(16) For a review, see A. L. Kwiram, *MTP Int. Rev. Sci.: Phys. Chem.*, Ser. One, **4**, 271 (1972).
(17) M. Kasha, *J. Chem. Phys.*, **20**, 71 (1952).
(18) G. G. Giachino and D. R. Kearns, *J. Chem. Phys.*, **52**, 2964 (1970); **53**, 3886 (1970).
(19) K. F. S. Luk, R. J. Hoover, and A. H. Maki, *J. Am. Chem. Soc.*, **97**, 1241 (1975).
(20) R. R. Anderson and A. H. Maki, *Photochem. Photobiol.*, **25**, 585 (1977).
(21) T. D. Waugh, H. F. Walton, and J. A. Laswick, *J. Phys. Chem.*, **59**, 395 (1955).
(22) A. H. Maki and T.-t. Co, *Biochemistry*, **15**, 1229 (1976).
(23) J. Schmidt, D. Antheunis, and J. H. van der Waals, *Mol. Phys.*, **22**, 1 (1971).
(24) J. Zuclich, J. U. von Schütz, and A. H. Maki, *J. Am. Chem. Soc.*, **96**, 710 (1974).
(25) C. J. Winscom and A. H. Maki, *Chem. Phys. Lett.*, **12**, 264 (1971).
(26) G. Schwartzenbach and M. Schellenberg, *Helv. Chim. Acta*, **48**, 28 (1965).
(27) C. A. Evans, D. L. Rabenstein, G. Geier, and I. Erni, *J. Am. Chem. Soc.*, **99**,

8106 (1977).
(28) A. Albert in "Physical Methods in Heterocyclic Chemistry", Vol. I, A. R. Katritzky, Ed., Academic Press, New York, N.Y., 1963, p 1.
(29) F. Bishai, E. Kuntz, and L. Augenstein, *Biochim. Biophys. Acta*, **140**, 381 (1967).
(30) R. F. Chen, *Arch. Biochem. Biophys.*, **166**, 584 (1975).
(31) P. Svejda, R. R. Anderson, and A. H. Maki, *J. Am. Chem. Soc.*, preceding paper in this issue.
(32) J. Zuclich, *J. Chem. Phys.*, **52**, 3586 (1970).
(33) A. H. Maki and J. Zuclich, *Top. Curr. Chem.*, **54**, 115 (1975).
(34) J. Zuclich, J. U. von Schütz, and A. H. Maki, *Mol. Phys.*, **28**, 33 (1974).
(35) T.-t. Co, R. J. Hoover, and A. H. Maki, *Chem. Phys. Lett.*, **27**, 5 (1974).
(36) D. L. Rabenstein, *Acc. Chem. Res.*, **11**, 100 (1978).
(37) G. W. Robinson, *J. Chem. Phys.*, **46**, 572 (1967).
(38) E. Kochanski and A. Pullman, *Int. J. Quantum Chem.*, **3**, 1055 (1969).
(39) E. T. Harrigan and N. Hirota, *J. Am. Chem. Soc.*, **97**, 6647 (1975).
(40) S. P. McGlynn, T. Azumi, and M. Kinoshita, "Molecular Spectroscopy of the Triplet State", Prentice-Hall, Englewood Cliffs, N.J., 1969, Chapters 9 and 10.

Picosecond Studies of the Fluorescence Probe Molecule 8-Anilino-1-naphthalenesulfonic Acid

G. W. Robinson,* R. J. Robbins, G. R. Fleming, J. M. Morris, A. E. W. Knight, and R. J. S. Morrison

Contribution from the Department of Physical Chemistry, University of Melbourne, Parkville, Victoria, Australia 3052. Received October 17, 1977

Abstract: Picosecond time-resolved fluorescence studies of 8-anilino-1-naphthalenesulfonic acid (ANS) have been carried out in a water–ethanol mixed-solvent system. Combined with quantum yield results, these data provide further insight into the nonradiative processes that can take place in this interesting fluorescence probe molecule. In a mixed solvent, the interchange of solvent molecules at the solvent shell level is slow compared with subnanosecond fluorescence decay, and thus excited-state equilibrium cannot be established. *Local* solvent relaxation around the excited-state molecule, on the other hand, has been found to occur on picosecond time scales for these solvents. No evidence was found for any unusually large interactions between the supposedly highly polar excited state of ANS and water (or ethanol) nor was there any propensity for 1:1 complex formation between excited ANS and either of these solvent molecules in this solvent system. Rather, a general "bulk solvent effect" is indicated by the data. Since photoionization is most likely the dominant nonradiative path in polar solvents, while intersystem crossing fills this role in nonpolar solvents, caution should be exercised in transferring conclusions for purely polar solvent systems, such as the one studied here, to nonpolar or to mixed polar–nonpolar solvent systems. The interpretation of results using ANS as a biological fluorescence probe molecule could be similarly affected.

