bound component. [f] Computed assuming relative photooxidation rate is due to 41% free EB and 59% bound EB.

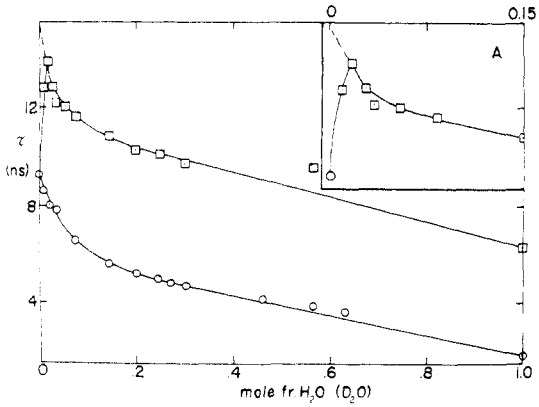

FIGURE 2: Variation in ethidium bromide fluorescence lifetime in acetone solution as a function of added H_2O (⊙) or D_2O (⊡). Dashed curve represents extrapolation of deuterated ethidium bromide lifetime to zero mole fraction D_2O. Inset A shows the effect of small amounts of added D_2O on an expanded mole-fraction scale.

both pairs of amino protons exchange within minutes upon addition of small amounts of D_2O). Earlier observations of a significant deuterium isotope effect on the fluorescence intensity and lifetime of ethidium bromide in water (Kajiwara and Kearns, unpublished results) prompted us to examine in more detail the effect of replacing the ethidium bromide amino protons with deuterons. Our observation is that, in every medium we have examined, the fluorescence intensity and lifetime are considerably enhanced upon deuteration.

Lifetime data for deuterated vs. protonated ethidium bromide are given in Table V. Additionally, we observed that, whereas the addition of ca. 1% H_2O to ethidium bromide solutions in Me_2SO, pyridine, ethanol, or acetone resulted in a fluorescence lifetime reduction of approximately 10%, additions of similar proportions of D_2O to these same solutions yielded significantly longer fluorescence lifetimes (acetone, 13.8 ns; pyridine, 8.6 ns; Me_2SO, 7.7 ns). In the case of acetone we systematically varied the H_2O or D_2O content to study fluorescence lifetime changes as a function of mole fraction water. These data (shown in Figure 2) demonstrate both a striking fluorescence enhancement upon addition of small amounts of D_2O and a sharp reduction in fluorescence when small amounts of water are present in the system. Our qualitative observations for other nonaqueous solvents indicate this is a general phenomenon.

When small amounts of D_2O were added to ethidium bromide solutions in dried acetone, the intensity of ethidium fluorescence displayed a time dependence which is illustrated in the upper portion of Figure 3. Immediately after addition of D_2O and mixing, a substantial quenching of fluorescence was

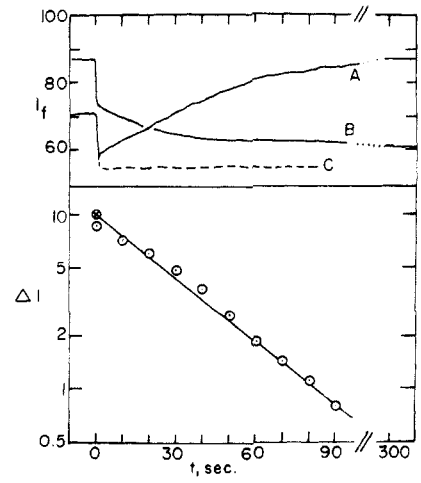

FIGURE 3: Time variation of fluorescence intensity of ethidium bromide in dry acetone after injection of 2 vol % H_2O or D_2O at time $t = 0$. (Curve A) Injection of D_2O into protonated solution; (curve B) injection of H_2O into a deuterium-exchanged solution; (curve C) injection of H_2O into protonated solution. Lower portion: semilogarithmic plot of $\Delta I = I_\infty - I_t$ vs. time for curve A. Cross at time $t = 0$ represents intensity change for H_2O injection (curve C).

observed (curve A), and the magnitude was comparable to that observed upon addition of the same quantity of H_2O to a solution in dried acetone (curve C). For solutions to which D_2O had been added, but not for those to which H_2O was added, the rapid appearance of substantial quenching was followed by a slow regeneration of fluorescence (curve A), with the fluorescence intensity finally reaching an asymptotic value whose ratio to the initial fluorescence *intensity* in dry acetone was equal to the fluorescence *lifetime* ratio for these solutions. If H_2O was subsequently added to D_2O–acetone solutions which had been allowed to equilibrate, a small initial quenching of fluorescence was followed by additional fluorescence quenching which increased at the same rate as the regeneration of fluorescence following the initial addition of D_2O. This effect is also shown in Figure 3, curve B. Both kinetic phenomena showed approximately first-order behavior (time constant ~30 s), as illustrated in the lower part of Figure 3.

