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Effect of Solvent–Water Mixtures on the Prototropic Equilibria of Fluorescein and on the Spectral Properties of the Monoanion¶

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ABSTRACT

A spectral resolution procedure was used to resolve the absorption, excitation and emission spectra of the fluorescein monoanion in a number of solvent–water mixtures. This permitted an analysis of the effect of the solvent environment on the spectral properties of the monoanion and on the lactone/monoanion/dianion transitions of fluorescein. The monoanion excitation and emission spectra show relatively small changes with changing environment, a behavior that is related to the hydrogen-bonding environment of the solvent–water mixtures. There is also a general increase in the quantum yield of the monoanion from 0.36 in water to values up to 0.49 in the solvent–water mixtures. The presence of solvent also results in a general increase in the lactone content and in the monoanion:dianion and lactone:monoanion ratios. General polarity effects alone cannot account for the observed effects on the prototropic transitions indicating that specific solute–solvent effects involving hydrogen bonding perturb the prototropic equilibria of fluorescein.

INTRODUCTION

Fluorescein is one of the most commonly used fluorescent probes in the biosciences. The chromophore can exist in a number of prototropic forms, each of which possesses its own distinct spectral properties. However, it is the dianion species (Fig. 1) that is usually observed, since this is the predominant species under neutral conditions and possesses a large extinction coefficient and quantum yield (1). The hydrogen-bonding environment surrounding the fluorophore (2,3) modulates the spectral properties of the dianion. In particular, the dianion absorption spectrum exhibits a substantial bathochromic shift and a reduction in bandwidth in solvent–water mixtures containing aprotic solvents as the hydrogen-bonding environment is decreased. As a result, the absorption maximum of the dianion is a useful index of the hydrogen-bonding environment experienced by the chromophore (2).

Detailed spectral analyses of the dianion can be compromised by the presence of the monoanion species (Fig. 1). The pK_a of the monoanion–dianion transition is 6.3 in water (1) but is substantially increased when the chromophore is present in media of lower polarity (4–8), at the lipid–water interface of micelles and bilayers (7–11) or conjugated to macromolecules (12–15). As a result, the monoanion contributes significantly to the spectroscopic signal in these instances. An understanding of the photochemistry of the monoanion is therefore necessary in order to interpret properly the spectral properties of fluorescein in biological systems.

Unlike the dianion species, it is not possible to observe the monoanion in the absence of other species (1). Kubista and coworkers have resolved the monoanion absorption and fluorescence spectrum in water by a global analysis of the pH-dependent spectra of fluorescein (16,17). Previous attempts to characterize the spectral properties of the monoanion in solvent–water mixtures have been restricted to pH regions where the monoanion is the predominant species (8) or have relied on pK_a determinations (4,6). In the present study, we have used a spectral resolution procedure (18) to resolve the monoanion excitation, emission and absorption spectra in a range of solvent–water mixtures in order to investigate the effect of environment on the spectral properties of the monoanion. This method does not require any *a priori* knowledge about the prototropic equilibria and permits the analysis of the monoanion spectra even under conditions where the dianion makes significant contributions to the absorbance and fluorescence. As we demonstrate, it also provides information on the prototropic equilibria.

MATERIALS AND METHODS

Chemicals. Solvents were of the purest grade available. Disodium fluorescein and 2,2,2-trifluoroethanol (TFE)‡ were from the Sigma Chemical Company (St. Louis, MO). The fluorescein sample showed a single fluorescent species on thin layer chromatography and was used without further purification. Ammonium hydroxide and acetonitrile (ACN) were from Malliinkrodt (Paris, KY), dimethylsulfoxide (DMSO) from Merck (Darmstadt, Germany) and tetrahydrofuran (THF) from May & Baker (Dagenham, UK). Methanol (MeOH) and

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‡*Abbreviations:* ACN, acetonitrile; AON, acetone; DMSO, dimethylsulfoxide; MeOH, methanol; THF, tetrahydrofuran; TFE 2,2,2-trifluoroethanol.