Introduction

Molecules with fluorescence spectra, quantum yields, and lifetimes that are sensitive to their environment have been used as probes for structure studies of biological macromolecules for many years.[1-3] Some of the most widely used molecules in this work are the anilinonaphthalenesulfonates (ANS) and related molecules. 1,8-ANS (8-anilino-1-naphthalenesulfonic acid) shows a dramatic decrease in fluorescence quantum yield when the solvent is changed from ethanol ($\phi_F = 0.4$) to water ($\phi_F = 0.003$).[4] Accompanying the decrease in fluorescence yield is a large red shift in the fluorescence emission maximum.[4,5] A number of different explanations of this huge solvent effect on the excited-state lifetime have been given.[2,6,7] Highly pertinent to this point is the suggestion by Fleming et al.[8] that the major nonradiative pathway in ANS may be one-photon ionization.

The study of ANS in *aqueous solvents* seems particularly relevant to biological probe studies, yet to our knowledge there have been no measurements of the fluorescence lifetimes of ANS and related molecules in such solvents, simply because of the subnanosecond time scales required. Consequently, discussions of the photophysics of aqueous ANS have up to now

involved experimentally observable absorption and fluorescence maxima and quantum yields, rather than the rates of radiative and nonradiative decay of the excited state. In this paper we present fluorescence lifetime data for 1,8-ANS in a series of water–ethanol mixtures. These data are combined with quantum yield determinations to evaluate the changes in both radiative and nonradiative decay constants as to the solvent composition is varied. A discussion of the results then follows.

Experimental Section

1,8-ANS obtained from Sigma was used as supplied. Spectroscopic grade ethanol (Merck) and triply distilled water were used as solvents. Absorption and emission spectra were measured on a Cary 17 spectrophotometer and a Perkin-Elmer MPF3 spectrofluorimeter, respectively. For quantum yield determinations, a quinine sulfate fluorescence standard (10^{-5} M, 1 M H_2SO_4, $\phi_F = 0.546$) was used.[9] A quadratic correction for refractive index variation was applied.[10]

Fluorescence lifetimes were measured on two instruments. Subnanosecond lifetimes were measured using the third harmonic (351 nm) of a mode-locked neodymium phosphate glass laser for excitation. An Electro-Photonics Photochron II streak camera coupled to a Princeton Applied Research optical multichannel analyzer system was used as detector. The design and operation of this equipment has been described in detail in other publications.[11,12] Right-angle detection geometry was used. Polarization bias was eliminated by using

* To whom correspondence should be addressed at the Department of Chemistry, Texas Tech University, Lubbock, Texas 79409.

Table I. Emission Maxima, Quantum Yields, Lifetimes, and Radiative and Nonradiative Rates for ANS in a Series of Water-Ethanol Solvent Mixtures

vol % EtOH	λ_{max}^{em}, nm	ΔE, cm^{-1}	ϕ_F	τ_F, ps	$10^7 k_r$, s^{-1}	$10^7 k_{nr}$, s^{-1}
100	464	0	0.41	11090	3.70	5.32
90	474	450	0.21	6490	3.24	12.17
80	480	720	0.12	4150	2.89	21.21
70	485	930	0.065	2760	2.36	33.88
60	488	1060	0.045	1750	2.57	54.57
50	490	1140	0.033	1480	2.23	65.34
40	496	1390	0.022	1200	1.83	81.50
30	501	1590	0.013	930	1.40	106.1
20	505	1750	0.008	550	1.45	180.4
10	512	2020	0.005	380	1.32	261.8
0	523	2430	0.003	250	1.20	398.8
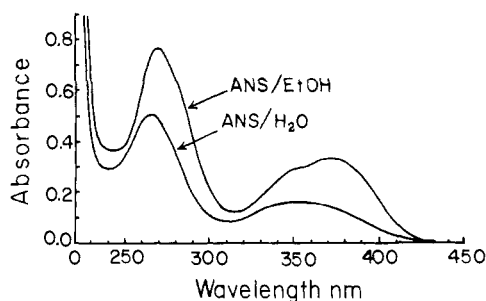
Figure 1. Absorption spectrum of ANS in pure water and pure ethanol solvents showing solvent effects on the first two singlet-singlet electronic transitions.

an analyzer polarizer set at 54.75° to the direction of polarization of the exciting light. The decay curves were analyzed by a nonlinear, least squares fitting procedure on a Nova 2-10 computer. The longer decays were measured by the time-correlated single photon counting technique using a Model SP2X Applied Photophysics Ltd. nanosecond spectrometer. Lifetimes were extracted from the data by iterative convolution analysis.[13]

Results

A. Absorption Spectra. The absorption spectrum of 1,8-ANS involving the two lowest excited singlet electronic states is similar to the spectra of most other α-substituted naphthalenes,[14,17] indicating that the naphthalene ring chromophore is primarily responsible for electronic changes in these transitions. Kosower et al.[6] on the basis of small changes in the absorption spectra of various 6-anilino-2-naphthalenesulfonates have also come to this conclusion but offer a much more complex picture of fluorescence. Camerman and Jensen,[18] from a crystal structure determination, suggest, however, that there is significant overlap between the aryl and the naphthalene π systems.