The initial rapid quenching of fluorescence when either D_2O or H_2O aliquots are added to acetone solutions of ethidium bromide appears to be the result of solvation of the fluorescent molecules by H_2O or D_2O. The slower processes—enhancement in the case of D_2O added to previously protonated ethidium bromide or additional quenching in the case of H_2O added to previously deuterated ethidium bromide—must be associated with the proton–deuteron exchange phenomenon, deuterated ethidium bromide showing enhanced fluorescence relative to protonated as already shown by the above-mentioned lifetime and intensity data.

Quenching Studies. In addition to the data given above which demonstrate that H_2O is an efficient quencher of ethidium bromide fluorescence in non-aqueous media, we have also examined the quenching effect of OH^- in aqueous solutions and acetate ion in ethanolic solution. Hydroxide ion, below pH 13, does not affect the absorption spectrum of the ethidium bromide, nor does it alter the shape of the emission spectrum; however, it does quench the fluorescence of ethidium bromide. Quenching obeys Stern–Volmer kinetics with a quenching constant of 2×10^{10} M^{-1} s^{-1} (i.e., diffusion controlled). Acetate ion in ethanol also causes efficient quenching, although the quenching displays a decreasing efficiency with increasing concentration which we attribute to deviations from ideality

for concentrated sodium acetate/ethanol solutions. The low concentration limiting slope of the Stern–Volmer plot for sodium acetate quenching of ethidium bromide fluorescence in ethanol gives a lower limit of 3.3×10^9 M^{-1} s^{-1} for the quenching rate.

Photooxidation. When methanolic solutions containing se Bengal ($\phi_T = 0.76$) (Gollnick, 1968) or ethidium bromide as sensitizer were irradiated with 546-nm light in the presence of 1,3-diphenylisobenzofuran (DPI) as singlet oxygen detector, sensitized photooxidation was observed with first-order rate of disappearance of DPI as shown in Figure 4. The rate of disappearance was some 30 times smaller for ethidium bromide as sensitizer than for Rose Bengal for equal optical densities and photon fluxes. Saturating the ethidium bromide containing solution with oxygen just prior to irradiation resulted in a reduction in initial DPI fluorescence intensity due to increased oxygen quenching of excited singlet DPI but did not have any effect on the rate of the reaction consuming DPI.

These results can be interpreted according to the following reaction scheme (Olmsted and Akashah, 1973; Kearns, 1971) ($^1S_0$, ^1S, and ^3S refer to ground state, first excited singlet, and triplet state excited sensitizer molecules, respectively; D = DPI, the singlet oxygen acceptor).

$$^1S_0 + h\nu \rightarrow {}^1S$$

$$^1S \xrightarrow{k_1} {}^1S_0\ (+h\nu_f)$$

$$^1S \xrightarrow{k_2} {}^3S\ (\phi_T)$$

$$^3S + O_2 \xrightarrow{k_3} {}^1S_0 + {}^1O_2$$

$$^3S \xrightarrow{k_4} {}^1S_0$$

$$^1O_2 \xrightarrow{k_5} O_2$$

$$^1O_2 + D \xrightarrow{k_6} DO_2$$

At $[D] \cong 10^{-6}$ M, $k_6[D] \cong 10^3$, whereas $k_5 = 1.4 \times 10^5$ s^{-1} (Merkel and Kearns, 1971); hence, at these concentrations the substrate negligibly perturbs the singlet oxygen concentration and first-order disappearance of substrate is observed. If, additionally, oxygen quenching of sensitizer triplets is efficient compared with unimolecular triplet decay, the observed rate of substrate disappearance depends directly on the efficiency ϕ_T of triplet sensitizer formation. Generally speaking, $k_3[O_2] \cong 10^6$ s^{-1}, whereas $k_4 \leqq 10^4$; for ethidium bromide, however, the rate of triplet decay is unknown. Nonetheless, our observation that a fivefold increase in oxygen concentration (oxygen vs. air saturation) has no measurable effect on the rate of the sensitized reaction shows this condition is satisfied for this system. Slope values from Figure 1 together with the Rose Bengal triplet yield give $\phi_T = 2.5 \times 10^{-3}$ for ethidium bromide in methanol solution.

