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Fluorescence Enhancement of Ethidium Bromide by DNA Bases and Nucleosides

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Fluorescence enhancements of ethidium bromide (EB) by solution species of low molecular weights such as DNA base molecules and nucleosides in water are reported. The degree of enhancements was determined by intensity as well as lifetime measurements for EB fluorescence. Experiments including solvent effects on absorbance and fluorescence spectra of EB, effects of protonation on the EB absorbance spectrum, and determination of equilibrium constants for EB-DNA bases have been performed to help explain the fluorescence enhancement. The results suggest that the excited state stabilization in the hydrophobic environment, the loss of torsional/vibrational energy of amino groups, and the change in the electronic transition characteristics are all responsible for the fluorescence enhancement.

Introduction

Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridium bromide; EB) is one of the most studied of the intercalating compounds. A great deal of interest in EB might be due to its biological reactivities and unique spectroscopic properties. Biologically, it is trypanocidal[1] and antiviral and inhibits DNA and/or RNA biosynthesis *in vivo*.[2-4] Spectroscopically, the red shifts shown in the absorption and fluorescence bands as well as fluorescence enhancement upon inter-

calation allow its use as a probe in DNA and RNA structural studies.[5,7] The majority of the reports are concerned with the biological/physiological aspects of EB and its derivatives. Some were found that dealt with the EB-DNA interaction mechanism and the utility of this as a structural probe for biomacromolecules. And yet, there are only a few studies reported on the fundamental properties of EB.

Several possible mechanisms have been proposed to explain the fluorescence enhancement of EB upon its intercalation into the base pairs of DNA. According to LePecq and Paoletti,[8] the fluorescence quantum yield of intercalated EB would be enhanced due to its hydrophobic environment, where the approach of aqueous solvent molecules is prohibited. In other words, the solvent molecule, *i.e.*, water, serves as an effective quencher. Hudson and Jacobs[9] performed theoretical calculations to show that a triplet excited state is located very close to the lowest singlet excited state resulting in interconversion of these states depending on the environment surrounding EB. In the study of Waleh *et al.*[10] and others, EB was modeled as a derivative of a phenylphenanthridium cation. This mechanism would predict a strong solvent effect on the transition energy as well as the transition probability (molar absorptivity) for the lowest transition. Finally, a comprehensive study by Olmsted and Kearns[11] indicates that the EB fluorescence is quenched by exchanging the amine proton with that in the solvent in the excited state. In an intercalated EB molecule, the proton exchange in the excited state is prohibited due to its hydrophobic environment. The most convincing observation for this mechanism came from the isotope effect on fluorescence quenching using H_2O or D_2O as a solvent.

We have been studying interactions of various organic compounds with DNA or DNA base molecules by generating their anion/cation redicals electrochemically,[12] by using fluorescence[13] and electrogenerated chemiluminescence measurements,[14,15] and with transient electrochemical techniques.[16-18] As part of our continued effort in this area we have studied the interaction of EB with a few DNA base molecules and nucleosides, and our preliminary results are reported here. From our results, new mechanisms for the fluorescence enhancement of EB are proposed.

Experimental

EB was purchased from Sigma and used as received. In most cases stock solutions of 10.0 mM EB were prepared in an appropriate solvent and diluted with or without other compounds to make solutions of 20.0 μM in EB for fluorescence measurements. Of solvents used, water was deionized and doubly distilled. Fisher's reagent grade dimethyl sulfoxide and formamide, HPLC grade methanol, certified spectrograde acetone, absolute ethanol, Sigma's ethylene glycol and glycerol (99%), and MCB's HPLC grade acetonitrile were used without further purification. For studies on fluorescence enhancements of EB by DNA bases and related compounds, Sigma's adenine, guanosine, 2',3'-isopropylidene adenosine (IPA), and 2',3'-isopropylidene guanosine (IPA), and Aldrich's cytosine and γ-cyclodextrin were used as received.