Participation of the amino nitrogen orbitals in these two low-lying transitions has been established, certainly for the 6-anilino-2-naphthalenesulfonates, but probably also for the 1,8 derivatives. Seliskar and Brand[19] showed that as the N substituents become more electron donating, the $S_1 \leftarrow S_0$ transition energy is lowered and the electronic transition dipole is increased. They conclude that because of charge transfer between the nitrogen lone-pair orbital and a naphthalene antibonding π orbital, it is "of questionable validity" to consider the two lowest transitions in these molecules as being derived from naphthalene $^1B_{3u}^-$ and $^1B_{2u}^+$ states. The theoretical work of Suzuki et al.[15] has shown that the first two transitions of the parent naphthalene ^1L_b (B_{3u}^-) and ^1L_a(B_{2u}^+) are somewhat mixed because of the presence of the amino group, and the intensity of the second strong transition is distributed between both excited states. The effects are larger in 2-anilinonaphthalene than 1-anilinonaphthalene, but in both cases only

a small amount of the charge-transfer state described by Seliskar and Brand is evident. However, increasing the electron-donating power of the N substituents and increasing the polarity of the solvent is expected to increase this effect. Even so, it still seems like the best way of describing the absorption spectrum of the two lowest excited states of ANS and related molecules is in terms of the parent naphthalene π orbitals together with some charge-transfer contribution and some mixing of the $^1B_{3u}^-$ and $^1B_{2u}^+$ states.

The absorption spectra of ANS and its derivatives in a variety of neat and mixed solvents have been reported by many authors.[6,19-21] In ethanol, peaks at 374, 352, 369, and 219 nm are evident in 1,8-ANS, but the one at 352 nm is detectable only as a shoulder on the 374-nm feature (Figure 1). In water the spectrum is slightly broadened, and the two long-wavelength bands merge into a single, broad, flat-topped absorption band. A small blue shift (~4 nm) occurs in aqueous solution compared with ethanol. In addition, the integrated absorbance (i.e., integrated optical density) is only about 30–50% that of 1,8-ANS in ethanol for the lowest two absorption bands. Because of the small absorption shifts in these two pure solvents, it is not surprising that the absorption spectrum of 1,8-ANS in EtOH-H_2O mixed solvents changes little across the entire range of solvent concentrations.

B. Emission Spectra. The results of fluorescence studies of 1,8-ANS in EtOH-H_2O mixed solvents are given in Table I. In contrast to the absorption results, the solvent dependence of the fluorescence emission maximum is very marked, a shift of 2430 cm^{-1} occurring between pure water and pure ethanol solutions. This shift is highly nonlinear with changes in solvent concentration in the mixed solvent. An interesting result is that in viscous media, ANS and its derivatives show nanosecond time-resolved emission red shifts.[22-24] Even in highly polar solvents, the red shift takes time to develop in very viscous media or it may not develop at all, spectra characteristic of a much less polar medium being observed.

Part of the mystery of the ANS fluorescence spectrum has concerned the exact nature of the solute-solvent interaction in the excited state. Brand and coworkers,[2,24] using theories of solvent reorientation due to Lippert[25] and Bakhshiev,[26] interpret the fluorescence shifts in terms of a large change of dipole moment between the ground and excited states. Kosower et al.[6] contend that the spectral shifts arise because of the charge-transfer nature of the emitting state. On the other hand, hydrogen-bonding interactions may be involved. As the proportion of water molecules in the solvent is increased, extra hydrogen-bond stabilization of the excited state may contribute to the fluorescence red shift. However, Förster and Rokos[27] have ruled out this explanation.

A recent paper by DeToma and Brand[28] has shed further light on the nature of the solvent binding in the excited state of ANS. Nanosecond fluorescence studies of 2-aminonaphthalene (2-AN) in a mixed cyclohexane-ethanol solvent
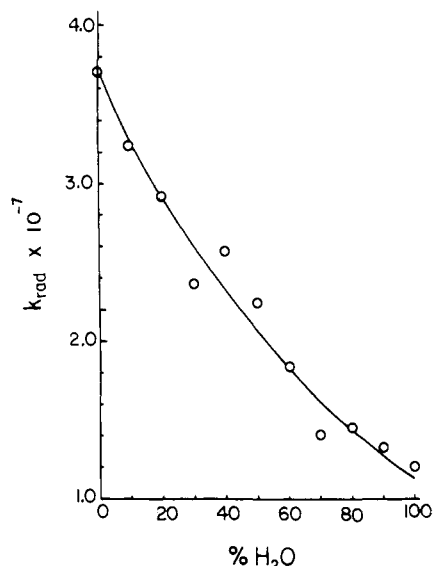
Figure 2. Variation of the radiative rate constant with volume % H_2O in H_2O–EtOH mixed solvents.

Figure 3. Variation of the nonradiative rate constant with volume % H_2O in H_2O–EtOH mixed solvents.

(0.1 M in ethanol) could be interpreted in terms of the formation dynamics of the solvation shell consisting of at least two parts, 1:1 photoassociation between the excited molecule and the polar solvent molecule, followed by a more general solvent reorganization around the excited-state molecule. Time-resolved fluorescence spectra further indicated that a major part of the solvent emission shift is caused by the 1:1 complex formation between 2-AN and ethanol. At 0.1 M ethanol in cyclohexane, there is an approximately 100:1 ratio between the nonpolar and polar solvent components. Therefore, unless there are ground-state equilibrium considerations bringing 2-AN and ethanol molecules together, the probability that the polar molecule has exactly the right location and orientation in the solvent shell of the solute at the instant of excitation is rather small. Thus, in the experiments of DeToma and Brand, the temporal evolution of the 1:1 complex would have to arise from molecular diffusion coupled with solvent exchange. Our own work[29] is beginning to show that the latter process can very well require a few nanoseconds in a nonviscous solvent (vide infra). Thus the 2 ns shown in Figure 3Aa of ref 28 for "half the emission shift" to develop coincides well with this kind of picture. If one takes seriously the 10% "static contribution", i.e., the contribution to the emission red shift at $t = 0$ for the 0.1 M ethanol-cyclohexane mixed solvent, then a propensity must also exist for some sort of ground-state "complex" between 2-AN and ethanol, since statistically the ethanol concentration is far too low to account for this result. One of the most interesting aspects of the DeToma and Brand paper is the conclusion that the 1:1 photoassociation process is reversible, suggesting that the polar nature of the solvent does not contribute much to the solvent–solute binding properties in the excited state of AN derivatives. This would seem to argue against a massive polar solvent attachment to the excited state of AN by virtue of a large dipole moment change. More will be said about this later.