The effect of deuteration of the ethidium bromide molecule on its intersystem crossing yield was investigated by irradiating solutions containing identical ethidium bromide and DPI substrate concentrations in dried acetone containing 10% (v/v) H_2O and dried acetone containing 10% (v/v) D_2O. Since singlet oxygen may show a substantial lifetime change upon the addition of small quantities of water to another solvent (Merkel and Kearns, 1972), control experiments were also performed on the same solvent systems using Rose Bengal as sensitizer in place of ethidium bromide. When Rose Bengal

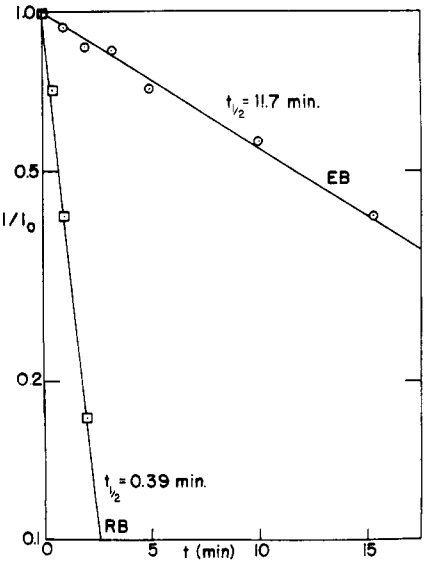

FIGURE 4: The triplet sensitized photooxidation of 1,3-diphenylisobenzofuran (DPI) by ethidium bromide (EB) and Rose Bengal (RB). Plot shows semilogarithmic variation of relative DPI fluorescence intensity with time of irradiation. See text for experimental details.

was the sensitizer, photooxidation of DPI in the D_2O-containing medium was 10% faster than in that containing H_2O, presumably due to a slightly longer singlet lifetime when D_2O rather than H_2O was present. When ethidium bromide was the sensitizer, photooxidation in the D_2O-containing medium was 2.2 times faster. When the 10% increase attributed to the effect of D_2O on singlet oxygen lifetime is taken into account, the enhancement of photooxidation exactly matches the twofold enhancement of the fluorescence intensity of ethidium bromide in these two solvent systems. This indicates that both fluorescence yield and triplet production are *equally* enhanced when ethidium bromide is deuterated.

When methanol–water (60:40) solutions containing free ethidium bromide or ethidium bromide bound to DNA were photolyzed in the presence of DPI, ethidium bromide bound to DNA was found to be a *better* photosensitizer than free ethidium bromide. Although reaction rates in this solvent system were substantially slower than in pure methanol, owing to the fact that singlet oxygen is shorter lived in aqueous media (Merkel and Kearns, 1972), the effect of binding ethidium bromide to DNA could be clearly seen as a better than twofold enhancement of the reaction rate. We also measured fluorescence lifetimes in this medium to verify intercalative binding and to estimate the fraction of ethidium bromide bound. The results are summarized in Table V.

Quantitative analysis of the data is rendered difficult by the presence of both bound and free ethidium bromide, and possibly some electrostatically bound ethidium bromide (as evidenced by the somewhat larger fluorescence lifetime of the "free" component) in DNA-containing samples. The possibility of kinetic effects due to the reduced accessibility of bound ethidium bromide to diffusing oxygen (Lakowicz and Weber, 1973) may also play a complicating role. Qualitatively, however, it is clear that binding ethidium bromide to DNA *increases* the probability of triplet state formation. From the rate enhancement together with the fraction of ethidium bromide bound (derived from the relative intensities of the two fluorescence components), a triplet production enhancement factor of 4.0 ± 0.5 upon binding to DNA can be estimated which corresponds well with the 4.3-fold enhancement of the fluorescence lifetime.