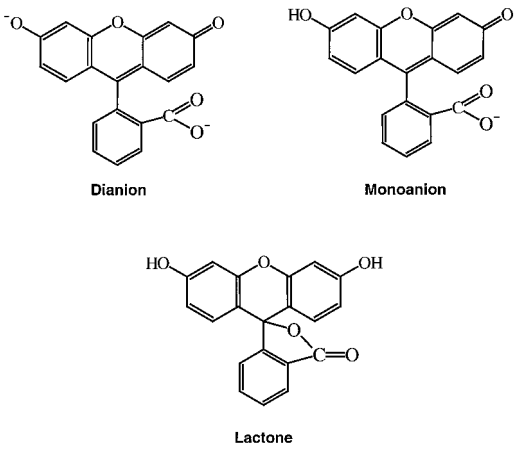

Figure 1. The dianion, monoanion and lactone forms of fluorescein.

acetone (AON) were from British Drug Houses (Poole, UK). The background fluorescence and absorbance of each solvent was negligible.

Spectroscopic measurements. Stock solutions of fluorescein were prepared in water and then diluted into the appropriate solvent–water mixture so that the maximum visible absorbance was less than 0.1. The fluorescein concentration of the stock was determined spectrophotometrically after dilution in 0.01 *M* NaOH using an extinction coefficient of 88 000 M^{-1} cm^{-1} at 490 nm (1). Absorption spectra were measured on a Cary 5 UV–VIS spectrophotometer. Fluorescence excitation and emission spectra were measured on a SPEX Fluorolog Tau-2 fluorometer equipped with a Rhodamine B quantum counter to correct for the wavelength-dependence of the exciting light. The excitation and emission spectra were corrected using correction factors supplied by SPEX. Quantum yields were calculated as previously described (2) using fluorescein in 0.01 *M* NaOH as the reference. All spectral measurements were performed at 20°C.

RESULTS

Resolution of monoanion absorption, excitation and emission spectra

A spectral resolution procedure was used to resolve the monoanion spectra in solvent–water mixtures where the dianion makes a significant contribution to the spectroscopic signal. We illustrate the approach adopted by reference to fluorescein in ACN–water.

The dianion is the only fluorescein species present under basic conditions. As shown in Fig. 2a, the fluorescence excitation and emission spectra of fluorescein in 50 mol% ACN–water mixtures in the presence of a base are independent of the respective excitation and emission wavelengths used to monitor the spectra, and the excitation spectrum reflects the absorption spectrum. In contrast, the excitation and emission spectra in the absence of the base are dependent on the respective emission and excitation wavelengths (Fig. 2b). These spectra and the corresponding absorption spectrum (Fig. 2b, inset) show that both the monoanion and dianion contribute to the spectroscopic signal in the absence of added base. By subtracting the appropriate amount of the dianion spectra recorded in the presence of base, it is possible to obtain excitation and emission spectra that are independent of the respective emission and excitation wavelengths and which represent the resolved monoanion contribution to the fluorescence (Fig. 2c). This procedure was performed iteratively on a spreadsheet by progressively