C. Fluorescence Quantum Yields and Lifetimes. The variation of fluorescence quantum yields and lifetimes with solvent composition in H_2O–EtOH mixed solvents is shown in Table I. The quantum yields in contrast to the emission shifts are in agreement with earlier work.[4] Also in Table I are the radiative decay rates (k_r) and nonradiative decay rates (k_{nr}) derived from the experimental data, assuming simple exponential decay. This latter assumption may not be correct for mixed solvents, as will be discussed more fully later in the paper.
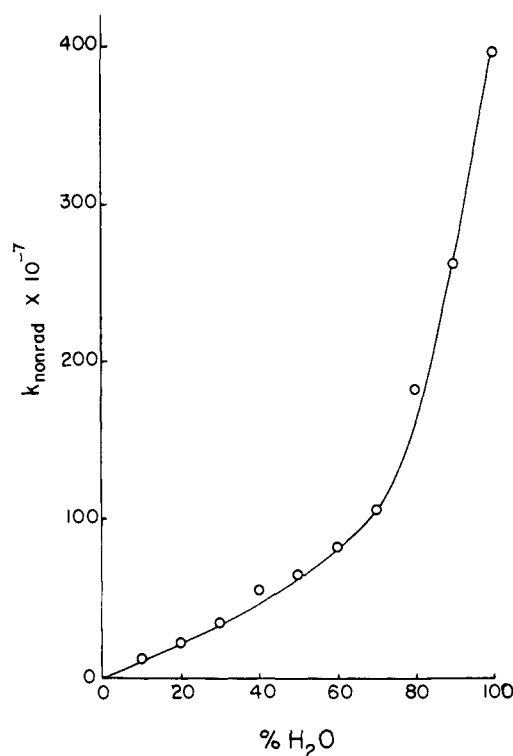
These decay rates are also plotted against solvent composition in Figures 2 and 3. The roughly three-fold decrease in the radiative rate of ANS in going from pure ethanol to pure water (both show purely exponential decay) is consistent with the ~2.5 ratio of integrated absorbance, together with an emission frequency ratio of ~1.13, which enters as a square.[30] Just as in the case of the fluorescence maxima discussed in the previous section, all these data are seen to change in a highly nonlinear way with solvent composition. In spite of these strong variations, a curiously simple relationship exists between the nonradiative rate k_{nr} and the shift ΔE in the fluorescence emission maximum. This can be seen by plotting log $(k_{nr}/k_{nr}{}^0)$ against ΔE, where $k_{nr}{}^0$ is the nonradiative rate in pure ethanol, and ΔE, zero for pure ethanol, is the red shift accompanying an increase in the water concentration. When ΔE is in cm^{-1}, the plot gives a straight line with slope of -8.4×10^{-4} (Figure 4). Also see ref 5.

Interestingly, a solvent deuterium effect on the fluorescence yield of ANS and related molecules has been reported by several authors.[4,27,31] This isotope effect cannot involve exchange or proton transfer since, for example, N-methyl-substituted anilinonaphthalenesulfonates behave in an identical way as the proton-substituted derivatives.[27] The isotope effects are not large but are easily measurable, the fluorescence yield being enhanced by two- to four-fold in D_2O and CH_3OD as compared with the respective protonated solvents. These effects have also been confirmed by lifetime measurements in our laboratory using picosecond techniques, showing, for example, a factor of about 2 increase of lifetime in D_2O as opposed to H_2O. The absorption and emission spectra are unchanged by solvent deuteration. Such effects have been attributed[27] to the poorer accepting mode characteristics of the O–D stretching vibration in an internal conversion process. In agreement with this view, there is no solvent deuterium effect on fluorescence yields of certain fluorescein derivatives,[32,33] where the main nonradiative pathway is intersystem crossing by way of a small energy gap. In ANS, however, deuterium-dependent rates may have to do with solvent-reorganization processes, the produc-
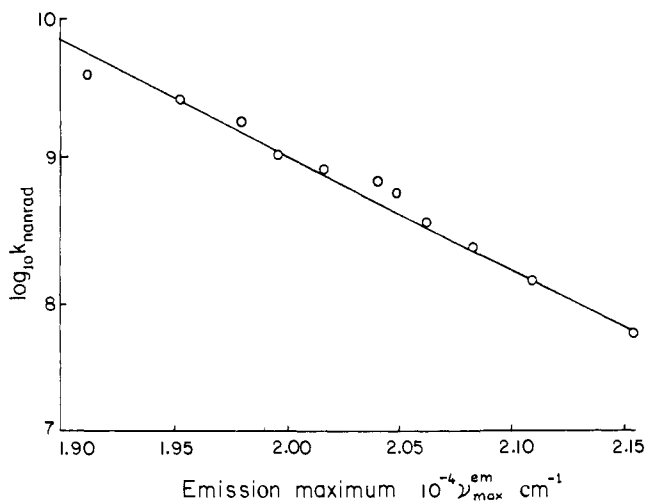
Figure 4. Relationship between nonradiative rate constant and emission maximum in H_2O-EtOH mixed solvents.

tion of the solvated electron in a photoionization process, or hydrogen-bonding effects.