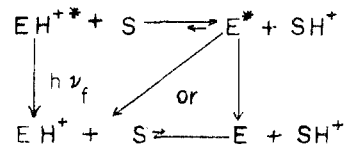

FIGURE 5: Schematic representation of mechanism for fluorescence quenching of ethidium bromide by proton transfer to solvent. EH^+, ground state ethidium bromide; EH^{+*}, excited state ethidium; E^*, excited state deprotonated ethidium bromide; S, solvent.

Discussion

Medium-induced variations in fluorescence properties can be caused by several different mechanisms: interchange of low-lying n,π^* and π,π^* electronic states [exemplified by polycyclic monoazines (Brederick et al., 1962)]; shifts in near isoenergetic singlet and triplet states with concomitant intersystem crossing rate variations [anthracene (Kellogg, 1966)]; viscosity-influenced conformational changes [malachite green (Pringsheim, 1949)]; and solvent quenching through complexation, hydrogen-bonding, or proton transfer (Förster, 1972; Stryer, 1966).

The mechanism for fluorescence enhancement of ethidium bromide upon bonding to nucleic acids must be in accordance with the following experimental observations: (1) fluorescence intensity and lifetime vary by the same factor upon binding, deuteration, or change of solvent; (2) lifetime variations with solvent and temperature do not seem to correlate well with known solvent properties; (3) deuteration of the exchangeable protons of ethidium bromide results in fluorescence enhancement in all media; (4) addition of small amounts of water to nonaqueous solvents significantly decreases fluorescence; (5) addition of D_2O to acetone solutions results in immediate quenching followed by slow regeneration and finally enhancement of fluorescence; (6) hydroxide ion (aqueous medium) and acetate ion (ethanolic medium) both quench fluorescence; (7) population of the ethidium bromide triplet state under photooxidizing conditions is inefficient ($\phi_T = 2.5 \times 10^{-3}$ in methanol); (8) the ethidium bromide triplet yield is enhanced upon deuteration; and (9) the ethidium bromide triplet yield is enhanced upon intercalative binding to DNA.

All of these observations can be straightforwardly accounted for by a mechanism of excited state quenching by solute-to-solvent proton transfer, depicted schematically in Figure 5. According to this mechanism, the low fluorescence intensity of free ethidium bromide in water is attributed to efficient quenching of excited state molecules by proton transfer to water molecules (quenching rate constant of $10^7\ s^{-1}\ M^{-1}$), and the enhancement of the fluorescence intensity on going to nonaqueous solvents, deuterated ethidium bromide, or binding to DNA is attributed to a reduction in the proton transfer rate.

This mechanism predicts that both fluorescence intensity and lifetime will change by the same factor upon going from one set of conditions to another since the intrinsic rate of fluorescence is not affected. It is, thus, in accord with observation 1. It is consistent with observation 2, although it cannot be conclusively tested with respect to solvent proton affinity inasmuch as proton affinities of different media cannot reliably be evaluated relative to one another (Bates, 1969). Nonetheless, it is qualitatively reasonable that water has a very high proton affinity, alcohols, pyridine, and Me_2SO somewhat lower values, and acetone a relatively low affinity (attempts to measure lifetimes in extremely low affinity solvents such as chloroform or methyl cyclohexane were unsuccessful due to the insolubility of ethidium bromide in these media). The deuteration effects we observe are similar in magnitude to those found for other dyes for which proton transfer to solvent has been implicated as a quenching mechanism (Förster, 1972; Stryer, 1966); thus, the mechanism is in accord with observation 3. It also readily explains observation 4 since small quantities of high proton affinity water should act as an efficient quencher in nonaqueous solvents. NMR data on ethidium bromide solutions in acetone show that, upon addition of ca. 1% water, one set of amino protons undergoes a measurable upfield shift, demonstrating there is an interaction between the amino protons and trace amounts of water. The time-dependent variation in fluorescence intensity upon addition of D_2O (observation 5) is exactly as predicted by the mechanism: when the protonated dye first encounters D_2O) molecules, the latter instantaneously serve as proton acceptors and thus cause quenching; however, at later times, dye protons exchange with deuterons from the added D_2O causing fluorescence enhancement since the deuterated dye has a reduced transfer rate compared with the protonated dye. Good proton-ption spectrum (observation 6), again in agreement with the mechanism (we are grateful to one of the referees for suggesting this positive test). The mechanism predicts that any change in conditions which leads to an increase in fluorescence intensity will also cause an increase in triplet yields, which will be low when the fluorescence yield is low. This is exactly in accord with our last three observations, not only qualitatively but also quantitatively since the triplet yield, as measured by photooxidation rate, increases by the same factor as predicted by the fluorescence change, for both deuterium substitutions and intercalative binding.