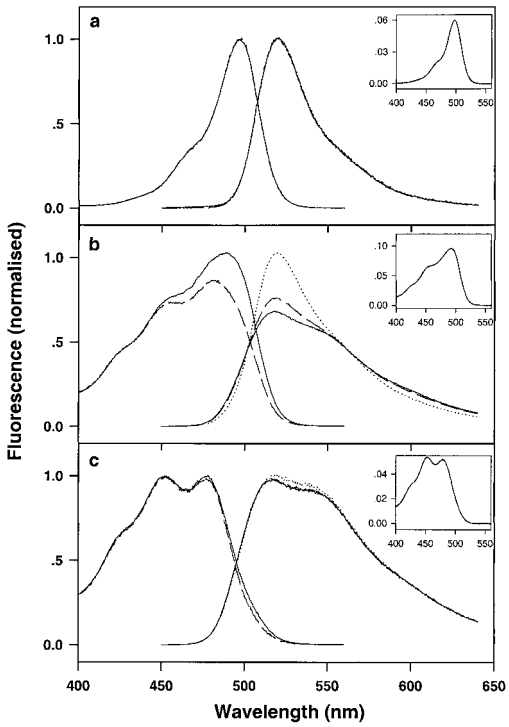

Figure 2. Resolution of monoanion excitation, emission and absorption spectrum in 50 mol% ACN–water mixture. Fluorescence spectra were measured in 50 mol% ACN with 650 n*M* fluorescein in the presence of 1% (vol/vol) ammonium hydroxide (a) and with 6.5 μ*M* fluorescein in the absence of base (b). Subtraction of 110% of the spectra in (a) from those in (b) produced the spectra shown in (c), which represent the resolved spectra of the monoanion. Excitation spectra were monitored at emission wavelengths of 600 (— —) and 640 (— —) nm. Emission spectra were monitored at excitation wavelengths of 420 (——), 440 (— —) and 475 (· · ·) nm. Insets: corresponding absorption spectra of samples. The resolved monoanion absorption spectrum (c) was obtained by subtracting 110% of the dianion spectrum (a) from the spectrum in (b).

subtracting the dianion spectra measured in the presence of base from the spectra recorded in the absence of base until the resultant normalized spectra were superimposable as judged by a visual inspection.

The amount of the dianion spectra subtracted in order to resolve the monoanion excitation and emission spectra represents the ground state concentration of the dianion in the solvent–water mixture. By performing a similar subtraction of the absorption spectra recorded in the absence and presence of base, an absorption spectrum that reflects the resolved excitation spectrum is produced (Fig. 2c, inset). These results show that the monoanion and dianion behave as simple noninteracting fluorophores and that the procedure permits the resolution of the monoanion spectrum and the subsequent characterization of its spectral properties.

This method was used to resolve the monoanion spectra in a range of solvent–water mixtures. The resolved spectra in solvent–water mixtures containing 50 mol% cosolvent are shown in Fig. 3 and the dianion contribution in the unresolved spectra is summarized in Table 1. In the case of THF–water mixtures, it was necessary to measure the unresolved spectra in the presence of 0.07% (vol/vol) ammo-

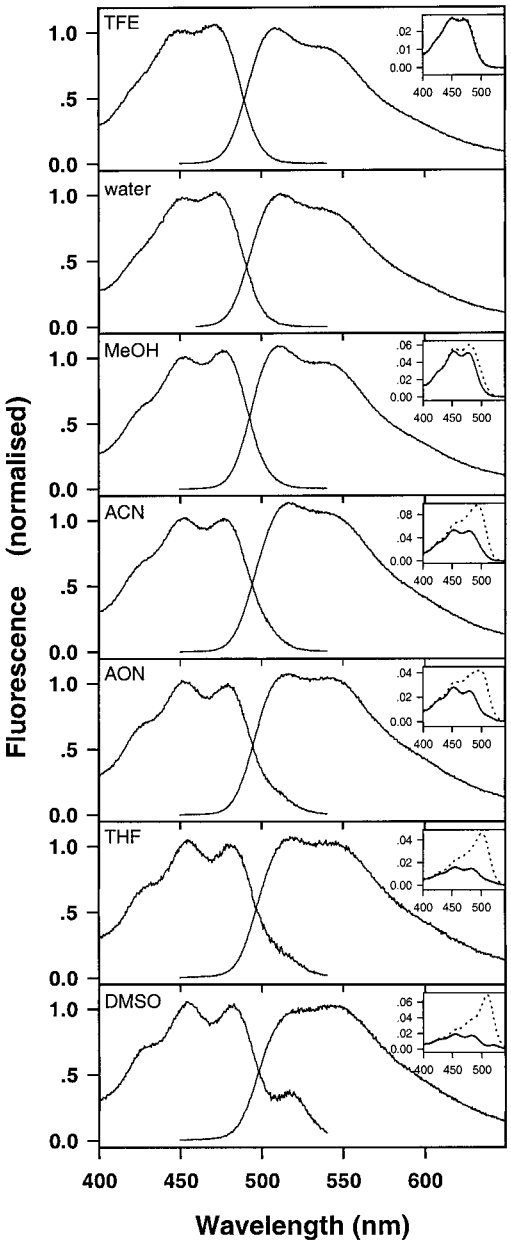

Figure 3. Fluorescence excitation and emission spectra of the fluorescein monoanion in water and in 50 mol% solvent–water mixtures at 20°C. The monoanion spectra in the solvent–water mixtures were resolved as described in Fig. 2 using spectra recorded in the absence and presence of 1% (vol/vol) ammonium hydroxide. The fluorescein concentrations used and the dianion content of the unresolved spectra in the absence of base are summarized in Table 1. The monoanion spectra in water are taken from Klonis and Sawyer (1). Inset: unresolved (· · ·) and resolved (——) monoanion absorption spectra in the corresponding solvent–water mixtures determined as described in Fig. 2.

nium hydroxide due to the predominance of the lactone form in the absence of base (see below).