Nonexponential Decays

Certainly in mixed solvents, but even in pure solvents, a range of solute environments exists at any instant. These environments give rise to inhomogeneous broadening in the electronic absorption spectroscopy of molecules in the condensed phase. If the time scale of the experiment is rapid enough, this varied solute environment in solution can also give rise to observable temporal effects. One can even imagine excitation exchange between different "sites" in fluid solution, much like that observed in solids,[34] and one can imagine "hole-burning" experiments[35] where narrow-line excitation depletes a certain part of the inhomogeneously broadened line, equilibrium being reestablished on picosecond or subpicosecond time scales. The more viscous the solution or the more different the environments or the less communication there is between them, the more slowly the temporal effects occur.

In the EtOH-H_2O mixed-solvent system used in the experiments described here, the solute fluorescence properties are quite sensitive to the local environment. An ANS molecule surrounded mainly by water molecules will have a lifetime shorter by a factor of nearly 50 than one surrounded mainly by ethanol molecules. *The ANS molecule can therefore in this sense act as a probe for local solvent structure.* This feature of water-ethanol mixed solvents is unlike that in the DeToma and Brand[28] work where the lifetime difference between pure cyclohexane and pure ethanol solvents is less than a factor of 2. If the solvent-solute interactions are sufficiently strong, then the time for breaking up and re-forming local solute-solvent structure in the excited state may be long enough for temporal inhomogeneities of this kind to be observed.

In fact, we have found the fluorescence decay curves of ANS in EtOH-H_2O mixed solvents to be nonexponential, while they are exact exponentials within experimental error when the solvent is either pure water or pure ethanol. The presence of any nonexponentiality in the decay and the probable explanation of it in terms of a range of emitting "sites" are quite interesting but can considerably complicate the analysis of the data. The rate constants k_r and k_{nr} presented in Table I, for example, have ignored this nonexponentiality. They have been derived by forcing the best exponential fit to the decay curves and determining the $1/e$ times. They therefore represent some kind of average over the various local solute environments.

Discussion

From our point of view, a most interesting development in the unravelling puzzle of ANS is some recent work carried out at the Royal Institution by Fleming et al.[8] Using nanosecond absorption spectroscopy, those authors have found evidence that the solvated electron is a photoproduct of excited ANS in solution, probably by way of a one-photon process. The solvated electron was found to be more efficiently produced in aqueous than in alcoholic solutions, but the yields are high in both cases (perhaps 100 and 50%, respectively). While these experiments have yet to be done on a picosecond time scale, where yields and build-up times of the solvated electron absorption can be followed and correlated with the subnanosecond fluorescence decays, the strong implication is that one-photon photoionization is an important nonradiative path for ANS. This should not be too surprising considering the past work of Jortner et al.,[36] Grossweiner et al.,[37] and others, which has shown that aromatic hydrocarbons containing electron-donating groups (N-aryl groups in ANS derivatives) are particularly susceptible to photoionization in aqueous solution. The presence of photoionizaton would have a strong bearing on the understanding of solvent effects in these systems.

The small solvent shift in the absorption spectrum is consistent with but does not prove a picture where the ground-state solute-solvent binding is relatively independent of solvent character. Using the small shift in the absorption maximum as an argument against a highly polar excited state, as some authors have done, is incorrect, since it is easy to draw potential energy diagrams[8,38] giving rise to little shift in absorption even though excited-state interactions are large. It would be equally easy to conceive of such diagrams if ground-state interactions were large. Without additional information, spectral shifts alone cannot say very much about the magnitude of interactions in one or the other of the combining states. The lack of a large absorption shift may simply be caused by accidental cancellation of a Franck-Condon displacement and a center-of-gravity shift (i.e., a shift of the 0-0 position).

Unfortunately, such uncertainties in interpretation also apply to the large emission red shifts for ANS-H_2O. The shifts can be due to a large solvent-solute *attractive* interaction in the excited state relative to the ground-state equilibrium structure, a large *repulsive* interaction in the ground state relative to the excited state equilibrium structure, or they could be caused by some sort of solvent-sensitive intramolecular rearrangement of ANS in the excited state. The latter is not easily distinguishable from the other types of solvent effects, however, where no large intramolecular structural changes take place. The relationship discovered in this work between emission red shift and nonradiative rate constant would imply mainly an upper state basis for the shift, but again this relationship may be accidental.

Even though no deep understanding about the origin of the large emission red shifts emerges from this work, it is of interest to examine the dependence of the shifts and the nonradiative rate constants on composition in the H_2O-EtOH mixed-solvent system. Of particular interest are the effects at low concentration of either one of the solvent components.