Fluorescence spectra were recorded using an Aminco-Bowman spectrophotofluorometer with Lynseis LY-1800 xy

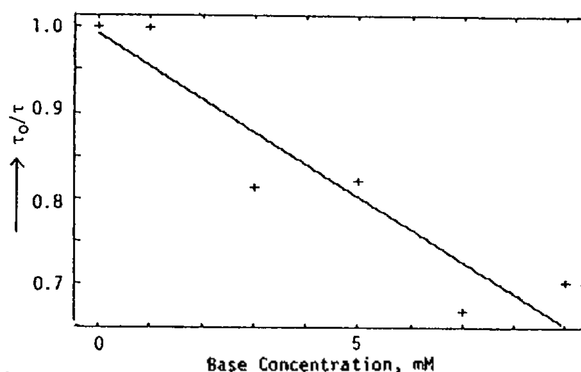


Figure 1. Fluorescence enhancement shown by ethidium bromide (EB) in the presence of various concentrations of adenine. The ratios of the fluorescence lifetimes in the absence (τ_0) to those in the presence of various DNA concentrations (τ) are plotted against the adenine concentrations. The EB concentration was 20 μM.

recorder. Band widths of excitation and emission monochromators were 7.7 nm. Absorbance spectra were measured with a Bausch and Lomb Spectronic 2000 spectrophotometer and Houston Instrument's Omnigraphic 2000 xy recorder. Fluorescence lifetimes were determined by pulse sampling methods, as described previously.[19] An Optitron model NR-10 Nanosecond Radiator with a model NR-10-T Electrical Trigger Pulse Generator (Torrance, California) was used as an excitation source. The pulse trains from the detector, Hammamatsu R-928 photomultiplier tube, was sampled with a Tektronix 5S14N sampling probe. The sampling oscilloscope was then interfaced with an APPLE II+ computer system through an APPLAB interface card (Interactive Microware, Inc., State College, PA). The fluorescence decay signal was then deconvoluted from the excitation signal employing the phase plane method. All the results were displayed on the computer monitor screen. This subnanosecond fluorimeter was shown to reliably measure fluorescence lifetimes down to approximately 120 ps.[19,20]

Results and Discussion

In order to investigate whether the EB fluorescence would be affected BY DNA base molecules or not, we made fluorescence intensity as well as lifetime measurements as a function of the base concentration. A typical result of the fluorescence enhancement is shown in Figure 1 for various concentrations of adenine, and results obtained from other DNA bases or nucleosides are summarized in Table 1. To our surprise, these intensity and lifetime data show that the EB fluorescence is enhanced by smaller solution species such as DNA bases or nucleosides. With the exception of γ-cyclodextrin, all the compounds enhanced EB fluorescence. We used the Stern-Volmer equation to compare the enhancement efficiency for each compound, *i.e.*,

$$\frac{\tau_0}{\tau} = 1 + k_q \tau_0 [B]$$

where τ_0 and τ are fluorescence lifetimes of EB without and with a quencher present, and k_q is the quenching rate constant. There is no theoretical justification for using this equation derived for fluorescence enhancement experiments, but at least the quenching or enhancing efficiencies can be com-

Table 1. EB Fluorescence Enhancement by DNA Bases and Related Compounds[a]

Compound	Concn., mM	τ, ns	k_e	I_f/I_f^0
cytosine	10.7	1.65	4.0×10^9	1.11
thymine	10.1	1.76	8.0×10^9	0.91
adenine	2.01	2.15	4.5×10^{10}	1.12
guanosine	1.06	1.76	2.8×10^{10}	1.05
IPA	3.25	2.05	5.0×10^{10}	1.16
IPG	2.01	1.80	4.7×10^{10}	1.10
γ-cyclodextrin	5.02	1.54	0	1.02
blank	---	1.54	---	---

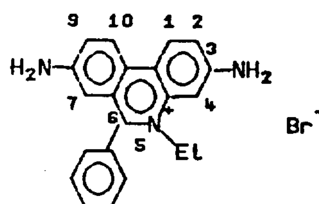
[a][EB] = 20 μM.

pared with each other. There are some scatters on the data points in the Stern-Volmer plot; it was difficult to obtain accurate results of fluorescence lifetimes of EB owing to its low intensity and the limitations of our instrument. The limitations of this type of instruments were pointed out by Good *et al.* for measurements of short fluorescence lifetimes.[21]

Pyrimidine group bases (cytosine and thymine) enhance approximately one order of magnitude less effectively than purine compounds. It can be discerned that adenine (or IPA) is a slightly more effective enhancer when compared to guanosine (or IPG), whereas guanosine has a larger equilibrium constant of complex formation (more on this below). This might be due to the higher electron donating ability of guanosine, which would quench fluorescence. Guanine is known to quench fluorescence of certain dyes such as acridines through an electron transfer mechanism.[22] To the best of our knowledge, our findings represent the first observations reported for the EB fluorescence enhancement by small molecules such as DNA bases and nucleosides. The EB-DNA base interaction is not large enough to describe the large fluorescence enhancement of intercalated EB in DNA (τ = 20 ns, Olmsted and Kearns[11]; Mandal *et al.*[23]), however.