In general, the excitation spectra are well resolved up to wavelengths of 490–500 nm. Superimposable spectra are not obtained at longer wavelengths in some of the solvents (*e.g.* THF and DMSO) due to the relatively large dianion contribution. The large dianion contribution also affects the accuracy of many of the resolved absorption spectra (compare unresolved and resolved absorption spectra in Fig. 3). The weighting of the dianion contribution is less in the fluorescence spectra since wavelengths were chosen to facilitate observation of the monoanion fluorescence. For this reason, the subsequent analysis is confined to excitation rather than absorption spectra since the excitation spectra are resolved with a greater degree of certainty.

The emission spectra are generally well resolved due to the choice of excitation wavelengths, which are biased towards excitation of the monoanion. However, the resolved emission spectra in THF and DMSO–water mixtures are less certain near 520 nm due to the greater contributions of the dianion in this region of the spectrum.

Table 1. Dianion contribution to the fluorescence spectra of fluorescein in the 50 mol% solvent–water mixtures determined from the resolution of the monoanion spectra*

Cosolvent	Total fluorescein† (μM)	Dianion contribution to spectra‡ (%)
TFE	1.3	2.9
MeOH	3.3	32
ACN	6.5	110§
AON	8.7	55
THF*	8.5	69
DMSO	6.5	90

* The resolved monoanion spectra are shown in Fig. 3. All the spectra (except those measured in THF) were resolved from spectra measured in 50 mol% solvent–water mixtures containing no added base. The monoanion spectra in THF–water mixtures were resolved from spectra measured in the presence of 0.07% (vol/vol) ammonium hydroxide.

† Total concentration of fluorescein present in the solvent–water mixtures from which the monoanion spectra were resolved.

‡ The proportion of the dianion spectra required to be subtracted from the spectra obtained in solvent–water mixtures in order to resolve the monoanion spectra (see text). The dianion spectra were measured with fluorescein (650 n*M*) in the corresponding solvent–water mixtures in the presence of 1% (vol/vol) ammonium hydroxide.

§ Value is greater than 100% due to the use of a 10-fold lower fluorescein concentration in the sample used to measure the dianion spectra.

Effect of solvent–water mixtures on the spectral properties of the fluorescein monoanion

The resolved monoanion spectra in Fig. 3 are arranged in series according to the effect of the solvent–water mixture on the dianion absorption maximum which reflects the hydrogen-bonding environment surrounding the fluorophore (2). The solvents affect the relative heights of the two peaks that comprise the characteristic double peak of the monoanion excitation spectrum (1). There is a relative increase in the 450 nm peak compared to the 475 nm peak in the series from TFE– to DMSO–water mixtures. The change in the excitation spectrum is also associated with a more pronounced shoulder at 420 nm, a modest redshift of 5–10 nm and a sharpening of the absorption bands. The emission spectrum shows a similar trend with respect to the main peak and the shoulder, although there does not appear to be any

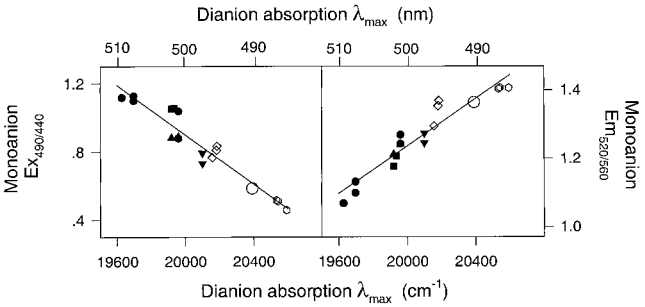

Figure 4. Correlation of the monoanion $Ex_{490/440}$ and $Em_{520/560}$ spectral parameters in solvent–water mixtures with the dianion absorption maximum in the corresponding solvent–water mixtures. The parameters are calculated from the resolved fluorescence excitation and emission spectra in various solvent–water mixtures as described in text. The symbols correspond to the following solvent–water mixtures: water (○), DMSO (●), THF (■), AON (▲), ACN (▼), MeOH (♦), TFE (○).

gross shift in the emission spectrum in the different solvent–water mixtures.