One would get the impression from the published discussions about the electronic properties of the excited state of ANS that the effects of adding a little H_2O to an ANS-EtOH solution would be much greater than those from adding a little ethanol to an ANS-H_2O solution. The data in Table I show definitely that this is not the case insofar as photostationary fluorescence maxima are concerned. For example, adding 10% (by volume) water to an ANS-EtOH solution causes the emission maximum to shift only 450 cm^{-1} to the "red", while adding 10% (by volume) ethanol to an ANS-H_2O solution causes a shift of 410 cm^{-1} to the "blue". Therefore, on this basis the effects are comparable. Using mole fraction or "surface fraction", either

of which may be at least as good a measure of relative concentrations in a "solvent shell", causes the H_2O effect to appear much *smaller* than the EtOH effect (10% ethanol by volume corresponds to a mole fraction of only about 0.03 and a "surface fraction" of about 0.07, while 10% water by volume corresponds to 0.265 and 0.151 for these same quantities, respectively).

In contradistinction to the results of DeToma and Brand[28] for cyclohexane–ethanol solvents, the emission shift data here do not seem readily explainable in terms of 1:1 "complex" formation. It seems reasonable that this picture would lead to peaking primarily in the regions of the pure water and pure ethanol spectra, rather than the general shift across the whole concentration range that is observed.

A more convincing way, perhaps, of showing the futility of employing a 1:1 "complex" picture in the H_2O–EtOH system is to examine the nonradiative rate data. Remember that the relevant rate processes are probably fast compared with solvent diffusion and exchange at the solvent shell level (vide infra). Now assume that each distinct ANS–solvent configuration containing m total solvent molecules, i of which are water, gives rise to a nonradiative rate k_i. The observed nonradiative decay $f(t)$ is an ensemble average of these:

$$f(t) = \sum_{i=0}^{m} a_i e^{-k_i t} \qquad (1)$$

The function $f(t)$ is, of course, nonexponential but has a well defined $1/e$ time. For the case of the 1:1 "complex", $m = 1$ and there are but two configurations. Statistically, $a_0 = x$, $a_1 = 1 - x$, $k_1 = k_{nr}$ (pure water), and $k_0 = k_{nr}$ (pure ethanol), where x is taken to be the volume fraction. The inverse of the $1/e$ time for $x = 0.1$ is then found to be 329×10^7 s^{-1} and for $x = 0.9$ is 5.95×10^7 s^{-1}. Each "calculated" value is far too close to that for the respective pure solvent, indicating the presence of some very nonlinear contribution in the actual experimental data. Using mole fraction or "surface fraction" instead of volume fraction or employing larger values of m does not help. The alternative to the statistical approach is a highly unlikely ground-state equilibrium, which favors the ANS–EtOH configuration at high water content and the ANS–H_2O configuration at high ethanol content! Clearly there is a severe nonlinearity in the response of k_{nr} to local solvent composition, defeating analysis by any method based on simple linear models. Again, the effect of a little EtOH on an ANS–H_2O solution is about as great *relatively* as that of a little H_2O added to an ANS–EtOH solution.

Solvent Interchange

In a mixed solvent two distinct processes at the solvent shell level have to be distinguished—*solvent relaxation* and *solvent interchange*. Both depend on solvent viscosity. The former occurs in pure solvents as well as in mixed solvents, while the latter can be detected only in mixed solvents. Solvent interchange depends on translational diffusion *to* the solvent shell as well as on other more complex interchange processes *at* the solvent shell. It is expected in general to be slower than solvent relaxation, which is merely the response of the *local solvent shell* to a change in the electronic properties of the solute. The recent paper by DeToma and Brand[28] has discussed similar ideas. Situations can arise of course (viscous solvents?) where the two processes have comparable rates and are not easily disentangled.

Some preliminary experiments have been carried out[29,39] in an attempt to separate these two processes in the H_2O–EtOH mixed solvent system. A ~10^{-4} M solution of 2,6-TNS (6-toluidinyl-2-naphthalenesulfonic acid) in either pure ethanol or pure water shows a purely exponential decay, with lifetimes of ~9 ns and ~60 ps, respectively. The greater than a factor of 100 difference is remarkable and is highly desirable

for sensitive solvent probe studies. The fluorescence spectrum of 2,6-TNS shifts from 430 nm in ethanol to 515 nm in water. Wavelength-dependent rise time studies, with resolution of a few picoseconds, in these two pure solvents indicate that the time for solvent *relaxation* into a new configuration around the excited state is extremely fast, <5 ps.[38] These results should be compared with the temporal behavior of ANS analogues in pure glycerol on the nanosecond time scale.[24] In mixed H_2O–EtOH solvent systems (30:70 by volume; mole fraction water 0.58), the temporal fluorescence is nonexponential. Observations in the "middle-wavelength range" ($400 < \lambda < 488$ nm) show that the early part of the decay approximately matches the lifetime of 2,6-TNS in water, while the later part matches that in ethanol. Observations in the far-red region show a "long" delay time of ~150 ps. This rise time has been interpreted in terms of a solvent-interchange phenomenon. At the observational wavelength used ($\lambda > 585$ nm), only those solute molecules mainly surrounded by H_2O in the mixed-solvent system are able to emit. Initially, however, there is a low statistical probability for the solvent shell to contain a high concentration of water molecules (providing more than 1:1 association is important, as discussed in the previous sections), and therefore at early times there is a low probability for emission in the far red. Thus the "water-like" emission of most 2,6-TNS molecules in the H_2O–EtOH mixed solvent can only appear after a delay time caused by solvent interchange. The delay corresponds to the time required for water molecules to replace ethanol, most probably by proximate translational diffusion combined with a concerted rotational interchange at the local solvent shell level of the excited solute molecule. This delay time in nonviscous solvents (~ 1 cP) is expected to range into the nanosecond regime if one of the solvent components is fairly dilute (cf. DeToma and Brand[28]) since the process then becomes primarily determined by "long-range" molecular translational diffusion.