The excited state proton-transfer mechanism for quenching of ethidium bromide fluorescence also reasonably accounts for the fluorescence lifetime changes observed upon deuteration and/or binding of the dye to DNA. From the fluorescence lifetime of free ethidium bromide in aqueous solution (1.8 ns) and the radiative lifetime of 73 ns (Genest et al., 1974), we compute that the pseudo-first-order rate constant for quenching of protonated dye in water is $5.55 \times 10^8\ s^{-1}$, assuming no significant contributions from unimolecular radiationless decay. Upon deuteration, this rate constant is reduced by a factor of 3.7. Upon binding to DNA, the quenching rate constant for protonated dye drops 18-fold to $3.0 \times 10^7\ s^{-1}$. Making the plausible assumption that the deuteration and binding effects operate independently and therefore the reduction in quenching associated with binding and deuteration will be the product of the above two factors, we predict that the fluorescence lifetime of deuterated ethidium bromide bound to DNA should be 45 ns. Given the number of assumptions involved, this prediction is in good agreement with the observed value of 38 ns.

The mechanism also allows a qualitative interpretation of the observation of different fluorescence lifetimes, despite similar absorption spectra for intercalative and (presumably) electrostatic binding. Intercalated ethidium bromide is substantially less accessible to water molecules than is outside-bound ethidium bromide (though both are considerably less accessible than is free ethidium bromide); hence, the lifetime in the intercalated sites is substantially longer than in electrostatically bound sites.

Why the end result of proton transfer should be deactivation to the ground state is at this stage a matter of speculation, but two reasonable possibilities can be entertained. One possibility is that the neutral species formed by proton transfer is predominantly quinone-like in its electronic structure (structure II) and undergoes deactivation similar to *p*-benzoquinone

which is known to display very little luminescence (Sidman, 1956). The other possibility is that the reverse proton-transfer process also occurs rapidly and leads directly to ground state ethidium bromide. Both of these possibilities are indicated in Figure 5.

Other mechanisms which might be responsible for the fluorescence intensity variations of ethidium bromide are interchange of n,π^* and π,π^* states (Burns, 1969), viscosity-induced conformational changes (Pringsheim, 1949), "base specific" interactions such as have been implicated for proflavin fluorescence variations (Thomes et al., 1969), and triplet yield variations (Hudson and Jacobs, 1975; Waleh et al., 1976). We believe that our observations argue convincingly against each of these possibilities.

Interchange of electronic states as implied by Burns (1969) would require that the radiative lifetime of the dye change as the fluorescence intensity changes, leading to differences between the intensity and lifetime ratios. This is contrary to our observations. Furthermore, deuteration would not be predicted to result in lifetime lengthening of both states (as observation 3 requires) nor would a smoothly varying reduction of fluorescence upon addition of water be expected (observation 4). Quenching by good proton acceptors like hydroxide and acetate is also not readily reconciled with this mechanism unless additional ad hoc assumptions are made.

Fluorescence enhancement by viscosity-induced conformational changes is argued against by observations 1 (since such changes generally would be expected to alter radiative lifetimes), 3 (since deuteration should not significantly alter conformations), 4 (since small amounts of water do not significantly change the viscosity of, e.g., ethanol) and by the fact that the fluorescence lifetime in glycerol is the same as that in methanol, despite a 2000-fold difference in viscosity.

"Base specific" interactions have been invoked of necessity to account for the observed fact that the fluorescence yield of proflavin is negligible when bound in the vicinity of guanosine but is relatively high when association with other nucleic acid bases. For ethidium bromide, on the contrary, our observations as well as those of others (LePecq and Paoletti, 1967; Pohl et al., 1972) demonstrate no difference in fluorescence intensity or lifetime for dye molecules bound between G-C or A-T base pairs. Furthermore, the "base specific" hypothesis fails to account for deuteration effects which are observed both in the presence and absence of nucleic acids.