The complexity of the monoanion spectrum makes it difficult to quantitate its spectral changes in the various solvent–water mixtures. Two parameters were defined to characterize these spectra: $Ex_{490/440}$ and $Em_{520/560}$ represent the fluorescence ratios at the indicated wavelengths of the resolved monoanion excitation and emission spectra, respectively. These represent a qualitative measure of the spectral structure and are sensitive to changes in the maxima, in the spectral bandwidths and in the relative heights of the two peaks. As shown in Fig. 4, a linear relationship exists between these two parameters and the dianion absorption maximum measured in the same solvent–water mixture. Thus, as with the dianion (2), the structure of the monoanion excitation and emission spectrum is sensitive to the hydrogen-bonding environment. Indeed, the redshift and the decreased bandwidth exhibited by the monoanion excitation spectrum in decreased hydrogen-bonding environment mirrors the effects observed in the dianion absorption spectrum.

The quantum yield of the monoanion in the various solvent–water mixtures was calculated using the resolved absorption and emission spectra (Table 2). The monoanion quantum yield in the presence of solvents (0.39–0.49) shows a general increase compared to water (0.36). The quantum yield of the monoanion in water is also lower than that of the dianionic and cationic forms of fluorescein, which possess quantum yields close to 1 (1). If it is assumed that the lower quantum yield is the result of a dynamic quenching mechanism, the radiative lifetime (τ_R) can be calculated according to:

$$\tau_R = \frac{\tau}{Q} \qquad (1)$$

where τ and Q are the measured lifetime and quantum yield, respectively. The radiative lifetime of the monoanion in water (9.4 ns) is similar to that determined in propylene glycol (9.2 ns; unpublished) even though the monoanion possesses a greater quantum yield in this solvent (0.50; unpublished). This implies that a dynamic quenching mechanism modulates the quantum yield and lifetime of the monoanion and that the dynamic quenching is more efficient in the presence of water compared with propylene glycol. The general increase of the monoanion quantum yield in solvent–water mixtures compared to water may therefore partly reflect the dilution of water.

Table 2. Quantum yield of the fluorescein monoanion in 50 mol% solvent–water mixtures at 20°C

Cosolvent	Quantum yield*
TFE	0.49
Water†	0.36
MeOH	0.49
ACN	0.46
AON	0.45
THF	0.39
DMSO	0.39

* Quantum yields were determined using the resolved emission spectra and the resolved absorbance obtained with 440 nm excitation.

Unlike the accompanying spectral changes, there does not appear to be any simple relationship between the magnitude of the quantum yield and either the hydrogen-bonding environment or the polarity of the solvent. Interestingly, if the quantum yield in water is omitted from the comparison, a relationship between the quantum yield and the hydrogen-bonding environment in the different solvent–water mixtures is apparent (Table 2). A dependence of lifetime on the hydrogen-bonding environment has also been noted in some fluorescein analogs (19). One explanation for these observations is that two processes are contributing to the monoanion quantum yield: one is related to the efficient quenching by water, the other to a general increase in fluorescence with an increase in the hydrogen-bonding environment.

Perturbation of prototropic equilibria in solvent–water mixtures

The technique used to resolve the fluorescence spectra of the monoanion in the solvent–water mixtures provides the ground-state concentration of the dianion in the mixture. It is therefore possible to estimate the monoanion concentration in the mixture using the resolved absorption spectrum assuming an extinction coefficient of 32 300 M^{-1} cm^{-1} at the absorption maximum (1). The combined monoanion and dianion concentration determined this way is always less than the total fluorescein concentration in the solvent–water mixtures. The discrepancy is attributed to the presence of the colorless lactone form of fluorescein (1) (Fig. 1). The contribution of each species (*i.e.* dianion, monoanion and lactone) to the total fluorescein population in the solvent–water mixtures is presented in Table 3.