One may note that the fluorescence lifetime of free EB in water was measured to be 1.54 ± 0.04 ns (Table 1), which is shorter than literature values of 1.80 ± 0.2 ns[11] and 1.75 ns.[22] We also measured its lifetime of 1.80 ± 0.03 ns[19] in 10 M LiCl, which is in excellent agreement with the value reported by Olmsted and Kearns[11] under similar experimental conditions. This discrepancy might be due to different experimental conditions used for these measurements; we used pure water as a solvent in our current experiments, while reported values mentioned above and the one we obtained previously were measured in 10 M LiCl or 1 M NaCl solutions containing small amounts of buffer and other salts.[11,22]

Also noted in the table is that both IPA and IPG cause a slightly larger enhancement compared to their corresponding base molecules. γ-Cyclodextrin was included in this study to test the possibility of fluorescence enhancement due to its hydrophobic environment. It has been reported to have a hydrophobic cavity, which can accommodate fluorescence molecules of proper size in an aqueous solution.[24,25] The negative result show here indicates either that hydrophobicity may not have much to do with the EB fluorescence enhancement, or that EB is too large to be trapped in the γ-dextrin cavity or its charge on the N5 position prevents it from getting into the hydrophobic cavity. Ethidium bromide has the following structure:



It is very likely that the charge on the N5 could be a factor in this case, since γ-dextrin has a cavity large enough to accommodate EB.

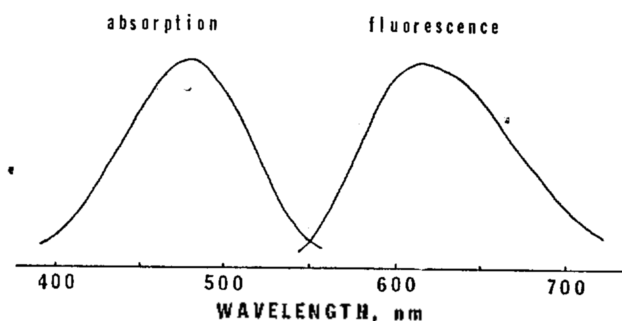


Figure 2. Absorbance and fluorescence spectra of EB with [EB] = 20 μM.

Table 2. Solvent Properties

Solvent	Donor[a] Number	Acceptor[a] Number	Dielectric[b] Constant	Refractive[b] Index	Polarity[b] Parameter
water	18.0	54.8	80.37	1.333	0.6402
methanol	19	41.3	33.62	1.329	0.6179
ethanol	20	37.1	24.3	1.359	0.5787
n-propanol	--	--	20.1	1.383	0.5489
ethylene glycol	--	--	37.7	1.4318	0.5490
glycerol	--	--	42.5	1.4746	0.5260
acetone	17.0	12.5	20.7	1.357	0.5699
formamide	24	39.8	109	1.4472	0.5645
dimethylformamide	26.6	16.0	36.7	1.4305	0.5488
propylene carbonate	15.1	18.3	64.4	1.4189	0.5737
dimethylsulfoxide	29.8	19.3	46.7	1.4770	0.5276
acetonitrile	14.1	9.3	37.45	1.34423	0.6107

[a]Taken from Gutman[28]. [b]CRC Handbook of Chemistry and Physics, 60th ed., CRC Press, 1980.

In an effort to understand fluorescence enhancements by a variety of molecules, we ran several experiments including solvent effects on both fluorescence and absorption spectra, effects of protonation of amines on EB, and complexation of EB with both adenine and guanosine. Results of these experiments are discussed to explain fluorescence enhancements.