The conclusion is that solvent interchange cannot always efficiently compete with fluorescence decay on nanosecond and subnanosecond time scales—even in nonviscous mixed solvents at high proportionate concentrations.

Summary

In summary, one can state a number of conclusions about the ANS–H_2O–EtOH system. Some of these conclusions may also be relevant to ANS in other solvents.

1. The time scale for decay of the excited singlet state of ANS is sufficiently short, particularly at high H_2O content (subnanosecond decay times), that diffusion to and solvent *interchange* at the solvent shell is not an efficient process. Thus, the initial ground-state solute–solvent configuration is not expected to change appreciably during the fluorescence lifetime, *irrespective of the equilibrium properties of the excited state.*

2. Solvent *relaxation,* on the other hand, has been seen to be short compared with picosecond time scales. The reason that this process is so rapid in these solvent systems is that it occurs primarily by way of low-amplitude nearest-neighbor translations and librations and does not require a bodily interchange of solvent molecules.

3. The nonradiative rate constant was found to vary much more strongly than the emission shift as the solvent composition is changed. In fact, the *logarithm* of the rate constant varies as fast (exactly) as the emission shift. If the fast nonradiative process in polar solvents is indeed photoionization as implied in ref 8, then this strong solvent dependence is reasonable.

4. The major clue that the *excited-state equilibrium* favors ANS–H_2O binding over ANS–EtOH binding is the 2430 cm^{-1} (6.95 kcal/mol) "red shift" in the photostationary emission spectrum. The relationship between these spectral shifts and log k_{nr} in mixed solvents suggests an excited-state

origin of the shifts. The shifts were found to vary in a gradual way on adding water to ANS-EtOH solutions or EtOH to ANS-H$_2$O solutions. Thus, a delocalized interaction with solvent over the surface of the solute or a bulk solvent effect is indicated by the data. In this regard our conclusions are similar to those of Förster and Rokos.[27] The binding energy is therefore smeared out over the solvent shell rather than being concentrated at a localized "active site" on ANS, as would be implied by 1:1 complex formation. The excess binding energy of ANS with an individual water molecule in H$_2$O-EtOH mixed solvents is therefore not very large.

5. Recent picosecond time-dependent rotational depolarization studies for ANS in pure water solution (see comments under ref 38) have shown a quick rise time followed by a ~70-ps decay of the rotational correlation function in the *excited state* of ANS in this medium. Using purely hydrodynamic considerations,[40] the decay time for a molecule the size and shape of ANS should be about 70 ps. This fortuitous agreement[41] implies that there is some solvent binding[11] in the excited state of ANS. However, a massive solvent "crystallization effect" around the supposedly highly polar solute molecule is not indicated by the data, though this was originally believed to be the case.[38]

6. The conclusions presented here are not necessarily transferable to ANS in other solvent systems. For example, in nonpolar solvents it is well-known[2,6,8] that intersystem crossing to a triplet state in ANS derivatives is an important nonradiative process. However, an unsuccessful search[8] for triplet-triplet absorption in ANS-H$_2$O and ANS-EtOH solutions has indicated that this process is very likely absent in polar solvents. Certainly, in pure H$_2$O, a *slow* intersystem crossing process could not compete. Thus the nonradiative transition is probably qualitatively different in polar and nonpolar solvents. It also may be different when ANS interacts with a single polar molecule in an otherwise nonpolar solvent environment,[28] as compared with bulk effects in a purely polar medium.