Although the lactone is the major neutral species of fluorescein in solution, it is not present at neutral pH and exists in appreciable quantities only under more acidic conditions. In contrast, the lactone is a significant species in solvent–water mixtures and in many cases represents the most abundant species (Table 3). In addition, there is a general increase in the lactone:monoanion and monoanion:dianion ratio in solvent–water mixtures compared to water. The perturbations in the prototropic equilibria show no obvious relation-

Table 3. Prototropic forms of fluorescein present in 50 mol% solvent–water mixtures at 20°C

Solvent	Fluorescein species			Monoanion: dianion ratio	Lactone: monoanion ratio
	Dianion* (%)	Monoanion† (%)	Lactone‡ (%)		
TFE	1.5	63	35.5	43	0.57
Water§	83	17	0	0.17	0
MeOH	6.3	48	45.7	7.6	0.96
ACN	11	25	64	2.3	2.6
AON	4.1	9.8	86.1	2.4	8.8
THF‖	0	0	100	ND¶	ND¶
DMSO	9.0	8.9	82.1	0.98	9.2

* The dianion contribution was determined by the amount of the dianion fluorescence spectra that was required to be subtracted from the unresolved spectra in order to resolve the monoanion spectra (see Table 1).
† The monoanion contribution was calculated based on the absorbance of the resolved monoanion absorption spectrum shown in Fig. 3 (insets). The extinction coefficient of the double peak was assumed to be the same as that of the monoanion in water (32 300 M^{-1} cm^{-1}; [1]).
‡ The lactone contribution was calculated based on the difference between the calculated monoanion and dianion concentrations and the total fluorescein concentration.
§ Values in water are calculated at pH 7 using a pK_a value of 6.3 for the monoanion–dianion transition (1).
‖ Essentially no absorbance is present in 50 mol% THF–water mixtures in the absence of base.
¶ Could not be determined.

ship to either the polarity or the hydrogen-bonding environment of the solvent–water mixtures.

DISCUSSION

Influence of polarity and hydrogen-bonding environment on the prototropic transitions of fluorescein

We have previously shown that the prototropic transitions of fluorescein in solution conform to the model shown in Fig. 5 (1). An examination of these transitions in media of varying polarity comprising a single solvent–water system has shown that the various transitions are affected to different extents by the polarity of the medium (4,6–8). The pK_a of the carboxyl group is particularly sensitive to the environment and shows relatively large *increases* with decreasing polarity. The pK_a values of the neutral xanthene and cationic xanthene groups exhibit more modest *increases* and *decreases* with decreasing polarity, respectively. These effects are consistent with other acid–base systems where a decrease in polarity favors the least charged form of the compound (20–25). Since the lactone is neutral and is the least polar of the fluorescein species, the pK_a of lactonization (the cation to lactone transition) would also be expected to *decrease* with decreasing polarity. Based on these trends, a medium of lower polarity should (1) promote the disappearance of the zwitterionic and neutral quinoid species so that the lactone is the only neutral species present; (2) promote the existence of the lactone form over a broader pH range; and (3) increase the pK_a of the monoanion–dianion transition. As a result, two transitions would be expected to exist under neutral conditions in solvent–water mixtures—one corresponding to the monoanion–dianion transition, the other to the lactone–monoanion transition. While the monoanion–dianion transition represents a simple prototropic equilibrium, the lactone–monoanion transition is described by a more complex mechanism (Fig. 5). The pK_a for this transition (pK_{lm}) can be related to the underlying mechanistic pK_a values:

$$pK_{lm} = pK_q + pK_c - pK_{cl} \quad (2)$$

where pK_q, pK_c and pK_{cl} are the pK_a values of the neutral quinoid–monoanion, cation–neutral quinoid and cation–lactone transitions, respectively. The net effect of decreasing the polarity of the medium should be to *increase* pK_{lm}.