Absorption and fluorescence spectra of 20.0 μM EB in water are shown in Figure 2. Ethidium bromide gave a featureless absorption spectrum at longer wavelength regions (400-550 nm), typical of a nitrogen containing heterocyclic aromatic amines.[26] Various solvents were used to study the solvent effect on absorption and fluorescence spectra. Solvent properties such as acceptor number (AN), donor number (DN), dielectric constant (D), and refractive index (n), are compiled in Table 2 along with the polarity parameter[27]

Table 3. Solvent Effects on EB Absorption and Fluorescence Spectra[a,b]

Solvent	λ_{abs}^{max},nm	ε_{max}, l.mole^{-1}cm^{-1}	λ_{fl}^{max},nm	I_{fl}/I_{fl}^{0}
water	478	5400	614	1.00
methanol	524	6050	630	2.93
ethanol	532	6060	640	4.74
n-propanol	536	5980	643	3.87
ethylene glycol	518	6060	636	3.70
glycerol	512	5190	626	4.92
acetone	519	6300	619	3.45
formamide	515	5980	629	2.54
dimethylformamide	533	6060	640	2.34
propylene carbonate	514	6450	616	7.48
dimethylsulfoxide	534	6130	646	--
acetonitrile	511	6310	611	4.12

[a] [EB] = 20.0 μM. [b] Fluorescence intensity ratio were obtained after correcting absorbance; aqueous solution was used as a reference.

[$f(D,n)$]. The polarity parameter [$f(D,n)$] is an index of the electrostatic property of the solvent originated from the dipole moment and the polarizability of the solvent and was calculated as follows:

$$f(D,\ n)=\frac{(D+1)}{(2D+1)}-\frac{(n^2-1)}{(2n^2+1)}$$

This parameter is a measure of the solvent-solute electrostatic interaction.[28]

The absorption and fluorescence spectra showed considerable hypsochromic shifts in polar solvents and the shifts are listed in Table 3. No significant correlation was found between the spectral shift and donor or acceptor numbers of solvents,[29] when plotted against each other. This indicates that the spectral shift due to the charge transfer interaction between solute and solvent molecules may not be very important. The fluorescence wavenumbers were plotted against the polarity parameter in Figure 3 according to the equation[27]:

$$\bar{\nu}=\bar{\nu}_0-\frac{2}{hc}\frac{(\Delta\mu)^2}{r^3}f(D,\ n),$$

where $\bar{\nu}_0$ and $\bar{\nu}$ are the fluorescence wavenumber at the maximum in the vacuum and in solution, respectively; $\Delta\mu$ is the change in dipole moments upon transition and r is Onsager's cavity radius. Although there is some scatter of data points, one can recognize a general trend of increasing fluorescence emission energies (blue shift) with increasing polarity parameter. The scatter might have resulted from different solvent properties such as the proton donating abilities among others, which were not taken into consideration in calculation of the polarity parameter. The change in dipole moments of this molecule upon excitation is estimated to be -4.6 Debye unit (DU) from the slope of the plot by assuming r = 4.9Å. The radius was estimated by measuring the density, d_1, of the m g/ml aqueous EB solution and calculated as follows:

$$\frac{4}{3}\pi r^3 N(d_1-d_0)=m$$

where d_0 is the density of water and N is Avogadro's number.

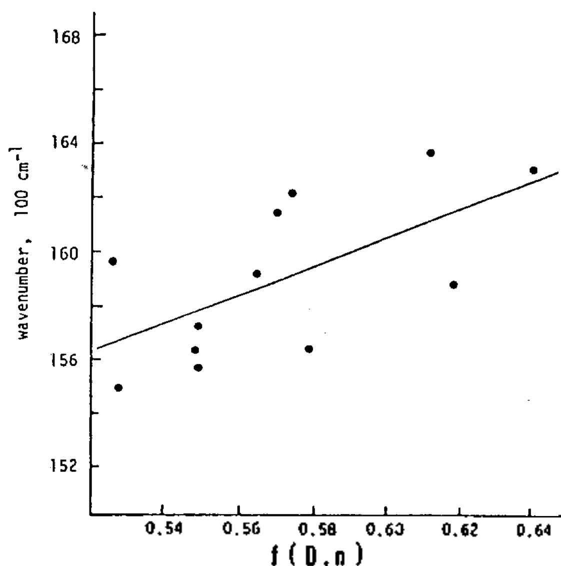


Figure 3. Plot of the fluorescence energies *vs.* the polarity parameters of various solvents. See the text for details.

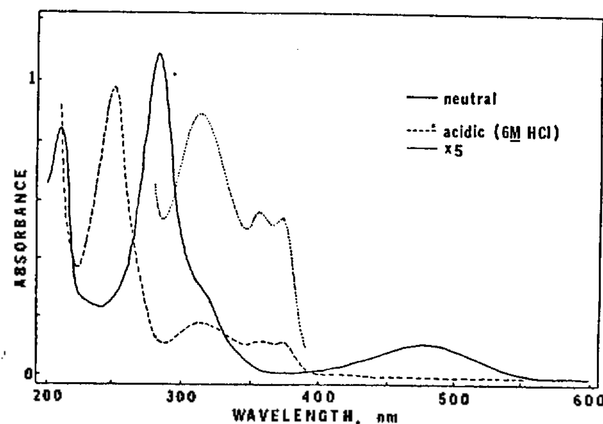


Figure 4. Absorbance spectra of EB in the ultraviolet-visible region in water with [EB] = 20μM.