References and Notes

(1) J. Yguerabide, *Methods Enzymology*, **26**, 498 (1972).
(2) L. Brand and J. R. Gohlke, *Annu. Rev. Biochem.*, **41**, 843 (1972).
(3) A. Azzi, *Q. Rev. Biophys.*, **8**, 237 (1975).
(4) D. C. Turner and L. Brand, *Biochemistry*, **7**, 3381 (1968).
(5) E. S. Bowen and D. Seaman in "Luminescence", H. P. Kallman and G. MarmorSpruch, Eds., Wiley, New York, N.Y., 1962, p 153.
(6) E. M. Kosower and K. Tanizawa, *Chem. Phys. Lett.*, **16**, 419 (1972); E. M. Kosower, H. Doduik, K. Tanizawa, M. Ottolenghi, and N. Orbach, *J. Am. Chem. Soc.*, **97**, 2167 (1975).
(7) G. R. Penzer, *Eur. J. Biochem.*, **25**, 18 (1972).
(8) G. R. Fleming, G. Porter, R. J. Robbins, and J. A. Synowiec, *Chem. Phys. Lett.*, **52**, 228 (1977).
(9) J. N. Demas and G. A. Crosby, *J. Phys. Chem.*, **75**, 991 (1971).
(10) J. B. Birks, "Photophysics of Aromatic Molecules", Wiley, New York, N.Y., 1970.
(11) G. R. Fleming, J. M. Morris, and G. W. Robinson, *Chem. Phys.*, **17**, 91 (1976).
(12) G. R. Fleming, J. M. Morris, and G. W. Robinson, *Aust. J. Chem.*, **30**, 2337 (1977).
(13) A. E. W. Knight and B. K. Selinger, *Spectrochim. Acta, Part A*, **27**, 1223 (1971).
(14) I. B. Berlman, "Handbook of Fluorescence Spectra of Aromatic Molecules", 2nd ed., Academic Press, New York, N.Y., 1971.
(15) S. Suzuki, T. Fujii, and H. Baba, *J. Mol. Spectrosc.*, **47**, 243 (1973).
(16) S. Suzuki, T. Fujii, and T. Ishikawa, *J. Mol. Spectrosc.*, **57**, 490 (1975).
(17) S. Suzuki and T. Fujii, *J. Mol. Spectrosc.*, **61**, 350 (1976).
(18) A. Camerman and L. H. Jensen, *J. Am. Chem. Soc.*, **92**, 4200 (1970).
(19) C. J. Seliskar and L. Brand, *J. Am. Chem. Soc.*, **93**, 5405 (1971).
(20) F. C. Greene, *Biochemistry*, **14**, 747 (1975).
(21) W. O. McClure and G. M. Edelman, *Biochemistry*, **5**, 1908 (1966).
(22) S. K. Chakrabarti and W. R. Ware, *J. Chem. Phys.*, **55**, 5494 (1971).
(23) L. Brand and J. R. Gohlke, *J. Biol. Chem.*, **246**, 2317 (1971).
(24) R. P. DeToma, J. H. Easter, and L. Brand, *J. Am. Chem. Soc.*, **98**, 5001 (1976).
(25) E. Lippert, *Z. Elektrochem.*, **61**, 962 (1957).
(26) N. G. Bakhshiev, *Opt. Spectrosc. (USSR)*, **16**, 821 (1964).
(27) T. Förster and K. Rokos, *Chem. Phys. Lett.*, **1**, 279 (1967).
(28) R. P. DeToma and L. Brand, *Chem. Phys. Lett.*, **47**, 231 (1977).
(29) G. W. Robinson, T. A. Caughey, R. A. Auerbach in "Advances in Laser Chemistry", A. H. Zewail, Ed., Springer, Berlin, Heidelberg, New York, 1978, p 108.
(30) J. G. Calvert and J. N. Pitts, Jr, "Photochemistry", Wiley, New York, N.Y., 1966, p 174. Note, however, that the frequency factor, through a typographical error, has been omitted from the first of the two listed equations.
(31) L. Stryer, *J. Mol. Biol.*, **13**, 482 (1965).
(32) G. R. Fleming, A. E. W. Knight, J. M. Morris, R. J. S. Morrison, and G. W. Robinson, *J. Am. Chem. Soc.*, **99**, 4306 (1977).
(33) M. Martin and L. Lindqvist, *Chem. Phys. Lett.*, **22**, 309 (1973).
(34) I. I. Abram, R. A. Auerbach, R. R. Birge, B. E. Kohler, and J. M. Stevenson, *J. Chem. Phys.*, **63**, 2473 (1975).
(35) B. M. Kharlamov, R. I. Personov, and L. A. Bykovskaya, *Opt. Commun.*, **12**, 191 (1974); G. Mourou, *IEEE J. Quantum Electron.*, QE-11 (1975) 1; M. R. Topp and H. B. Lin, *Chem. Phys. Lett.*, **50**, 412 (1977).
(36) J. Jortner, M. Ottolenghi, and G. Stein, *J. Am. Chem. Soc.*, **85**, 2712 (1963).
(37) L. I. Grossweiner and H.-I. Joschek, *Adv. Chem. Ser.*, **No. 50**, 279 (1965), and other papers.
(38) G. W. Robinson, J. M. Morris, R. J. Robbins, and G. R. Fleming, *SPIE Annu. Tech. Symp., Proc., 22nd*, 113, 13–24 (1977). While the "potential energy diagram." (Figure 2) in this paper seems reasonable in light of recent experiments, some of the statements made on the basis of preliminary work in the last paragraph, p 20, have not been confirmed. The rotational correlation function has been remeasured at the University of Melbourne by one of the present authors (R. J. R.) and was found to be normal with about a 70-ps decay time. No massive solvent attachment is therefore suggested. Nor is there a long solvent relaxation time for ANS-H$_2$O. This is in agreement with the results of this paper and with more recent data. Reference 31 of the SPIE paper and ref 7 of the Fleming et al. paper (our ref 8) are also confusing. Because of the difficulty of meeting a deadline during a time when most of the authors were moving, an ANS paper was never published in the *Israel Journal of Chemistry*.
(39) R. J. Robbins and G. W. Robinson, unpublished work.
(40) T. Tao, *Biopolymers*, **8**, 609 (1969).
(41) J. T. Hynes, *Annu. Rev. Phys. Chem.*, **28**, 301 (1977).