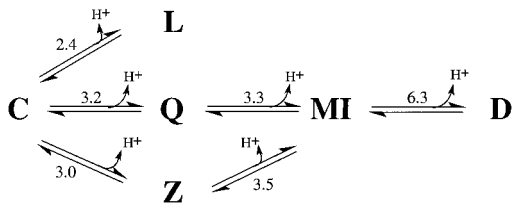

Figure 5. The prototropic transitions of fluorescein in aqueous solution. The model and nomenclature are taken from Klonis and Sawyer (1). The pK_a of each transition is indicated in the figure. (C, cation; L, lactone; Q, neutral quinoid; Z, zwitterion; MI, monoanion; D, dianion).

General polarity effects can account for the general increase in the lactone species and in the lactone:monoanion and monoanion:dianion ratios in the solvent–water mixtures compared to water. However, they cannot account for the trends observed *between* the solvent–water mixtures (Table 3). For example, the lactone:monoanion ratio is greater in the presence of DMSO compared to TFE even though TFE is less polar and a stronger acid. Similarly, the monoanion:dianion ratio is greater in TFE compared to the less polar AON. The inability to correlate the perturbations in the transitions with the polarity of the medium may reflect the underlying role of specific interactions in stabilizing certain species. Such specific effects involving dipolar and hydrogen bond interactions between the solvent and one component of an acid–base system are known to be significant in certain acid–base systems (20,22).

The data presented in Table 3 provide some evidence that hydrogen-bonding effects influence the prototropic equilibria of fluorescein. A relationship between the monoanion:dianion and lactone:monoanion ratios and the hydrogen-bonding environment of the solvent–water mixtures is apparent

if the ratios obtained in water are omitted from the comparison. The monoanion is favored over the dianion and the lactone in solvents that produce an increase the hydrogen-bonding environment. Although the trend in the monoanion: dianion ratio can be partly attributed to the relative acidities and basicities of the solvents, this cannot explain the trends in the lactone:monoanion ratio since the strongest acids (*e.g.* TFE) favor the monoanion species. Mchedelov-Petrossyan and coworkers have similarly concluded that hydrogen-bonding effects perturb the prototropic equilibria of fluorescein by comparing the pK_a shifts in a range of solvent–water mixtures possessing the same polarity (4).

Implications of present results for applications involving fluorescein

Fluorescein is commonly employed as a pH probe utilizing the spectral changes associated with the monoanion–dianion transition. The sensitivity of the spectral properties to the environment—through perturbations in the spectral properties of the individual species and through perturbations in the prototropic equilibria—means that an altered environment can itself produce spectral changes independent of the pH. As a result, the nature of the probe environment needs to be considered for the proper interpretation of pH effects.

Perturbations in the prototropic equilibria of probes are commonly related to the polarity of the probe microenvironment by comparing the pK_a shifts with those obtained in solvent–water mixtures (7,8,21,23–25). In the case of fluorescein, such perturbations are observed when it is located at a lipid–water interface (7–11) and when it is conjugated to macromolecules (12–15). However, the sensitivity of the prototropic equilibria to the hydrogen-bonding environment complicates the interpretation of the pK_a shifts. Thus, while the pK_a shifts may be used as indicators of a change in environment, they cannot be unambiguously related to the polarity of the microenvironment. This can account for the inability to correlate the pK_a shifts of three fluorescein transitions at a lipid–water interface with those in a single solvent–water mixture of defined polarity (7,8). In contrast, other acid–base indicators provide consistent values for the polarity at the interface (23–26).

While the structure of the monoanion excitation and emission spectrum reflects the hydrogen-bonding environment, the complex nature of the spectrum and the inability to observe the monoanion in isolation makes it difficult to quantitate its spectral properties. The spectral properties of the fluorescein dianion are easier to quantitate and to relate to the hydrogen-bonding environment of the fluorophore. However, the change in the environment of fluorescein upon its association with macromolecules means that the monoanion can potentially make a significant contribution to the measured absorbance and fluorescence, a feature that is promoted by the increase in its quantum yield. This has a major consequence in the determination of the spectral parameters of the dianion since monoanionic contributions will produce an apparent blueshift and an apparent widening of the bandwidth of the dianion. A more complete spectral characterization of fluorescein is required in such instances to ensure that the spectral properties determined represent those of the dianion. Measurements of the pH dependence of the spectral parameters, a comparison of absorption and excitation spectra or measurements of the fluorescence spectra at multiple wavelengths are simple ways of determining whether a single species is being observed.

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