The negative change in the dipole moment observed in this experiment indicates that the positive charge at the N5 position of the ground state EB molecule becomes delocalized upon excitation. Molar absorption coefficients were in the range of 5200-6200 mole^{-1}cm^{-1} for the solvents used in this study. These large spectral shifts and lower absorption coefficients indicate that the electronic transition is not of an ordinary π^*-π type[30] (more on this follows).

On the contrary, the blue shift shown by EB in polar solvents can be attributed to the positive charge on N5, which gives a high dipole moment to the ground state. The dipole moment becomes smaller as a negative charge is transferred to the ring system from amino nitrogen atoms upon excitation. Dipole moments of ground and excited states were calculated to be 7.68 DU and 3.38 DU, respectively[31], which are consistent with our observations (the change in dipole moments of -4.6 DU change from solvent dependency measurements of fluorescence spectra).

To further investigate the nature of the electronic transition of EB, absorption spectra were recorded before and after the protonation of amine groups on EB. These spectra are shown in Figure 4. The UV-vis absorption spectrum of

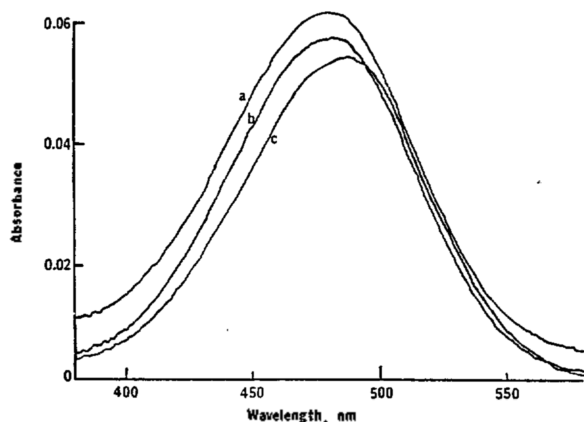


Figure 5. Absorbance spectra of (a) EB alone (10.4μM), (b) 9.1μM EB + 10.0 mM adenine, and (c) 9.7μM EB + 1.0 mM guanosine.

EB (Figure 4) in neutral water shows a weak broad band at 478.5 nm, a shoulder at 324.4 nm, and two strong maxima at 283.3 nm and 211.1 nm. The broad maximum at 478.5 nm is a typical feature of an amino derivative of aromatic compounds.[30,32] When two amino groups of EB are protonated in 6 M HCl, EB shows a drastic change in its spectral shape (Figure 4). The broad band at 478.5 nm disappears while new peaks appear at 373.3 nm and 355.0 nm. The shoulder at 324.4 nm is resolved with 10 nm blue shift and the band at 283.3 nm is blue shifted to 251.3 nm. This EB spectrum in 6 M HCl shows exactly the same features as found in a spectrum of the 5-ethylphenanthridium cation in ethanol.[33] It was shown that bands in the range of 350-370 nm were red shifted with a loss of resolution to longer wavelengths as amino groups were substituted progressively into 3- and 8-positions of 5-ethylphenanthridium cation.[31,33] This results are in agreement with that of an analogous series of acridine.[32] Thus it is clear that EB should be modeled as an amino derivative of a phenanthridium cation, not a phenanthridium cation itself.

Aromatic compounds with amino group(s) show different spectroscopic characteristics depending on the interaction of the lone pair orbital of the amino nitrogen and the π electron system of the ring. Coplanar compounds, in which planes of the π electron system and the amino ($-NH_2$) groups are on the same plane, allow a good overlap of the π system and the lone pair orbital. This overlap extends the π system to the amino nitrogen atom and gives a characteristic π^*-π electronic transition. When two planes are rotated by 90 degrees, however, the orbital overlap cannot occur. In this case, the compound shows a typical π^*-n transition with very low transition probabilities, *i.e.*, low molar absorptivities. Aromatic amines that have a conformation between these two extremes display intermediate property of considerable charge transfer characters. This intramolecular charge transfer transition is classified as $a\pi^*$-l to differentiate from normal π^*-π or π^*-n type transitions.[34] Since aromatic amines have a charge transfer characteristics, their spectra exhibit red shifts in polar solvents.[30]

Aromatic amines have lower fluorescence quantum yields due to the vibrational (torsional and flipping) degree of freedom, since the vibrational motions quench the fluorescence effectively.[30,35,36] The low fluorescence quantum yield of EB can be attributed to the presence of two amino groups in the ring system. The greater part of the fluorescence enhancement may thus be achieved by reducing the quenching effect due to vibrations of amino groups.