According to the triplet manifold mechanism, efficient quenching of ethidium bromide fluorescence in water is attributed to rapid intersystem crossing which effectively competes with radiative decay; intersystem crossing is then postulated, without proof, to become substantially less efficient when the dye is bound (Hudson et al., 1969). This mechanism and the proton transfer mechanism yield contrary predictions concerning the way in which the triplet yield changes with fluorescence yield. According to the intersystem crossing hypothesis, an increase in the fluorescence yield must *decrease* the triplet yield since one process takes place at the expense of the other. However, according to the proton transfer quenching hypothesis, fluorescence *increases* are predicted to be accompanied by triplet yield *increases* since both are in competition with the quenching such that a reduction in quenching enhances all other processes. The photooxidation experiments critically test these predictions. Our observations are that photooxidation rate is low under conditions where the proton transfer mechanism predicts a low triplet yield, but the intersystem crossing mechanism predicts a high triplet yield. Furthermore, changes (deuteration, binding to DNA) which enhance the fluorescence yield also equally enhance the photooxidation yield. These experimental observations only admit two possibilities: the intersystem crossing mechanism is incorrect or intersystem crossing is taking place with high efficiency, but the photooxidation experiments are not monitoring triplet state production. In order for photooxidation not to monitor triplet state production, it would be necessary for the triplet state of ethidium bromide to decay unimolecularly more rapidly than it is intercepted by oxygen molecules. Our observation that oxygen-saturated solutions react no more rapidly than do air-saturated solutions sets a lower limit to this postulated unimolecular decay rate of 3×10^8 s^{-1}. This rate is some six orders of magnitude larger than the normal triplet–singlet deactivation rate for aromatic molecules (Siebrand, 1967) and four orders of magnitude larger than maximum phosphorescence decay rates for aromatic $^3n,\pi^*$ states (Becker, 1969). Moreover, it would be necessary for excited ethidium bromide molecules to sensitize diphenylisobenzofuran oxidation by some alternative mechanism, involving a photochemical intermediate that was formed in a twofold higher yield upon deuteration and fourfold upon binding to DNA, thus fortuitously paralleling the fluorescence yield changes which we have observed. A further difficulty with the intersystem crossing mechanism is that the enhancement of fluorescence upon deuteration would have to be attributed to isotope effects on the intersystem crossing rate constant even though such effects have not been found for singlet-to-triplet processes in aromatic molecules where pronounced triplet-to-ground singlet isotope effects (Laposa et al., 1965) are observed. In short, the intersystem crossing mechanism for fluorescence enhancement requires several additional ad hoc assumptions to account for our observations, whereas the excited state proton transfer mechanism accounts for all observations without any additional assumptions. We believe that the experiments reported in this paper are satisfactorily explained only by excited state proton transfer.

The very substantial fluorescence enhancement of ethidium bromide on binding is due in large part to its remarkably long radiative lifetime (73 ns) (Genest et al., 1974) which makes its excited singlet state much more susceptible to quenching than similar dyes with much shorter radiative lifetime. This suggests that the search for other intercalating compounds with behavior similar to that of ethidium bromide might profitably focus on compounds like pyrenes which are expected to have particularly long radiative lifetimes.

The mechanism proposed here suggests that intercalation sites on different biopolymers may yield different fluorescence lifetimes, depending on the degree of exposure to solvent of the intercalated ethidium bromide molecule; and preliminary experiments indicate that such is, indeed, the case (Olmsted and Kearns, unpublished results). There are also several indications in the literature that the fluorescence quantum yield and/or lifetime of ethidium bromide is different when bound to tRNA then when bound to DNA (LePecq and Paoletti, 1967, Burns, 1969; Tao et al., 1970).

Since a longer lifetime indicates less exposure to solvent, detailed investigations of lifetime variations hold promise of yielding information about local site conformations, and it is also possible that the presence or absence of lifetime variations when ethidium bromide labeled nucleic acids interact with other biopolymers may shed light on the nature of these interactions. At the same time, however, the observation that the fluorescence yield from intercalated sites is not independent of conditions makes it imperative to have experimental knowledge about lifetime variations before proceeding to interpret fluorescence intensity variations in terms of greater or lesser amounts of double helical structure.

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