Finally, to assess the role of the complexation of EB with these small base molecules in their ground state, the EB absorption spectrum was recorded in the presence of adenine and guanosine. The results obtained from these measurements are shown in Figure 5. Assuming that EB and adenine or guanosine interact with a (1:1) stoichiometry, one obtains equilibrium constans of 14.8 M^{-1} and 68.2 M^{-1}, respectively, for adenine and guanosine from the reduction in absorbance values of EB due to the presence of these DNA bases.

This result is consistent with observations of EB intercalation preference of GC to AT sites in DNA.[37] These equilibrium constants can be compared to reported values of 3.2×10^4 M^{-1} for EB-DNA[23], 2.2×10^4 M^{-1} for EB-dinucleotide and 100 M^{-1} for EB-nucleotide.[38] The general trend of the equilibrium constant for the molecular size can be found in this series of interaction; smaller molecules give smaller equilibrium constants. The large equilibrium constant for the EB-DNA interaction might be due to concerted effects of the affinity of EB to DNA base, *i.e.*, the dipole-dipole attraction of opposite charges in the ethidium cation and phosphate moiety in the DNA, the hydrogen bond formation between EB and hydroxyl groups in ribose, and the relatively rigid protective DNA (or RNA) structure.[39]

Our results suggest that the presence of DNA bases in water along with EB may change the dielectric constant near the EB-base association site. This local change in the dielectric constant may be partially responsible for the fluorescence enhancement. The nature of the association may not be predominantly of charge-transfer. The charge-transfer process involved in this type of aggregation appears to reduce its ability to enhance fluorescence. This is shown by lower enhancing abilities by pyrimidine bases than by purine bases. Results obtained from adenine and guanosine also support this postulate. We doubt, however, that the presence of DNA bases in solution would possibly change the characteristics of the electronic transition of EB, although this effect might be very important when EB is intercalated. It may still be likely that these base molecules somehow stabilize the excited state EB by sharing the charge after forming an exciplex. This possibility has not been examined in our study.

Conclusion

From the results discussed thus far, a few important conclusions can be reached. These are significant in that the fluorescence enhancement mechanisms for ethidium bromide may need to be modified.

First, the fluorescence enhancement of EB does not require its intercalation into the DNA base pairs as previously postulated by many investigators. The EB fluorescence is enhanced even in the presence of small solution species, *i.e.*, DNA bases or nucleosides. The enhancement cannot be explained by the formation of charge-transfer (CT) complexes, since guanosine, which forms a stronger CT complex with EB than adenine shows a lower degree of enhancement. The nature of interaction responsible for the fluorescence enhancement is not understood. We speculate that the formation of some aggregates between these molecules through

non-CT processes may cause the enhancement. Further investigations are warranted to understand the phenomenon.

Secondly, the phenanthridinium cation does not model EB molecules accurately. We believe that it is much more reasonable to model EB as an aromatic amine, since anomalies involved in the fluorescence behavior of EB are mostly related to the broad band with the lowest energy. While this view appears somewhat obvious, it has not been pointed out in the literature.

Finally, our results indicate that the EB fluorescence would be enhanced in any media stabilizing its excited state. The stabilization of the excited EB molecule would result from the low polarity of the solvent due to a decrease in the dipole moment in its excited state. Further, the reduction in the vibrational flexibility of the amine protons in a restrictive environment should increase the fluorescence quantum yield. Another parameter, which keeps the amino groups on the same plane as the aromatic π system, would increase the fluorescence quantum yield significantly due to the increased π^*-π characteristics in its electronic transition.

We believe that all three are responsible for the fluorescence enhancement shown by intercalated EB. That is, the hydrophobicity stabilizes the excited state of intercalated EB. Also, the loss in structural flexibility upon intercalation prevents the internal fluorescence quenching through torsional vibration and also increases π^*-π characteristics of the transition.

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